PLANT GROWTH REGULATION

Fourth International Conference on Plant Growth Regulation

Sponsored by

The Boyce Thompson Institute
for Plant Research

The New York Botanical Garden

The Brooklyn Botanic Garden

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Dedication of Volume to

PERCY W. ZIMMERMAN

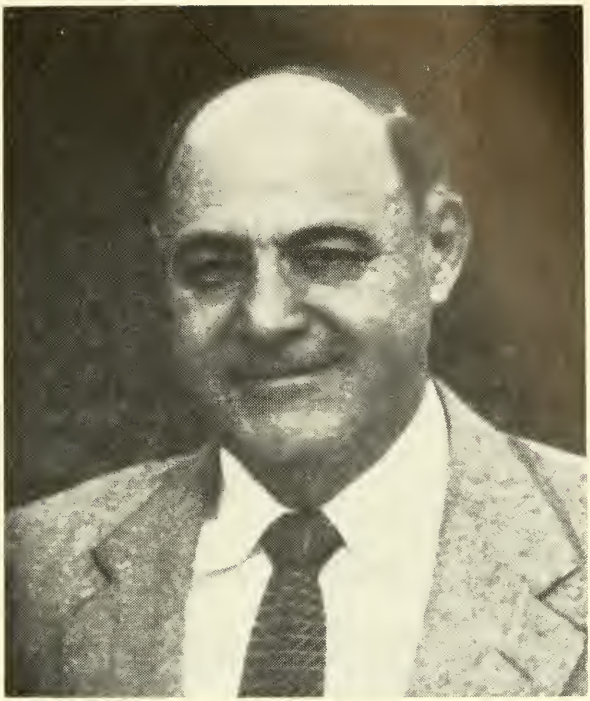
EARLY IN ITS DELIBERATIONS, the Organization Committee decided that Percy W. Zimmerman would be designated as honorary chairman of the Fourth International Conference on Plant Growth Regulation. Fate intervened, and this opportunity to provide a small token of honor and appreciation for a lifetime of faithful service to the study of plant growth was denied. He succumbed to an embolism following an emergency operation at Wenatchee, Washington, on August 14, 1958. The dinner meeting which he was to have addressed during the Conference was changed to a memorial meeting in his honor, and Dr. W. J. Robbins was asked to give the address.

From Dr. Zimmerman's laboratory, in collaboration with Dr. A. E. Hitchcock, there came an ever-expanding series of discoveries over a period of a quarter of a century. They found the means of using indole-3-butyric acid to root cuttings of species difficult to propagate and discovered the growth regulating properties of 2,4-dichlorophenoxyacetic acid, α-naphthaleneacetic acid, and the halogenated benzoic acids. These and many other similar findings were not fortuitous accidents. They came from prolonged and diligent search that was guided by certain lines of provocative thinking.

To know "Zim" was to respect him even when you differed with his ideas. He was unflinching in his examination of the validity of every new idea and every piece of data. He insisted on testing new concepts extensively on many kinds of plants under all sorts of conditions before accepting an observation as factual. He was just as relentless in questioning the ideas of his colleagues in research and administration as he was in scrutinizing his own progress.

His blunt questioning of all theories that were superficially drafted, or that left unanswered questions, did not necessarily endear him to his colleagues. He believed in the use of theory in the research laboratory but he was distressed that tenuous theories should be published as semi-facts where they might lead neophytes into the wrong channels of learning. The pages of history show that his skeptical attitude has been vindicated as the auxin a and auxin b concept has been laid to rest, the concept of the ubiquitous role of "auxin" in all plant functions has been drastically changed, and the superficial theories on the relation of chemical structure to growth regulant ability of molecules have been disintegrated by wider knowledge.

Such was the unwavering honesty and loyalty of the man to his dedicated purposes in life. He spent 32 years in the laboratories of the Boyce Thompson Institute, breathing life into its ideal of acquiring useful new knowledge on plant life. It was a richer place for having had him as an associate. The genial smile, the warm interest in everything and every person around him, and the ever youthful approach and un-



PERCY W. ZIMMERMAN

wavering interest in all the problems of life gave him an enduring place in the hearts of his colleagues even as they recognized his occasional very human frailties.

Dr. Zimmerman was born at Manito, Illinois, on February 23, 1884. He was trained at the University of Chicago from which he received the B.S. degree in 1915, M.S. in 1916, and Ph.D. in 1925. His professional career included a period as public school teacher and Superintendent of Schools at Westville, Illinois (1910–13), Associate Professor of Botany and Dean of the College of Agriculture at the University of Maryland (1916–25), and Plant Physiologist at the Boyce Thompson Institute from 1925 until his death in 1958.

This volume is affectionately dedicated to him as one of the pioneers who opened the door on this exciting new area of plant research. Without the foresight, persistent effort, and dedication to research of men such as he, there would have been no assemblage in this conference of representatives from so many lands. No more fitting memorial could be offered to his memory than this volume wherein are assembled the best ideas of the distinguished leaders of today.

George L. McNew

Preface

The Fourth International Conference on Plant Growth Regulation was held at the Boyce Thompson Institute for Plant Research in Yonkers, New York, from August 10 through August 14, 1959. The Conference was sponsored jointly by the Boyce Thompson Institute, The New York Botanical Garden, and the Brooklyn Botanic Garden through an Organization Committee headed by Dr. George L. McNew, Managing Director of the Boyce Thompson Institute.

One hundred and forty scientists from eighteen countries were invited to participate through the presentation of formal papers, in organized discussion groups, or via informal question and answer periods. This volume reports the proceedings of the Conference.

The program was arranged by a committee headed by Dr. A. J. Vlitos, who first suggested the Conference. Housing, transportation, and the memorable luncheons and dinners were handled by other committees.

An international conference of this size is an expensive undertaking requiring funds to assist participants who came from abroad, to cover the costs of food, transportation, and the many other obligations which had to be met. Our desire to hold the cost of this book to a reasonable figure meant it had to be subsidized. Financial support was generously provided by The Rockefeller Foundation, the National Science Foundation, and the following corporations.

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Shell Development Co., Agricultural Research Division, Modesto, California.
United Fruit Co., Boston, Massachusetts.
Upjohn Co., Research Division, Kalamazoo, Michigan.

Many individuals from the sponsoring institutions were necessarily involved in making the Conference a success. Mrs. Bettie Brooks, Executive Assistant to the Managing Director, Dr. Clyde Chandler, and Mrs. Florence Flemion of the Boyce Thompson Institute and Mrs. Eileen Kene and Miss Bernice Winkler of The New York Botanical Garden did a great deal of the indispensable behind-the-scenes work that resulted in a smoothly running Conference. The excellent work of our recording engineer, Mr. William R. Begany, permitted the transcription of the discussion periods. Mrs. George L. McNew and Mrs. William C. Steere arranged programs for the wives and families of the participants. The heroic efforts of Dr. McNew for obtaining the necessary financial support, for making special arrangements for just about everything, and for being, withall, a charming host, warrants a rising vote of thanks.

Publication of these proceedings required considerable cooperation and forbearance from the many participants. The invaluable assistance of Dr. S. E. A. McCallan with text and figures is most gratefully acknowledged. Mr. William G. Smith, Jr., head of the Illustration Division of Boyce Thompson Institute, did yeoman service in preparing the figures for the book. Special thanks are due Mrs. Bettie Brooks for reading both the galley and page proof with painstaking thoroughness. The editors are also indebted to Dr. R. E. Buchanan for careful and conscientious preparation of a meaningful, comprehensive index. The concerted efforts of the Editorial Committee and of Mr. Marshall Townsend, Manager of the Iowa State University Press, made the chairman's task almost too easy.

Richard M. Klein
Chairman, Editorial Committee

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Introduction

GEORGE L. McNEW
Boyce Thompson Institute

The Broader Concepts of Plant Growth Regulation

Men and plants have come a long way together down through the ages. The association has long since changed from the casual contact of a nomad with a quick meal to that of almost complete interdependence. One of the major goals of civilization has always been to improve the usefulness and reliability of plants in promoting human welfare. Those societies that have failed to achieve this improvement in proportion to the material needs of a growing population have crumbled and perished from the earth.

THE PLASTICITY OF THE GROWING PLANT

The plant scientist of the twentieth century has come to look upon the major crop plants as so many plastic materials of life that can be shaped and altered by skilled hands. Much of the altered design of plant development has been achieved by genetics — first by studying natural variants and selecting the preferred races and varieties, and more lately by selection of suitable building blocks for synthesizing new varieties with highly specialized attributes.

This synthetic process of hastening or diverting the ordinary processes of evolution has come to be considered entirely inadequate. The natural processes of inherited growth regulation fail in so many respects that the geneticist has sought new tools such as gene mutation by irradiation or induction of polyploidy by chemicals or other means. The heritable processes of plant regulation are very desirable in that they are spontaneously self-reproducible and hence very economical to use once they are properly established.

As men have gradually unravelled the mysteries surrounding normal metabolism and growth processes in plants it has become clear that nearly all regulatory processes depend upon underlying chemical

activities of the cell. It has become increasingly self-evident that anyone who understands the chemical processes of the living cell has the potential power of regulating that cell's activities and its ultimate incorporation into a tissue and thence into a functional organ. The person who can control the activities of the living cell without destroying its life can determine the ultimate fate of the individual plant.

This breath-taking concept has long since dropped from the realm of human fancy and daydreaming into the reality of agricultural practice. There is no really valid reason why the physiologists and chemists should not eventually design molecules that will duplicate, circumvent, block, or accelerate any and all the activities of the gene. The great problem is to design such a molecule so it will operate gently but specifically in the desired manner without disrupting major vital processes.

Probably the greatest handicap to achieving this utopia of chemical control has been the simple difficulty of properly administering the material so its effects will be felt over a prolonged period of time. Exogenous chemicals have not yet duplicated the effect of genes because they are applied crudely in massive doses that are dissipated or detoxified in very short order. What is needed is a relatively inert chemical that will generate the proper regulant over a long period of time as it is required in cell functions.

The idea of a chemical that would generate a plant growth regulant over a sustained period is not an impossibility. Those people working with the dithiocarbamate fungicides, for example, have done this very thing. Relatively inert dithiocarbamates that can be piled on foliage in heavy doses without injury to the crop but which generate highly fungitoxic isothiocyanates as they are needed are now being used to control fungous diseases of plants by the tens of millions of pounds each year. The protectants are so ephemeral they cannot readily accumulate in sufficient amount to injure plants as may occur with a more stable material such as a copper fungicide. There is substantial evidence that several other organic sulfur molecules generate toxicants for fungi and nematodes in situ. There is good reason to believe that the entomologists have comparable tools in the organic phosphate insecticides and miticides that serve as systemic eradicants.

THE NATURE OF PLANT REGULANTS NOW IN EXISTENCE

Great achievements in plant growth regulation have come into being by the use of relatively simple chemicals that have been dis-

covered and developed in the past quarter century. Weeds may be removed selectively from crops, the dormancy of buds can be prolonged or disrupted, the processes of abscission can be instigated or retarded, and the interconversion of starches and sugars can be directed one way or the other. Frequently the magnitude or even the direction of these interconversions can be regulated very specifically by the dosage applied and the maturity of the tissues at the time of application.

It is of more than passing interest that a major proportion of the plant regulants discovered to date are organic acids. Many others contain strong electronegative or alcohol, ester, and ether groups that could be converted into carboxyl groups by relatively simple processes of hydrolysis or oxidation. The predominant presence of acidic moieties in the molecule raises serious questions as to the nature of their effect. One soon comes to suspect that they may be primarily involved in altering the nature of cell walls since they present wonderful possibilities for affecting the synthesis of cellulose, lignin, and pectin, the very materials that lead to restriction of cell expansion and define tissue integrity.

If most of the mechanism of plant regulation by these acidic substances is proven to be associated with cell wall deposition, then it becomes obvious that the science of auxinology is really in a very primitive state. Only the surface of the problem has been scratched, and the really great practical achievement must still be ahead of us. The great potential of the cell lies in the activities of the protoplast and especially in its nucleus rather than in the behavior of the cell membrane and its structural support in the wall.

Therefore, it is not out of order to propose that attention must be directed to creating chemicals that will penetrate the living cell and enter into the vital processes of the natural cell-regulating system. A study of the analogues and homologues of nucleic acid components and those materials that will alter the processes of protein synthesis and activation should in due time provide fruitful leads to new plant regulants.

Unfortunately this is a complicated area of cell function to attack. However, with the sweeping progress being made today in protein chemistry and the understanding of DNA and RNA synthesis it is not too much to expect that before long we will see chemicals that will generate a directed cytoplasmic structure that will regulate vital processes of metabolism on a more restricted self-sustaining basis. The closest analogy to this material known today is the plant viruses. At least one of these (breaking of tulips) has been associated with production of a desirable horticultural property.

THE DESIGN OF A PLANT REGULATING MOLECULE

Unfortunately, the golden era of the 1940's and 1950's in practical achievements of horticulture has not been matched by comparable progress in understanding the underlying principles. Too many people have developed pet theories without having first turned to the living cell to seek proper orientation of their ideas. The secret lies inside the living cell, and it will be unlocked only by careful analysis of the cell constituents on a quantitative basis or by tracing the metabolic fate of the plant growth incitant. Fortunately, with the wonderful new tools that are coming into use for tracing minute quantities of chemicals and unravelling local changes in metabolism of a few cells, perhaps this neglected area will be investigated more fully, as it should be.

There has been much written about the design requirements of a plant regulating molecule. These specifications have been modified from time to time — almost every time a new group of compounds has been introduced. Unfortunately, the theories have done very little to promote progress because they failed to analyze completely the various factors involved. Any change in chemical structure must be analyzed completely from the viewpoint of five effects on the chemical and physical attributes of the molecule; namely, selective solubility involved in cell permeation, translocatability, reactivity with specific cell metabolites, detoxication by extraneous reactions or physical or chemical binding to nonvital cell constituents, and type of degradation products formed during its metabolism by the cell.

Very rarely have plant physiologists stopped, for example, to consider the effect of lengthening a carbon chain or adding a parachlorophenyl group on lipide solubility. There are ample data on the effects of such changes in fungicides and bactericides on the partition coefficients between lipides and aqueous components of a mixed system. Undoubtedly any such change is going to affect the rate and completeness with which a molecule can penetrate cuticularized barriers from an aqueous spray dispersion and pass from the interstitial spaces in the cell wall through the lipoprotein barrier of the cell membrane. However, if its lipophylic-hydrophilic balance is not very carefully adjusted, it cannot pass from the lipide phase of the cell membrane into the aqueous substratum of the cytoplasm and thence to the site of enzyme activity, possibly by contact with the lipide phase of mitochondria. It is obvious that the first consideration in evaluation of any change in chemical structure on growth-regulant activity must be upon the simple physical attribute of selective solubilities in a complex medium of various lipides and aqueous solutions.

The potential capacity of the molecule to disperse through tissues

and enter into the translocation streams becomes a major consideration. The very fact that so many vital processes of the living plant are concentrated in the apical meristem, the phloem parenchyma, and the root system which are relatively well protected from direct exposure to chemicals applied to plant surfaces is warning enough that this factor must be considered. There is very substantial evidence that the effective plant regulants do move readily in plant tissues and very often in the general direction of food translocation.

To this extent they resemble the viruses that move strongly toward and into the roots of perennials in late summer or fall and upward into the growing shoots and expanding buds in the spring. The general rule for viruses, such as in the yellows disease of beets, is that they move from the areas of ample food reserves to deficient areas where assimilation into tissues is proceeding most rapidly. It has been shown that translocation of foreign bodies into and through the phloem can be accelerated along with sugar by addition of boron.

The changes in chemical reactivity attendant to changes in chemical structure have never been properly assayed because it is not clear what vital processes are changed by growth regulants. The materials are reactive and hence undoubtedly affect many enzyme systems. The great problem in perfecting better herbicides, growth stimulants, and retardants is to find methods of accentuating specific reactivities without promoting the indiscriminate reactions that are meaningless to cell regulation but exhaust and detoxify the regulant chemical before it can reach the proper site of activity.

Probably no area of research in the entire pesticide and plant regulant field is more deficient than this one of the proper site of action for a molecule. There is no easy road to its solution because of the terrifically dynamic processes of the functioning and growing cell. However, the experimental approaches must take one of three directions. The physiologist must do everything possible to measure the quantitative changes in cell activity in various tissues of the plant and to determine the change in biochemical activities to see where metabolic processes are accentuated or blocked. This will lead to many blind or false alleys, but eventually a pathway should be uncovered that has real fundamental significance. The second approach is to label or otherwise find the means of tracing the molecule of interest as it reacts with cell components or is metabolized into degradation products. Already great progress is being made in this direction. It is somewhat like looking for a needle in a haystack to follow the course of a few millions or thousands of millions of molecules through their various activities in the complex medium of the living cell but it must be done by one means or another.

The third direction is to study the kinetics of the reaction of var-

ious homologues and analogues in a class of active compounds with representative cell constituents in vitro. By defining types of reactions that molecules may be expected to enter into under specific conditions, a suggestion may be revealed as to what the investigator should be looking for in the living cell. This approach to the study of fungicides by Dr. Burchfield and Miss Storrs at Boyce Thompson Institute is revealing new analytical concepts. For example, one idea is that there probably are microenvironments within each cell which may very well determine whether the protein moiety of an enzyme may react through the amino or sulfhydryl group with an alkylating agent.

It is only by prying into the living cell that the real answers to growth-regulant processes of a molecule can be determined reliably. Theories and reasoning from analogy are scarcely worth printing until the treated living cell has been examined to verify the new idea. A minor change in chemical structure in synthesizing a new member of a class of regulants modifies so many attributes simultaneously that chances for error in reasoning are tremendous. Compound this error by exposure to the multitudinous environmental factors in the cell and one becomes most humble in recognition of his own ignorance.

To be blunt, there is no easy road open to determining the nature of the relationship of chemical structure to activity. It is among one of the more complicated mental exercises of modern science, and more biologists should accept the situation in humility and determination to obtain many divergent lines of evidence before being too positive in any one theory.

THE GREAT NEED FOR BETTER PLANT REGULATION

The opportunities to serve men better through science probably are greater in this field than in almost any area of scientific endeavor. We work to improve the material comforts of man, stabilize his society, and permit the continued growth and development of his economy in a world where the physical resources for plant culture are drastically limiting. The things that can be done, and will be done, in this area are tremendous. Is there any harm to dream of what we may do in the years ahead?

If we could free certain valuable crop plants from their dependence upon rigid photoperiod requirements, the barrier of geographical latitude might be broken so many unused or poorly used areas could be sown to much needed crops. Furthermore, there are many areas where two seed crops might be grown in place of one each year

if day length were not a dominating consideration. If we could but suppress the power of reproductive processes in plants, the useful life of forage crops and leafy vegetables could be prolonged by delaying senescence. The concentration of nutritious, palatable materials in the foliage and stems could be increased, and the yield of harvestable material could be multiplied by prolonged growth.

The day men learn to regulate physiological activity of plants to match the ecological situation of that season will mark the first practical great break-through in the age-old conquest of environment. There is no reason why drought resistance and winter hardiness, for example, should not be modified by chemical treatment so as to meet unusual local conditions and to expand plant culture out toward the desert, up the mountain, and over the tundra. Such regulation by chemicals should be more serviceable than the genetic modification that we now depend upon. No one has been able to predict all the fluctuations of environment or safeguard against them by breeding or selecting resistant sorts of crops. What is needed are treatments that can be applied to meet each season's development and whose intensity can be multiplied to match the fury or deficiency of the weather elements. This will never be done adequately by genetics alone but it could be done by combining the best in breeding and chemical treatment.

The fight against crop pests has been grievously handicapped by modern genetics, standardized cultural practices, and repetitive intensive cultivation of crops in selected areas where they will thrive. The complete standardization of genetical composition and cultural conditions has given the parasitic fungi, bacteria, nematodes, insects, and viruses a field day at our expense. The pests have become perfectly adjusted to destroying every plant in a field each year, provided the weather does not hamper them. We cannot abandon the agronomic practices that have encouraged this situation because they are necessary for great efficiency in crop production. Neither can we tolerate the loss of 21 per cent of all crop productivity such as occurs in the United States now, in 1960.

What is more important, we cannot afford, in a highly organized civilized society where factors of production are so delicately balanced with human need, to risk the possibility of a widespread outbreak of any major pest. For example, in the battle against black stem rust of wheat, there has been periodic development of new parasitic races that attack the prevalent disease resistant varieties. After half a century of breeding for disease resistance we are forced to conclude that we merely go from one crisis to another. In the past 50 years the

wheat crop was reasonably protected only 20 years according to no less authority than Stakman and Harrar (*Plant Pathology,* Ronald Press, N. Y. 1957, page 507).

The use of fungicides to meet these recurring crises comes to mind at once, but keeping all the wheat fields covered with a protective fungicide throughout the season is economically unsound. The real need is for a growth regulant that would alter the innate susceptibility of a plant for 30 or even 10 days. Such material could be applied when the need for it was apparent, and only then. If the activity of a single gene will impart immunity to a specific race of rust fungus, is it too much to expect someone to find a chemical with equal resistance-regulation capacity?

Looking toward other outlets for research in plant regulation, we become aware that the quality of plant products could be improved tremendously. For too many years the plant scientist has been overly concerned with quantity of produce rather than its quality. Most botanists are fully aware that by use of hybridization the average yield of maize was increased about 90 per cent in the United States within two decades. However, the yield of protein per acre held almost constant. The achievement was primarily in production of starch. There is no reason why chemical controls should not be available to increase nitrogen assimilation in proteinaceous crops, balancing of sucrose and other sugars with organic acids in fruits, or storage of starch or sugar in root crops.

There is little need to dwell on these and other areas of potential service to agriculture. Great opportunities lie ahead if we can only develop sound concepts as to how cells function and grow.

If we are to regulate plant growth in specific directions as indicated here, we must have a clear concept of what constitutes plant growth. In the broad sense it is the sum total of all cell activities that lead to normal expansion, differentiation, and multiplication of cells so that they may be incorporated into new, functional tissues and organs. Growth, therefore, starts in the processes of cell division, progresses with cell enlargement, and culminates in cell differentiation.

The course and rapidity of any one of these stages of growth will be determined by the balance existing between cell constituents. These balances are both chemical and physical; for example, hydrostatic pressure vs. strength of cell wall, pectic substances vs. lignin deposition, food reserve vs. water supply, etc. It would be unwise to believe that any one chemical that we ordinary mortals might synthesize will be all-powerful in determining the course of cell multiplication, enlargment, or differentiation. The most it can do is to

change the delicately adjusted balance that exists between thousands of components in the cell.

Growth regulation, therefore, becomes a matter of modifying the balance between components of the cell. It is probably misleading to expect one massive disruptive force such as the blocking of a single enzyme system to regulate rate of flowering, abscission, stem elongation, food conversion, etc. into specific desired channels. Many chemicals with many regulatory effects must operate in cells to keep this balanced system functional. It is our purpose to locate these multitudinous natural coordinators of cell functions and gently re-enforce or retard their activities with specifically designed molecules.

WILLIAM J. ROBBINS
The New York Botanical Garden

The Expanding Concepts of Plant Growth Regulation

The Origin of Species by Means of Natural Selection or the Preservation of Favoured Races in the Struggle for Life, written by Charles Darwin, was published in 1859 — one hundred years ago. The centennial observances attest to the stature Darwin has attained.

Charles Darwin was a botanist and, had he never written the Origin of Species, would be remembered today as one of the major botanical scientists of the nineteenth century. In fact, Charles Darwin's investigations of the sensitiveness of plants to light and gravity may justly be considered, in many ways, to have laid the foundations for our knowledge of plant growth regulators.

His observations and conclusions were published in 1880 in a book entitled *The Power of Movement in Plants* (4). Chapter IX deals with the Sensitiveness of Plants to Light: Its Transmitted Effect. Chapter XI deals with Localized Sensitiveness to Gravitation and Its Transmitted Effect.

In a series of ingenious experiments Darwin explored the responses to light of the coleoptiles of seedlings of *Phalaris canariensis* and *Avena sativa,* which had been decapitated or had been covered with caps of tinfoil; gold beater's skin, either transparent or painted so as to be impermeable to light; pipes of very thin glass or quills — some blackened; bandages of tinfoil applied to various parts of the coleoptile; coats of India ink and other procedures. The results of his experiments led irresistibly to the conclusion that the stimulus of light was perceived by the tip of the coleoptile and transmitted to the base where movement occurred.

Darwin says, "From these several sets of experiments, including those with the glass tubes, and those where the tips were cut off, we may infer that the exclusion of light from the upper part of the coty-

ledons (coleoptiles) of *Phalaris* prevents the lower part, though fully exposed to a lateral light, from becoming curved.... We must, therefore, conclude that when seedlings are freely exposed to a lateral light some influence is transmitted from the upper to the lower part, causing the latter to bend.... These results seem to imply the presence of some matter in the upper part which is acted upon by light and which transmits its effects to the lower part."

He concluded also that stimuli were perceived by the root tips and transmitted to the adjoining upper part where the bending occurred. To quote from his own words: "In the case of the radicles of several, probably of all seedling plants, sensitiveness to gravitation is confined to the tip, which transmits an influence to the adjoining upper part, causing it to bend toward the center of the earth. That there is transmission of this kind was proved in an interesting manner when horizontally extended radicles of the bean were exposed to the attraction of gravity for 1 or 1½ hours and their tips were then amputated. Within this time no trace of curvature was exhibited and the radicles were now placed pointing vertically downwards; but an influence had already been transmitted from the tip to the adjoining part, for it soon became bent to one side, in the same manner as would have occurred had the radicle remained horizontal and been still acted on by geotropism.... To see anything of the above kind in the animal kingdom, we should have to suppose that an animal whilst lying down determined to rise up in some particular direction; and that after its head had been cut off, an impulse continued to travel very slowly along the nerves to the proper muscles; so that after several hours the headless animal rose up in the predetermined direction.... We believe that there is no structure in plants more wonderful, so far as its functions are concerned, than the tip of the radicle."

If you have not recently read this volume on *The Power of Movement in Plants,* and especially the two chapters on light and gravity, I recommend them to you. You will feel as though you yourself were participating in the experiments as you note how he covered soil around the seedlings with black paper to prevent upward reflection of light, records the number of plants in many of the experiments which reacted or failed to do so, and discusses alternate explanations for his results. For example, he says, "When the upper halves of the cotyledons of *Phalaris* and *Avena* were enclosed in little pipes of tinfoil or blackened glass. . . . the lower and unenclosed part did not bend when exposed to lateral light, and it occurred to us that this fact might be due not to the exclusion of the light from the upper part, but to some necessity of the bending gradually travelling down

the cotyledons, so that unless the upper part first became bent, the lower could not bend, however much it might be stimulated. It was necessary for our purposes to ascertain whether this notion was true, and it was proved false; for the lower halves of several cotyledons became bowed to the light, although their upper halves were enclosed in little glass tubes (not blackened) which prevented, as far as we could judge, their bending. Nevertheless, as the part within the tube might possibly bend very little, fine rigid rods or flat splinters of thin glass were cemented with shellac to one side of the upper part of 15 cotyledons; and in six cases they were in addition tied on with threads. They were thus forced to remain quite straight. The result was that the lower halves of all became bowed to the light, but generally not in so great a degree as the corresponding part of the free seedlings in the same pots, and this may be accounted for by some slight degree of injury having been caused by a considerable surface having been smeared with shellac."

But before you read Darwin's account of his experiments, may I suggest that you visit his home, Down House, near the tiny village of Downe, some 20 miles from London where he and his sons, Francis and George, did the experiments about which he writes so engagingly. There you can see the remains of the simple greenhouses in which his plants were grown, his study, still much as it was when he used it, and the Sand Walk where he daily took his constitutional and reviewed the experiments he and his sons had made, and planned new ones.

Darwin did not express an opinion on how the stimulus of light was transmitted from the tip to the base. He was inclined to consider changes in turgescence as responsible for the movement but says, "In what manner light, gravitation, etc., act on the cells is not known."

During the next 30 years investigators confirmed and extended the major observations and conclusions Darwin had made. The sensitiveness to light was defined in terms of light units, the sensitive area was more clearly limited, the effective wave lengths were studied, equipment for subjecting coleoptiles to light of known intensity and composition was devised, but the fundamental question — how was the stimulus transmitted from the tip to the coleoptile base — remained unanswered. Did light induce electric currents which, moving downward, caused unequal growth and bending? Did light induce changes in electric potential, changes in turgor, in permeability, in sap reactions which were propagated downward? Or did it cause the formation of growth inhibitors, destroy growth accelerators, influence polarity, modify the movement of food, or water, or act in some ill-defined way?

In 1910 Boysen Jensen (1) of Copenhagen reported some simple but most illuminating experiments performed in a truly Darwinian manner which clearly indicated that phototropism resulted from the movement of a water-soluble substance or substances from the illuminated tip. He made horizontal cuts about halfway through the coleoptile tip 3 or 4 mm. from the apex. In some he inserted a thin piece of mica or platinum and then illuminated the tip; others were left with open cuts. If the cut was on the illuminated side, bending toward the light occurred. If the cut was on the shaded side, there was no response. The conclusion drawn was that something which would not penetrate the platinum, mica, or a dry cut passed from the illuminated tip down the shaded side, causing lengthening of that side and the bending toward light. In addition to these experiments, Boysen Jensen performed another still more critical and decisive. He severed the tip completely, covered the decapitated base with gelatin, and then replaced the tip. When the tip of such a plant was illuminated, the plant bent toward the light. This proved that the effect of the stimulus was transmitted over a discontinuity.

It is worth noting that these experiments were performed, although Fitting (5) had reported 3 years earlier some similar experiments but with negative results, probably because the experimental plants were kept in too moist an atmosphere. Under such conditions the cut filled with exuded water through which the active material diffused.

Paál (11, 12), Stark (22), Stark and Drechsel (23), Purdy (14), Söding (21), Snow (20), Seubert (19), Boysen Jensen and Nielsen (2), and others confirmed and extended these significant observations.

Although Boysen Jensen's experiments of 1910 now seem so convincing, not everyone was willing to accept them and the interpretations placed upon them. Brauner (3) considered the process of bending in response to light to involve: (1) increase in permeability and increase of growth inhibitors on the illuminated side, (2) movement of a growth inhibitor down the lighted side to the growth zones, (3) inhibition of growth on the illuminated side, (4) bending toward the lighted side.

Priestley (13) said, "It may be permissible to point out what a pyramid of conceptions are struggling to maintain themselves upon one general experimental fact — the phototropic response of a coleoptile stump when its severed apex is replaced and alone laterally illuminated." Priestley then points out the frequency of the exudation of drops of water from coleoptile tips (guttation). He assumed the permeability of the apical tissues of the coleoptile to be increased by light and, therefore, light falling on the apex to increase apical guttation. Lateral light increased guttation on the lighted side, decreased the

turgor, and caused in his opinion the bending toward light. The results of the decapitation experiments he considered explainable on the basis that decapitation opened the veins and increased water loss. It is a little difficult to understand how so able a physiologist as Priestley could have read the results of Darwin, Boysen Jensen, and those who followed, and then could propose an explanation for the phototropism of coleoptiles which so obviously was inadequate and in error.

The final and indisputable proof of the existence of potent growth regulators for plants was given by F. W. Went (25), son of the botanist F. A. F. C. Went, whose laboratories in Utrecht had for many years been concerned with a careful and extended study of phototropism. Went demonstrated that active material would diffuse from a coleoptile tip into a block of gelatin which would then act as the tip itself did. From the diffusion rate he calculated the molecular weight of the compound to be in the vicinity of 376. It was thermostable and withstood drying. In addition to the final and convincing proof of the existence of a growth regulator, the great contribution made by Went was his method of quantitative determination of the growth regulator by using gelatin or agar blocks placed laterally on the decapitated base of oat coleoptiles under controlled conditions.

A preliminary report of the experiments of Fritz Went was made by his father in a notable address on *Plant Movements* at the International Congress of Plant Sciences at Ithaca, New York, in 1926 (24). I well remember hearing rumors that evidence would be presented for a thermostable, water-soluble substance involved in phototropism and the skepticism freely expressed by many of my colleagues.

However, from this time on, a widening circle of investigators busied themselves with the problem. The discovery of a wide range of synthetic compounds which have effects similar to those of the naturally occurring auxins has engaged the attention of many investigators, among others those at the Boyce Thompson Institute. The application of the auxins and similar compounds to the induction of root formation, weed control, prevention of preharvest drop of apples and other fruits, increasing fruit set, inducing seedless fruit formation, thinning of fruit, regulating flowering, increasing fruit size, and hastening ripening has become increasingly important.

Although the practical applications of the auxins are not to be underestimated, the great importance of their discovery was to give impetus to the concept that minute amounts of naturally occurring organic substances could profoundly influence plant growth and development.

One of the few comforts of reaching what some charitably refer

to as the more mature years is the privilege it gives of looking back and seeing the changes and advances which have occurred in a lifetime. Plant physiology in my graduate student years, more than 40 years ago, consisted mainly of a consideration of mineral nutrition, transpiration, water requirements, osmosis and osmotic pressure, photosynthesis, nitrogen relations, especially nitrogen fixation, respiration, toxicity, antagonism, and balanced solutions, conduction of water and translocation of organic food, and similar fundamental processes. Any phenomenon not readily explainable on some other basis was assigned to that universal solvent of all problems, changes in permeability. That naturally occurring specific organic substances in minute amounts might materially influence the growth and development of a plant had little evidence to support it and received scant attention from teachers, students, or investigators.

It is true that in 1858–1860 Pasteur had observed that the development of yeast and of lactic acid bacteria in a synthetic medium was favored by the addition of small amounts of natural products. Sachs (17) had proposed the concept but not the term hormone. Wildiers (26) reported that minute amounts of Bios, a concentrate of unknown composition, was of great importance for the growth of some races of yeast. Ludwig Jost, in his Plant Physiology (7) was quite prepared to account for the formation of insect galls by the action of some definite substance which diffuses out from the larva and stimulates the cells to hypertrophy. Loeb (8) had suggested that hormones produced by the leaf of *Bryophyllum* played a role in the formation of new plants.

But these reports and others like them could be and were explained on some other basis than the action of specific compounds. It required something as decisive and dramatic as the auxins and their effects to change the viewpoint of plant physiologists interested in the problems of growth and development.

For some years the auxins were the only growth regulators to which botanists devoted much serious attention. But new knowledge of the existence of vitamins, hormones, and similar substances, their chemistry and their action in animal physiology, together with the discovery that there were some problems in the development of plants not solved by auxins alone, led to a substantial expansion of our concepts of plant growth regulators.

Schopfer's discovery (18) that *Phycomyces blakesleeanus* required for growth an external supply of small amounts of thiamin and investigations on the growth requirements of yeast led to the inclusion of the vitamins, the purine and pyrimidine bases, and specific amino acids among the growth regulators. Kinetin (9) and the gibberellins have been added more recently.

We have become convinced that growth regulators are involved in the flowering of plants even though their chemical nature still eludes us. The sex hormones of the water mold *Achlya* first reported by Raper (15) must also be admitted to the growing list of plant growth regulators, together with the antheridium-inducing factor from fern prothallia recently investigated by Näf (10). I would include also among the plant growth regulators, acrasin, the autogenic chemical substance involved in the organization of some of the slime molds (16).

To refer to all the plant growth regulators, that is, all the specific chemical substances which in minute quantities affect, even determine, the rate and pattern of plant growth and also those for which there is convincing evidence, though they have not yet been isolated and identified, would make a long list indeed and one which is increasing rapidly. This is not the place to undertake such a review.

How delicate the dynamic equilibrium of the metabolic systems of a living organism is. What profound effects on the rate, amount and character of growth can be produced by minute amounts of a specific organic compound. A few molecules of vitamin B_{12} determine whether *Euglena* will grow and how fast. *p*-Chlorophenoxyacetic acid causes the apex of *Kalanchöe* to develop into a spathe-like organ which can be cut off and rooted. The *ortho-* and *meta*-chlorophenoxyacetic acids are inactive (Zimmerman and Hitchcock, 27).

At the same time, how stable the systems are: like the treasures in a safe-deposit vault the course of metabolism which results in normal growth and normal form is protected against all the agents which impinge upon it, except for those which have the right configuration, which are the keys capable of unlocking the vault door.

This is the area which concerned Percy W. Zimmerman most. It is true that his botanical interests were broad. He developed hardy strains of *Camellia japonica* and of the American and English holly. He was a pioneer in the study of the effects of air pollution on plants, and because of long experience and extensive researches he was recognized as an authority in this field. But his great and abiding interest was the search for compounds which modified the normal growth pattern of a plant.

Dr. Zimmerman was not a locksmith. He cared less for the mechanism of the lock and how the key turned the tumblers than he did for the key and the treasures in the vault. What keys he found, and what treasures were revealed!

In cooperation with his close associate, Dr. A. E. Hitchcock, he investigated the properties of more than 500 compounds as growth regulators. β-Indole-3-butyric acid, 1-naphthaleneacetic acid, substi-

tuted phenoxy acids, including 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and chloro-substituted benzoic acids were some of those first tested in his laboratories.

However, Percy Zimmerman was not a narrow botanical and horticultural specialist. He served wherever his service would benefit his friends and neighbors and actively participated in a wide variety of community activities (6).

We have come a long way since Darwin at his country home outside London satisfied, so far as he was able, his curiosity about the response to light of the coleoptiles of *Phalaris* and *Avena*. He had the aid of tinfoil, India ink, flat splinters of glass, thread, and similar pieces of "complex apparatus," but above all, a clear, inquiring, and logical mind, and the serenity of a peaceful household managed by a devoted wife. A long succession of able and dedicated investigators, among whom we include Percy W. Zimmerman, have answered some of the questions Darwin's investigations raised and carried on into areas of which Darwin never dreamed. Not the least of these areas is that of the effects of minute quantities of specific organic compounds — call them auxins, vitamins, hormones, growth regulators, or what you will — on the growth and development of plants. Investigation of these substances promises to be for some time to come one of the most important fields in plant physiology. To those who have and are contributing to this subject, we might well say in the words of Swinburne:

Thy works and mine are ripples on the sea.
Take heart, I say; we know not their end.

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Naturally Occurring Plant Growth Substances

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Some Investigations on Interconvertible Naturally Occurring Auxins

Modern techniques in plant hormone analysis, in particular the use of paper chromatography for the purification of extracts, followed by bioassay, have revealed a number of as yet chemically unidentified auxins in various plant species. This paper is concerned with one particular group, which has come into prominence recently and about which very little is known — that is, the group of so-called interconvertible auxins. A group of three interconvertible auxins was first reported in the ether-insoluble fraction of tomato roots (3), followed by a report of similar substances in pea roots (1). The present investigation deals with a group of interconvertible auxins located in various species of algae. The algae were chosen as a field of investigation for two reasons. Firstly, there is an ecological problem; there is considerable evidence that the growth and distribution of both marine and freshwater algae may be governed, at least in part, by minute traces of metabolites in their aqueous environment; these metabolites may range from toxins to vitamins and hormones (8, 9). It is of interest to investigate whether the algae do in fact produce hormones of the auxin type and excrete them to the media in which they are growing. Secondly, the single-celled algae are free from the problems of differentiation encountered in the higher plants. Since the auxins affect fundamental aspects of growth, for example cell elongation and cell division, their primary effect appears to be on the individual cell, and it is therefore of interest to investigate their occurrence in single-celled organisms.

MATERIALS

The following materials have been examined. I am grateful to the various people, indicated in the text, who generously supplied material.

1. *Oscillatoria* spp. (Cyanophyceae) from Dr. A. J. Brook, Freshwater Fisheries Laboratory, Pitlochry. A sample of approximately 150 g. fresh weight was collected from a small Scottish lake, where it occurs as a persistent bloom and as practically a unialgal culture.

2. *Chlorella pyrenoidosa* (Chlorophyceae). 118 g. fresh material was received frozen from Dr. G. E. Fogg, University College, London. This was an 11-day-old culture and was extracted immediately.

3. *Ochromonas malhamensis* (Chrysophyceae). Two samples, consisting of 3.3 and 2.1 g., respectively, of freeze-dried cells, were received from Dr. J. E. Ford, National Institute for Research in Dairying, Reading.

EXPERIMENTAL TECHNIQUES

Extraction

1. *Oscillatoria* spp. The material was frozen (—30° C.), then thawed, acidified to pH 5.0, and extracted with ether. The dried ether extract was partitioned between 95 per cent methanol and petroleum ether (40° to 60° C.), and the petrol fraction rejected. The methanol fraction was separated into acidic and neutral components.

2. *Chlorella pyrenoidosa* and *Ochromonas malhamensis*. The material was extracted at pH 4.0 by stirring with 70 per cent aqueous ethanol. After neutralization, the alcohol was distilled in vacuo and the re-acidified residue extracted with petroleum ether (40° to 60° C.) and then ether. The ether extract was separated into acid and neutral fractions. The aqueous residues were saponified with *N* NaOH (15 min. at 15 lb. pressure), extracted with ether and the ether extract separated into acid and neutral fractions.

Chromatography

Extracts were usually purified by preliminary paper chromatography using water as eluant; the pigments remained on the starting line, which was rejected. The remainder of the paper was flushed with ethanol and the recovered material chromatographed usually in isopropanol-ammonia-water (10:1:1) or water. Chromatograms were examined under filtered ultraviolet light (2537Å) and used either for color tests [usually Ehrlich, Salkowski and nitrous-nitric acid reagents, using the techniques of Jepson (6)] or for bioassay. Chromatograms to be bioassayed were cut into ten equal portions and the portions eluted with water for testing.

Assay Technique

The *Avena* straight-growth method was used (2), with some modifications which give greater sensitivity. It is possible to use 10 sec-

tions (10 mm.) with only 1 ml. of test solution in 3.5 cm. petri dishes, instead of 10 ml. in 5 cm. petri dishes, as used previously. Under these conditions, best growth is obtained with the addition of 1 per cent sucrose and phosphate-citrate buffer (10^{-2} *M* K_2HPO_4 and 0.5 $\times$ 10^{-2} *M* citric acid at pH 5.0. With these modifications, the sensitivity of the test can be increased 10 to 100-fold over that previously obtained.

RESULTS

Oscillatoria spp.

Chromatography and bioassay of the acidic ether fraction showed clear-cut activity in two zones, which have been called zones X and Z (Figure 1A). These zones are still clearly defined even on diluting the extracts 10 times. There is a spot giving a purple Ehrlich, pink Salkowski, and pink nitrous-nitric acid reaction in zone Z. When zone X is eluted and rechromatographed in either alkaline or neutral conditions [*n*-butanol-ethanol-1.5 *N* NH_3 (6:2:2) or water, Figures 1B and 1C, respectively], zone Z appears in addition to X. There is also evidence of an intermediate zone (Y) in Figure 1B. This nomenclature of the zones is considered further in the discussion. Similarly, when Z is eluted and rechromatographed in isopropanol-ammonia, X appears (Figure 1D). Thus, a mutual interconvertibility between X and Z can be demonstrated, similar to the interconvertibility demonstrated in tomato roots (3) and pea roots (1). It should be noted that the conversion of one zone to the other is never complete — both zones can be picked up on the paper and there appears to be an equilibrium between them. Zone Y is more transient in the algae and not so easily demonstrated.

Chlorella pyrenoidosa

All fractions received preliminary purification by chromatography in water, and rejection of the pigment zone.

Color tests of the acidic ether fraction chromatographed in isopropanol-ammonia gave a spot with unusual color reactions, a rapid dark-blue Ehrlich reaction and faint yellow nitrous-nitric acid reaction, at R_f approximately 0.8. This spot, which is also found in corresponding fractions of dried *Chlorella,* is referred to in the discussion as *Chlorella* 5. Solutions from this zone rechromatographed after bioassay gave a faint pink Ehrlich reaction in the same region.

Bioassay of the acidic ether fraction after chromatography in isopropanol-ammonia showed that there was activity in this region and also in the IAA region (Figure 2A). Rechromatography of zone Z in

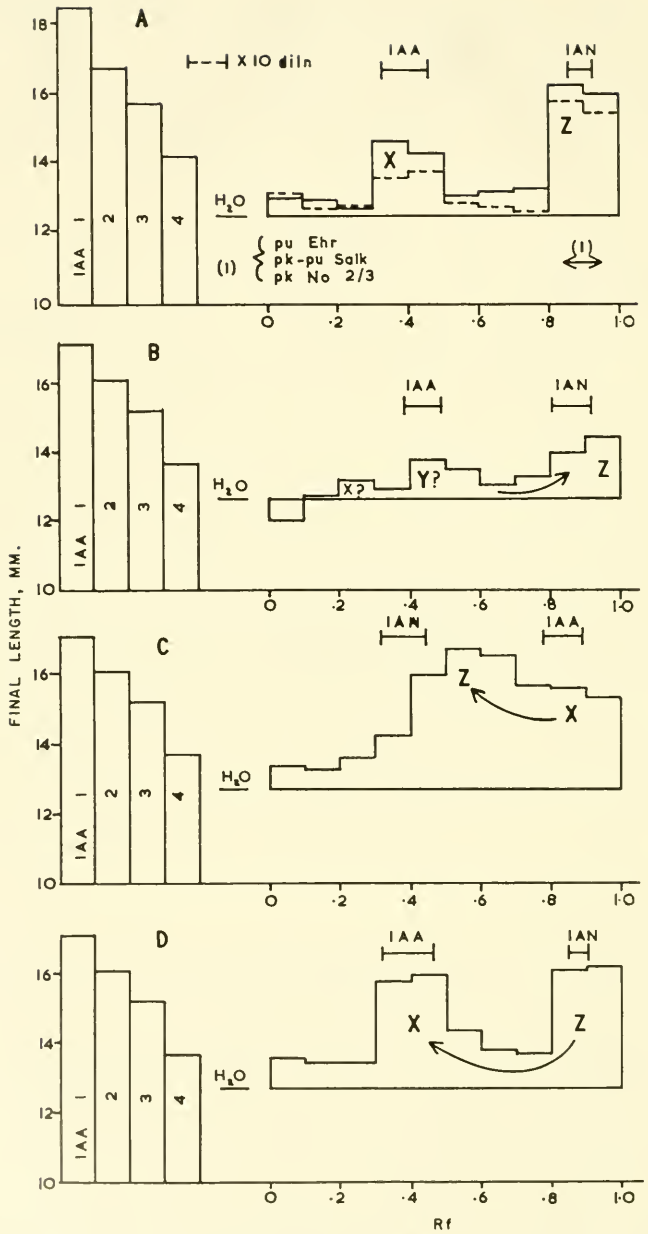

Fig. 1. Acidic ether fraction of *Oscillatoria* spp. **A.** Chromatographed in isopropanol-ammonia: extract = 10 g. fresh wt. of original material. **B.** Zone R_f 0.3 to 0.5 of 1A eluted and rechromatographed in *n*-butanol-ethanol-1.5 *N* NH_3 (6:2:2). **C.** Zone R_f 0.3 to 0.5 of 1A eluted and rechromatographed in water. **D.** Zone R_f 0.8 to 1.0 of 1A eluted and rechromatographed in isopropanol-ammonia. Controls of IAA are 1 = 5×10^{-6} g/ml and successive $\times$ 10 dilutions.

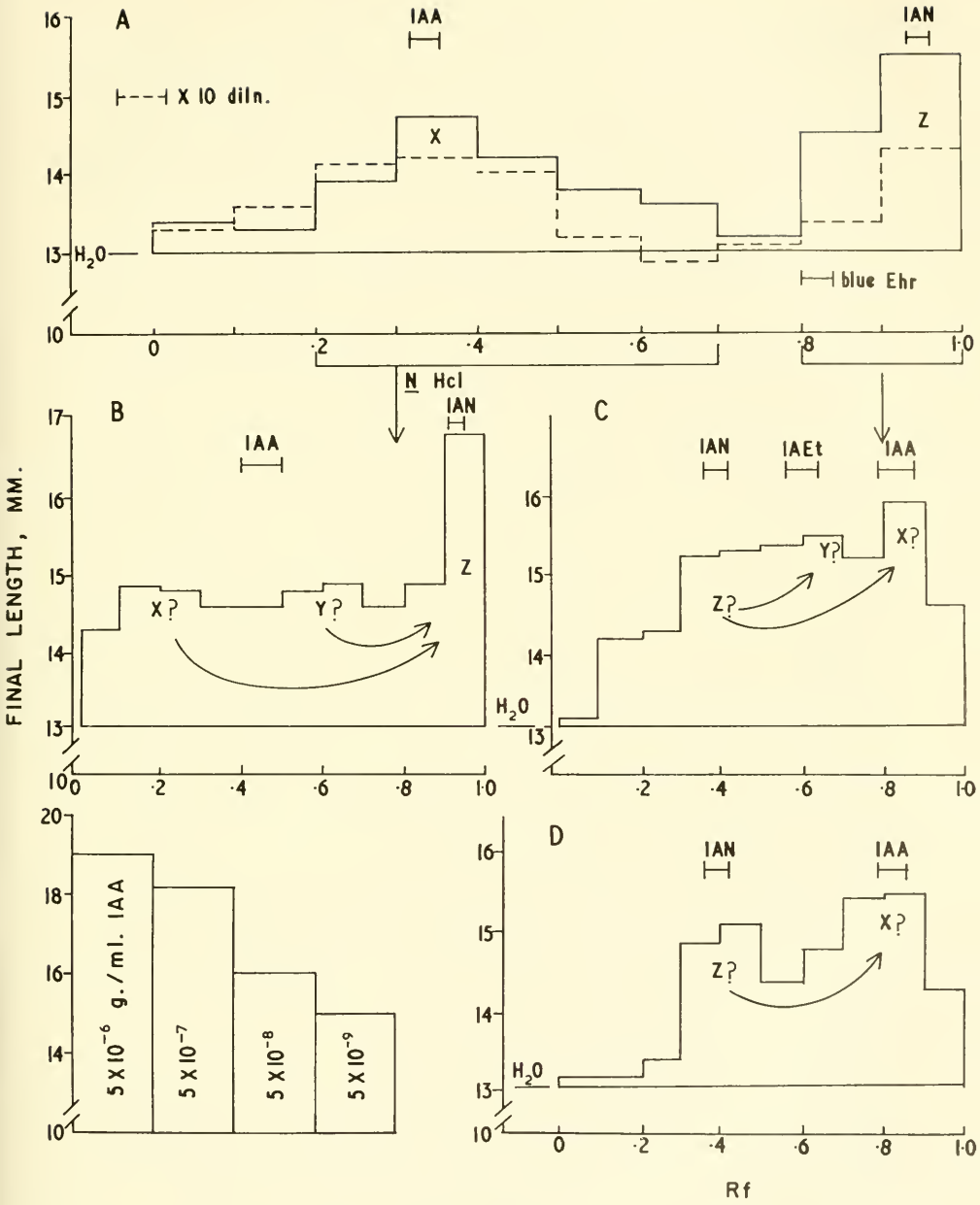

Fig. 2. Acidic ether fraction of *Chlorella pyrenoidosa*. **A.** Chromatographed in isopropanol-ammonia; extract = 7.5 g. fresh wt. of original material. **B.** Zone R_f 0.2 to 0.7 of 2A treated with *N* HCl, extracted with ether and rechromatographed in ammoniacal isopropanol. **C.** Zone R_f 0.8 to 1.0 of 2A eluted and rechromatographed in water. **D.** Zone R_f 0.8 to 1.0 of 2A rechromatographed in water after bioassay. Controls of IAA are as in Figure 1.

water showed high activity, with the production of peaks which are possibly zones X and Y (Figure 2C).

Rechromatography of zone Z (from Figure 2A) in water after bioassay clearly showed two peaks of activity (Figure 2D). If it is assumed that X and Z behave similarly to IAA and IAN respectively on chromatography in the solvents used here, then the peak near the solvent front in Figures 2C and 2D will be X, released from Z, which is running at an R_f of approximately 0.4.

Treatment of zone X (Figure 2A) with *N* HCl and rechromatography in isopropanol-ammonia gave a large peak in zone Z (Figure 2B) and a hint of zone Y. Thus, a mutual interconversion of X and Z has again been demonstrated as in *Oscillatoria,* and X, Y, and Z appear to be stable to treatment with *N* HCl.

The possibility was considered that the active substances were not completely separating during chromatography, and that dips in the histograms between peaks of activity might be due to the presence of inhibitors, although true inhibition (below the level of the water controls) is rarely obtained. Frequently, however, bioassay of dilutions of the original solutions (*e.g.,* Figures 1A and 2A) show the same general pattern of distribution of activity. Inhibitory effects generally disappear very much more quickly on dilution than growth-promoting effects, and if an inhibitor had been present at R_f 0.6 to 0.8 in Figure 2A in sufficient quantities to cause a dip in the level of activity, one would expect that bioassay of tenfold dilutions would show over-all activity along the length of the paper from X to Z. This does not happen, and the original promoting peaks are still clearly defined. It is thus more than likely that peaks X and Z represent true peaks due to discrete substances. The over-all activity sometimes obtained on papers such as those illustrated in Figure 2B and Figure 2C is more likely to be due to the activation of precursors on the paper during the various operations to which it is subjected. Evidence is continually turning up that there are inactive precursors on the papers.

Ochromonas malhamensis

The petroleum ether fractions were inactive and were rejected. The acidic ether fractions of both samples contained active substances, which were concentrated in zone Z near the solvent front in the first sample (Figure 3A), but which separated into three well-defined zones in the second sample (Figures 3B and 3C, zones X, Y and Z).

Ochromonas 1 extracts were chromatographed directly in isopropanol-ammonia, but *Ochromonas* 2 fractions were first purified by line-loaded chromatography in water, because of the large amounts of pigments in the extracts. The base line portion where most of the

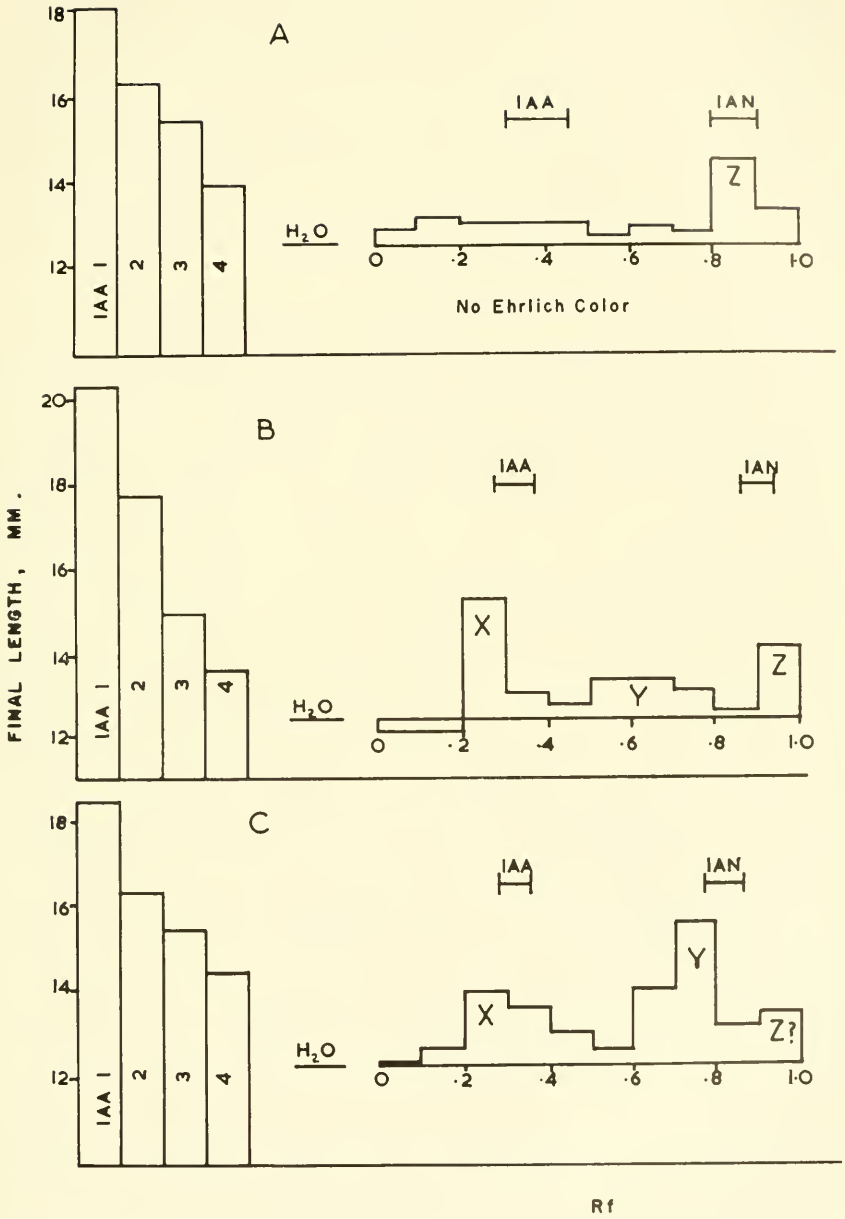

Fig. 3. Chromatography of acidic ether fractions of *Ochromonas malhamensis* in ammoniacal isopropanol. **A.** *Ochromonas* 1 (= 0.8 g. dry wt.) **B.** *Ochromonas* 2 (= 1.0 g. dry wt.) first eluate after chromatography in water. **C.** *Ochromonas* 2 second eluate after chromatography in water. Controls of IAA are 1 = 10^{-6} g/ml; 2 = 10^{-7}; 3 = 10^{-8}; 4 = 10^{-9}.

pigments remained was rejected. The rest of the chromatogram was eluted with ether or alcohol, the eluate evaporated to a small volume and then chromatographed. Figure 3B represents an extract chromatographed once in water, and Figure 3C represents a second washing of the same base line in water. It is evident that all the active substances are not removed in the first washing. This has been noted several times with heavily pigmented extracts. All activity is usually removed from the base line by two washings. The difference in treatments between *Ochromonas* 1 and 2 may explain why zones X and Y were not detected in *Ochromonas* 1.

Zone Z was eluted from chromatograms run at the same time as that of Figure 3A, and the eluates were rechromatographed in both ammoniacal isopropanol and water (Figures 4A and B, respectively). In both figures there is evidence that Z gives rise to another zone of activity, probably zone X. Separation is not good in chromatography in water, but if it is assumed that zone Z runs similarly to indole-3-acetonitrile (IAN), then Z will be the large zone in the center of the paper, and there is evidence of trailing to the solvent front, which may be the production of zone X running at approximately the same position as indole-3-acetic acid (IAA).

The interconvertible auxins located in tomato and pea roots were found in the ether-insoluble aqueous fraction, whereas the auxins in the algae occur in the ether fraction. The ether-insoluble aqueous fraction in the algae is very difficult to examine, as it is heavily pigmented, besides containing large quantities of carbohydrates, amino acids and other substances which interfere with the bioassays. Preliminary experiments on the aqueous fraction suggested that the ether-soluble auxins may be released to a limited extent from an unstable water-soluble complex by manipulative treatments during extraction. To speed up this process, the aqueous fractions were treated with alkali (*N* NaOH at 15 lb. pressure for 15 min.) and then extracted with ether. The resulting fractions are referred to as the saponified aqueous fractions. Under these conditions bioassay has shown that no ether-extractable auxins are obtained from tryptophan.

Chromatography of the saponified aqueous fraction of *Ochromonas* 1 gave a zone similar to zone Z (Figure 5A). On elution and rechromatography, this zone gave rise to a zone similar to zone X (Figures 5B and C). Chromatography of the saponified aqueous fraction of *Chlorella* and of *Ochromonas* 2 gave exactly similar results.

Thus, ether-soluble compounds similar in behavior to those occurring in the acidic ether fraction can be obtained from the aqueous ether-insoluble fraction after treatment with alkali.

It was thought that the interconvertible auxins were acidic, as

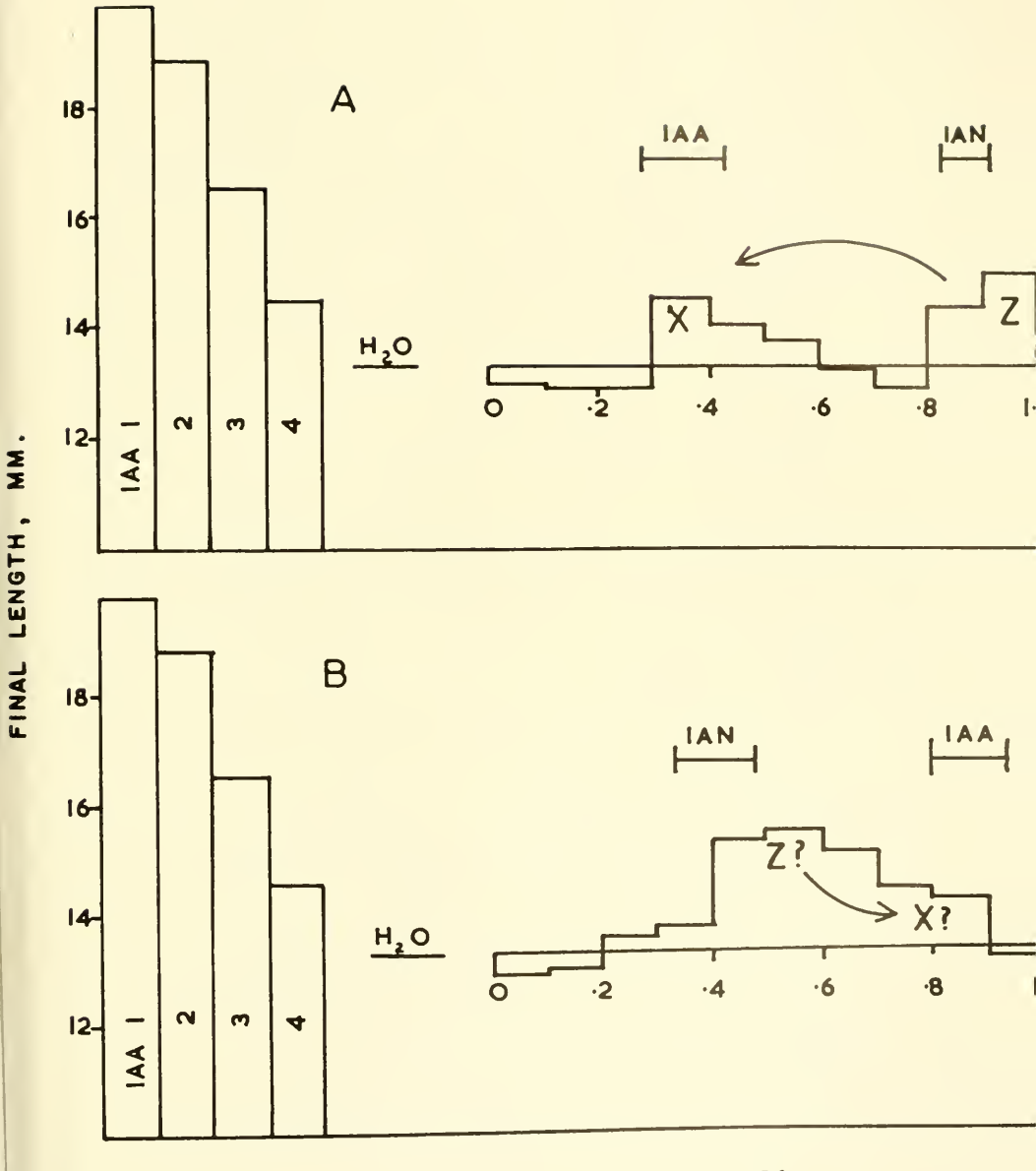

Fig. 4. **A.** Zone R_f 0.8 to 1.0 of 3A rechromatographed in isopropanol-ammonia. **B.** Zone R_f 0.8 to 1.0 of 3A rechromatographed in water. IAA controls as in Figure 3.

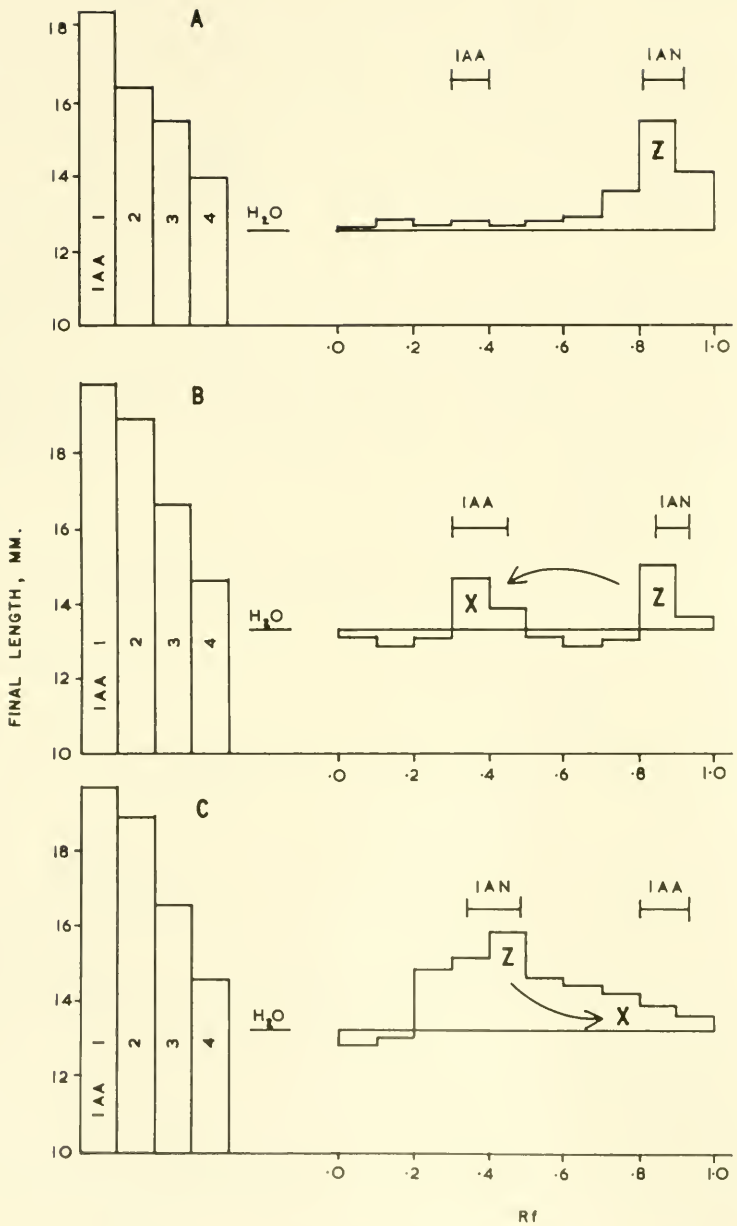

Fig. 5. A. Chromatography of saponified aqueous fraction of *Ochromonas* 1 (= 0.8 g. dry wt.) in isopropanol ammonia. B. Zone R_f 0.7 to 1.0 of 5A rechromatographed in isopropanol ammonia. C. Zone R_f 0.7 to 1.0 of 5A rechromatographed in water. IAA controls as in Figure 3.

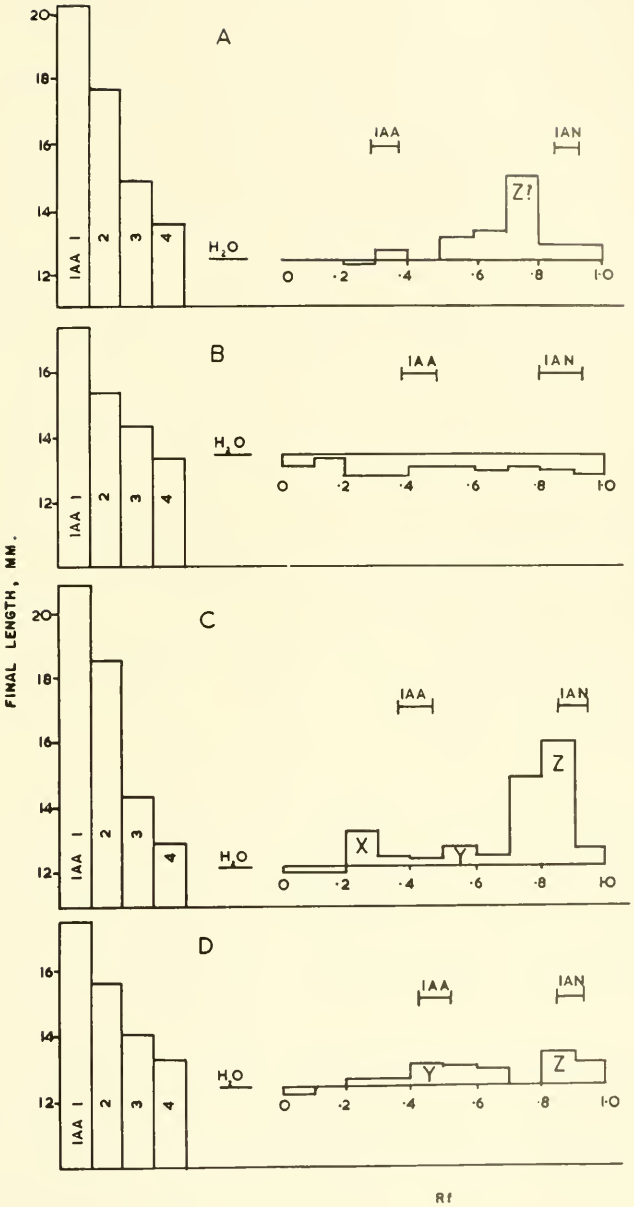

Fig. 6. Neutral fractions of *Ochromonas* 2 chromatographed in isopropanol-ammonia. **A.** Neutral ether fraction. **B.** Neutral saponified aqueous fraction. **C.** Zone R_f 0.8 to 1.0 of 6B rechromatographed. **D.** Zone R_f 0 to 0.8 of 6B rechromatographed. IAA controls as in Figure 3.

they occur largely in the acid fractions. There is, however, a growing body of evidence on the occurrence of neutral auxins [*e.g.*, Fukui *et al.* (4, 5), on a neutral auxin in immature corn kernels and in corn pollen]. The neutral ether and neutral saponified aqueous fractions of *Ochromonas* 1 were inactive when chromatographed in isopropanol-ammonia. The neutral ether fraction of *Ochromonas* 2, however, after preliminary chromatography in water, showed a well-marked zone of activity on chromatography in isopropanol-ammonia (Figure 6A). The neutral saponified aqueous fraction showed slight inhibition along the length of the paper (Figure 6B), but since it was thought that this might be due to something toxic, the chromatogram was eluted in two portions and rechromatographed (R_f 0.8 to 1.0 in Figure 6C; R_f 0 to 0.8 in Figure 6D). Surprisingly, considerable activity was located, again in zone Z, with hints of activity in zones X and Y. Either something inhibitory is preventing the activity from showing in Figure 6B, or else there are inactive precursors on the paper, which release the active compounds X, Y, and Z on further manipulation and chromatography of the extracts. The appearance of these compounds in the neutral fractions may be due to incomplete separation from the acidic fraction, or possibly a precursor of X, Y, and Z may be neutral or amphoteric. In any event, the active compounds occur in much smaller amounts in the neutral fractions than in the acid fraction. For example, no activity could be obtained from the original base lines on second chromatography in water, whereas in acid fractions it often can.

DISCUSSION

The foregoing results establish that a number of algae, covering several classes, contain biologically active substances with activity similar to the auxins of the higher plants. The main bulk of the activity is located in two zones, which have been called zones X and Z, although these zones do not necessarily represent the same substances in all the species examined. Zone X runs usually at an R_f of approximately 0.3 to 0.5, and Z at R_f approximately 0.9, in ammoniacal isopropanol. The activity in the ether extracts of saponified fractions indicates that there are water-soluble, ether-insoluble auxin precursors in the algae. These ether-insoluble compounds yield ether-soluble auxins on treatment with alkali. Under the conditions chosen, bioassay has shown that no ether-soluble auxins are produced from tryptophan. It is possible that the active compounds located in the saponified fractions are the same substances as the ether-soluble auxins present in the algae originally (the acidic ether fractions), as both sets show a similar mutual interconvertibility. It is possible that X and

Z are released to a limited extent from an unstable water-soluble complex by manipulative treatments during extraction, and so would be found in the acidic ether fraction without any alkaline treatment.

The interconvertibility of X and Z may be related to a similar phenomenon observed in extracts of tomato roots, in which three compounds, X, Y, and Z, were shown to be mutually interconvertible (3). The same phenomenon is also reported in extracts of pea roots (1). The behavior of the tomato X and Z on chromatograms corresponds to the behavior of the algal X and Z considered here. There is also evidence on several of the algal chromatograms of the existence of zone Y, though this is only transient. This may be because chromatography in the present work was usually in ammoniacal isopropanol. It was shown in work on tomato roots that X and Y may not be separated in this solvent mixture, whereas they are easily separated in ammoniacal butanol. Zone X in tomato, when separated from zone Y, usually ran behind IAA; this has been noted in the present work also.

If the tomato and pea auxins are the same substances as the algal hormones, we are obviously dealing with auxins of wide distribution in the plant kingdom and probably of importance in fundamental cell metabolism. It is clearly important that their chemical constitutions should be investigated further. The interconversions demonstrated between X, Y, and Z suggest that the compounds are probably closely related chemically.

The two zones of major activity (X and Z) on the chromatograms correspond to the positions of IAA and IAN on chromatography in ammoniacal isopropanol, so that it is obviously necessary to determine whether these two latter compounds are present. Zone X usually survives autoclaving with *N* HCl (*e.g.*, Figure 2B). IAA, which is popularly supposed to be destroyed by acid, is only partially decomposed under the conditions used here. However, IAA does not give activity in zone Z on rechromatography, as zone X does. This fact does not rule out the possibility that some IAA may be present in addition to another compound.

Zone Z contains one or more indole compounds, but probably not indole-3-acetonitrile, since it does not give the same color reactions as IAN. Z sometimes gives a pink or purple Ehrlich reaction, a pink Salkowski reaction, and pink nitrite-nitric acid reaction. Indole-3-acetonitrile gives a purple Ehrlich, blue-purple Salkowski, and fading blue nitrite-nitric acid reactions.

Chlorella 5, which occurs in the acidic ether fraction and sometimes the acidic saponified fraction of *Chlorella* and which runs at an R_f of 0.8 to 0.9 in ammoniacal isopropanol, gives markedly char-

acteristic color reactions, in particular a dark blue Ehrlich reaction. It has not been located in any of the other algae. A blue Ehrlich reaction usually denotes that there is substitution of an OH- or OR-group at position 5 of the indole nucleus.

Another interesting indole compound was located in *Chlorella*. This gives a purple Ehrlich reaction, a red Salkowski and orange nitrous-nitric acid reaction. It has an unusually low R_f of approximately 0.12 in ammoniacal isopropanol. It runs at R_f 0.5 in water, with some streaking, which suggests that it may be polyionic. It is not indole-3-acetylaspartic acid, which runs at R_f 0.83 in water, nor is it malonyltrytophan, which has an R_f of 0.95 in water, and gives a brown Salkowski reaction. Nor is it likely to be indole-3-pyruvic acid, which remains very close to the starting line on chromatography in water. From the Ehrlich reaction, it is unlikely to be a 5-hydroxy indole compound.

Some consideration was given to whether indole-3-acetamide occurred in zone Z, since this has been shown to be produced during ammoniacal chromatography of indole-3-pyruvic acid and other natural indoles (7). Indole-3-acetamide has only slight biological activity (about 1 per cent that of IAA), which is unlikely to account for all the activity located in zone Z. Also it runs at a slightly lower R_f than zone Z in ammoniacal isopropanol, and no activity is located in the IAA region (equivalent to zone X) on rechromatography.

It is noteworthy that there is no evidence of either the α-accelerator (close to the starting line) or the β-inhibitor (just ahead of IAA) in any of the algal extracts examined.

SUMMARY

Auxin activity has been located in extracts of the following—*Chlorella pyrenoidosa, Oscillatoria* spp., and *Ochromonas malhamensis*.

Two fractions of the extracts have been examined in detail, the acidic ether-soluble fraction, and the aqueous ether-insoluble fraction after saponification in alkali and extraction with ether. Two active zones, tentatively labeled X and Z, have been located in both fractions of all the species examined. These are not necessarily due to the same substances in both fractions but this appears likely, since X and Z in both fractions show a mutual interconvertibility. The interconvertibility suggests that the compounds may be closely related chemically. There is also evidence of activity in a zone (Y) intermediate between X and Z. The interconvertibility of zones X, Y, and Z has been noted previously in extracts of tomato roots, and a similar phenomenon is reported recently by workers using pea roots. These

compounds possibly all come from an ether-insoluble precursor which breaks down spontaneously during the extraction process, or more completely on treatment with alkali.

It is not clear whether these substances are indole compounds, although several indole substances are present in the extracts. The behavior of X and Z on chromatography and their R_f values in different solvent mixtures indicate that these are not any of the known indole hormones or indole complexes.

ACKNOWLEDGMENT

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DISCUSSION

Dr. Bennet-Clark: Do you always get both zones? This looks like an association phenomenon, or is a chemical equilibrium involved?

Dr. Bentley: You can always pick up both zones. You never get a complete conversion which suggests an equilibrium between the two. When extracts are kept in a deep freeze the two zones will still be obtained after a year or more, indicating stability under these conditions.

Dr. Kefford: I would like to make my comments rather generally in the field of natural auxins, and I do this because I feel that the technique that Prof. Bennet-Clark and I have pioneered and which we felt would be of great assistance in helping to clarify our thoughts on natural auxins has now become a curse. Not only has it revealed numerous substances in plant extracts which can produce a growth reaction, but it appears that the very act of chromatography itself can produce new compounds. It has become apparent that we can't avoid the very tedious task of having to isolate and identify the compounds that turn up on chromatograms. But since this is a tedious task, I think it is up to us to make some efforts to decide which are the physiologically interesting substances that appear on our chromatograms. It is becoming apparent that unless we are dealing with a reaction of rather rare promiscuity, not all these substances which are able to make *Avena* sections grow are acting at the same reaction site. So I would suggest that the first step in helping us to limit the field of substances which we must identify would be to know something about the primary site of action of these substances. And if we are going to be interested in compounds that are active in correlation phenomena, we must be concerned with those that can be transported through the plant. And here, at least in the auxin transport system, we have a reaction with, at last, a respectable chemical specificity. To date, in the published data there is only one contender for a natural substance that is transported, and that is indole-3-acetic acid. So I would suggest that maybe tests which tell us something about the transport of these substances would help us to limit the field.

Dr. Thimann: When you hydrolyze the aqueous fractions with alkali to get the auxins, do you get both X and Z, or do you only get X, or do you get indole-3-acetic acid only?

Dr. Bentley: No, you get substantially the same pictures as with the acidic ether extract. You get both X and Z and you can demonstrate this interchangeability. I wouldn't say categorically that they are the same compounds, but they do behave the same and show the same incompatibility. There's no more evidence for the presence of indole-3-acetic acid in the substance we obtained from the aqueous fraction, than from the ether fraction. On the subject of transport which Dr. Kefford raised, there are other people here more qualified than I am to speak on the gibberellins, but I believe that they don't show this polar transport. And if we have the gibberellins involved, then we have to think even further than just looking for polar transport, in our compound.

Dr. Fawcett: I'd just like to say that from some advice that Dr. Bennet-Clark gave a year or two ago we switched from using ether to ethyl acetate for the extraction of indole compounds. Although we ran into some problems with emulsions, we found that we got a much better and wider spectrum of the indole compounds. I wonder if Dr. Bentley had any experience in using ethyl acetate with the algae.

Dr. Bentley: No, I haven't used ethyl acetate and I couldn't say anything about it really.

Dr. Nitsch: May I ask Dr. Bentley a point of technique which is very important? Could you tell me if you leave the solvent in the tank and use it over and over again?

Dr. Bentley: No, we mostly renew it. We found that the percentage of ammonia changes.

Dr. Nitsch: Do you use fresh solvent every time?

Dr. Bentley: No, not necessarily every time.

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The Occurrence of Indole-3-acetaldehyde in Certain Plant Extracts

In 1939 (19) the senior writer detected a neutral growth substance in extracts of etiolated epicotyls of *Pisum sativum* and *Vicia faba* and in hypocotyls of *Helianthus*. Subsequent investigations of this substance (20, 21) led to the conclusion that it was identical with indole-3-acetaldehyde (IAAld). Since the active material was not isolated in the chemically pure state, its identification rested on indirect evidence. Similar evidence for the occurrence of IAAld in extracts of other plants was subsequently provided by Hemberg in 1947 (16), Gordon and Sanchez Nieva in 1949 (11, 12), Yamaki and Nakamura (31), and others. The literature concerning the occurrence and possible function of IAAld in plants has been reviewed by Larsen, 1951 (22), and Gordon, 1954 (8). Among the more recent studies of these problems may be mentioned the ones by Wiedow-Pätzold, 1955 (30), Gordon, 1956 (9, 10), and Clarke and Mann, 1957 (7).

The only other natural auxin that had been claimed to play a role in plant growth regulation was Kögl's auxin a lactone. As it became more and more unlikely that this compound (or auxin a or b) occurred in plants, it became customary to ascribe auxin activity found in nonacidic fractions of plant extracts to the action of IAAld. In 1952, however, IAAld was synthesized by Brown, Henbest, and Jones (6), and in the same year, indole-3-acetonitrile (IAN) was isolated from cabbage in the chemically pure state by Jones, Henbest, Smith, and Bentley (18). The biological activities of the two neutral synthetic auxins were studied by Bentley and Housley (4) and by Bentley and Bickle (3).

Two neutral auxins had now to be considered, and since the occurrence in plants of the IAAld still rested on indirect evidence only, it was natural for some workers to suspect that in the past the aldehyde

might sometimes have been confused with the nitrile (1, 2). In certain cases, however, the nitrile can be excluded as the active material in the neutral preparations, namely when the auxin activity is determined in the *Avena* coleoptile curvature test and found to be increased several-fold by conversion of the active material to an acid. The nitrile is already as active as indole-3-acetic acid (IAA) or even more so (3, 24, and our own data); preparations containing it cannot be made more active by any change in the nitrile molecule. Only a concomitant removal of a growth inhibitor could explain the increase in activity if the nitrile were the active, neutral material in such cases.

In the early work by the senior writer (20, 21), only extracts of etiolated pea epicotyls were thoroughly investigated, and the evidence for the occurrence of IAAld actually pertained only to *Pisum*. When the presence of a neutral growth substance in extracts of cabbage could be demonstrated (21), it was only by analogy that the identity of this with IAAld was inferred. No attempts were actually made to establish the aldehyde nature of this material. In light of the work of Bentley and her co-workers, it would now be highly desirable to reinvestigate the status of IAAld as an auxin. For several years we were discouraged from resuming the work on IAAld, however, primarily on account of the reported extreme lability of the compound, not to mention the fact that in spite of its synthesis in 1952 it remained inaccessible for research purposes. Recently, a number of findings have been reported which should be favorable for a successful attempt to demonstrate the presence of IAAld in plant extracts.

First, a simplified method for obtaining chemically pure IAAld was published by Gray in 1958–59 (13, 14) utilizing Langheld's method in treating tryptophan with sodium hypochlorite, but introducing the refinement of trapping the aldehyde in benzene before it would become destroyed in the reaction mixture. According to Gray, the aldehyde is not nearly as labile as had been believed; and its sodium hydrosulfite addition product proved to be stable for many years.

In the meantime, Nitsch and Nitsch, 1955 (28), and Nitsch, 1956 (27), had been searching for a chromatographic solvent system that would separate various neutral auxins on filter paper. As a result of their extensive investigations they recommended *n*-hexane or a practical grade hexane to be used in a water-saturated atmosphere. Such a system, which contains no acid or alkaline component and very little water, should be considerably less harmful to an unstable compound like IAAld than most of the systems previously preferred in chromatographic work on auxins. In addition, the hexane gives a much better separation of known neutral auxins.

Finally, a new, sensitive spray reagent for indole derivatives was described by Harley-Mason and Archer in 1958 (15). This reagent is *p*-dimethylaminocinnamaldehyde (DMCA), a vinyl compound corresponding to Ehrlich's reagent, *p*-dimethylaminobenzaldehyde (DMBA). DMCA, possessing an additional double bond, was expected to give more intensely colored condensation products with the indole compounds and is reported to be about ten times as sensitive as Ehrlich's reagent as a detector of indole and tryptophan.

Basing our procedures on the work of Gray, Nitsch, and Harley-Mason and Archer, we hoped to be able to clarify the situation with respect to the occurrence of IAAld and IAN in ether extracts of etiolated pea epicotyls and heads of cabbage.

MATERIAL AND METHODS

The compound, IAAld-$NaHSO_3$, was prepared from tryptophan and NaOCl, following exactly the directions given by Gray (14), except that all quantities of material were reduced to one-half (starting with 1.5 g. of tryptophan instead of 3 g., etc.). Free IAAld, IAA, and IAN were chromatographed (descending) on filter paper in the same system as was used by Gray, namely *n*-butanol saturated with 2.8 per cent aqueous ammonia. After 7 hrs. the paper was dried and sprayed with a 1 per cent solution of dimethylaminobenzaldehyde in 1 *N* HCl. IAAld and IAA gave practically the same R_f values as reported by Gray who used the ascending technique, namely 0.92 to 0.95 and 0.23 to 0.25, respectively (Gray: 0.93 and 0.28 to 0.29). IAN, not tested by Gray, ran at R_f 0.89. The color reaction of IAAld with DMBA is bluish purple, changing to brownish. Weller, Wittwer, and Sell (29), who obtained a sample of IAAld-$NaHSO_3$ from Gray, report that IAAld runs at R_f 0.88 in 1-butanol saturated with 5 per cent NH_4OH and in 1-propanol:concentrated NH_4OH:H_2O (60:30:10).

The free aldehyde was obtained by adding 4 ml. water and 1 ml. 0.5 *M* Na_2CO_3 to 5 ml. of an aqueous solution containing 0.5 or 1.0 mg. of IAAld-$NaHSO_3$. The alkaline solution was shaken three times with 8 ml. of peroxide-free ether; the ether, containing the aldehyde, was adjusted to a volume of 25 ml. It is not yet known whether all of the original IAAld is secured in the ether fraction by this procedure, so quantities of IAAld are given as relative values only in the following:

IAN. This compound was purchased from the Aldrich Chemical Company, 3747 N. Booth St., Milwaukee 12, Wisconsin. It was used without further purification.

p-Dimethylaminocinnamaldehyde (DMCA) (Aldrich, see above). The reagent was prepared as directed by Harley-Mason and Archer

(15) : 2 g. of DMCA were dissolved in 100 ml. 6 *N* HCl plus 100 ml. 96 per cent ethanol.

Plant Extracts

Extracts were made as described by Larsen (21).

Pisum. 15 to 50 g. samples of the upper 10 to 15 cm. portions of epicotyls of etiolated 7- to 9-day-old pea seedlings 'Alaska' were extracted at room temperature with 25 to 100 ml. portions of peroxide-free ether. The ether was renewed twice after 24 or 48 hr. periods. The 3 portions of ether were reduced in volume and combined. A suitable number of such extracts were combined, adjusted to a volume of about 12 ml., and shaken three times with 10 ml. H_2O + 1 ml. 8 per cent $NaHCO_3$. The aqueous, alkaline fraction was discarded. The ether fraction, now containing only the nonacidic materials, may be tested in the *Avena* curvature test, but contains fatty materials in quantities too high for a satisfactory application of concentrated droplets to the starting line of a paper chromatogram. Most of these fatty materials were removed by the following method. The ether was evaporated to dryness in a 50 ml. Erlenmeyer flask. The residue is a smooth layer, covering the bottom of the flask. This residue was dissolved in 2.5 ml. 96 per cent ethanol which was then evaporated off at about 45° C. Probably on account of the water present in the ethanol, the residue is now in the form of small flakes, which can be suspended in water. Eight ml. water were added to the flask which was then shaken for 10 min. at 45° C. and cooled to 5 to 7° C. The suspension was filtered through a coarse Pyrex sintered glass filter, using suction. The flask was rinsed three times in this manner. The combined slightly opaque filtrates, about 24 ml., were shaken with three 12 ml. portions of ether, and the combined ether fractions were adjusted to a volume of 25 ml. The content of fat in this ether extract caused no difficulties in chromatographic work.

Brassica. 50 g. of the inner, etiolated leaves of a head of cabbage were extracted and treated as described above for *Pisum*, except that removal of fat by carrying the neutral fraction through water was omitted. These extracts were used at much lower concentrations than those of *Pisum*.

***Avena* Coleoptile Curvature Tests**

These tests were carried out as described by Larsen (23). *Avena* seedlings were grown individually in soil in vials and decapitated twice. Ether extracts were transferred to 0.1 ml. agar platelets by the ether-dropping method.

Paper Chromatography

All the chromatography was carried out on Whatman No. 1 filter paper. Except for a few control experiments, the plant extracts and synthetic samples were chromatographed in hexane (27, 28). The descending technique was used. The solvent (*n*-hexane, Merck) was shaken with water before use, but no water was placed in the upper trough. The atmosphere in the tank was kept saturated with water vapor by means of a sheet of filter paper dipping into a separate trough containing water.

Nitsch and Nitsch (27, 28) emphasize that the water conditions during the experiment have a pronounced influence on the R_f values of IAN and ethyl indole-3-acetate (IAEt). Our R_f values for IAN and IAAld were low, but reproducible. They were 0.10 and 0.055, respectively. R_f values for IAEt were variable (0.38 to 0.57), and more streaking occurred behind the spot. In order to get good separation of IAAld and IAN, the chromatogram was run for 18 hrs. or more. Under these conditions, of course, the front would run off the paper and for a direct determination of R_f values, chromatograms had to be run for a shorter time, e.g., 3½ hrs. IAN and IAAld were used as reference markers in the establishment of R_f values for other compounds in the 18 hr. runs.

RESULTS

Synthetic compounds. Chromatography. A list of R_f values in water-saturated *n*-hexane and color reactions with DMCA given by IAA and a few neutral indole derivatives is given in Table 1. It will be seen that the two compounds with which we were most directly concerned, IAN and IAAld, can be distinguished without difficulty. These two auxins became well separated when applied in mixture to the same starting point.

Table 1. R_f values in water-saturated *n*-hexane (Merck) and color reactions with dimethylaminocinnamaldehyde (DMCA) given by IAA and some neutral indole derivatives.

Compound	R_f	Color Reaction With DMCA
Indole-3-acetic acid (IAA)	0.0	Bluish purple
Ethyl-3-indoleacetate (IAEt)	variable, 0.38 to 0.57	Bluish purple
Indole-3-acetaldehyde (IAAld)	0.055	Clear blue
Indole-3-ethanol (= tryptophol)	0.04	Bluish purple
Indole-3-acetamide	0.0	Reddish purple
Indole-3-acetonitrile (IAN)	0.10	Purple

Bioassay

One of the findings that indicated the aldehyde nature of the neutral auxin in *Pisum* extracts in earlier work was the fact that it could be converted to IAA by treatment with soil (21). The activity of a soil-treated preparation was many times higher than that of an untreated, neutral fraction. Using the *Avena* coleoptile curvature test, we therefore determined the auxin activity of synthetic IAAld and IAN both directly and after treatment with soil. The soil treatment was carried out as described by Larsen (21): The auxin was transferred to a 1-mm. thick agar platelet (1 sq. cm.) by the ether-dropping method. The 1-mm. platelet was covered with an agar platelet of the same area, but only 0.5 mm. thick. A suitable amount of soil was placed on top of the cover platelet. The soil was always taken from one of the vials in which the day's test plants were grown. This means that the physical and biological conditions in the soil samples were standardized. After the lapse of 90 min. the cover platelet with the soil was removed and the lower 1-mm. platelet cut into test blocks, 2 $\times$ 2 $\times$ 1 mm., which were later applied to twice-decapitated test plants.

The curves marked IAAld in Figure 1 represent the results of tests with this compound. The solid line is the concentration-activity curve obtained directly, without soil treatment, and the broken line, the corresponding curve for soil-treated material. It will be noted that the activity of the preparation was increased by several hundred per cent by the soil treatment. In this respect the synthetic IAAld re-

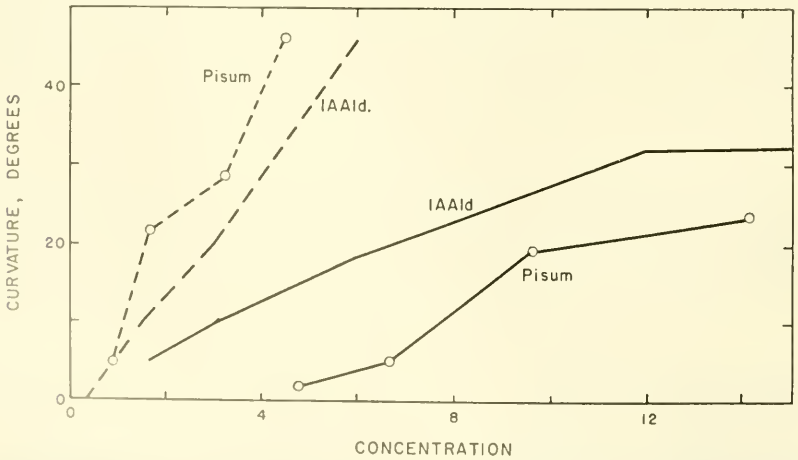

Fig. 1. Effect of soil treatments on auxin activity of *Pisum* extracts and of indole-3-acetaldehyde. Solid lines represent activity obtained directly and the broken lines activity after treatment with soil.

sembles the neutral auxin from *Pisum* and *Vicia faba* and the auxin prepared from isatin and tryptophan (21).

Synthetic IAN, when tested at low concentrations, proved to have about the same activity per microgram as IAA in the *Avena* coleoptile curvature test. This confirms the results of Bentley and Bickle (3). The standard soil treatment (see above) almost completely removed the activity of the preparation. Examples: IAN, 80 μg/l, 30.4° curvature; soil-treated, 0° curvature; IAN, 8 μg/l, 8.5° curvature; soil-treated, 0° curvature. IAN and IAAld can thus be distinguished by their different reactions to the standard soil treatment.

Plant Extracts

Pisum. Samples of neutral, defatted fractions of pea epicotyl extract, each representing 11 to 12 g. of fresh plant material, were evaporated as spots on the starting line of the chromatographic paper. Samples of synthetic IAAld and IAN (8 to 10 μg.) were applied at separate spots to serve as markers. The chromatograms were developed by spraying with DMCA after about 18 hrs. A colored spot at the starting line was reddish purple and a 40 mm. long spot around R_f 0.055 (IAAld location) was clear blue. Faint blue streaking was visible behind the blue spot at R_f 0.055, both in the pea extract and in the synthetic IAAld preparation. No other coloration was detected on the chromatogram. Suitable zones (see Figure 2) were cut out of two parallel, unsprayed strips of the paper. Each of these zones was cut to smaller pieces and extracted with three changes of ether over a period of 3 days. Each of these eluates was made to a volume of 25 ml. and tested at various concentrations in the *Avena* coleoptile curvature test. The results are given in Figure 2. Auxin activities are expressed in relative units, based on the amount of plant material needed. The concentration yielding 10° curvature was interpolated on the ascending part of concentration activity curves (example in Figure 1).

The solid lines in Figure 2 represent direct tests without soil treatment. By far the highest activity is present in zone III containing the clear blue spot at R_f 0.055. Considerably less was present in zones I and II, and no activity was detected in the other parts of the chromatogram down to R_f 0.31, although concentrations representing up to 110 kg. fresh plant material per liter of agar were tested.

The broken lines (Figure 2) indicate the activity of the eluates after soil treatment. In zone III the auxin activity was increased several-fold by the soil treatment (see also Figure 1). Also in zone I the activity was increased, but to a smaller extent. The eluate from zone II was not treated with soil. Soil treatment did not produce any activity in zones IV and V.

From these results, the identity of the neutral auxin in etiolated pea epicotyls with IAAld is indicated by R_f (0.055), color reaction with DMCA (clear blue), and reaction toward soil treatment (several-fold increase in auxin activity). Obviously, this auxin cannot possibly be identical with IAN.

Brassica. Samples representing 0.5 to 0.6 g. fresh weight were applied as described above for *Pisum,* and the chromatograms were treated and eluted in the same manner. Results are shown in Figure 2.

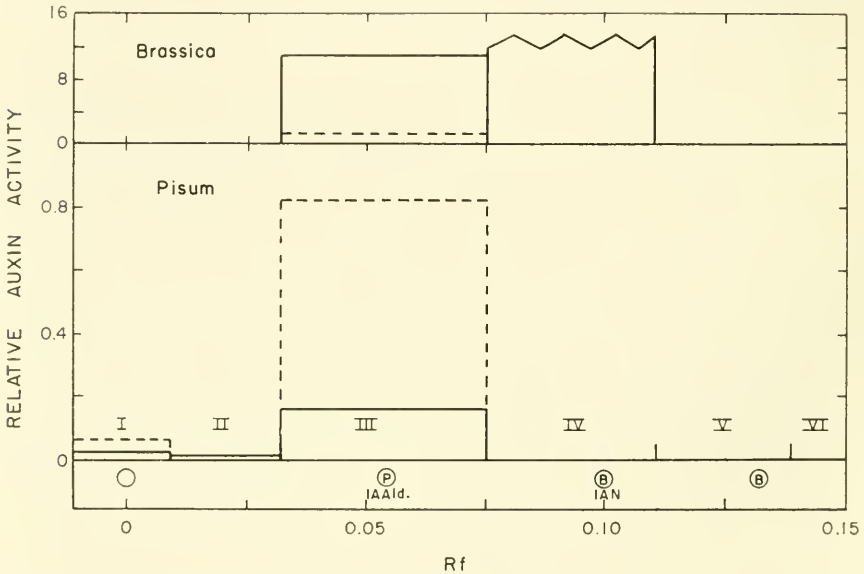

Fig. 2. Auxin activity of eluates of chromatograms of extracts of *Pisum* and *Brassica.* Solid lines represent activity obtained directly and broken lines after treatment with soil. Areas corresponding to the indole-3-acetaldehyde in *Pisum* show a considerable increase in activity after treatment in soil. For further explanation see text.

Colored spots developed in the following places after spraying with DMCA: starting line (reddish purple), R_f 0.10 (purple), R_f 0.135 (purple). In addition to these spots a faint, reddish-purple spot was detected at R_f 0.4 on a chromatogram which was run for 3¼ hrs. instead of 18 hrs., and in which the front had moved to 32.7 cm.

Bioassays were made on zones III and IV in the 18 hr. chromatogram (Figure 2) and very high activity was found in both. (Note the different scales for *Brassica* and *Pisum.*) Supramaximum curvatures were obtained from zone IV at the concentrations tested, but no at-

tempt was made to determine the activity in this zone more accurately. The auxin present here is undoubtedly IAN. Zone III, which includes the locus of IAAld, contained no definite colored spot, but only a very faint purple streaking. The auxin activity found here was much higher than expected from the faint coloration, and it seems probable that a nonindole compound is responsible for this activity. Such nonindole auxins were also reported to be present in members of the Cruciferae by Linser and Machek (26) and by Housley and Bentley (17). Since this activity occurred in the zone containing the locus of IAAld, we first thought that it was due to this compound, but soil treatment proved definitely that this was not the case. The activity was reduced to less than one-tenth by the soil treatment, whereas it would have been increased several-fold had it been due to IAAld. These results, however, do not exclude the occurrence of IAAld in cabbage since the starting material on the chromatogram represented less than 1/20 of the amount of plant material used in the case of *Pisum*. Linser, Kiermayer, and Youssef (25) studied the auxins in *Brassica napus* and three varieties of *Brassica oleracea*. Extracts of seeds, leaves, stems, and roots of these plants were chromatographed in propanol, water, ammonia (80:15:5) on filter paper. In stems of one of these plants, *B. oleracea* var. *sabauda* (Wirsingkohl), auxin activity coincided with the locus, giving a yellow color reaction with ferric chloride and perchloric acid at R_f 0.77. The authors report that the color reaction and position of this locus agree with those of synthetic IAAld, unfortunately, however, without mentioning the origin of their sample of synthetic IAAld. As regards our ether extracts of heads of cabbage, it can be concluded that, if present at all, IAAld occurs.

The occurrence of IAN in members of the Cruciferae has been established beyond doubt by isolation and chemical characterization. As regards extracts of other plants, the identification of IAN rests exclusively on chromatographic data. A survey of the literature on chromatographic separation of auxins shows that IAN and a number of other neutral auxins, now also including IAAld, run rather close together in the majority of systems containing an aliphatic alcohol, water, and ammonia. [The statement by Blommaert (5) that IAAld runs at R_f 0.40 to 0.42 in *n*-butanol saturated with 2 *N* ammonia is probably erroneous since IAN ran at R_f 0.87 to 1.0 in Blommaert's system. Blommaert does not mention the source of his sample of IAAld.] Biological activity in the "IAN zone" of such systems does not unequivocally indicate the presence of IAN. The activity may as well have been due to other neutral auxins unless more specific tests for IAN have also been carried out.

SUMMARY

Preparations of synthetic indole-3-acetaldehyde (IAAld) and indole-3-acetonitrile (IAN) were chromatographed (descending) on Whatman No. 1 filter paper in water-saturated *n*-hexane. Chromatograms were developed by spraying with dimethylaminocinnamaldehyde (DMCA). The colors obtained with this reagent are clear blue for IAAld and purple for IAN. R_f values, determined after 3½ hrs. at 20° C., were 0.055 for IAAld and 0.10 for IAN. Good separation was obtained by running the chromatograms for 18 hrs., letting the front run off the paper.

Ether extracts of etiolated epicotyls of pea (*Pisum*) and of the etiolated, inner leaves of a head of cabbage (*Brassica*) were chromatographed under the same conditions as the synthetic compounds. *Pisum* yielded a clear blue spot at the same location as synthetic IAAld, but no spot at the IAN locus. *Brassica* yielded a purple spot at the same location as IAN, but no spot at the IAAld locus. With *Brassica,* however, colored spots were also observed at R_f 0.135 (purple) and R_f 0.4 (reddish purple).

The auxin activity (*Avena* coleoptile curvature test) of synthetic IAAld and of the material from the zone containing the IAAld locus in *Pisum* could be increased several-fold by treatment with soil, whereas the activity of IAN almost disappears by the same treatment. With *Brassica,* auxin activity was found both in the IAN zone and in the zone corresponding to the locus of IAAld. No colored spot, however, was developed in the latter, and the auxin activity in this zone was reduced very considerably by treatment with soil.

It is concluded that the *Pisum* extracts contain IAAld, but very little, if any, IAN, and that the *Brassica* extracts contain large amounts of IAN, but considerably less IAAld, if any.

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DISCUSSION

Dr. Thimann: How do you account for the disappearance of activity in the various fractions? What happens in the soil?

Dr. Larsen: As regards IAAld, I imagine that it diffuses from the agar platelet into the soil where we know it becomes oxidized to IAA. IAA will diffuse from the soil to the agar platelet, thereby increasing the auxin activity of the agar. But if we leave the soil in contact with the agar for a time considerably longer than 90 minutes, the auxin activity decreases again, indicating that IAA is being inactivated by the soil. The inactivation must be assumed to take place also during the time when the auxin activity in the agar is increasing, but we have a steady state (IAAld → IAA → inactive products) for some time. Plotted against time, the auxin activity shows a broad optimum around 90 minutes after application of the soil.

As regards IAN, there are two possibilities. (1) If we assume that IAN is converted to IAA by the soil, this conversion will not be manifested as an increase in activity, because these two substances are equally active in our test. On the contrary, as soon as some IAA has been formed, it will be subject to inactivation, thus lowering the total auxin activity of the agar-soil system. (2) IAN may be inactivated without a preceding conversion to IAA. In both cases the auxin activity will be steadily decreasing.

Dr. Bennet-Clark: Why did you use soil in preference to one of the conventional oxidizing agents such as alkaline iodine solutions or hydrogen peroxide?

Dr. Larsen: Because the soil treatment was a simple procedure, and because we wanted to show that the synthetic IAAld reacted in the same way as the material in plant extracts which we had studied in the past. But of course it will be important to accomplish the oxidation also by other means, such as aldehyde dehydrogenase or inorganic oxidizing agents. Gray has shown that his synthetic IAAld can be

converted to IAA by oxidation with permanganate, silver oxide, or hydrogen peroxide, but the IAA is subject to break-down by the chemicals employed. In my own previous work with neutral pea extracts, inorganic oxidants have failed to yield IAA in quantities that could be demonstrated, but the chances may be better now that we have a method to isolate IAAld from plant material by means of paper chromatography.

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New Auxins From 'Maryland Mammoth' Tobacco

Indole derivatives have held a position of pre-eminence in the field of natural auxins for many years. In fact, they have been regarded by many workers to constitute probably the only group of growth regulators to occur in nature. At the present time, the only two indole derivatives possessing pronounced growth-promoting activity to have been isolated from plants are indole-3-acetic acid (IAA) and indole-3-acetonitrile (IAN), although there is evidence for the occurrence of a variety of other 3-substituted indoles.

Paper chromatography has emerged as a versatile method for the resolution of the complex chemical mixtures occurring in plant extracts. With the development of sensitive and rapid techniques for the bioassay of growth substances, this has resulted in our ability to measure "hormone profiles" which present an indication of the extent to which different growth-regulating substances occur in extracts of plant material. Considerable effort has been made to ascribe the areas of growth stimulation of such profiles to IAA, IAN, and the other indoles.

An increasing number of hormone profiles has been determined for different plant species growing under a wide variety of conditions. However, continued refinement of technique has caused their interpretation in terms of the known indolic growth substances to become increasingly difficult. Many investigations have failed to show the presence of the known indoles, while others have revealed growth activity in chromatogram areas which do not correspond to classical growth substances (1). The work described in this paper, which started with an investigation of one such case, has led to the discovery of two

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new types of naturally occurring nonindolic growth substances which easily might be confused with IAA and IAN on paper chromatograms.

AUXINS IN 'MARYLAND MAMMOTH' TOBACCO

During the period of 1954 to 1956, Vlitos and co-workers (10, 11) studied, with the aid of paper chromatography, the relationship of naturally occurring indole compounds to flowering in photoinduced plants. It was found that a substantial increase in the levels of IAA and indole-3-pyruvic acid occurred after photoinduction of a short-day soybean (*Glycine max,* 'Biloxi'). However, extension of the investigation to a short-day variety of tobacco (*Nicotiana tabacum,* 'Maryland Mammoth') revealed that neither IAA nor IAN could be detected in leaves and apical tissue of these plants. A material similar to, but not identical with, IAN in its chromatographic and colorimetric characteristics was observed, and bioassay of chromatogram areas containing this substance indicated the presence of a growth stimulant.

Several factors were responsible for the decision to attempt isolation of the growth-promoting substances of 'Maryland Mammoth' tobacco. The unusual vigor and rapid growth rate of this variety have long been intriguing. Inability to detect the classical auxins, and the presence of the unidentified indole, suggested that an unusual type of hormonal growth regulation might be in operation.

In order to obtain sufficient material for the isolation, seedling tobacco plants were transplanted to a farm near Winfield, West Virginia. After 3 months of growth, the leaves and apical tissue were harvested, frozen in solid carbon dioxide, and transported to the research laboratories of the Union Carbide Chemicals Company. The frozen material was ground in a large, cooled mill, extracted with absolute alcohol, and the extract was treated by a modification of the methods reported previously (9, 11). The total fresh weight of the tobacco used was 2,300 lbs.

The bioassay method of Nitsch and Nitsch (7) was used for measurement of growth-promoting activity. Sections of first internodes of dark-grown oat seedlings (*Avena sativa,* 'Brighton'), 4 mm. in length, were rotated for about 20 hours in citrate-phosphate buffer (pH 5.0) containing 2 per cent sucrose. The final length of the sections was measured with the aid of a photographic enlarger, and the resulting data were subjected to statistical analysis — a step which was found to be very important in obtaining significant hormone profiles. The shaded areas on histograms drawn from these data indicate responses significant at the 1 per cent level.

Hormone profiles of 'Maryland Mammoth' tobacco were obtained by extraction of the homogenized leaves and apical tissue in the usual

way with ethanol, followed by removal of the alcohol, extraction of the residual solution with ether, and chromatography of the two layers separately on paper using isopropanol-ammonia-water (80:5:15) as solvent. Figure 1 reveals the presence of ether-soluble growth substances having R_f values identical with those of IAA and IAN. However, the area of greatest growth-promoting activity (R_f 0.5 to 0.6) failed to give the typical indole color reactions, while the areas above R_f 0.9 gave only a very faint color, as described previously (11).

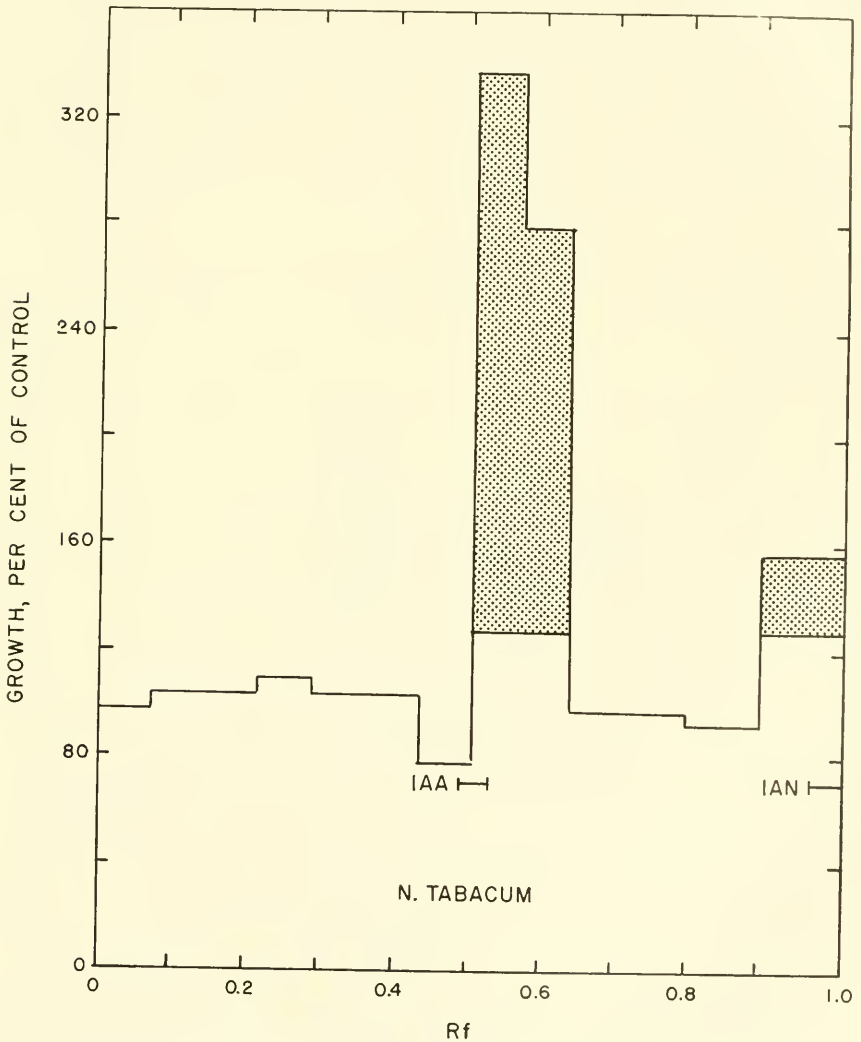

Fig. 1. Ether-soluble growth substances from *N. tabacum* L., 'Maryland Mammoth,' as detected by the *Avena* first internode assay. The shaded areas indicate responses significant at the 1 per cent level.

The profile of the water fraction (Figure 2) is very simple. Based on results to be described later in this paper, there is reason to believe that the activity observed at R_f 0.5 to 0.6 is due to the same substance responsible for that at the same place on chromatograms of the ether fraction. The cause of the activity at R_f 0.3 is not yet known. No indolic compounds could be detected on the chromatograms.

The materials responsible for activity in these two areas were concentrated on paper chromatograms, eluted with methanol, and rechromatographed in both water and isopropanol-ammonia-water. R_f values of the active zones still corresponded to those of IAA and IAN. The material having an R_f above 0.9 (called A) could be isolated on Whatman No. 1 paper by ascending chromatography, and on a Grycksbo filter paper column by descending chromatography. The material at R_f 0.5 to 0.6 was further concentrated on Whatman No. 17 paper.

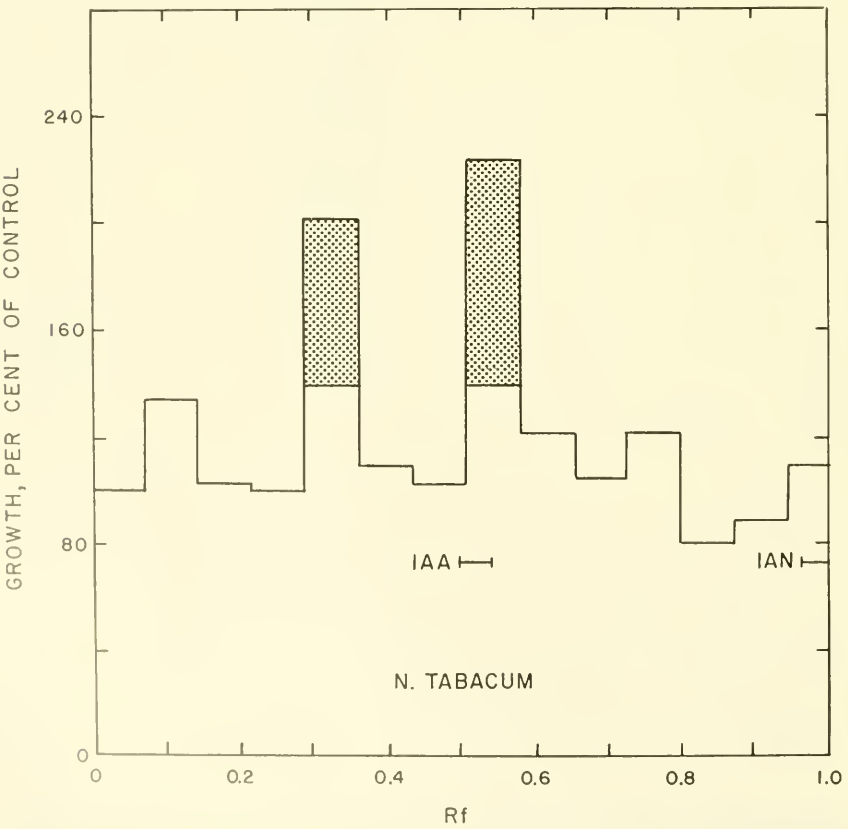

Fig. 2. Water-soluble growth substances from *N. tabacum* L., 'Maryland Mammoth.'

After repeated fractional crystallization from anhydrous ether, concentrate A provided a small amount of waxy white solid which melted at 70 to 72° C. and contained only carbon, hydrogen, and oxygen. Its infrared and ultraviolet spectra were those characteristic of a long-chain unbranched fatty alcohol such as 1-docosanol (melting point 73° C.). Color tests for the indole nucleus were negative. Figure 3 shows the results of bioassays of the tobacco isolate and several of the common naturally occurring alcohols. All together, more than 60 long-chain compounds were examined, and Figure 4 shows the activity of one of these, 2-heptadecanol, at several concentrations. With two exceptions, activity was found to be restricted to alcohols containing from 17 to 22 carbon atoms and their acidic inorganic esters (5).

Repeated precipitation of concentrate B from methanol solution with absolute ether resulted in a white crystalline material which possessed pronounced auxin activity, but which rapidly became dark and gummy, even in the cold, with complete loss of activity. The crystalline material did not melt below 300° C., and also contained no nitrogen, phosphorus, sulfur, or halogen. Chemical studies and data from infrared, ultraviolet, and emission spectra revealed that the active material was the sodium salt of a long-chain unsaturated fatty acid. A variety of other fatty acid salts has now been bioassayed, and some

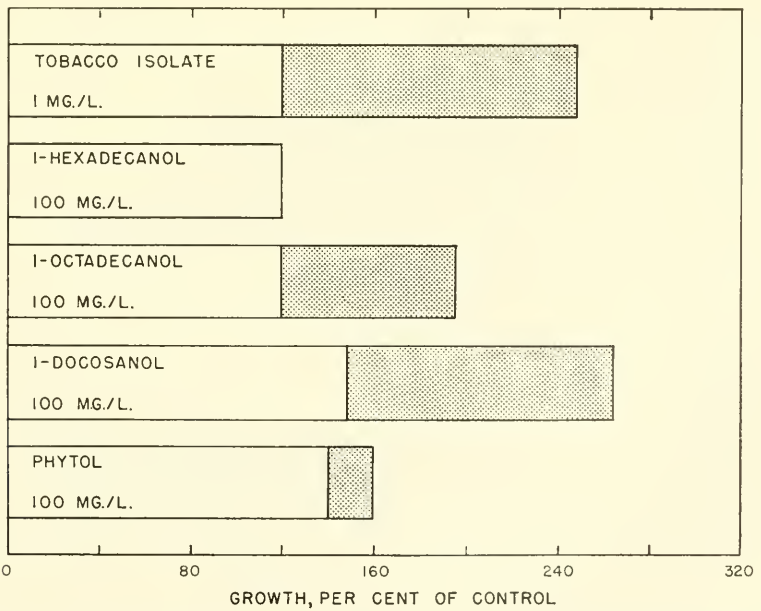

Fig. 3. Growth-promoting activity of several naturally occurring long-chain alcohols.

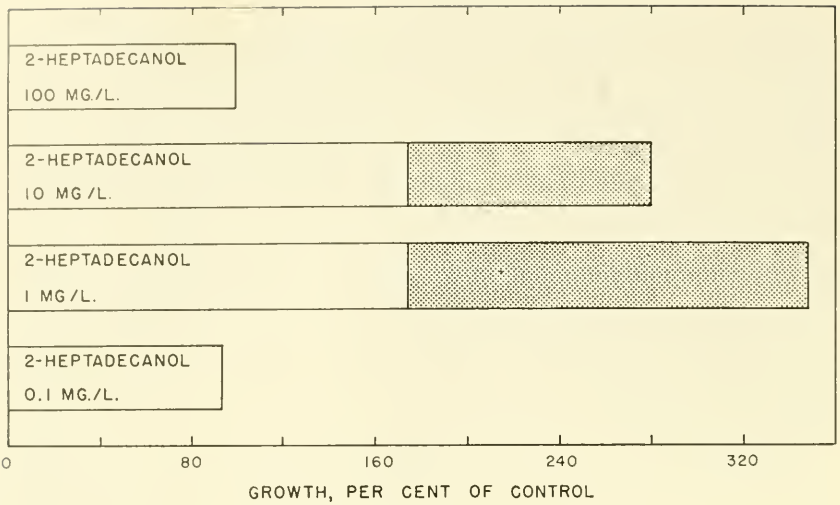

Fig. 4. Growth-promoting activity of 2-heptadecanol.

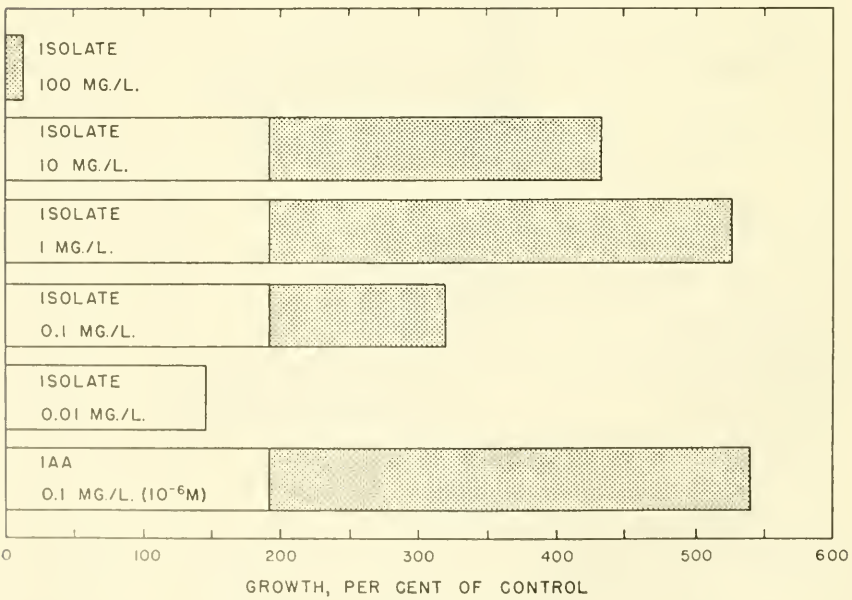

Fig. 5. Growth-promoting activity of the long-chain fatty acid from *N. tabacum* L., 'Maryland Mammoth.'

show significant growth promotion. Figure 5 indicates the activity of the tobacco compound, although the values are only relatively quantitative due to the probable presence of impurities in the sample.

The identity of the substance which lay behind all this effort — the elusive indolic material similar to IAN — has not yet been determined. So far, all attempts to isolate it have failed, and, in fact, it frequently has even escaped detection. The continued search for this substance may yield an exciting story in itself some day.

DISCUSSION

A number of instances have been reported (1) in which the presence of IAA and IAN was suspected on the basis of bioassay and chromatographic data, but in which the characteristic color reactions of the indole nucleus could not be detected. Undoubtedly, many more such cases have gone unreported. The fact that the substances responsible for growth promotion on these occasions were not extensively investigated indicates the dominating influence which knowledge of the naturally occurring indoles has had on auxin studies. It is quite possible that the substances detected in previous examples are similar to, or perhaps even identical with, the growth promoters in 'Maryland Mammoth' tobacco.

The similarity of the chromatographic characteristics of these nonindolic compounds to those of IAA and IAN certainly is coincidental. However, such similarity cannot be considered unlikely, since the precision of resolution in the usual hormone profile is only 0.1 to 0.2 R_f units. Despite the great value of paper chromatography to the plant hormone field, other severe limitations of this method for the determination of hormone profiles have become apparent in the present investigation. Since a biologically active area may contain more than one growth-stimulating substance, extensive efforts must be made to separate the chemical factors in each case. Certainly, R_f data cannot be used as proof, nor generally even as critical evidence of the chemical structures involved.

"Specific" color tests also may be deceiving. For example, an inactive indole may have the same R_f as a growth-promoting nonindolic compound, or, in the case of an active indole, the two biological effects may be superimposed. Although a great deal of valuable information obviously can be obtained by thorough chromatographic analysis of plant materials, rigorous proof of the structure of the compounds present probably can come only through actual isolation and chemical study.

It is apparent from the examination of 'Maryland Mammoth' tobacco that nonindolic growth-promoting compounds may be iso-

lated from plant extracts. Since it has not been possible to demonstrate the presence of either of the two principal known indolic auxins, this tobacco variety may present one example of a hormone system in which indoles are not involved, or in which at least they do not play a major role.

Other examples have been found in rapidly growing species of bamboo. Figure 6 presents a hormone profile of *Bambusa multiplex* obtained in the same way as those of tobacco. In this case, the ether fraction showed neither activity nor the presence of indolic compounds. The aqueous fraction represented in the figure exhibited a high level of activity, again at about R_f 0.5, although chromogenic reactions suggested the presence of an indole at R_f 0.41. The hormone profile of an aqueous acetone extract of the bamboo *Sinocalamus oldhami* was very similar to that of *B. multiplex*.

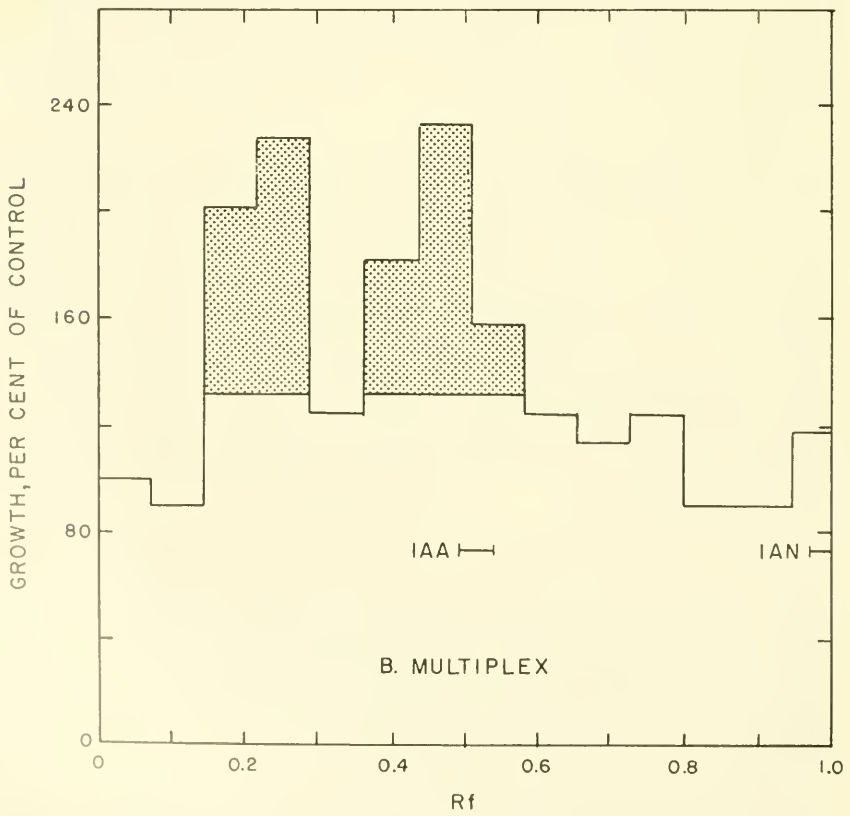

Fig. 6. Water-soluble growth substances from *Bambusa multiplex*.

The possibility that the growth-promoting substances isolated from 'Maryland Mammoth' tobacco are artifacts cannot yet be excluded. The extensive work of Chibnall *et al.* (4) and others has shown that long-chain alcohols occur widely in both the cuticle and cellular waxes of plants (12), although the wax of tobacco was found to be associated primarily with the cell (4). The occurrence of a great variety of unsaturated fatty acids in plant tissues also is well established. However, these two types of substances are interesting in their own right. The comparatively narrow range of activity in the series of long-chain alcohols is noteworthy, since there exists very little difference in solubility, chemical reactivity, and other physical and chemical properties between the inactive 1-hexadecanol and the active 1-octadecanol.

The physiological effects of long-chain fatty acids have been reported previously by several workers. Haagen-Smit and Viglierchio (6) found that several of these compounds, such as myristic acid and linoleic acid, were active in the Wehnelt bean test for wound hormones. Stowe (8) reported that several long-chain fatty esters, including a preparation isolated from 'Alaska' peas, were active in stimulating growth in pea epicotyl sections, although they were inactive on oat coleoptiles. However, the degree of activity of the tobacco isolate is unusual and suggests that it may possess peculiar structural features not now appreciated as being important to growth-regulatory activity.

The importance of long-chain unsaturated fatty acids such as linolenic acid, arachidonic acid, and docosahexaenoic acid in animal nutrition is becoming increasingly evident upon continued investigation. In recent months, two similar compounds important to the lives of insects have been reported: Butenandt *et al.* (2) have shown that the sexual attractant of the silk worm (*Bombyx mori*) is the unsaturated, primary alcohol 10,12-hexadecadiene-1-ol, while the active constituent of the royal jelly of the honey bee has been identified as 10-hydroxydecenoic acid (3). The results of the investigation of 'Maryland Mammoth' tobacco strongly indicate that this type of compound may prove to hold an equally important place in the plant world.

SUMMARY

A paper-chromatographic study of growth-promoting substances from 'Maryland Mammoth' tobacco (*Nicotiana tabacum* L.) revealed the presence of two compounds which, although they exhibited chromatographic behavior similar to that of indole-3-acetic acid and indole-3-acetonitrile, were found to be nonindolic. Isolation and characterization provided evidence that one active material consisted

principally of the long-chain alcohol 1-docosanol, while the other was the sodium salt of a long-chain unsaturated fatty acid. These findings emphasize the necessity for actual isolation and chemical study of natural growth substances and the importance of long-chain compounds to plant life.

ACKNOWLEDGMENT

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DISCUSSION

Dr. Stowe: I am interested in this report because we obtained much the same results in pea straight-growth assays, testing a number of higher alkyl lipide compounds. In the pea section-growth assay, it is necessary for auxin to be present if growth acceleration is to be found. May I ask if IAA or other auxins were introduced in your assay?

Dr. Crosby: No exogenous IAA was ever introduced.

Dr. Wain: Reference has been made to the occurrence of plant waxes. We all know that these are in the main esters of long-chain fatty acids and long-chain monohydric alcohols. Obviously, with this large amount of material a good deal of wax would be extracted from the tobacco. It is rather significant that one gets here not only an alcohol but an acid. Does the possibility exist that these two compounds have arisen from the cuticular wax?

Dr. Crosby: From its configuration, our long-chain fatty acid must be an extremely unstable one. It is possible, but I would be surprised if this were the case. We would never say that these materials positively had an importance to the plant, but they are of interest to us in that they stimulate growth.

Dr. Kefford: My particular interest in the auxins of 'Maryland Mammoth' tobacco is the presence or absence of IAA. The most specific auxin test is the *Avena* curvature test and, to date, of the naturally occurring auxins only IAA has been found to give this test. I have been able to detect activity with this test in extracts of 'Maryland Mammoth' tobacco following repeated chromatography. But the activity is very small — about 1/40 of the activity found in other tobacco varieties.

Dr. Vlitos: I would like to say that we have not tested the fatty alcohols in the *Avena* curvature test, although Dr. Kefford, through correspondence, has many times urged us to do so. I feel that we have used the most rigorous type of chemical evidence to identify a naturally occurring auxin, and I have not felt it necessary to rely on what is perhaps a nonspecific biological assay. Since we were using the *Avena* first internode test which seemed to be responsive to the alcohols, and it looked as though we were getting a classical growth dosage response in that particular assay, we relied entirely on chemical identification. I would say I feel that there are very few instances where growth substances have been isolated in crystalline form and shown by a series of rigorous chemical methods to be a particular substance. We relied neither on a bioassay nor on a colorimetric test.

Dr. Galston: We have been interested in the differences in growth characteristics between completely etiolated pea sections and those which have received a prior exposure to morphogenically active red light. It has been reported that the surface characteristics of peas and of other plants could be markedly affected by the degree of prior exposure to red light. By an ingenious carbon-casting technique, flakes and scales were seen on the surface of plants which were waxes. They may contain some of these growth-active materials. Are we, by virtue of prior red light exposure, inducing the synthesis of more cuticular wax on the surface of these sections? Are we then producing, in a sense, artifactual growth promotions or inhibitions?

Dr. Crosby: Dr. Galston, I think this is likely. One problem which we have had in dealing with compounds such as docosanol and octadecanol is that we have no way to measure their aqueous solubility. They are extremely insoluble; even surface tension measurements cannot determine their solubility. How, then, can they act as growth stimulants? In our bioassay, we rotate the sections for 20 hrs. and, certainly, each section must become evenly coated with the alcohol as it comes to the surface. It is interesting that we get a very definite change of activity with concentration. This has been repeated many times and on different occasions over a period of many months. What is the cause of this growth stimulation? Perhaps the thickness of the waxy material influences directly the growth of the sections. We don't know.

Dr. Thimann: Would any of these active materials be present in lanolin that is normally used in the laboratory? Lanolin has long been known to produce some small amount of growth and cell division.

Dr. Crosby: The esters of the long-chain fatty acids and straight-chain fatty alcohols would be present as minor constituents in lanolin.

Dr. Bitancourt: Is there any interaction between the fatty acids and IAA? I ask because in attempts to get solutions of IAA that would not decompose over a long period, we used air-free water covered by a layer of paraffin oil. Instead of getting the stability that we expected, we found that our solutions decomposed more rapidly or as rapidly as those that hadn't been protected. I am wondering whether there could be some chemical change in IAA induced by the hydrocarbons from paraffin oils. The decomposition was clearly different from that which occurred in aerated solutions where we got a brownish coloration, whereas we got a beautiful red coloration in the solution that was protected by paraffin oil.

Dr. Crosby: We have not tried such experiments on a chemical basis. One would not expect a long-chain fatty alcohol such as docosanol to stabilize IAA. On the other hand, salts of our long-chain

unsaturated fatty acid might be expected to do so. These compounds, particularly in the configuration we believe ours has, do act as antioxidants.

Dr. Housley: Is it possible that this type of compound influences cell division and that the growth you have been getting has reflected this action rather than an effect on cell elongation?

Dr. Crosby: We have not carried out microscopic examination of these materials. We do note that the sections elongate; they do not seem to grow in bulk.

Dr. Osborne: Do the substances which have been isolated from 'Maryland Mammoth' tobacco have any effect on accelerating abscission? In the Agriculture Department in Oxford we are trying, with Prof. E. R. H. Jones, to isolate this abscission-accelerating factor. It seems likely, from what we know so far of the compound, that it might fit in with your findings. As a subsidiary question, may I ask if there was a large number of very old leaves in the ton of material you extracted?

Dr. Crosby: The plants were close to the flowering stage. We took only the younger, bright green leaves and the apical tissue. We have not investigated the effects of these materials on abscission.

Dr. Fawcett: Activity in the wheat coleoptile test is shown by certain compounds which do not possess a ring structure (Nature. 178: 972. 1956). Ethanol, ethylenediaminetetraacetic acid, certain xanthates, and chloroalkanecarboxylic acids were cited as examples. All these compounds had the same low order of activity and their optimum activity was given at a concentration just below the level at which toxic symptoms were observed. Since they were inactive in the pea curvature and tomato leaf epinasty tests and showed some other common features, their growth-regulating activity was regarded as nontypical — possibly resulting from subacute toxicity. S-carboxymethyl *N,N*-dimethylaminodithiocarbamate, however, was highly active in the wheat, pea, and tomato tests, and we think this type of nonring structure can induce a typical auxin response. Have you tested the lower members of this homologous series of alcohols?

Dr. Crosby: No, we started our examination with the C-10 alcohol. I think the physical and chemical properties of the alcohols below C-10 fall somewhat into one class, and the properties of those greater than C-10 fall into a different class.

Dr. Thimann: It was shown recently that many algae are extremely sensitive to ethanol and respond to quite low concentrations. The indication was that it perhaps acts as a nutrient but I would say in several instances, effects on algal growth ascribed to IAA were really due to the ethanol in which it was dissolved.

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Chromatographic Investigations on the Metabolism of Certain Indole Acids and Their Amides, Nitriles, and Methyl Esters in Wheat and Pea Tissues[2]

Research at Wye has been concerned for several years with the metabolism in various plant tissues of homologous series of chloro- and methyl-substituted phenoxyalkanecarboxylic acids (4, 5, 9). Chemical and biological evidence has been obtained that the side-chain of such ω-substituted fatty acids can be degraded by β-oxidation, and although the capacity to effect this breakdown is common to many plants, specificity has been observed in some species to the members of certain series. In this connection, the results indicate that the number and orientation of the nuclear substituents affect the ease with which β-oxidation occurs at the shorter side-chain lengths, and these findings provide an explanation for the different patterns of growth-regulating activity shown by the many homologous series examined.

In a related study the growth-regulating activity and metabolism in wheat and pea tissues of the first six members of the homologous series of ω-(2,4-dichlorophenoxy)alkanecarboxylic acids and their corresponding amides and nitriles were investigated (6,7). The pattern of activity shown by the acid and amide series was found to be similar, and chromatographic examination of the treated tissues showed that the amides, after hydrolysis to the corresponding acid, were degraded by β-oxidation in a manner identical to that observed with

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[2] This paper was read at the Conference by F. Wightman.

members of the acid series. The nitriles, however, showed exceptional behavior in both the tissues employed. In pea tissue, only the first member of the series (i.e., 2,4-dichlorophenoxyacetonitrile) showed evidence of hydrolysis to the corresponding carboxylic acid, whereas in wheat tissue, all homologues produced not only the corresponding acid, but also the next lower carboxylic acid. In the case of the higher nitriles, subsequent β-oxidation of one or other of these acids resulted in the production of the highly active 2,4-dichlorophenoxyacetic acid. This behavior of the nitriles in wheat tissue is explicable in terms of an initial modification of the $-CH_2CN$ group by two mechanisms, namely, either hydrolysis to the corresponding acid or conversion to the lower carboxylic acid with the loss of a one-carbon fragment. The latter type of breakdown was referred to as α-oxidation of nitriles, and further evidence for this reaction in plant metabolism has been obtained from studies on the degradation of indole-3-acetonitrile in wheat, pea, tomato, maize, and celery tissues (8). α-Oxidation of this nitrile yields indole-3-carboxylic acid as end product, and the occurrence of indole-3-aldehyde as an intermediate has been established (7).

In view of the importance of indole compounds and in particular indole-3-acetic acid and indole-3-acetonitrile in relation to the hormonal control of plant growth, it was logical to extend the above investigations by examining the growth-regulating activity of a homologous series of indole-3-alkanecarboxylic acids together with the corresponding amides, nitriles, and methyl esters. This has been carried out using the wheat cylinder, pea segment, and pea curvature tests, and a study has been made of the metabolism of these compounds in pea and wheat tissues. It is with the results from the latter aspect of the work that this paper is mainly concerned. Briefly the metabolism experiments involved exposing solutions of the various indole compounds to wheat coleoptile or pea stem tissues with subsequent extraction and paper chromatographic separation of the metabolic products present in the tissues and in the residual solution. After development, the chromatograms were examined by chromogenic and biological methods.

MATERIALS AND METHODS

The compounds examined are given in Table 1 together with their uncorrected melting points, R_f values in two different solvent systems, ultraviolet fluorescence characteristics, and chromogenic reactions with three reagents.

The growth-regulating activity of each of these substances was assessed in the wheat cylinder, pea segment, and pea curvature tests.

Table 1. Melting points, R_f values, fluorescence in ultraviolet, and color reactions of ω-(indole-3-)alkanecarboxylic acids and their corresponding carbonamides, methyl esters, and nitriles.

Compound	Melting Point °C.	Mean R_f in Solvents		Ultraviolet fluorescence	Color Reactions With Various Reagents		
		Iso-propanol-ammonia-water	*n*-Butanol-ammonia-water		Ehrlich	Salkowski	Nitrous acid
Indole-3-carboxylic acid (ICA)	222–223	0.28	0.19	Blue	Pink(m) *	Crimson(s)	Red(s)
Indole-3-acetic acid (IAA)	165–166	0.39	0.25	Light blue	Grayish-blue(m)	Pink(r)	Red(r)
β-(Indole-3-)propionic acid (IPA)	133–134	0.46	0.31	Light blue	Bluish-purple(r)	Orange-brown(m)	Orange(m)
γ-(Indole-3-)butyric acid (IBA)	123–124	0.54	0.38	Light blue	Bluish-purple(r)	Orange-brown(m)	Orange(m)
δ-(Indole-3-)valeric acid (IVA)	105	0.64	0.45	Light blue	Blue(r)	Orange-brown(m)	Orange(m)
ϵ-(Indole-3-)caproic acid (ICAPA)	139–140	0.70	0.53	Light blue	Blue(r)	Orange-brown(m)	Orange(m)
ζ-(Indole-3-)heptanoic acid (IHA)	90.5–91	0.75	0.60	Light blue	Blue(r)	Orange-brown(m)	Orange(m)
Indole-3-carbonamide ($CoNH_2$)	201–202	0.76	0.72	...	Yellow(s)	...	...
Indole-3-acetamide (ANH_2)	152	0.79	0.75	Light blue	Blue(r)	Pink(r)	Pinkish-mauve(r)
β-(Indole-3-)propionamide (PNH_2)	130–131.5	0.82	0.78	Blue	Bluish-purple(r)	Pinkish-gray(m)	Orange-gray(m)
γ-(Indole-3-)butyramide (BNH_2)	94–96	0.86	0.82	Blue	Blue(r)	Orange-brown(m)	Orange(m)
δ-(Indole-3-)valeramide (VNH_2)	129–130.5	0.89	0.86	Light blue	Blue(r)	Orange-brown(m)	Orange(m)
Methyl indole-3-carboxylate (CoMe)	143–145	0.86	0.90	...	Faint yellow(s)	...	..
Methyl indole-3-acetate (AMe)	51–52	0.87	0.92	Greenish blue	Blue(r)	Violet(r)	Mauve(r)
Methyl β (indole-3-)propionate (PMe)	78.5–79.5	0.88	0.93	Blue	Bluish-purple(r)	Orange-brown(r)	Orange(m)
Methyl γ (indole-3-)butyrate (BMe)	73–74	0.90	0.94	Blue	Bluish-purple(r)	Orange-brown(r)	Orange(m)
Methyl δ (indole-3-)valerate (VMe)	56–57	0.91	0.94	Blue	Bluish-purple(r)	Orange-brown(r)	Orange(m)
Indole-3-nitrile (CN)	179.5–180.5	0.85	0.91	Violet	Yellow(s)	Light gray(s)	Light brown(s)
Indole-3-acetonitrile (ACN)	35–36	0.86	0.92	Greenish-blue	Greenish-blue(m)	Greenish-gray(m)	Brownish-purple(r)
β-(Indole-3-)propionitrile (PCN)	67–68	0.87	0.93	Blue	Purple(r)	Orange-brown(m)	Orange(m)
γ-(Indole-3-)butyronitrile (BCN)	b.p. 170/0.1 mm.Hg.	0.89	0.94	Light blue	Bluish-purple(r)	Orange-brown(m)	Orange(m)
δ-(Indole-3-)valeronitrile (VCN)	54–60	0.91	0.95	Light blue	Blue(r)	Orange-brown(m)	Orange(m)
ϵ-(Indole-3-)capronitrile (CCN)	b.p. 195/0.1 mm.Hg.	0.93	0.96	Light blue	Blue(r)	Orange-brown(m)	Orange(m)
ζ-(Indole-3-)heptanonitrile (HCN)	b.p. 210/0.1 mm.Hg.	0.94	0.96	Light blue	Blue(r)	Orange-brown(m)	Orange(m)

* The rate of color development after spraying chromatogram with the above reagents is indicated in parentheses; (r) denotes rapid color development (within 0 to 2 min.), (m) denotes a medium rate of development (5 to 30 min.), (s) denotes a slow rate of development (longer than 30 min).

The compounds were dissolved in water containing 0.1 per cent acetone and were examined at five concentrations ranging from $10^{-4}M$ to $10^{-8}M$.

Wheat Cylinder Test

Ten-mm. sections excised from 2-cm. coleoptiles of 3-day-old wheat seedlings ('Eclipse') were used as the experimental material. The sections were threaded on glass capillaries and floated on the test solutions contained in petri dishes, ten sections per dish. After 24 hrs. treatment at 25° C., the length of the test sections was determined and the results are presented as a percentage of the final length of water controls.

Pea Segment Test

Five-mm. segments excised from the second internode of 6-day-old pea plants ('Alaska') were used as test material. The seedlings were grown in sand under red light at 25° C., and the second internode was approximately 2 cm. in length when the test segment was removed. Batches of ten segments were placed in petri dishes on filter-paper bridges supported by two glass rods, the ends of the filter paper dipping into the test solution and serving as a wick for supplying solution to the segments. After 24 hrs. treatment at 25° C., the segments were measured under a microscope and the results are again presented as a percentage of the final length of water controls.

Pea Curvature Test

Three-cm. segments were excised from the second internode of young pea plants ('Alaska') grown for 7 days as described above. Each segment was split longitudinally with a razor blade for approximately 2 cm. through its upper elongating region, and after washing in water for 2 hrs., five split segments were placed in each test solution for 24 hrs. at 25° C. The curvatures induced were assessed by a numerical scale from 0 (inactive) to 6 (highly active) similar to that suggested by Went and Thimann (10).

In the metabolism experiments, solutions of each compound were exposed to wheat coleoptile or pea stem tissue with subsequent extraction and paper chromatographic analysis of the products present in the tissue and in the residual solution. For each treatment, 100 1-cm. coleoptile segments or 50 1-cm. pea stem segments were placed in a petri dish containing 1,000 μg. of the compound in 50 ml. of distilled water, the solutions being then incubated in the dark for 48 hrs. at 25° C. It was usual to metabolize 4,000 μg. of each compound

in one experiment, this amount being evenly distributed among four petri dishes. A tissue-in-water treatment and a solution of each compound untreated with tissue were included as controls in each experiment. Bacterial contamination was negligible in all treatments since streptomycin at a concentration of $10^{-4}M$ was included in all solutions. After 48 hrs. the solutions together with tissue were frozen overnight at —10° C. On the following day the solutions were thawed and the four identically treated solutions of each compound combined. During this process the tissue was removed and rapidly ground to a fine paste and then recombined with the residual solutions. Compounds present in this homogenate were removed by acidifying the system to pH 2.5 and extracting three times with 200-ml. quantities of ethyl acetate. The combined extract was dried over anhydrous sodium sulfate and concentrated for analysis by paper chromatography.

Chromatographic analysis of each extract was carried out by the ascending method using Whatman No. 1 paper in all glass tanks. The solvent used in most instances was a mixture of *n*-butanol, ammonia (0.880), and water (100:3:18 v/v), although occasionally isopropanol, ammonia (0.880), and water (10:1:1 v/v) was employed for comparative purposes. Temperature was controlled at 20° C. and each chromatogram was developed for 12 hrs. Chromatograms for chromogenic analysis were spotted with amounts of ethyl acetate extract equivalent to 1,000 μg. of the compound in the original solution. After development, the papers were dried in air and in most cases sprayed with Ehrlich's reagent applied as 1 per cent *p*-dimethylaminobenzaldehyde dissolved in 50 per cent alcoholic HCl. Other sprays, such as the Salkowski reagent and nitrous acid reagent which were prepared by conventional methods, were also used.

When preparing chromatograms for biological examination, ethyl acetate extract equivalent to 2,000 μg. of the original compound was evenly distributed over 20 spots on a 10-inch wide sheet of Whatman No. 1 paper. After development, a longitudinal strip containing two spots was removed from one side of the chromatogram and sprayed with Ehrlich's reagent to establish the position of indole compounds. The rest of the sheet was cut transversely into twenty strips of equal size each corresponding to one-twentieth of the distance travelled by the solvent front. Each strip was thus equal to half an R_f unit, i.e., 0 to 0.05, 0.05 to 0.1, 0.1 to 0.15, etc. The strips were placed in petri dishes, one per dish, containing 10 ml. of distilled water, and the biological activity of any compound present in each strip was then determined by the wheat cylinder test. The activity of a control strip taken from above the starting line of the chromatogram was deter-

mined in each experiment. The results obtained are recorded in histogram form which shows clearly the position on each chromatogram of compounds with growth-promoting activity.

EXPERIMENTAL RESULTS AND DISCUSSION

The activity shown by members of the series of acids, amides, nitriles, and methyl esters in the wheat and pea tests are shown in Table 2. For convenience, the results for each series will be discussed below in separate sections together with the evidence obtained in chromatographic studies on the metabolism of these compounds in wheat and pea tissues.

Chromatographic analysis of the extracts of solutions of all the compounds incubated for 48 hrs. at 25° C. in the absence of tissue showed that with the exception of certain methyl esters, all were effectively stable under these conditions. In the case of the ester series, indications were obtained to show that several of these compounds will hydrolyze to a slight extent to the corresponding carboxylic acid when solutions are left in an incubator for 48 hrs. The production of the corresponding carboxylic acid in untreated solutions, however, was in no way comparable with the amount produced when the solutions were exposed to wheat or pea tissues.

ω-(Indole-3-)alkanecarboxylic Acids

With the exception of indole-3-carboxylic acid, all members of this series are highly active in the wheat cylinder, pea segment, and pea curvature tests (Table 2). The acetic, butyric, and caproic homologues are the most active members of the series, and this result is consistent with the probable degradation of the side-chain of the latter two acids by β-oxidation to yield the highly active acetic derivative. The activity shown by the propionic, valeric, and heptanoic derivatives appears, at first, to be inexplicable in terms of β-oxidation of the side-chain within the test tissues since the expected end-product, namely indole-3-carboxylic acid, has no growth-promoting activity.

When solutions of all these acids were exposed to wheat coleoptile or pea stem tissues, the chromatograms obtained using extracts of the residual tissues and solutions provided clear evidence that β-oxidation of the side-chain of the higher homologues had occurred in both tissues employed (Figure 1). Thus both chromatograms showed evidence of a blue spot corresponding to the acetic acid in extracts from treated butyric and caproic acid solutions, and furthermore, the caproic derivative also yielded the butyric acid presumably as an intermediate degradation product. Similarly, a blue spot corresponding to the propionic acid was obtained in both tissues from the valeric and heptanoic acids, the latter acid also producing the

Table 2. Growth regulating activities of ω-(3-indole-3-)alkanecarboxylic acids, carbonamides, methyl esters, and nitriles in the wheat cylinder, pea segment, and pea curvature tests.*

Compound	Response as Per Cent of Control at Molar Concentrations Indicated										Response in Pea Curvature Test in Arbitrary Units (Molars)				
	Wheat Cylinder Test					Pea Segment Test									
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Indole-3-carboxylic acid	102	103	100	100	98	100	101	101	102	103	0	0	0	0	0
Indole-3-acetic acid	120	142	156	160	158	102	107	127	130	132	0	1	2	4	6
β-(Indole-3-)propionic acid	102	103	102	125	156	99	101	107	126	132	0	0	3	5	6
γ-(Indole-3-)butyric acid	117	135	160	161	157	101	110	124	132	135	0	2	4	6	6
δ-(Indole-3-)valeric acid	103	102	102	113	143	99	102	104	122	130	0	0	1	4	6
ϵ-(Indole-3-)caproic acid	115	135	150	161	147	103	112	124	130	136	0	2	4	6	6
ζ-(Indole-3-)heptanoic acid	100	99	102	124	149	101	103	104	121	130	0	0	0	3	6
Indole-3-carbonamide	100	101	101	101	100	100	101	103	101	100	0	0	0	0	0
Indole-3-acetamide	102	103	101	102	141	101	103	109	122	132	0	0	0	1	3
β-(Indole-3-)propionamide	98	98	101	100	123	101	103	102	104	124	0	0	0	0	3
γ-(Indole-3-)butyramide	100	101	109	148	166	103	110	120	128	132	0	1	3	5	6
δ-(Indole-3-)valeramide	101	99	101	105	124	98	102	101	106	125	0	0	0	1	4
Methyl indole-3-carboxylate	100	102	101	100	102	99	100	100	100	97	0	0	0	0	0
Methyl indole-3-acetate	126	147	156	164	156	111	120	125	127	130	1	2	3	4	6
Methyl β-(indole-3-)propionate	99	98	114	158	153	102	104	107	124	130	0	0	2	5	6
Methyl γ-(indole-3-)butyrate	115	155	165	158	151	104	108	120	130	132	1	2	3	5	6
Methyl δ-(indole-3-)valerate	98	100	116	156	152	98	100	105	112	126	0	0	2	4	6
Indole-3-nitrile	99	99	100	101	105	99	99	100	99	101	0	0	0	0	0
Indole-3-acetonitrile	131	160	167	170	166	100	102	103	111	114	0	0	0	1	2
β-(Indole-3-)propionitrile	99	99	98	98	126	101	100	103	103	106	0	0	0	0	1
γ-(Indole-3-)butyronitrile	100	115	152	174	164	99	98	99	99	100	0	0	0	0	0
δ-(Indole-3-)valeronitrile	102	99	100	105	148	100	99	101	98	98	0	0	0	0	0
ϵ-(Indole-3-)capronitrile	121	155	168	174	160	99	98	98	98	99	0	0	0	0	0
ζ-(Indole-3-)heptanonitrile	107	113	135	162	160	101	100	104	108	119	0	0	1	2	4

* In the wheat cylinder test values at 109 are significant at 5 per cent level, 113 at the 0.1 per cent level. In the pea segment test 105 is significant at 5 per cent, 107 at 1 per cent, and 108 at 0.1 per cent.

valeric acid as an intermediate product. Further proof of the presence of one or more of these active acids in each extract was obtained from the bioassay of separate chromatograms of each extract, the results of which are presented in Figure 2.

Although, on the basis of β-oxidation, the propionic, valeric, and heptanoic acids would be expected to yield indole-3-carboxylic acid as end-product, no evidence was obtained in these experiments for the production of this acid during the 48-hr. treatment period employed. During this time interval, however, the valeric and heptanoic acids were readily converted in both wheat and pea tissues to the propionic homologue (Figure 1), and this fact must be considered in relation to the high growth-regulating activity shown by these compounds (Table 2). When duplicate chromatograms containing extracts of the wheat or pea-treated propionic acid were sprayed with Ehrlich's reagent and examined by the wheat cylinder bioassay technique, substantial quantities of this acid were found to be present in each extract and only one zone of high growth-promoting activity was revealed, which coincided exactly with the region of the chromatogram containing the unchanged propionic acid. These results clearly indicate that the side-chain of β-(indole-3-)propionic acid is not readily degraded in wheat or pea tissue and that this molecule, like that of indole-3-acetic acid, can show growth-regulating activity *per se*.

On chromatograms from extracts of both wheat and pea-treated propionic, valeric, and heptanoic acids a greenish-orange spot (R_f 0.25) developed with Ehrlich's reagent immediately above the position of the propionic acid (R_f 0.35). The identity of this compound is under investigation. It is inactive in the wheat cylinder, pea segment, and pea curvature tests and, although its color reaction with Ehrlich's reagent and R_f in butanol-ammonia-water suggest that it is β-(indole-3-)acrylic acid, this has not been confirmed in further work.

In pea metabolism experiments, the pattern of metabolites on the chromatogram (Figure 1) as revealed by the Ehrlich's reagent is similar to that obtained with extracts from wheat-treated solutions. There is, however, one important difference, this being the appearance on chromatograms from pea-treated solutions of distinct blue spots with low R_f values. These substances are closely similar in R_f and color reaction to those reported for indole-3-acetylaspartic and indole-3-propionylaspartic acids which Andreae and Good (1, 2) have shown to occur in pea metabolism experiments with indole-3-acetic and indole-3-propionic acids. Moreover, Fang *et al.* (3) have also recently confirmed by means of a tracer technique that the major metabolic product obtained in pea tissue from exogenously supplied indole-3-acetic acid-1-C^{14} is indole-3-acetylaspartic acid. It is very probable, there-

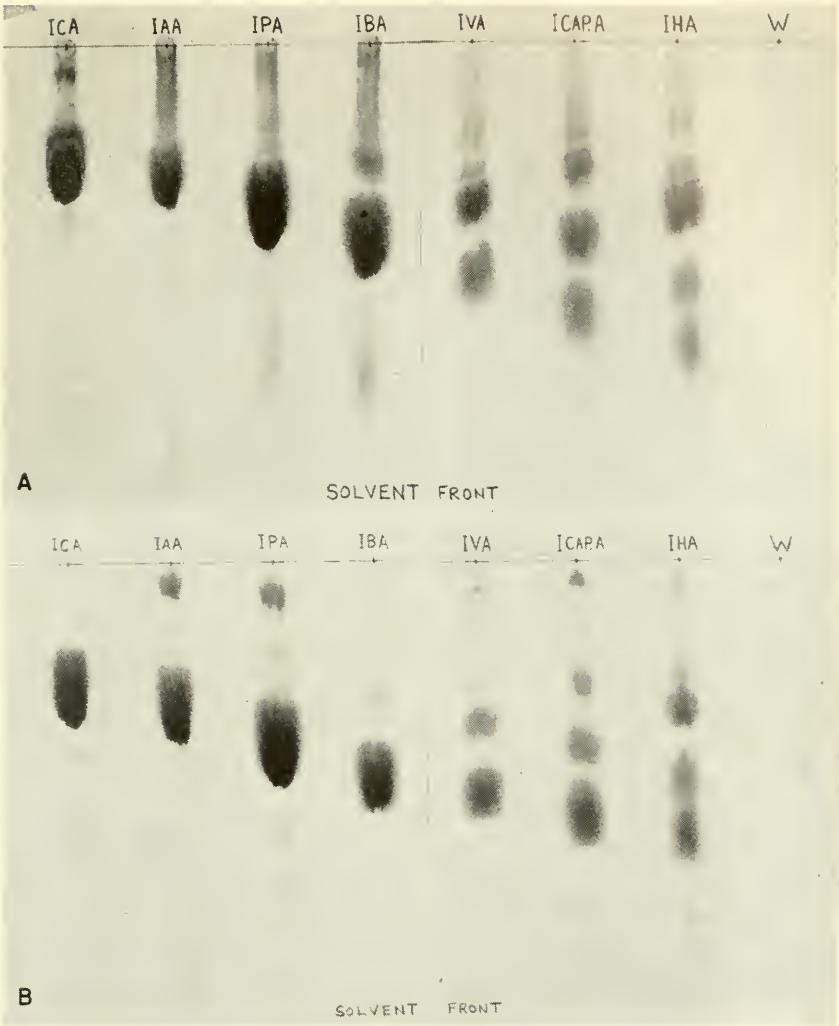

Fig. 1. Chromatograms of extracts of (**A**) wheat-treated and (**B**) pea-treated solutions of ω-(indole-3-)alkanecarboxylic acids sprayed with Ehrlich's reagent. See Table 1 for chemical names corresponding to the abbreviations.

fore, that the compounds of low R_f present in our extracts of pea-treated acetic and propionic acids represent the products of an in vivo condensation reaction between aspartic acid and each of these indole acids. In support of this, the compound obtained from the metabolized acetic acid was shown on further chromatographic analysis to behave as authentic indole-3-acetylaspartic acid. Furthermore, bioassay of a chromatogram containing the extract from the metabolized

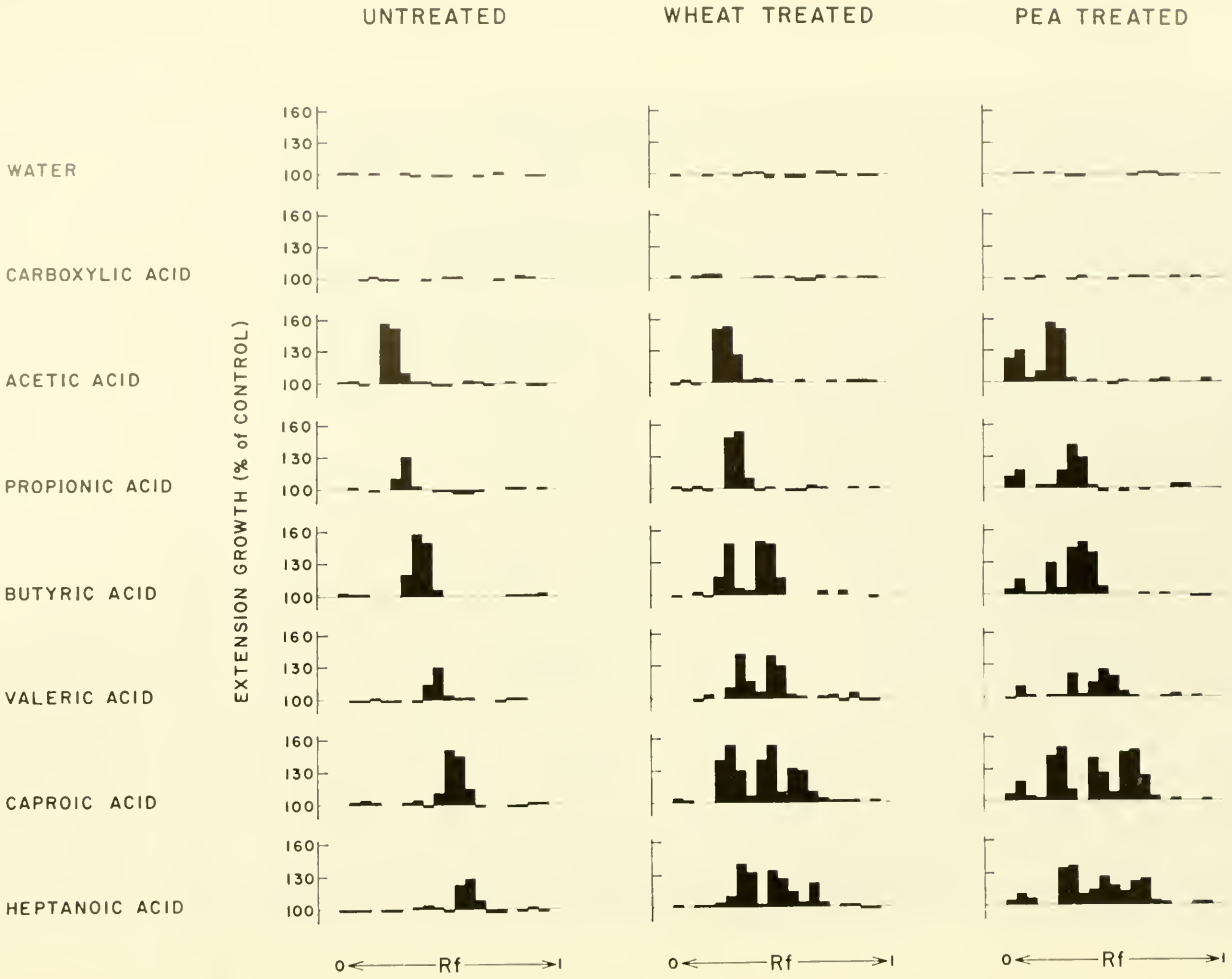

Fig. 2. Histograms showing activities in the wheat cylinder test of chromatograms of extracts from untreated, wheat-treated and pea-treated solutions of ω-(indole-3-)alkanecarboxylic acids.

acetic acid showed that the compound occurring at low R_f possessed growth-promoting activity (Figure 2), a result which is consistent with the activity previously reported for authentic indole-3-acetylaspartic acid (1). With the ethyl-acetate extraction technique used in this series of experiments, it has been possible to demonstrate the presence of one or more of these aspartic acid condensation products in the extracts of all the pea-treated indole acids, except in that obtained from indole-3-carboxylic acid. Thus, in addition to the metabolites produced in the β-oxidative degradation of these acids in pea tissue, all the higher homologues yielded small blue spots with low R_f values which corresponded chromatographically with either indole-3-acetyl- or indole-3-propionylaspartic acids (Figure 1).

ω-(Indole-3-)alkanecarbonamides

With the exception of indole-3-carbonamide, all members of this series showed growth-promoting activity in the three tests employed (Table 2). Such activity is consistent with hydrolysis of these amides with the test tissue to the corresponding carboxylic acid, which may then be active *per se* or converted to an active product by β-oxidation. Although no conversion of amide to acid occurred in the absence of tissue, clear evidence for this was found on the chromatograms obtained from metabolism experiments with both wheat and pea tissues (Figure 3) and from the bioassay results of comparable chromatograms (Figure 4). Thus, the chromatogram of metabolized acetamide and propionamide showed distinct blue spots (at R_f 0.24 and 0.30) when sprayed with Ehrlich's reagent, which corresponded respectively to indole-3-acetic and β-(indole-3-) propionic acids. Each chromatogram also showed a second spot with a high R_f value which represented the unchanged amide present in the extract. The metabolized butyramide and valeramide yielded three large blue spots on the chromatograms which corresponded with three major peaks of activity in the bioassay results. In the case of the butyramide, the first two spots correspond respectively to indole-3-acetic and -butyric acids, whereas the third spot near the solvent front was the residual amide. Similarly, on the valeramide chromatograms, the first two major spots correspond respectively to indole-3-propionic and -valeric acids and the third spot was again due to the residual amide. This amide also yielded appreciable quantities in the extracts from both wheat and pea-treated solutions of the unknown acidic compound at R_f 0.25 which gave a distinct greenish-orange spot with Ehrlich's reagent. The presence of aspartic acid conjugation products with low R_f values was again observed on the chromatograms from pea metabolism experiments (Figure 3).

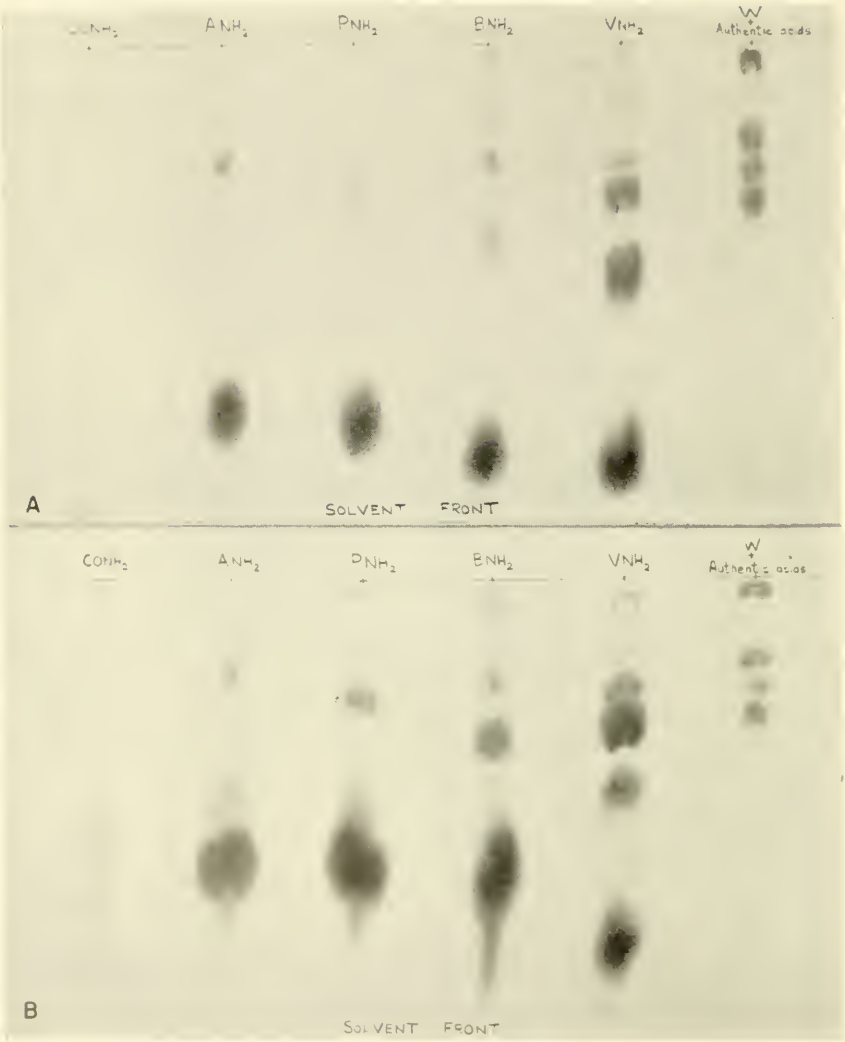

Fig. 3. Chromatograms of extracts of (**A**) wheat-treated and (**B**) pea-treated solutions of ω-(indole-3-)alkanecarbonamides sprayed with Ehrlich's reagent. The authentic acids chromatographed in the water plus tissue extract (**W**) are, in descending order, indole-3-acetylaspartic, indole-3-carboxylic, indole-3-acetic, and β-(indole-3-)propionic acids. See Table I for chemical names corresponding to the abbreviations.

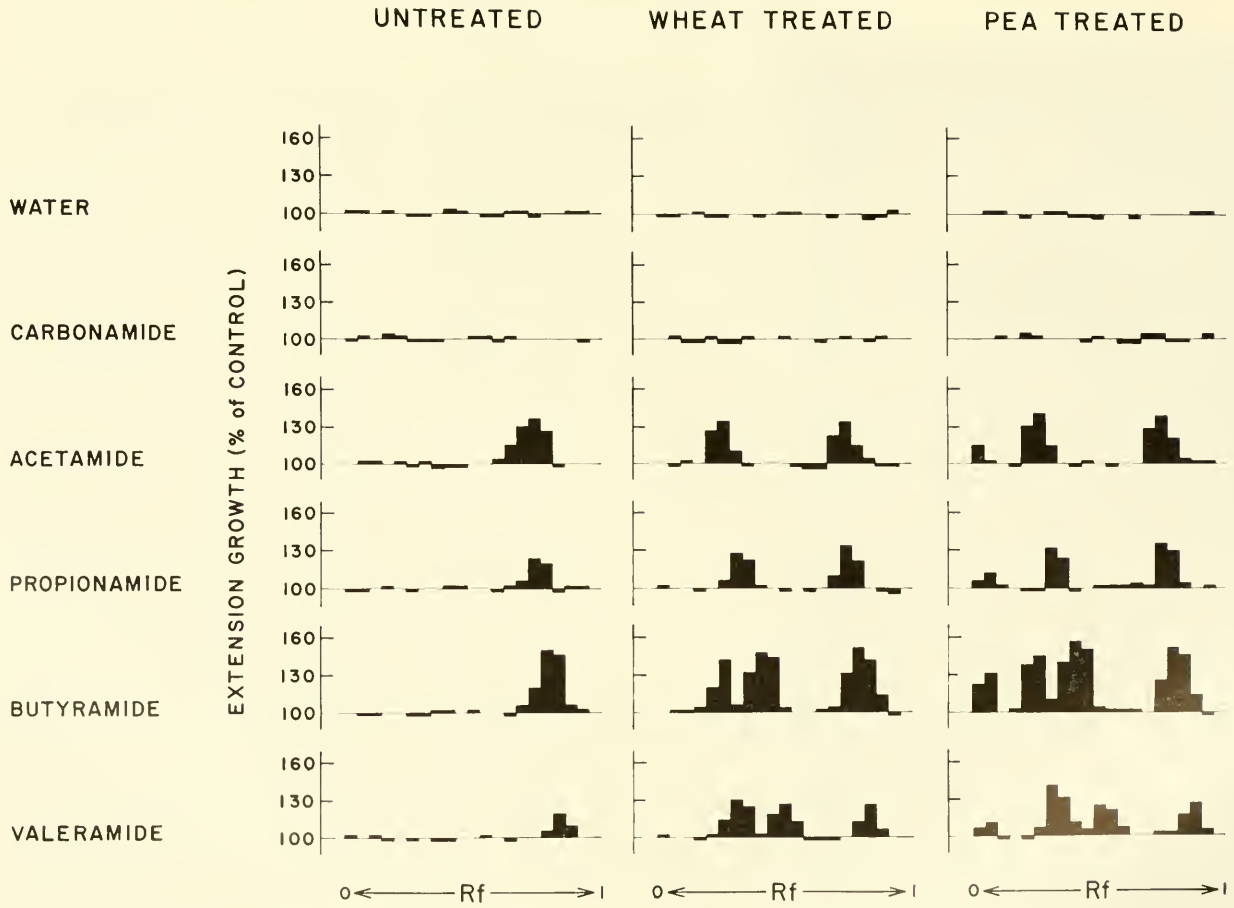

Fig. 4. Histograms showing activities in the wheat cylinder test of chromatograms of extracts from untreated, wheat-treated, and pea-treated solutions of ω-(indole-3-)alkanecarbonamides.

From the quantities of acids detected on these chromatograms, as revealed by their spot size and intensity of color, it is apparent that the members of this homologous series of amides vary in their susceptibility to hydrolase action in wheat and pea tissues. The results indicate that the differences observed are related to the distance between the ring system and the terminal amide group. When these are directly attached to each other, as in 3-indolecarbonamide, only slight production of the corresponding carboxylic acid occurred, whereas when a comparatively long side-chain separates the amide group from the indole ring, as for example in δ-(indole-3-)valeramide, hydrolysis of $-CONH_2 \longrightarrow -COOH$ readily occurred.

Methyl Esters of ω-(indole-3-)alkanecarboxylic Acids

The pattern of biological activity shown by this homologous series of esters is similar to that already observed with the corresponding acids and carbonamides. All members of the series, except methyl indole-3-carboxylate, showed high growth-promoting activity in the three tests employed (Table 2). The activity of the acetate and the higher homologues is consistent with their probable conversion within the test tissue to the corresponding carboxylic acid which is followed, in the case of the butyrate and valerate, by β-oxidation of this acid to yield respectively indole-3-acetic or β-(indole-3-)propionic acids. The chromatograms (Figure 5) and the bioassay results (Figure 6) obtained from metabolism experiments show clear evidence for this type of degradation in wheat and pea tissues. Furthermore, the chromatographic results indicate that methyl indole-3-carboxylate is also hydrolyzed to the corresponding carboxylic acid in both these tissues.

The results from pea metabolism experiments again provide evidence for the formation of aspartic acid derivatives in this tissue. For example, in extracts of the metabolized acetate, a large blue spot was obtained at R_f 0.05 which was chromatographically identical with authentic indole-3-acetylaspartic acid. Other substances produced in metabolism experiments with this series of esters include the greenish-orange compound at R_f 0.25 which appeared in extracts of both wheat and pea-treated propionate and valerate and also indole-3-carboxylic acid, which occurred not only on the chromatogram from the treated carboxylate, but was also apparent in slight quantities in extracts from metabolized indole-3-acetate.

The chromatographic results (Figure 5) obtained with these esters indicate that conversion of $-COOCH_3 \longrightarrow -COOH$ occurs more readily as the series is ascended. When the side-chain is short, only a small amount of the corresponding carboxylic acid is produced,

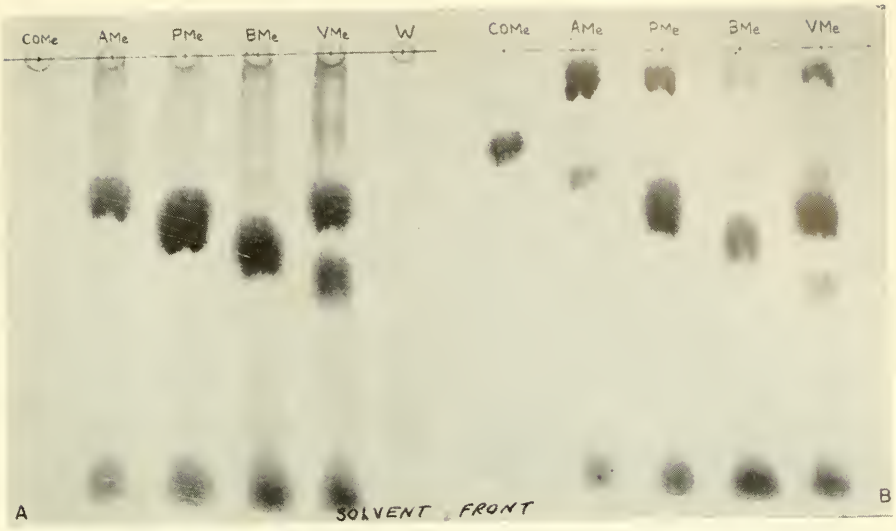

Fig. 5. Chromatograms of extracts of (**A**) wheat-treated and (**B**) pea-treated solutions of the methyl esters of ω-(indole-3-)alkanecarboxylic acids sprayed with Ehrlich's reagent. See page 73 for chemical names corresponding to the abbreviations.

whereas when the side-chain is comparatively long, hydrolysis to yield the corresponding acid readily occurs. This situation, like that already observed in the corresponding homologous series of carbonamides, clearly suggests that enzymatic hydrolysis of the terminal ester grouping is influenced by the proximity of the indole ring.

ω-(Indole-3-)alkanenitriles

All of the nitrile series, except indole-3-nitrile, showed activity in the wheat cylinder test (Table 2, p. 77). This result suggests that hydrolysis of -CN ⟶ -COOH, followed in the case of higher members of the series by β-oxidation to yield either the highly active acetic or propionic acids, is the most probable degradation pathway for members of this series in wheat tissue. The chromatographic and bioassay results (Figures 7 and 8) obtained in wheat metabolism experiments support this view. Thus, in extracts of the metabolized nitrile, acetonitrile, and propionitrile, the corresponding carboxylic, acetic, and propionic acids were clearly apparent on the chromatogram (Figure 7). Similarly, the butyro- and valero-nitriles were shown to produce not only the corresponding butyric and valeric acids, but also, respectively, the acetic and propionic acids by β-oxidation. With the capro- and heptano-nitriles, however, no indication of the presence of the corresponding caproic and heptanoic acids was observed on the chromatogram, but the presence of their lower alternate homologues, which could have arisen from the β-oxidation of these two

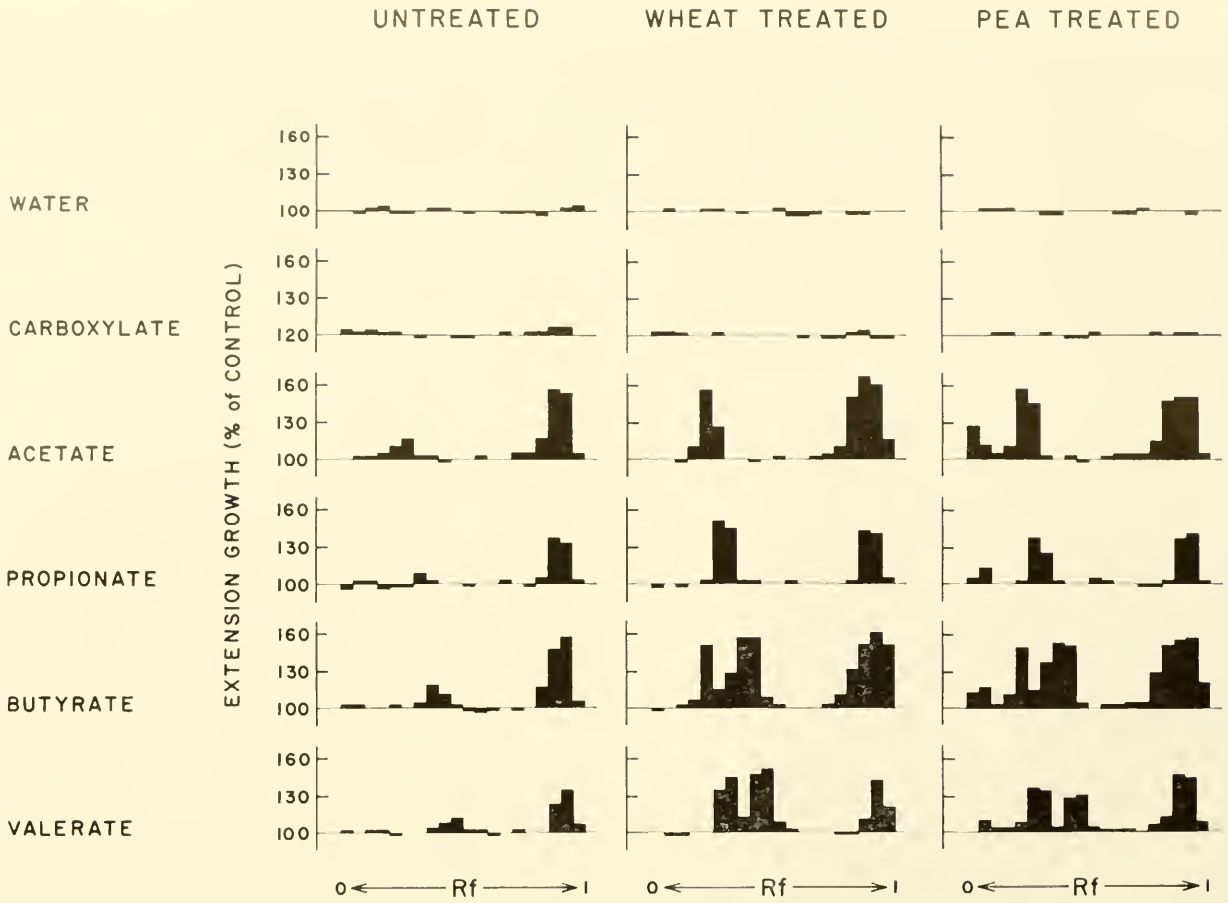

Fig. 6. Histograms showing activities in the wheat cylinder test of chromatograms of extracts from untreated, wheat-treated, and pea-treated solutions of the methyl esters of ω-(indole-3-)alkanecarboxylic acids.

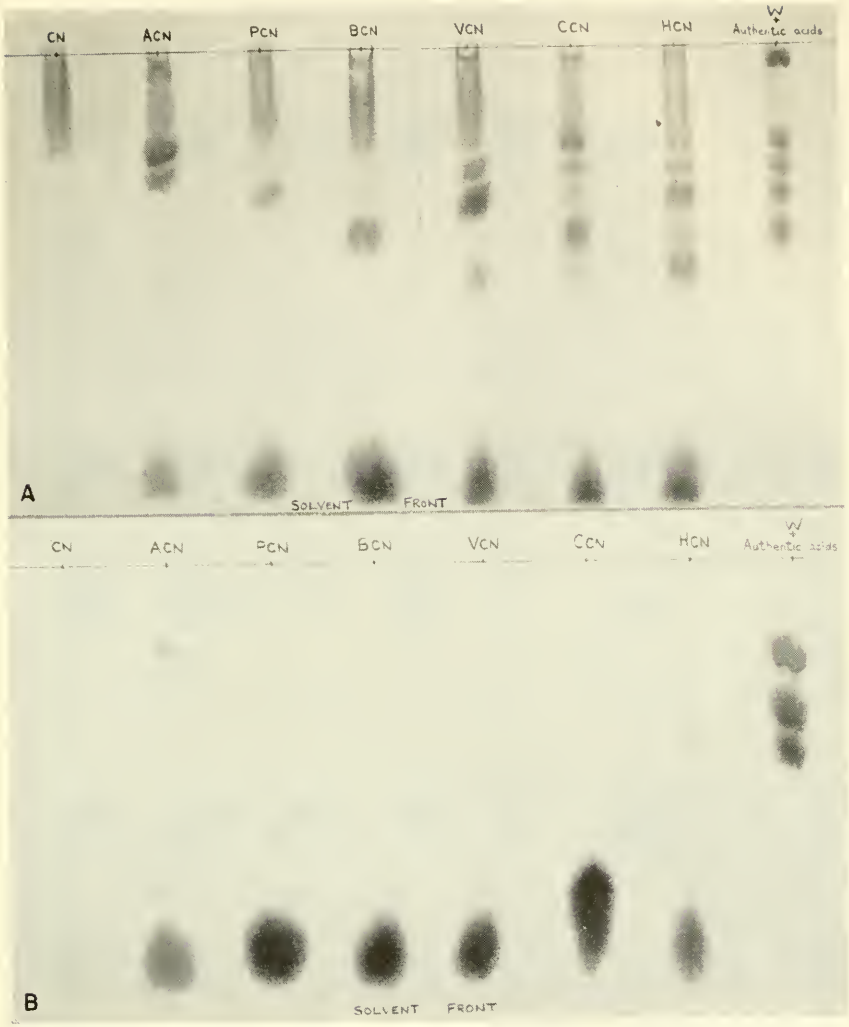

Fig. 7. Chromatograms of extracts of (**A**) wheat-treated and (**B**) pea-treated solutions of ω-(indole-3-)alkanenitriles sprayed with Ehrlich's reagent. See Table 1 for chemical names corresponding to the abbreviations.

higher acids, was clearly revealed. It is of further interest to note that the chromatographic and bioassay results with the wheat-treated aceto-, butyro- and capro-nitriles revealed traces of a compound at R_f 0.04 which from its R_f, color reactions and biological activity would appear to be indole-3-acetylaspartic acid.

As previously observed (7, 8), indole-3-acetonitrile is converted in wheat tissue not only to the corresponding acetic acid but also to indole-3-carboxylic acid. The latter reaction, which involves the conver-

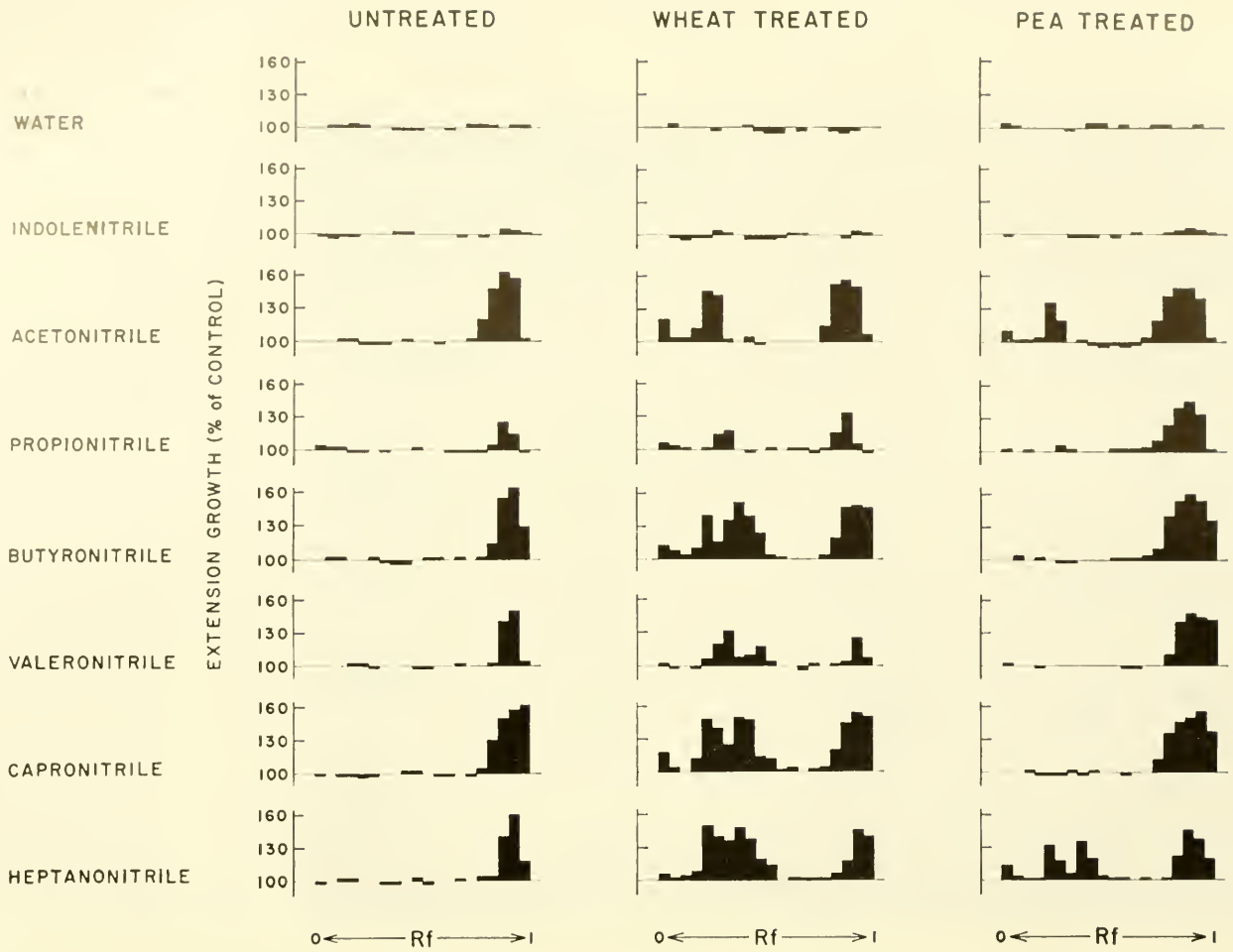

Fig. 8. Histograms showing activities in the wheat cylinder test of chromatograms of extracts from untreated, wheat-treated, and pea-treated solutions of ω-(indole-3-)alkanenitriles. The authentic acids chromatographed in the water plus tissue extract (W) are, in descending order, indole-3-acetylaspartic, indole-3-carboxylic, indole-3-acetic, β-(indole-3-)propionic, and γ-(indole-3-)butyric acids.

sion of $-CH_2CN \longrightarrow -COOH$ and is considered an example of the α-oxidation of nitriles, might be expected to occur with all the higher homologues of the nitrile series when metabolized in wheat tissue. In each case, the $-CH_2CN$ grouping would be converted to -COOH and the resulting acid would then be subjected to β-oxidation. The chromatographic results (Figure 7) support this possibility and indicate that the higher indole nitriles are metabolized in wheat not only by a sequence involving initial hydrolysis of the nitrile to the corresponding carboxylic acid and subsequent β-oxidation, but also by a sequence involving an initial α-oxidation of the nitrile to the next lower carboxylic acid which then undergoes β-oxidation. Evidence for these two distinct pathways is most clearly revealed on chromatograms of the metabolized capro- and heptano-nitriles. Thus, for example, the extract of wheat-treated capronitrile yielded spots at R_f 0.26 and 0.40 which were identified as the acetic and butyric acids respectively and presumably arose via the hydrolysis followed by β-oxidation pathway. Distinct blue spots were also evident on the chromatogram at R_f 0.32 and 0.49, and from their R_f value, color reactions, and biological activity the compounds were identified as the propionic and valeric acids, respectively. The latter acid could arise from α-oxidation of the capronitrile, and the propionic acid would then be produced by β-oxidation. A similar explanation could account for the degradation products observed on the chromatogram obtained from the metabolized heptanonitrile.

In the extracts of both of these higher nitriles a further compound was apparent which from its R_f value (0.20), characteristic color reactions, and absence of biological activity was identified as indole-3-carboxylic acid. Although this acid is the logical end product of the heptanoic acid $\longrightarrow$ valeric acid $\longrightarrow$ propionic acid β-oxidation sequence, earlier metabolism experiments using the authentic acids produced no evidence that this final stage occurred in wheat tissue (Figure 1). In the present experiments, however, in addition to its appearance in appreciable quantities during the metabolism of the capro- and heptano-nitriles, a small amount of indole-3-carboxylic acid was also observed on the chromatogram of the metabolized valeronitrile. Hence there is a possibility that in wheat tissue these higher nitriles are degraded by another mechanism which involves oxidation of the methylene group adjacent to the indole ring. This type of degradation reaction may be referred to as ω-oxidation.

The following are the results obtained with this series of nitriles in the pea segment and pea curvature tests (Table 2). Only indole-3-acetonitrile and ζ-(indole-3-)heptanonitrile were active, suggesting that hydrolysis of $-CN \longrightarrow -COOH$ does not readily occur with all mem-

bers of this series in pea tissue. The chromatographic (Figure 7) and bioassay results (Figure 8) from metabolism experiments with pea tissue sustain this conclusion, only the acetonitrile and heptanonitrile being found to yield acidic compounds with high growth-promoting activity. The metabolized acetonitrile gave rise to three major spots on the chromatogram at R_f 0.21, 0.26, and 0.93 which were identified as indole-3-carboxylic acid, indole-3-acetic acid and indole-3-acetonitrile respectively. Production of the acetic acid confirms the view that the activity of indole-3-acetonitrile in the pea tests is related to its conversion to this highly active acid, and the appearance of appreciable quantities of indole-3-carboxylic acid indicates that the conversion $-CH_2CN \longrightarrow -COOH$ also readily occurs in pea tissue. Of the higher nitriles, only the heptanonitrile yielded evidence of acidic degradation products, and from their R_f value, color reaction, and biological activity these compounds were identified as indole-3-acetylaspartic acid (R_f 0.05), indole-3-carboxylic acid (R_f 0.20), indole-3-acetic acid (R_f 0.25), and γ-(indole-3-)butyric acid (R_f 0.38). The acetic and butyric acids presumably arose by β-oxidation of $_e$-(indole-3-)caproic acid produced by α-oxidation of the heptanonitrile. The appearance of indole-3-carboxylic acid may be due to ω-oxidation of this nitrile as occurred in wheat tissue.

SUMMARY

It would appear from the metabolism experiments that the growth-regulating activity shown by all the higher indole acids in wheat and pea tissue is due to the breakdown of the side-chain of each acid by β-oxidation to yield either the highly active acetic or propionic acids as end product. The activity shown by the higher amides and methyl esters can be similarly explained, except that with these compounds hydrolysis of the amide or ester grouping to the corresponding carboxylic acid precedes the β-oxidative degradation reactions. The contrasting behavior of the nitrile series in the three standard tests is evidently due primarily to the different abilities of wheat and pea tissue to convert the higher nitriles either by hydrolysis to the corresponding acid or by α-oxidation to the next lower carboxylic acid. Except in the case of the heptanonitrile, neither of these reactions appears to proceed in pea tissue with the higher members of this series, and in consequence further degradation of the side-chain does not occur. On the other hand, in wheat tissue the higher nitriles are subject to two and possibly three distinct degradation pathways, namely (a) hydrolysis to the corresponding acid followed by β-oxidation, (b) α-oxidation to the next lower carboxylic acid followed by β-oxidation, and (c) ω-oxidation to yield in all instances indole-3-carboxylic acid.

All these results provide further evidence that, although the tissues of different plants can often carry out the same degradation reactions, thus suggesting the presence of similar enzyme systems, these enzymes can nevertheless show different behavior towards a specific substrate. Studies of this type are of use in defining some of the biochemical reactions which are important in plant metabolism and which may be involved in the regulation of plant growth by synthetic chemicals.

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DISCUSSION

Dr. Thimann: Your bioassay pictures appear to settle the question as to whether indole-3-butyric and indole-3-caproic acids are active *per se* or only on conversion to indole-3-acetic acid. They are apparently active *per se*. Would you agree with that? There is clearly more than one peak.

Dr. Wightman: We have often discussed this question at Wye, particularly with regard to whether the activity of indole-3-butyric acid

is due solely to its conversion to indole-3-acetic acid in the test tissues or whether it is due partly to the production of the acetic acid and partly to the fact that the butyric acid molecule itself possesses growth-promoting activity. I favor the view that indole-3-butyric acid is active *per se,* because I find it difficult to explain the high activity of this acid in the wheat and pea tests in terms of the small amount of IAA produced in metabolism experiments.

Dr. Thimann: It would mean 100 per cent conversion.

Dr. Wightman: Yes, it would, because if you examine the activity of indole-3-acetic and indole-3-butyric acids over a wide range of concentrations, you find that the butyric acid is more active than the acetic acid at lower concentrations. Unless you assume that the butyric acid penetrates the tissue more readily and is then converted almost 100 per cent to the acetic acid, it is difficult to explain the high activity of the butyric acid at low concentrations, solely in terms of its conversion to IAA.

Dr. Thimann: But still you do have two peaks in the bioassay of the chromatogram of metabolized indole-3-butyric acid.

Dr. Wightman: That is correct. The first peak is due to the small amount of indole-3-acetic acid produced from the metabolism of the butyric acid and the second peak is due to the residual, unchanged butyric acid. Now, as we have already discussed, the activity shown by the residual butyric acid may be due either to the compound itself or the result of its conversion to IAA in the tissue used in the bioassay.

Dr. Andreae: We tried solutions and could never find indole-3-acetylaspartic acid. Do you analyze solutions only, not the tissues?

Dr. Wightman: No, we do analyze both the residual solutions and the treated tissue. In our extraction procedure we first remove the segments of tissue from the residual solution and immediately grind them up with acid-washed sand. The macerated tissue is then added back to the residual solution and after acidification the homogenate is extracted several times with ethyl acetate. One point I would like to make is that our pea metabolism experiments differed from yours in that we used much less tissue. According to your papers, you used 100 g. of pea stem tissue per treatment, whereas we used only approximately 1 g. of tissue per treatment. The reason why we extracted both the treated tissue and the residual solution was because we found in earlier metabolism experiments with phenoxy compounds that we obtained metabolites in the solution, and since we were anxious to obtain as much of these metabolites as possible, we extracted not only the tissue but also the residual solution. This, of course, resulted in

our including quite a considerable amount of the unchanged compounds in the extract.

Dr. Andreae: Whenever we applied the material to our chromatograms we used aliquots not exceeding 3 g. Did you ever get the amide?

Dr. Wightman: No, we found no evidence at all on our chromatograms for the formation of indole-3-acetamide in wheat tissue. This may be due to the fact that we did not use sufficient tissue in our treatments to produce enough indole-3-acetamide to give a color reaction on the chromatogram when sprayed with Ehrlich's reagent or a peak of activity in the bioassay. Certainly this amide is not as sensitive to Ehrlich's as many of the other indole compounds used in this investigation and is only active at fairly high concentrations in the wheat cylinder bioassay technique. Our results, therefore, do not preclude the possibility that indole-3-acetamide is formed during the metabolism of IAA in wheat tissue, but they do indicate that it is certainly not formed to any appreciable extent.

Dr. Thimann: We can confirm that too, because our work on barley tissue and also with the extracted enzyme shows not only that the amide is not formed in any appreciable amount but that if it were formed it is not acted on by the enzyme at any appreciable rate.

Dr. Wain: I think the most important thing that Dr. Wightman has mentioned is this conversion of the CH_2CN group to COOH, which is quite a new reaction. As he said, this is an alpha-oxidation of nitrile and involves the breaking of a carbon carbon bond with the loss of one carbon fragment. It's not strictly analogous to the breakdown of the cyanhydrin, as for example, the ones produced from glycosides. Recently we have shown that you can take cell-free extracts from pea tissue and effect alpha-oxidation of nitriles very readily. Here is the same type of breakdown which occurs in the animal since compounds like *p*-chlorobenzylnitrile fed to dogs are excreted as derivatives of *p*-chlorobenzoic acid.

Dr. Jepson: We must not forget that the whole of this indole story as related to plants sprang from investigations that weren't really on plants at all, but on human beings — the indole-3-acetic acid isolated by Kögl and Haagensmit from the urine of their laboratory assistant came not from the plants he ate but from the metabolism of his dietary tryptophan. I want to suggest that further information on plant indoles may well be obtained from studies of indoles obtained directly from animals, though of course in general they come initially from plants via tryptophan. For example, one is able to find in human urine two of the compounds that Dr. Wightman wanted to

find in the experiments that he's just related to us — indole-3-acrylic acid and indole-3-carboxylic acid. Indole-3-acrylic acid is a normal component of human urine as its glycine derivative. The small amount in all human urine from subjects on a normal mixed diet disappears on fruit-free and plant-free diets. It must be derived from some unknown indolic component, probably with a 3-carbon side chain, present in plant or fruit products. It is not derived from tryptophan, because a single subject can take as much as 10 g. of oral tryptophan (a procedure I don't recommend) without causing any indole-3-acrylic acid or its derivative to appear in the urine. But it is found in urine if Dr. Wightman's compound indole-3-propionic acid is fed, as reported by Decker. Indole-3-propionic acid has never been found, as far as I'm aware, in plants, but it may have some precursor which may give rise to it on ingestion. Another possibility, currently being investigated with Dr. K. N. F. Shaw, is that indole-3-pyruvic acid gives rise to the acrylic acid. Indole-3-pyruvic acid has had a checkered history in this connection, and it may be that there is some plant component other than tryptophan which gives rise to indole-3-pyruvic acid, say through the action of bacteria in the gut, and from that to indole-3-acrylic acid. These are probably acetic materials. They may be auxins, we do not know.

There are a lot of indoles which occur in plants which may well have important influences on animal metabolism. Serotonin is found in many plants, in banana fruit for example, in tremendous amounts physiologically speaking, together with smaller amounts of related amines; tomato contains a large amount of tryptamine, which will normally give rise to indole-3-acetic acid.

At the National Institutes of Health, we are giving drugs to patients to prevent or divert the normal oxidative metabolism of amines including tryptamine and serotonin. If patients on these drugs eat a lot of bananas, the accumulated indolic amines will materially affect their physiology. So we may find that this topic of indole auxins, which is branching out so rapidly into all fields of plant and agricultural physiology, may well find itself in human physiology as well.

Dr. Fawcett: There are two papers which give evidence that indolepropionic acid occurs in *Brassica* species (Planta 44: 103, 1954; Planta 50: 557, 1958). Although conversion of indolepropionic acid to indoleacrylic acid occurs in human metabolism, there is no evidence from our work that this occurs in pea or wheat metabolism. I have synthesized indoleacrylic acid, and when it is chromatographed on paper with an aqueous 20 per cent potassium chloride solution, it has an R_f of 0.14. The unknown compound we found in the metabolism experiments has a much higher R_f in this solvent (about 0.67) so that it apparently is not indoleacrylic acid.

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Growth Substance and Inhibitor Changes in Buds and Seeds in Response to Chilling

During the past 50 years, a number of hypotheses have been put forward to account for the phenomena of dormancy in plant organs. Since the discovery and isolation of plant growth hormones, however, many workers have attempted to explain dormancy in terms of these substances. Since the ability for growth is in some way arrested in dormant tissues, it is clear that dormancy is closely linked with general problems of growth control, and it is reasonable to consider how far the alternating cycles of growth and dormancy shown by many plants are controlled by specific growth substances.

Now dormancy can be envisaged as being due either to the lack of certain essential growth factors or to the presence of active growth inhibitors. Certain earlier authors suggested that the inability of dormant tissues to grow may be due to lack of auxin, and indeed several workers found that auxin appears in buds only in the latter part of the winter (2, 4, 24). It seems unlikely that dormancy is controlled primarily by auxin deficiency, however, since application of exogenous auxin is generally not effective in breaking dormancy. On the other hand, others have concluded that dormancy of buds is caused by the presence of supraoptimal concentrations of auxin (7, 17), but this view is difficult to reconcile with the fact that auxin levels are very low during the early stages of bud dormancy.

The view that bud dormancy may be due to specific growth-inhibiting substances was first put forward by Hemberg (10 to 14), who showed that the peel of dormant potatoes and the bud scales of *Fraxinus excelsior* contain growth-inhibiting substances, and that when dormancy is broken by chilling or treatment with ethylene chlorhy-

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drin, there is an associated decrease in the level of the endogenous inhibitors. Various workers have since investigated the changes in inhibitor content of buds during chilling, and most of these have confirmed that the inhibitor level gradually decreases during the winter, reaching a minimum in the spring when the buds are expanding (5, 9, 15). Phillips and Wareing (20) investigated the changes in inhibitor level of buds of *Acer pseudoplatanus* throughout the year and observed that the amount of inhibitor gradually increased in developing buds in the late summer, reaching a maximum in October. The inhibitor level decreased gradually during the winter, reaching a minimum in April when the buds were expanding. There is no doubt, therefore, that in this species there is a marked annual variation in inhibitor level which is correlated with the state of dormancy of the buds. Such a correlation does not, of course, necessarily imply a causal relationship, and studies of this sort on tree buds under natural conditions do not readily lend themselves to experimental techniques designed to elucidate whether the changes in inhibitor level *control* the changes in states of dormancy. For this latter purpose, seeds are much more suitable objects of study since they can readily be maintained under controlled conditions and can be more easily exposed to various chemical and other treatments.

Studies on changes of inhibitor levels in seeds in response to chilling have been very few. Barton and Solt (3) and Luckwill (19) observed some reduction in the inhibitor content of seeds of *Sorbus aucuparia* and apple, respectively, in response to chilling. Lasheen and Blackhurst (18) observed that ether-soluble inhibitors disappeared from seeds of *Rubus* during chilling, and the disappearance of the inhibitor was correlated with the breaking of dormancy and ability of the seeds to germinate. On the other hand, there was little correlation between the inhibitor content of the embryos and their state of dormancy. Several workers have studied dormancy in seeds of *Fraxinus* in relation to inhibitors. Ferenczy (8), using crude extracts of the various parts of the fruit of *F. excelsior,* concluded that most of the inhibitory material is present in a mucilaginous layer surrounding the seeds. He found a decrease in this inhibitory material during moist storage at both 20° and 5° C. Using ether and aqueous extracts of *F. spaethiana,* Asakawa (1) found that there was some inhibitory activity in the pericarps, but little in the seeds. The pericarp inhibitors decreased during moist storage at both 2° C. and laboratory temperatures, probably by leaching.

Studies With Seed of *Fraxinus excelsior*

During the past 3 years we have carried out a detailed study of dormancy in seeds of *F. excelsior,* particularly in relation to growth

substances and inhibitors. Ripe fruits of this species are in a very deep state of dormancy, and neither intact fruits nor seeds will germinate over a period of 16 months if they are maintained moist at laboratory temperatures. When the seed is shed, the embryos are morphologically complete, but they need to undergo a further period of maturation, during which there is considerable growth, involving both cell division and cell extension. This maturation takes place more rapidly at laboratory temperatures than at chilling temperatures. The embryos themselves are found to be dormant, when dissected out of the seeds. In order to obtain germination of the intact seeds or fruits, a period of chilling is required, and this is only effective after the embryo has undergone maturation at a higher temperature. The minimum treatment times for the seeds are 1 month at laboratory temperatures, followed by 5 to 6 months of chilling.

If the dry seed is extracted, first with ether and then with water, then a well-marked growth promoter is found in the ether fraction at R_f 0.8 to 0.9. This is found to be a neutral substance and it appears to be indole-3-acetonitrile (IAN), on the grounds of its R_f value in several solvents and color reactions. This promoter is gradually reduced as the embryo undergoes maturation at laboratory temperatures (Figure 1). There is evidence in some extracts (especially of the endosperm) of a second ether-soluble promoter, which may be IAA from its R_f value.

The aqueous extracts contain certain promoting regions at low

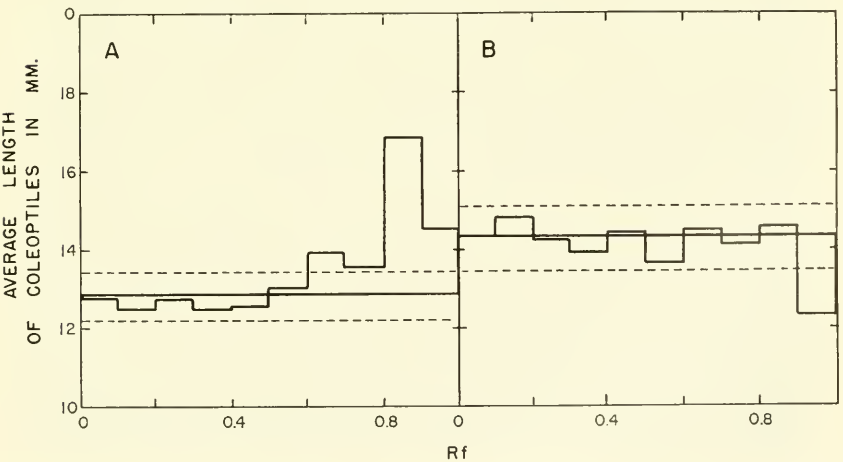

Fig. 1. Ether extracts of seeds of *Fraxinus excelsior* assayed by the *Avena* coleoptile test. **A** — fresh (untreated) seeds; **B** — seeds imbibed 3 months (embryo full size). Running solvent 99 parts 80 per cent aqueous isopropanol: 1 part ammonium hydroxide (S.G. 0.88); descending chromatography. Solid horizontal line indicates water control.

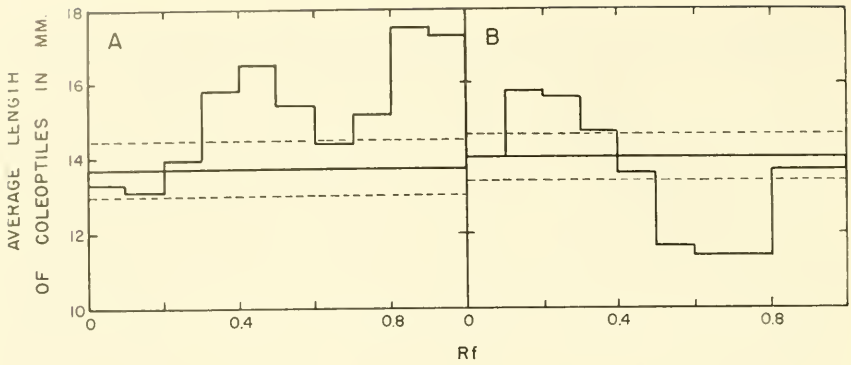

Fig. 2. Aqueous extract of *F. excelsior* seeds assayed by the *Avena* coleoptile test. **A** — extract of dry seeds; **B** — extract of seeds imbibed for 48 hrs. Solid horizontal line indicates water control. (Running solvent as for Figure 1.)

R_f values, but aqueous extracts of the dry seed contain no inhibitors. If, however, the seed is allowed to imbibe water at laboratory temperatures for 24 hrs. or more, then an inhibitory region appears on the chromatograms at R_f 0.7 to 0.9 (Figure 2). Evidently this inhibitor is metabolically produced, since it does not appear during moist storage at 0° C. This inhibitor, which is water soluble and ether insoluble, not only inhibits *Avena* coleoptile sections, but also cress roots, lettuce seeds, and embryos of *F. excelsior* (Figure 3). On germination the level of this water-soluble inhibitor appears to be depressed slightly and an ether-soluble inhibitor of the same R_f as the β-inhibitor of other workers appears.

The presence of this growth inhibitor raises two questions, viz. (1) Does the inhibitor play any significant role in the dormancy of the seed? (2) Does chilling-treatment remove the dormancy by bring-

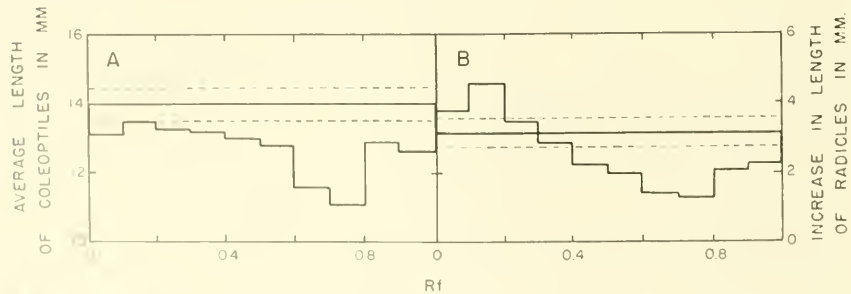

Fig. 3. Assay of aqueous extract of embryos of *F. excelsior*. Chromatogram halved longitudinally, **A** assayed with *Avena* coleoptile test, **B** assayed with leached *F. excelsior* embryos. Solid horizontal line indicates water control. (Running solvent as for Figure 1.)

ing about a reduction in the level of inhibitor? If the inability of dormant embryos to grow is due to the presence of the inhibitor, then it ought to be possible to show that removal of the inhibitor permits growth. Conversely, it ought to be possible to show that application of inhibitor to nondormant embryos prevents their growth.

An experiment was carried out to determine whether leaching of the dormant embryos would enable them to germinate. The embryos were dissected out from the seeds which had been imbibed at laboratory temperatures for some time. These embryos and an equal number of intact seeds from the same sample were then soaked in water for 48 hrs. The embryos were then dissected out from the washed seeds, both series of embryos were then placed directly on the glass of a petri dish, and wet filter paper was placed inside the lid of the upper dish. In this way any further leaching of inhibitor during the germination test was precluded. Within 3 days the embryos from which the endosperm and testa had been removed before soaking had germinated, whereas the embryos which had been removed from the seeds after the period of soaking remained dormant (Figure 4). It was found that *chilled* embryos were capable of germinating under the same conditions even without leaching, so that it seems unlikely

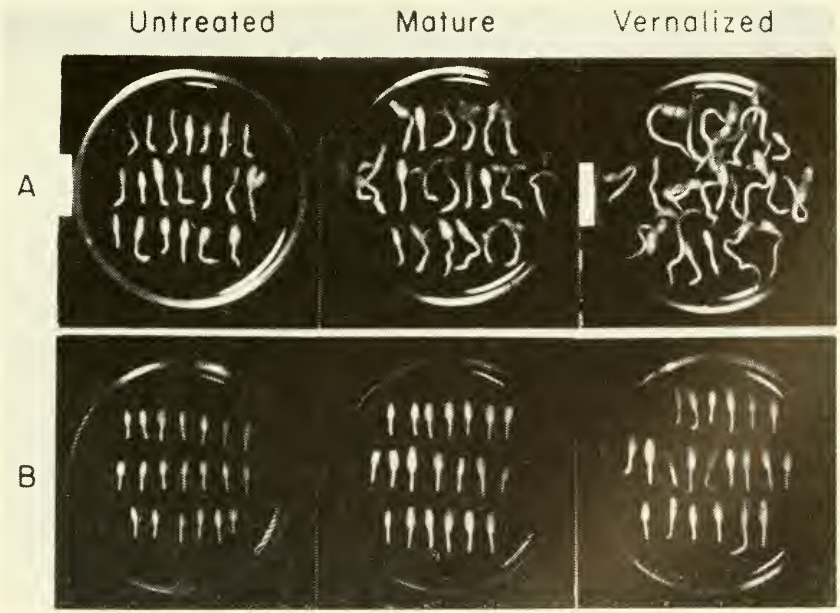

Fig. 4. Effect of leaching on dormancy of embryos of *F. excelsior*. Embryos in row **A** were soaked naked 48 hrs. whereas those in row **B** were derived from intact seeds which had been soaked 48 hrs. and the embryos excised *after* the leaching treatment.

that the failure to grow of unleached, unchilled embryos was due to an inadequate water content or unfavorable moisture conditions during the germination test.

It seemed probable that the leaching treatment would result in a reduction of the level of inhibitor in the embryos, as was found for *Xanthium pennsylvanicum* (23), but no consistent differences could be detected between the inhibitor contents of leached and unleached embryos. The mechanism of the leaching effect therefore remains obscure, but it is difficult to see how leaching can be effective in overcoming the dormancy of unchilled embryos except by removing some block to growth. It was observed that the subsequent growth of the seedlings from leached, unchilled embryos is stunted, whereas chilled embryos give rise to normal seedlings even if leached.

Experiments were also carried out to determine whether the application of the inhibitor to nondormant embryos prevented their growth. For this purpose, unchilled embryos were first leached for 48 hrs., and half were then planted on filter paper moistened with the inhibitor, and the remainder on filter paper moistened with water. The latter readily germinated, whereas very few of those planted on inhibitor solution showed any growth. Thus, the application of the inhibitor to excised leached embryos restores their dormancy. It seems probable, therefore, that the inhibitor must play an important role in controlling the dormancy of the embryos in vivo.

The question then arises as to whether chilling treatment is effective in breaking dormancy because it results in a reduction of the inhibitor level. To test this possibility, extracts were made of seeds which had been chilled at 0 to 1° C. for varying periods up to 6 months. It was found that there was very little change in the inhibitor content of the seeds (endosperm and embryo) over this period, despite the fact that seeds which had been chilled for 6 months readily germinated when transferred to warm conditions. No evidence could thus be obtained of an appreciable reduction of inhibitor level as a result of chilling.

Thiourea is also effective in stimulating the germination of dormant, unleached embryos; if the embryos are placed on filter paper moistened with 0.5 per cent thiourea, they germinate rapidly. In order to determine whether thiourea affects the level of inhibitor, embryos treated with 0.5 per cent thiourea for 48 hrs. were extracted and the inhibitor content compared with that of untreated embryos; no appreciable difference in inhibitor content could be detected. Neither chilling nor thiourea, therefore, brings about a significant reduction in the inhibitor content. It remains possible that chilling results in an increase in an endogenous promoter. No evidence could be

obtained, however, of any increase in auxins active in the *Avena* coleoptile test as a result of chilling. The discovery of mature gibberellins in higher plants opens up yet another possibility, since it is well known that gibberellic acid will break the dormancy of potato tubers (21), of resting-buds of woody species (6), and of certain seeds (16). Gibberellic acid was also found to stimulate the germination of dormant, unleached embryos of *F. excelsior*.

The possibility that chilling might bring about an increase in growth promoters other than auxins was therefore investigated, dormant, unleached embryos of *F. excelsior* being used as test objects. Aqueous extracts of chilled and unchilled embryos were chromatographed, and the dried chromatograms moistened with distilled water. Dormant, unleached embryos were then planted directly onto the different sections of the chromatograms. It was found that with the extract of chilled embryos, 8 of 10 embryos germinated within 48 hrs. in the region R_f 0.2 to 0.3, and there was some germination at R_f 0.1 to 0.2. No germination occurred with the extracts of unchilled embryos or in the water controls (Figure 5). This germination stimulator is present only in the *embryo* and not in the endosperm of

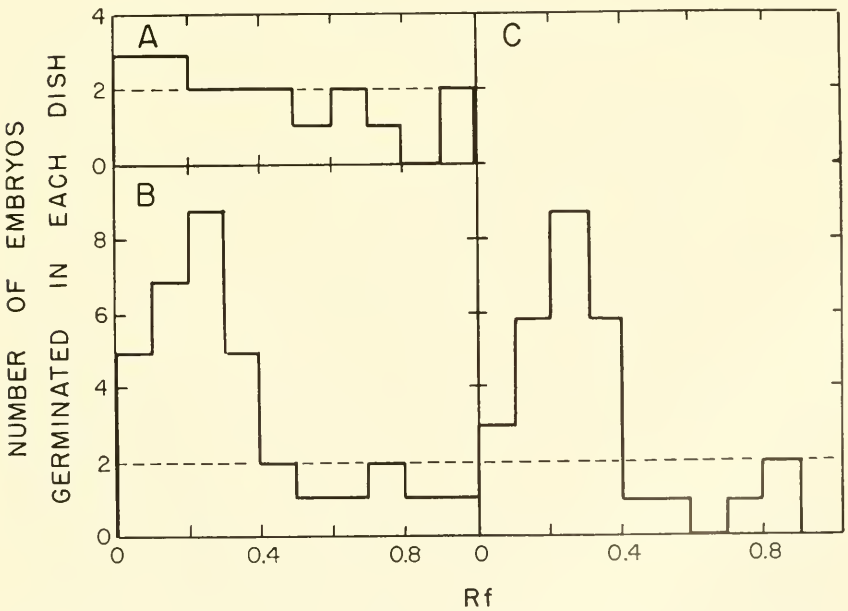

Fig. 5. Effect of aqueous embryo extracts of *Fraxinus excelsior* seeds on germination of unchilled embryos. **A** — extract of unchilled embryos. **B** — extract of chilled, nongerminating embryos. **C** — extract of chilled, germinating embryos. Dotted horizontal line indicates water control.

chilled seeds. The subsequent growth of unchilled embryos stimulated to germinate in this way appears to be normal.

It seems clear that the extracts of chilled embryos contain certain substances not present in unchilled embryos, which are capable of inducing germination in the latter. It would seem, therefore, that the effect of chilling is not to reduce the level of inhibitor but to increase the level of an endogenous germination stimulator which overcomes the effect of the inhibitor; some evidence in support of this hypothesis was obtained in the following experiment. The region of a chromatogram containing the inhibitor (R_f 0.65 to 0.80) was cut into four strips and each strip moistened with one of the following: (1) eluate from R_f 0.2 to 0.3 of a chromatogram of extract of 10 unchilled embryos; (2) eluate from R_f 0.2 to 0.3 of a chromatogram of extract of 10 chilled embryos; (3) distilled water (control); (4) 0.25 per cent thiourea. A further control of filter paper moistened with distilled water only was set up (i. e., no inhibitor). Leached (nondormant) embryos were then planted on the paper. It was found that there was little germination either on the paper containing only inhibitor or inhibitor plus extract of unchilled embryos (Table 1). On the

Table 1. Interaction between inhibitor and germination promoter in embryos of *F. excelsior*.

Germination Medium*	Percentage Germination
Water only	95
Inhibitor only	30
Inhibitor + eluate from zone R_f 0.1 to 0.3 of extract of *unchilled* embryos	45
Inhibitor + 0.5 per cent thiourea	85
Inhibitor + eluate from zone R_f 0.1 to 0.3 of extract of *chilled* embryos	100

* Leached, unchilled embryos were placed on filter paper moistened with solutions indicated—20 embryos per dish.

other hand, the presence of extract from chilled embryos enabled the test embryos to overcome the effect of the inhibitor, and thiourea was almost equally effective. Embryos receiving only water produced stunted seedlings, those exposed to the inhibitor did not grow further, while those exposed to the eluate from chilled embryos produced normal seedlings. It would seem, therefore, that the effect of chilling is to lead to the accumulation of a germination promoter which enables the embryo to overcome the effects of the inhibitor.

DISCUSSION

From the foregoing evidence, it would seem that both promoters and inhibitors are involved in the control of dormancy in embryos of *F. excelsior*. Although it appears that the changes resulting from chill-

ing involve primarily the germination promoter, nevertheless there is good evidence that an inhibitor plays an important part in the dormancy of the seed. Indeed, the responses of the seed appear to involve interaction between the promoter and the inhibitor, and the hypothesis is suggested that dormancy is due to the presence of the inhibitor and that emergence from dormancy involves the accumulation of the promoter to a level which overcomes the effect of the inhibitor. Since the greater part of the inhibitor present in the seed is contained in the endosperm, whereas the promotor is confined to the embryo, it would seem that a build-up of promoter is necessary to enable the embryo to overcome the inhibitory effect of the endosperm.

Since gibberellins are able to break the dormancy of various resting organs, including the unchilled embryos of *F. excelsior,* the question arises whether the germination promotor present in the chilled embryos is a gibberellin. Several pieces of evidence suggest that this is not the case; for example, the very small amount of tissue required to be extracted for its detection contrasts markedly with the relatively large amounts of tissue generally required for the detection of gibberellins. Moreover, the embryo promoter is capable of removing the stunting of seedlings derived from unchilled embryos, whereas gibberellic acid does not have this effect.

Whatever the nature of this promoter may prove to be, it would seem very probable that it functions as such in the intact seed, for the great merit of the work with *Fraxinus* seeds is that detection of the germination promoter was carried out with embryos of the same species, so that there is strong presumptive evidence that the in vitro experimental results are equally applicable in vivo.

If these results with seed of *F. excelsior* prove to be of more general application to buds and other resting organs, then it would seem that the reduced inhibitor level, reported to occur in the buds of several species in response to chilling, is not the only or even the primary cause of emergence from dormancy of these buds. It is thus desirable to investigate whether there is any accumulation of dormancy breaking substances during the chilling of buds. The existence of such substances in buds of *Tilia* and *Fraxinus* has, in fact, already been reported (22).

SUMMARY

Dormancy can be envisaged as being due either to a lack of certain essential growth factors or to the presence of active growth inhibitors. Studies of the changes in the levels of growth inhibitors in resting buds have shown a correlation between the state of dormancy and the level of inhibitors, and suggest that the effect of winter chilling in removing dormancy is to reduce the level of inhibitor.

A study of dormancy in seeds of *Fraxinus excelsior,* in relation to growth substances and inhibitors, is reported. In order to remove the dormancy of these seeds, a period of maturation for 4 to 6 weeks at warm temperatures is required, followed by chilling treatment for 5 to 6 months. The dry seeds contain no inhibitors, but after they have been permitted to imbibe water for 24 hrs., a water-soluble inhibitor is present in the endosperm and embryo, and is apparently metabolically produced. The unchilled embryos themselves are dormant, but their dormancy can be removed by leaching the excised embryos for 48 hrs. Seedlings derived from leached, unchilled embryos show stunted growth. Application of inhibitor to leached embryos restores their dormancy. Thus, it appears that dormancy in these seeds is due to the presence of the inhibitor in the endosperm and embryo.

Chilling treatment results in no appreciable reduction in the level of inhibitor. On the other hand, it is found that extracts of chilled embryos contain a germination promoter which is capable of overcoming the dormancy of unchilled embryos. The promoter is able to overcome the effects of the inhibitor when both substances are added to the germination medium. It appears that dormancy in *Fraxinus* seed is controlled by interaction between the inhibitor and the promoter, and that chilling results in an increase in the concentration of promoter to a level which overcomes the effect of the inhibitor. The promoter is also effective in removing the stunting of seedlings from unchilled embryos.

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DISCUSSION

Dr. Torrey: Is it possible to say from your assays whether your inhibitor affects cell elongation or cell division in the embryos? You gave evidence to suggest that the accelerator affected the acceleration of cell enlargement in roots. Can you distinguish between the two processes?

Dr. Wareing: I am afraid not. We have looked at cell division and cell extension at certain stages, but I am afraid I can't specifically answer that question with regard to the promoter on root growth.

Dr. Tukey: Dr. Wareing, you spoke about the inhibitor zone. This is the endosperm, is it? [Yes.] There is a very interesting situation in peach that falls right in line with what you are suggesting.

Of course, the peach seed must be after-ripened before it will germinate, but if the integuments are removed, the naked embryo

will germinate without after-ripening. Now, if just the chalazal region of the integuments is removed, the seed will not germinate. In fact, if all the integuments are removed, excepting for 2 or 3 mm. at the micropylar end, the seed will not develop. On the other hand, if this 2 or 3 mm. portion of the integuments at the micropylar end is removed, the seed will germinate even though the remainder of the integuments is left intact. If you examine the peach seed morphologically, you will observe that there are rudiments of endosperm tissue immediately adjacent to the radicle, or the hypocotyledonary axis in the micropylar region. When the integuments are removed, this endosperm tissue comes away with it. From all of this, one might speculate that an inhibitor lies in the endosperm of the peach seed at the micropylar end, and he might be prompted to look for it there.

Dr. Wareing: In the case of ash, of course, the endosperm completely surrounds the embryo, but what Dr. Tukey said ties up also with observations on *Iris*. Randolph and Cox (Proc. Amer. Soc. Hort. Sci. 43: 284, 1943) showed that the endosperm has a very strong inhibitory effect which is probably due to a specific inhibitor.

Dr. Evenari: I would like to compare this situation with the case of lettuce seeds. Dry lettuce seeds are full of inhibitors and there is apparently no promoter present. After root growth has started, the inhibitors disappear and a number of promoters (the chemical nature of which is unknown) appear. This disappearance of inhibitor and appearance of promoters occurs quite late insofar as the germination process is concerned and occurs only after germination has, in reality, finished and root growth has already started. In this case, at least, it will be difficult to correlate the so-called dormancy of the seeds with the presence of the inhibitor. I think we have to be careful here in differentiating between what we call germination inhibitors and growth inhibitors, as apparently these two are different from each other.

Dr. Wareing: First of all, when Professor Evenari speaks of promoters, he's talking of promoters revealed in the *Avena* coleoptile test which presumably means auxins and, therefore, I would not be surprised to find no particular correlation between dormancy in lettuce seed and changes in auxins. On the question of correlations between inhibitors and dormancy, the whole subject is fraught with pitfalls because so many things will stop *Avena* coleoptile growth or, for that matter, will inhibit germination, and the real crux of this problem is to sort out purely toxic substances from functional inhibitors. Professor Evenari said that in lettuce seed he cannot get a correlation between the inhibitor content and the state of dormancy.

On the other hand, in the seed of cocklebur, we were able to get a very marked correlation. For example, oxygen will break the dormancy of dormant cocklebur, but the inhibitor disappears 30 hrs. after you have put them into oxygen, before there are any visible signs of germination. There, the inhibitor disappears before germination.

Dr. Burström: Do your results imply that your promoters and inhibitors are active in the soluble state, since you have only studied the soluble fractions, contrary to auxins which probably are active in some bound form or other?

Dr. Wareing: Well, it's very difficult to answer Professor Burström's question in the present state of our knowledge. In fact, I would almost be inclined to throw it back at him and ask him how he would demonstrate this.

Dr. Wain: Just a very small point on technique here. In the early part of your report, the dry seeds were extracted with water and no inhibitor was found. Then when you soaked the seed and extracted with water, you found an inhibitor. It does seem to me that this might be explicable in terms of ease of extraction.

Dr. Wareing: I don't think so, Professor Wain. If you extract the dry seed at 0° C., you get no inhibitor. If, however, you keep the seeds soaking for 24 hrs. at laboratory temperature, you get an inhibitor. On the other hand, if you keep them soaking for 24 hrs. at 0° C., no inhibitor appears. We were very conscious of the possibility that these effects were simply the result of extraction technique, but further consideration seems to leave no doubt that the inhibitor is metabolically produced shortly after the seed is soaked at laboratory temperatures.

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On the Adaptation of Pea Roots to Auxins and Auxin Homologues

The phenomenon of adaptation whereby enzyme balance in an actively functioning cell may be modified or even transformed by a change of exogenous metabolite or the administration of a growth repressant has been widely studied in microorganisms. It now seems likely that enzyme induction by *endogenous* substrates or by structurally related molecules in the cell is a universal phenomenon whereby the adjustment of constitutive enzyme levels is normally accomplished (5). The fundamental significance of these new concepts for the biochemical, and hence structural, differentiation of the organism needs no stressing. Recently, these ideas from microbial behavior have been applied to higher plants in which, it has been suggested by Galston (6), indole-3-acetic acid-oxidase may be induced to form in this way by its own substrate. A certain amount of experimental evidence supports this claim, and the implications, in terms of the auxin control of plant growth, are, as Galston has pointed out, far-reaching. But Burström (4), who has demonstrated that without doubt growing root cells show progressively adaptive changes in their response to indole-3-acetic acid (IAA) when grown continuously in dilute solutions of that substance, does not think that the induced augmentation of IAA-oxidase activity can explain those changes. He visualized a switch in the mechanism of cell extension involving, as we interpret his meaning, other enzyme systems, probably those concerned in the incorporation of cellulose into the growing wall. This being so, it might be expected that changes in growth response to other homologous plant growth regulators, which could be expected to act in the same cell wall system, but which are not metabolized by IAA-oxidase, might result from IAA adaptation. Furthermore, the system might also be expected to adapt

directly to these IAA homologues, either with or without changes in IAA-oxidase, depending on whether Galston's or Burström's interpretation of adaptive growth changes are correct.

The purpose of the present investigation therefore was to investigate adaptive changes in pea roots, not only to IAA but also to its synthetic homologue, 2,4-dichlorophenoxyacetic acid (2,4-D), and to two other compounds, both suspected of interfering with growth via the auxin system. One was 2,3,5-triiodobenzoic acid (TIBA), which, among other things, is claimed to increase IAA-oxidase activity in *pea stem tissue* (7), and lowers IAA concentrations in pea roots (3). The other was 2,4-dichloroanisole (DCA), which has certain claims to being an auxin antagonist by direct competition at the growth centers (8). The changes studied were of two kinds: (a) growth responses to depressive concentrations not only of the adapting molecule but also of the other substances; and (b) changes in IAA-oxidase activity during adapting treatments.

METHODS

Seeds of 'Meteor' pea were germinated and grown from the second to the fourth day with their roots in a dilute solution of the chosen growth substance at concentrations that induced small inhibitions of elongation. Tests of the sensitivity of these roots to IAA and other growth substances were made by the excised-segment technique (2). Segments 1.7 to 2.0 mm. long were cut 1 mm. behind the apex of both treated and normal roots of the same age and then grown in aerated solutions of 0.5 per cent sucrose. Total extension of these segments was determined over the subsequent 48 hrs. Complete factorial experiments involving growth substances at different concentrations allowed the growth responses of segments from treated roots to be compared with those from normal roots. In each factorial experiment samples consisted of 10 root segments each and were replicated once. Furthermore, each experiment was exactly repeated a number of times on different occasions and the data thus acquired were subjected to an analysis of variance to determine the consistency of the responses and of their dependence on the adaptation treatment. Residual errors from these analyses were used to determine the least significant differences to be used as a basis of comparison in the results to follow.

Since the length of the meristem of adapted roots usually differs somewhat from that of normal roots (e.g., with IAA adaptation it is slightly shorter, as shown by Burström in wheat roots), it is clear that by taking the same length of segment at the same distance from the apex, the segments from normal and adapted roots will have

somewhat different cellular constitutions; indeed there will be some variations even within groups of segments cut from the same root sample. Obviously it is not possible to allow for such differences in the cutting of the segments and so, to supplement observations on the over-all growth, studies were made of the extension of individual epidermal cells of representative segments, so that the behavior of comparable cells in segments from normal and treated roots could be studied. Such observations were made on 8 μ sections cut from segments fixed in Navashin's fixative and stained in hematoxylin. Samples of five segments were taken for each treatment and lengths of individual cells measured from basal to apical end. Taking the cells in successive groups of 10 (i. e., groups of 50 cells for the sample of five segments), mean lengths and their standard errors were determined for the construction of cell length distribution curves.

Finally IAA-oxidase content of seedling roots similar to those used for the cutting of segments was determined from enzyme extracts by methods described by Galston and Dalberg (7). Average rates of IAA destruction, eliminating the small inconsistent initial lag and the enzyme inactivation which sets in as the reaction proceeds, were determined from a number of replicate samples by a statistical method, which also gave estimates of over-all errors for the evaluation of confidence limits.

RESULTS

Segments From Roots Grown in 10^{-7} G/Ml of Indole-3-acetic Acid

Responses to inhibiting concetrations of IAA (10^{-8} and 10^{-7} g/ml). In Figure 1A are plotted the mean percentage extensions of samples from eight identical factorial experiments. In sucrose, IAA-grown segments extend less (i. e., about 15 per cent) than normal segments. On the other hand, they extend very much more than normal segments exposed directly to the adapting concentrations. If the reduced extension of these adapted segments is due to a carry-over of IAA from the adapting solution, then either its concentration must be much lower than in the adapting medium or cell sensitivity to IAA has been lowered by the adaptation treatment. Inspection of the cell extension graphs (Figure 1C, D) shows that this reduced growth is not a property of all cells in the adapted segment. In normal segments in sucrose all cells extend, but the less mature cells at the apical end of the segment, where extension had not commenced at the time of excision, do not attain the same final length as the more mature cells at the basal end of the segment. In IAA-grown segments, these more mature basal cells extend as much as the corresponding cells in normal segments while the growth of the

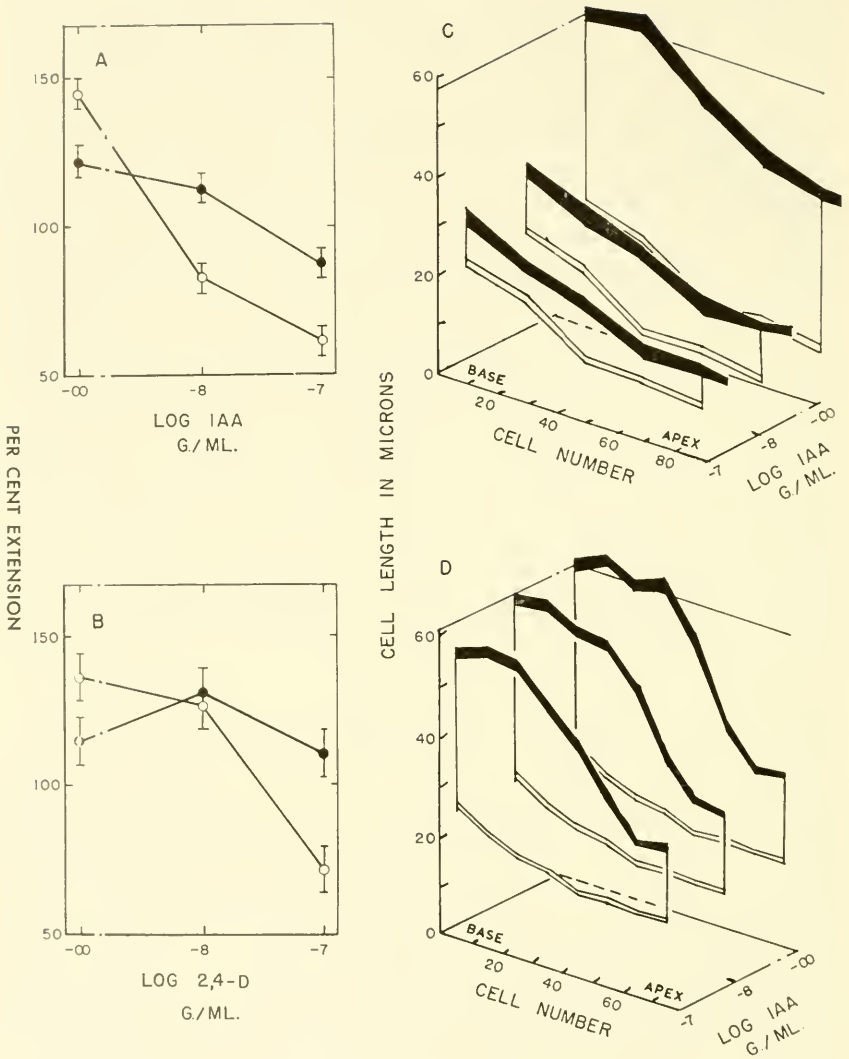

Fig. 1. Growth responses of segments from roots grown in indole-3-acetic acid solutions (10^{-7} g/ml). **A** and **B**. Total extension in 48 hrs. of normal (open circles) and IAA-grown (solid circles) segments. (**A**) Responses to IAA. (**B**) Responses to 2,4-D. Vertical lines indicate the least significant difference (5 per cent) between means. **C** and **D**. Epidermal cell length distribution before and after 48 hrs. of extension. (**C**) Normal segment responses to IAA. (**D**) IAA-grown segment responses to IAA. Open parallel lines—segments immediately after excision. Blacked-in parallel lines—segments after 18 hrs. in 0.5 per cent sucrose solution. These lines are plotted at distance of $\sigma/\sqrt{N}$ above and below the respective mean of successive groups of cells.

less mature apical cells is much more restricted than that of the corresponding normal cells. It seems likely that cells in all segments may be limited in their extension by some growth factor or factors (not auxins) coming either from the root tip or from the mature regions of the root, since they never reach the size attained by cells in intact roots. In this respect the immature cells would tend to suffer most, since the more mature cells would make earlier inroads into this limited growth-factor supply. On the other hand, one might equally well postulate that growth limitation may be set by the accumulation of a staling factor, which in intact roots might be removed upwards. Again immature cells would be the most affected since, being the last to commence extension, they would be most affected by the accumulating products from the maturer cells. It is difficult to see how IAA adaptation could aggravate this restriction of the extension of younger cells except perhaps by augmenting the accumulation of staling products, of which it might even be the precursor.

The response of normal segments to the direct action of IAA at the concentration used for adaption is a reduction of over-all extension of 58 per cent (Figure 1A) which is seen to affect all the cells of the segment to virtually the same extent (Figure 1C). IAA-grown segments are, however, very much less sensitive. Figure 1A shows that in normal segments 10^{-8} g/ml IAA can produce a growth reduction which, in adapted segments, requires a concentration of 10^{-7} g/ml, i. e., a drop in sensitivity of at least ten times. The cell distribution analysis of Figure 1D shows that this loss of sensitivity takes place over the whole segment but is most marked in the immature apical cells, which extend to virtually the same extent whether IAA is present in the medium or not. The small inhibition which does occur is confined to the mature, more completely extended cells.

Such behavior might be explained in terms of enhanced IAA-oxidase of the adapted cells. Direct estimations for these roots (see Table 1) show that extracts of treated roots inactivated IAA almost 50 per cent faster than those of normal roots, an increase that is highly significant. Although no enzyme distribution studies were made, the results in Figure 1D suggest that possibly inactivation was more complete in the immature apical cells which were virtually unresponsive to exogenous IAA.

This, however, is a very much oversimplified picture of the situation, since IAA adaptation also involves changes in sensitivity to 2,4-D, which is certainly not destroyed by IAA-oxidase.

Table 1. Rates of inactivation of IAA by root breis.

Roots Grown for 48 Hrs. Before Harvest	No. of Root Samples	Rate of IAA Inactivation mg/g Fresh Wt /10 Min (Regression Coefficient)	Standard Error of Regression Coefficient	"t" (Difference Between Regression Adapted-Water-grown)	P	Ratio of Rates, Adapted-Normal
Water	6	3.77	.146	...	....	....
IAA, 10^{-7} g/ml.		5.54	.297	5.35	.001	1.47
2,4-D 5×10^{-8} g/ml.		4.22	.422	1.31	.2	1.12
Water	5	2.92	.156	....	....	....
TIBA 5×10^{-6} g/ml.		6.09	.274	10.06	.001	2.16
Water	6	3.60	.155	....	....	....
DCA 10^{-5} g/ml.		7.20	.803	4.40	.001	2.00

Responses to inhibitory concentrations of 2,4-D (10^{-9} g/ml and 10^{-8} g/ml). Here only total segment extension has been measured, and results are recorded in Figure 1B. Growth of normal segments is not significantly reduced by the lower but is reduced by about 48 per cent by the higher concentration. IAA-grown segments, on the other hand, are not significantly affected by these 2,4-D concentrations, the total extension being the same as in sucrose alone. Unfortunately, time has not permitted analysis of growth on a cell basis for these 2,4-D-inhibited segments, but it is clear from these results alone that IAA adaptation has markedly reduced the sensitivity of cells to 2,4-D inhibition. It might be suggested that the lack of response to 2,4-D is due to the domination of the growth centers by IAA carried over from the adapting solutions, but this would certainly not be so if IAA-oxidase were at a high level in the cells. It would be much more logical to suppose that here we are dealing with a change in the growth centers themselves involving a great loss in sensitivity not only to 2,4-D but also most probably to IAA itself. It is very unlikely that IAA-oxidase induction plays any direct part in these 2,4-D sensitivity changes.

Segments From Roots Grown in 2,4-D (3×10^{-8} g/ml)

In this series of nine identical factorial experiments we tested the responses of normal and 2,4-D-grown segments to three IAA concentrations (10^{-11}, 10^{-9}, and 10^{-7} g/ml). The pooled results of these experiments are drawn in Figure 2A. Normal control segments show, contrary to earlier experiments by Audus and Das (1), no stimulation in 10^{-11} g/ml IAA but a progressive inhibition in higher concentrations, reaching about 27 per cent in 10^{-7} g/ml. The pattern of cell extension in these normal segments (Figure 2C) closely resembles that already described, with restricted growth of the cells at the apical end. IAA (10^{-7} g/ml) reduces the extension of all cells in the normal segment, with a proportionately greater effect on the more rapidly extending maturer basal cells.

Root segments grown in 2,4-D extend much less (about 27 per cent) in sucrose than normal segments (Figure 2A). The analysis of cell length distribution (Figure 2D) shows that this restriction of extension applies to all cells, with perhaps a slightly greater effect (as for IAA adaptation) on the immature apical cells. Such a reduced extension could undoubtedly be explained in terms of residual 2,4-D left associated with the growth centers after excision and exerting, at least in part during subsequent extension, a continuing growth inhibition. But this reduction in extension is much less than that produced by the *direct* action of the adapting concentration (3×10^{-8}

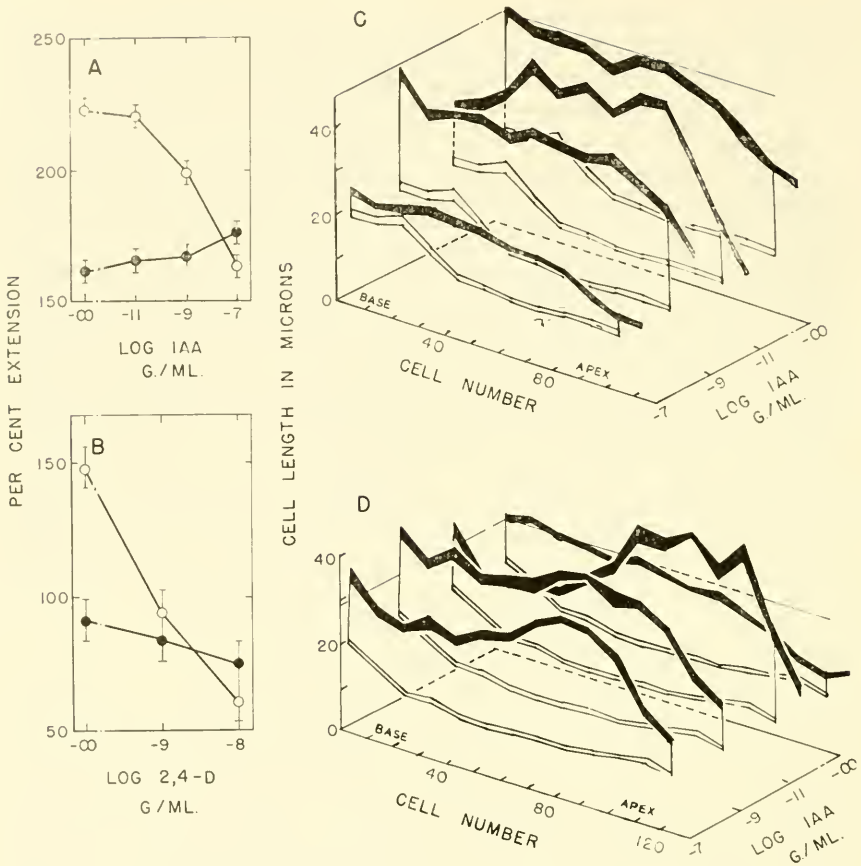

Fig. 2. Growth response of segments from roots grown in 2,4-dichlorophenoxyacetic acid solution (3×10^{-6} g/ml). **A** and **B**. Total extension in 48 hrs. of normal (open circles) and 2,4-D-grown (solid circles) segments. (**A**) Responses to IAA. (**B**) Responses to 2,4-D. Vertical lines indicate the least significant difference (5 per cent) between means. **C** and **D**. Epidermal cell length distribution before and after 48 hrs. of extension. (**C**) Normal segment responses to IAA. (**D**) 2,4-D-grown segment responses to IAA. Open parallel lines—segments immediately after excision. Blacked-in parallel lines—segments after 48 hrs. in 0.5 per cent sucrose solution. These lines are plotted at distance of $\sigma/\sqrt{N}$ above and below the respective mean of successive groups of cells.

g/ml) on normal segments, which is of the order of 60 per cent. This suggests that either 2,4-D is rapidly lost from the segments under growth conditions in sucrose, about 80 per cent loss being necessary to explain the much lower inhibition, or that there might have occurred a direct loss of sensitivity to the persistent 2,4-D, of a nature reminiscent of bacterial adaptation to drug action. The latter alternative is somewhat favored by observations on the growth of such

adapted segments in a range of 2,4-D solutions (Figure 2B). In these experiments concentrations up to 10^{-8} g/ml had only slight additional depressive effects and the growth of these segments was slightly (although not significantly) greater than normal segments in the same concentration (10^{-8} g/ml).

Convincing evidence of a change in auxin sensitivity comes from the response of 2,4-D-grown segments to IAA. Here, instead of reduction, there is a progressive increase in segment extension which in 10^{-7} g/ml reaches 19 per cent over those in sucrose, a difference which is highly significant (Figure 2A). The cell length distribution results of Figure 2D show a surprising feature: the mature basal cells of the adapted segment are completely insensitive to these high IAA concentrations, but in the immature cells at the apical end extension is stimulated, their growth equaling that of corresponding normal cells and being at least twice that of corresponding adapted cells in sucrose.

In the first place, it is very unlikely that changes in IAA-oxidase activity of the 2,4-D-grown cells have any part to play in these changed responses. Direct measurement of IAA inactivation by extracts of adapted roots gives mean rates only 12 per cent above those of normal roots, the difference being completely nonsignificant (Table 1). This is supported by previous observations by Audus and Thresh (3), which showed that such 2,4-D treatments had no effect on the internal levels of IAA in pea roots. It seems possible that the lack of sensitivity of the more mature basal cells could be caused at least in part by the retention of 2,4-D at the growth centers of the treated cells. If all these centers were occupied by 2,4-D to the exclusion of the IAA penetrating from the medium, then one would expect the results obtained. Even if IAA were to replace 2,4-D, no great change in growth would be expected since, molecule for molecule, they have the same order of inhibiting effect on root cells.

But the almost normal growth of the younger apical cells is very much more puzzling. The following speculations may be worth consideration. First, there might be a mutual, and therefore chemical neutralization of these two substances in apical meristematic cells. This would mean that 2,4-D adaptation involved the generation of an enzyme catalyzing this reaction and would necessitate that this enzyme should disappear as soon as extension commenced; this would explain the persistence of inhibition (either by 2,4-D or IAA or both) in cells just beginning to extend at the time of excision and IAA treatment. From what is known at present of the chemical properties and biochemical behavior of these two substances, this would seem most unlikely. Alternatively, we could explain this virtually

normal growth by invoking changes at the growth centers in both the sensitivity to and the affinity for growth substances in these immature adapted cells. It would be necessary to postulate that residual 2,4-D is easily pushed off these cells by the IAA entering from the medium but, at the same time, the sensitivity of the growth centers to IAA is so reduced that it has virtually no effect and normal extension ensues. Furthermore, there would have to be a rapid increase in 2,4-D affinity at the commencement of extension in order to explain the lack of IAA effect in the basal cells starting to extend at the time of excision.

Segments From Roots Grown in TIBA (5×10^{-6} g/ml)

A series of five identical factorial experiments has been carried out to test the responses of these roots to 10^{-11}, 10^{-8}, and 10^{-7} g/ml IAA solutions. The pooled treatment means are drawn in Figure 3A. The behavior of control segments is as in previous experiments, IAA at 10^{-7} g/ml giving an inhibition of extension of about 18 per cent. TIBA-grown segments extend about 19 per cent less than normal segments in sucrose. The cell length distribution curves of Figure 3B and C show that this is due, as in the case of IAA-adapted segments, to a marked restriction of the extension of the youngest apical cells. Although, for some unknown reason, the mature basal cells of this particular sample of control segments grow less than usual, thus emphasizing these differences, corresponding cells of the TIBA-grown segments appear to extend more than normal, reaching a significantly higher final length.

The responses of these treated segments to IAA are very slight (Figure 3A), amounting to a reduction of only 9 per cent in 10^{-7} g/ml, as compared with those of corresponding segments in sucrose only. Whereas IAA inhibition is exerted along the whole length of the normal segment, in TIBA-treated segments, the much reduced inhibition is exerted mainly on the maturer basal cells. In all respects the growth behavior of TIBA-adapted segments closely resembles that of IAA-adapted segments and the similarity also extends to augmented IAA-oxidase activity. Enzyme extracts of TIBA-treated roots gave mean IAA destruction rates well over twice those of normal roots, the difference being very highly significant (Table 1). TIBA in low concentrations appears to have a direct activating effect on IAA oxidase in vitro. But such small effects (12 per cent at 10^{-6} g/ml) of TIBA in the extracts of treated roots could hardly explain these large activity increases. It would seem that here we are dealing with IAA-oxidase induction by TIBA.

The lowered sensitivity of TIBA-treated segments to applied IAA

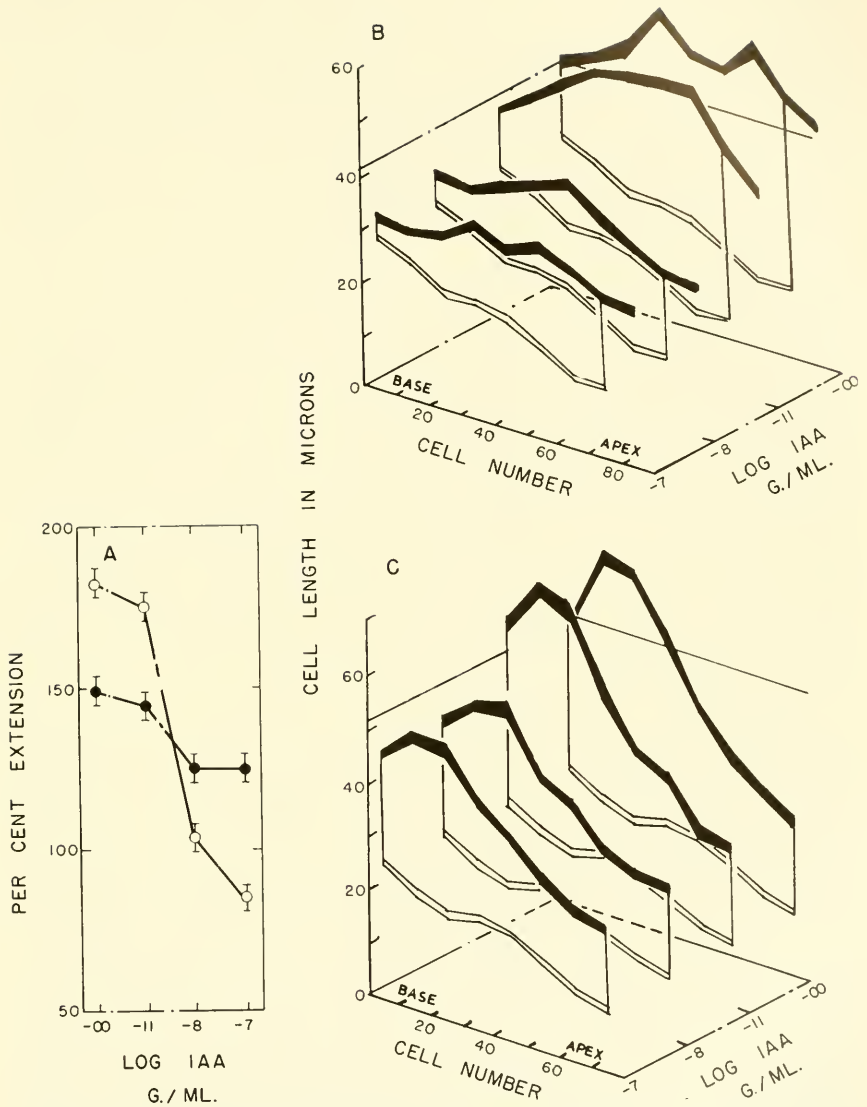

Fig. 3. Growth responses of segments from roots grown in 2,3,5-triiodobenzoic acid solutions (5×10^{-6} g/ml). (**A**)Total extension in 48 hrs. of normal (open circle) and TIBA-grown (solid circle) segments. Response to IAA. Vertical lines indicate least significant difference (5 per cent) between means. **B** and **C**. Epidermal cell length distribution before and after 48 hrs. of extension. (**B**) Normal segment responses to IAA. (**C**) TIBA-grown segment responses to IAA. Open parallel lines—segments immediately after excision. Blacked-in parallel lines—segments after 48 hrs. in 0.5 per cent sucrose solution. These lines are plotted at distance of $\sigma/\sqrt{N}$ above and below the respective mean of successive groups of cells.

could certainly be explained on a basis of heightened IAA-oxidase content which, like that in the IAA-adapted roots, appears to be greater in the meristematic end of the segment. Furthermore the very close similarities in the curves of cell growth distribution shown by TIBA- and IAA-adapted segments raise the question whether they might not have similar underlying causes linked with the high induced IAA-oxidase activity. It could be suggested that under these conditions endogenous auxin concentration is lowered far below the supraoptimal levels supposed to exist in roots and that the marked difference in growth at the two ends is a reflection of differences in IAA concentration maintained by the oxidase at the two ends. At the basal end it is near optimal, accounting therefore for the apparently real stimulation of extension over the corresponding cells of normal segments. At the apical end it is markedly suboptimal, giving much reduced growth. But if this were so, one would have expected applied IAA, even though most of it were oxidized, to have given at least some slight stimulation of extension in this apical region. Since there is no sign of this, the lower growth cannot be due to IAA deficiency. As previously suggested for IAA adaptation, it might more probably be due to the more rapid accumulation there of a staling product from IAA oxidation in the apical cells.

Segments From Roots Grown in DCA (10^{-5} g/ml)

Nine identical factorial experiments have tested the sensitivity of DCA-grown segments to IAA concentrations of 5×10^{-8} and 10^{-7} g/ml. From the mean segment extension data of Figure 4A it will be seen that DCA-grown segments extend in sucrose only about half as much as normal segments. But examination of Figures 4B and C will show that adapted cells are not so restricted in their extension as these figures would suggest. The reason for this discrepancy is that roots grown in this DCA concentration have shorter meristems and segments thus contain fewer cells, many more of which have already started to extend at the time of excision. The over-all extension of these segments is therefore correspondingly smaller. There is no indication here that this growth limitation is restricted to any part of the segment, and, as with 2,4-D, it is most rational to explain this uniformly reduced growth as a result of the direct inhibiting action of residual DCA retained adsorbed at the growth centers. On grounds of molecular structure one might expect the rings to show similar affinities for the growth centers.

The action of IAA is to produce only slight reduction of adapted segment growth, amounting to 11 per cent at 10^{-7} g/ml. Although this is significant over the whole set of experiments, in the particular

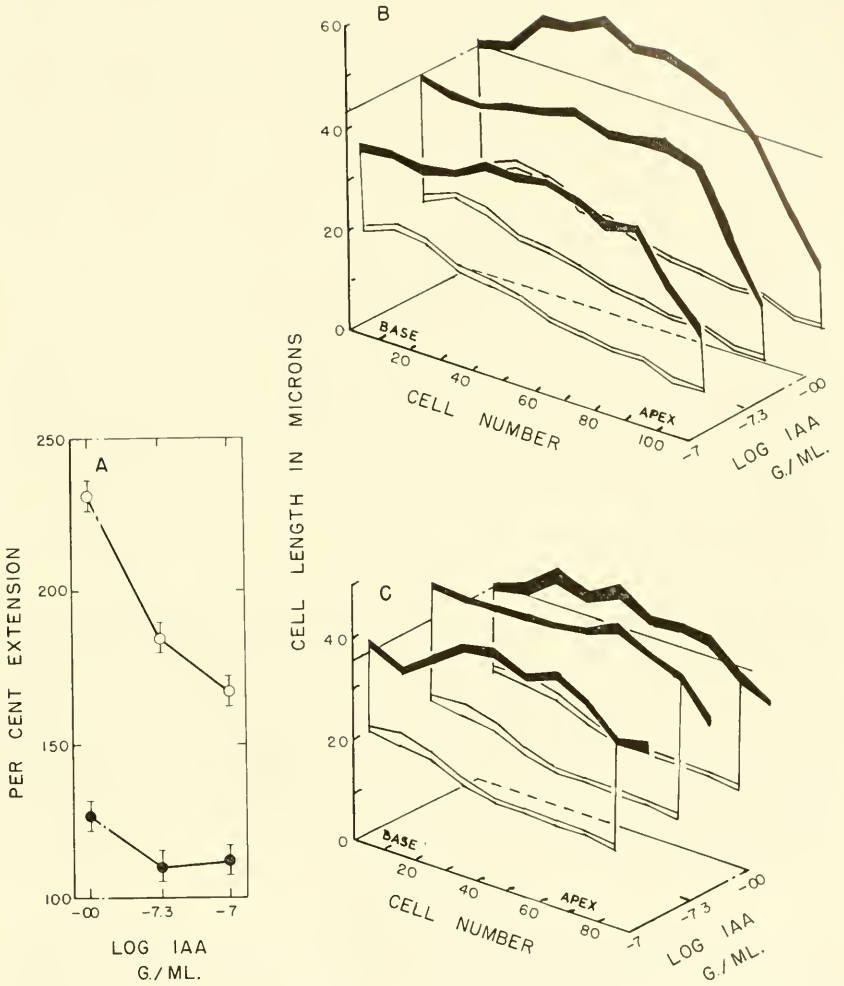

Fig. 4. Growth responses of segments from roots grown in 2,4-dichloroanisole (DCA) (10^{-5} g/ml). (**A**) Total extension in 48 hrs. of normal (open circles) and DCA-grown (solid circle) segments. Response to IAA. Vertical lines indicate the least significant difference (5 per cent) between means. **B** and **C**. Epidermal cell length distribution before and after 48 hrs. of extension. (**B**) Normal segment responses to IAA. (**C**) DCA-grown segment responses to IAA. Open parallel lines—segments immediately after excision. Blacked-in parallel lines—segments after 48 hrs. in 0.5 per cent sucrose solution. These lines are plotted at distance of $\sigma/\sqrt{N}$ above and below the respective mean of successive groups of cells.

batch of DCA-grown segments in which cell lengths were studied, IAA at this concentration produces no appreciable reduction in cell extension.

There are two possible reasons for this lack of response to IAA. First, it might be argued that DCA, being an auxin antagonist and being retained in the adapted segment, completely antagonizes auxin action and therefore prevents its inhibition of cell extension. This seems unlikely, since previous work by Audus and Shipton (2), has shown no evidence for such auxin antagonism in roots, in which DCA and 2,4-D inhibitions seem strictly additive. Secondly, it could be due to the complete destruction of the applied IAA by heightened IAA-oxidase, since DCA-adapted roots have about twice the oxidase content of normal roots, a difference which is highly significant (Table 1, p. 114). Some of this stimulation may be caused by a direct activation of the oxidase already present as shown by in vitro studies, in which a 40 per cent increase in activity was obtained by the adapting concentration of 10^{-5} g/ml. Evidence for IAA-oxidase induction is thus less convincing than for TIBA. Lack of IAA response would seem then to be more likely the result of heightened IAA-oxidase activity.

It is interesting then to compare the cell extension behavior of DCA-grown segments with those from IAA-grown and TIBA-grown roots in which IAA-oxidase activity is also increased. The differences are mainly in the maturer basal cells, which, unlike the corresponding IAA- and TIBA-grown cells, extend much less than normal, owing, we have presumed, to depressive DCA retained at the growth centers. By implication this suggests that TIBA has no direct inhibiting action on roots at this concentration, its sole effect being exerted via IAA metabolism (3).

CONCLUSIONS

It may be concluded from the experiments described that adaptation of roots to exogenous growth substances takes place in the cells of the meristem before extension commences and is the result of induced changes in the balance of enzyme complexes or of endogenous growth factors or of both, and that these changes persist during subsequent extension and therefore determine its pattern. With some adapting molecules (e.g., IAA, TIBA, and DCA), a marked augmentation of IAA-oxidase activity may play an important part in IAA-response changes, but, as in 2,4-D adaptation, a real change in the sensitivity of the growth centers is undoubtedly involved. Here the picture may be complicated by the competitive action of the adapting molecules which remain adsorbed to the growth centers

after excision and during subsequent extension. The pattern of adsorption and of growth-substance competition may also change from meristem to extending cell, as was indicated by the 2,4-D adaptation results. These great changes in cell behavior at the time when extension is commencing and the variety of effects of different adapting molecules emphasize the great complexities of the phenomena and the dependence of any phase in the growth of a cell on the growth conditions of the previous phase. Future studies of adaptation phenomena cannot ignore this.

A word of warning must also be sounded on the dangers involved in the interpretation of responses of intact organs to growth-modifying substances. It is usual to assume that any such responses of the extension growth of the cell invoked by an exogenous growth substance arise from *direct* effects on the extension processes themselves. This may be true if responses can be recorded immediately after application of the regulator, but observations over an extended period may well involve indirect effects acting via the meristem similar to those just described. On the other hand, elimination of the indirect effect of the meristem by the excised segment technique also has its dangers since, as we have just seen, marked differences in behavior can occur at the two ends of what is all too frequently assumed to be a homogeneous segment, a situation undoubtedly complicated by the traumatic effects of excision. Furthermore, the same total extension of differently treated segments may conceal quite different behaviors of the various constituent cells and hence lead to oversimplified and erroneous conclusions. To avoid such pitfalls, therefore, future research will have to concern itself more and more with following the behavior and biochemical properties of the *individual cell* through the successive stages of its growth *in intact organs.*

SUMMARY

An attempt has been made to study by direct methods adaptive changes in the sensitivity of roots to auxins and auxin homologues. The technique involved the measurement of the extension of 1.7 to 2.0 mm. segments excised from just behind the root tip and grown in 0.5 per cent sucrose solution with or without auxin. The extension pattern of the individual epidermal cells in these segments was also investigated. The behavior of segments from auxin-grown roots was compared with control segments from water-grown roots in all cases.

Roots grown in inhibiting concentrations (10^{-7} g/ml) of indole-3-acetic acid (IAA) show a greatly reduced sensitivity to IAA, which varies with the age of the cell in the segment. These roots have a

heightened IAA-oxidase content but this cannot alone account for sensitivity reduction since the responses of these roots to 2,4-D, which is not destroyed by IAA-oxidase, is similarly lowered.

Roots grown in low 2,4-D concentrations (3×10^{-8} g/ml) show similar reduction in over-all sensitivity of segments to both IAA and 2,4-D. Here auxin destruction can play no part, since these roots have an IAA-oxidase content not significantly different from that of normal roots. Analysis of the growth pattern of individual epidermal cells reveals several striking sensitivity changes, particularly in the youngest, immature cells of the segment. These 2,4-D-adapted cells are stimulated to extend as much as normal cells in sucrose alone by IAA concentrations which halve the growth of normal cells.

Roots adapted in solutions of 2,3,5-triiodobenzoic acid show growth behavior to IAA treatment very similar to that of IAA-grown roots and also a much enhanced IAA-oxidase content. On the other hand, 2,4-dichloroanisole-grown roots, which also have a much heightened IAA-oxidase content, exhibit a somewhat different pattern of sensitivity changes.

It is concluded that these changes in growth response sensitivity are predetermined in the meristem and their effects persist during subsequent extension. They are complex in nature and in all cases due, at least in part, to real changes in the reactivity of the growth centers to the auxin molecules.

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DISCUSSION

Dr. Galston: I am very happy to learn of Dr. Audus' confirmation of our report on the enhanced activity of indoleacetic acid

oxidase after pre-incubation of intact tissue with indoleacetic acid. This, as he pointed out, could possibly be due to the induced formation of an enzyme as we had originally suggested. It could also be due to an alteration of the cofactors which are known to be required for the operation of this enzyme.

One of my graduate students, Mr. Masaki Furuya, has carried out a few experiments which have convinced us that the so-called inductive or adaptive changes can be demonstrated very easily in vitro under conditions in which protein synthesis is impossible. Thus, we now think that at least part of the adaptive changes are due to changes in the cofactors and not in the enzyme itself.

As you know, there are two kinds of cofactors for the IAA-oxidase of peas. One is the manganous ion. If an etiolated pea homogenate containing IAA-oxidase activity is pre-incubated with *ca.* 10^{-4} Mn^{++} for various periods of time prior to the addition of the IAA substrate, the activity of the enzyme will be found to rise markedly with time. These changes leading to enhanced activity are temperature and oxygen sensitive. We have evidence that they are due to destruction of an inhibitor of the IAA-oxidase contained in the homogenate.

The second cofactor is a yet unknown material the action of which can be imitated by 2,4-dichlorophenol (DCP). If the IAA-oxidase is incubated with 10^{-4} *M* DCP, the enzymatic activity becomes successively lower and lower with increasing pre-incubation time. This inhibitory effect of pre-incubation with DCP is reversible by a subsequent incubation with Mn^{++}. If both cofactors are added simultaneously to the pre-incubation mixture, the first effect noted is the inhibitory action of DCP, which is later reversed by Mn^{++}. These results lead us to the belief that at least part of the induced changes in IAA-oxidase activity noted by Galston and Dalberg and also by Audus are not true enzymatic inductions, but are, rather, due to changes in the cofactor levels for the enzyme.

Dr. Audus: I don't know how this affects Dr. Galston's ideas, but all these observations of ours were made in the presence of DCP added to the breis.

Dr. Burström: You said that you measured the elongation from the start of the elongation of the cells. I perhaps didn't quite see the figures on your diagrams but I got the impression that you had the intial cell lengths at apical ends of your sections of about 40 microns. Is that so?

Dr. Audus: No; they are 10 microns long. The segment included about 75 per cent of cells which hadn't started to elongate at the time of excision.

Dr. Burström: I agree with you completely that we must follow the progress of elongation more carefully than is usually done. I

also want to emphasize that if we measure elongation for a period of 15 hours we measure only initial and end result — we don't know what is happening in between. We found as the basis of our assumption of an adaptation, that the growth curve of the cells didn't change in the way we had expected. I wonder if you could construct such time curves from the frequency diagrams of your cells?

Dr. Audus: No, with this technique it is impossible to do that. It would be nice to think that one day one might be able to study changes like the ones described without damaging or mechanically affecting the cells in any way. In this excised segment technique, we still don't know the traumatic effect involved. We have another example before us in the split-pea segment tests, where the inner and outer cells respond differently to auxins. We still don't know for certain what causes this differential response. We know from the work of Schneider that some of it at least is due to trauma; but what are the interactions between trauma and indoleacetic acid action?

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Some Biological Characteristics of an Inhibitor Extracted From Woody Shoots

Since the adoption of chromatography for the separation of natural plant growth regulators, many workers have reported the presence of substances which inhibit the extension of coleoptile sections. Similarity in position on many chromatograms of diverse plant extracts has tended to give the impression that all these inhibitory zones exert a physiologically similar effect on the test object by which they are located in the first place. There are, however, certainly two strongly contrasting types: those in which the inhibition is readily reversible, as described by the present authors (2), and more recently by Van Steveninck (16), and those which are merely toxic, such as the substances isolated by Housley and Taylor (8). Within these two types — and perhaps only the nontoxic one commands much interest — several modes of action may be involved, not all of which will necessarily produce inhibition in all tests. This paper presents the results of several biological tests carried out with a material separated from extracts of plum shoots, and found to inhibit coleoptile section extension, and the variation in behavior in different assays suggests that it is rash to make generalizations about the growth regulatory properties of a particular substance on the basis of any one biological test.

MATERIALS AND METHODS

The active material used in the tests to be described, and subsequently referred to simply as "the inhibitor," is a substance, or mixture of substances, found on chromatograms as detailed below, which causes a reduction in the extension of wheat coleoptile sections compared with their growth in water; at no concentration does it promote extension. The action of the inhibitor is largely reversible,

i. e., if sections have not been too drastically inhibited, they will recover after washing with water.

The inhibitor was obtained in the following way: current year's shoots of the plum rootstock 'Myrobalan B,' grown on a layerbed, were cut into short lengths and twice extracted for 24 hrs. at 3° to 5° C. with peroxide-free diethyl ether. The inhibitor was originally found in the acid fraction (9) on descending chromatograms run in butanol-propanol-ammonia-water, 5:5:1:1, at R_f 0.6 to 0.7, but was obtained in larger amounts by a method which cleared the extract of fatty material, allowing heavier loading of the chromatograms. The ether extract was evaporated down in the presence of water, precipitating the chlorophyll and fats; the aqueous filtrate (*ca.* pH 4) was shaken out three times with ether, and after concentration, this was applied as a band to Whatman 3 MM paper, and run in plain water. The inhibitor occurred at R_f 0.85 to 0.95, and after elution and re-running in either water or the organic solvent mixture, a reasonably clean product was obtained. The concentration of the solutions used in the tests is given as "internodes per ml." (ints/ml); one internode was on the average about 1 g. fresh weight.

The auxin used throughout was the sodium salt of indole-3-acetic acid (NaIAA), diluted from a stock solution of 1,000 p.p.m. adjusted to pH 7.0. Upon dilution the pH falls to that of the water used (about pH 5.6). The inhibitor solution at 100 ints/ml was of pH 5.0.

Test methods will be briefly described along with the results in the next section.

BIOLOGICAL CHARACTERISTICS

Cell Extension in Coleoptile Sections

In the usual section test in this laboratory, five 10-mm. sections of wheat coleoptiles are used in 0.5 ml. of solution in a small vial which is rotated horizontally about its axis for the growth period of about 20 hrs. (3). Neither a buffer nor sucrose is added. By definition the inhibitor reduces the extension of sections under these conditions, but does so similarly when the test is carried out in KH_2PO_4 buffer at pH 4.6 or in 2 per cent sucrose.

Extension due to an exogenous growth promoter (NaIAA) is also reduced by the inhibitor, as shown by Figure 1, which represents the growth of coleoptile sections in a range of concentrations of NaIAA and inhibitor; the left rear edge of the diagram shows the typical concentration-response curve for NaIAA of sections under our conditions, with concentration on a logarithmic scale increasing from left to right; (wheat coleoptile sections are remarkably tolerant of high concentrations of NaIAA (6), and 500 p.p.m. in this experi-

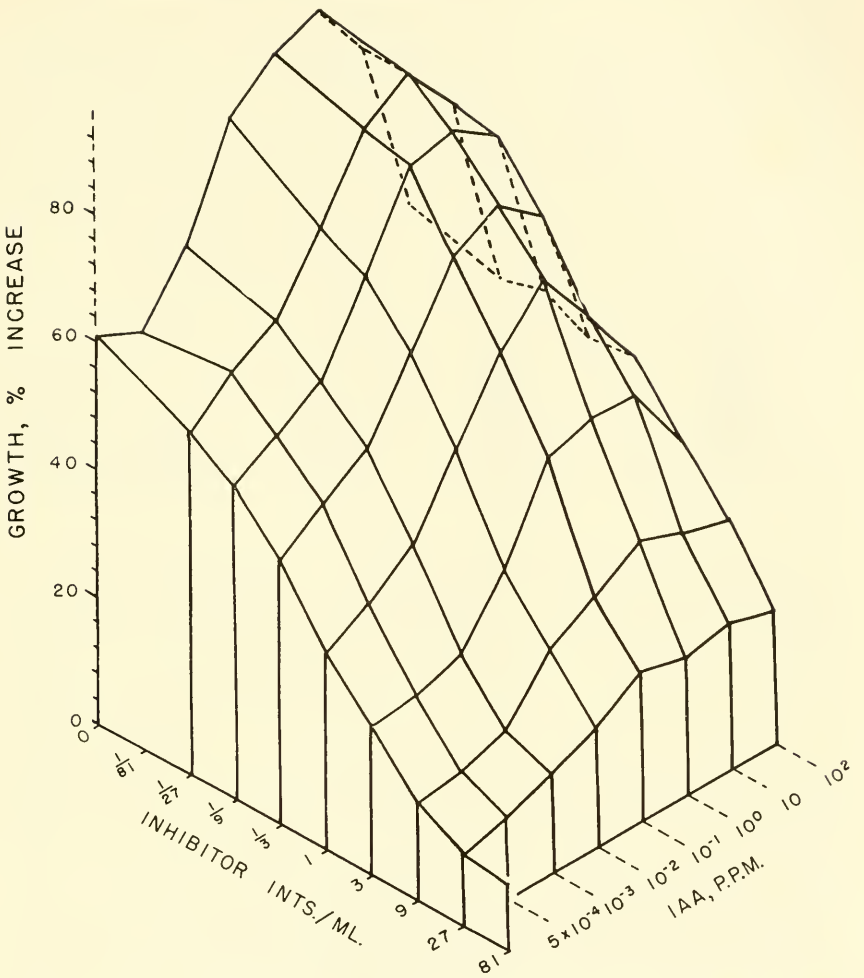

Fig. 1. Growth of wheat coleoptile sections in mixtures of NaIAA and inhibitor. Concentration of IAA in p.p.m. shown on the right hand edge increasing from front to back; concentration of inhibitor in ints/ml shown on the left hand edge increasing from back to front. Growth shown on the vertical scale as per cent increase over original length.

ment, though supra-optimal, resulted in section growth only just below the controls). The left front edge shows the response to inhibitor (also on a logarithmic scale) with concentration increasing from back to front.

While the general effect of increasing concentrations of inhibitor is clearly to reduce growth in each auxin concentration, the detailed interactions of the two substances are somewhat obscured by variability in response; up to the optimum NaIAA (5 p.p.m.) a given concentration of inhibitor has a fairly similar effect at all auxin levels,

(either expressed as an actual or a relative difference in length between treatments with and without inhibitor); at concentrations above the optimum, NaIAA reduces the effect of the inhibitor, e.g., the average effect of the highest inhibitor dosage (27 ints/ml) over all concentrations of NaIAA from 0 to 5 p.p.m. was a 32.6 per cent reduction below similar solutions without inhibitor, but in 500 p.p.m. NaIAA the reduction was only 21.1 per cent. Conversely, the effect of NaIAA (as a percentage of the response without auxin) was in general the same at all inhibitor levels, except that at 500 p.p.m. NaIAA the depressing effect of the high auxin concentration was relieved by adding inhibitor, e.g. with increasing inhibitor from 0, 1/27 . . 27 ints/ml the percentage differences between 0 and 500 p.p.m. NaIAA were respectively—4.4, —2.6, +1.2, 1.7, 12.9, 12.5, 13.5, and 8.4. In another experiment the response in 500 p.p.m. NaIAA was 1.3 per cent above that in water, while in successive inhibitor concentrations of 2, 10, and 50 ints/ml it was respectively 1.8, 11.3, and 11.5 per cent above.

Time-course studies have been made in some detail and will be presented elsewhere; here it is sufficient to say that during the first 3 hrs. of growth under our conditions, there seems to be an increase in length of up to 10 per cent, which is not reduced by this inhibitor, low temperature (1), or low oxygen tensions; after this phase is over, and the sections are growing quickly, the inhibitor has a rapid effect upon extension, a reduction in growth rate being detectable within 1 hr.; conversely, inhibited sections transferred to water or NaIAA show rapid recovery by an increased growth rate. The final lengths achieved by such sections depend upon the degree and period of inhibition, as shown in Figure 2. Sections were grown in water, and inhibitor at 0.5, 1, and 10 ints/ml for 0, 3, 8, 13, and 19 hrs., and then washed in running water (representing about 100 times the original volume of solution) and transferred to 5 p.p.m. NaIAA; after a further 24 hrs. final lengths had been attained. Open tubes were used to avoid the complications of uncorking the tubes after different times. Growth up to the time of transfer is shown by the basal portions of the histograms and the subsequent growth in NaIAA by the upper plain part; it will be seen that although the final length attained falls with increasing time in any of the solutions, including water, the growth made in NaIAA is about the same after transfer from inhibitor as from water at 3 and 8 hrs., slightly more at 13 hrs., and considerably more at 19 hrs.

A similar situation was found on transfer to 0.5 p.p.m. NaIAA, except that the amount of growth made after transfer was not so great, particularly from the highest inhibitor concentration; this trend was even more marked on transfer to water.

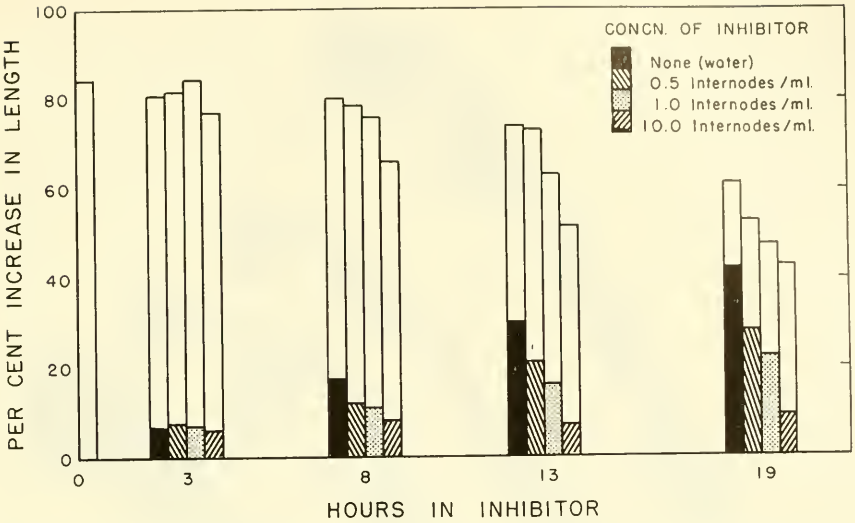

Fig. 2. Percentage increase in growth of wheat coleoptile sections during exposure to different concentrations of inhibitor (shaded portion at base of each column) and after washing in water and subsequent incubation in 5 p.p.m. solution of sodium indole-3-acetate (unshaded). From left to right at each time interval the concentration of inhibitor was 0 (water control), 0.5, 1.0, and 10.0 ints/ml.

Coleoptile Curvature

While *Avena* coleoptile sections are affected by the inhibitor in the same way as wheat sections, curvature due to IAA is much less curtailed, as seen from the examples in Table 1. The inhibitor

Table 1. The effect of inhibitor on *Avena* coleoptile curvature induced by 0.1 p.p.m. IAA.

Average Curvature, Degrees				
Concentration of inhibitor (ints/ml)				
0	1	10	20	40
10.9	....	8.9	8.0	....
16.5	14.2	13.7	12.9	11.6

causes no curvature alone, its effect being estimated by comparing the curvatures induced by agar blocks containing NaIAA with or without inhibitor; the test method employed was that of Rawes and Hatcher (13).

Wheat Leaf-Base Extension

Using a modification of Radley's method (12), 10 mm. sections were cut 2 mm. from the base of wheat coleoptiles 4 to 6 cm. long,

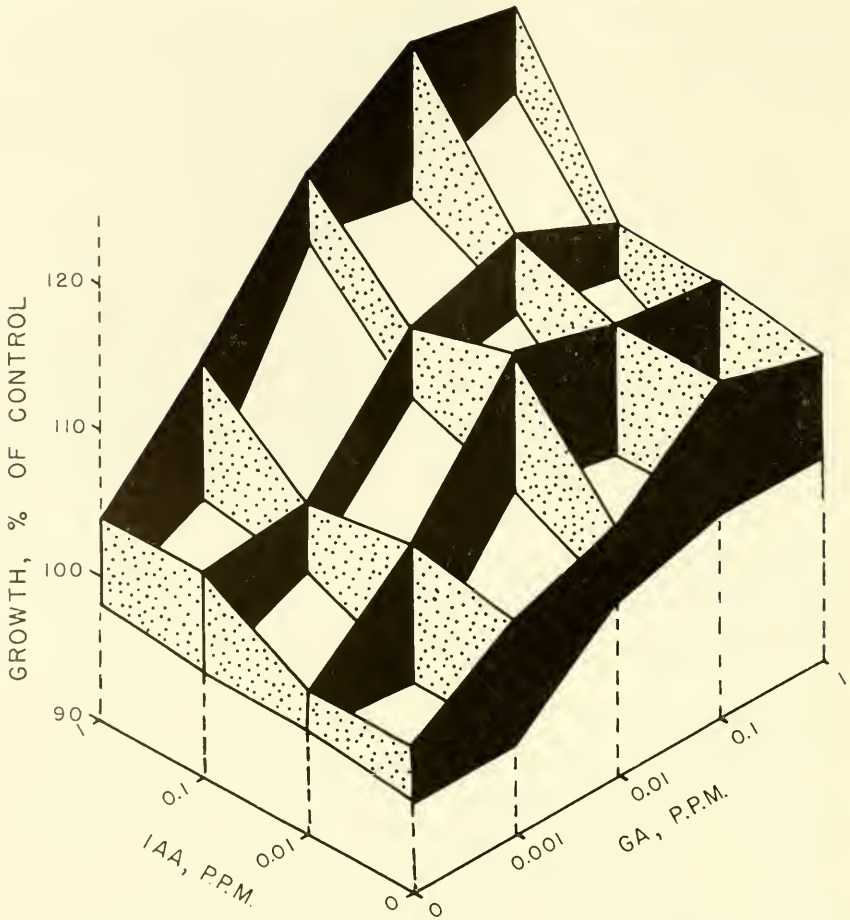

Fig. 3. Growth (as a percentage of controls in water) of wheat leaf bases in various combinations of sodium indole-3-acetate and gibberellic acid in the presence or absence of the inhibitor. The shaded portion represents the reduction in growth due to the inhibitor (5 ints/ml).

and five sections put into small vials with 0.2 ml. solution. As in our coleoptile section test, these tubes were rotated horizontally for the growth period of *ca.* 20 hrs. (though a longer period is probably advantageous in this test). Mixtures of NaIAA and GA (gibberellic acid) were used with and without inhibitor at a concentration of 5 ints/ml. Figure 3 shows the results, from which it is clear that the inhibitor reduces leaf extension in all solutions about equally — it is not only the IAA-induced growth which is affected.

Slit Pea Stem Curvature

Sections 4 cm. long were cut from the third internode of 'Alaska' pea seedlings grown in the dark with short daily doses of white light

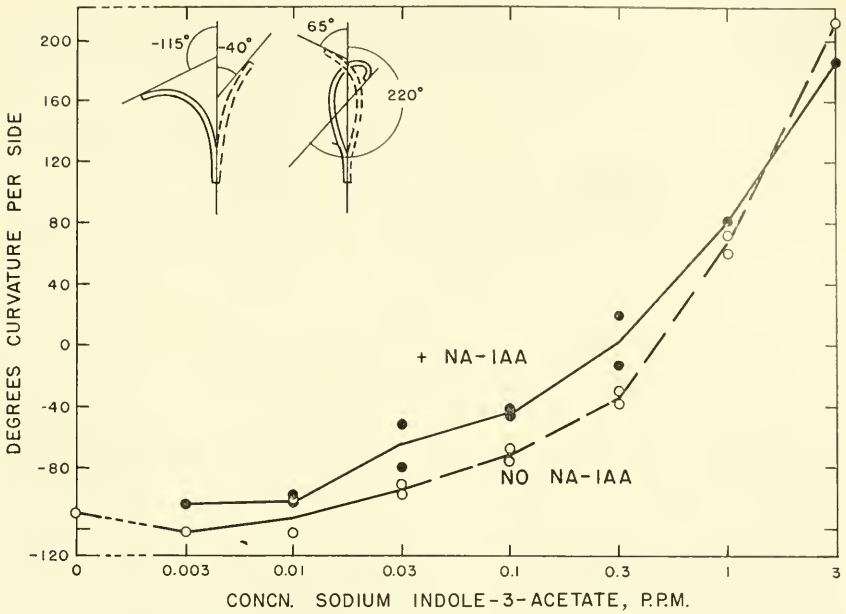

Fig. 4. Average curvature per side of slit pea stems in response to NaIAA with (broken lines), or without (solid lines), inhibitor at 10 ints/ml. The curve shows the average response from two separate experiments, the individual values being shown as open and closed circles for sections with and without inhibitor respectively. The method of measuring the curvature is shown in the insets.

(15); they were slit to within 1 cm. of the base, washed in running tap water for a few hours, and then dispensed six to a petri dish containing 3 ml. solution. After *ca.* 20 hrs. tracings were made of the segments, and the amount of curvature measured as shown in the inset on Figure 4 (14). The graphs show the steadily increasing inward curvature with increasing concentration of NaIAA, and an *augmentation* of this effect by inhibitor at 10 ints/ml except at the highest auxin level used (3 p.p.m.).

Interpretation of the results of the slit pea stem test is difficult (15), and without further work it is impossible to say whether the inhibitor has had an "auxin-sparing" effect, making each auxin concentration in effect higher, or has inhibited the inner tissue more than the outer, so resulting in a greater net inward bending for a given elongation of the outer tissues. *Straight growth* of pea stem sections is certainly inhibited, but only a few tests have been performed and comparable data for inner and outer tissues — the critical point in this connection — have not been obtained.

Pollen-Tube Extension

The germination of apple pollen was not greatly affected by even quite high concentrations of inhibitor, but the length of the tubes

Table 2. The effect of different inhibitor concentrations on the length (μ) of pollen tubes of two apple varieties growing in 10 per cent sucrose; average of solutions containing 0, 1, and 5 p.p.m. 1-naphthaleneacetic acid.

Inhibitor Ints/Ml	'Edward VII'	'Lord Lambourne'
0	35.4	40.3
1	34.3	24.7
5	21.0	16.6
50	18.2	14.3

was reduced. Table 2 shows the results of two experiments averaged together.

It is of interest that the highest inhibitor concentration, which would have a drastic effect on coleoptile section extension, has allowed quite an amount of growth by pollen tubes. These test objects were too delicate to wash and transfer to inhibitor-free solution to find out if recovery could occur.

Extension of Cress Roots

Cress seeds were grown on damp filter paper at 25° C. for 2 days, and those with radicles 5 to 7 mm. long selected (11), and placed, five in a line, on filter paper in petri dishes sloped at *ca.* 45°; 2 ml. solutions were applied to the papers, and the root length measured after 48 hrs. at 25° C. In another method, 20 seeds were sown directly onto filter paper wetted with the solutions, an extra ml. being added to allow for the water taken up by the gelatinous seed coats.

Results with either method have always been very variable, but from a large number of tests it is possible to say with certainty that the inhibitor does not function as an anti-auxin, neither promoting root growth at any concentration, nor relieving the inhibition due to NaIAA.

Fungal Growth

The inhibitor, even at 100 ints/ml, had no effect on the growth of *Pullularia pullulans* (a saprophytic ascomycete), *Verticillium albo-atrum* (a pathogenic ascomycete), or an unidentified yeast, in tests kindly carried out by the Plant Pathology Section of this station.

Abscission

Three experiments, with three clones of *Coleus* sp. in each, failed to detect any effect of inhibitor on the abscission of debladed petioles

on detached half nodes in a humid chamber (10), either treated with various concentrations of α-naphthaleneacetic acid or not. Some tests on bean explants[1] similarly showed no definite effect of the inhibitor.

Respiration of Coleoptile Sections

No differences could be detected either in oxygen uptake or carbon dioxide output after tipping inhibitor into the Warburg flasks, although growth was greatly reduced; the sections were in K_2HPO_4 buffer at pH 4.6 at 20° C. This inhibitor evidently does not affect growth by a general reduction in metabolic activity.

Permeability of Cells to Water

The short-term effect of inhibitor on water exchange, which was independent of growth, was studied by observing the rate of loss of fresh weight by potato discs in 0.5 *M* mannitol solution with or without inhibitor at 10 ints/ml, and the rate of recovery of plasmolyzed discs on their return to water with or without inhibitor. In the former case a loss of 32 per cent of the initial weight, and in the latter a regain of 29 per cent, were unaffected by the presence of inhibitor, indicating that the inhibitor does not operate by altering the permeability of the protoplast to water.

Transport of NaIAA and Inhibitor Through Coleoptile Sections

Agar plates $12 \times 9 \times 1$ mm. were prepared containing NaIAA at 0.25 p.p.m., inhibitor at 10 ints/ml, both, or neither. Coleoptile sections 1 cm. long were cut, and stored on a klinostat at 3° C.; at appropriate times samples were taken, the leaf removed, and three sections of 2 mm. cut from the middle region of each 1 cm. section. Three sets of three of these rings were placed lower ends downward onto an agar plate on a glass slide, and a second plate applied on top of the rings; this upper plate was covered by a thin sheet of polyethylene, and the whole system stored under humid conditions at 20° C. for times varying from 2 to 23 hrs. Either the upper or the lower plate was a "donor," containing the substance to be transported, and the opposite one a "receptor" of plain agar; at the end of the period the plates were separated, cut into three strips, and placed in the standard section test vials with 0.25 ml. water for testing with five coleoptile sections as usual. The rings used for transport were fixed, and subsequently measured under the microscope.

Figure 5 shows the response of sections to the material present in donor or receptor plates after periods of transport of 0, 2, 4, 8, 15, or 23 hrs. The apical plate is shown on the left of each pair of histograms; the donor plate is shaded and the receptor plain. It is clear that the NaIAA has behaved "classically," the donor plate in row

[1] Kindly carried out for us by Dr. D. J. Osborne at Oxford.

losing promoter and the receptor gaining it with increasing time of transport; in row 2, with the donor plate at the base of the section, there has been no movement of promoter. Inhibitor has not moved either way (rows 3 and 4), nor has it prevented the basipetal movement of promoter; with the mixture of auxin and inhibitor giving a response about the same as the controls to begin with, when applied apically (row 5) the promoter moves out of the donor block, so that it becomes more and more inhibitory, and into the receptor block which becomes more and more promoting. Basal application of the mixture allows no movement so that the net effect of the plate remains at about the control level.

It is of great interest, however, that the sections used for transport have responded equally to promoter applied at apex or base, the length of the rings after 23 hrs. transport time being 108.5 per cent and 109.5 per cent, respectively, of the length of rings between plain agar plates; they also responded similarly to apical or basal supplies of inhibitor (90.5 and 90.6 per cent) or the mixture of inhibitor and promoter (94.0 and 94.6 per cent), implying that these substances can *enter* the coleoptile at either surface, but only the promoter can leave it, and then only from the basal end.

The entry at either end to produce the expected promotion or inhibition of growth has been demonstrated in another series of experiments with 1 cm. coleoptile sections. Short lengths of polyvinyl chloride tubing of a bore slightly larger than a coleoptile were held in two parallel strips of plasticene on 3 inch x 2 inch glass slides so that pairs of tubes were in line, with their ends 8 mm. apart; a coleoptile section was held between the tubes with 1 mm. inserted in each. There were ten pairs of tubes per slide, the left-hand row always receiving the apical end of the section; solutions were dispensed into the appropriate tubes from a micrometer syringe, and after loading with coleoptile sections the slides were placed on a klinostat so that the sections rotated horizontally about their long axes (to keep them straight). Table 3 shows the results of two experiments averaged together, and indicates that inhibitor at *either* end reduces growth about equally. Because NaIAA is rather more effective supplied apically than basally (d vs. b, g vs. c) [confirmed by other experiments here, and with other techniques by Housley, Bentley, and Bickle (7) and Choudhuri (5)], only strictly comparable treatments must be considered, e.g., compared with water, inhibitor at either end causes a reduction of 8 per cent (e — f, e — h), but in the presence of promoter the real effects of inhibitor are represented not by a — c, and a — g, but by the difference between water and inhibitor at a given end, i.e., b — c = 12 per cent for apically supplied inhibitor, and d — g = 13 per cent when supplied basally.

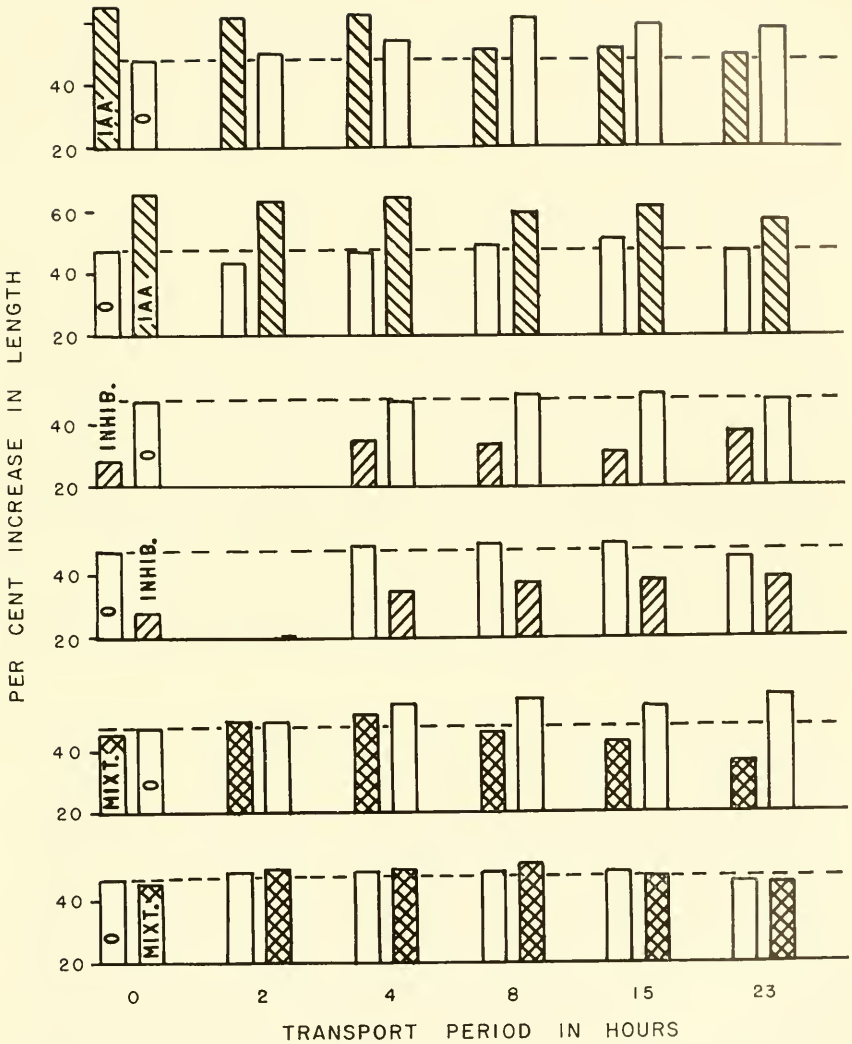

Fig. 5. The response of coleoptile sections (percentage increase over original length) to materials transported through coleoptile tissue from a "donor" agar plate (shaded histograms) to a "receptor" plate (unshaded); the donor plate contained NaIAA, inhibitor, or a mixture of the two. The response to the apically applied plate is shown on the left of each pair of histograms, that to the basal application on the right. Transport periods were 0, 2, 4, 8, 15, and 23 hrs. (For further details see text.)

Table 3. Percentage increase over initial length (10 mm.) of coleoptile sections given separate apical and basal supplies of NaIAA 1 p.p.m., inhibitor 20 ints/ml, or water. Mixture of NaIAA and inhibitor at both ends = 17.

Basal Supply	Apical Supply					
	NaIAA		Water		Inhibitor	
NaIAA	a.	48	b.	38	c.	26
Water	d.	50	e.	24	f.	16
Inhibitor	g.	37	h.	16	j.	12

DISCUSSION

Although the concentration response curves differ according to the test objects employed, this inhibitor reduces extension in the following: coleoptile sections with or without an exogenous source of auxin, pea epicotyl sections, wheat leaf bases, with or without added auxin or GA, cress roots, and pollen tubes; it also reduces the auxin-induced curvature of *Avena* coleoptiles. It increases the effect of auxin in causing inward curvature of slit pea stem sections, but its action here may not necessarily be truly synergistic. It has no effect in our tests on abscission, respiration, or permeability of the protoplast to water; the inhibitor does not move out of coleoptile sections into agar in either direction; transport of NaIAA through coleoptile sections is not affected.

How then does this material effect a reduction in cell extension? These experiments do not provide an answer, but they do indicate the desirability of testing substances for biological activity in a variety of ways before calling them "inhibitors" or "promoters," or at least of keeping the definition of their activity continually in mind. Had we assayed our chromatograms by the slit pea stem test, we should have found a "synergist of IAA"; by an abscission or respiration test the same region would have been "inactive," while by many other tests it would have appeared as "inhibitory." Which of these roles, if any, does this material play in the physiology of the plant from which it has been extracted (Cf. 4)? This is the basic and disturbing question which applies to many growth substance studies, and which has seldom been answered unequivocally, particularly for these enigmatic "inhibitors" of coleoptile section extension.

It is obvious that the nature, mode of action on the cell, and function in the plant, of such substances, warrant intensive study by chemists and biochemists, at least to the same extent as that devoted to auxins and gibberellins.

SUMMARY

Shoots of the plum rootstock 'Myrobalan B' extracted with ether yielded material which, when separated on paper chromatograms, was inhibitory to coleoptile extension. This material was tested in various ways, with the following results.

Inhibition: coleoptile section extension with or without added promoter (sodium salt of indole-3-acetic acid = NaIAA) and when supplied to either end of the section; coleoptile curvature due to unilaterally applied auxin (but less sensitive than section growth); wheat leaf-base extension in the presence or absence of NaIAA or GA (gibberellic acid); pea stem section extension; apple pollen tube extension; cress root extension.

Augmentation of auxin effect: slit pea stem inward curvature due to auxin was increased by inhibitor.

No effect: abscission of *Coleus* and *Phaseolus* explants; respiration of coleoptile sections; water loss during plasmolysis of potato discs or water uptake after plasmolysis; transport of NaIAA basipetally through coleoptile sections was not hindered by inhibitor.

Recovery of coleoptile sections is considerable, and depends on the severity of the inhibitor treatment before releasing the inhibition by washing in water.

Our lack of knowledge on the nature, mode of action, and function in the plant of such inhibitors is stressed, and study of these substances by chemists and biochemists called for.

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The Mechanisms of Auxin Activation and Inactivation

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The Indole-3-acetic Acid Oxidase-Peroxidase of Peas

I want to give a brief outline of the structure and properties of the enzyme known as indoleacetic acid oxidase from peas. This enzyme system has suffered somewhat from the publication of experiments which are *almost* relevant, and from unsympathetic comparisons of preparations from diverse tissues, with the result that the literature is conflicting, both in substance and in detail. I want to try to reconcile some of these divergences, and present a generalized picture.

Firstly, indole-3-acetic acid (IAA) is inactivated by plant tissues.

Secondly, macerates from many plant tissues can inactivate IAA by oxidation (9, 17, 19). Whether such preparations have real significance for the growth of the plant or are artifacts of maceration (1, 2) is a question discussed elsewhere.

Thirdly, it seems likely that there exists a *number* of possible ways in which IAA is enzymatically destroyed. Evidence is accumulating that there are several alternative systems. These may vary from tissue to tissue or may coexist to varying degrees in the same tissue (8, 9). Any postulated role assigned IAA oxidase for a particular tissue must take into account the properties of those enzyme systems present in that tissue.

The IAA oxidase system of etiolated pea epicotyls or roots is present entirely in the nonparticulate fraction (8). Each mole of IAA disappearing is accompanied by the consumption of one mole of oxygen and the production of one mole of carbon dioxide (17, 19). The stepwise reduction of oxygen, with the intermediate formation and subsequent utilization of hydrogen peroxide, is inferred from the total inhibition of IAA destruction by peroxide-consuming addenda.

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such as catalase (4, 6), certain peroxidase substrates (6, 13), or manganese (4) in the presence of the peroxidase of the preparation. The notion that peroxidase is obligatorily implicated in the process is supported by the total inhibitions produced by cyanide, azide, and other agents which bind heavy metals (17, 19).

Thus, we may picture that IAA is subject to two successive oxidative steps, one mediated directly or indirectly by oxygen, the other by hydrogen peroxide:

$$\begin{array}{lll} \mathrm{IAA} + \mathrm{H_2O_2} \longrightarrow \mathrm{P_1} & & \text{(peroxidase)} \\ \uparrow \qquad\qquad\quad | & & \\ \mathrm{P_1} + \mathrm{O_2} \longrightarrow \mathrm{P_2} + \mathrm{H_2O_2} & & \text{(oxidase)} \end{array}$$

The peroxide must be produced in one of the steps in the oxidation of IAA itself, for if the oxidation of an exogenous metabolite were involved obligatorily, two and not one mole of oxygen per mole of IAA would be consumed. Since catalase can inhibit oxygen uptake completely, we may infer that the peroxidase moiety deals with IAA itself and the oxidase with the reaction product. Otherwise we would expect a maximum inhibition of 50 per cent oxygen uptake while the oxidase step continued independently.

Dialysis (8) or ultrafiltration (13) of enzyme preparations reveals that activity is enhanced by the presence of a diffusible cofactor. However, exhaustive dialysis may not reduce the activity to *zero,* and for a particular tissue, there remains a consistent residue of activity which is not cofactor-dependent (8). This residue may amount to about 40 per cent in macerates from the epicotyls of peas grown in weak red light, 10 per cent for those grown in darkness, and zero for pea roots. The pineapple enzyme appears to have no cofactor requirement (9). This suggests that there are at least two systems for IAA destruction, acting in parallel. However, as oxidation of IAA is *totally* inhibitable by catalase, cyanide, or guaiacol, the *total* activity depends on peroxidation. Activity lost by dialysis can be replaced, or even increased above the original level, by adding a monohydric phenol, e.g., 2,4-dichlorophenol (DCP), optimum concentration 3×10^{-5} *M*. As DCP can overcome the inhibition by catalase (6, 7), it is concluded that it stimulates production of peroxide or promotes its more effective utilization.

A property of great interest in the pea epicotyl preparation is its activation by light. Though there is considerable activity in darkness, up to fourfold promotion may be obtained by 250 foot candles of white light (4). The action spectrum for the increment of activity

corresponds closely to that for the photosensitized oxidation of IAA by riboflavin and to the absorption spectrum of riboflavin (3). It has therefore been postulated by Galston *et al.* (5) that a flavine enzyme is involved in the light-promoted step. And because light overcomes the inhibitory effect of added catalase, and in view of the well-known production of hydrogen peroxide upon autoxidation of flavines, it was proposed (5) that the oxidase step, i.e., the peroxide-generating step, was mediated by a flavine enzyme.

Other workers (9, 20) interpret the light promotion as being due to photolysis of a natural inhibitor, for the promotion occurs only on undialyzed preparations.

Notwithstanding the activation by light and by a dialyzable cofactor, it has been demonstrated (5, 13) that crystalline horseradish peroxidase alone can catalyze the oxidation of IAA. (Acting on its classical substrates, this enzyme requires no cofactor, nor light.) Oxygen is consumed, and peroxide is again obligatorily involved. There may be a lag phase in the reaction, which can be abolished by adding hydrogen peroxide, or natural cofactor, or DCP. The reaction scheme evolved by Kenten (13), and generally supported in its essentials by others, is seen in Figure 1.

Manganous ions have been variously reported to promote (9, 13, 19, 20) or inhibit (4, 8, 10) the oxidation of IAA. It now appears (11, 14)

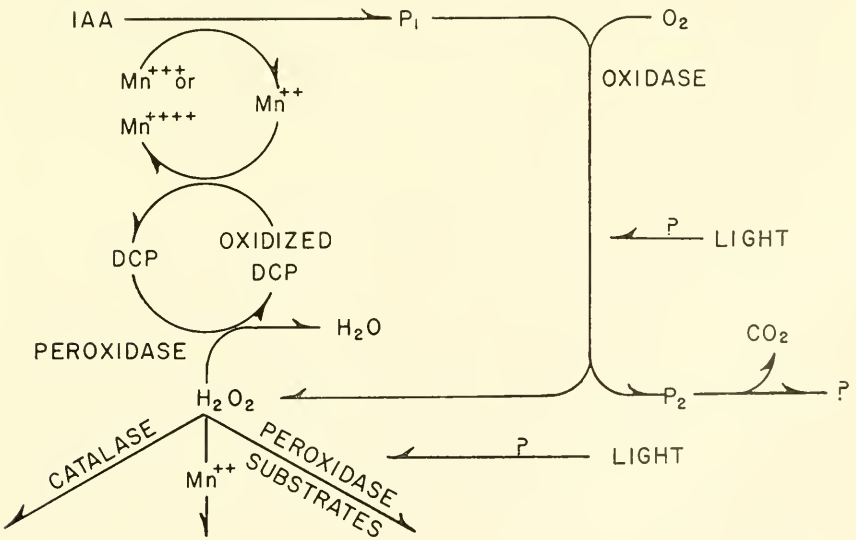

Fig. 1. Reaction scheme evolved by Kenten for the enzymatic oxidation of indole-3-acetic acid.

that inhibition occurs in the absence of natural cofactor or added phenolic carrier due to the catalytic diversion of peroxide:

$$MnO \longrightarrow MnO_2$$

$$MnO_2 + H_2O_2 \longrightarrow MnO + O_2 + H_2O$$

$$MnO + MnO_2 \longrightarrow Mn_2O_3$$

$$Mn_2O_3 + H_2O_2 \longrightarrow 2MnO + O_2 + H_2O$$

Promotion occurs in the presence of natural cofactor or DCP by facilitating utilization of peroxide (13, 20).

Thus, it seems that purified peroxidase can mediate the peroxidation of IAA and the oxidation (by oxygen) of its product. This is not an unique instance, highly purified peroxidases acting similarly toward dihydroxymaleic acid (16), tryptophan (15), phenylacetaldehyde (12), and certain dicarboxylic acids (14).

It is now established (11, 18) that plant tissues contain several separable peroxidases, differing in substrate specificities. It would be desirable to compare the peroxidase components of tissues under study, determine which can mediate IAA oxidation, and examine whether these separated components show any differential response to light or DCP.

The *total* IAA oxidase activity of pea epicotyl brei may be contributed to by a number of enzymatic components, and modified by the presence of manganese, natural cofactors and inhibitors, and light. The important task facing us is not only to resolve the descriptive biochemistry of the macerates, *especially for each tissue under consideration,* but to interpret the meaning of this IAA-destroying activity for the plant.

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Inhibition and Retardation of the Enzymatically Catalyzed Oxidation of Indole-3-acetic Acid

From studies described previously (5, 6, 9) on the enzymatic and nonenzymatic breakdown of indole-3-acetic acid (IAA) in vitro, a chain oxidative reaction sequence has been proposed for the peroxidase catalyzed decarboxylation and oxidation of IAA.

The chain reaction is initiated by a system consisting of either peroxidase or catalase and a specific type of phenolic cofactor which oxidizes manganous (Mn^{+2}) to manganic ions (Mn^{+3}); propagation of the chain is brought about by a reaction between IAA ($S{\cdot}COOH$) ($S =$ skatole) which results in its spontaneous decarboxylation and the consumption of one equivalent of oxygen to form a skatole peroxy-radical as follows:

$$Mn^{+3} + S{\cdot}COOH \longrightarrow Mn^{+2} + H^{+} + CO_2 + S{\cdot}$$
$$S{\cdot} + O_2 \longrightarrow SO_2{\cdot}$$

The skatole peroxy-radical is stabilized by an enzyme-controlled peroxidation involving the phenolic cofactor (ROH) as hydrogen donor, resulting in the latter's oxidation and the formation of an end product of empirical formula (SO_2H); *viz.*

$$SO_2{\cdot} + ROH \xrightarrow[\text{catalase}]{\text{peroxidase}} SO_2H + RO{\cdot}$$

The phenolic radical (semiquinol) is capable of oxidizing manganese and regenerating the reduced cofactor by the Kenten-Mann reaction (4) until the supply of IAA is exhausted, e.g.,

$$Mn^{+2} + H^{+} + RO{\cdot} \longrightarrow Mn^{+3} + ROH$$

In the present communication the inhibitory and retarding effects of various substances on IAA oxidation are interpreted in terms of

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a chain-stopping or chain-transferring mechanism. It should be noted that the destruction of IAA differs from standard chain oxidations (7) by being dependent on peroxidase. Consequently, substances that inhibit peroxidase or catalase, e.g., cyanide, also inhibit IAA oxidation. Such enzyme poisons are to be distinguished from inhibitors and retarders of the chain oxidation of IAA and are not considered in this paper.

METHODS

The preparation of wheat leaf extracts and horseradish peroxidase, the reaction conditions, and the experimental techniques used in the present study have been described previously (6, 9). Unless otherwise stated, standard systems with resorcinol or dichlorophenol (DCP) as cofactor have been used to catalyze the oxidation of IAA. These contain the following components: 0.50 ml. wheat leaf enzyme (ca. 0.2 mg. protein N); 3.0 μM $MnCl_2$; 1.5 μM resorcinol or 2,4-dichlorophenol (DCP); 150 μM orthophosphate, pH 6.0; 6.6 μM IAA, ammonium or sodium salt, pH 6.0 = 158 μl. O_2; vol. 3.0 ml.; 29.5° C.

EXPERIMENTAL RESULTS

Typical progress curves of the oxygen uptake during IAA oxidation catalyzed by extracts from winter-grown wheat leaves exhibited

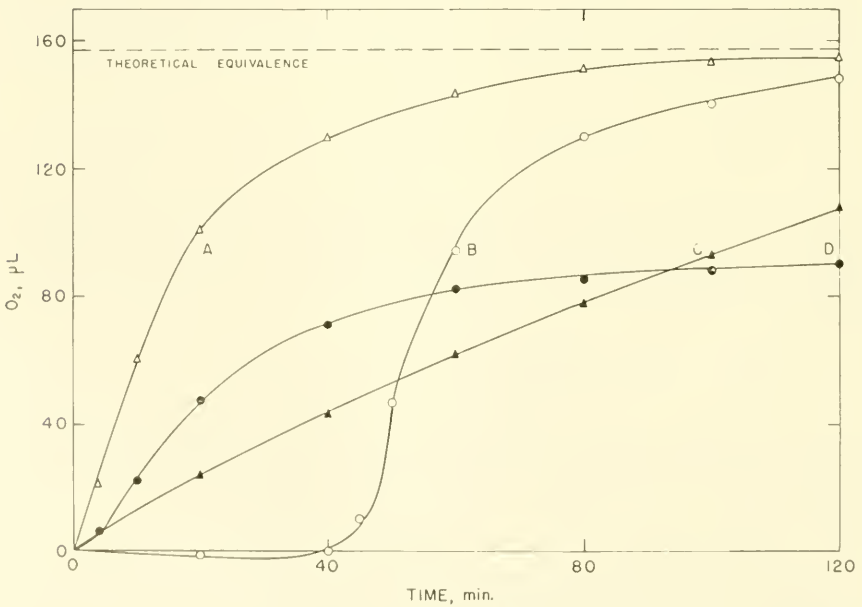

Fig. 1. Retardation and inhibition of indole-3-acetic acid oxidation. Systems standard with: **A**, no additions; **B**, 0.03 μM (10^{-5} M) catechol; **C**, 0.63 μM (2.1×10^{-4} M) riboflavinphosphate; **D**, 0.045 μM (1.5×10^{-5} M) hydroquinone.

Table 1. Inhibition and retardation of indole-3-acetic acid oxidation in the presence of catechol, hydroquinone, and riboflavinphosphate.*

Cofactor	Induction Period in the Presence of Catechol, (Minutes)	Percentage of Control Rate		
		Hydroquinone	Riboflavinphosphate	
			Dark	Light
Resorcinol	46.5	55	35	100
Dichlorophenol	24.0	59	28	97
Phenol	26.5	45	31	91
Maleic hydrazide	30.0	53	33	94
Natural factor	41.0	42	40	147

* Concentrations: Catechol 1.5×10^{-2} μM; hydroquinone 6×10^{-2} μM; riboflavinphosphate 3.3×10^{-1} μM; resorcinol, DCP, phenol, 1.5 μM; maleic hydrazide 30 μM, natural factor 0.15 ml. Vol. 3.0 ml. blue light, 220 foot candles.

a short induction period and a subsequent rapid rate of oxidation that gradually decelerated until theoretical molar equivalence was attained (Figure 1A). The rate of oxidation was influenced by substances that either extended the induction period, e.g., catechol at 10^{-5} M (Figure 1B), or retarded the rate of oxidation from the outset, e.g., riboflavinphosphate at 2.1×10^{-4} M (Figure 1C) or hydroquinone at 1.5×10^{-5} M (Figure 1D).

The above experiments were performed using resorcinol as cofactor of the oxidation, but the effects of these inhibitors and retarders were the same in the presence of other cofactors (Table 1). Catechol resulted in an extension of the induction period the length of which depended on the cofactor used. On the other hand, the degree to which hydroquinone and riboflavinphosphate retarded the oxidation of IAA was almost independent of the nature of the cofactor. The systems retarded by riboflavinphosphate, but not those retarded by hydroquinone, were rendered fully active by illumination (Table 1).

Extension of the Induction Phase (Inhibitors)

The relationship between the concentration of catechol in the system and the length of the induction period is shown in Figure 2. The data show that the oxidation of IAA would never occur above a catechol concentration of 1.9×10^{-5} M. However, this value can be regarded only as approximate since the age of the solutions and the enzyme used were found to influence the length of the lag phase induced by catechol (9).

A similar inhibition was caused by pyrogallol and the flavonoid pigment rutin, both of which extended the lag phase by 25 min. at

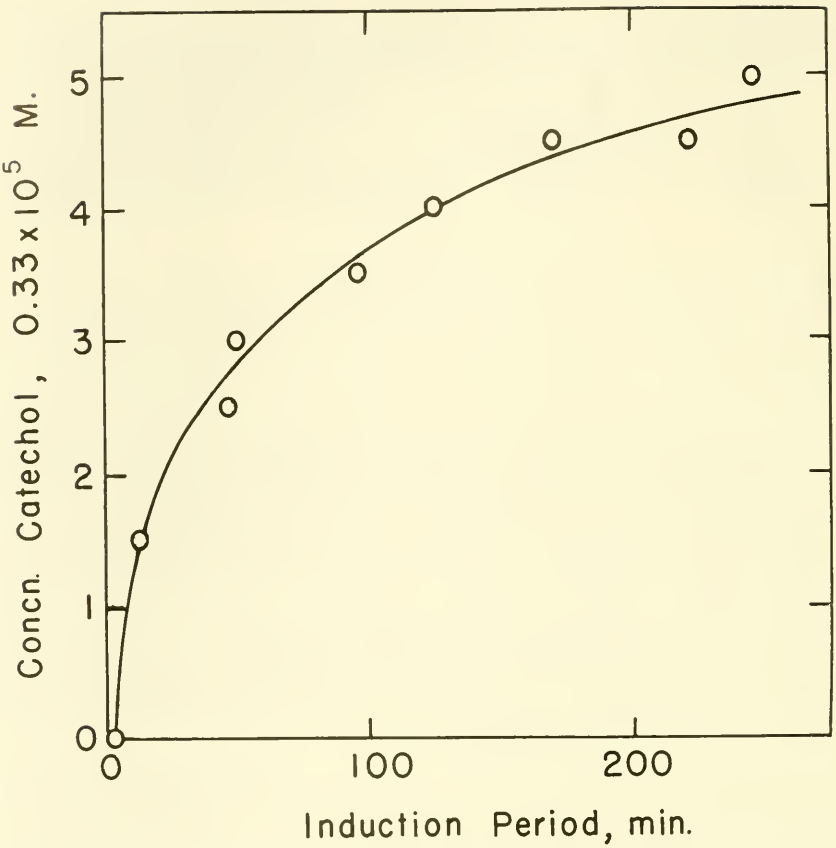

Fig. 2. Induction period in indole-3-acetic acid oxidation caused by catechol. Systems standard, catechol added prior to IAA.

concentrations of 1.7×10^{-5} *M* and 0.5×10^{-5} *M* respectively. Catechol and pyrogallol were reported also to inhibit the oxidation of IAA catalyzed by horseradish peroxidase (3). Ascorbic acid is another inhibitor that extended the lag by 45 min. at 3.33×10^{-5} *M*. It is noteworthy that an appreciable lag phase was induced by these inhibitors at concentrations as low as 10^{-5} *M*.

Retarders

Typical of the progress curves of oxygen uptake at varying concentrations of retarder are those shown in Figure 3, where riboflavin-phosphate was progressively more inhibitory in darkness as its concentration was increased above 10^{-5} *M*. The progress curves for hydroquinone retardation were similar except that the systems attained a low equilibrium (Figure 1). This was not due to enzyme or cofactor destruction, but apparently to a decline in the concen-

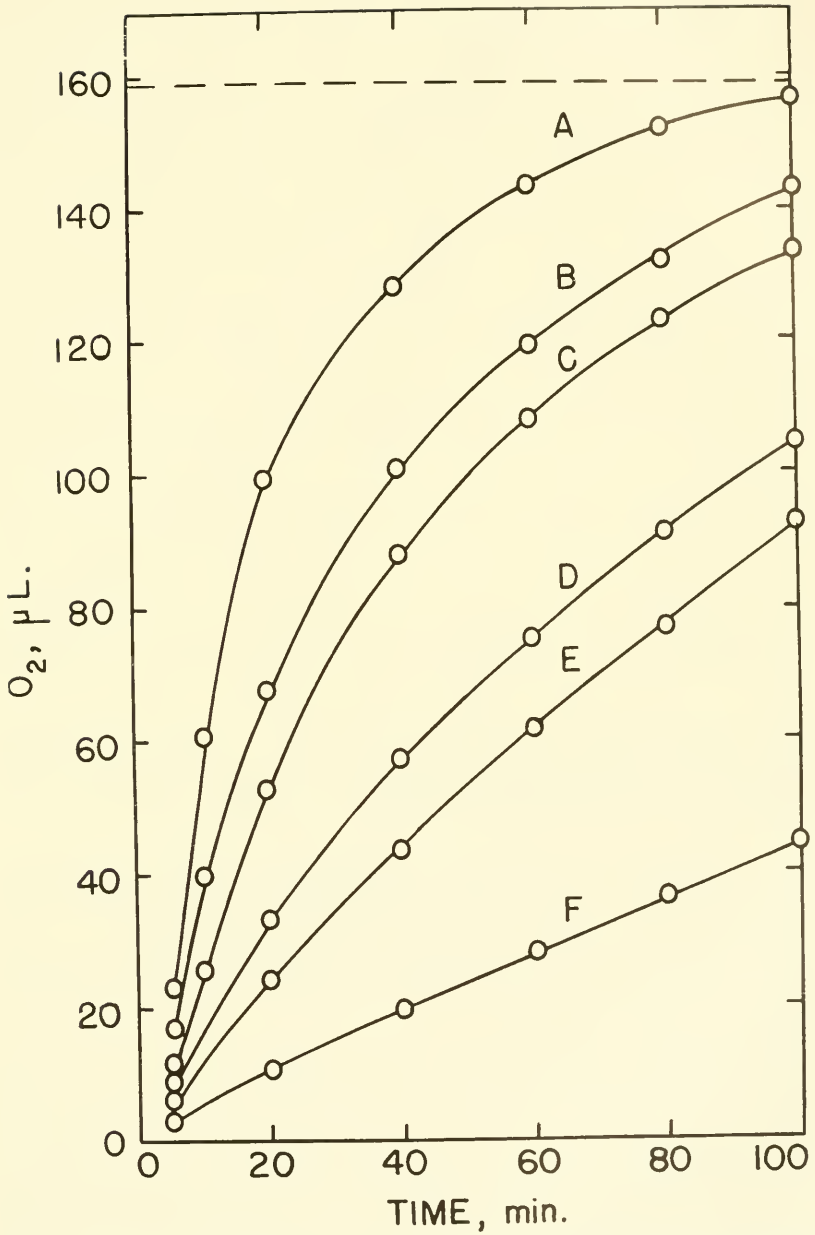

Fig. 3. Progress of indole-3-acetic acid oxidation in the presence of riboflavinphosphate in darkness. Systems standard, riboflavinphosphate concentration: **A**, 0; **B**, 0.126 μM; **C**, 0.188 μM; **D**, 0.375 μM; **E**, 0.63 μM; **F**, 3.65 μM.

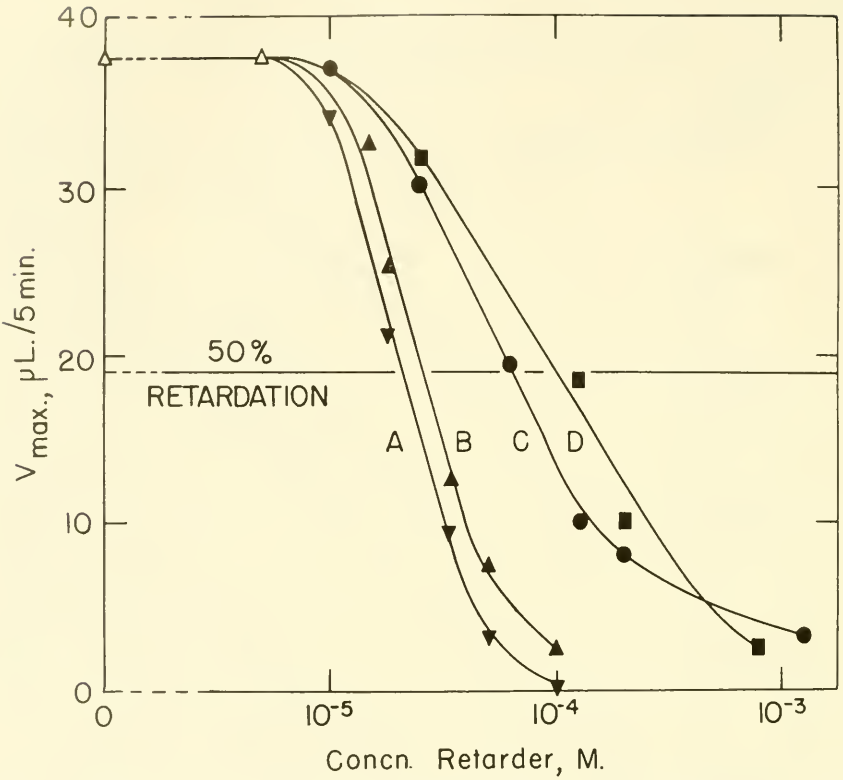

Fig. 4. Relative rates of indole-3-acetic acid oxidation in the presence of various concentrations of retarders. Systems standard containing: **A**, hydroquinone; **B**, *p*-quinone; **C**, riboflavinphosphate; **D**, scopoletin.

tration of IAA, since the further addition of IAA at equilibrium caused a resumption of oxygen uptake. Both hydroquinone and its oxidation product *p*-benzoquinone retarded the oxidation to the same extent. The coumarin derivative scopoletin, already shown to be a competitive inhibitor of IAA oxidation (1), had effects resembling riboflavinphosphate.

The maximum initial rates of IAA oxidation in the presence of each of these retarders are compared as a function of retarder concentration in Figure 4. Unlike the inhibition induced by catechol, etc., inhibition caused by retarders did not appear to depend on the age of reagents, enzyme, etc., but only on the concentration. Irrespective of the absolute rate of the unretarded control, close to 50 per cent retardation occurred at the following concentrations: hydroquinone 3.0×10^{-5} *M*; *p*-quinone 3.5×10^{-5} *M*; riboflavinphosphate 7.5×10^{-5} *M*; scopoletin 1.25×10^{-4} *M*. As indicated

Table 2. Retardation of indole-3-acetic acid oxidation* by riboflavinphosphate. The effect of enzyme, manganese, dichlorophenol, and indole-3-acetic acid concentrations.

Concentration of Varying Component				Percentage Retardation			
Enzyme, ml.	Mn^{+2}, μM	DCP, μM	IAA, μM	Enzyme	Mn^{+2}	DCP	IAA
0.1	1.5	0.5	1.66	10	75.5	24	42
0.25	3	1.0	3.33	44	75	39	53.5
0.50	6	1.5	5	52	76.5	59	60.5
0.75	30	4	6.66	60	77	70	67.5
1.0	60	6	10	58	69	77	75.5

* Standard DCP-horseradish peroxidase systems containing 0.6 μM riboflavinphosphate. Components were varied individually.

previously (Table 1) the extent to which systems were retarded by hydroquinone and riboflavinphosphate was almost independent of the nature of the cofactors.

In order to elucidate the mechanism by which riboflavin or its phosphate exerts a retarding effect on the system in darkness, kinetic studies were undertaken to determine the effect of the concentration of the individual components on the degree of retardation. The data in Table 2 show the effects of varying the concentration of enzyme, substrate, manganese, and dichlorophenol on the retardation brought about by riboflavinphosphate at a concentration of 0.6 μM. Standard DCP-horseradish peroxidase systems were employed. The data indicate that under these experimental conditions an increased concentration of any of the components was unable to overcome the retardation by riboflavin. However, it has been shown previously (6) that it would be more accurate to describe the optimum concentration of the phenolic cofactor relative to the substrate concentration since in this delicately balanced system its activity is dependent on the concentration ratio, e.g., IAA/resorcinol = 2.2, and IAA/DCP = 4.4. In subsequent kinetic experiments it was shown that when this ratio was maintained in the case of IAA/DCP and the concentration of IAA increased as in the experiments in Table 2, then the retardation by riboflavinphosphate could be partially overcome. In these standard DCP systems with 6.6 μM of IAA the rate was retarded 60 per cent by 0.5 μM of riboflavinphosphate. When the concentration of IAA was increased to 13.2 μM and 16.5 μM and the concentration ratio IAA/DCP maintained at 4.4, the retardation was 42 per cent and 24 per cent, respectively.

The Effect of Light and Riboflavin

As was demonstrated previously with the catalase-dichlorophenol system (8), blue light, absorbed by riboflavin, overcame the retarding effect of riboflavinphosphate on the wheat leaf system in the presence of any one of the cofactors (Table 1). The rate of oxidation of illuminated systems containing riboflavin was no greater than the rate in darkness without riboflavin except when the natural factor (9) was used. In the absence of riboflavin, light had no effect on the system. The augmentation of the dark rate in the illuminated system containing the natural factor and riboflavin may have been due to the combined effect of riboflavin and light in overcoming an inhibitor present in the partially purified extract of the former (cf. 8).

The alleviation of the riboflavin effect depended on the quality

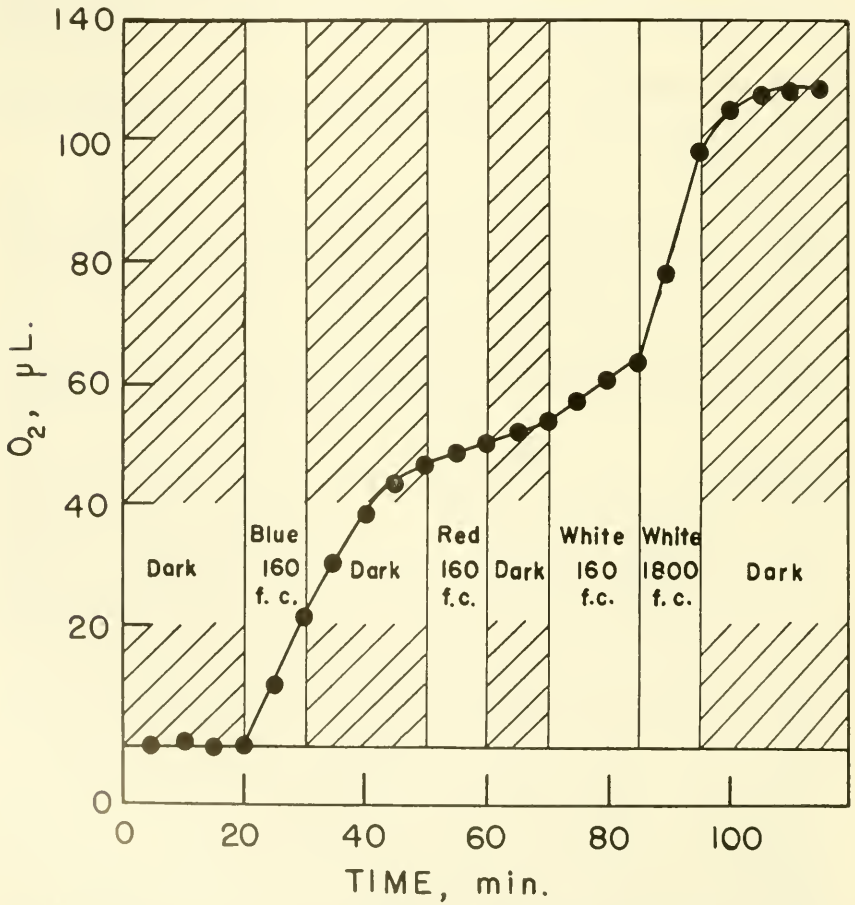

Fig. 5. Effect of quality and intensity of light on riboflavin-inhibited indole-3-acetic acid oxidation. Standard system plus 6 μM riboflavinphosphate. Color and intensity of light (in foot candles at surface of reaction vessel) indicated in diagram.

and intensity of illumination. Figure 5 shows the progress of the oxidation with various kinds of illumination. As would be expected from the absorption spectrum of riboflavin, blue light was most effective, whereas red light was totally ineffective and white light was required at a higher intensity to give the same effect as blue. In systems containing 0.33 μM riboflavinphosphate, light saturation was attained at an intensity of 110 foot candles, under which conditions the rate of oxidation attained a maximum equal to the dark rate without riboflavin. Oxygen uptake always proceeded well past the theoretical oxygen equivalence for IAA (Table 3). It is noteworthy that after the light had been switched off (Figure 5), rapid oxidation of IAA continued for some time, indicating a residual effect of light except when IAA became limiting.

The oxygen consumed in the breakdown of IAA by catalase systems was observed to exceed the theoretical molar equivalence of IAA when illuminated in the presence of riboflavin (8). This also occurred with the wheat leaf system (Figure 6A and Table 3). In the experiment (Table 3) carried on for 400 min., 199 μl. of oxygen were consumed by an illuminated standard system containing 6.0 μM riboflavin and the equivalence of 3.3 μM or 79 μl. IAA. The rate of oxygen uptake showed no signs of abating, and similar results were obtained when DCP or maleic hydrazide was present in place of resorcinol. The amount of riboflavin used in this experiment was approximately 30 times greater than that required to produce 50 per cent inhibition.

In the absence of IAA (Figure 6C), oxygen was consumed by these systems at a slower initial rate, but at the same final rate as with IAA (Figure 6A). The difference between the oxygen consumed with and without IAA (Figure 6B) always exceeded at equilibrium the molar oxygen equivalence of IAA. The difference was greater at

Table 3. Oxygen consumed by illuminated systems containing riboflavin.*

Cofactor	Oxygen Uptake (μl/400 min)		
	Plus IAA	Minus IAA	Difference
Resorcinol	199	70	129
Dichlorophenol	181	53	128
Maleic hydrazide	149	32	117

* Standard wheat leaf systems containing: 3.33 μM IAA; 6.0 μM riboflavinphosphate; blue light, 220 foot candles. Molar oxygen equivalence of IAA = 79 μl.; riboflavinphosphate = 133 μl.; resorcinol and dichlorophenol = 35.5 μl.; maleic hydrazide = 712 μl.

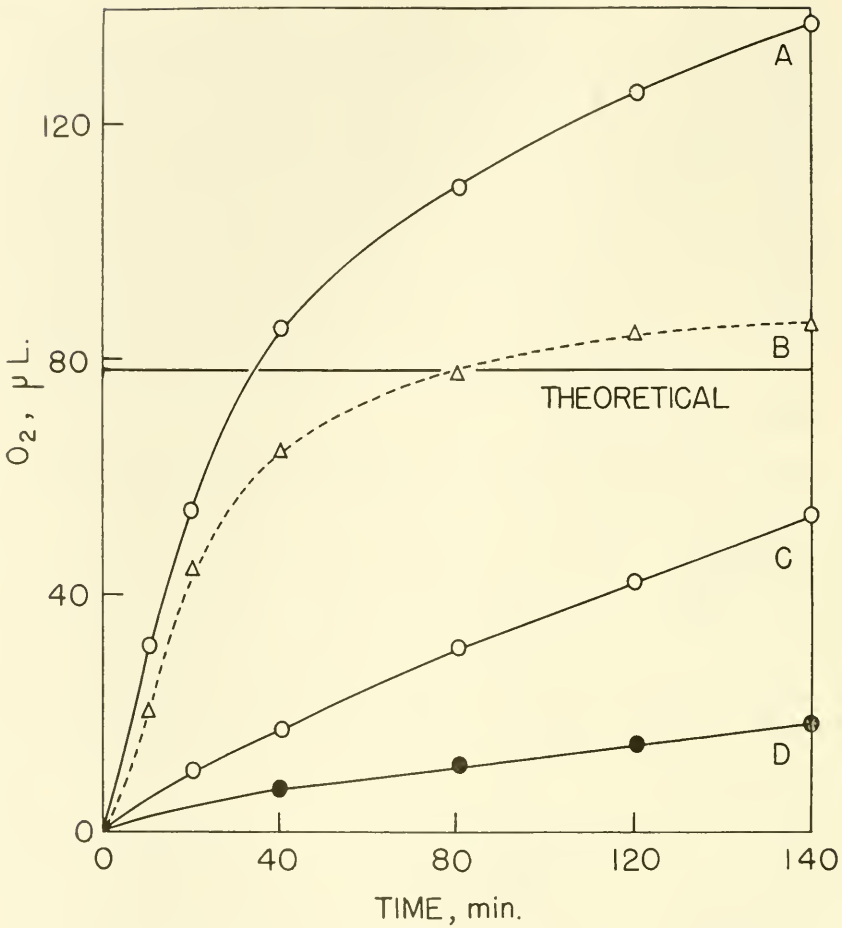

Fig. 6. Oxygen uptake of systems containing excess riboflavin with and without indole-3-acetic acid: **A**, standard system plus 6 μM riboflavinphosphate in blue light (220 foot candles) indole-3-acetic acid = 3.33 μM = 79 μl. O_2 (molar equivalence); **B**, oxygen uptake of **A** minus uptake of **C**; **C**, same as **A** minus indole-3-acetic acid; **D**, same as **C** in darkness.

higher light intensities or when the riboflavin concentration was increased, e.g., to twice the molar concentration of IAA.

DISCUSSION

In order to inhibit such an autoxidation sequence a substance must interrupt a chain of interdependent steps by reacting with an essential intermediate of the system. Inhibition may be expressed in either of two ways: (1) as an extension of the induction period which occurs in all autoxidations; or (2) as a retardation of reaction velocity. As pointed out by Waters and Wickham-Jones (7) these ef-

fects are caused by agents which interfere in the reaction sequence in fundamentally different ways and which may be described as chain-stopping and chain-transferring agents respectively.

In the presence of an inhibitor or chain-stopping agent the initiation of autoxidation is prevented. At low inhibitor concentrations the reaction may commence abruptly after a long induction period and attain a rate equal to that of the control in the absence of inhibitor. Such temporary inhibition or lag-extension indicates that the inhibitor is irreversibly changed during the induction period to a product that is not inhibitory to the reaction. Its destruction must occur at the expense of an essential intermediate of the system, but it also may be aided by side reactions, e.g., by autodestruction. Thus, the system would never operate in the presence of excessive amounts of the inhibitor or if the latter were not destroyed.

On the other hand, in the presence of retarding agents, the reaction may proceed to completion at a reduced rate without an induction phase. This could occur only if the retarder acted as a chain-transferring agent by substituting a sequence of slow reactions within the rapid sequence of the control. In order to exert its retarding effect continuously, the substance must be reformed following reaction with an essential intermediate. Thereby the retarder inserts a shunt into the normal reaction sequence which slows down the speed of propagation.

Mechanism of Inhibition by Chain-stopping Agents

According to the foregoing definition, it may be validly assumed that the catechol-type inhibitor is a chain-stopping agent and is destroyed by an intermediate prior to or during the first reaction step of IAA oxidation. The length of the catechol-induced lag period is dependent on its concentration, and when an amount of catechol that would normally cause a 45 min. lag phase is added during the oxidation only a brief staggering of the rate resulted. This was also true of the inhibitor found in boiled undialyzed preparations of commercial catalase (8). Such effects would be expected if the destroying agent is an essential intermediate present at a higher concentration after the oxidation has commenced. Catechol, pyrogallol, ascorbic acid, and probably rutin are all readily oxidized and could temporarily inhibit the oxidation of IAA by reducing manganic ions, a cofactor radical, or possibly a skatole radical.

We have suggested previously (9) that manganic ions arising during the initiation and propagation reactions preferentially oxidize catechol. This is supported by the findings that MnO_2, PbO_2, H_2O_2, or an oxygen atmosphere may partially or completely overcome catechol inhibition since these are known to lead to the formation

of Mn^{+3} from Mn^{+2}. Manganiversene is rapidly reduced by catechol, and pre-incubation of catechol with manganiversene or even manganous ions also destroys its inhibitory effect, presumably by promoting oxidation of catechol. The evolution of carbon dioxide is also inhibited by catechol, and this lends support to the hypothesis that catechol exerts its inhibition in the initiation reactions or in the first step of propagation in which reactions manganic ions are involved.

Nevertheless, the possibility cannot be excluded that the inhibition by catechol is caused by stabilization of a cofactor or skatole radical which in turn results in its destruction. The fact that the catechol-induced lag period varies in length with the cofactor used (Table 1) suggests that the cofactors or their radicals may compete at different rates for catechol, but this could also be interpreted in terms of their varying reactivity in manganigenesis. If, on the other hand, catechol were involved in a reaction with skatole radicals which are involved solely in propagation, it would be more likely to exert a retarding effect rather than an inhibition at least at lower concentrations. The most probable explanation is that catechol reacts with manganic ions produced during the initiation reactions and temporarily blocks the decarboxylation of IAA. The same argument would apply to pyrogallol, ascorbic acid, and possibly rutin.

Mechanism of Retardation by Chain-transferring Agents

The retarding effects of hydroquinone or its oxidation product, *p*-benzoquinone, riboflavin, or its phosphate, and scopoletin persisted even though several hundred moles of oxygen were consumed per mole of retarder present. This persistence indicates that although these retarders are changed by interfering in the reaction sequence they must also be reformed probably by participating in reversible redox system, a property that they would all share.

Hydroquinone p-benzoquinone. Systems containing hydroquinone differed from the others in the lower final equilibrium attained (Figure 1). This was due to a decline in IAA concentration and indicates a side reaction of the retarder with IAA. The oxygen uptake of systems containing either hydroquinone or *p*-benzoquinone differed only in the initial velocity, which was always slightly greater with the latter. Thereafter the progress curves continued parallel as would be expected if a steady state equilibrium was established between the oxidized and reduced forms.

Since hydroquinone rapidly reduces manganiversene it is probable that it competes successfully with IAA for Mn^{+3} in the same way as catechol, but differs in that the oxidation product *p*-quinone is not without effect on the system, but enters into another reaction

in which it is reduced back to hydroquinone and indirectly causes the further production of Mn^{+3}. The only readily oxidizable component that could take part in such a reversible reaction is the phenolic cofactor. Resorcinol forms an insoluble oxidation product merely on standing with *p*-quinone. Dichlorophenol and maleic hydrazide did not show any visible reaction, probably because their oxidized radicals (RO·) do not condense readily to colored products. Nevertheless, when *p*-quinone was incubated with pyrophosphate or citrate, the addition of either maleic hydrazide or dichlorophenol resulted in the oxidation of manganese. Manganic ions were rapidly produced on warming and were detected as pink manganipyrophosphate or orange manganicitrate. This is essentially the Kenten-Mann reaction for the oxidation of manganese (4), but differs in that quinone replaces peroxide plus peroxidase. Assuming that hydroquinone [$Q\ (-OH)_2$] competes with IAA for Mn^{+3}, then the mechanism of this retardation may be considered due to the partial substitution in the chain of the following series of slower reactions, where ROH represents the monohydric phenolic cofactor.

$$Q\ (-OH)_2 + Mn^{+3} \rightarrow Q\ (=O)_2 + Mn^{+2} + 2H^+$$
$$Q\ (=O)_2 + 2ROH \rightleftarrows Q\ (-OH)_2 + 2RO\cdot$$
$$RO\cdot + Mn^{+2} + H^+ \rightleftarrows ROH + Mn^{+3}$$

Riboflavin. In contrast to hydroquinone, riboflavin (Rb) is added in the oxidized form only. Consequently, if it were not involved in a reversible redox system, it would have no effect on the system, but in this respect it resembles *p*-benzoquinone. We have implied previously that the redox system ($Rb \rightleftarrows Rb\cdot 2H$) is established and that most probably the oxidized form indirectly generates Mn^{+3} by reacting with the phenolic cofactor and reduced riboflavin ($Rb\cdot 2H$) would compete with IAA for Mn^{+3}. This would have the effect of retarding the oxidation from the outset by a chain transfer mechanism.

The evidence in support of the manganigenic properties of riboflavin is as follows. Riboflavin partially overcomes the catechol-induced lag period of IAA oxidation in the same manner as manganiversene, MnO_2, PbO_2, H_2O_2, and oxygen which effects have been interpreted as manganigenic. Riboflavin, as well as these other agencies, also overcomes the inherent lag period of resorcinol oxidation catalyzed by Mn-peroxidase system (6). This oxidation appears to be dependent on the generation of Mn^{+3} since it is completely inhibited by pyrophosphate and citrate as is IAA oxidation when catalyzed by Mn-peroxidase-resorcinol systems (9). The only readily oxidizable component of the IAA oxidation system is the phenolic

cofactor, e.g., resorcinol or DCP, etc., and presumably riboflavin oxidizes resorcinol, for example, to its semiquinol which can in turn oxidize Mn^{+2} to Mn^{+3} by the Kenten-Mann reaction (4). Quite apart from this work, Andreae (2) has demonstrated that light-activated riboflavin generates Mn^{+3} from Mn^{+2} in the presence of monohydric and polyhydric phenols which do not readily form quinones on oxidation, and he has postulated a similar reaction. The ease with which riboflavin forms free radicals when illuminated suggests that this would occur not too infrequently in the presence of metal ions in darkness or in the diffuse light of the Warburg bath.

In the absence of IAA, standard Mn-phenol-peroxidase systems containing 6 μM of riboflavin consumed oxygen in the dark, a phenomenon that was accelerated by illumination. The oxygen consumed by these systems could not have been due to irreversible cofactor oxidation entirely since it appeared to be capable of continuing indefinitely and by 400 min. had exceeded the molar oxygen equivalence of the cofactors. Since no oxygen uptake occurred in the absence of a cofactor or manganese it is suggested that the product of the reaction in light or darkness must be oxidized manganese formed by reversible cofactor oxidation. From the work of Kenten and Mann (4) it is known that manganic ions are to some extent stable in orthophosphate, and there is also the possibility that MnO_2 could be produced. A mechanism whereby riboflavin (Rb), a redox catalyst (ROH), and peroxidase could interact to oxidize manganese is summarized as follows:

$$\mathrm{Rb} + 2\mathrm{ROH} \rightarrow \mathrm{Rb{\cdot}2H} + 2\mathrm{RO}\cdot \qquad \text{(A)}$$

$$\mathrm{Rb{\cdot}2H} + \mathrm{O_2} \rightarrow \mathrm{Rb} + \mathrm{H_2O_2} \qquad \text{(B)}$$

$$\mathrm{H_2O_2} + 2\mathrm{ROH} \xrightarrow[\text{or catalase}]{\text{peroxidase}} 2\mathrm{H_2O} + 2\mathrm{RO}\cdot \qquad \text{(C)}$$

$$\left.\begin{aligned} &\mathrm{RO}\cdot + \mathrm{Mn^{+2}} + \mathrm{H^{+}} \rightleftharpoons \mathrm{ROH} + \mathrm{Mn^{+3}} \\ &\mathrm{RO}\cdot + \mathrm{Mn^{+3}} + \mathrm{H^{+}} \rightarrow \mathrm{ROH} + \mathrm{Mn^{+4}} \end{aligned}\right) \qquad \text{(D)}$$

Reaction (B) is a well-known spontaneous reaction, (C) is normal peroxidation, and (D) the Kenten-Mann reaction. This reaction sequence is similar to that proposed by Andreae (2) with the exception that peroxidase and catalase are considered here to perform a peroxidatic reaction (C) and thus dispose of hydrogen peroxide which would otherwise speed the decomposition of oxidized manganese. In the absence of enzyme some oxygen uptake occurred (6) and manganic ions were formed (2), though to a lesser extent.

It is important to note that light absorbed by riboflavin is not an absolute requirement, but merely an accelerator of these reactions. In darkness, manganic ions were still produced by Andreae's

system and oxygen was consumed at a slow rate by the system described here. When these systems were illuminated, the production of manganic ions was increased (2) as was also oxygen uptake in our system.

With respect to the mechanism by which light activates the reaction sequence, tests were made on the effect of light on the oxygen uptake of riboflavin-resorcinol systems (reaction A), and on the spontaneous oxidation of reduced riboflavin in air (reaction B). In the absence of manganese and enzyme, very little reaction was found to occur between riboflavin and resorcinol in light or darkness, and hence an activation by light of reaction A as proposed by Andreae (2) appears unlikely. On the other hand, white light (980 foot candles) almost doubled the rate of oxygen consumed by solutions of riboflavinphosphate that had been reduced by dithionite. Therefore, we suggest that light activated Andreae's system and the system described herein at the stage of reaction B.

Oxygen uptake in this system appeared to continue indefinitely and in all probability could exceed the combined theoretical molar equivalence of riboflavin and cofactor. This is possibly due to the cyclical nature of the reaction in which the over-all reaction is an oxidation of Mn^{+2} to Mn^{+4}, but the intermediate valence stage Mn^{+3} may compete with oxygen for reduced riboflavin as follows:

$$Rb \cdot 2H + 2Mn^{+3} \rightarrow Rb + 2Mn^{+2} + 2H^{+}$$

When the system is illuminated, reduced riboflavin reacts preferentially with oxygen owing to light activation at this stage (reaction B). This would explain the increased production of manganipyrophosphate as found by Andreae (2) and the increased oxygen uptake of our system under illumination.

The significant points emerging from the experiments of Andreae (2) and those reported here are that riboflavin can indirectly generate manganic ions in this system and that reduced riboflavin may possibly react with Mn^{+3}. This would provide an explanation not only for the retardation of the indole-3-acetic acid system by riboflavin, but also for the alleviation of this retardation by light. Evidently the reduced form of the retarder which is a necessary product of manganigenesis must be the immediate cause of retardation by being involved in a second redox system with an essential oxidized intermediate. The degree of retardation would depend on the equilibrium established between the oxidized and reduced forms of riboflavin, the latter diverting an oxidized intermediate from its normal function as a chain reactant in indole-3-acetic acid autoxidation.

The intermediate with which reduced riboflavin reacts is most

probably the manganic ion. This is supported by the following evidence. Riboflavin suppresses oxygen uptake and carbon dioxide evolution equally; therefore, it must compete with an oxidized intermediate in the initiation reactions or in the first step of propagation. Reduced riboflavin decolorizes manganiversene instantaneously, and kinetic evidence suggests that no reaction occurs with other oxidized intermediates. For example, any reaction of reduced riboflavin with oxidized cofactor radical (reverse of reaction A) could occur only at the expense of the normal reaction of the cofactor radical with Mn^{+2}. Since an increase in Mn^{+2} concentration failed to counteract the retardation by riboflavin in darkness, then manganese and riboflavin probably do not compete for the cofactor radical. Similarly riboflavin does not compete with the cofactor for the skatole peroxy-radical; otherwise different cofactors would have a significant effect on the degree of retardation and an increased con centration of cofactor by itself should counteract the retardation.

The alternative explanation is that reduced riboflavin competes with IAA for Mn^{+3}, but increased concentrations of IAA alone do not overcome the retardation. However, because the critical optimum concentration of the cofactor is dependent on the concentration of IAA, the unbalance caused by varying the concentration of IAA independently of the cofactor may be the cause of the ineffectiveness of IAA in overcoming the retardation by itself (Table 2). When the concentration ratio IAA/DCP was maintained constant at 4.4, increasing concentrations of IAA were able to overcome the inhibition. Such evidence makes it appear most probable that Mn^{+3} is implicated in a reaction with reduced riboflavin.

Accordingly it is possible to formulate a hypothesis for the mechanism of retardation as follows:

In darkness (retardation).

$$Rb + 2ROH \rightarrow Rb{\cdot}2H + 2RO\cdot$$
$$RO\cdot + Mn^{+2} + H^{+} \rightleftarrows ROH + Mn^{+3}$$
$$Rb{\cdot}2H + 2Mn^{+3} \rightarrow Rb + 2Mn^{+2} + 2H^{+}$$

This series of slow reactions would be substituted in part for the normal sequence of reactions. Some Rb·2H possibly cannot escape reaction with oxygen, but it would more likely be preferentially oxidized by Mn^{+3}.

In light. The retardation is overcome since Rb·2H reacts preferentially with O_2 (light activated) and the H_2O_2 produced supports the production of cofactor radicals for manganigenesis in the Kenten-Mann reaction as follows:

$$Rb + 2ROH \longrightarrow Rb\cdot 2H + 2RO\cdot$$

$$Rb\cdot 2H + O_2 \xrightarrow{\text{light}} Rb + H_2O_2$$

$$H_2O_2 + 2ROH \xrightarrow[\text{catalase}]{\text{peroxidase}} 2RO\cdot + 2H_2O$$

In this illuminated system riboflavin would be maintained in the oxidized state by oxygen rather than by Mn^{+3}; thus it would not interfere in the reaction sequence of IAA oxidation and cause a transfer of the chain reaction. Also oxygen consumption would continue well past the theoretical for IAA oxidation since it would revert to a slower cyclical reaction in which Mn^{+4} (MnO_2) may be the eventual end product accompanied by the uptake of oxygen as discussed earlier.

Implicit in the interpretation of the mechanism of action of inhibitors and retarders in this paper is the essential correctness of the scheme proposed by the present authors to explain the catalytic action of wheat leaf enzymes, horseradish peroxidase, and beef liver catalase on the oxidation of IAA in vitro. The fact that all components of the system exist or are readily available in vitro justifies consideration of the physiological significance of the system in the control of plant growth, in which IAA apparently plays a central role.

From a biochemical viewpoint, the enzymically catalyzed free radical mechanism proposed for the oxidation of IAA represents a departure from classical interpretations of reaction mechanisms in plant physiology. Such phenomena as the photoreversible inhibition of IAA oxidation by riboflavin, as well as providing a useful system to study the kinetics and mechanism of photochemical reactions, may also prove, in their interpretation, to strengthen our knowledge of probable biochemical mechanisms underlying the photocontrol of plant growth.

ACKNOWLEDGMENTS

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Auxins and the Process of Aging in Root Cells

The relationship between the aging of plant tissues and auxin metabolism has been discussed in a number of papers by several investigators (5, 14, 18, 30). Among these, the work of Galston and Dalberg (6) is of special interest, for they have shown that the ability of pea seedling cells to destroy the native auxins increases as the cell ages. The purpose of this paper is to analyze various biochemical properties of root cells in relation to indole-3-acetic acid (IAA) destruction. The advantage of working with root sections is that they possess, simultaneously, both very young tissues (meristem) and older ones (root tip).

MATERIALS AND METHODS

The experimental material used was *Lens culinaris* Med. The seeds were selected and nearly 100 per cent germination occurred after 38 hrs. Seeds were first soaked in de-ionized water for 12 hrs., then washed, and finally placed on wet filter paper in petri dishes in darkness at $22 \pm 0.5°$ C. The first selection was made after 24 hrs., and only seedlings measuring 3 ± 1 mm. were kept and replaced in the above conditions. The seedlings were removed for treatment when they had reached a length of 18 ± 2 mm., for this has been found to be the period of optimal growth (11, 29). A series of cytological analyses has shown that the tip, composed of older cells, is located between the extreme point and 0.5 mm. from it while the meristem, containing the young cells, is situated in the region from 0.5 to 3.0 mm. The work on the process of aging was done exclusively on these two types of fragments. In order to prepare these two root sections, a small guillotine (22) was developed (Figure 1); it is a modified version of a design by Linser and Kiermayer (10).

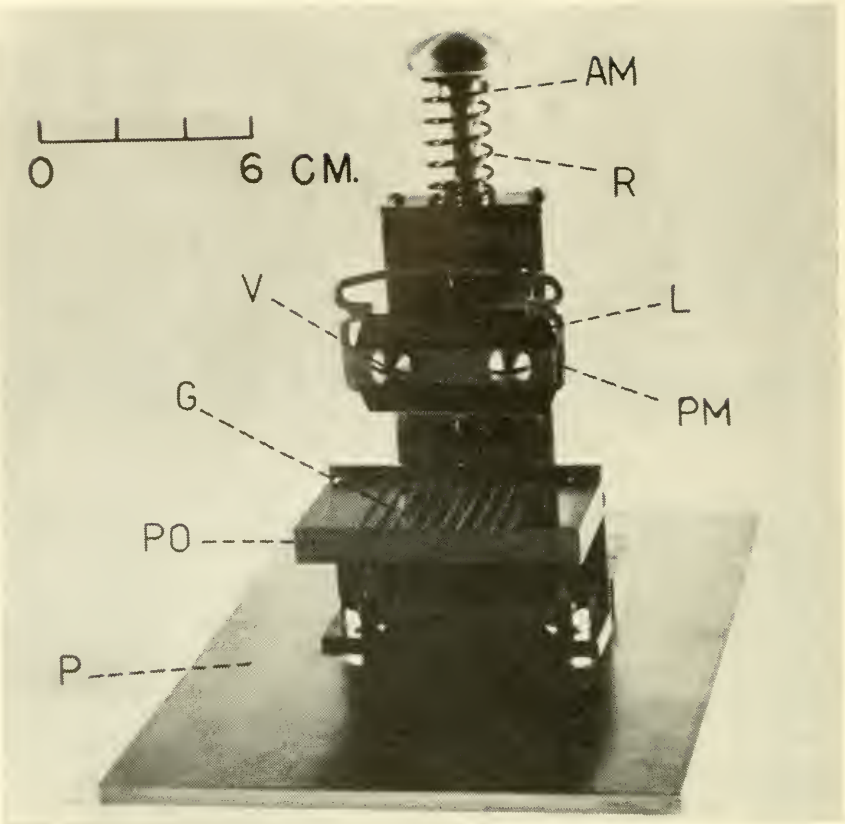

Fig. 1. Guillotine. With the use of this apparatus, the exact root sections (0–0.5; 0.5–3.0 mm.) can be easily obtained; the roots, 18 mm. in length, are placed on a grooved (**G**) plate (**PO**) with the tips just touching a vertical upright. A quick depression of the handle (**AM, R**) lowers two blades (**L**) which cleanly cut the roots to size, producing the desired fragments.

BIOLOGICAL ANALYSES

Auxin Content

The extraction of auxins from the roots (an acid fraction of extracts obtained with peroxide-free $CHCl_3$) was carried out as described elsewhere (11), with subsequent measurements of activity by the *Avena* test. The auxin content was determined in the two regions previously mentioned and expressed (in terms of equivalent μg. IAA) as a function of the age of the roots (length). Table 1 shows that the concentration of auxins in the meristem is consistently greater than that in the tip. Furthermore, the level of auxins increases as the roots age.

These facts, together with many other observations (12), suggest

an interpretation concerning the phenomenon of root growth and aging in relation to the strength of native auxins. Figure 2 gives the essential points of this theory. The speed of growth passes a maximum at the moment when the level of auxins, which increase with increasing age, becomes supra-optimal. When the root is young, IAA treatment induces an increase in elongation, whereas exposure to light inhibits growth. These facts can be explained if, at this moment, the roots possess only very low levels of auxins. If the roots are old, however, this phenomenon is reversed, for auxin treatment inhibits the elongation while illumination stimulates the growth. Needless to say, the period of stimulation is extremely brief and it can be supposed that in certain cases it is practically non-existent (4,32).

Auxin Destruction

Data on IAA destruction, measured by a colorimetric technique (15, 26), are plotted in Figure 3. They indicate that IAA destruction is greater in old than in young tissues. The suggestion can be made that IAA-oxidases determine the endogenous auxin level: high enzyme activity meaning low auxin content (old cells) and vice versa. Meanwhile, recent observations do not exactly confirm this hypothesis. If the IAA destruction is expressed in terms of growth gradients, simple relations between auxin catabolism and endogenous auxins are not so evident. [See P. E. Pilet, Gradients de croissance et problèmes auxiniques. Bul. Soc. Bot. Suisse. 70: (in press) 1960.]

In both regions, this IAA destruction increases with the increasing age of the roots. The fact, however, that IAA destruction increases with age would seem at first sight to contradict the material discussed above (which shows that the auxin content increases with increasing age). Nevertheless, it can be supposed that these two

Table 1. Auxin content (acid fraction free auxins) in μg IAA/100 mg fresh weight of root segments.

Length of Roots, Mm.	Auxin Content		
	Old cells	Young cells	Total
2 ± 0.5	0.07	6.84	6.91
6 ± 0.8	0.10	17.12	17.22
10 ± 1.0	0.14	20.04	20.18
18 ± 2.0	0.13	21.00	21.13
32 ± 3.0	0.15	22.07	22.22

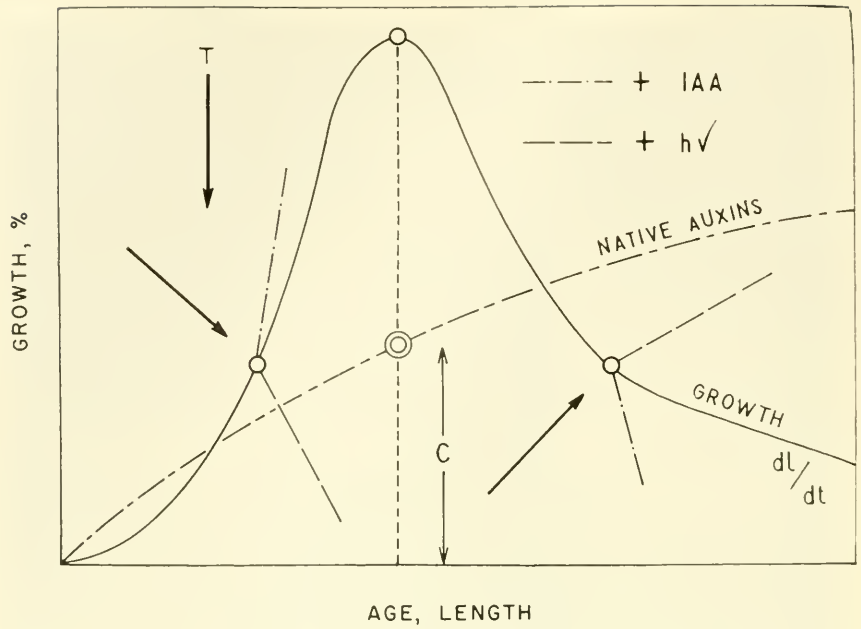

Fig. 2. Auxin content and growth (control, with IAA and light) of *Lens* roots in relation to their age (length).

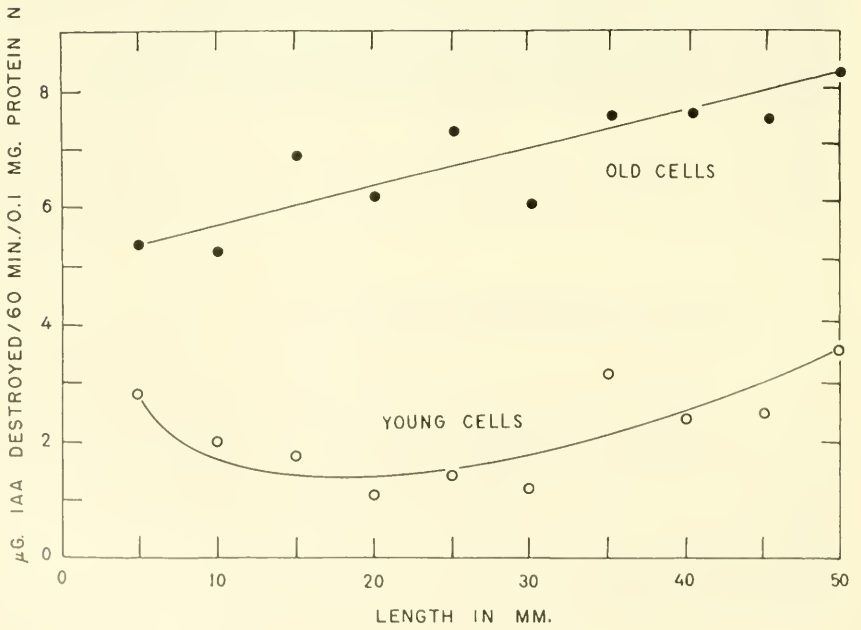

Fig. 3. IAA destruction for the two types of fragments (root tip: old cells as filled circles; meristem: young cells as open circles) of roots from different lengths.

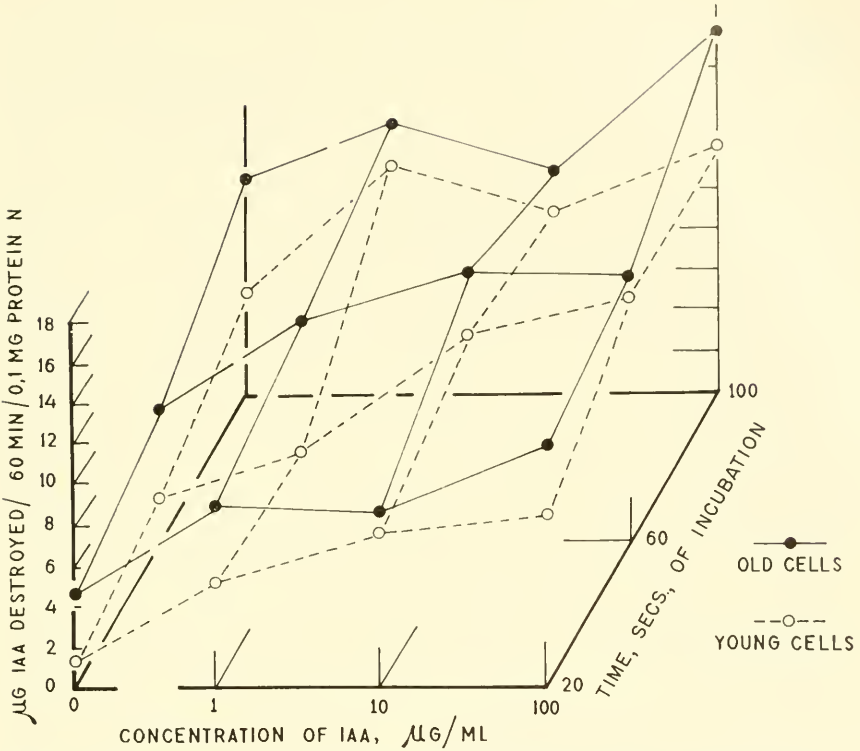

Fig. 4. IAA destruction for the two types of fragments pretreated by IAA at different concentrations for 20, 60, and 100 seconds.

processes operate simultaneously. In other words, the tissues greatly increase their ability to destroy native auxins in proportion to the age of the roots at the same time as the accumulation of auxins is increasing. Even if the destruction is greater, the final auxin content rises because auxins are produced faster than they are destroyed. These observations suggest a process of enzyme adaptation, or induction, and work of a similar nature (6) performed on a different material clarifies this phenomenon. If, before making the enzyme extracts, the tissues are treated with IAA of increasing concentrations and increasing time of incubation, the destructive power of the extracts (which remains stronger for the tissues of the root cap in relation to those of the meristem) increases slightly more for the young than for the old cells (24, Figure 4).

Several substances have been used which induced stimulation or inhibition of the in vitro IAA destruction by *Lens* root tissues: 2,4-dichlorophenol, 2,4-dinitrophenol, 2,4-dinitro-*o*-cresol and Mn^{+2} (13), maleic hydrazide (16), gibberellic acid (17), glutathione (20), and in-

Table 2. Stimulation or inhibition of IAA destruction in *Lens* roots, 18 mm. in length.

Treatment	Molar Concentration	IAA Destroyed/60 Min/100 Mg Fresh Weight			
		Old cells		Young cells	
		μG.	Per cent*	μG.	Per cent
Control		75	0	21	0
2,4-Dinitro-*o*-cresol	1×10^{-6}**	97	+29	84	+300
2,4-Dinitrophenol	1×10^{-6}**	88	+18	81	+285
2,4-Dichlorophenol	5×10^{-5}**	84	+12	76	+262
Maleic hydrazide	1×10^{-2}†	89	+19	44	+105
Mn^{+2} ($MnCl_2$)	2×10^{-6}	73	− 3	27	+ 29
Gibberellic acid	5×10^{-5}	57	−24	18	− 14
Glutathione	1×10^{-4}	52	−31	17	− 19
Indole	4×10^{-5}	48	−36	18	− 14
3-Methoxy-4-hydroxy-cinnamic acid	1×10^{-4}	31	−59	15	− 29

* Per cent $= \frac{\text{Treated-Control}}{\text{Control}} \times 10^2$.

** Optimal concentration.

† No effect up to 5×10^{-4} *M*.

Table 3. Biochemical gradients in *Lens* roots, 18 mm. in length.

Determination Made	Expressed As:	Old Cells	Young Cells
Fresh weight	Mg/100 fragments	28.4	163.7
Total N	μg/100 mg. fresh wt.	185.3	97.9
	Mg/10^8 cells	10.08	3.58
Protein N	μg/10 mg. fresh wt.	89.7	47.4
	Mg/10^8 cells	8.12	1.22
Auxin in equivalent micrograms IAA	Per 100 mg. fresh wt.	0.13	21.00
	Per mg. N total N	0.12	24.10
	Per mg. protein N	0.16	70.61
	In 10^8 cells	1.26	86.38
Auxin destruction	Per 100 mg. fresh wt.	75	21
	Per mg. total N	73	24
	Per mg. protein N	90	71
	In 10^8 cells	73.3	8.6

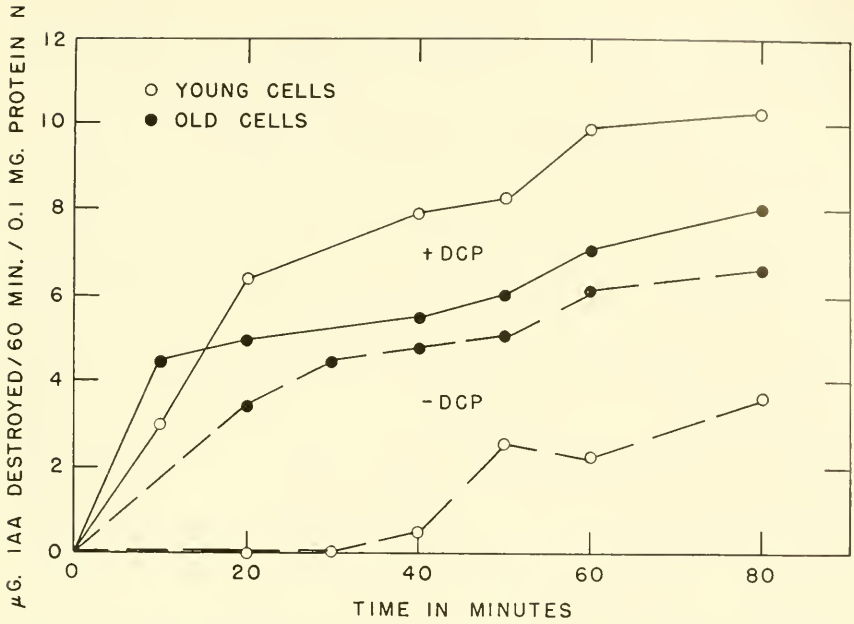

Fig. 5. IAA destruction for two types of fragments with or without 2,4-dichlorophenol (DCP) at $1 \times 10^{-5} M$, in relation to time.

dole (19). It is a general observation (Table 2) that the stimulation is stronger when the activity is stronger.

Study of the progress of IAA destruction (Figure 5) shows that in the case of the tip tissues, the decomposition of IAA is almost immediate, although, for the meristem, there is a certain time lag (6). Treatment with 2,4-dichlorophenol (DCP) alters this process, since it induces an immediate stimulation of IAA destruction in the two root fragments. This seems to indicate that IAA-oxidase induction cannot be expressed in terms of adaptive formation of enzyme since, in young cells, DCP (substance without action on nonenzymatic IAA destruction) produces immediately a dramatic stimulation of IAA-oxidase which was already present in the tissues but probably bound.

BIOCHEMICAL ANALYSES

It was essential to establish, for the analyzed tissues, the nature and importance of some biochemical gradients: first, to see if a correlation existed between these gradients and the aging of cells (19, 28); secondly, to express more logically and to compare on the same basis (23, 30) the native auxin content and IAA-destroying activity. Data are shown in Table 3, and it can be seen that, regardless of

the criterion used, (1) auxin concentration is greater in the meristem than in the root tip, and (2) auxin destruction has a higher value for the tissues of the root tip than for those of the meristem.

BIOCHROMATOGRAPHIC ANALYSES

The study of the active factors has been undertaken on the acid fractions of free auxins extracted by peroxide-free ether (8). For the two types of tissues, the biological measurements (21) were obtained by two different tests: sections of *Lens* root (R) and fragments of *Lens* stem (T) (25). It seemed best to use equivalent material and, thus, the roots utilized for the measurements (the 5 mm. root tips from the 18 mm. roots) were identical to those which were used to prepare the extracts. Examination of the resulting histograms (Figure 6) clearly shows the existence of IAA in a concentration which produces an inhibition of the R test and a stimulation of the T test. Further, the level of IAA is, according to the previous results, greater in the meristem than in the root tip. Additionally, it shows the presence of two factors already described (2, 3, 9, 21) and discussed (1) elsewhere: an inhibitor and an accelerator (probably an artifact) (7).

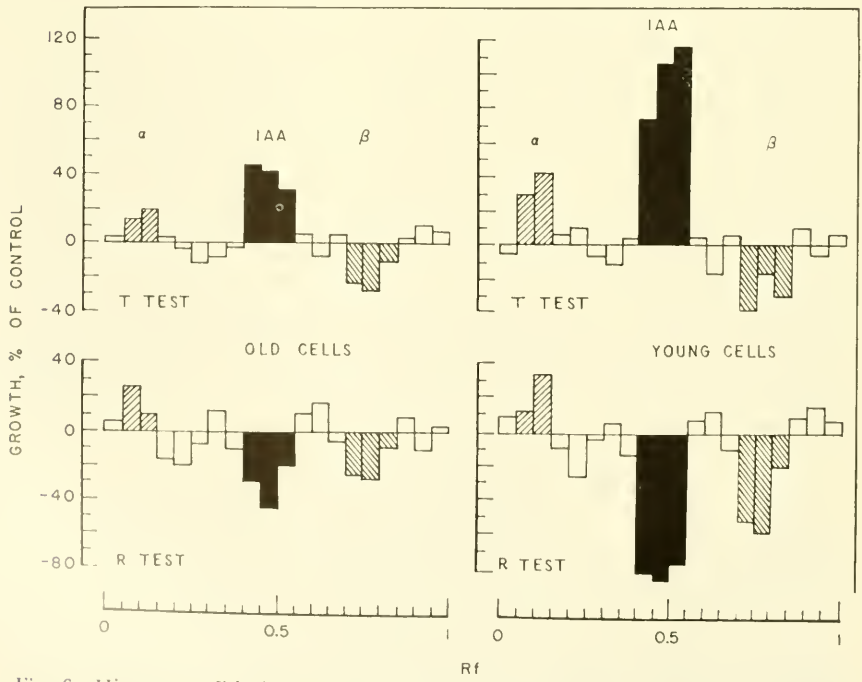

Fig. 6. Histograms [biochromatograms: root (**R**) and stem (**T**) tests] for two types of fragments.

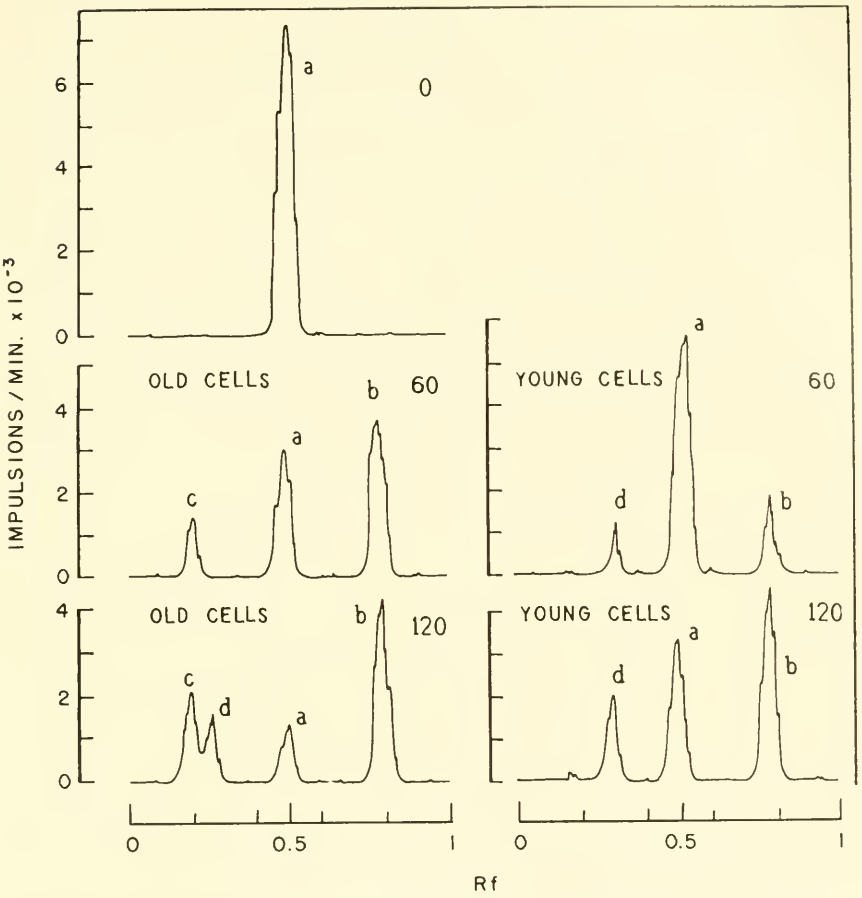

Fig. 7. Radiochromatograms (IAA labeled with C^{14} in the 2-carbon atom of the ring, applied to the enzyme extracts) for two types of fragments. Collections made at 0, 60, and 120 min.

RADIOCHROMATOGRAPHIC ANALYSES

The use of IAA labeled with C^{14} in the 2-carbon atom of the heterocyclic ring has made it possible to determine the nature of IAA destruction (31) in the two types of tissue. Examination of the radiochromatograms (obtained with extracts corresponding at different times of incubation) indicates (Figure 7) that the speed of IAA destruction is faster for the tissues of the tip. Furthermore, the degradation products are rather different. Table 4 furnishes some quantitative relations (27) on this subject. The study of the chemical nature of the products resulting from the IAA (labeled with C^{14}) destruction by the root extracts will be presented in detail elsewhere. [See P. E. Pilet, Physiol. Plantarum. (in press) 1960.]

Table 4. Concentration of the products labeled with C^{14}, resulting from IAA enzymatic destruction expressed in terms of area in mm^2. (A) measured by planimetric method from counting pulses. See Figure 7.

Material	Time of Incubation, Min.	Products									
		IAA		b		c		d		Total	
		A	per cent	A	per cent	A	per cent	A	per cent	A	per cent
Old cells	0	412	100	...	..	..	..	..	..	412	100
	60	144	35	172	41	37	9	..	..	353	85
	120	56	13	188	45	76	18	48	12	368	88
Young cells	0	412	100	...	..	..	..	..	..	412	100
	60	277	66	54	13	..	..	38	9	369	88
	120	148	36	176	42	..	..	59	14	383	92

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DISCUSSION

Dr. Andreae: In our studies on IAA metabolism, we became particularly interested in the fate of IAA applied to intact tissues. Studies during the past few years have shown that part of the applied IAA accumulates in the tissues as indoleacetylaspartic acid; this has been confirmed by other workers (Fang *et al.,* Plant Physiol. 34: 26. 1959). Fawcett *et al.* (Nature 181: 1387. 1958) were unable to find any evidence for this reaction. The failure of the latter workers can be ascribed to the inadequate extraction procedure used in their studies.

Conjugation of IAA with aspartic acid has only so far been observed in intact tissues. Cell-free breis entirely lose their ability to conjugate IAA with aspartic acid although such breis may retain IAA-oxidase activity.

We found that indoleacetylaspartic acid accumulates in pea tissues as the major Salkowski-reactive IAA metabolite, but could find no evidence of a metabolically produced, Salkowski-reactive, IAA-protein complex reported by Siegel and Galston (Proc. Nat. Acad. Sci. 39: 1111. 1953). These workers found that during the initial hours of incubation, IAA is rapidly bound to the pea root proteins and precipitated by trichloroacetic acid. In our studies, the Salkowski-reactive material in pea roots can be extracted with ethanol and consits entirely of IAA and indoleacetylaspartic acid (Figure 1). We therefore believe that the IAA-protein complex of Siegel and Galston is not a product of plant metabolism, but an artifact of the trichloroacetic acid precipitation procedure.

The time course of IAA conjugation with aspartic acid in pea roots is shown in Figure 1. Roots were removed from solution, washed, homogenized, and extracted with ethanol. The entire extract

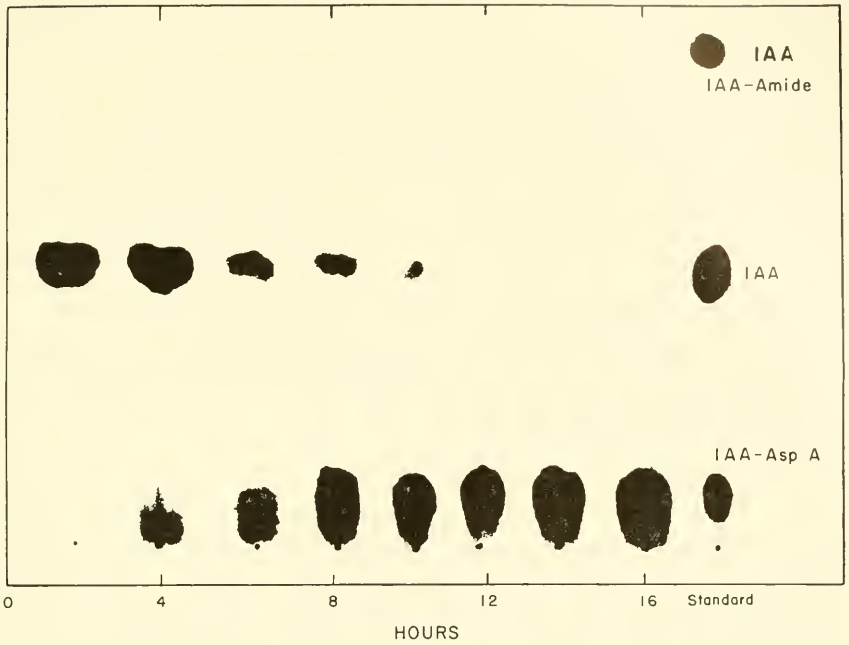

Fig. 1. Chromatogram of the Ehrlich-positive substances extracted by ethanol from pea root tips following incubation in auxin for different periods of time. Five mm. root tips from 2-day-old seedlings incubated in solution containing IAA, 1×10^{-4} *M*; sucrose, 0.5 per cent; $Ca(NO_3)_2$, 1×10^{-3}*M*; potassium phosphate, 5×10^{-3} *M*; streptomycin, 30 p.p.m.; penicillin, 15 p.p.m.; pH 5.2.

was chromatographed in *iso*-propanol-ammonia-water (80:10:15). Three phases can be differentiated. During the initial phase, lasting about two hours, free IAA rapidly accumulates in the tissues. Quantitively, it has been found that the initial concentration of IAA reaches ten times that of the external solution and that during this time all the IAA lost from solution can be accounted for as accumulated IAA. During the second phase, lasting from about the second to the twelfth hour, the IAA content declines and conjugation commences. In the third phase, free IAA is no longer detectable in the tissues, while the indoleacetylaspartic acid content continues to increase. The formation of indoleacetylaspartic acid appears to be a detoxication process and not a physiological activation of the IAA molecule.

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Pathways of Decomposition (Catabolic Lattice) of Indole Derivatives

Plant tumors induced by *Agrobacterium tumefaciens* usually have a higher content of free auxin than the corresponding normal tissue (4, 13, 16, 17). This may be due to increased auxin synthesis in tumors or more active auxin destruction in the corresponding normal tissue (3). The formation and destruction of auxins in general, and of indole-3-acetic acid in particular, have therefore an important bearing on the etiology of plant cancer and in 1954 we started a study of the metabolism of indole derivatives (30). We soon noticed that destruction of indole substrates occurred in our blanks as well as during extraction and chromatographic procedures, due to spontaneous decomposition. We concluded that a knowledge of the spontaneous process was necessary for the interpretation of metabolic studies. Since 1956 we have studied the spontaneous or induced decomposition, by ultraviolet radiation, of 14 indole derivatives[1], most of which are involved in the classical scheme of indole metabolism (8, 11, 20) represented in the central part of the diagram of Figure 1.

[1]The following indole derivatives were used in these studies: tryptophan (TRPH), 5-hydroxytryptophan (5-HTRPH), indole-3-pyruvic acid (IPA), tryptamine (TRAM), 5-hydroxytryptamine (5-HTRAM), indole-3-acetaldehyde (IAAL), indole-3-acetic acid (IAA), 5-hydroxyindole-3-acetic acid (5-HIAA), N-hydroxyindole-3-acetic acid (N-HIAA), indole-3-glycolic acid (IGCA), indole-3-glyoxylic acid (IGXA), indole-3-aldehyde (IA), skatole (SK), and indole (IN).

After the meetings in 1959, we were able to compare the sample of indole-3-aldehyde used in these studies (Bios Laboratories, Inc.) with one purchased from Aldrich Chemical Co., Inc. The two samples are obviously different, although both are aldehydic indole derivatives. The Aldrich sample is the only one that agrees with the physical and chemical properties of indole-3-aldehyde reported in the literature. It is therefore obvious that the substance mentioned under the initials of IA in this paper is not indole-3-aldehyde, but it is possibly one of its hydroxy derivatives.

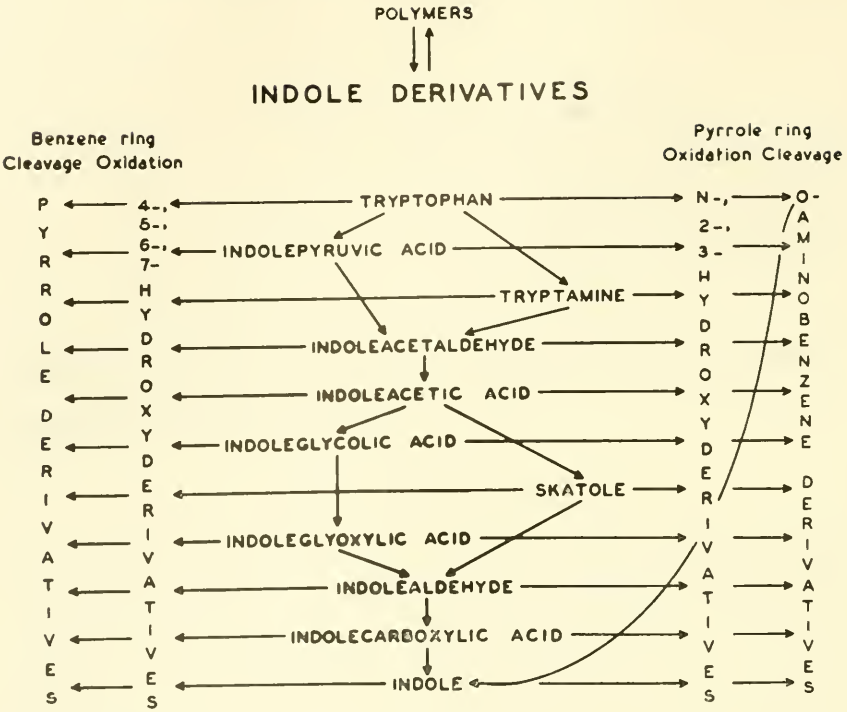
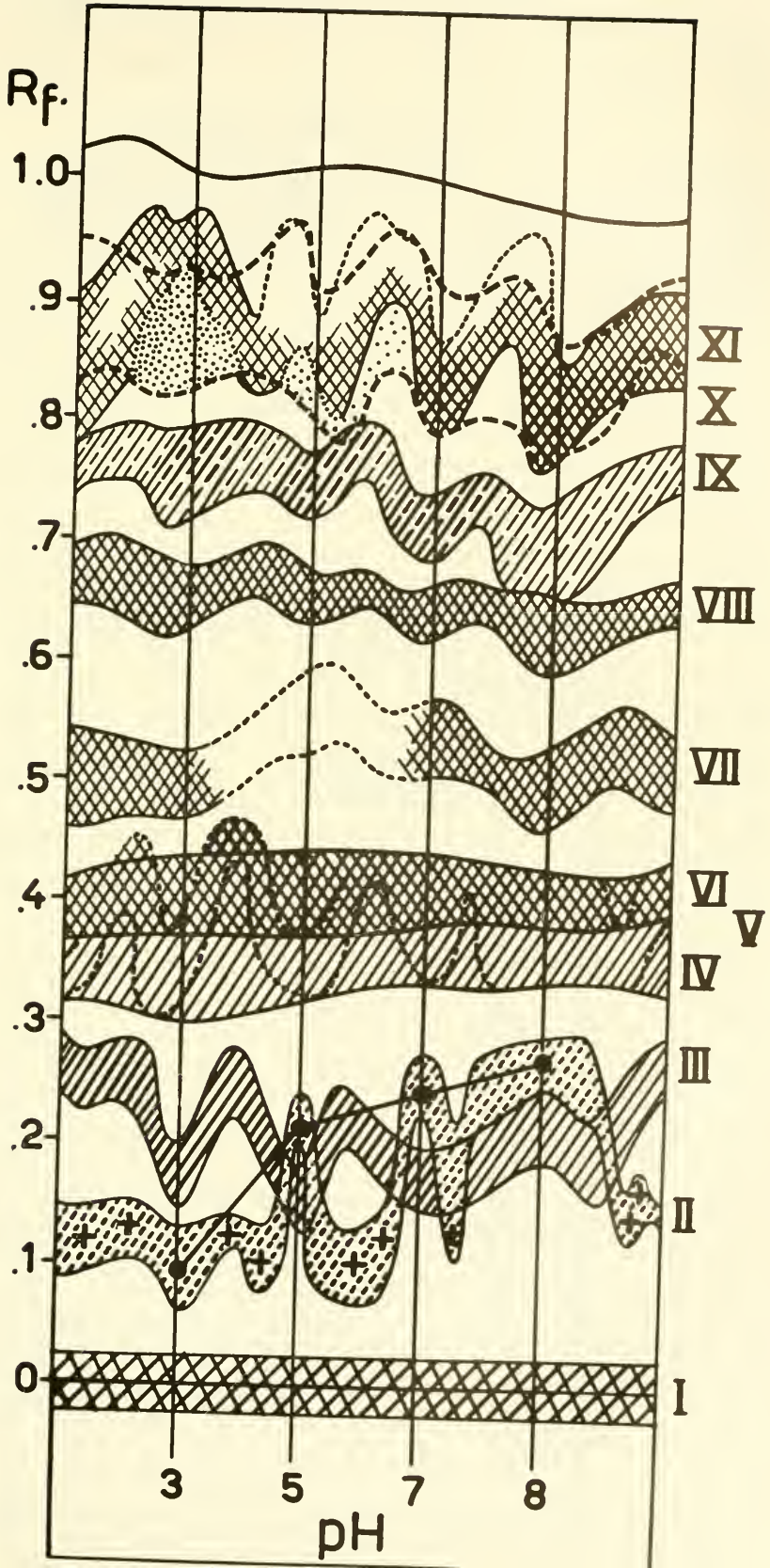


Fig. 1. Catabolic lattice of indole derivatives.

In the course of these studies we had the opportunity to establish or improve a number of useful chromatographic methods, notably that of double chromatography (27) which, in the case of eminently labile substances like some indole derivatives, furnish valuable evidence on the nature of the decomposition products and their pathways of decomposition.

The complete studies are published or are in preparation for publication (7, 23, 26, 28, 29), and this paper presents a condensation of the principal results.

Fig. 2. Chromatogram of ultraviolet decomposition products of indole-3-acetic acid. Ascending chromatography. Acetone and water 8:2. Citric acid-disodium phosphate buffer solutions (0.1 *M*) at pH 3, 5, 7, and 8 were applied along the vertical lines before chromatography. The decomposed solution was applied at I. The short-wave ultraviolet absorption of some portions of zone X (IAA) is indicated by stippling. The complete outline of this zone as revealed by Ehrlich's reagent, is indicated by two interrupted lines. Dots and crosses indicate the center of zone II at the level of pH lines and in between lines, respectively. Conventional hatching indicates approximate fluorescence colors as in Figure 7.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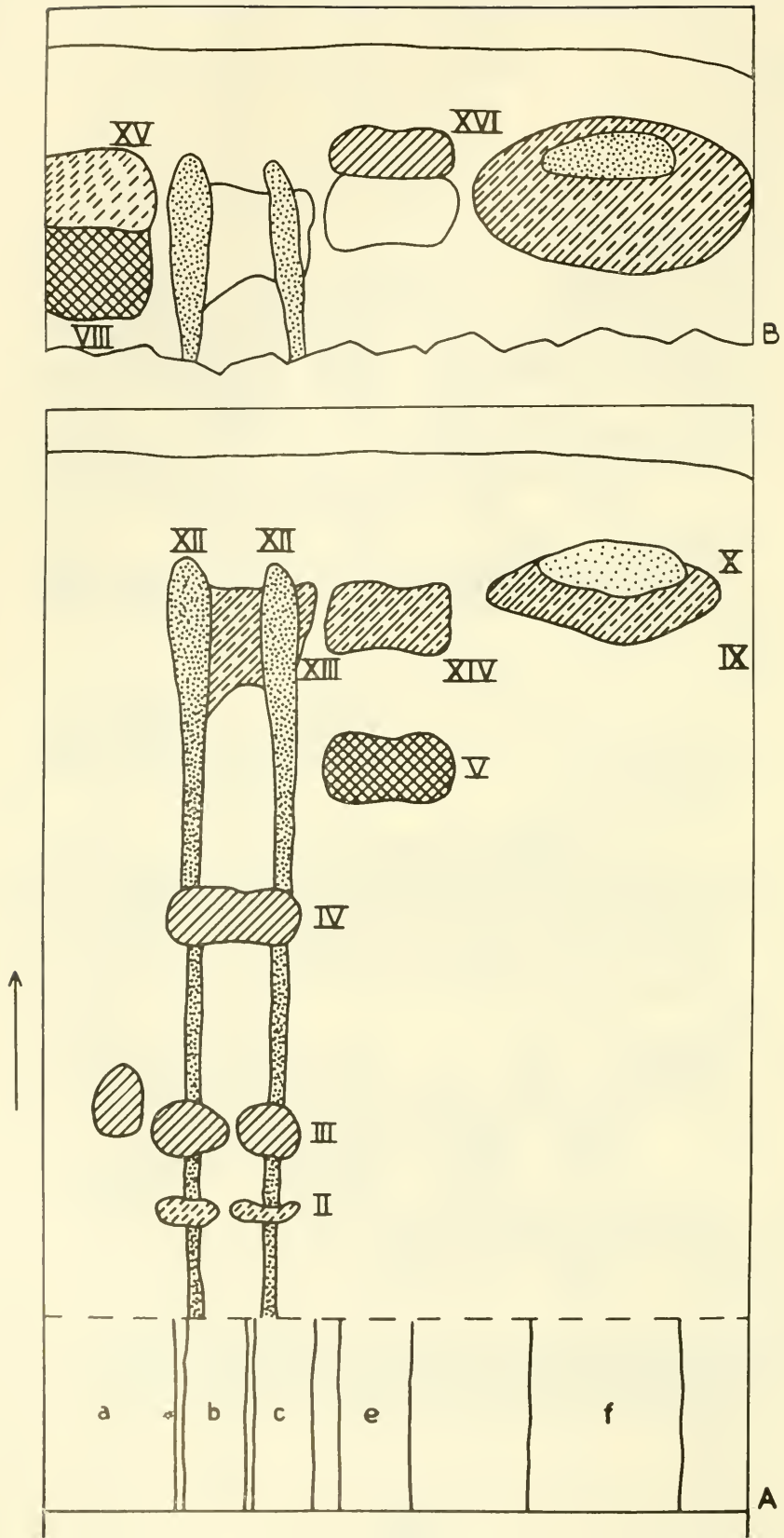
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MATERIAL AND METHODS

Chemicals

Some of the indole derivatives were purchased, others were generously supplied by Drs. J. A. Bentley, D. Von Denffer, A. W. Galston, R. A. Gray, Q. Mingoia, G. F. Smith, and K. V. Thimann. N-hydroxyindole-3-acetic acid was synthesized according to Houff *et al.* (14).

Decomposition of aqueous solutions at a concentration of 0.1 per cent was induced by aging (minutes, hours, days, or years, according to the substance), heating, ultraviolet radiation (exposure for several minutes to a Hanovia Utility ultraviolet lamp) or by oxidants like ferric chloride. The decomposed solution was either directly applied to chromatograms and ionograms or extracted first in *n*-butyl alcohol. In some cases ether fractionation of the neutral and acid decomposition products was performed.

Chromatography and Electrophoresis

Uni- and bi-dimensional ascending chromatography was performed with Whatman No. 1 paper and the following solvents: (a) water, (b) acetone and water (8:2), (c) 16 per cent NaCl and 2 per cent acetic acid (1:1), (d) 25 per cent acetic acid, and (e) isopropanol, 28 per cent ammonia and water (8:1:1). In the case of (a) and (b), the chromatogram was usually run in an atmosphere saturated with vapors of acetic acid. Addition of a few ml. of acetone on the walls of the tank improved some of the separations in the case of (a). In

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Fig. 3. **A**, Ionochromatogram of ultraviolet decomposition products of indole-3-acetic acid. The limits of the fluorescent zones (**a**, **c**, **e**) and of the ultraviolet-absorbing zone **f** (IAA) in the ionogram were traced with a pencil (cf. Figure 4). The substances which remained at the origin (O) formed a reddish brown double line (zone **b**) due to chromatographing during the repeated application of the decomposed solution on the ionogram. The ionogram was next machine-sewed onto two filter paper strips. The sewing line on the upper strip is shown as a horizontal interrupted line on top of the ionogram at the bottom of the figure. Ascending chromatography with acetone and water 8:2. Spots II, III, and XII and its tail are double as they originate from the double line of zone **b**. Their slight displacement to the right of that line is probably due to the holes of the sewing line which diverted the flow of the solvent during chromatography. **B**, Upper part of the chromatogram after exposure to ultraviolet radiation. Spots XIII and XIV faded considerably and new fluorescent spots (VIII, XV, XVI) appeared. After treatment of the chromatogram with *N* HCl, spots IX, X, XII and its tails, XIII, XV, and XVI exhibited different shades of red. VIII was slightly yellowish. Conventional hatching indicating approximate fluorescence colors as in Figure 7.

order to avoid decomposition during chromatography, chromatograms were always short (10 cm.) and were sometimes run in the cold (5 to 8° C.) or in an atmosphere of nitrogen. The inconveniences of this decomposition were put to a marked advantage in double chromatograms, i.e., two-dimensional chromatograms in which the same solvent is used in both directions (27).

Multiple levels of pH were established in some chromatograms (6) by drawing longitudinal, evenly spaced, lines with a straight-edge and a capillary pipette filled with buffer solutions (Figure 2).

Electrophoresis was initially carried out in an Elphor apparatus, with a voltage of 140 volts, and later in a Pheromatic II apparatus with 200 volts. Whatman No. 1 filter paper strips, 4 x 40 cm., received the solution on a 5 mm. wide transversal band in the middle of the strip. The strip was moistened with a 0.1 *M* phosphate buffer solution and placed in the apparatus filled with the same buffer. The operation usually lasted 6 to 8 hrs.

A combination of electrophoresis and chromatography was used in several instances. For this purpose after electrophoresis, the ionogram, reduced to 3 cm. width, was machine sewed onto two strips of filter paper, one of them for dipping in the chromatographic solvent and the other one for running the chromatogram (Figure 3).

Inspection of Chromatograms and Ionograms and Color Reactions

Chromatograms and ionograms were examined under long-wave (366 mμ) and short-wave (254 mμ) ultraviolet light and fluorescent and ultraviolet-absorbing spots and zones were outlined with a pencil. The contrast between ultraviolet-absorbing spots and the surrounding paper was greatly enhanced by spraying the chromatogram with a 0.02 per cent alcoholic solution of safranin. Under short-wave ultraviolet light, absorbing spots appeared dark blue in contrast to the brilliant red of safranin. Significant changes of the color or intensity of fluorescence of some of the spots were produced by exposure of the chromatograms and ionograms to ultraviolet radiation for a few minutes. This also induced fluorescence in some of the nonfluorescent, short-wave, ultraviolet-absorbing spots.

The following reagents were applied to the chromatograms and ionograms: $FeCl_3$ reagents (aqueous solutions, Salkowski, Gordon-Weber); *p*-dimethylaminobenzaldehyde (Ehrlich); safranin; *N* HCl; 2,6-dichlorophenol-indophenol (DCPIP); 2,4-dinitrophenylhydrazine (DNPH); benzidine (Van Eck's); ammoniacal $AgNO_3$. In many cases more than one reagent was applied to the same chromatogram or ionogram which for that purpose was cut into several longitudinal strips, one for each reagent (Figure 4). The composition of the reagents is given elsewhere (29).

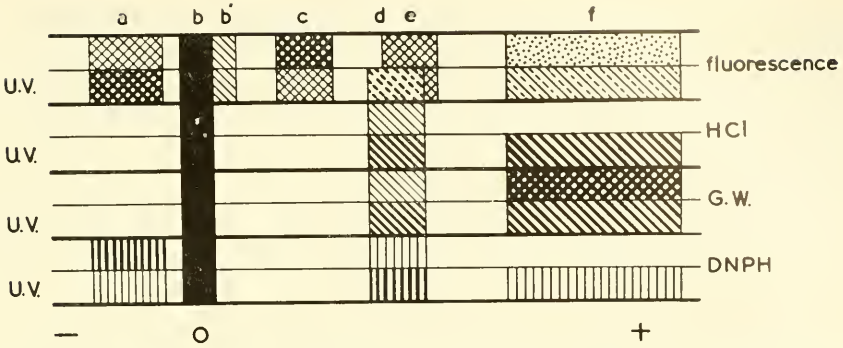

Fig. 4. Ionogram of the ultraviolet decomposition products of indole-3-acetic acid. Phosphate buffer 0.1 *M*, pH 7; 6 hrs., 140 volts. All the colored substances (reddish brown) of the decomposed solution applied at the origin O remained there (zone **b**). The ionogram was inspected under long-wave ultraviolet light which revealed fluorescent zones **a**, **b'**, **c**, and **e**. Under short-wave ultraviolet light zone **f** (IAA) was seen as a dark zone due to the quenching of the blue fluorescence of the filter paper. The ionogram was next exposed to ultraviolet radiation through a stencil so that parallel longitudinal exposed bands alternated with unexposed bands. As a result of ultraviolet exposure the blue fluorescence of zone **a** was markedly intensified, that of zone **c** decreased and a bright turquoise green fluorescence appeared in zone **f**. A new zone, **d**, with strong yellowish fluorescence appeared, in part overlapping zone **e**. The ionogram was then cut longitudinally in four strips, each of which contained an exposed and an unexposed band. *N* HCl, Gordon-Weber reagent, and dinitrophenylhydrazine were applied to three of the strips. *N* HCl produced a red color in zone **d**, greatly intensified in the irradiated portion and a pinkish buff coloration in the irradiated portion of zone **f**. Almost the same colorations were observed in the strip which received the Gordon-Weber reagent which in addition produced a purplish coloration in the unexposed portion of zone **f**. Dinitrophenylhydrazine produced a brown color in zones **a**, **d**, and the exposed portion of **f**. The color was intensified in the unexposed portion of **a** and in the exposed portion of **d**. Conventional hatching indicating approximate fluorescence colors as in Figure 7.

Sublimation Test

The production of volatile indole derivatives like skatole and indole in solutions applied to a strip of filter paper and decomposed by exposure of the dry strip to ultraviolet radiation was detected by sublimation onto a clean strip of the same paper. The exposed and the clean strips, separated by a strip of Japanese lens paper, were placed between two glass plates and the whole was set on a hot plate at a temperature of 85° C. for 15 min. The clean sheet was then sprayed with Ehrlich's reagent.

RESULTS

Spontaneous and Induced Decomposition

Judging from the discoloration of their aqueous solutions, several of the indole derivatives of this study decompose spontaneously in a few minutes, hours, days, or years. This was observed with TRPH,

IPA, IAAL, IAA, N-HIAA, IGCA, IA, SK, and IN. This decomposition is accelerated by heating and exposure to visible light and occurs in a matter of minutes with ultraviolet radiation. Results comparable to this radiation are obtained with minute quantities of an oxidant like ferric chloride.

Salkowski and Ehrlich Reacting Spots

In general only a small proportion of the spots and zones detected in our chromatograms and ionograms gave colored reactions with these reagents. IPA and IAA gave six spots, IGXA gave two, and TRPH and IGCA one each, besides their own spots. Three out of the six spots of IPA turned red with the application of *N* HCl which shows that the color reaction with Salkowski and Ehrlich's reagents is mostly due to the acid they contain. The same occurred with six spots of IAA and one of IGCA. A number of spots gave yellow or orange reactions with Ehrlich's reagent, in contrast with the purple or pink reactions of most of the indole derivatives.

Fluorescent and Ultraviolet Absorbing Spots

In contrast to the color reagents, ultraviolet light revealed many spots in our chromatograms and ionograms. Most of these spots are fluorescent and revealed by long-wave (366 mμ) ultraviolet light. Unlike many of their products of decomposition, only a few indole derivatives are fluorescent but they become so on chromatograms under several influences (aging, heating, ultraviolet radiation, alkalinization, application of oxidants). Nonfluorescent indole derivatives are usually short-wave ultraviolet-light absorbing substances and quench the blue fluorescence of the filter paper induced by that light. Most of the fluorescent spots and zones of our chromatograms and ionograms do not give any of the reactions of the preceding section.

Dinitrophenylhydrazine Reacting Spots

One spot or zone in the case of IGXA and IA and two in that of IPA, besides their own spots, gave a brown reaction with the carbonyl reagent DHPH. TRPH and IGCA gave one spot each and IAA two (zones *a* and *d*, Figure 4). In the latter case, however, zone *a* showed a weaker reaction after ultraviolet exposure and zone *d* an increased reaction, showing that, as a result of exposure, the substance of *a* was partially destroyed while that of zone *d* was transformed into a DNPH-reacting substance.

Volatile Substances

The sublimation test was positive in the case of SK and IN, as expected, and also in that of IAA and IAAL decomposed by ultraviolet radiation on the paper.

Substances Separated by Electrophoresis

Electropositive or basic substances and electronegative or acidic substances were detected in ionograms at pH 7 (Figure 4). Substances which remained at the starting line were mostly insoluble in the phosphate buffer at that pH. Some, however, may have been neutral or amphoteric. In chromatograms with multiple levels of pH (Figure 2) acidic, basic, and amphoteric substances exhibited a wavy pattern due to the different R_f values of the forms of dissoci-

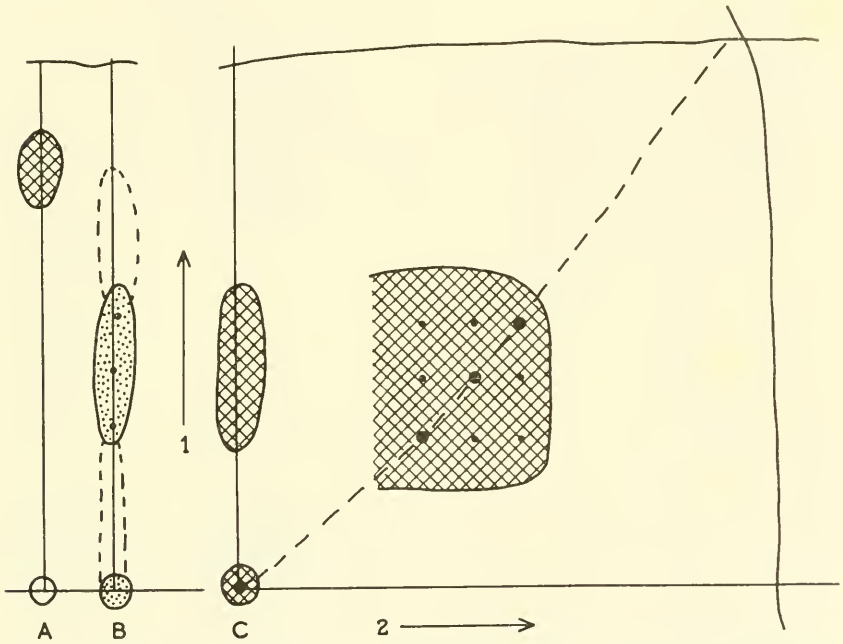

Fig. 5. Double chromatogram of indole-3-acetaldehyde. Ascending chromatography, with water, in an atmosphere saturated with vapors of acetic acid. **A–B**, distribution of spots after the first run; **A**, IAAL-sodium bisulfite addition compound, **B**, ether extract of a solution of the same compound. After the first run **A** was detached and sprayed with Ehrlich's reagent which revealed a purplish spot of IAAL or of the addition compound. In **B** IAAL was seen under short-wave ultraviolet light as a dark elongated area. Three dots indicate the center of the spots of the presumed polymers. It is suggested that the ultraviolet absorbing spot that remained at the origin is also a polymer. Interrupted lines indicate a faint yellow fluorescent tail above and below the large spot of IAAL. **C**, same chromatogram after the second run, sprayed with Ehrlich's reagent. IAAL now constitutes the large square spot near the center of the chromatogram. Left behind is another spot at the starting line, corresponding to the large spot of the first run in **B** but probably of the same polymer as at the origin. All three spots exhibited the same light blue color of IAAL in the Ehrlich reaction. In the square, large dots on the diagonal indicate the center of primary spots of the three presumed polymers; small dots indicate the secondary spots of the same polymers formed by polymerization and depolymerization during chromatography.

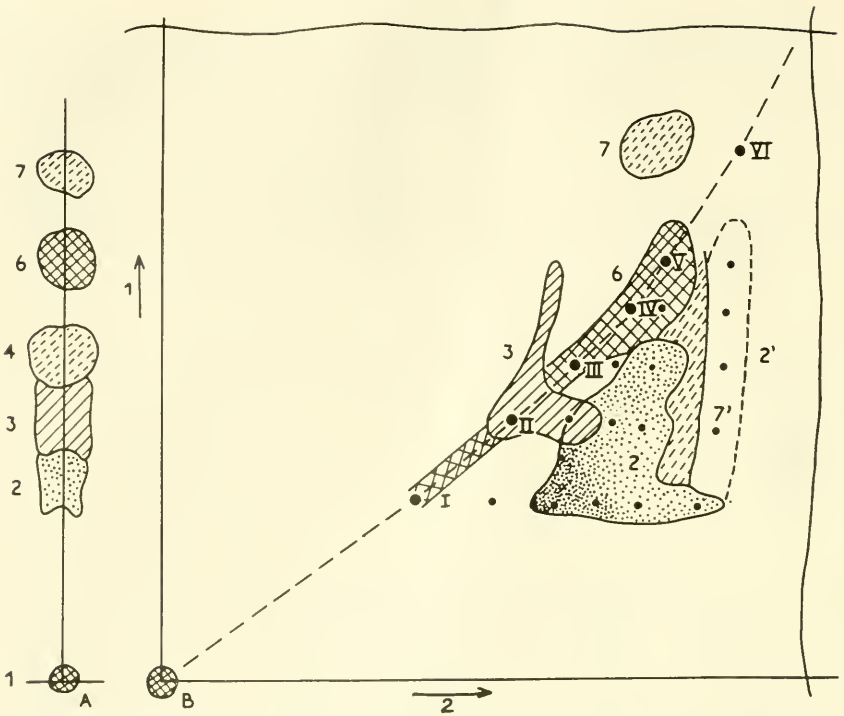

Fig. 6. Double chromatogram of ultraviolet decomposition products of an aldehydic indole derivative (IA, see footnote 1). Ascending chromatography. Acetone and water 8:2 in both directions in an atmosphere saturated with vapors of acetic acid. **A**, distribution of spots after the first run. **B**, after the second run. The diagonal of the chromatogram is indicated by an interrupted line. The center of the primary spots of the presumed polymers of IA is indicated by large dots, numbered I to VI; that of the secondary spots (of polymers formed by polymerization or depolymerization during chromatography) by small dots. IA occupies the large spot 2 and its extension 2′. The outline of spot 2 was traced thanks to its quenching of the blue fluorescence of the filter paper under short-wave ultraviolet light. After spraying the chromatogram with Salkowski's reagent, the whole area of spot 2 and its extension 2′, inclusive of the part with a yellow fluorescence (spot 7′), was colored orange-yellow. No reaction was produced at the sites of the primary spots and some of the secondary spots of the presumable polymers showing that they were completely transformed by polymerization or depolymerization during the second chromatography. Conventional hatching indicating approximate fluorescence colors of the decomposition products (spots 1, 3, 4, 6 and 7) as in Figure 7.

ation. Neutral substances which are not affected by pH form a straight zone (Figure 2). Wavy patterns were also exhibited by compounds like IAAL and IA with no strong acidic or basic groups and IAA with water as the solvent, showed at least three different R_f values. These two results are interpreted as possibly indicating forms of dissociation of the carbonyl and imino groups.

Ether or ethyl acetate fractionation of the decomposed solutions

and of the hydrazones of the DNPH reaction in the case of IPA has, on a few occasions, furnished additional evidence of the acidic or neutral nature of some of the products of decomposition.

Tautomerization and Polymerization

The presence of two tautomers in chromatograms of IPA was demonstrated (26, 27, 29). It is possible that the presence of two forms of IA with different R_f values evidenced by the wavy pattern in

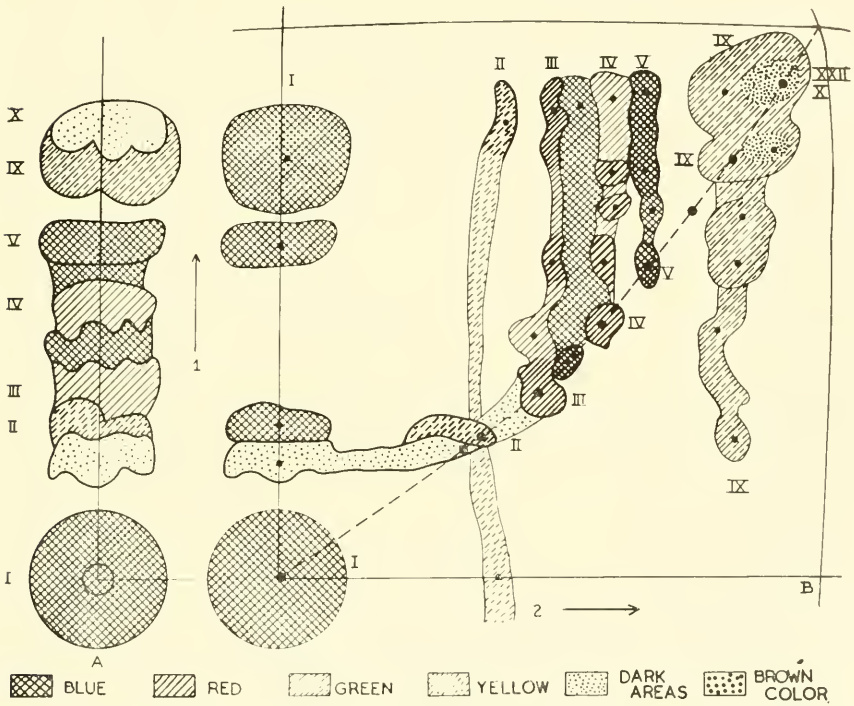

Fig. 7. Double chromatogram of ultraviolet decomposition products of indole-3-acetic acid. Ascending chromatography. Acetone and water 8:2 in both directions, in an atmosphere saturated with vapors of acetic acid. **A**, distribution of spots after the first run. **B**, after the second run. The solution was applied at the origin in the small circle (I) and the sheet was left overnight for equilibration in the chromatographic tank. This resulted in diffusion up to the limit of the greater circle at the origin. The spots between I and II and between III and IV seldom appeared in other chromatograms and were not numbered. The diagonal of the chromatogram in **B** is indicated as an interrupted line. The center of primary spots (substances present in the solution) is indicated as large dots, that of secondary spots (formed during chromatography from the substances whose primary spot is on the same horizontal line) as small dots. The isolated spot on the diagonal, in between the tails of spot V and IX is from a substance presumably present in the solution but entirely decomposed during the second chromatography. The secondary spots of IV, V and IX at the same horizontal level presumably originated from this substance. Fluorescence colors, ultraviolet absorption, and visible color indicated by conventional hatching. Heavier hatching indicates stronger intensity of fluorescence.

Table 1. Number of spots detected by several methods in chromatograms and ionograms of decomposition products of indole derivatives.*

Method of Detection	TRPH	5-H TRPH	TRAM	5-H TRAM	IPA	IAAL	IAA	5-H IAA	N-H IAA	IGCA	IGXA	IA	SK	IN
Fluorescence														
before u.v. radiation	4	1	3	1	11	5	7	1		7	4	4	4	6
after u.v. radiation	2		1		2		7	1		2	2	1	2	
Color (red or brown)					1	1	1	1	1	2	2	1	1	
Ultraviolet absorption	1	1	1	1	3	1	2	1	1	2	2	1	2	1
N HCl	1				3		6		1	1				
Salkowski and Gordon Weber	2	1	1		7	1	7	1	1	2	3	1	1	1
Ehrlich {purple			2	3	1	2	1						1	
Ehrlich {pink					2		1			2	3	1		1
Ehrlich {yellow				1	3	1	1				1	1	1	1
DNPH	1				3	1	2			1	2	2		
Electrophoretic displacement to														
positive pole	2				6	4	5	1	1	2	3			1
negative pole	1		2		1?	2	1	1		1	1		3	2
no displacement	1		1		2	1	10			5	2	7	3	3
Double chromatography														
Tautomers					2	?						?		
Polymers					2	4?	3					7?		
Sublimation at 85° C.						1	1						1	1
Identifiable products	IPA IAA IA?				IAAL IAA IGCA IA?	IAA IA?	IGCA SK			IGXA?	IA?			
Total number of spots	8	1	5	4	14	6	16	2	?	8	6	7	6	6

* See footnote on page 181 for compounds abbreviations represent.

multiple pH chromatograms results from tautomerization at high pH values (22).

The two aldehydic compounds investigated in this study, IAAL and IA, produce in chromatography unusually large spots (Figures 5 and 6). Thanks to the properties of double chromatograms (27) those spots can be interpreted in terms of different states of polymerization of these compounds. Spontaneous precipitation occurring in aged solutions of IAAL and IA may also result from polymerization which probably occurs through the side-chain as in the corresponding aliphatic aldehydes. Solutions of IAA (0.1 per cent) in boiled (air-free) water exposed to ultraviolet radiation do not produce the brown discoloration of ordinary solutions but instead become milky. This also may be due to polymerization of which there is evidence in double chromatograms (Figure 7). It is seen that the spot in the upper right-hand corner is in reality quadruple and composed of two primary spots IX and X on the diagonal and two secondary spots, one of them a spot of IX produced from the substance of spot X and the other one, a spot of X, produced from the substance of spot XI. The best interpretation of this composite spot seems to be that IX is a polymer of X (IAA).

In Table 1 is shown the number of spots and zones of decomposition products of indole derivatives in chromatograms and ionograms which exhibited the properties just described. The derivatives themselves are included in the number whenever they possessed the corresponding property.

DISCUSSION

The most significant result of these studies is perhaps the small number of spots and zones of our chromatograms and ionograms that give the Salkowski and the Gordon-Weber reactions. Only in the case of TRPH, IPA, and IAA was it possible to demonstrate the presence of the immediate product of degradation of the side-chain, IPA, IAAL and IGCA, respectively. The demonstration of IGXA in the case of IGCA and of IA in that of IGXA was doubtful. The products of more advanced degradation of the side-chain are even more rare, and only IAA, among the decomposition products of TRPH and, with IGCA, among those of IPA, is detectable in some of our chromatograms. The identification of IA in the chromatograms of TRPH, IPA, IAAL and IGXA is doubtful in view of the possible confusion with *o*-aminobenzene derivatives with the same R_f. It seems, therefore, that degradation of the side-chain is not a major pathway in the spontaneous decomposition (aging) or the decomposition induced by ultraviolet radiation[2] of the indole derivatives.

Other workers, however, have been more successful than we have in demonstrating the presence of indolic products of decomposition in chromatograms and ionograms of decomposed solutions of indole derivatives. Mayr (19), von Denffer and his collaborators (10), especially Melchior who made the most extensive investigation in this field (20), and recently Kaper and Veldstra (15) were able to identify several indole derivatives or at least show the presence of several Salkowski reacting substances which we have failed to demonstrate. Except in the case of IAA and IAAL, we were not able to detect the presence of skatole, and much less of indole, which several of these authors have mentioned among the decomposition products of many of the indole derivatives of the currently accepted scheme of decomposition (Figure 1). With a much more refined sublimation method than the test we have devised, Behrens and Fischer (2) were able to demonstrate the presence of IA, skatole, and indole among the photolytic decomposition products of IAA.

All these authors, however, have made little if any use of ultraviolet light to detect the presence of fluorescent substances among the decomposition products of indole derivatives and have overlooked those we find in our chromatograms and ionograms.

Substances giving a yellow or orange-yellow color with Ehrlich's reagent but not Salkowski and Gordon-Weber reactions were often found in our chromatograms and ionograms. Presumably they are primary amines, especially *o*-aminobenzene derivatives, which could result from cleavage of the pyrrole ring. Cleavage of that ring in tryptophan with the production of kynurenine, one of those derivatives, is well known and, at least in the case of enzymatic decomposition, has been shown to occur also with other indolic substances (18). Gordon and Weber (12) state that in excess of air inactivation of IAA by X-radiation appears chiefly due to ring opening.

Another evidence of ring opening, with the production of an electropositive amino group is given by the presence of zones on the negative side of ionograms (pH 7) of practically all the derivatives we studied. If the substances corresponding to those zones are *o*-aminobenzene derivatives, the group in the *ortho* position must be neutral, which is suggested in the case of zone *a* of ionograms of IAA by its strong reaction with DNPH (Figure 4). One possibility is that zone *a* be *o*-aminophenylacetaldehyde or one of its hydroxyderivatives. The *o*-aminobenzene derivative resulting from the opening of the pyrrole ring of indole in a reaction similar to that which produces kynurenine from tryptophan is *o*-aminobenzaldehyde, a substance which also gives a positive reaction with DNPH. None of the

[2] Enzymatic decomposition and decomposition induced by visible light in the presence of fluorescent substances were beyond the scope of the present studies and will not be discussed here.

spots and zones giving a yellow reaction with Ehrlich's reagent were identified as being anthranilic acid, an *o*-aminobenzene derivative which was found among the decomposition products of indole derivatives by other workers (20).

In contrast with substances reacting with Salkowski, Gordon-Weber, and Ehrlich's reagents, fluorescent substances which do not give those reactions were numerous. It was rarely possible to establish the correspondence of fluorescent spots of one indole derivative with those of another one. Spot II of the chromatograms of IAA (Figure 7) is an exception in that it was also apparently present in chromatograms of TRPH, IPA, and IAAL. It would seem, therefore, that most of the fluorescent substances originated separately from products resulting from degradation or changes in the nucleus of each derivative, rather than from degradation of the side-chain. In this connection, the possibility of an opening of the benzene ring, yielding a pyrrole derivative (5) which to our knowledge has not been considered to date, is worthy of attention.

Whether degradation of indole derivatives starts by the side-chain, the pyrrole ring or the benzene ring, the final products must be aliphatic compounds. Meyer and Pohl (21) claim that the end product of photolysis of IAA by riboflavin is anthranilic acid which, in ultraviolet light, decomposes further into glycine and formic acid with oxalic acid as an intermediate of the latter. It is possible that some of our fluorescent substances are indeed aliphatic compounds and represent final stages of decomposition of indole derivatives.

The condensation of indole compounds with the formation of colored dimers like indigo and its inducement by ultraviolet radiation are well known (1). The possibility that dimerization occurs during the oxidation of IAA has been discussed by Ray and Thimann (25). Houff *et al.* (14) have shown that a colored dimer of IAA is an intermediate in the formation of N-HIAA, the main product of the Salkowski reaction. Evidence of polymerization in our chromatograms might be due to such dimers. In Figure 7, spot IX might be the dimer of Houff *et al.* but we were not able to identify N-HIAA among the decomposition products of IAA because that substance diffuses extensively and quickly disappears in chromatograms.

The case of IAAL and IA (Figures 5 and 6) is different in that polymerization probably occurs as a result of the condensation of the side-chains of the molecules involved, in a manner similar to that of the corresponding aliphatic aldehydes.

Summing up, we may say that spontaneous decomposition, or the decomposition induced by ultraviolet radiation, of indole derivatives may follow four main courses and their combinations: (1) oxidation and other changes in the side-chain, (2) oxidation and opening of the

pyrrole ring, (3) oxidation and possibly opening of the benzene ring, (4) polymerization.

These possibilities have been represented in the diagram of Figure 1. In such a diagram the concept of pathway is all but lost. In the interpretation of the facts this concept constitutes a serious drawback. We suggest that the intricate network of pathways be called a lattice and since it is a representation of all the possible ways in which indolic compounds may be decomposed, spontaneously or under the influence of pyhsical, chemical, or biochemical factors, it can be called the catabolic lattice of indole derivatives.

SUMMARY

Solutions of 14 indole derivatives decomposed by aging, heating, ultraviolet radiation, or oxidants like $FeCl_3$ were studied chromatographically and electrophoretically. Fluorescence and ultraviolet absorption of spots and zones were detected under long-wave and short-wave ultraviolet light respectively. Significant changes in fluorescence and absorption were induced by exposure of the chromatograms and ionograms to ultraviolet radiation. Salkowski, Gordon-Weber, Ehrlich, and Van Eck reagents, safranin, *N* HCl, dinitrophenylhydrazine, dichlorophenolindophenol, and ammoniacal $AgNO_3$ were applied to the chromatograms and ionograms, either to the whole or to longitudinal strips cut out from them so that more than one reagent could be used at a time.

Only a small proportion of the decomposition products were indole derivatives reacting with Salkowski and Gordon-Weber reagents. A few of them were identified as the immediate product of degradation of the side-chain. Of those which did not react, a number of spots and zones gave yellow or orange color reactions with Ehrlich's reagent and are probably *o*-aminobenzene derivatives but not anthranilic acid. Among them some moved to the negative side of the electrophoretic strips so that the side group in the *ortho* position must have been neutral.

Double chromatograms produced large spots of IAA, IAAL, and IA which are interpreted as made up of from 2 to 6 polymers, present in the solution or formed during chromatography.

The majority of spots were fluorescent and did not react with any of the reagents. It is suggested that they might be pyrrole derivatives resulting from oxidation and cleavage of the benzene ring of the indole compounds, and aliphatic compounds, the last stages of decomposition of those substances.

It is concluded that decomposition of indole derivatives may follow four main courses or a combination of them.

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The Interpretation of Rates of Indole-3-acetic Acid Oxidation

Although several fundamentally different mechanisms have been proposed to explain enzymatic oxidation of indole-3-acetic acid (IAA) by peroxidase-containing preparations, a feature common to all of these is that the oxidation is interpreted as a cyclical process involving several reactions. Goldacre (2) and Galston, Bonner, and Baker (1) suggested that H_2O_2 is consumed in a peroxidase-catalyzed step, and reformed from O_2 in an oxidase-catalyzed step. In the scheme proposed by Maclachlan and Waygood (3), IAA is supposed to be oxidized by Mn^{+3}, giving a radical which reacts with O_2; the resulting oxidant is used by peroxidase to oxidize a phenol, which in turn reoxidizes Mn^{+2} to Mn^{+3}. Another possible mechanism, suggested recently by Yamazaki and Souzu (6), is that IAA is oxidized directly by peroxidase in a one-electron oxidation ($\frac{1}{2}$ H_2O_2), giving a radical which reacts with oxygen to regenerate H_2O_2.

I do not feel that any of the specific proposals about the mechanism of enzymatic IAA oxidation has been established unequivocally, and I think it is not yet possible to say whether some of the different enzyme preparations which have been studied actually operate by different reaction mechanisms. It may even be that some or all of the reactions of the formally distinct mechanisms actually go on together in a single reaction medium, and contribute to varying degrees to the over-all oxidation depending upon the conditions, the addition of phenols or of Mn, and so forth. In all of the suggested mechanisms, and very likely in any others which may be conceived, the reaction rates must depend upon concentrations of intermediates such as H_2O_2 and radicals or other partially oxidized products derived from IAA. The purpose of this discussion is to explain the important bearing of this upon the meaning of IAA oxidation rates, and of progress

curves of IAA destruction, since such measurements are used widely not only as a basis for interpreting the mechanism of IAA oxidation, but also in physiological experiments in which IAA destruction is involved. The phenomena have been investigated in experiments performed with the IAA-oxidizing enzyme of *Omphalia flavida* (4, 5).

When IAA is added to a peroxidase capable of oxidizing it, a characteristic lag or induction phase precedes attainment of a rapid reaction rate. This is illustrated with the solid line in Figure 1. It appears to be due to the fact that the reaction medium does not contain, initially, concentrations of the above-mentioned oxidation intermediates (such as H_2O_2) great enough to allow a substantial rate of oxidation. If H_2O_2 is added initially, no induction phase is observed; instead the reaction is occurring rapidly at the earliest possible observation, as shown by the broken line in Figure 1. Evidently H_2O_2 is either a reaction intermediate, or it can give rise to the actual intermediates rapidly.

Under different conditions and with different enzyme preparations it is to be expected that the initial content of oxidation intermediates will vary. The initially observed rates of IAA destruction, as well as how pronounced the induction effect is, will vary accordingly, quite apart from variations in enzyme activity and effects of cofactors

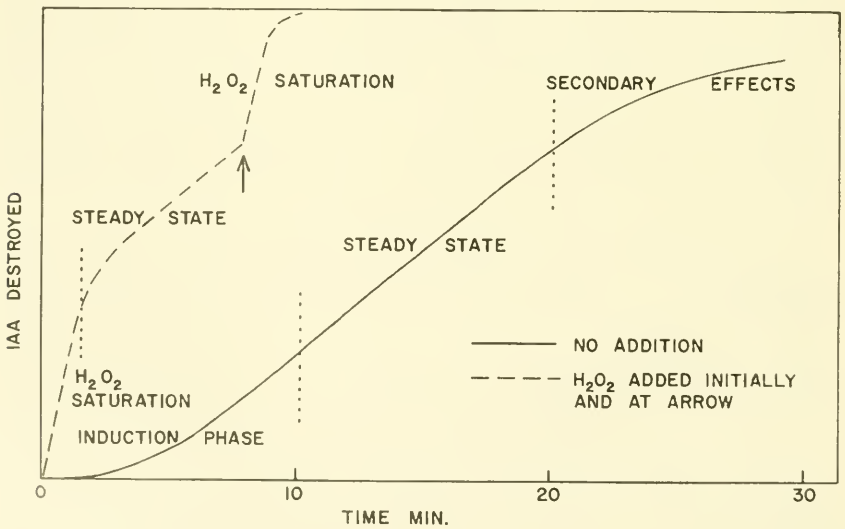

Fig. 1. Illustration of the kinetic phases of enzymatic IAA oxidation observed with and without addition of H_2O_2. In this example, the initial IAA concentration was $2 \times 10^{-4}M$, and the top of the figure represents destruction of all the IAA. At zero time and at the arrow $5 \times 10^{-6}M$ H_2O_2 was added to the sample shown by the broken line.

and inhibitors. This is an inescapable source of possible confusion in comparative experiments on IAA oxidation.

The gradual increase in reaction rate which occurs during the induction phase must be ascribed to some process by which the total concentration of oxidation intermediates in the medium increases. The proposals mentioned concerning the IAA oxidation mechanism do not provide for such an increase in concentration, since every intermediate must be consumed and produced in equal amounts to maintain the catalytic cycle and account for the reaction stoichiometry. Two principal types of processes can be considered in looking for an explanation of why the reaction rate increases during the induction phase: (a) a minor reaction occurs, separately from the main IAA oxidation process, which forms an IAA oxidation intermediate as a product, or (b) the IAA oxidation reaction itself forms a product which is or can give rise to one of the intermediates, so that as the cycle operates one of the intermediates is formed in excess of the amount consumed. We shall call processes such as (a) or (b) *initiation* reactions; they initiate a catalytic cycle dependent upon oxidation intermediates. Types (a) and (b) are fundamentally different kinetically, for (b) is tied to the occurrence of the IAA oxidation reaction while (a) is not. With process (b) the reaction would appear to be autocatalytic, whereas with (a) it would not. With (a) the amount of IAA destroyed at time t after the start of reaction should be a linear function of t^2; with (b) the log of the amount of IAA destroyed at time t should increase linearly with t once an appreciable rate of reaction is observed. We have concluded that the *Omphalia* enzyme reaction is in fact autocatalytic, that is, alternative (b). It should be noted that a positive distinction between initiation reactions (a) and (b) is not easy to make, as it must be based upon close study of the early part of the induction phase, because further complications in the kinetics, to be discussed below, appear as the reaction rate increases.

By quantitative comparison between the kinetics of the induction phase and the rate effects of added H_2O_2, one can get some indication as to the concentrations of intermediates which must actually be formed during induction. It appears that the amounts formed are much less than the amount of IAA which undergoes oxidation during this period, which suggests that the autocatalytic effect does not arise from a major product of IAA oxidation, but rather by a minor side reaction in the oxidation cycle.

The IAA oxidation rate does not continue to increase indefinitely, but leads gradually into a steady maximum rate of oxidation which

is maintained until exhaustion of substrate, or occurrence of secondary complications such as enzyme inactivation, cause it to fall. These phases are also illustrated in Figure 1 (solid line). The steady rate is, however, considerably lower than can be obtained by adding H_2O_2 to the medium. The rate increases, with increasing concentrations of added H_2O_2, up to a maximum which we call the H_2O_2 saturation rate, and which we presume is due to saturation of the peroxidase with H_2O_2. This occurs at about $2 \times 10^{-6}M$ H_2O_2 with the *Omphalia* enzyme, and is illustrated by the first part of the broken curve in Figure 1. It is apparent that in the absence of added H_2O_2, the enzyme does not become saturated even when the rate has become steady, after induction. A further fact of significance is that when saturating amounts of H_2O_2 are added, it can be demonstrated that H_2O_2 disappears gradually from the system, since after a time the rate falls to the same value as would be reached, after induction, if H_2O_2 had not been added, and at this point the H_2O_2 saturation rate can be restored by adding more H_2O_2. These effects are shown in the broken curve of Figure 1. They indicate that processes are also occurring which lead to loss of oxidation intermediates from the system, processes which can be called termination reactions by analogy with organic auto-oxidation processes. A probable explanation of the steady rate of oxidation which is reached after induction is that it represents a steady state in which the rates of formation (initiation) and disappearance (termination) of oxidation intermediates have become equal — due, for example, to termination increasing more rapidly than initiation as the reaction rate rises (termination is evidently more rapid than initiation at the high rate attained by saturation with H_2O_2). Examples of possible types of termination reactions are (a) a catalase-like reaction involving removal of H_2O_2, and (b) a reaction between free-radical intermediates such as $P\cdot + PO_2\cdot \rightarrow PO_2P$.

It will be evident that the IAA oxidation rate observed at steady state will be influenced not only by effects on enzyme activity and the main catalytic cycle, but also by any factors which influence the initiation or termination processes, and thereby the steady state concentrations of intermediates. There is thus considerable danger in interpreting rate effects of experimental treatments as if the observed effects related only to enzyme activity or to the cyclic IAA oxidation mechanism. To proceed further with interpretation of rate effects on IAA oxidation it will be essential to develop a kinetic analysis of the proposed mechanism which includes assumptions about the nature of initiation and termination reactions.

In our experience, the rates of IAA oxidation attainable by sat-

uration with H_2O_2 have proved to be a much more reliable and reproducible means of measuring enzyme activity than the steady state rates, presumably because the H_2O_2 saturation rate is not affected by variations in the rates of initiation and termination.

Finally, several examples exist in the literature of effects on IAA oxidation rates which involve secondary complications on the steady state rate, particularly effects on enzyme inactivation. Such effects must be distinguished carefully from true kinetic effects, because they cannot properly be used, in a kinetic sense, as evidence of the reaction mechanism, and they may also have quite different significance in physiology.

It must be noticed that the early reaction phases illustrated in Figure 1 take place over amounts of IAA oxidation too small to be measured accurately by usual manometric methods, so that in manometry one is dealing usually with the steady state (whether or not H_2O_2 is added) or with secondary rate complications. This limits the usefulness of manometry in investigation of IAA oxidation.

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A Model Chemical System for the Study of the Oxidation of Indole-3-acetic Acid by Peroxidase

The oxidative degradation of indole-3-acetic acid (IAA) by plant extracts was studied intensively during the 1950's (9). Despite the advances made in understanding the enzymatic components, the required cofactors, and the physiological significance of the reaction, elucidation of the complete process has remained elusive, in good part because the changes undergone by the organic substrate, IAA, are still unknown. It is the purpose of work under way in these laboratories to determine the identity of the products formed from IAA when acted upon by the IAA oxidase system. We have found that hydrogen peroxide in acidic media converts IAA to products the spectra of which resemble closely those of the products formed in the oxidation of IAA by peroxidase in the absence of added hydrogen peroxide. Since it is now almost certain that the effective component of the IAA oxidase of higher plants is a peroxidase (9), the H_2O_2/H^+ system has considerable significance in relation to the biological activity of IAA. We are using the chemical system as a model to aid in investigating the chemistry of the oxidation of IAA by peroxidase.

Previous attempts to identify the products of the oxidation of IAA by IAA oxidase have depended mainly on isolation of the products either in the free state or on paper chromatograms. Because of the multiplicity of products actually isolated (7, 10), and the possibility that the product formed initially may undergo decomposition under the conditions of isolation, we have chosen to study the nature of the product(s) by following the changes in the ultraviolet spectrum of IAA during its reaction with peroxidase and with the H_2O_2/H^+ system. Following the course of these reactions by spectrophotometric techniques has one other advantage over techniques in which only residual IAA is determined. It is quite possible that IAA could be

degraded by different pathways under different conditions, and that oxygen could be consumed and carbon dioxide evolved in each. Differences in these pathways could not be detected by assaying for residual IAA, but could be discerned by following the changes in the substrate as the reaction proceeds. For this purpose the spectrophotometric technique is particularly well suited.

This paper is a preliminary account of the chemistry of the model system, its relationship to the oxidations effected by peroxidase, and the spectrophotometric techniques employed in following the reactions.

EXPERIMENTAL

Changes in the ultraviolet absorption spectra were followed in quartz cuvettes of 1 cm. light path by means of a Beckman DK-2 recording spectrophotometer, equipped with the usual accessories for work in the ultraviolet region. The reference cell in each experiment contained the buffer system or solvent used in the sample cell. Corrections were made for the weak absorptions above 230 mμ of peroxidase and hydrogen peroxide by subtraction of the absorbancies of blanks from those of the spectra being recorded.

The reactions were carried out by pipetting solutions of hydrogen peroxide or peroxidase in buffer into the cuvette, which contained a solution of IAA or some other substrate in the same buffer. The solution was stirred and the spectrum scanned. A typical enzymatic reaction mixture contained IAA (final concn. $10^{-4}M$) and peroxidase ($10^{-6}M$) in acetate buffer ($0.1M$ sodium acetate + $0.04M$ acetic acid) at pH 5. A typical reaction in the peroxide system involved IAA ($10^{-4}M$) and hydrogen peroxide ($10^{-3}M$) in a buffer of $0.097M$ hydrochloric acid and $0.05M$ KCl (pH 1). Reactions were allowed to continue until changes in the spectra had ceased or were very slow, requiring from a few hours to several days depending on the reaction.

Crystalline horseradish root peroxidase was obtained from Nutritional Biochemicals Corporation (Lot no. 7625). 2-Phenylindole-3-acetic acid was synthesized by the method of Bauer and Andersag (1), and 2-phenylskatole by the method of Kissman *et al.* (5). Oxindole-3-acetic acid was a gift of Dr. Percy Julian, The Julian Laboratories, Chicago, Illinois. Other chemicals were purified grades.

THE PEROXIDASE SYSTEM

The spectral changes which took place when IAA was treated with peroxidase under the conditions described above are shown in Figure 1. There was a very rapid increase in absorbancy in the 240

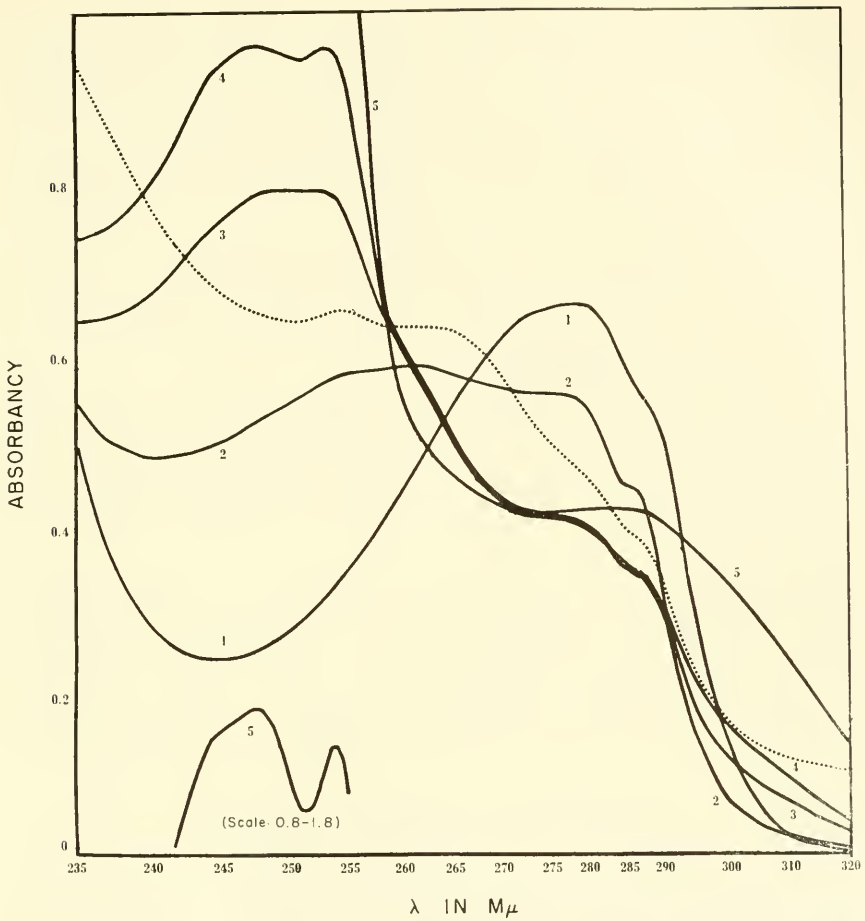


Fig. 1. Changes in the ultraviolet spectrum during the oxidation of IAA ($10^{-4}M$) by peroxidase ($10^{-6}M$). Solid line, in acetate buffer (pH 5); 1, 35 sec.; 2, 10 min.; 3, 30 min.; 4, 4 hrs.; 5, 24 hrs.; broken line in phosphate buffer, pH 7.5, after 24 hrs. Continuation of curve 5 over range 0.8 to 1.8 absorbancy is given in reduced scale at lower left.

to 260 mμ region during the first 10 min., after which the rate of increase of absorbancy gradually fell off. The peaks at 248 and 254 mμ could first be distinguished after about an hour, and reached their maximum in about 20 hrs. After that the peaks decreased slightly (0.06 unit) during the next 3 days. In the indole region (280 mμ) absorption fell from 0.61 to 0.41 in the first 40 min., remained constant for the next 3 hrs., and then slowly fell as a new maximum formed at 285 mμ during the ensuing 3 days.

The changes observed resemble closely those recorded by Ray (8) for the action on IAA of the IAA oxidase from *Omphalia,* and

of a crude peroxidase from horseradish root, both at pH 3.7. The principal differences are at 254 mμ, where Ray observed only a shoulder, and at 285 mμ, where a new peak appeared in our experiments after 3 days. Since Ray's experiments were for periods under 2 hrs., it cannot be determined whether the maximum at 285 mμ would have appeared. These minor differences may be due in part to differences in pH (at pH 7.5, Figure 1, the spectrum of the product(s) of IAA and peroxidase is quite different from that at pH 5), or to the presence of hydrogen peroxide in Ray's experiments. We found that the presence of hydrogen peroxide in the mixture resulted in a general blurring of the spectrum, particularly in the 250 mμ region, where the peak at 254 mμ became a shoulder.

Despite these minor differences the otherwise striking resemblance of the spectra from the peroxidase reaction and those from the IAA oxidase reaction confirm again the close relationship between the two, and the relevancy of using peroxidase as a model enzymatic system for studies of IAA oxidase.

In addition to IAA, a number of related indoles were examined as possible substrates for peroxidase in the absence of added hydrogen peroxide. Under the conditions described for IAA in the experimental section, the spectra of β-(indole-3-)propionic acid (IPA), γ-(indole-3-)-*n*-butyric acid (IBA), α,α-dimethylindole-3-acetic acid, tryptophan, tryptamine, and skatole were unchanged during periods extending up to 90 hrs.

THE MODEL CHEMICAL SYSTEM

From other studies of the oxidation of indoles we have found that the rate of oxidation of some indoles by oxygen or hydrogen peroxide is greatly accelerated by the presence of acid. The action of oxygen on IAA in aqueous solution at pH 1 produced red pigments, but there was little change in the ultraviolet portion of the spectrum. When IAA and hydrogen peroxide were mixed at pH 1, however, the spectrum of IAA underwent immediate changes, as shown in Figure 2. The peaks at 245 to 248 and 254 mμ are remarkably like those observed in the experiments with peroxidase. At pH 1 maximum absorbancy was attained by the two peaks in the 250 mμ region in about 24 hrs., after which a slow decrease occurred. The indole peak at 279 mμ decreased very slowly while a new maximum appeared at 297 mμ, complete in 72 to 90 hrs. In short, rapid changes took place at first in the 250 mμ region while the characteristic indole spectrum (278 to 288 mμ) underwent little change. Then, much more slowly, the indole portion of the spectrum changed completely, with little disturbance of the peaks which had previously appeared in the 250 mμ region.

Fig. 2. Changes in the ultraviolet spectrum during the reaction of IAA ($10^{-4}M$) and H_2O_2 ($10^{-2}M$) at pH 1. Solid lines: curve 1, 30 sec.; 2, 1 hr.; 3, 5.5 hrs.; 4, 28 hrs.; 5, 72 hrs. Broken line IAA ($10^{-4}M$). Continuation of curves 4 and 5 over range 0.8 to 1.8 absorbancy is given in reduced scale at lower left.

The reaction of hydrogen peroxide with IAA is pH dependent. At pH 1 the initial reaction is very rapid; the peak at 254 mμ is present as soon as the spectrum is scanned (about 30 sec.). From the accompanying table it is clear that the reaction rate decreases markedly with increasing pH. In neutral or alkaline solution there is no change in the spectrum of mixtures of IAA and hydrogen peroxide. This agrees with Ray's (8) finding and confirms the earlier work of Siegel and Weintraub (12), who showed by other methods that IAA is not attacked appreciably by peroxides in neutral solution.

When glacial acetic acid was used as the acidic medium, the peaks at 248 and 254 mμ appeared, but the spectral changes were much slower than in aqueous hydrochloric acid. However, in a 2:1 (v/v)

Table 1. Rates of oxidation of indole-3-acetic acid (IAA) and γ-(indole-3-)-*n*-butyric acid (IBA) in various media.

Substrate*	pH or Source of H^+	Time Required for First Appearance of Peak at 254 mμ	Max. Absorbancy Attained at 254 mμ	Time (hrs.) Required To Attain Max. Absorbancy at 254 mμ
IAA	1	30 sec.	1.11	28
IAA	3	1 hr.	0.72	25
IAA	5	4 hrs.	0.67	46
IAA	6.6	73 hrs.	0.28	166
IAA	8			
IAA	glacial acetic acid	90 min.	0.79	23
IAA	67% acetic acid	15 min.	0.86	19
IAA	5†	30 min.	1.29	24
IBA	1	90 min.	0.90	48

* Initial concentration of substrate = $10^{-4}M$. Ratio of H_2O_2 to substrate = 10 in all experiments.

† In the presence of $10^{-6}M$ peroxidase; no H_2O_2 added.

mixture of acetic acid and water the rate of increase of the peaks was about the same as in the mineral acid system, although the initial appearance of the peaks was still much slower (see Table 1).

Decreasing the ratio of H_2O_2/IAA from 10:1 (which was the ratio used for most of the experiments) to 1:1 did not affect significantly the rate of growth of the peaks in the 250 mμ region, although the initial appearance of the peak at 254 mμ required somewhat longer (5 min. when the ratio of 1:1). At a ratio of 1:2 the rate fell off much more, although the final height reached by the peaks was about the same as that of higher ratios, indicating that hydrogen peroxide was not limiting. At still lower ratios of oxidant to IAA hydrogen peroxide was limiting, as shown by the fact that the maximum absorption was much less than that attained at higher ratios. It is not known whether the system also consumes oxygen, but from these results it seems unlikely.

SPECIFICITY OF THE MODEL SYSTEM

One of the most striking features of the H_2O_2/H^+ system is its specificity. It appears to attack only those indoles which have a carboxyl group (or a potential carboxyl group) in the side chain at the 3-position, and for greatest reactivity the carboxyl carbon must be

separated from the indole ring by one other carbon atom, as in IAA. This generalization is based on the following observations. (All experiments were carried out at pH 1 with a mole ratio of H_2O_2/IAA of 10:1.)

(1) The spectrum of skatole remained unchanged for 25 hrs.

(2) The spectrum of γ-(indole-3-)-*n*-butyric acid (IBA) underwent relatively slow changes, but peaks at 248 and 254 mμ were distinguishable after 12 hrs. and reached maximum absorbancy in 48 hrs. (Figure 3). A shoulder with its center at 290 mμ appeared between 24 and 48 hrs., corresponding to the maximum observed at 295 mμ when IAA was subjected to prolonged treatment with the H_2O_2/H^+ system.

(3) The spectrum of β-(indole-3-)propionic acid (IPA) underwent a very slow increase in absorption in the 230 to 260 mμ region, while the indole peak at 280 mμ slowly declined. No specific peaks appeared (Figure 3).

(4) The spectrum of α,α-dimethylindole-3-acetic acid in which, as in IAA, the carboxyl group and the ring are separated by one carbon, changed more rapidly than did the spectrum of IAA, and was complete in 18 hrs. The new peaks were at 248, 259, and 293 mμ (Figure 3). In this case the peak at 293 mμ was formed about as rapidly as those at lower wave lengths, whereas with IAA the lower peaks reached their maximum heights first, and the peak at 295 mμ required an additional 48 hrs. for complete formation (Figure 2).

(5) The spectrum of 2-phenylindole-3-acetic acid changed rapidly and was complete in about 4 hrs. The spectral shifts are not directly comparable to those of the IAA reactions because the absorption spectra of IAA and of 2-phenyl-IAA are quite different, as are those of their oxidation products. Nevertheless, it is significant that the spectrum of 2-phenylskatole, which lacks the carboxyl group, was unchanged after 25 hrs. in the H_2O_2/H^+ system.

It is clear from these results that the H_2O_2/H^+ system is selective in its attack of indoles. Any reaction which is limited to IAA and related compounds would be of interest in considering the biological activity of IAA. The close relationship of the products of the chemical reaction and of the enzymatic reaction makes the chemical system considerably more significant.

THE COURSE OF THE CHEMICAL REACTION AND THE NATURE OF THE PRODUCTS

Although final answers have not been obtained to the questions of the detailed pathway of IAA oxidation in the H_2O_2/H^+ system and

Fig. 3. Ultraviolet spectra of various indole-3-alkanoic acids after prolonged oxidation. 1. IAA + peroxidase, 96 hrs. 2. IAA + H_2O_2 at pH 1, 72 hrs. 3. IBA + H_2O_2 at pH 1, 48 hrs. 4. IPA + H_2O_2 at pH 1, 72 hrs. 5. α,α-Dimethyl-IAA + H_2O_2 at pH 1, 44 hrs. Continuation of curves 1 and 2 over range 0,8 to 1.8 absorbancy is given in reduced scale at lower left.

of the nature of the products, the available data suggest areas in which these answers may be found. It should be noted at the outset that we have no idea of the number of products formed in any of these reactions.

The pH dependency of the reaction can be accounted for in a number of ways. The indole ring may undergo protonation, producing a reactive species which subsequently reacts with the oxidizing agent, or the hydrogen peroxide may be protonated to yield a reactive fragment such as $H_3O_2^+$ or ^+OH. A third possibility is that the carboxyl group is converted to a peracid, since peracids can generally

be formed from carboxylic acids and hydrogen peroxide in the presence of strong acids. The last possibility does not seem likely, however, since the reaction of IAA and hydrogen peroxide occurs in acetic acid, where one would expect that the peroxide would be consumed in forming peracetic acid.

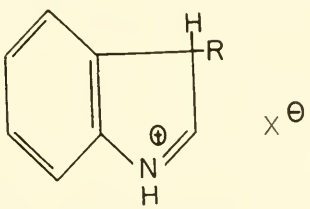
Since the spectrum of the indoles used changed little even at pH 1, there is probably only a small amount of protonated species present.[1] On the other hand a large part of the hydrogen peroxide may be protonated at pH 1 (11, p. 379). At the present time, therefore, we prefer the interpretation that the pH dependency arises from the necessity of protonating hydrogen peroxide to produce a reactive species.

Assuming that OH^+ or an equivalent is the active species facilitates discussion of the possible points of attack of IAA. For any cationic species the most likely point of attack in an indole is the 3-position[1] (4). Attack of the side chain in the initial step is unlikely, since α,α-dimethylindole-3-acetic acid undergoes the reaction even more rapidly than IAA. From a theoretical point of view, the α-hydrogens of IAA would *not* be active toward an attacking cationic species, since they are already more than normally positive because of electron-withdrawal by the carboxyl group and the ring system.

Although attack of the 3-position accords well with most of the known oxidations of indoles (15), oxidation at the 2-position might take place either directly (e.g., if IAA were protonated first and hydrogen peroxide attacked the protonated species[1] at the 2-position) or by rearrangement of a group from the 3-position.[2]

Oxidation at the 2-position might yield oxindole-3-acetic[2] acid as the final product or as an intermediate. This pathway was ruled

[1] Protonation of the indole nucleus would certainly cause significant changes in the ultraviolet spectrum because of the formation of indolenine salts:

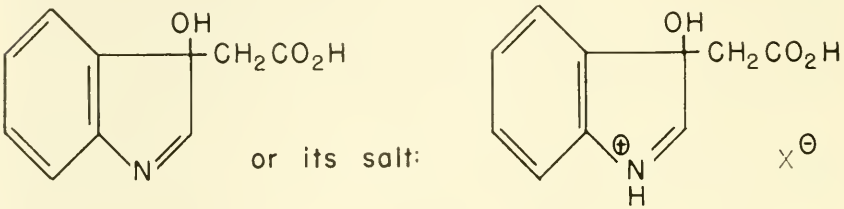


Contrary to the commonly written form, protonation of an indole probably occurs at the 3-position rather than at the nitrogen, in agreement with the well-established behavior of α,β-unsaturated amines (6). Protonation at the 3-position also accounts for the dimers obtained from indole (13) and from skatole (unpublished).

[2] Chemical precedent for the oxidation of the 2-position has been provided by Witkop (14), who converted skatole and tryptophan to the corresponding oxindoles by the use of peracetic acid. More recently, Dalgliesh and Kelly (2) have obtained oxindoles from indoles by means of potassium persulfate. There is no reason to assume, however, that the *initial* oxidation takes place at the 2-position (3).

out, however, because an authentic specimen of oxindole-3-acetic acid showed the typical spectrum of an oxindole [λ_{max} (0.1*N* HCl) 249 mμ, shoulder 280; log ε_{max} 4.00, 3.35], and underwent no change when treated with hydrogen peroxide at pH 1.

The available information leads us to believe that the intermediate formed first in the reaction of IAA and hydrogen peroxide in acidic media is:



Neither of these is the final product since the spectra of known compounds of this type (16) do not correspond to those observed in this reaction. Moreover, such intermediates do not explain, of themselves, the requirement of a two-carbon carboxyl-bearing side chain. They do, however, afford a variety of potential pathways, such as cyclization, dehydration, decarboxylation, etc., in which the carboxyl group may participate.[3] These pathways are now under investigation.

A number of attempts have been made to scale up the reaction of IAA and hydrogen peroxide at pH 1 and in acetic acid to permit isolation of the products. At concentrations of 0.1*M*, however, the spectra of the products showed only broad general absorption in the 240 to 260 mμ region. The products themselves were intractable tars.

The spectra of a variety of other compounds which might have been among the products have been compared to those obtained in the reactions described herein. *o*-Aminoacetophenones, Bz-hydroxy-*o*-aminoacetophenones, anthranilic acid, hydroxyanthranilic acids, 2- and 4-quinolones, dihydro-2- and -4-quinolones, indolenines, and dioxindoles were considered, but their spectra differed in significant ways from those reported herein.

COMPARISON OF THE PEROXIDASE SYSTEM AND THE H_2O_2/H^+ SYSTEM

As shown in Figure 3, the similarity of the spectra observed near the ends of the two reactions is striking. There are, however, certain differences to be noted. Following the disappearance of the indole

[3] In considering the possibility that reaction occurs by oxidation of the benzene ring, it is difficult to see how the carboxyl group could participate selectively in such a reaction. The most important consideration in ruling out this pathway, however, is the known preference of most reagents for the hetero ring (4).

peaks in the H_2O_2/H^+ system, a new maximum appears at 297 mμ. In the peroxidase system, on the other hand, the maximum is at 285 mμ. For a given quantity of substrate, the peaks at 248 and 254 mμ attain higher absorbancies in the peroxidase system than in the H_2O_2/H^+ system.

More marked are the differences between the spectra during the course of the reaction, as shown in Figures 1 and 2. The appearance of the peak at 254 mμ is almost instantaneous in the peroxide system, but much slower in the enzyme system, a clear separation showing only after 45 min. Although the peak at 254 does not appear as quickly in the peroxidase system, the general increase in absorption in the 240 to 260 mμ region is more rapid, so that the absorbancy of the enzyme system soon surpasses that of the peroxide system. In the indole region of the spectrum absorbancy falls off rapidly during the first hour in the enzyme system. The decrease is much slower in the peroxide system. These differences in the changing spectra of the two systems indicate that the final products, though similar, are formed by different mechanisms, as might be expected. The difference in the specificities of the two systems has already been pointed out.

Future work will be devoted to elucidation of the chemistry of the H_2O_2/H^+ system, which may enable us to predict the probable structure of the product or products of the reaction. It is hoped that this knowledge will permit us to select the proper conditions for isolation of the products and for their synthesis.

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The Synthetic Growth Regulants

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Some New Aspects of the Growth Regulating Effects of Phenoxy Compounds

The present study utilized 79 phenoxy derivatives, all of which were tested for their growth regulating effects on oat coleoptile cylinders, wheat roots and flax roots following previous methods (4). The data have been condensed in Table 1. Full details have been given for some of the substances by Åberg (6) and will follow for the others. In the present connection only the effects of certain especially interesting substances or series of substances will be illustrated by concentration-response curves and more detailed data for their interaction with other types of growth regulators.

Among the substances studied, most of which have been kindly supplied by Professor A. Fredga and Dr. M. Matell, are 17 pairs of optically active α-propionic acids. In order to save space the following basic abbreviations will be used: — POA: phenoxyacetic acid, $C_6H_5 \cdot O \cdot CH_2 \cdot COOH$; POP: α-(phenoxy)propionic acid; POB: α-(phenoxy)-*n*-butyric acid; POiB: α-(phenoxy)isobutyric acid; POV: α-(phenoxy)-*n*-valeric acid; POiV: α-(phenoxy)isovaleric acid; POC: α-(phenoxy)-*n*-caproic acid. Substituents of the phenyl ring will be indicated in the usual manner; 2,4-dichlorophenoxyacetic acid will thus be abbreviated 2,4-Cl_2POA (or shorter: 2,4-D). For the halogens the chemical symbols (F, Cl, Br, I) are used, but some other groups are further abbreviated in the following manner: Me: methyl, Et: ethyl, iP: isopropyl, tB: *tert*-butyl, $-C(CH_3)_3$, MeO: the methoxy group, $-OCH_3$, Ni: the nitro group, $-NO_2$.

The optical activity is usually indicated by the sign of rotation, dextrorotatory forms by (+) and laevorotatory forms by (—). The (+)-forms generally belong to the D series, with one notable excep-

Table 1. Growth effects of various phenoxy compounds. *

Substance	Oat Coleoptile		Wheat Root		Flax Root			Type of Activity	
	pC_I 20%	pC_S 40%	pC_S 20%	pC_I 50%	pC_I 50%	Effect of 1-NMSP	Effect on 2,4-D	Auxin	Anti-auxin
IA	–	6.8	–	7.7	8.2	++	–	+++	0
2-NMSeA	6.3†	–	7.7	–	4.7†	–	+++	0	+++
1-NMSP	4.7†	–	5.9	–	– †	–	+++	0	+++
2-NOA	6.0†	4.1	S	4.8	7.1	+++	–	++	+
1-NOA	5.5†	R	6.3	–	4.2	0	+++	(+)	+++
POA	I	S	5.4	3.2	3.4	+	+	+	++
(+)POP	–	5.5	–	4.9	5.9	+++	–	++	0
(–)POP	5.0	–	S	–	4.0	–	+	0	+
POiB	4.7	–	5.8	3.3	3.5†	–	++	0	+++
(+)POB	–	5.6	–	5.1	5.9	+++	–	++	0
(–)POB	4.9	–	S	–	4.1	–	+§	0	+
(+)POV	4.9	R	–	–	5.3	++	0	+	(+)
(–)POV	4.1	–	4.9	–	– †	–	++	0	++
rPOiV	5.0	–	–	–	3.6	–	++	0	+
(+)POC	4.1	–	5.1	–	–	–	++	0	++
(–)POC	<4	–	5.1	–	–	–	++	0	++
4-FPOA	–	5.4	–	4.2	6.1	++	–	++	0
(+)4-FPOP	–	5.4	–	4.9	6.0	+++	–	++	0
(–)4-FPOP	5.6	–	5.1	3.4	4.0†	–	0	0	+
2-ClPOA	5.3	3.6	S	4.0	5.0	+++	0	+	+
3-ClPOA	I	5.0	6.0	4.6	6.0	++	–	++	+
4-ClPOA	–	5.6	S	5.4	6.8	+++	–	+++	(+)
(+)4-ClPOP	–	5.9	–	5.4	6.5	+++	–	+++	0
(–)4-ClPOP	6.0	–	6.5	–	4.4	–	(+)	0	+
4-ClPOiB	6.0†	(R)	6.7	–	4.3†	–	+++	(+)	+++
2-BrPOA	5.6†	3.1	–	4.5	4.5	+++	+	+	(+)
3-BrPOA	–	5.6	S	4.8	6.4	++	–	++	(+)
4-BrPOA	6.5	4.8	S	5.0	6.4	+++	–	++	+
(+)4-BrPOP	I	5.3	S	5.2	6.6	+++	–	++	+
(–)4-BrPOP	6.5	–	7.0	–	4.5†	(+)	++	0	++
2-IPOA	5.1	S	–‖	4.6	4.2	–	(+)	+	+
(–)2-IPOP	–	5.4	–	5.7	6.0	+++	–	++	0
(+)2-IPOP	5.1†	(R)	S	–	5.0	+	++	(+)	+
3-IPOA	–	5.5	S	5.2	6.5	++	–	++	(+)
(+)3-IPOP	–	6.3	7.6	5.8	7.4	+++	–	+++	(+)
(–)3-IPOP	5.0†	R	6.4	–	5.1†	+	+	(+)	+
4-IPOA	6.0†	R	6.8	4.0	5.1	+	+++	(+)	+++
(+)4-IPOP	–	S	S	4.7	6.2	+++	–	+	+
(–)4-IPOP	6.6	(R)	7.3	–	– †	–	+++	0	+++
2,4-I_2POA	6.2	(R)	–	5.3	5.1	(+)	+	(+)	+
2-MePOA	6.2	3.7	–	5.0	5.4	+++	0	+	(+)
3-MePOA	5.6	S	6.0	–	4.7	++	+	+	+
4-MePOA	4.7	R	5.8	–	5.2	+++	+	(+)	+
(+)4-MePOP	6.0†	3.5	S	4.5	5.9	+++	–	+	+
(–)4-MePOP	5.8	–	5.8	–	– †	–	++	0	++
4-EtPOA	5.3†	(R)	6.3	–	4.5	+	++	(+)	++
4-iPPOA	4.9	–	5.8	–	3.7†	–	+++	0	+++
4-tBPOA	4.9	–	5.8	–	– †	–	+++	0	+++
2-MeOPOA	3.5	–	4.1	–	3.1	–	+	0	+
3-MeOPOA	5.0	3.8	4.9	–	4.8	++	0	+	+
4-MeOPOA	4.5	S	5.5	–	5.4	+++	0	+	+
2-NiPOA	(I)	(S)	5.4	–	–	–	++	0	++
3-NiPOA	(I)	4.3	4.5	–	4.2	–	0	++	+
4-NiPOA	3.7	–	4.9	–	3.5	–	+++	0	++

Table 1. Growth effects of various phenoxy compounds* (*continued*).

Substance	Oat Coleoptile		Wheat Root		Flax Root			Type of Activity	
	pC_I 20%	pC_S 40%	pC_S 20%	pC_I 50%	pC_I 50%	Effect of 1-NMSP	Effect on 2,4-D	Auxin	Anti-auxin
2,4-Cl_2PO-A	–	6.5	–	6.7	7.4	+++	–	+++	0
(+)P	–	6.4	–	6.7	7.5	+++	–	+++	0
(–)P	5.5†	–	6.9	–	4.9	+	+	0	++
3,4-Cl_2PO-A	I	6.1	7.0	5.3	7.2	++	–	++	+
(+)P	–	6.6	–	6.4	7.6	+++	–	+++	0
(–)P	6.2†	–	7.2	–	5.4	+	(+)	0	++
2,5-Cl_2PO-A	I	5.4	–	5.4	6.0	++	–	++	(+)
(+)P	–	6.3	–	6.2	7.3	+++	–	+++	0
(–)P	4.9†	–	–‖	–	5.0	(+)	0	0	(+)
2,3-Cl_2PO-A	5.1	R	6.2	4.1	4.5	0	+++	(+)	++
(+)P	6.1	S	–	4.5	6.5	+++	–	+	(+)
(–)P	5.3	(R)	S	4.1	5.0†	0	++	0	++
2,6-Cl_2PO-A	5.1	R	–	3.6	4.5	–	++	(+)	+
(+)P	5.5	S	–	4.6	5.6	+	0	+	(+)
(–)P	5.5	R	–	4.2	5.4	0	+++	(+)	++
3,5-Cl_2PO-A	4.3	–	6.3	–	4.2	–	+++	0	++
(+)P	I	R	7.0	–	5.4	+	+	+	+
(–)P	4.7	–	6.0	–	4.8	0	+++	0	++
iB	5.6	(R)	6.2	4.2	4.3†	–	+++	(+)	+++
2,4,5-Cl_3PO-A	–	6.5	–	6.1	6.6	+++	–	+++	0
(+)P	–	7.1	–	7.1	7.5	+++	–	+++	0
(–)P	5.6	–	6.9	4.3	5.0	(+)	++	0	++
2,4,6-Cl_3PO-A	4.5	–	5.0	–	4.1	–	+++	0	++
(+)P	4.9	–	6.5	–	5.1	0	+++	0	+++
(–)P	5.7	–	7.1	–	4.6†	–	+++	0	+++
2,4,5,6-Cl_4PO-									
(+)P	4.7	–	6.3	4.5	4.7	0	+++	0	+++
(–)P	5.3	–	6.6	5.1	4.6	–	+++	0	+++
2-iP,4-Cl,5-MePO-A	4.3	–	5.6	4.2	–†	–	+++	0	++
(+)P	5.8	(R)	5.9‡	4.7	4.3‖	–	+++	(+)‡	+++
(–)P	5.0	–	7.1	–	–†	–	+++	0	+++

* Stimulating (C_S) and inhibiting (C_I) concentrations (moles per liter) indicated as their negative logarithms ($pC_S = -\log C_S$). Effects not reaching the levels shown in the head of the table and some other information are given in the following way. *Oat coleoptile cylinder test*: **I** indicates that a significant inhibition occurs but does not reach 20 per cent. pC_I-values have only been given in cases in which no stimulation at low concentrations precedes the inhibition. Even typical auxins naturally cause a final inhibition at high concentrations.

† At a pC_I-value denotes that the inhibition is weakened or eliminated by the presence of IAA. but this has been tested only for a limited number of substances. **S** indicates that a significant stimulation occurs but does not reach 40 per cent. **R** indicates that some reversal of the initial inhibition occurs at higher concentrations, but that the growth rate does not reach the control level. *Wheat root test:* **S** indicates that a significant stimulation occurs but does not reach 20 per cent. All substances certainly give 50 per cent inhibition when applied in sufficiently high concentration. In the case of the antiauxins, this inhibition is presumably due to nonspecific toxicity, and pC_I-values below 4.5 have usually not been determined. *Flax root test:* † following a pC_I-value denotes that the inhibition is preceded by a significant stimulation at low concentrations. When the pC_I-values fall below 4.5 they have sometimes not been determined. The column "Effect of 1-NMSP" indicates the strength of the restorative effect of the antiauxin 1-NMSP on the growth of flax roots inhibited by the substance at issue, and the column "Effect on 2,4-D" the strength of the restorative effect of the substance at issue upon flax roots inhibited by 2,4-D. *Type of activity*: The method of characterization is schematical and semiquantitative. In cases where different tests indicate different types or degrees of activity, the formulation is the result of a compromise.

‡ The stimulation of wheat root growth is preceded by a strong inhibition at low concentrations (see Figure 4), which is possibly indicative of some type of auxin synergism.

§ The restorative effect is preceded by a synergistic one at lower concentrations.

‖ Action curve of unusual form, possibly indicating a synergistic component of the activity.

tion: (+)2-IPOP belongs to the L series (13, 14, 16, and personal communication from A. Fredga).

Some growth regulating chemicals, not belonging to the phenoxy group, will be abbreviated as follows: IAA: indole-3-acetic acid, 1-NMSP: α-(1-naphthylmethylthio)propionic acid. $1\text{-}C_{10}H_7 \cdot CH_2 \cdot S \cdot CH(CH_3) \cdot COOH$, 2-NMSeA: 2-naphthylmethylselenoacetic acid, 1- and 2-NOA: 1- and 2-naphthoxyacetic acid, respectively.

GENERAL CONSIDERATIONS

At present we are far from a detailed understanding of the auxin action (or actions). We know a large number of substances, all of which affect various growth processes in the same manner as does IAA, and this has led to the assumption of a common receptor in the auxin responses. Other substances, the structure of which is often very closely related to some auxin, cause growth effects of a different sign and also counteract the effect of externally applied auxin. These substances may be assumed to compete with the auxins at the receptor site, but they may also interfere with auxin uptake and transport, and with auxin metabolism. Nor should a direct effect at the receptor sites of a sign opposite to that of the auxins be excluded at the present stage of knowledge (5). Such substances have generally been called antiauxins, and for the present it seems best to retain this usage. The explanation of the antiauxin action is still largely hypothetical, and an antiauxin in the strict sense (19) could easily be specified by the prefix competitive.

In order to illustrate the growth effects of auxins and antiauxins as delimited in the present study, some results with the standard auxin IAA and with the antiauxin 2-NMSeA are shown in Figure 1. The most powerful antiauxin known to the present author, 2-NMSeA, strongly stimulates the growth of wheat seedling roots; it also stimulates the growth of flax roots but gives only growth depression when tested on oat coleoptile sections. The inhibition of flax root growth caused by the auxin 2,4-D is effectively restored by 2-NMSeA. In a series of 5 independent experiments, the growth in $10^{-7}M$ 2,4-D was 23.4, and in $10^{-7}M$ 2,4-D + $3 \times 10^{-6}M$ 2-NMSeA 67.0 per cent of the control growth. Also the growth of IAA-inhibited flax roots is restored, but to a somewhat less extent. On the other hand, the inhibition of coleoptile growth caused by 10^{-6} to $10^{-5}M$ 2-NMSeA is completely annihilated in the presence of $10^{-6}M$ IAA.

The situation is complicated, however, by the existence of substances intermediate between the typical auxins and the typical antiauxins (1, 4, 6, 8). These substances normally give an inhibition of oat coleoptile cylinder growth at low concentrations, which inhibition

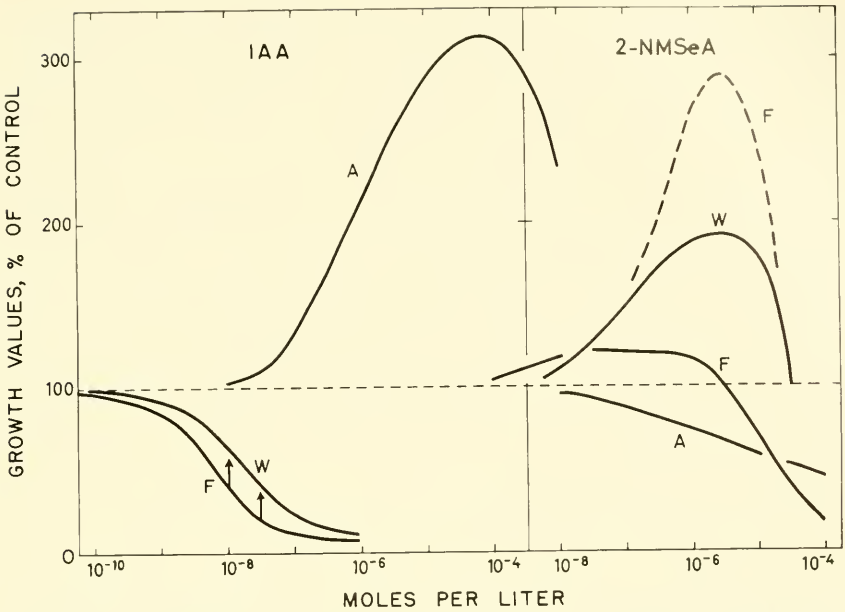

Fig. 1. Growth effects upon *Avena* coleoptile cylinders (**A**), wheat roots (**W**), and flax roots (**F**) of indole-3-acetic acid (IAA) and 2-naphthylmethylselenoacetic acid (2-NMSeA). Growth values expressed as per cent control. The dashed F-curve shows effect of 2-MNSeA on flax roots inhibited by 2,4-D (growth in $10^{-7}M$ 2,4-D alone is 100). Vertical arrows indicate growth restoration obtained by addition of $10^{-5}M$ α-(1-naphthylmethylthio)propionic acid (1-NMSP).

is eliminated by the presence of $10^{-6}M$ IAA. At higher concentrations the growth rises again and may reach a level considerably exceeding the control growth (Figure 2). To the initial inhibition of oat coleoptile cylinder growth corresponds an initial stimulation of wheat root growth. Flax root growth is inhibited, and the inhibition is usually restored to a less extent by the antiauxin 1-NMSP than is the inhibition caused by a typical auxin. The growth of 2,4-D-inhibited flax roots is usually stimulated.

As mentioned, a typical auxin usually causes a long series of growth responses, and a substance giving one of these responses can normally be expected to give also the other ones. The same is also valid for the typical antiauxins. The occurrence and strength of the auxin, or antiauxin, responses do not show an absolute connection, however, and for the intermediate substances the behavior is sometimes irregular. No stimulation of wheat root growth could thus be detected for 2-BrPOA and 2-MePOA in spite of their clearly intermediate character in the oat cylinder test.

Apparently such irregularities may to some extent be caused by

differences in regulator uptake and translocation, by effects on auxin metabolism, on other metabolic processes, and so on, but species differences in the auxin system may also be at work (4, 11). Assuming an attachment to a protein receptor by many weak bonds as the initial phase in auxin action, the additional hypothesis of slight differences in the structure of the receptor in different species and organs seems fairly natural. It would also be in good keeping with known facts about organ and species specificity of isodynamic enzymes (15). Minor differences in the adsorption areas of enzymes of different origin may result in catalysis of the same reaction of the same substrate with very different rates, and may bring about a different relative, or even absolute, specificity toward some substrates. The assumption of a cooperation of several similar, but not identical, auxin receptors in the same system (17) is a further step in the same direction that may facilitate the interpretation of the synergistic phenomena and also of the behavior of the intermediate regulators.

In the way now outlined, the basic uniformity of auxin regulation of growth is understood as well as the existence of many deviations from the common scheme. The connection between the primary auxin action and the growth responses of different type remains enigmatical, however.

THE INTERMEDIATE REGULATORS

The existence of intermediate regulators can be inferred already from their interactions with typical auxins and antiauxins (1, 3), but further substantial support comes from their composite concentration-response curves which are particularly instructive in the case of oat coleoptile cylinders. With increasing concentration the growth is at first inhibited, but then the inhibition subsides and may give place to a quite considerable stimulation (Figure 2). The antiauxin nature of the initial inhibition is indicated by the occurrence of a corresponding initial stimulation of wheat root growth, and also by its disappearance in the presence of IAA.

It is very interesting to observe the occurrence of intermediate regulators in series of substances, in which a gradual shift in the chemical structure causes a shift from auxin to antiauxin character. One series of this type is represented by the phenoxyacetic acids with *para*-substituents of growing size (6). In 4-BrPOA the intermediate character is quite pronounced, in 4-MePOA, 4-IPOA, and 4-EtPOA the auxin component is gradually lessened, and, with 4-iP-, 4-Ni-, and 4-tB-POA substances with pure or almost pure antiauxin activity are reached (Figure 3). Another example is given by the homologous (+)-α-phenoxyalkylcarboxylic acids (Table 1, Figure 3). The pro-

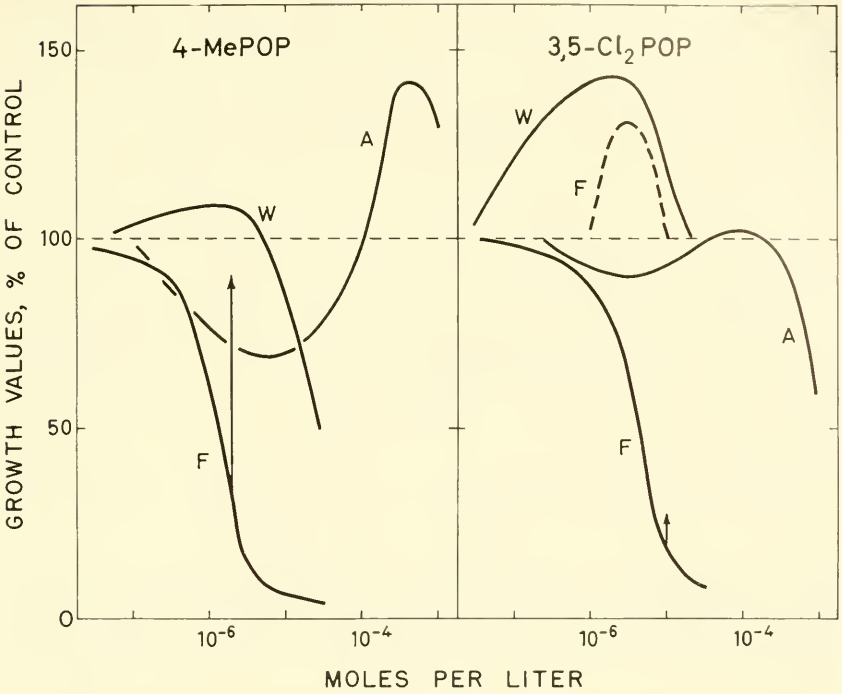

Fig. 2. Growth effects upon *Avena* coleoptile cylinders (**A**), wheat roots (**W**), and flax roots (**F**) of (+)α-(4-methylphenoxy)propionic acid (4-MePOP) and (+)α-(3, 5-dichlorophenoxy)propionic acid. Data presented as in Figure 1.

pionic and butyric acids are auxins of similar strength, the valeric acid shows intermediate character (at least in the oat cylinder test), while only antiauxin properties are revealed for the caproic acid.

The presence of an auxin component in the activity of intermediate substances like 2-naphthoxyacetic acid (4) or 4-BrPOA can hardly be disputed. In view of the gradual transition from such substances to those for which the restoration of oat coleoptile growth at higher concentrations remains well below the control level (e.g., 4-MePOA, Figure 3), the author does not hesitate to accept even a slight tendency to such a restoration as a sign of weak auxin activity. Such signs are often paralleled by indications of auxin activity in the flax root test. It should also be noted that the tendency to growth restoration at high concentrations of 4-MePOA is changed to a real stimulation for the nearly related substance (+)4-MePOP (Figures 2 and 3).

The quantitative elucidation of the action of intermediate substances along the lines used for the pure auxins and antiauxins meets with difficulties. It seems probable, however, that these can be obvi-

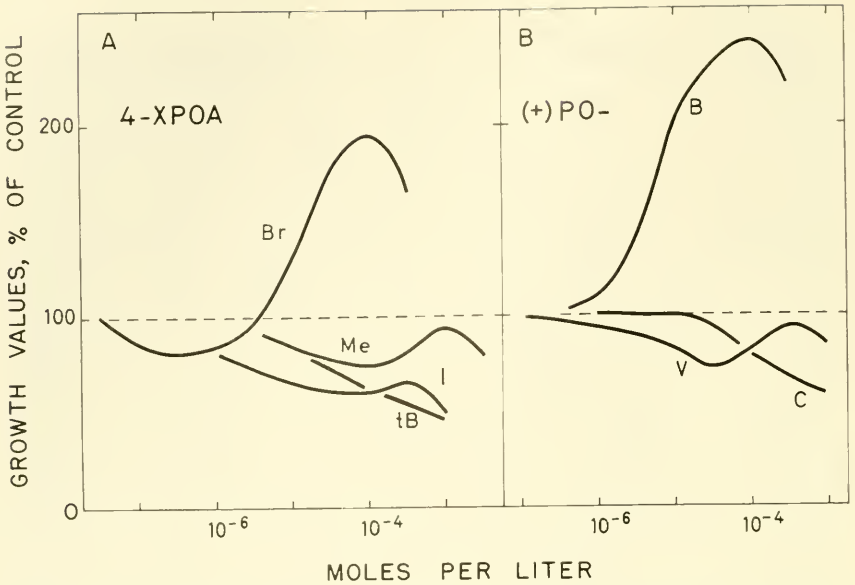

Fig. 3. Growth effects on *Avena* coleoptile of: **A**. Some phenoxyacetic acids with different *para*-substituents (X), i.e. bromo (Br), methyl (Me), iodo (I), and tertiary butyl (tB). **B**. Some (+)phenoxyalkylcarboxylic acids, i.e. α-substituted *n*-butyric acid (B), *n*-valeric acid (V), and *n*-caproic acid (C). Data presented as in Figure 1.

ated by the assumption of the cooperation of several similar, but not identical, auxin receptors.

An alternative way would be to assume independent systems for auxin and antiauxin actions, but it seems that such an hypothesis generates more problems than it solves. If the initial inhibition of oat coleoptile cylinders is not related to the auxin system, it is difficult to understand why it should be annihilated by the presence of IAA. The final stimulation, on the other hand, corresponds to an inhibition of flax root growth which is relieved by antiauxin application, and nothing is as yet known which makes it possible to differentiate this phase of the action from an ordinary auxin effect.

THE EFFECTS OF SUBSTITUENTS IN DIFFERENT POSITIONS

The effect of *para*-substitution has been discussed earlier (4, 6). 4-F- and 4-ClPOA show a considerably augmented auxin activity compared to the unsubstituted POA. With increasing size of the *para*-substituent a change in the antiauxin direction takes place and is complete with 4-iP-POA and 4-NiPOA. In the *meta*-substituted POA series a conspicuous antiauxin component of the activity is apparent already for 3-ClPOA, but, on the other hand, a strong auxin

component is still present in 3-IPOA and 3-NiPOA. It is suggested that the *meta* compounds and, for example, 3,4-Cl_2POA are closely related to 2-NOA. The *ortho*-substituted compounds generally show a fairly weak activity with both auxin and antiauxin components. In 2,3-Cl_2POA the antiauxin component is fairly strong, and it is suggested that this substance is closely related to 1-NOA. The lack of stimulating effects on wheat root growth observed for 2-ClPOA reappears for 2,6-Cl_2POA, which may otherwise be characterized as an intermediate growth regulator. The presence of an auxin component in its activity is further indicated by the positive effects obtained in the pea curvature test (18). In 3,5-Cl_2POA the antiauxin component is wholly prevailing, but the appearance of a clear auxin component in (+)3,5-Cl_2POP Figure 2 shows that the presence of two *meta* substituents is not wholly incompatible with auxin activity. The racemic 3,5-Cl_2POP shows very weak activity in the pea curvature test according to Toothill *et al.* (18), but these authors hold that side-chain substitution has a negligible effect on 3,5-derivatives. It seems to be an interesting task to study further how far 3,5-Cl_2POA is related to phenoxyacetic acids with large *para*-substituents. The induction of auxin activity by further substitution in the ring of 3,5-Cl_2POA (18), however, seems to indicate that simple dimensional factors can hardly be at work.

Rather unexpectedly, 2,4,6-Cl_3POA shows a purer antiauxin character than does 2,6-Cl_2POA, and both α-propionic acids are stronger antiauxins than the acetic acid (Figure 4B). As the racemic 2,4,6-Cl_3POP is reported to show clear auxin activity in the pea curvature test (12, 18), this is apparently not true for all types of tests. The thymoxyacetic acid (2-iP, 4-Cl, 5-MePOA) exerts only antiauxin effects, presumably due to the large *ortho*-substituent. The corresponding (+) propionic acid, however, is a regulator with quite unusual properties (Figure 4A). Wheat roots are strongly inhibited at $10^{-7}M$, but at 10^{-6} to $3 \times 10^{-6}M$ the inhibition has changed into a conspicuous stimulation. It is possible that this type of concentration-response curve can be related to that of 2,3,5-triiodobenzoic acid (TIBA) (7), but no stimulation in the oat cylinder test corresponding to that obtained with TIBA is apparent. A response of wheat roots similar to that for (+)2-iP, 4-Cl, 5-MePOP has been observed for (+)α-(2-naphthoxy)-*n*-valeric acid which belongs to the intermediate growth regulators (unpublished data).

THE EFFECTS OF OPTICAL ACTIVITY

The effect of optical isomerism in plant growth regulators is not absolute, but is related to the molecular structure as a whole. The slightest effect is found in the α-(indole-3)propionic acid, and the

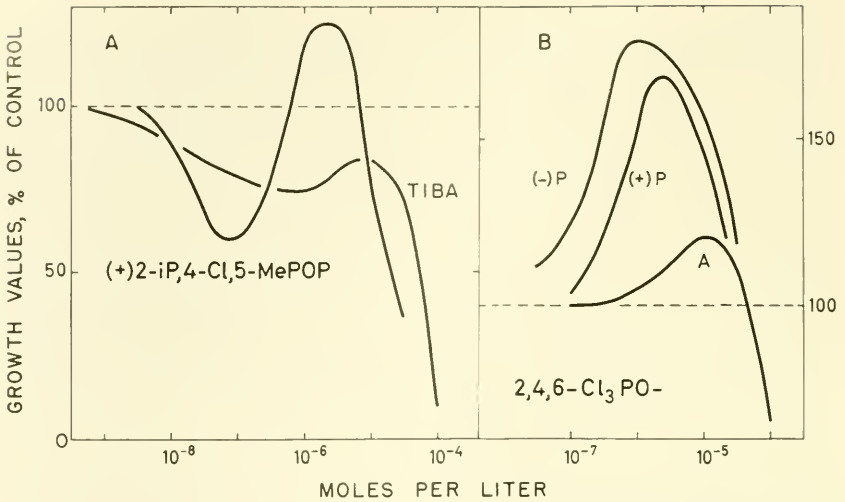

Fig. 4. Growth effects on wheat roots of: **A.** (+)Chlorothymoxypropionic acid [(+)2-IP,4-Cl,5-MePOP] and triiodobenzoic acid (TIBA); **B.** Of 2,4,6-trichlorophenoxyacetic acid (2,4,6-Cl_3POA) and the corresponding propionic acids, i.e., dextrorotatory propionic acid [(+)P], and laevorotatory [(—)P]. Data presented as in Figure 1.

strongest effects among the phenoxy- and naphthoxy-propionic acids (see 8), where the change from D to L form does often result in a complete change from auxin to antiauxin activity. It is of interest that such effects are also found among the structurally unique thiocarbamic acid derivatives (unpublished data).

The usually strong effect of optical isomerism among the phenoxy derivatives is strongly weakened or almost completely abolished either by the insertion of a large substituent in the α-position in the side chain (e.g., POC) or by some types of nuclear substitution (e.g., 2,4,6-Cl_3POP, 2,4,5,6-Cl_4POP). For 2-iP, 4-Cl, and 5-MePOP the situation is complicated by the occurrence of an unusual, possibly synergistic, component in the activity of the (+)-form.

In the case of the 2,4,6-Cl_3-phenoxy compounds (Figure 4), both propionic acids show a strongly increased stimulating effect on wheat root growth in comparison with the acetic acid, which might indicate that the introduction of an α-methyl group in the side chain compensates for an otherwise weak affinity to the receptor sites of this material.

The D-propionic acids related to acetic acids of intermediate character often show stronger and purer auxin activity than the corresponding acetic acids [e.g., (+)POP, (+)4-IPOP, (+)2,3-Cl_2POP]. Weak auxin activity may appear even if the acetic acid is purely anti-

auxinic [e.g., (+)3,5-Cl_2POP] and an already strong auxin activity can be considerably strengthened [e.g., (+)2,4,5-Cl_3POP]. This points to an active role of the D methyl group in the attachment of the regulator to the receptor site and in its function (2). Such a situation is not universal, however, as the D forms of the α-naphthylpropionic acids have weaker auxin components in their activity than the corresponding acetic acids (9).

For all phenoxyacetic acids possessing clear auxin character the introduction of an α-methyl group in the L position results in a pronounced change in the antiauxin direction. In other cases the effect is weaker and less clear.

Unfortunately, only a few of the α-isobutyric acid derivatives have been included in the present study. POiB shows a rather pure antiauxin activity, while slight traces of auxin activity are possibly present in 4-ClPOiB and 3,5-Cl_2POiB as judged from the type of concentration-response curves in the oat cylinder test. This may be seen in connection with the occurrence of very slight activity of 4-ClPOiB also in the pea curvature test, and with the rather conspicuous activity of 2,4,5-Cl_3POiB both in the wheat cylinder and the pea curvature tests (12). This latter case is especially interesting, as the corresponding (—)-propionic acid shows a rather pure antiauxin character. Both α-methyl groups may perhaps influence the binding and position of the side chain, and in 2,4,5-Cl_3POiB the influence of the group in the D position would predominate. The presence of an auxin component in the activity of this substance is analogous with its presence in α-(2-naphthoxy)isobutyric acid (10, and unpublished data). Its presence in α-(indole-3)isobutyric acid, on the other hand, represents another situation, as in this case both corresponding α-propionic acids show strong auxin activity (8).

SUMMARY

It was found that a rather large number of the studied phenoxy compounds cannot simply be classified as auxins or antiauxins, but show intermediate character. In discussing the relation between chemical structure and physiological activity this must be appreciated, and the existence of the intermediate regulators might also be able to shed some light on the difficult question of the mechanism of auxin action.

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DISCUSSION

Dr. Wain: Dr. Wightman showed very striking competitive antagonism effects with one stereoisomer against another. The main hypothesis underlying this work was that the ring system anchors the auxin down to some surface at what you might call the primary site of action. Therefore, compounds of similar structure with the same ring system might very well anchor down without producing the growth response. Very striking effects on competitive antagonism were shown in this work both in the wheat cylinder elongation test and pea curvature test.

Dr. Hansch: I'd like to comment on this paper with respect to 2-point attachment in a somewhat opposite fashion from what Dr. Wain has done. It seems to us that the data can be explained rather nicely in terms of the 2-point contact idea. Take for example the 4-methylphenoxyacetic acid which is very weakly active as an auxin, and compare this end with the 4-bromo compound. Dr. Wain suggested that the first thing was attachment of the ring and then this prevents a further more active ring from attaching. If you look at it the other way around — first, attachment of the carboxyl by rapid reactions of amidization or salt formation, then the inability of the ring to react as fast, say, in the 4-methyl as it would in the 4-bromo — this can be explained very nicely for the whole series of compounds on the basis that the ring is less electron-rich. Electron-poor rings with halogens would latch down more tightly so that groups like methyl and iodo would give antiauxins. These compounds would be attached first by the carboxyl, but you would get the second-point attachment to the ring only very slowly as, with phenoxyacetic acid. Now, then, if you put IAA in the system, the carboxyls attach at about the same rate. But the phenoxyacetic acid would not permit IAA to come in as fast as it normally would for 2-point attachment.

Dr. Wain: Just one word on that. I was not doing Dr. Wightman justice in those very few remarks. He did dissect out all these factors and studied a wide range of compounds which could produce what we thought were the specific groupings. The significant thing is that these acids which do not have this highly active ring system are not competitive antagonists at all. He thinks that palmitic acid or any of the aliphatic acids which can provide the carboxyl group are not attached. The point is, that in relation to Dr. Åberg's paper I think it is possibly a little dangerous to use, as antagonists, compounds which themselves possess auxin activity. The bromophenoxyacetic acid has positive activity and it can at best be only in the intermediate range.

Dr. Nitsch: I have been very much interested by the S-shaped curves Dr. Åberg showed us. They reminded me of some work done by McRae and collaborators with 2,4-D (D. H. McRae, R. J. Foster, and J. Bonner. Kinetics of auxin interaction. Plant Physiol. 28: 343-355, 1953) and by Barlow *et al.* (H. W. B. Barlow, C. R. Hancock, and H. J. Lacey. Studies on extension growth in coleoptile sections. I. The influence of age of coleoptile upon the response of sections to IAA. Ann. Bot., N. S. 21: 257–271, 1957). McRae *et al.* observed that the addition of very low concentrations of 2,4-D in the presence of low concentrations of IAA reduced growth below that obtained with IAA alone. Barlow *et al.* showed that low concentrations of IAA have a depressive effect upon the elongation of oat coleoptile

sections as compared with the water controls. We have observed the same phenomenon with oat first internodes. At very low concentrations, IAA depresses the elongation of the sections below the value obtained in buffer plus sucrose alone. The effect is small, and sometimes hard to justify statistically. However it does occur time and again. In fact it is the factor which prevented us from increasing the sensitivity of the first internode test beyond 0.3 to 1 μg. of IAA per liter.

Dr. Åberg: As yet I have not observed the inhibition described by Dr. Nitsch for *Avena* coleoptile sections grown in very low concentrations of IAA, but further experiments will be made. An explanation is not easily given, but I want to point to the negative after-effects of IAA applications which have repeatedly been found (Åberg, Ann. Rev. Plant Physiol. 8: 168, 1957; Osborne, Plant Physiol. 33: 46–57, 1958). If the IAA amount is very low, it may rapidly disappear from the medium, and the negative after-effect may come to the fore. Possibly the final growth result might then indicate an inhibition. This tentative explanation seems to gain considerable support from the data of Barlow, Hancock, and Lacey (Ann. Bot. 21: 257–271, 1957) which show that a low IAA concentration (0.01 p.p.m.) may stimulate the growth of wheat coleoptile sections during the first hours, even if the final result after 20 hrs. is a slight growth inhibition.

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A New Physiological Approach to the Selective Action of 2,4-Dichlorophenoxyacetic Acid

At the conference in 1955, a preliminary account (1) was given of the first experiments on the uptake of growth substances, particularly 2,4-dichlorophenoxyacetic acid (2,4-D) by the aquatic plant *Lemna minor*. Subsequently the investigations were extended and the results have been described in a more recent paper (4). Since the present study stemmed from this work, it is necessary to recapitulate some of the main findings.

When *L. minor* is grown under conditions of constant light and temperature and 2,4-D, labeled with carbon-14 in the carboxyl group, is added to the culture solution, there is an initial phase of rapid uptake in the first 30 min., followed by a second phase when the rate decreases progressively to zero after 2 to 3 hrs., and finally by a third phase, extending up to 24 hrs., when there is a net loss of activity from the tissues. This loss of carbon-14 from the plants is not due to decarboxylation and the escape of $C^{14}O_2$ but to the egress of 2,4-D into the external solution. This is not a toxic effect, since many of the combinations of concentration and length of exposure investigated either have no effect on, or significantly increase, the growth rate when plants are subsequently placed in culture solution.

If plants, after exposure to the labeled compound for 30 to 60 min., are transferred to culture solution, then within 4.5 hrs. over 90 per cent of the labeled 2,4-D originally present in the tissues is found in the external medium, but if this medium contains unlabeled 2,4-D, the rate of loss is retarded. The outward movement is not affected by the external pH, is slowed down but not arrested at 1.25° C., and up to 22.5° C. the Q_{10} is 1.6 to 1.9. Turning to the initial phase of uptake,

the rate is closely linked with the external concentration, is highly sensitive to the pH of the medium, and between 7.5 and 30° C. the Q_{10} is 2.3 to 2.6.

At the time these investigations were being completed, papers by other workers on the uptake of 2,4-D by segments of *Avena* coleoptiles (5) and *Chlorella* (7) demonstrated that the patterns of uptake are strikingly different. In *Avena* coleoptiles after an initial high rate of uptake there is a continued steady accumulation with no suggestion of a change from a positive to negative rate. Again, when coleoptiles or *Chlorella* after treatment with labeled 2,4-D are transferred to culture solution, there is no marked loss to the solution, while the amount lost is enhanced if unlabeled 2,4-D has been added to the external solution. Although these trends were similar, there were also divergences. For *Chlorella* the magnitude of the changes in the initial rate of absorption induced by an alteration in pH indicated that entry was largely in the molecular form, but for *Avena* the influence of pH was far less.

The evidence of these investigations clearly established that the external and internal factors governing the course of uptake of 2,4-D were dependent on the tissue under examination, and the questions that came to mind were the extent to which these differences were due to experimental conditions, the nature of the tissue, or the specific physiological differences at cell level. Previous investigations (2) had demonstrated that in terms of the concentration of 2,4-D causing phytotoxic effects *L. minor* must be regarded as a susceptible plant, and it seemed of high significance that *Avena* from an agronomic point of view is a resistant species. To test the hypothesis that resistance and susceptibility are linked with the physiological processes underlying absorption, the first approach has been to examine uptake through the roots of intact plants of selected species known in agricultural practice to have a wide range of tolerance to 2,4-D. The next step has been to establish whether the observed specific differences in root uptake also held for stem tissue. Lastly, some account is given of the results so far obtained in the analysis of the specific variation in the mechanisms involved.

UPTAKE OF 2,4-DICHLOROPHENOXYACETIC ACID BY THE ROOTS OF INTACT PLANTS OF DIFFERENT SPECIES

Seeds of the individual species, after being allowed to germinate in moist sterile sand, were transferred to a modified Steinberg (2) culture solution. The solution was adjusted to pH 5.6, continuously aerated, and maintained at 25° C. The plants were illuminated with an intensity of 750 foot candles for 18 hrs. a day by a bank of daylight

fluorescent tubes. At the end of 6 to 8 days, matched plants in pairs were transferred to a series of modified specimen tubes, each of which contained an aerating device which prevented splashing but ensured adequate stirring of the same culture solution (pH 5.6) containing 1 mg/l of 2,4-D labeled in the carboxyl group with carbon-14. For the experiment the tubes were kept at 25° C. in a water bath, and the shoots were continuously illuminated at an intensity of 500 foot candles. Replicated samples were withdrawn at 0.5, 1, 2, 4, 8, 16, and 32 hrs., the roots washed twice for a few seconds each in a large volume of distilled water, the surplus water removed by blotting between filter papers, the plants divided into root and shoot, and the remains of the seed or the cotyledons separated. The parts were dried at 90° C. and then assayed separately. The method of assay involved liquid combustion and the conversion of the carbon dioxide to barium carbonate which was filtered onto a disk of filter paper. The disk was then secured in a planchette by a brass ring and counted under constant geometry with an end-window Geiger counter (4).

As examples of the course of uptake exhibited by graminaceous species, acknowledged agronomically to be resistant to 2,4-D, four species have been included in Figure 1, namely *Triticum vulgare,* 'Eclipse'; *Hordeum vulgare,* 'Proctor'; *Avena sativa,* 'Victory'; and *Oryza sativa,* 'Heenati 3224.'

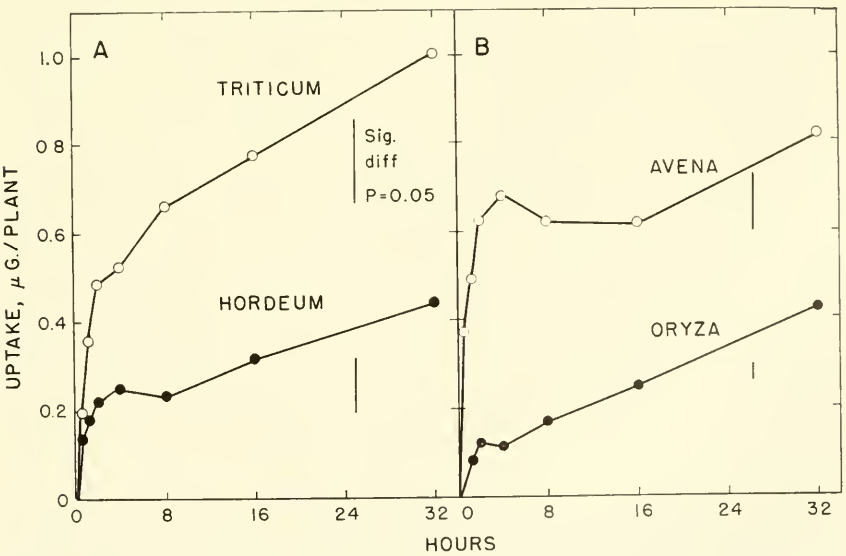

Fig. 1. The course of uptake of 2,4-D from a solution containing 1 mg/l by roots of intact plants of *T. vulgare, H. vulgare, A. sativa,* and *O. sativa.* The significant differences given in this and subsequent figures refer to the differences between any two treatments.

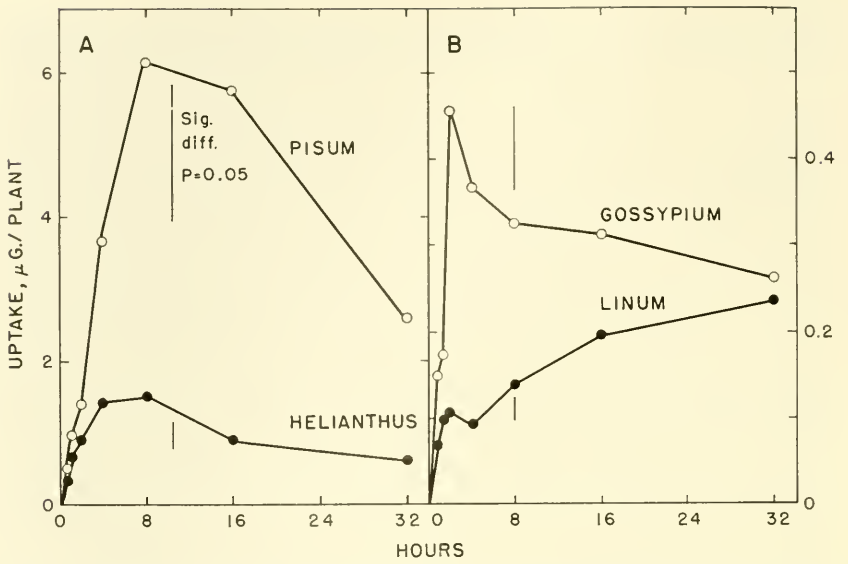

Fig. 2. The course of uptake of 2,4-D from a solution containing 1 mg/l by the roots of intact plants of *P. sativum, H. annuus, G. hirsutum,* and *L. usitatissimum.*

It is apparent that the basic pattern is the same: The plants continue to accumulate 2,4-D over the 32 hrs. For each species absorption is most rapid in the first hour, but the subsequent fall in the rate is dependent upon the species. With *Hordeum* and *Avena* there is a second phase when uptake comes to a stop, followed by a third phase when it recommences. There is some suggestion that this interruption of absorption may also take place with *Oryza.*

Figure 2 contains the results for four dicotyledonous species: *Pisum sativum,* 'Alaska'; *Helianthus annuus,* 'Pole Star'; *Gossypium hirsutum,* 'Samarus 26J'; and *Linum usitatissimum,* 'Royal.' The first three species are all well known to be killed or severely injured by field applications of 2,4-D which are innocuous to the four species of Figure 1, while 2,4-D, as long as the dose is limited, is widely employed in the United States for selective weed control in *Linum.* It is therefore noteworthy that the course of uptake of the one resistant species is quite different, following closely the pattern of *Hordeum* and *Oryza.*

The pattern of the three susceptible species only varies in the time that elapses before uptake changes from a positive to a negative rate. In parentheses it should be recorded that in the case of *Brassica alba,* another susceptible species, uptake ceases within 4 hrs., but there is no subsequent egress of 2,4-D into the external solution.

It might be advanced that since the concentration of 2,4-D in the external solution was only 1 mg/l, the difference between susceptible and resistant species would not be maintained if the concentration were increased. This aspect will be more fully explored in the next section. At present it is sufficient to add that when the concentration is raised by fivefold, the pattern of uptake for *Hordeum* is in no way altered.

UPTAKE OF 2,4-DICHLOROPHENOXYACETIC ACID BY STEM SEGMENTS OF DIFFERENT SPECIES

In the studies of uptake by stem tissues the procedures of obtaining suitable segments followed closely those employed in the standard extension growth tests, i.e., *Avena* coleoptile, wheat cylinder, and pea internode, using the varieties noted above. The only departure was that the first leaf was not removed from the segments of *Avena* coleoptiles. For other species, such as *Gossypium* and *Helianthus,* the plants were also raised in sterile sand in the dark at 24.5° C. and segments (10 mm.) excised from just below the cotyledons 6 days after the plants were sown.

Batches of segments selected at random were placed individually in a series of glass tubes immersed in a water bath maintained at 25° C. These tubes contained the same culture solution (adjusted to pH 5.1) as that used in the root studies. To ensure an adequate oxygen supply and effective stirring, the solutions were vigorously aerated with water-saturated air. At each sampling occasion the segments were washed twice for a few seconds each in distilled water, blotted, weighed on a torsion balance, dried at 90° C., reweighed, and the carbon-14 assayed by the methods already described.

It seemed of interest to investigate to what extent any specific differences in the course of uptake are dependent on the concentration of 2,4-D, more particularly when in terms of extension growth the concentrations are suboptimal, optimal, and supraoptimal. On the basis of unpublished work by S. Novoa in this department, it was apparent that for several of these species the optimal concentration lay within the range of 2 to 9 mg/l, and to allow for effective comparison, the concentrations of labeled 2,4-D were standardized at 0.5, 3, and 45 mg/l.

The results for *Avena* and *Triticum* are given in Figure 3. At each concentration the *Avena* segments continue to accumulate 2,4-D throughout the 24 hrs. It will be observed that irrespective of concentration the tissues increase in fresh weight and that though the lowest and highest concentrations are clearly suboptimal and supra-

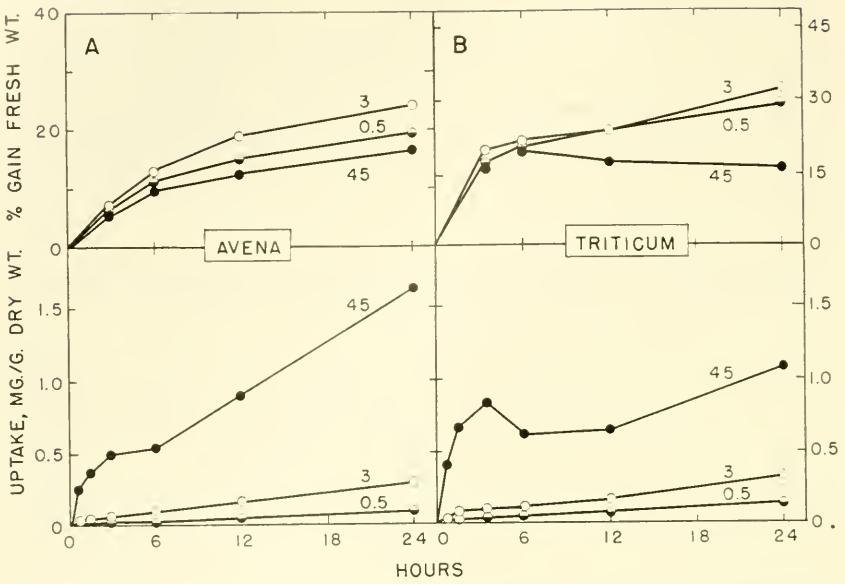

Fig. 3. The course of uptake of 2,4-D and the gain in fresh weight by coleoptile segments of (A) *A. sativa* and (B) *T. vulgare,* treated with concentrations of 0.5, 3, and 45 mg/l.

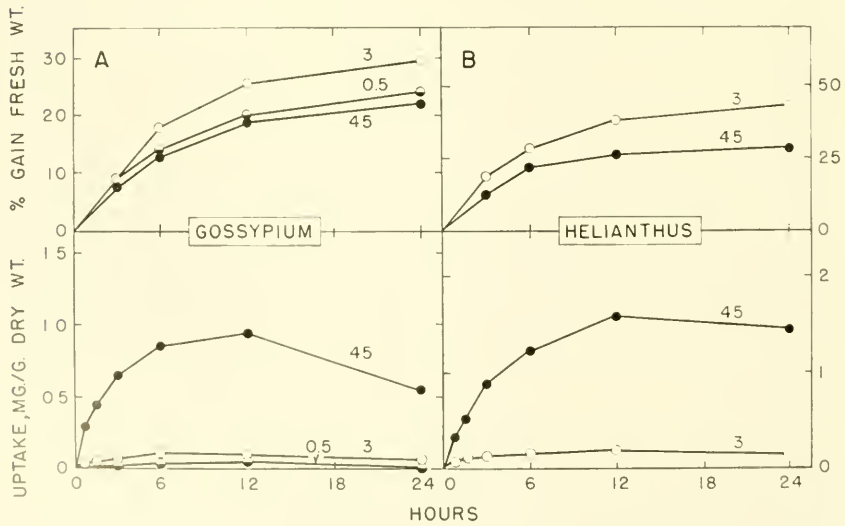

Fig. 4. The course of uptake of 2,4-D and the gain in fresh weight by etiolated stem segments of (A) *G. hirsutum* and (B) *H. annuus,* treated with concentrations of 0.5, 3, and 45 mg/l.

optimal, the trends for uptake are similar. For *Triticum* at 0.5 and 3 mg/l there is again a steady accumulation; at 45 mg/l between 3 and 6 hrs., 2,4-D is lost from the tissues, but subsequently uptake recommences. From the fresh-weight data it is to be observed that after being subjected for 6 hrs. to the highest concentration the tissues start to lose water, and though the negative rate of uptake between 3 and 6 hrs. could reasonably be ascribed to the onset of these conditions, it is somewhat surprising that uptake should proceed at an appreciable rate when water is being lost from the tissues.

Comparison of Figure 3 with Figure 4 reveals that just as in the case of uptake by the roots the pattern of uptake for *Gossypium* and *Helianthus* segments is strikingly divergent from that of *Avena* and *Triticum*. There are the three characteristic phases: an initial high rate of uptake, a period when the rate falls to zero at *ca.* 12 hrs., and a third phase when there is a loss from the tissues. Though the changes are most evident at 45 mg/l, statistical analysis shows that for both species the losses between 12 and 24 hrs. at the lower concentrations are significant. It should also be noted that at each concentration there is a progressive gain in fresh weight over the 24 hrs. For *Gossypium* the concentration range is from sub- to supraoptimal.

Supporting evidence that other susceptible species can be characterized by the pattern of uptake is provided in Figure 5. For both

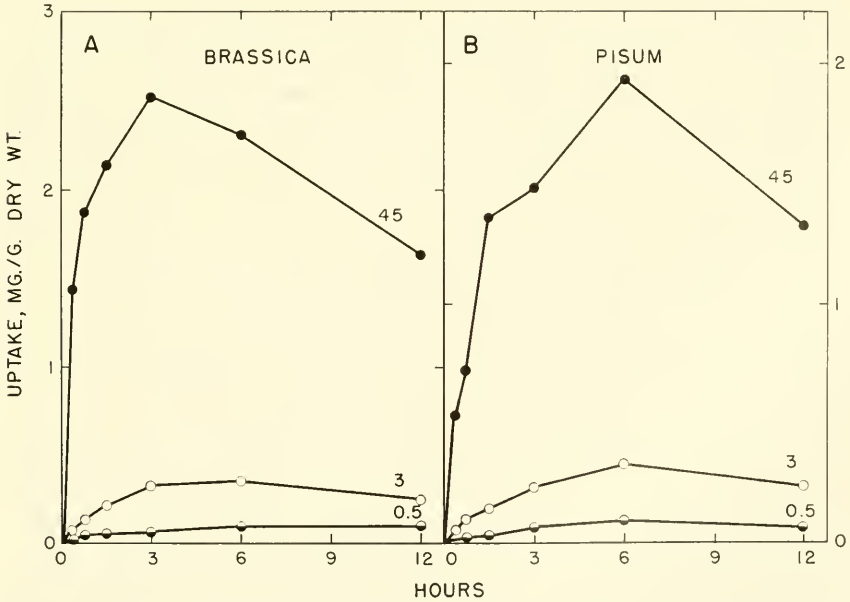

Fig. 5. The course of uptake of 2,4-D by etiolated stem segments of (**A**) *B. alba* and (**B**) *P. sativum*, treated with concentrations of 0.5, 3, and 45 mg/l.

Brassica and *Pisum* the rates of absorption change with time from a positive to a negative value. With the *Brassica* segments there is an interaction between the negative rate and the external concentration. At 45 and 3 mg/l the loss between 6 and 12 hrs. is significant, but there is no loss at 0.5 mg/l. In contrast, the losses from segments of pea stem are all significant.

SPECIFIC DIFFERENCES IN THE MECHANISM CONTROLLING THE UPTAKE OF 2,4-DICHLOROPHENOXYACETIC ACID

In considering the possible physiological factors which might account for specific differences in the course of uptake, there were clearly many experimental approaches. In the light of previous studies on *L. minor*, it was evident that the influences of temperature and external concentration on the rates of entry and egress would be of interest, while on the basis of the investigations of Johnson and Bonner (5) the effects of enzyme inhibitors ought also to be assessed. In weighing up the most likely point of attack, it was recalled (4) that the pattern of uptake of phenoxyacetic acid (POA) by *L. minor* was quite unlike that of 2,4-D since there was a continuous accumulation over the 24 hrs.; indeed, the general trend was akin to that of the uptake of 2,4-D by a resistant species, such as *Avena*. If for *L. minor* the variations in the pattern of uptake brought about by alterations in chemical structure are linked with shifts in the pathways of absorption and changes in the nature of the reactive sites, it could be further postulated that the specific differences in the course of uptake of 2,4-D are due to the same basic causes. Thus, where there is a continuous accumulation, e.g., *Avena*, the sites are unaffected or do not become affected as the auxin accumulates; that is, they are like those in *L. minor* which permit the accumulation of POA. In contrast, in a susceptible species, such as *Gossypium*, at least a proportion of the sites are different in the sense that they do not react similarly with POA and 2,4-D. On these postulates it could be further advanced that when POA is added to the external solution, its competitive influence on the uptake of 2,4-D would be greater for a resistant than a susceptible species.

To test this hypothesis the first experiment aimed at determining the influence of pretreatment with POA on the subsequent uptake of 2,4-D by segments of *Avena* coleoptiles and *Gossypium* stem, employing the procedures described in the previous section. Preliminary experiments demonstrated that in terms of gain in fresh weight the optimal concentrations of POA lay between 200 and 400 mg/l. Accordingly, some segments were immersed in buffer solution contain-

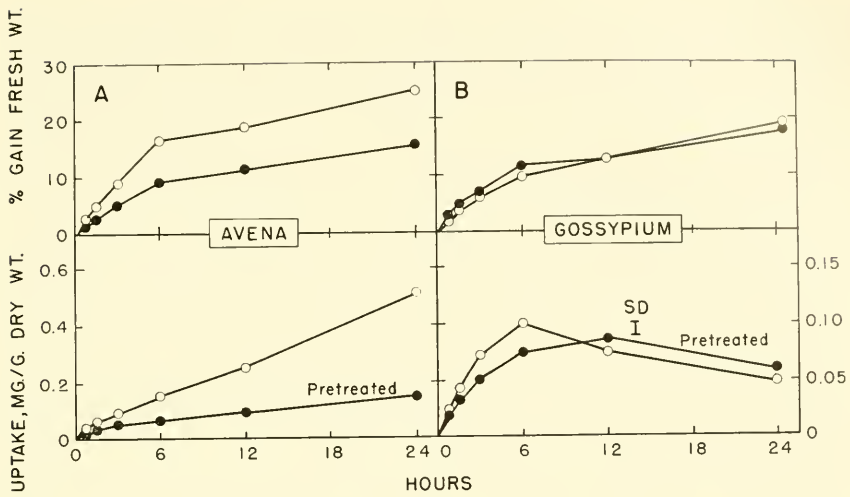

Fig. 6. The influence of pretreatment (filled in circles) with phenoxyacetic acid on the subsequent course of uptake of 2,4-D and the gain in fresh weight by segments of (**A**) *A. sativa* coleoptile and (**B**) *G. hirsutum* stem.

ing 300 mg/l of POA for 3 hrs. before they were transferred to a solution containing 3 mg/l of labeled 2,4-D plus the POA, while others received no pretreatment but were put directly into 3 mg/l of 2,4-D.

From Figure 6 there is no doubt that there is a major specific difference in the influence of POA on the absorption of 2,4-D. For *Avena* the reduction attains 72 per cent at the end of 24 hrs. In contrast, the pattern of uptake by *Gossypium* is not materially altered by POA, save that the time when the rate becomes negative is shifted from 6 to 12 hrs.

Table 1. The uptake of 2,4-dichlorophenoxyacetic acid by segments of *Avena* coleoptile and *Gossypium* stem in the presence and absence of phenoxyacetic acid (POA).

Species	Concn. of POA, Mg/L	Uptake of 2,4-D, μg/G Dry Wt.	
		3 Hrs.	9 Hrs.
Avena	0	59	135
	150	45	72
Gossypium	0	53	91
	150	52	76
Sign. diff. (P = 0.05)		9	

The next step was to investigate the competitive influence of POA without pretreatment. Table 1 gives the results of an experiment in which the uptake of 2,4-D (3 mg/l) in the presence and absence of POA (150 mg/l) was recorded for segments of *Avena* and *Gossypium*. It is once more apparent that the effect exerted by POA is dependent on the species: The induced depression in the rate of entry of 2,4-D is again much greater for *Avena*.

A possible explanation for the disparate reactions of the two species is that *Avena* coleoptiles absorb far more POA than *Gossypium* tissues and that in consequence there is a greater competition between POA and 2,4-D for internal sites. Therefore, experiments were undertaken to ascertain the rates of absorption of labeled POA. The data of Figure 7A demonstrate that in fact the accumulation of POA by *Avena* coleoptiles is somewhat greater.

If the action of POA in inhibiting the uptake of 2,4-D by *Avena* coleoptiles is largely confined to competition for internal sites then it would be expected that as the ratio of the external concentrations of 2,4-D to POA is raised, so the balance will shift in favor of the uptake of 2,4-D. That this is so is evident in Figure 7B. When the external concentration of POA is kept constant at 150 mg/l and the concentration of 2,4-D raised from 3 to 27 mg/l, the inhibitory influence of POA on 2,4-D uptake vanishes.

Experiments on the competitive uptake of POA and 2,4-D by

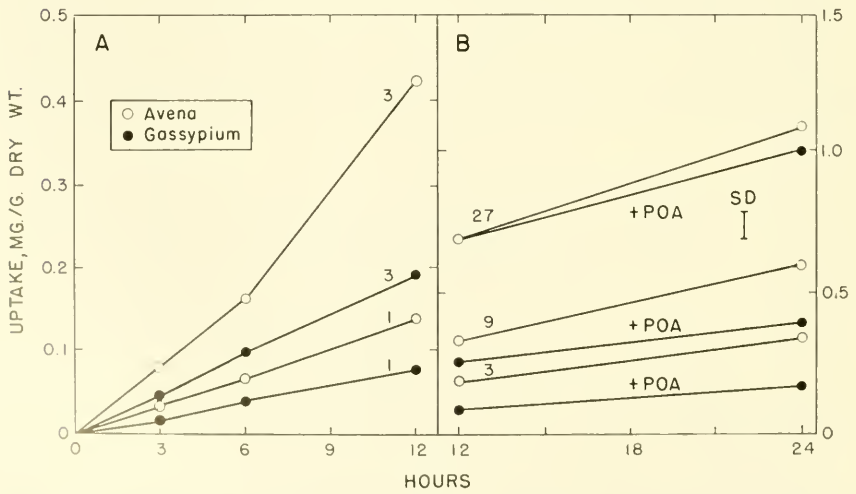

Fig. 7. (A) The uptake of phenoxyacetic acid by segments of *A. sativa* coleoptile and *G. hirsutum* stem from concentrations of 1 and 3 mg/l. (B) The effect of POA (150 mg/l) on the uptake of 2,4-D (3, 9, and 27 mg/l) by segments of *A. sativa* coleoptile.

Avena and *Gossypium* tissues have been described in some detail because they provide clear-cut evidence of specific differences in the physiological processes involved. Other experiments, which can only be reported briefly, aim at a further analysis of differences between *Avena* and *Pisum*.

Comparative experiments have been undertaken to examine the influence of anaerobic conditions on uptake. If nitrogen free from oxygen rather than air is bubbled through a solution containing either segments of *Avena* coleoptiles or *Pisum* stems, then the diminution in the rate of uptake of 2,4-D which is observed after 30 min. is greater for *Pisum*.

A further approach has been to compare the rates of loss of 2,4-D from segments after they have first been allowed to take up labeled auxin before transference to culture solution. In this instance the specific differences are striking; segments of *Pisum* stem lose more than *Avena* coleoptiles.

In conclusion, the purpose of this paper has been to draw attention to the potential value of comparative studies of the pattern of auxin uptake in elucidating specific differences in the mechanisms of action at all levels. At the present stage neither time nor knowledge permits of a detailed analysis of the implication of these findings. The factors which may contribute to the progressive change from a positive to a negative rate of absorption have been considered both in the previous paper (4) and in a further paper (3) in which it has been shown that the pattern of uptake of 2,3,5-triiodobenzoic acid (TIBA) by *L. minor* has many features in common with that of 2,4-D.

It is generally accepted that, apart from the mode of action at cell level, the selectivity of a herbicidal application is dependent on differential retention, the amounts entering either through the shoot or the root, coupled with the subsequent distribution and accumulation in the different parts of the plant. Weintraub and others (8) have followed the level of penetration into leaves, the degree of breakdown, and the extent of the movement of 2,4-D and related compounds in a range of susceptible and resistant species. They concluded that the major difference between the two categories was in the amount transported from the treated leaf to the shoot and that the separation was particularly clear-cut between susceptible and resistant inbred lines of *Zea mays*. At first sight the present findings that 2,4-D after absorption can be readily released from the stem tissues of susceptible, but not resistant, species are compatible with the view that a restriction of movement after penetration into the shoot may in part confer resistance. On the other hand, the studies of root uptake have shown that the amount of radioactive material transported to the shoot at

the end of 32 hrs. bears little relationship to the pattern of uptake. For example, the proportion of the total amount absorbed transferred to the shoots of the five resistant species (Figures 1 and 2) varied from 3.8 per cent (*Triticum*) to 10.9 per cent (*Oryza*), while the corresponding figures for the susceptible species ranged from 1.2 per cent (*Helianthus*) to 25.0 per cent (*Brassica*). However, on the basis of preliminary experiments on the uptake of 2,4-D by susceptible and resistant inbred lines of *Zea,* it would appear that between inbred lines the pathways or mechanisms operating in the transport of 2,4-D out of the leaf may be different, while those controlling the uptake and movement from the roots through the endodermis into the transportation stream are similar.

Woodford *et al.* (9), in their review, have emphasized that 2,4-D can affect a wide range of enzyme systems and cellular processes, but that no individual effect can satisfactorily account for selective action at cell level. It is more reasonable to suppose that selective action rests on the extent to which the auxin interferes with the organized pattern of cell growth and differentiation. Such a general interference could be brought about by changes in the physical and chemical properties of the interfaces or barriers within the individual cells. The present investigation has demonstrated that the complex of factors which are grouped under the aegis of permeability allows the movement of 2,4-D out of one species more than another. Previous research (1, 3) has shown that 2,4-D and TIBA respectively can reorientate the course of uptake of, for example, potassium and cerium-144. Similarly, Sacher and Glasziou (6) have found that indole-3-acetic acid (IAA) and 1-naphthaleneacetic acid inhibit both the injection of the intercellular spaces with water and the exudation of sugars and salts by segments of deseeded bean pods at a time when the cells are still capable of being plasmolyzed and deplasmolyzed. It would seem that, if the auxin which is introduced persists in the tissues, then on the basis of information theory only very small changes between or within cells are demanded to bring about ordered or disordered cellular development.

ACKNOWLEDGMENT

Many of the results reported in this paper have been obtained by E. Abeyaratne during his research for a higher degree at Oxford.

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DISCUSSION

Dr. Henderson: Have you measured the IAA content of these plants after treatment?

Professor Blackman: I haven't measured the IAA content. In fact, I don't know how I would measure the content of IAA which is physiologically active.

Dr. Henderson: In a variety of plants, we found that 2,4-D reduced the rate of disappearance of IAA, while in others auxin disappearance was increased by 2,4-D.

Professor Blackman: With TIBA, if you add IAA to the external solution at concentrations of 30 to 50 mg/l, you can prevent the loss of TIBA but not from roots. That's the only case I know where there is a clear-cut effect of averting the rate of loss by adding IAA.

Dr. Burström: With the high addition of 2,4-D to your susceptible species, as well as in one of the cereals, you had the peak of accumulation after 8 to 12 hrs. In these species have you ever observed any cytological disturbances which may account for the inability of the tissues to retain the amounts of 2,4-D initially taken up in these concentrations?

Professor Blackman: In the root uptake investigations we only used one mg/l, a very low concentration unlikely to cause cytological disturbances in a few hours. It is true that we have observed in *Brassica alba* that you can have injection of water into the intercellular spaces at high concentrations and still have 2,4-D being taken up by the stem segments. In this instance, this type of disturbance does not prevent accumulation of 2,4-D. For *Triticum* I do not know whether there was

visible injury, but I would again stress that although the segments at high concentrations eventually lost water, they still took up 2,4-D.

Dr. Bennet-Clark: In our laboratory we have extremely similar results which are to be published in the Journal of Experimental Botany. IAA accumulates in disks of tissue in essentially the same way that Dr. Blackman has described for 2,4-D. It rises to a maximum concentration and decreases again. The disappearance of IAA from the tissue, which at one time we thought was due to the metabolism of IAA, the formation of conjugates, oxidation to CO_2, etc., is partly due to metabolism. Part is due to the output into the external solution.

The point of interest in this connection is that the uptake process is oxygen-dependent, aerobic respiration-dependent, as Dr. Blackman shows for 2,4-D. The output is not dependent on aerobic respiration. The second feature of interest, as Dr. Blackman showed, is that the gross concentration of 2,4-D in the tissue was smaller than the gross concentration outside, and yet the material came out. This is definitely also the case in our IAA experiments. If you consider that the IAA concentration inside is the weight of IAA per unit weight of tissue water, this gross concentration of IAA inside is smaller than the concentration of IAA outside during the phase at which the IAA is coming out.

And yet this output of IAA is not an active secretion process; it is completely unaffected by cyanide, azide, anaerobic conditions, etc. And, therefore, I would judge that the gross IAA concentration inside and, by implication, the gross concentration of Dr. Blackman's 2,4-D inside, is not equal to the concentration of these substances in the effective site at which it is concentrated, and I would guess that it is concentrated in the cytoplasm rather than the vacuole. I would quite like to hear if that agrees with Professor Blackman's data.

Professor Blackman: Yes, our ideas are very much like those of Bennet-Clark. We do not think that 2,4-D has got past the cytoplasm. We do, however, think that we have certainly obtained some very puzzling results with *Lemna*. Thus, if *Lemna* is first allowed to take up labeled 2,4-D and then transferred to culture solution, more, not less, 2,4-D comes out if the external solution contains cold 2,4-D (Blackman *et al.,* Jour. Exper. Bot., 10: 33, 1959). Egress is clearly not a simple exchange reaction. My feeling is that the 2,4-D accumulating in the cytoplasm exerts a different effect from that located in the outer cytoplasm cell wall interface, and it is the concentration here which controls extension growth rather than what piles up behind the cytoplasm. Johnson and Bonner (Physiol. Plant. 9: 102, 1956) did

touch on this problem that the increased rate of extension growth of *Avena* coleoptiles is dependent on the external concentration of 2,4-D but that at each concentration the response is linear with time over several hours. If the rate of extension growth is dependent on the total internal concentration of 2,4-D, then the rate of extension growth should be curvilinear, not linear, since 2,4-D is steadily accumulating in the tissues.

Dr. Andreae: In connection with Dr. Bennet-Clark's interesting observations on IAA uptake and secretion, I should like to report our findings with IAA-treated tissues. Pea root tips accumulate IAA to ten times the external concentration. IAA, once inside the root tips, is metabolized and, according to our quantitative data, is not lost again to the external solution.

Dr. Bonner: Prof. Blackman has shown us some most interesting results. We can conclude that the 2,4-D is actively accumulated by many kinds of cells and that from some kinds of cells it leaks out again. He has also shown us, beyond any doubt, that the uptake of one auxin can be inhibited by another. I think everyone that has investigated this subject would agree that the various auxins and anti-auxins are competitive with one another in their uptake into the cell. Now, I feel quite confident that someone is thinking that the way auxins interact with one another in controlling growth or the way anti-auxins inhibit the growth-promoting activities of the auxins, is merely by preventing the uptake of the active material into the cell. Well, I wish to state that this isn't so. The effect of anti-auxins in inhibiting growth is due to some different phenomenon than the effect of anti-auxins on inhibiting the uptake of active auxins. That this is so can be shown by simple quantitative relationships. The concentration of IAA which is required to half-inhibit the growth promotion caused by 2,4-D in *Avena* coleoptile section, is somewhat lower than the 2,4-D concentration used. The concentration of IAA required to inhibit by half the rate of 2,4-D uptake by the *Avena* coleoptile section is of the order of one thousand times the concentration of the 2,4-D used. The 2,4-D uptake is less sensitive to the presence of IAA than is the promotion of growth of the section by 2,4-D. So apparently there are two entirely different processes.

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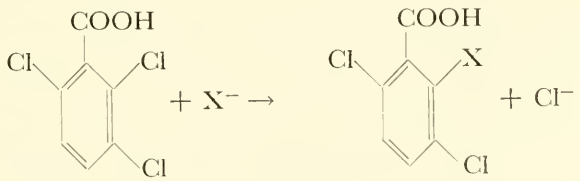
Chemical Structure and Growth-Activity of Substituted Benzoic Acids

Since the discovery by Zimmerman and Hitchcock in 1942 (12) that substituted benzoic acids would induce growth responses in plants, many studies have been made of the effects of these compounds in a wide variety of plant responses. In these investigations the usual finding is that unless the benzoic acid has a halogen substituent in one or both *ortho* positions it is inactive except in the case of a few compounds with methyl substituents in the *ortho* position (7, 8, 9, 11). The activating effect of an electronegative substituent in the *ortho* position may be explained by the electronic theory of organic reactions in that the low electron density of the benzene ring would promote displacement of the electronegative radical in reaction with a nucleophilic substance. This reaction may be represented for 2,3,6-trichlorobenzoic acid which Bentley (1) found to be very active in promoting cell elongation as follows:



In this interpretation the activity of the benzoic acid structure is dependent upon a suitable electron density at the *ortho* position. Recently Fukui *et al.* (2) have calculated the pi-electron distribution for various benzoic acid derivatives by the molecular-orbital method. The frontier electron density in the lowest vacant orbital of the ground state is calculated as the approximate superdelocalizability ($S_r'^{[N]}$) for a reaction with a nucleophilic reagent. Some of these values

Table 1. Chemical reactivity and auxin activity indices of substituted benzoic acids.

Substituent on Benzoic Acid	$S_r'^{(N)}$*	Auxin Activity Index†
2,5-Dichloro-	0.7771	1.0
2,4-Dichloro-	0.7186	0.0
2,3,6-Trichloro-	0.7108	*Ca.* 10.0
2-Chloro-	0.7093	0.05
2,3,5-Triiodo-	0.7074	50.0
2,4,6-Trichloro-	0.6918	0.0
2-Bromo-	0.6825	0.1
2-Iodo-	0.6810	0.0
2,6-Dichloro-	0.6693	0.1
2,6-Dibromo-	0.6501	0.0
4-Chloro	0.6199	0.0
2-Fluoro-	0.6096	0.0
3,4,5-Triiodo-	0.6079	0.0
4-Fluoro-	0.5564	0.0

* Approximate superdelocalizability at *ortho* position for reaction with a nucleophilic reagent [After Fukui *et al.* (2)].

† $\frac{\text{Molar concn. of indole-3-acetic acid inducing an elongation of 0.15 mm.}}{\text{Molar concn. of growth regulator inducing an elongation of 0.15 mm.}} \times 100$

are given in Table 1 together with our values for the activity of the various benzoic acids in promoting elongation at low concentrations compared with indole-3-acetic acid at 100.

Compounds with $S_r'^{(N)}$ values less than 0.6693 at the *ortho* position do not promote elongation while those with a value of 0.6693 or greater do except for some such as those substituted in the *para* position. The inactivating effect of substitution in the *para* position has been recognized for a long time. Veldstra (10) has suggested that since 2,3,6-trichloro-4-fluorobenzoic acid is active, the inactivation by substitution at the *para* position is due to the size of the substituent and the small size of fluorine permits activity. Since the $S_r'^{(N)}$ values for 2,4-dichloro- and 2,4,6-trichloro- benzoic acids are high but the compounds are inactive, some steric hindrance of the chlorine atom at the *para* position must exist. However, fluorine in the *para* position may also result in an inactive compound, for we have found 2-chloro-4-fluorobenzoic acid (IX) to be completely inactive in promoting elongation.

In general the introduction of fluorine into the benzoic acid structure has an inactivating effect as far as the promotion of elongation is concerned. We have examined a number of fluorine-substituted benzoic acids and all except 2-fluoro-6-chlorobenzoic acid have been inactive. Of particular significance is the effect of replacing the chlorine

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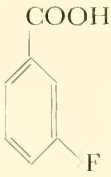

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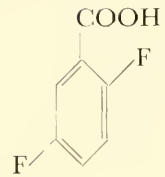

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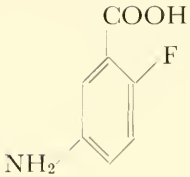

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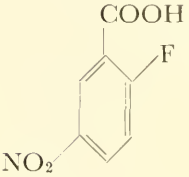

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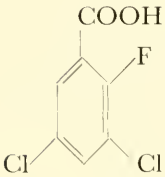

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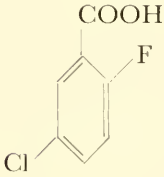

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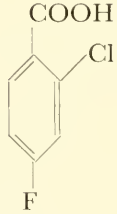

IX

at the 2 position in 2,5-dichlorobenzoic acid with fluorine. Although the 2,5-dichloro compound has the highest $S_r'^{(N)}$ value yet calculated for the series and is one of the more active compounds, the 2-fluoro-5-chlorobenzoic acid (VIII) is completely inactive.

Professor Fukui has kindly sent us his calculations of $S_r'^{(N)}$ values for the 2-chloro-4-fluoro- and 2-fluoro-6-chloro- compounds and these are given in Figure I. It is readily apparent that the effect of fluorine is not a consequence of its size but of its contribution to the electron density at the *ortho* position. Fluorine in the *para* position reduces the $S_r'^{(N)}$ value at the *ortho* position of the chlorine atom to 0.6614, below the value associated with activity in elongation, and the compound is completely inactive. With fluorine at the *ortho* position the $S_r'^{(N)}$ value for the other *ortho* position is increased to 0.7152. Thus 2-fluoro-6-chlorobenzoic acid has an auxin activity index of 0.1, which is the same as the value for 2,6-dichlorobenzoic acid.

The $S_r'^{(N)}$ calculations are of interest also in connection with an apparent effect of the size of the iodine atom on activity. Although

the $S_r'^{(N)}$ value for *ortho* iodobenzoic acid is higher than 0.6693, the compound is inactive, presumably because of the large size of iodine and the consequent steric hindrance in displacement. If, however, the $S_r'^{(N)}$ value is increased by additional iodine substitution in the 3 and 5 positions giving 2,3,5-triiodobenzoic acid, then the compound is very active.

Fukui *et al.* (2) have calculated approximate superdelocalizability at the *ortho* positions for reaction of the benzoic acids with an electrophilic reagent and with a radical reagent. No relation is found between these values and the auxin activity of the molecules. Thus our

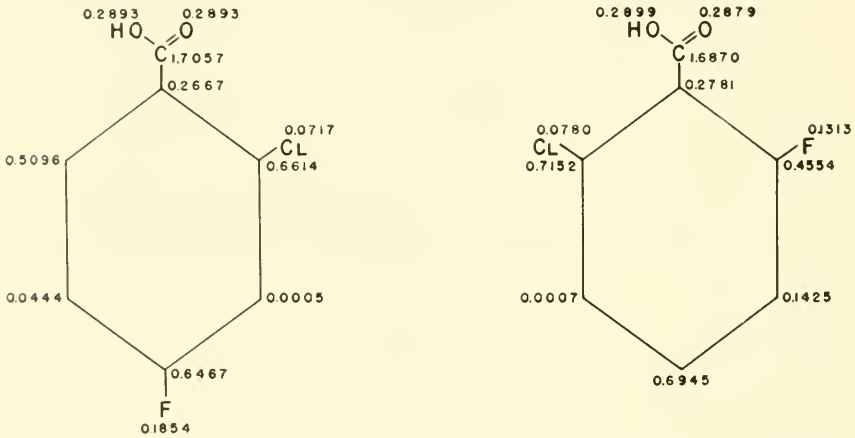

Fig. 1. Approximate superdelocalizability values ($S_r'^{(N)}$) as calculated by Fukui for 2-chloro-4-fluorobenzoic acid (**left**) and 2-fluoro-6-chlorobenzoic acid (**right**).

suggestion of the nucleophilic character of the substrate with which the growth regulators react (3, 6) has been substantiated by the theoretical chemistry of molecular orbitals.

In the course of examining substituted benzoic acids for auxin activity, a few have been found to inhibit the elongation of cells of *Avena* coleoptiles at low concentrations. Of these, 4-ethyl-3-mercaptobenzoic acid (EMBA) (4) has been the most interesting. It was found to inhibit elongation by 40 per cent at $10^{-4}M$ and to repress the effects of growth regulators promoting elongation at concentrations as low as $10^{-5}M$. The interaction of EMBA and these growth regulators is shown in Table 2. The growth induced by the synthetic regulators appears to be much more sensitive to the effect of EMBA than the growth induced by the natural hormone, indole-3-acetic acid. In general, the degree of the inhibitory action of EMBA appears to be inversely related to the relative activity of the regulators in promoting elongation. Similar and lesser inhibition is demonstrated for the

Table 2. Percentage inhibition of growth of *Avena* coleoptiles induced by growth regulators in the presence of $2 \times 10^{-5} M$ 4-ethyl-3-mercaptobenzoic acid ($5 \times 10^{-5} M$ with IAA).

Growth Regulator	Molar Concn.	Per Cent Growth Inhibition
Indole-3-acetic acid	2×10^{-7}	65
	5×10^{-7}	45
	2×10^{-6}	35
2,4-Dichlorophenoxyacetic acid	5×10^{-7}	80
	5×10^{-6}	55
	2×10^{-6}	30
2,4,5-Trichlorophenoxyacetic acid	5×10^{-7}	85
	5×10^{-6}	45
Indole-3-butyric acid	5×10^{-7}	85
	5×10^{-6}	85
1-Naphthaleneacetic acid	5×10^{-7}	45
	5×10^{-6}	40
1-Naphthaleneacetonitrile	2×10^{-7}	50
	5×10^{-7}	50
2,6-Dimethyl-3-bromobenzoic acid	5×10^{-6}	50
	1×10^{-5}	25
	2×10^{-5}	10
2,6-Dichlorobenzoic acid	5×10^{-5}	100
	1×10^{-4}	65
	2×10^{-4}	30

elongation induced by indole-3-acetic acid, 1-naphthaleneacetic acid and 1-naphthaleneacetonitrile, the compounds most active in promoting elongation. Greater inhibition occurs in the case of 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, and indole-3-butyric acid, which are less active. The greatest inhibitory effect is found with 2,6-dichloro- and 2,6-dimethyl-3-bromo- benzoic acids, which have the lowest activity as auxins. EMBA, at one-tenth of the concentration of 2,6-dichlorobenzoic acid, results in an inhibition of 30 per cent of the elongation effect of the latter.

These results suggest a competitive type of inhibition, and Lineweaver-Burk plots of the reciprocals of elongation and regulator concentration (5) conform to expectation for the interaction of EMBA and indole-3-acetic acid. The interaction with 2,4-dichlorophenoxyacetic acid (Figure 2) and 2,6-dimethyl-3-bromobenzoic acid appears to be competitive at high concentrations of the auxin, but at low concentrations the inhibition tends to be complete. The inhibition by EMBA also appeared to be reversible in an experiment in which in-

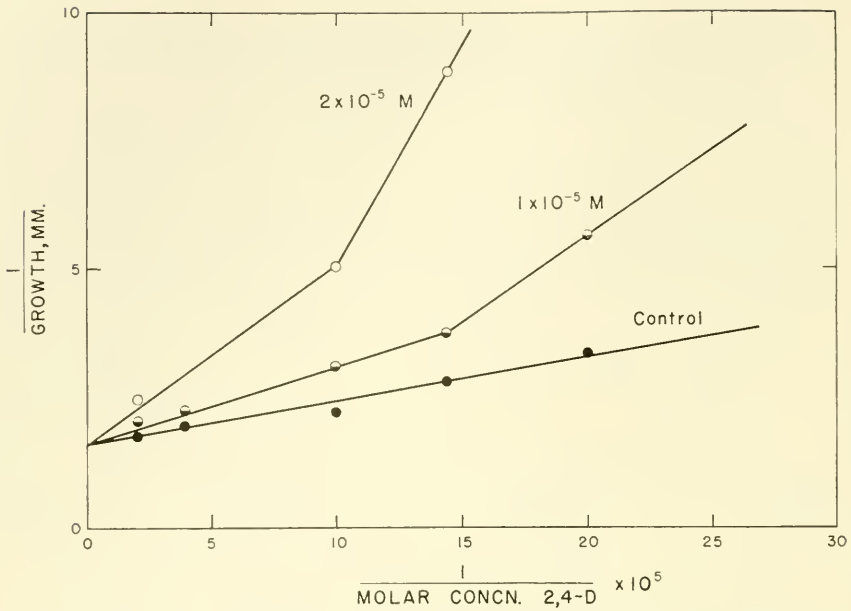

Fig. 2. Inhibiting effect of 4-ethyl-3-mercaptobenzoic acid at $1 \times 10^{-5}M$ and $2 \times 10^{-5}M$ on the growth of *Avena* coleoptile sections induced by 2,4-dichlorophenoxyacetic acid.

dole-3-acetic acid at $2 \times 10^{-7}M$ and EMBA at $5 \times 10^{-5}M$ were replaced after 5 hrs. by EMBA at the same concentration and indole-3-acetic acid at $4 \times 10^{-6}M$. The higher concentration of indole-3-acetic acid resulted in a growth rate nearly the same as that of the tissue in the absence of EMBA.

To evaluate the chemical grouping responsible for the inhibition brought about by EMBA, the effects of several related compounds were examined and the data are given in Table 3. The S-methyl ether

Table 3. Percentage inhibition of elongation of *Avena* coleoptiles produced by indole-3-acetic acid in presence of substituted benzoic acids.

Substituted Benzoic Acid	Molar Concn.	Molar Concn. of IAA		
		2×10^{-7}	5×10^{-7}	2×10^{-6}
3-Mercapto-4-ethyl-	5×10^{-5}	65	45	35
3-Methylmercapto-4-ethyl-	5×10^{-5}	20	20	10
2-Mercapto-	2×10^{-4}	10	5	5
3-Mercapto-	2×10^{-4}	15	10	10
4-Mercapto-	2×10^{-4}	50	40	40
4-Ethyl-	2×10^{-4}	35	30	20
4-Chloro-	1×10^{-4}	35	30	30
2,4-Dichloro-	1×10^{-4}	15	20	20

derivative is not as effective in inhibiting the elongation induced by indole-3-acetic acid, even though being less polar it should penetrate more rapidly to the reaction site. Thus the reaction at the thiol group must be the primary element in the inhibition effect. The aromatic thiol group itself, however, even in the *meta* position, gives rise to only slight inhibitory effect for the benzoic acid structure. The inhibition by *para*-substituted benzoic acids is, in general, of the same order for thiol, chlorine, and ethyl groups and only about one-fourth or less of the inhibition brought about by EMBA. Thus the action of EMBA appears to be quite specific and to depend on the effect of the ethyl substituent on the thiol group. This effect is probably electronic in character, with the ethyl substituent releasing electrons to the ring and rendering the thiol group S^- with a greater potential for reaction.

On the basis of this study of the inhibitory action of 4-ethyl-3-mercaptobenzoic acid on elongation, the following inferences may be formed: (1) Since the degree of inhibition in interaction with the various auxins is related to the activity of the auxins, the EMBA molecule reacts at the substrate site where the auxins react and induce growth. (2) Since the reaction involves the thiol group, it is possible that the substrate site is a sulfhydryl, as has been suggested on the basis of considerations involving only the auxin structure.

SUMMARY

The growth activity of substituted benzoic acids appears to be dependent primarily upon electronic characteristics determined by the substituents, but these electronic characteristics may be counteracted in part by steric factors.

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DISCUSSION

Dr. Smith: We have studied the effect of dichloro- and dimethyl-substitution of benzoic acids, and I would like to show you some of our results in relation to those obtained by Drs. Muir and Hansch. These assays utilized three test systems (Ann. Appl. Biol. 47: 173. 1959), but the data from the pea test are representative of our results. As seen in Table 1, and as stated by Dr. Hansch, the monochloro acids are inactive. However, not only is the 2,6-dichloro- active, but marked activity is also shown by the 2,3-, 2,5- and 2,3,6- derivatives. Similar, but less marked results, were obtaind with mono- and dimethylbenzoic acids. Here again, mono-substitution renders the compound inactive, but some activity is shown by the 2,3-, 2,5-, and 2,6- acids.

Dr. Wain: Dr. Smith has raised an interesting point. In structure activity relationships it is important to study whole series. One finding that must be noted is the quite high activity of the 2,3- compounds which to date has been overlooked. In view of this activity, one has to ask whether a removal of the 2- or 6- substituent is essential in the growth reaction. I think a very ingenious hypothesis has been put forward here, but I am rather dubious about it myself, especially when one has to accept the elimination of a methyl group from the 2 or 6 position, in order to explain the activity of the 2,6-dimethyl- and 2,3,6-trimethylbenzoic acids.

Table 1. Activities of chloro- and methyl-substituted benzoic acids in the pea curvature test.*

Substituted Benzoic Acid	Molar Concentrations				
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
2-Chloro-	0	0	0	0	0
3-Chloro-	0	0	0	0	0
4-Chloro-	0	0	0	0	0
2,3-Dichloro-	0	0	0	2	4
2,4-Dichloro-	0	0	0	0	0
2,5-Dichloro-	0	0	3	5	5
2,6-Dichloro-	0	0	0	1	3
3,4-Dichloro-	0	0	0	0	0
3,5-Dichloro-	0	0	0	0	0
2,3,6-Trichloro-	0	3	4	5	6
2-Methyl-	0	0	0	0	0
3-Methyl-	0	0	0	0	0
4-Methyl-	0	0	0	0	0
2,3-Dimethyl-	0	0	0	1	4
2,4-Dimethyl-	0	0	0	0	0
2,5-Dimethyl-	0	0	0	0	2
2,6-Dimethyl-	0	0	0	0	1
3,4-Dimethyl-	0	0	0	0	0
3,5-Dimethyl-	0	0	0	0	0

* Activity on an arbitrary scale: 0 = inactive; 6 = highly active.

Dr. Muir: I would like to make it clear that in the first place we were looking at a series of compounds which we were trying to explain. We found that in the fluorine series we had a rather unusual situation as far as halogen substituents were concerned, and our discourse was primarily concerned with them. I don't see, except for a few compounds which Dr. Smith studied, any real difference from our position other than in the methyl-substituted compounds. These may yield wholly different results, and until we have further electronic data to analyze this picture, I don't believe they constitute too serious an objection. Our primary purpose was to explain the relationship of structure as it is shown in the effects of these compounds on *Avena* coleoptile tissue.

Dr. Wain: I would just like to say again what Dr. Smith has said. All these results have been obtained in three different tests by Dr. Wightman so that the results are of general applicability as far as we can see in these different tests, using different types of tissue.

Dr. Fawcett: In his discussion of the highly active 2,3,6-trichlorobenzoic acid, Dr. Muir postulates a nucleophilic reaction involving a replacement of the chlorine atom in the 6 position. In 2,3,5,6-tetrachlorobenzoic acid, which has similar activity, a nucleophilic replacement of either of the *ortho* chlorine atoms would be subject to con-

siderable steric hindrance. Usually, in these circumstances the replacement rate becomes very small.

Dr. Muir: The selection of the *ortho* position for the attack as shown in the reaction of 2,3,6-trichlorobenzoic acid is determined by the $S_r'^{(N)}$ values. Where there is only one halogen-substituted position, that one is it. In the cases where there are two *ortho* substituents, as Fukui, Nagata, and Yonezawa have indicated, the one with the greater $S_r'^{(N)}$ would be most likely to be displaced in the reaction. Their calculations of superdelocalizability show that one may be of a different value from the other, depending upon the substituents in the ring.

Dr. Wain: We are not really concerned necessarily with reaction at the set 2 or 6 position. We were under the impression from all of your papers that it is the 2 or 6 position which was involved. But I now take it that any other position will do, providing that it is suitably activated. Is that right?

Dr. Muir: Partially. Where there is steric hindrance, even though the electronic value or density is favorable, the steric hindrance will make the molecule inactive.

Dr. Ray: In relation to the idea that 4-ethyl-3-mercaptobenzoic acid is reacting with the same site as do the auxins, I wonder if there is any way that you can explain why you get reversal of this inhibition by some auxins but not by others? It seems that, from the data you show, in the case of some auxins the inhibition was quite independent of the auxin concentration.

Dr. Muir: My explanation would be that in not all cases did we have the appropriate range in which to demonstrate competition. In some instances this was outside of the ranges in which the reversibility is most easily shown.

Dr. Wain: I would like to ask whether 4-ethyl-3-mercaptobenzoic acid reacts chemically with any of these auxins. For instance, have melting point curves been constructed to show whether you get compound formation or a simple eutectic?

Dr. Hansch: The answer is no.

Dr. Wain: One further point. The pH is very important in relation to all thiol compounds which are readily oxidized to disulfides under alkaline condition. The pH here was on the acid side, wasn't it?

Dr. Muir: It was controlled at 5.6.

Dr. Hansch: The values for superdelocalizability which you have just seen and which Professor Fukui was so kind to furnish us represent only a small number of those he has made. A more extensive list has been published (Jour. Amer. Chem. Soc. 80: 2267. 1958).

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Relationship of Molecular Structure to Biological Activity in the N-Arylphthalamic Acids[3]

The activity of a number of substituted *N*-phenylphthalamic acids in stimulating fruit set of tomato was reported by Hoffmann and Smith in 1949 (8). Since then, these chemicals have received only infrequent attention as fruit setting agents (3), although the closely related *N*-1-naphthylphthalamic acid has shown considerable promise as a selective herbicide (4). The action of *N*-*m*-tolylphthalamic acid in causing increased flower formation in the tomato (23) has led to the practical use of this chemical to obtain increased flower and fruit numbers and, thereby, higher yields of greenhouse grown tomatoes (33). It was subsequently found that a number of the chloro- and methyl-substituted *N*-phenylphthalamic acids possessed flower forming activity and that the structural requirements of these compounds for altering flower formation apparently differed from those for auxin activity in the substituted benzoic and phenoxyacetic acids (24).

At present, only two other synthetic growth regulators, α-(2-naphthoxy)phenylacetic acid (17) and the 2,3,5-triiodobenzoic acid (6, 31, 34, 35), have been clearly demonstrated to increase flower formation in the tomato. Although 2,3,5-triiodobenzoic acid has been considered an auxin antagonist (5), or auxin synergist (26), it also has auxin properties (1, 13). Similarly, α-(2-naphthoxy)phenylacetic acid is highly active in stimulating parthenocarpic fruit set (18) but has not, to our knowledge, been evaluated for auxin activity in any of the more classical assays. This report deals with the molecular structure of the

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substituted *N*-arylphthalamic acids as related to their biological activity in tomato flower formation, and as auxins in the stimulation of tomato parthenocarpy and elongation of *Avena* coleoptile sections.

MATERIALS AND METHODS

Chemical[4]

Most of the substituted *N*-arylphthalamic acids used in these investigations were prepared by reacting equal molar amounts of phthalic anhydride and the appropriate aromatic amine in benzene at 30 to 80° C. The products were dissolved in dilute base, reprecipitated with hydrochloric acid, washed thoroughly with water, and dried. The sterically hindered 2,6- and 2,4,6-substituted phenyl derivatives were prepared by heating the amine and phthalic anhydride together without solvent at 150 to 200° C. to form the imide, then hydrolyzing to the phthalamic acid with one equivalent of sodium hydroxide. Purity of the preparation was checked by titration to obtain the equivalent weight, and good agreement with theoretical values was found. Neutral equivalents were a better criterion of purity than melting points since most of the *N*-arylphthalamic acids decomposed as they melted, liberating water and forming the imide.

Tomato flower formation. The procedure was essentially that developed by Teubner and Wittwer (24) and was based on the observation that the first inflorescence of the tomato (*Lycopersicon esculentum,* 'Michigan-Ohio Hybrid') is initiated 9 days after cotyledon expansion (21). The inflorescence is most susceptible to modification by chemical treatment when the first flower primordium is differentiating, which, in the test variety, corresponded to plants having two plumule leaves and a third leaf one-half to one inch in length (24, 33). Plants treated with the *N*-arylphthalamic acids at this stage developed branched flower clusters with five to seven flowers on each branch. Over a limited concentration range, which varied for each active chemical, the flowering response was directly proportional to concentration. The maximum responses obtained with this method have been clusters consisting of three to four branches and 28 flowers. Higher concentrations either reduced branch and flower numbers or produced severe formative effects.

Solutions of the various *N*-arylphthalamic acids were prepared just prior to treatment by dissolving weighed amounts of each chemical in a small quantity of acetone and diluting to the appropriate volume with water. No deleterious effects were observed for concentrations of acetone up to 5 per cent, the maximum employed in the studies. The

[4] Personal communication from Dr. A. E. Smith, Agricultural Chemicals, Naugatuck Chemical Division, United States Rubber Company.

solutions were applied with a compressed air sprayer and plants were sprayed to the drip point. In other studies, responses equivalent to those from spraying have been obtained by applying 0.01 ml. of each concentration to the apex of the plant (21). Flower numbers were determined after 6 weeks or when the final flower bud was clearly visible.

Tomato parthenocarpy. Luckwill's test (11) was employed, and 0.01 ml. of each solution was applied directly to ovaries of tomato flowers emasculated just prior to anthesis. Six ovaries were treated with each concentration of a chemical. Solutions were prepared as described above, and ovaries treated with 5 per cent acetone did not differ from nontreated ovaries. Ovary diameters were measured to the nearest 0.1 mm. with a vernier caliper 5 days after treatment.

Avena *straight growth.* The procedure used for growing and sectioning *Avena* coleoptiles was that described by Leopold (10) using 'Brighton' oats. Coleoptiles 2 to 3 cm. in length were selected and a single 5 mm. section was cut 3 mm. below the tip. The sections were floated, ten to a dish, without removing the leaf, in 10 ml. of the test solutions which contained a phosphate-citrate buffer (pH 5.0) and 3 per cent sucrose (15). Since most of the substituted *N*-arylphthalamic acids dissolve only with difficulty below pH 6.0, the solutions were prepared by dissolving appropriate amounts of each chemical in the K_2HPO_4 buffer component (pH 8.5) and then adding the citric acid and sucrose. No visible precipitation occurred in 10^{-3} *M* solutions prepared in this manner. Lengths of the *Avena* sections were measured after 20 hrs. to obtain maximum differences between the treated and the control sections (16), and to minimize hydrolysis of the phthalamic acids at pH 5.0 (3). Measurements were made to the nearest 0.5 mm. Elongation was expressed as per cent of the final length of control sections grown in the phosphate-citrate-sucrose mixture.

Statistical

The statistical significance of the differences between the means of control and treated samples was based on the "t" test as outlined by Goulden for nonpaired variates (7).

RESULTS

Activity in Tomato Flowering

In preliminary studies tomato plants were treated with a number of phenyl-substituted *N*-phenylphthalamic acids at concentrations ranging from 5 to 500 p.p.m. in order to establish an effective range for each. It was found that substitution of chloro- or methyl- groups

at the *ortho* or *meta* positions enhanced flower forming activity while hydroxyl- or nitro- groups at either the *ortho, meta,* or *para* positions in the phenyl ring rendered the weakly active *N*-phenylphthalamic acid inactive. Substitution of chloro- or methyl- at the *para* position did not abolish the flower-forming activity of *N*-phenylphthalamic acid, although these derivatives had a high degree of phytotoxicity which often masked their weak flower-forming activity. On the other hand, substitution of bromo-, carboxyl-, amino-, dimethylamino-, or acetyl- groups at the *para* position resulted in derivatives which were inactive at concentrations up to 500 p.p.m. The *N*-2,5-dimethoxy- and *N*-2-methyl-5-isopropylphenylphthalamic acids were also inactive in the preliminary tests.

Replacement of the phenyl ring with a 1-naphthyl group (*N*-1-naphthylphthalamic acid) resulted in high phytotoxicity and formative effects, but no increase in flower numbers. On the other hand, *N*-2-naphthylphthalamic acid exhibited no phytotoxic or formative effects and did give a slight increase in flower numbers.[5]

On the basis of these preliminary tests, all of the monochloro- and dichloro- together with four of the six possible trichloro- derivatives were evaluated in the tomato flowering test over their most effective concentration ranges. In the same test *N*-phenylphthalamic acid was included together with its monomethyl- and several dimethyl- derivatives. The results obtained are presented in Table 1 together with their statistical significance. It is apparent that mono-*ortho* and mono-*meta* substitutions on the phenyl ring are far more effective in enhancing the tomato flower forming activity of *N*-phenylphthalamic acid than is *para* substitution. Furthermore, monomethyl substitution is as effective, or nearly so, as monochloro substitution. The positional effects of the mono-substituted derivatives are even more apparent if the relative activities of the disubstituted derivatives are examined. Thus, *N*-2,3-dichlorophenylphthalamic acid reflects the activating effect of both *ortho* and *meta* substitution and is the most active derivative examined to date.

In contrast to the effect of monomethyl substitution, the 2,3-dimethyl derivative has surprisingly low activity. Similarly, 2,5-disubstitution results in a slight depression of activity relative to the mono-substituted derivatives. The relatively low activity of the *para*-chloro and *para*-methyl derivatives is enhanced considerably by *ortho* substitution and less so by a *meta* substituent. In these cases, however, the relative activity of the disubstituted derivative is somewhat less than

[5] Only a single test of the naphthyl derivatives has been conducted so that these results should be considered as tentative.

Table 1. Activity of chloro- and methyl-substituted *N*-phenylphthalamic acids in promoting increased flower formation in the first cluster of tomato plants. Numbers of flowers as means of ten plants. *

Phenyl Substituent in *N*-phenylphthalamic Acid	Molar Concentrations Tested †							
	10^{-5}	2×10^{-5}	5×10^{-5}	10^{-4}	2×10^{-4}	5×10^{-4}	10^{-3}	2×10^{-3}
None	....	....	....	....	6.3	6.2	8.3	12.0‡
2-Chloro-	....	....	7.0	7.5	7.5	13.7§	21.0‖	....
3-Chloro-	....	....	....	7.3	8.0	11.7‡	13.0§	17.0‖
4-Chloro-	....	....	....	6.3	6.2	7.3	8.1	13.8§
2,3-Dichloro-	7.3	10.3‡	20.3‖	26.0‖	....	....	....	....
2,4-Dichloro-	....	....	....	7.1	6.5	8.8	12.5§	28.0‖
2,5-Dichloro-	....	....	....	6.3	6.8	7.5	11.7‡	17.0‖
2,6-Dichloro-	....	....	....	....	5.7	6.3	6.0	6.3
3,4-Dichloro-	....	....	....	....	7.7	7.3	9.7	17.8§
3,5-Dichloro-	....	....	....	7.1	6.5	16.0‖	20.8‖	....
2,3,5-Trichloro-	....	....	8.0	11.3‡	11.0‡	18.0‖	23.4‖	....
2,4,5-Trichloro-	....	....	....	....	6.3	8.2	15.0§	21.6‖
2,4,6-Trichloro-	....	....	....	....	5.5	6.3	6.8	5.8
3,4,5-Trichloro-	....	....	....	....	6.2	12.0‡	15.2§	19.8‖
2-Methyl-	....	....	....	8.3	8.8	11.9‡	17.7‖	....
3-Methyl-	....	....	....	6.7	7.8	10.7‡	19.8‖	....
4-Methyl-	....	....	....	....	6.7	5.8	6.8	10.3‡
2,3-Dimethyl-	....	....	....	6.8	6.5	9.8	12.9§	....
2,5-Dimethyl-	....	....	....	....	6.5	7.8	9.8	15.2§
2,6-Dimethyl-	....	....	....	....	6.2	6.7	5.4	5.9
3,4-Dimethyl-	....	....	....	....	6.7	7.3	7.3	12.4§

* Control (nontreated) plants averaged 6.3 flowers in the first cluster.

† Flower numbers significantly greater than control: ‡ at 5 per cent, § at 1 per cent, ‖ at 0.1 per cent levels

the corresponding mono-*ortho* or mono-*meta* compound. These relationships can also be applied to the trisubstituted *N*-phenylphthalamic acids which, in general, exhibit somewhat lower activity than the most active of the related disubstituted forms. It is of particular interest that di-*ortho*-substituted forms (*N*-2,6-dichlorophenyl-, *N*-2,6-dimethylphenyl-, *N*-2,4,6-trichlorophenylphthalamic acids) are entirely devoid of activity.

Activity in Tomato Parthenocarpy

While stimulation of tomato ovary development is not considered a specific test for auxin activity (10), it has proved a convenient tool for assessing the relative activities of a number of synthetic auxins (12, 18). After preliminary tests to establish the appropriate effective concentration range, the various substituted chloro- and methyl-derivatives of *N*-phenylphthalamic acid were evaluated relative to their ability to stimulate development of tomato ovaries. The results presented in Table 2 indicate that activity of the various substituted *N*-phenylphthalamic acids in stimulating parthenocarpy is almost identical to that obtained for tomato flower formation. Ovaries treated with *para*-chlorophenoxyacetic acid, a typical auxin, had diameters of 5.4 and 8.3 mm. at 10^{-4} and $10^{-3}M$, respectively. This activity compared favorably with that of *N*-3-chlorophenylphthalamic acid, but was far less than that of the 2,3-dichloro- and 2,3,5-trichlorophenylphthalamic acids. Indole-3-acetic and 2-naphthoxyacetic acids were only one-tenth as active as *para*-chlorophenoxyacetic acid, and showed an appreciable growth rate only at the highest concentration ($10^{-3}M$) tested.

In tomato ovary growth as with flower formation di-*ortho* substitution abolished activity. The low activity of the mono-*para* derivatives in flower formation was also reflected in their failure to promote parthenocarpic fruit development. In the latter case, there was a stimulation of abscission of the young ovaries after 2 days which may have masked any growth promoting effects. Non-treated ovaries did not abscise during the test period of 5 days. The abscission caused by mono-*para* substitution did not occur with the di-*ortho* derivatives, although the 3,4-disubstituted chloro- and methyl-compounds showed similar but less pronounced effects than the mono-*para* derivatives.

Activity in *Avena* Section Straight Growth

The high activity of some of the substituted *N*-phenylphthalamic acids in stimulating fruit development prompted an evaluation of their activities in the *Avena* section test. Earlier studies conducted primarily with *N*-phenylphthalamic acid had given variable results

Table 2. Activity of chloro- and methyl-substituted *N*-phenylphthalamic acids in stimulating parthenocarpic development of tomato ovaries. Ovary diameter (mm.) 5 days after treatment as means of six ovaries.*

Phenyl Substituent in *N*-phenylphthalamic Acid	Molar Concentrations Tested†							
	10^{-5}	2×10^{-5}	5×10^{-5}	10^{-4}	2×10^{-4}	5×10^{-4}	10^{-3}	2×10^{-3}
None	....	....	....	....	2.8	4.7‡	4.5‡	6.6‖
2-Chloro-	....	....	2.5	2.7	4.6‡	6.7‖	....	....
3-Chloro-	....	....	....	5.5§	7.8‖	7.1‖	8.1‖	....
4-Chloro-	....	....	....	....	2.5	2.7	2.6	2.8
2,3-Dichloro-	6.0§	7.0‖	7.7‖	8.1‖	....	....	....	....
2,4-Dichloro-	....	....	2.7	3.0	6.2§	7.6‖	....	....
2,5-Dichloro-	....	....	2.6	3.4	4.5‡	7.8‖	....	....
2,6-Dichloro-	....	....	....	....	2.5	2.5	2.5	2.5
3,4-Dichloro-	....	....	....	....	3.7	5.9§	6.6§	7.2‖
3,5-Dichloro-	....	2.7	3.5	6.9‖	8.0‖	....	....	....
2,3,5-Trichloro-	....	4.5‡	6.5§	8.0‖	8.2‖	....	....	....
2,4,5-Trichloro-	....	....	....	2.5	3.0	5.4§	7.1‖	....
2,4,6-Trichloro-	....	....	....	....	2.5	2.5	2.4	2.5
3,4,5-Trichloro-	....	....	2.7	2.5	4.7‡	7.7‖	....	....
2-Methyl-	....	....	....	2.5	2.5	4.6‡	6.8‖	....
3-Methyl-	....	....	2.5	2.7	5.4§	6.1§	....	....
4-Methyl-	....	....	....	....	2.5	2.4	2.4	2.4
2,3-Dimethyl-	....	2.5	2.4	4.2	5.9§	....	....	....
2,5-Dimethyl-	....	....	....	2.6	2.5	3.0	5.9§	....
2,6-Dimethyl-	....	....	....	....	2.5	2.5	2.5	2.4
3,4-Dimethyl-	....	....	....	....	2.5	2.4	4.7‡	5.7§

* Diameter of control ovaries at 5 days: Nontreated, 2.5 mm.; Pollinated, 8.8 mm.

† Diameters significantly greater than nontreated control: ‡ at 5 per cent, § at 1 per cent, ‖ at 0.1 per cent level.

because of solubility difficulties, loss of activity of solutions on standing, and the possible hydrolysis of the amide liberating the aromatic amine and phthalic acid (3). The measures taken to avoid these difficulties are given under Methods.

The results presented in Table 3 are those of a single test, but have been confirmed for the chloro-substituted derivatives in at least two other tests. The per cent elongation relative to controls shows essentially the same structure-activity relationships that were apparent in both flower formation and fruit parthenocarpy. Mono-*para* and di-*ortho* substitutions either abolish or have no effect on the activity of *N*-phenylphthalamic acid. The most active compounds were the *N*-2,3-dichloro-, *N*-2,3,5-trichloro-, and *N*-3,5-dichlorophenylphthalamic acids. The high activity of the latter was surprising in view of its lower activity in tomato flower formation and ovary development. In addition to the derivatives listed in Table 3, the three (*ortho, meta,* and *para*) mono-hydroxy and mono-nitro derivatives were tested and were inactive. The closely related *N*-1-naphthylphthalamic acid was almost as active as *N*-2-chlorophenylphthalamic acid at 10^{-4} and 10^{-5} *M*, giving 115 and 117 per cent elongation, respectively. *N*-1-naphthylphthalamic acid did not, however, stimulate the growth of *Avena* sections at concentrations lower than $10^{-5}M$ and retardation (88 per cent of controls) occurred at $10^{-3}M$. On the other hand, *N*-2-naphthylphthalamic acid was one of the more active derivatives tested with 133, 125, and 120 per cent elongation at 10^{-3}, 10^{-4}, and $10^{-5}M$ concentration, respectively. It should be noted (Table 3) that the maximum elongation obtained with any of these derivatives was only 129 per cent, while comparable results with indole-3-acetic acid at $10^{-5}M$ were 147 per cent of the final length of control sections.

DISCUSSION

The results clearly indicate that modifications of structure, through substitution of chloro- and methyl- groups on the phenyl ring, have precisely the same effects on auxin activity (Tables 2 and 3) as on activity in tomato flower formation (Table 1). It is not possible, at present, to resolve the question of whether these chemicals function as auxins, or in some other manner, in the control of tomato flower formation. However, it is evident that only those derivatives which have activity in stimulating *Avena* straight growth and tomato parthenocarpy are able to increase flower numbers in the tomato.

Consideration of the structural relationships of the substituted *N*-phenylphthalamic acids on the basis of their activity in *Avena* straight growth (Table 3) indicates requirements for activity distinctly dif-

Table 3. Activity of chloro- and methyl-substituted *N*-phenylphthalamic acids in the *Avena* straight growth test. Mean of ten sections as per cent final length of control. *

Phenyl Substituents in *N*-phenylphthalamic Acid	Molar Concentrations Tested †					
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
None	103	105	104	102	107‡	103
2-Chloro-	105	100	106	118‖	122‖	. . .
3-Chloro-	100	103	102	108‡	114‖	. . .
4-Chloro-	104	103	103	101	98	. .
2,3-Dichloro-	106	116‖	129‖	115‖	110§	. . .
2,4-Dichloro-	104	102	105	113‖	101	. . .
2,5-Dichloro-	100	102	102	107‡	104	. . .
2,6-Dichloro-	100	100	99	99	96	. .
3,4-Dichloro-	100	100	101	106	109§	. . .
3,5-Dichloro-	106	117‖	125‖	122‖	118‖	.
2,3,5-Trichloro-	106	121‖	121‖	114‖	95	
2,4,5-Trichloro-	105	111§	112‖	111§	95	. .
2,4,6-Trichloro-	100	99	102	99	94	. .
3,4,5-Trichloro-	106	112‖	112‖	112‖	106	. . .
2-Methyl-	. . .	102	106‡	108§	112‖	106‡
3-Methyl-	. . .	105	106‡	108§	117‖	103
4-Methyl-	. . .	101	102	101	107‡	105
2,3-Dimethyl-	.	102	106‡	108§	114‖	97
2,5-Dimethyl-	. . .	105	106‡	106‡	107‡	98
2,6-Dimethyl-	. .	100	100	100	100	101
3,4-Dimethyl-	. . .	100	102	105	109§	91

* Phosphate-citrate buffer, 3 per cent sucrose. Elongation in $10^{-5}M$ IAA, 147 per cent.
† Per cent elongation significant: ‡ at 5 per cent, § at 1 per cent, ‖ at 0.1 per cent level.

ferent from those found for the substituted benzoic, phenoxyacetic, and phenylacetic acids. In the benzoic acids it was originally considered that the *para* position must not be substituted (13, 28, 29, 36). It has been shown, however, that with the highly active 2,3,6-trichlorobenzoic acid further substitution of either chloro- or fluoro- at the 4-position reduces, but does not abolish, activity (19, 30). This is similar to the effects of *para* substitution of chloro- or methyl- groups on the activity of several active *N*-phenylphthalamic acid derivatives. Unfortunately the 2,3,4-trichloro derivative which would best illustrate this effect was not available for these tests. The high activity obtained with 2,3-dichloro substitution in *N*-phenylphthalamic acid is also similar to that found with phenylacetic acid and to a lesser degree with benzoic acid (20). The resemblance between the benzoic and phenylacetic acid auxins and the *N*-arylphthalamic acids, however, is dissimilar when di-*ortho* or 2,6-substitution is considered. Normally, these derivatives are active both in the phenylacetic and benzoic acid series (2, 19, 20, 28, 36). In contrast, di-*ortho* substitution results in inactive derivatives of *N*-phenylphthalamic acid. Here again it will be of considerable importance to establish if this is a total effect or if a second *ortho* substituent will merely reduce the activity of the highly active *N*-2,3-dichlorophenylphthalamic acid. As yet, the 2,3,6-trichloro derivative is not available for study. The loss of activity with di-*ortho* substitution is similar to that found for the substituted phenoxyacetic acid auxins (9, 14, 22). However, this is an effect which is subject to some qualifications (25, 27, 32).

The benzoic acid and phenoxyacetic acid auxins have in common the inactivity of their 3,5-disubstituted derivatives. Here again, however, the effect of di-*meta* substitution is not an absolute one since slight activity is exhibited by 2,3,5-trichlorobenzoic acid (28), 2,3,5,6-tetrachlorobenzoic acid (19), and by the 2,3,6-trichloro-, the 2,3,5-trimethyl- and trichloro-, the 3,4,5-trichloro-, and the 2,3,4,5-tetrachlorophenoxyacetic acids (20, 27). This is in distinct contrast to the high activities of the 3,5-dichloro- and the 2,3,5- and 3,4,5-trichlorophenylphthalamic acids (Table 3). It will be of interest to learn if the studies, which Pybus *et al.* (20) refer to, on nuclear substitution in the phenylacetic acids show similar structure-activity relationships.

SUMMARY

The activities of several chloro- and methyl- derivatives of *N*-phenylphthalamic acid have been evaluated with respect to tomato flower formation, parthenocarpic development of tomato ovaries, and straight growth of *Avena* sections.

Modifications in molecular structure had almost identical effects

on activity in all three biological responses. Both *ortho* and *meta* substitution of chloro- or methyl- groups enhanced the activity of *N*-phenylphthalamic acid, and the 2,3- and 3,5-dichloro-, and the 2,3,5-trichlorophenylphthalamic acids were the most active tested. In contrast, *para* and di-*ortho* substituents either abolished or had no effect on biological activity. While some of these substitutive effects suggested similarities to either the benzoic, phenylacetic, or phenoxyacetic acids, others showed distinct differences.

In view of the molecular structure-biological activity relationships in the *N*-arylphthalamic acids, continued study of this group of compounds should provide valuable information on the fundamental chemical structure requirements for plant growth regulating activity.

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DISCUSSION

Dr. Nitsch: Does your compound also stimulate flower formation in other species?

Dr. Teubner: We have only cursorily examined the effect on other species. The tomato has a sympodial flowering habit and this may account for its response. We're not completely sure whether this is a direct effect of the material on the developing floral primordium or whether it is an indirect effect through inhibition of the sympodial bud as Dr. Leopold has suggested in his recent review. However, histological evidence indicates that it is a direct effect on the floral meristem. The strawberry has a corymbose raceme which can be monochotomous, dichotomous, or polychotomous. While it is more difficult to work with clonal lines in this species, for large, uniform populations we have observed an appreciable effect on flowering. We obtained flower clusters of 50 to 100 flowers. Other plants which have been examined are snapdragon and petunia. Neither of these responded, but of course we had little knowledge of the stage of development at which floral initiation takes place. The time of application is very critical at lower concentrations with the tomato and we're sure this holds with other species. We know there is no response from low concentrations applied 2 or 3 days before the inflorescence is laid down or initiated. We previously reported this in Science (Science 122: 74, 1955). Apparently these compounds are so labile that when low concentrations are applied prior to initiation, the amount present when the flower cluster is developing is insufficient to produce the response. With higher concentrations we obtained a different effect if we apply 2 or 3 days prior to initiation, and reduce the number of nodes which antecede the flower cluster. Similar effects are obtained with 2,3,5-triiodobenzoic and α-(2-naphthoxy)phenylacetic acids. Whether or not this is induction we are not in a position to state. If you accept Dr. Lang's (Ann. Rev. Plant Physiol. 3: 265. 1952) thesis that reduced node number is the criterion for flowering, then we are inducing flowering with these chemicals. But we have to apply very early, at cotyledon expansion, and at high concentrations. If you ac-

cept the thesis that time of flowering is the essential criterion, as Gorter (Proc. Kon. Ned. Akad. v. Wetensch. 52: 1185. 1949) apparently does in her studies with triiodobenzoic acid, then we are not affecting flower initiation.

Dr. Gowing: Dr. Nitsch might be interested to know that the material does not induce flowering in pineapple, in contrast to a number of other compounds with auxin activity. However, it does have very marked formative effects. It leads to a closed tube of leaf tissue rather than the normal open channel-like leaf of the pineapple plant. If dichlorophenylphthalamic acid is applied about the time of natural inflorescence differentiation, it leads to a reduction in the number of fruitlets contributing to the multiple fruit, and a corking between the fruitlet tissues. The effect is somewhat similar to that reported by Muzik and Cruzado (Plant Physiol. 31: 81. 1956) for maleic hydrazide on pineapple in Puerto Rico a few years ago.

Dr. Wain: Have any compounds been made from substituted phthalic anhydrides?

Dr. Teubner: We are investigating a number of these, with chloro- and nitro-substituents on the phthalic ring. These have not been studied with respect to activity in all three tests, since the tomato flowering tests and parthenocarpic fruit tests are quite time consuming. As Dr. Wain pointed out earlier, it's best to examine a whole series of compounds before drawing conclusions as to structure-activity relationships, and we have insufficient information to comment on the effect of substitution in the phthalic ring.

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The Uptake and Fate of C^{14}-labeled 2,4-Dichlorophenoxyacetic Acid in Bean Stem Sections

Ever since the first reports of the involvement of indole-3-acetic acid (IAA) in the control of plant growth, considerable effort has been expended in attempts to explain its mode of action. The relative structural simplicity of the plant growth regulators, as compared to the animal hormones, seemed to lend encouragement to the view that the control mechanisms in plants might be comparatively simple. The morphological manifestations of the action of growth regulators are numerous indeed, but can be divided into three main categories: Effects due to cell elongation, effects due to nondifferentiative cell division, and effects due to cell division accompanied by differentiation. Systems which show a cell elongation response to auxin application have been studied extensively, and the results suggest an effect on the cell wall and/or a stimulation of water uptake as the primary mode of action (7). However, from a fundamental point of view, cell elongation is only the second step of growth, since it must be preceded by cell division. Furthermore, since cell elongation can be largely explained by water uptake, while cell division requires the de novo synthesis of new cell material (i.e., proteins and nucleic acids), the latter process has much more appeal to the biochemist. Under physiological conditions the auxins not only influence cell division, but also the differentiation and the formation of new organs (e.g., rooting, flowering, fruit set, etc.). These changes are very difficult to study without a clear understanding of the control of simple mitosis. In several tissues, however, plant growth regulators are known to promote cell division and callus formation without differentiation when

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applied at relatively high concentrations (3). Under such circumstances, the application of plant growth regulators must disrupt the control mechanisms which are normally existent and cause the proliferative growth of the tissue. It seemed, therefore, that such a system might be suitable for an investigation of the immediate biochemical changes which take place upon addition of a plant growth regulator, in the hope of finding the chemical causes for the initiation of proliferative growth. It is recognized, of course, that these changes cannot be equated to the physiological mode of action of the plant growth regulators. However, the interest here is primarily in the chemical or enzymatic changes which are involved in the disruption of normal growth and the initiation of proliferation, and the use of plant growth regulators to bring about these changes is somewhat incidental. Indeed, the initiation of proliferation by a variety of chemicals (not necessarily plant growth regulators) has been recognized for a long time (6).

The main difficulty in such an investigation is the critical differentiation between the causes and the effects of proliferation. Most of the reported effects of 2,4-dichlorophenoxyacetic acid (2,4-D) on biochemical systems (4, 15, 16, 24) have involved lengthy in vivo pretreatments. It is not at all clear whether these effects are the consequences rather than the primary causes of the morphological and physiological changes which are induced by this compound. These difficulties can be circumvented by approaching the biochemical studies from a kinetic point of view — investigating the rate at which the biochemical changes take place in the very early stages of proliferation, with a view to establishing a precursor-product relationship between the changes themselves. However, before such a study can be undertaken, it is essential that the time interval involved in the initiation of proliferation be clearly defined. To this end it must be established how long and in what form the inducing agent persists in the tissue, what concentrations of inducing agent are required, and what conditions must be met for the initiation of proliferation to take place.

This paper will describe some of our results in trying to define these variables. Since beans are particularly susceptible to callus formation (20, 21), they were used in this study.

MATERIALS AND METHODS

Phaseolus vulgaris, 'Giant Stringless Pod Bean,' was grown in the greenhouse in potting soil between 18 and 33° C. until 3 or 4 trifoliate leaves had fully developed. The plants were harvested just before use, the leaves removed, and the stems immersed in water until used.

In general, the second and third internodes above the primary leaf were used for investigation. The stems were scrubbed in water, cut into sections 7 to 10 mm. in length, and groups of five sections were weighed on a Roller-Smith balance and planted, basal end down, in modified White's medium. The 2,4-D was either incorporated into the medium or applied in small agar blocks to the apical ends of the stems. These blocks were formed either by cutting a thin layer of agar containing 2,4-D into small squares in a petri plate or by drawing the liquefied agar into glass tubing of 4 mm. inside diameter, extruding the agar after solidification, and cutting the agar into sections 2 mm. thick. Very uniform blocks were obtained in this manner. The medium used in these experiments was a modification of White's medium (27) as obtained from Dr. Jacques Lipetz of Yale University. The constituents in g/l were: $Ca(NO_3)_2 \cdot 4H_2O$, 0.2; Na_2SO_4, 0.2; KCl, 0.065; NaH_2PO_4, 0.017; $MgSO_4 \cdot 7H_2O$, 0.36; Hoagland's A-Z solution (14), 1 ml.; Klein and Manos' iron versenate solution (19), 3 ml.; sucrose, 30; and agar, 15. The pH was adjusted to 6.5 to 7.0 before autoclaving. It should be noted that vitamins and glycine were omitted, since it was found that the bean stems proliferated in their presence even without the addition of 2,4-D. In experiments which lasted longer than 3 days, the stems were surface sterilized by immersion in a 1:10 dilution of Clorox for 1.5 min., followed by two rinsings in sterile distilled water. All succeeding operations were carried out under aseptic conditions.

The bean stems were incubated at 25 to 28° C. and 70 to 80 per cent humidity in the dark for 10 days for the development of calluses. They were then harvested and the groups of five stems weighed. Gains in weight were calculated on a fresh weight basis. Preliminary experiments have shown that equivalent results were obtained when using either fresh or dry weights.

The carboxyl-labeled 2,4-D had a specific activity of 22.3 mc/millimole. It was found to be at least 96 per cent radiochemically pure by isotope dilution analysis, and showed only single radioactive spots when chromatographed on Whatman No. 1 paper in isopropanol-ammonia water (25), butanol-propionic acid-water (18), or 90 per cent aqueous butanol (26). Depending on the experiment, the 2,4-D was used undiluted, or was diluted up to tenfold with unlabeled material It was stored as a $10^{-2}M$ solution in acetone in the deep freeze. No evidence of radio-decomposition could be found after 8 months. The final concentration of 2,4-D used was always $10^{-4}M$ unless otherwise specified. At the end of the experiment the stems were rinsed quickly in ice water, placed in test tubes, and frozen in a dry ice-acetone mixture. When necessary, they were stored at —20° C. The radioactive

material was extracted by homogenizing the stems twice in 70 per cent aqueous ethanol in a Lourdes Multi-Mixer. The extracts were freed from fibers by centrifugation, concentrated in vacuo, adjusted to a small volume, and aliquots plated on copper planchets according to the method of Bergmann *et al.* (5). Extraction of the radioactive material was better than 99 per cent by this method. The samples were counted under a thin-window Geiger counter to a reliability of at least 5 per cent and the results corrected for the usual factors and expressed as infinitely thick samples. For the analysis of the radioactive products formed from 2,4-D, the solvent of Jaworski *et al.* (18) and Whatman No. 1 paper were used. The radioactivity of the 1-inch-wide chromatogram strips was detected with the aid of a Micromil window Geiger counter tube on a Nuclear Chicago Corp. Actigraph II. All scans were counted with a full scale reading of 1,000 counts per min. or less, a resolution time of 50 sec., a slit of ⅛ inch, and a scanning speed of 3 to 6 inches per hr. The distribution of the radioactivity in the various regions of the chromatograms was calculated from the average of three determinations of their areas with a planimeter. Preliminary experiments had shown a linear correlation between radioactivity and area without need of corrections for the self absorption of the paper.

The same scanning instrument was used for the determination of the distribution of radioactivity in the bean stems after various conditions and periods of exposure to 1-C^{14}-2,4-D. Sections 10 mm. long were exposed to undiluted radioactive 2,4-D applied at their apical ends. They were harvested and washed as described, and then mounted on glass slides with rubber cement. They were frozen by immersion in liquid nitrogen and dried in vacuo. The dried stems, which resembled hollow cylinders, were slit open longitudinally and mounted side by side on ¾-inch-wide strips of "Scotch" brand cellophane tape, making sure that all the apical ends were perfectly aligned. Marker spots were prepared by cutting 1/16-inch-wide strips of filter paper impregnated with a C^{14}-containing ink. Markers were mounted at known distances ahead of the apical end of the stems and after the basal end. The rest of the tape was covered with filter paper, and the assembly pressed briefly between two metal plates. The strips were then passed through the Actigraph at a rate of ¾ inch per hr. while the recorder was run at 6 inches per hr. In this manner an eightfold scale expansion was achieved. An especially narrow (0.5 mm.) slit-width was used for maximum resolution. Experiments were scanned in triplicate and the averages of the three results determined graphically.

Carbohydrate material was detected on the chromatograms with

a naphthoresorcinol spray (8) and amino acids with a 0.2 per cent ethanolic ninhydrin spray.

RESULTS AND DISCUSSION

The Effect of 2,4-D Concentration, Exposure Time, and the Origin of the Bean Stem Internode Used

In all the previously reported studies on the induction of callus formation in bean stems in vitro, the tissue was maintained in the presence of the growth regulator for the full duration of the experiment (2, 12, 22). Since it would be impossible to conclude when induction of proliferation took place under those conditions, it was important to establish the minimum effective concentration of 2,4-D, and how long the exposure to 2,4-D had to last. In initial qualitative experiments it was found that the shortest exposure time tested, 1 day, was sufficient for the induction of fully developed proliferations, if a concentration of $10^{-4}M$ 2,4-D was supplied. When the 2,4-D concentration was decreased to $10^{-5}M$ an exposure of 3 days was necessary.

While it is recognized that the calluses which are formed as a result of the application of 2,4-D to the bean stems are not true tumors, in that they are not auxin autonomous, it was, nevertheless, of interest to determine if any potentiation of the callus formation took place when the tissue was cut and planted for varying lengths of time prior to the application of 2,4-D. For this purpose, stem sections were first planted on plain White's medium, exposed to 2,4-D for 1 day, and then returned to White's medium. Preliminary results indicate an increase in proliferation when the stems are pre-incubated for 1 to 3 days in this fashion prior to the application of 2,4-D.

Figure 1 shows the time course of the uptake of 1-C^{14}-2,4-D. Each point is the average of four determinations. The radioactivity of the stem increased very rapidly and reached a plateau 2 to 3 hrs. after application. At this time about 10 to 20 per cent of the radioactivity initially present in the agar blocks had been taken up into the stems. The over-all concentration of the 2,4-D in the sap of the stems was about $3 \times 10^{-5}M$ at that time. When the agar blocks were removed at the end of 3 hrs. (arrow on the figure) and the stems allowed to stand for a continued period of time, the results depicted by the dotted line in Figure 1 were obtained. It will be noted that while the initial drop in radioactivity was very rapid, further changes were very slow. In a separate experiment it was found that at the end of 18 days some 60 per cent of the radioactivity initially present was still found in the stems.

It was of interest to identify the factors controlling the uptake of 2,4-D. Table 1 summarizes the results of these experiments. Ten-

mm.-long bean stems were used for the first six lines of this table, and the results are the averages of four replicate determinations. The stems were exposed to $10^{-5}M$ radioactive 2,4-D and varying amounts of unlabeled 2,4-D to the final concentrations given for a period of 6 hrs. As can be seen, the incorporated radioactivity remains constant above the lowest concentration of 2,4-D supplied, indicating that the uptake of 2,4-D was directly dependent on the concentration applied. The last two lines of this table depict the effect of the cross-sectional area of exposed surface on the uptake of 2,4-D. The same weights of tissue were used in both these conditions, but in the upper line the stems were cut into 10 mm. lengths and on the lower line into twice as many 5 mm. lengths. It can be seen clearly that the uptake is directly dependent on the exposed surface.

One would expect to find exposure to 2,4-D in liquid a much more effective method of inducing proliferation, since the area ex-

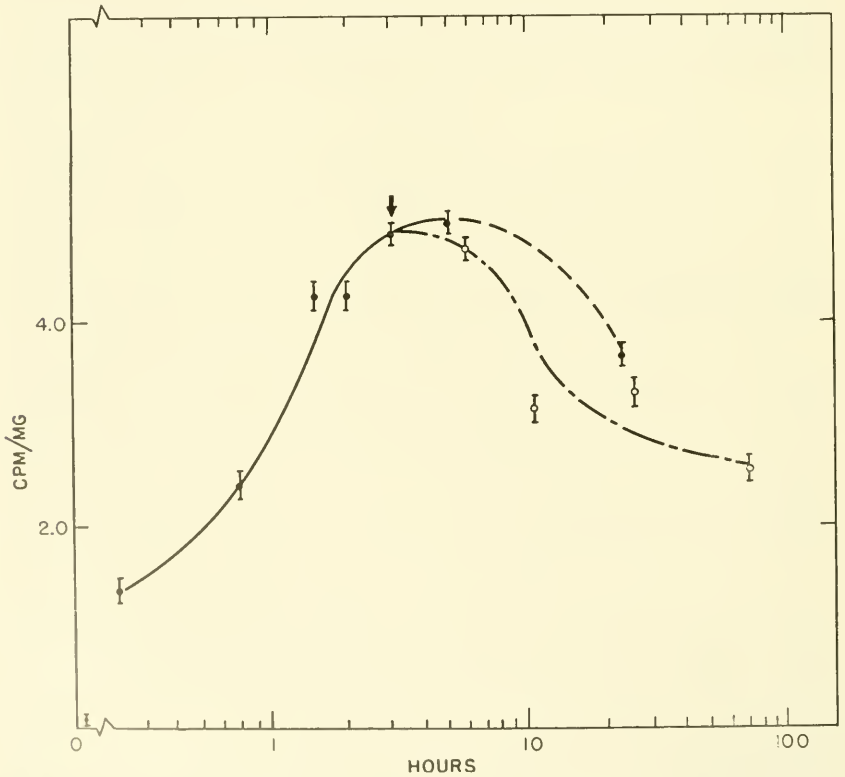

Fig. 1. The over-all changes in radioactivity of bean stem sections with time after application of 1-C^{14} 2,4-D. Closed circles, stems in contact with 2,4-D for the entire experiment. Open circles, 2,4-D removed at the end of 3 hrs.

Table 1. The effect of 2,4-D concentration and exposed area on 2,4-D uptake by bean stem sections.

Molar Concentration of 2,4-D	No. of Stems Per Sample	Stem Length, Mm.	Uptake, CPM/Mg Fresh Wt.
1×10^{-5}	5	10	0.372 ± .030
3×10^{-5}	5	10	0.118 ± .017
1×10^{-4}	5	10	0.124 ± .028
2×10^{-4}	5	10	0.118 ± .008
5×10^{-4}	5	10	0.124 ± .012
1×10^{-3}	5	10	0.147 ± .0087
1×10^{-4}	5	10	0.076 ± .004
1×10^{-4}	10	5	0.133 ± .013

posed would be much greater. However, when stems were exposed to 2,4-D in liquid, no proliferation took place if they were maintained in liquid (even in the absence of 2,4-D after the initial exposure). If such stems were planted on agar after exposure to 2,4-D in liquid, proliferation did take place. The reason for this difference between liquid and solid culture is not clear and is being investigated further.

No clear-cut differences could be established between the effect of 2,4-D on stem sections taken from various internodes of the plants.

Polarity of Uptake and Transport, and Localization of Radioactivity

No effect of polarity on the uptake of 2,4-D could be found. The average of eight replicate determinations of ten stem sections each showed a specific activity of 0.303 ± 0.022 cpm/mg fresh weight when the stems were planted with their apical ends down, and 0.307 ± 0.031 cpm/mg fresh weight when planted with the basal end down. Figures 2 and 3 show the effects of various lengths of incubation with labeled and unlabeled 2,4-D on the distribution of the radioactivity along the stems. To permit a better comparison of the distribution curves shown in Figure 2, the results were factored to adjust all the peaks of radioactivity to the same level (1.0). A clear broadening of the radioactive zone with time is apparent, and the peak of activity appears to shift about 0.5 mm. down the stem in the first 4 hrs. If application of labeled 2,4-D is preceded by application of unlabeled 2,4-D the pattern obtained very closely resembles the one for the equivalent exposure to radioactive 2,4-D (Figure 3). Removal of the 2,4-D, and particularly application of unlabeled 2,4-D after the radioactive material, resulted in a distinct spreading of the peaks. The unlabeled material appears to accelerate the loss of the radioactive material from the stems. However, it is of interest that the position of the radioactive peak remains 2

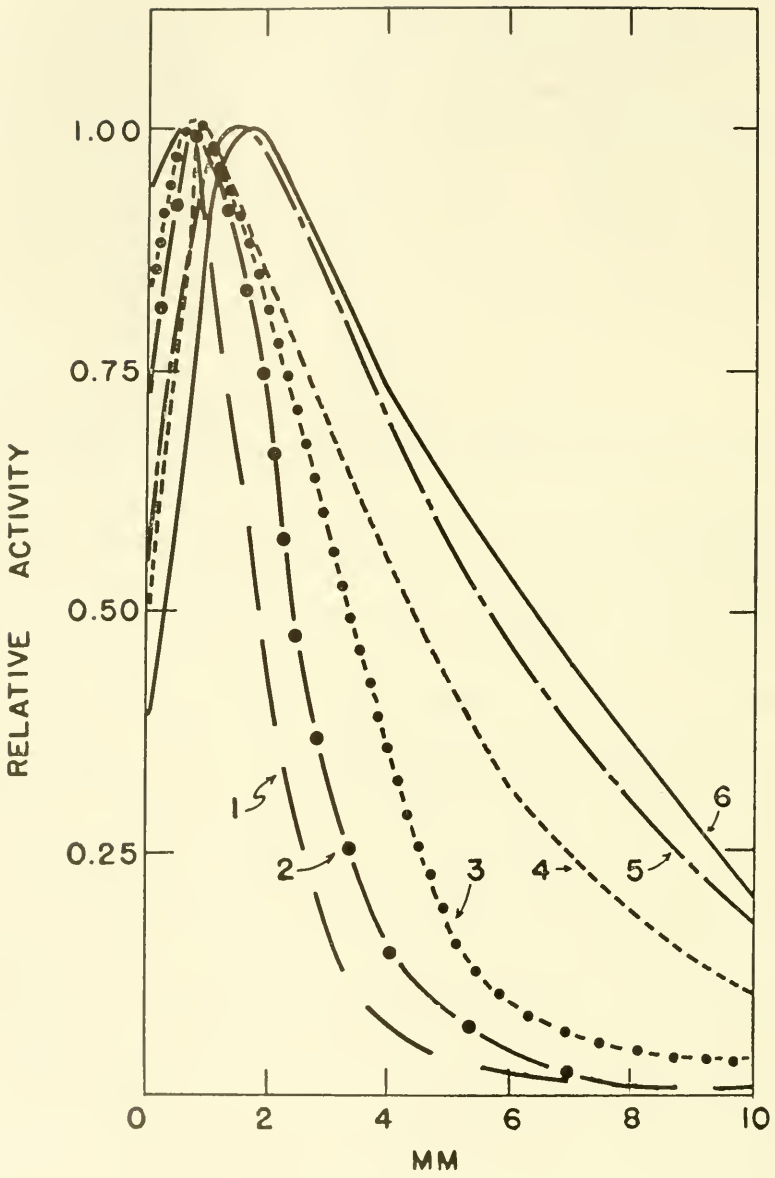

Fig. 2. The relative distribution of radioactivity in 10 mm. bean stem sections as a function of duration of exposure to 1-C^{14}-2,4-D. Curve 1, 1-hr. exposure. Curve 2, 2 hrs. Curve 3, 4 hrs. Curve 4, 8 hrs. Curve 5, 24 hrs. Curve 6, 72 hrs.

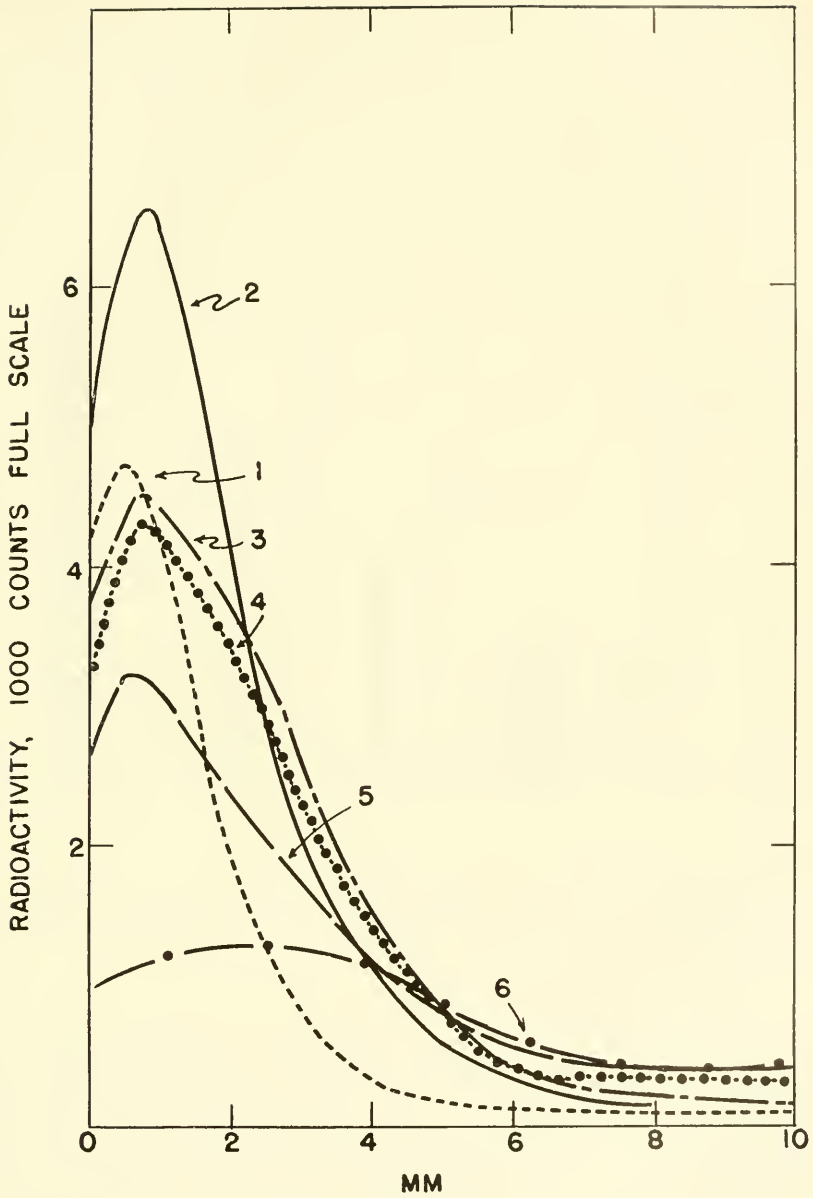

Fig. 3. The effect of various treatments on the localization of radioactivity in bean stem sections following exposure to 1-C^{14}-2,4-D. Curve 1, 1-hr. exposure. Curve 2, 2-hr. exposure. Curve 3, 4-hr. exposure. Curve 4, 2-hr. exposure to unlabeled 2,4-D followed by 2-hr. exposure to radioactive 2,4-D. Curve 5, 2-hr. exposure to radioactive 2,4-D followed by 2 hrs., with no additions. Curve 6, 2-hr. exposure to radioactive 2,4-D followed by 2-hr. exposure to unlabeled 2,4-D.

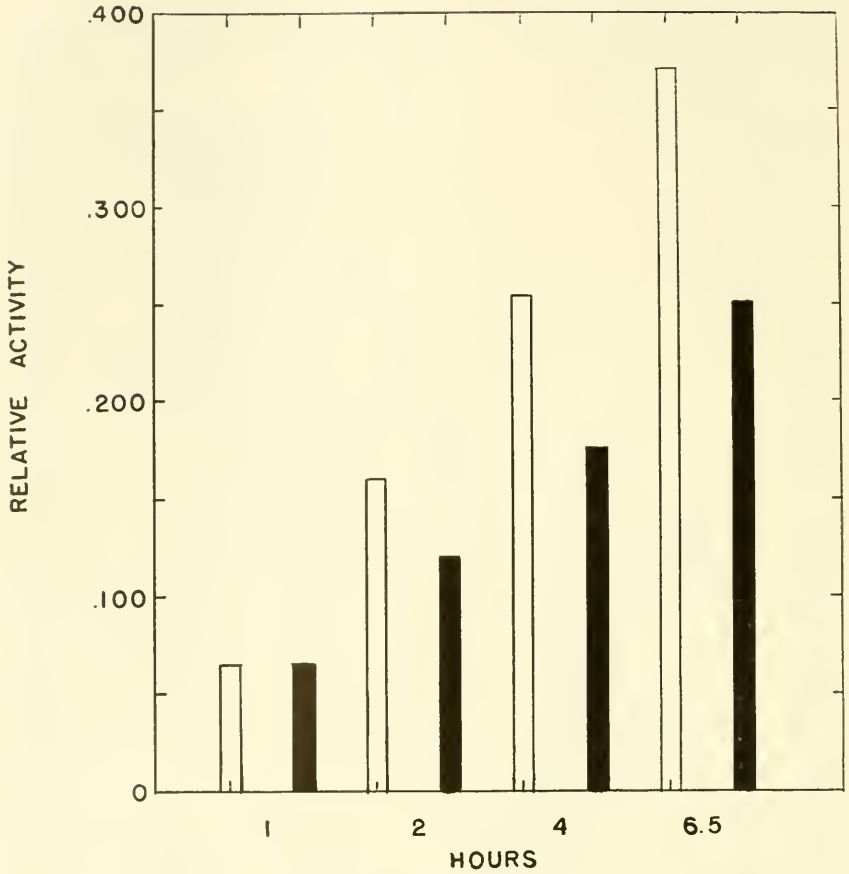

Fig. 4. The effect of polarity on the localization of radioactivity in bean stems following exposure to 1-C^{14}-2,4-D. The relative radioactivities 5 mm. from the point of application are compared. Open bars, planted basal end down. Closed bars, planted apical end down.

to 3 mm. from the point of application, even in the presence of the unlabeled 2,4-D. This suggests that the dilution of label in this area was very gradual, perhaps because the radioactive material was bound in some way to the cellular material. Figure 4 shows the effect of polarity on the transport of radioactive material through the stem sections by comparing the relative radioactivities 5 mm. from the point of application of 1-C^{14}-2,4-D. The results were factored as in Figure 2. In contrast to the absence of polarity effects on the total uptake of 2,4-D, the translocation of radioactivity in the sections was clearly faster in the apex to base direction.

The Fate of Radioactive 2,4-D in the Stem Sections

As has been reported by Fang *et al.* (11), 2,4-D is relatively immune to oxidative attack by plant tissues. Accordingly, less than 1 per cent of the radioactivity which was taken up by the stems could be recovered as CO_2 in a KOH trap. Thus the activity which is lost from the stems must be presumed to be liberated into the agar medium at the basal end of the stems. However, as would be expected from the results shown in Figures 2 and 3, it proved very difficult to demonstrate a diffusion of radioactivity from the stems into the agar. The gradient established in the stems results in very little radioactivity near their basal ends. Undoubtedly the concentration in the agar immediately adjacent to the stems cannot exceed the concentration in the stems at that point. For technical reasons it has not proved possible to concentrate the radioactivity from sufficiently large volumes of agar to get significant counts, or to identify the compounds involved. Experiments which are designed to establish a complete balance sheet for the radioactivity applied to the stems are now in progress.

In agreement with the results of Jaworski *et al.* (18), and of Butts and Fang (9), who used whole plants, we found that the stem sections converted 2,4-D to at least three compounds resolvable by paper chromatography. One of these, having an R_f of 0.5 in the solvent of Jaworski *et al.* (18), was formed in the largest amount, and, after 3 days' incubation, comprised up to 60 per cent of the total radioactivity in the extracts. Figure 5 shows the distribution of radioactivity

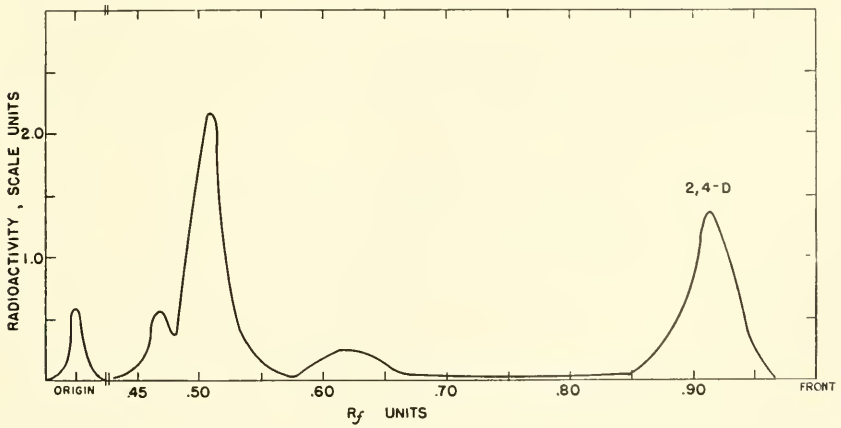

Fig. 5. The resolution of the radioactive products formed from 1-C^{14}-2,4-D in beans by paper chromatography.

on a typical chromatogram. The compound having an R_f of 0.5 was eluted and rechromatographed. A single radioactive peak with the same R_f was found. As was reported by Jaworski and Butts (17), refluxing in 2*N* HCl for 2 hrs. liberated a compound having an R_f similar to that of 2,4-D in the phenol-water and butanol (17) solvent systems. Addition of 1-C^{14}-2,4-D during the isolation procedure did not give rise to any radioactive peaks on the chromatograms other than that of 2,4-D itself. It must be concluded, therefore, that this material is a compound formed from 2,4-D by the plants. The suggestions that this compound is a glycoside (17) or a peptide (9) of 2,4-D must be viewed with some doubt, particularly since the band of R_f 0.5 on the chromatograms does contain carbohydrate as well as ninhydrin positive material. These ninhydrin and carbohydrate containing bands do not, however, coincide with the radioactive band on rechromatography.

The Biological Activity of the Product Formed From 2,4-D

In order to define the time during which the initiation of proliferation takes place, it was important to establish whether the compound of R_f 0.5 which is formed from 2,4-D by the bean stem sections has the capacity to induce proliferation, or, indeed, possesses any other biological activity. Table 2 summarizes the results obtained when $10^{-3}M$ concentrations of this material (as calculated from radioactivity) were applied to bean stem sections. The material used had been obtained by mass isolation using the paper chromatography technique described above, and thus contained several nonradioactive contaminants of unknown concentration. Upon rechromatography it showed only one radioactive spot (R_f 0.5). It will be seen that this material was completely inactive in causing the proliferation of the bean stems, even though it was applied at ten times the equivalent concentration which would be necessary for the maximum ef-

Table 2. The activity of a crude preparation of the main radioactive product formed by beans from carboxyl-C^{14}-2,4-D in the bean stem proliferation assay.

Sample	Gain in Fresh Weight, Per Cent
Control	24 ± 6
$10^{-4}M$ 2,4-D	166 ± 14
$10^{-3}M$ R_f 0.5 band	14 ± 7

fectiveness of 2,4-D. Furthermore, the material appeared to be slightly inhibitory both in the bean stem sections and in the *Avena* internode test (23). In bean stems no evidence of callus formation at the apical cut surface could be found in the sections which had been exposed to this preparation, while the control stems always show some swelling at this point. In the *Avena* test a concentration of $3 \times 10^{-6}M$ of this material was slightly inhibitory to growth, while lower concentrations were totally inactive. The maximal activity of 2,4-D in the *Avena* assay is found between 10^{-6} and $10^{-7}M$. It appears likely, therefore, that this material is a detoxification product of 2,4-D and does not have the biological properties of the auxin itself. This conclusion is also supported by the findings of Fang *et al.* (10) that 3-(*p*-chlorophenyl)-1,1-dimethylurea (CMU) is converted to a similar inactive product in beans and by those of Hay and Thimann (13) on the fate of 2,4-D in vivo. The findings of Andreae and Good (1) that 2,4-D is not detoxified by the formation of the aspartyl derivative nearly as readily as are other herbicides, seem to be contradictory to these findings. However, in the absence of any reliable information on the nature of this material, and in view of the large differences of experimental conditions employed, the two findings may not be incompatible.

SUMMARY

Using an in vitro incubation system it was shown that exposure to $10^{-4}M$ 2,4-D for 4 hrs. is sufficient to cause the initiation of extensive callus formation in bean stem sections. The uptake of 2,4-D during this period is dependent on the concentration of 2,4-D supplied and on the area exposed. Further, no clear-cut difference between the various internodes of young bean plants could be demonstrated, nor could a difference in uptake from the apical or basal end of the stems be shown.

After apical application of 1-C^{14}-2,4-D to the stems, the peak of radioactivity was a few mm. below the point of application and traveled down with time. The width of the peak increased markedly at longer exposures. Apical application of unlabeled 2,4-D accelerated the disappearance of radioactivity from stems which had previously been exposed to C^{14}-2,4-D, although the location of the peak of radioactivity remained essentially unchanged. Decarboxylation and release of $C^{14}O_2$ appear to be a minor path in the metabolism of 2,4-D by the bean stems. Most of the radioactivity was recovered in the form of a complex of unknown structure which yielded 2,4-D upon acid hydrolysis.

ACKNOWLEDGMENTS

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Some Physical-Chemical Aspects of Synthetic Auxins With Respect to Their Mode of Action[1]

It has become very apparent that the mechanism of action of synthetic plant growth regulators and the relation of their chemical structure to this activity is inextricably interwoven. Many attempts have been made to relate structure of the compound to activity, or to afford an explanation of the mechanism of action. Either case involved the necessity of considering the other facet of these interrelated problems. Thus, in attempting to explain why the varying of the organic structure of a synthetic plant growth regulator modifies its activity, it became necessary to invoke a mechanism of action compatible with the observations.

Most of the work attempting to relate structure of synthetic growth substances to their plant growth regulating activity has been from the standpoint of the organic chemistry of these molecules (1, 9, 13, 17, 26). It has been found that the number and kind of atoms in the molecule as well as their arrangement in relation to each other are of prime importance in determining whether or not the molecular species will be active. However, it must be remembered that the physical properties and geometry of the molecule are related to activity also. This paper is concerned with an attempt to relate some physical chemical aspects of synthetic plant growth substances to the biological action of these chemicals.

[1] Taken in part from a doctoral dissertation submitted to the Graduate School, University of Oregon, by senior author. Also Technical Paper No. 1263, Oregon Agricultural Experiment Station.

Shortly after the isolation and identification of indole-3-acetic acid, it was discovered that certain synthetic acids possessed the ability to produce responses from plants similar to that of IAA. Koepfli *et al.* (17) examined the activity of a number of indole derivatives, and this was followed in turn by the discovery of the activity of 1-naphthaleneacetic acid (36) and the phenoxyacetic acids (37, 38) as well as the biological activity of a number of related substances (33, 38). It was only natural that in the course of such studies attempts would be made to relate the structure of these molecules to their activity and to speculate as to their possible mode of action. It is nearly a requirement that any proposed theory of the relationship of structure to growth regulating activity must be premised on a mechanism of action consonant with the molecular structures involved (14).

A number of interesting theories concerning the mode of action of these substances and the significance of molecular structure relationships to activity have been proposed (3, 5, 9, 11, 18, 19, 22, 23). Most of the theories, wherein the organic structure of molecules was considered, propose a mechanism of action involving a chemical reaction between an enzyme molecule and the growth regulator (10, 11, 18). It would probably be naive to assume that the action of these chemicals could come about in any way than through their effect on the enzymes of the organism. On the other hand it becomes a more difficult task to obtain conclusive evidence as to the mechanism by which a growth regulator interacts with an enzyme. Various mechanisms have been proposed, ranging from the suggestion that a growth regulator serves as a prosthetic group involving a two-point attachment to the enzyme surface (10, 11), and that the growth regulators are chelating agents for essential metals (15).

The interaction of growth regulating chemicals with the enzymes of the protoplasm by physical rather than chemical forces has been considered in the theory of Veldstra (30) and the work of Brian and Rideal (8). Some support for this theory comes from the detailed consideration of dosage-response curves obtained with growth regulating chemicals. Northern's observation of a reduced viscosity of the cytoplasm upon treatment with a growth regulator was explained on the basis of disassociation of the proteins of the cytoplasm (24). This observation would be consonant with the theory of adsorption on the surface of select proteins, weakening the intermolecular bonds and resulting in a loss of viscosity of the gel of the cytoplasm. The increased streaming of cytoplasm noted upon treatment with plant growth regulators would also tend to bear this out (33).

Consideration of possible physical interaction of the growth regulating phenoxyacetic acid with proteins of the organism came about

through the reports indicating failure to demonstrate the competitive effects of 2,4-D on enzymes (27, 28). While many enzymes have been shown to be affected by the presence of this chemical, there seems to be a lack of evidence of a clear-cut competitive effect of sufficient order of magnitude to account for the rather magnificent biological responses induced by the chemical.

BIOLOGICAL ACTIVITY OF CHLOROPHENOXYACETIC ACIDS

In order to evaluate any physical differences that may be significant in the relation of structure to activity and mode of action of the chlorophenoxyacetic acids, it was necessary to assess and rate the biological activity of members of this series. Such rating was done by means of the root growth test using corn and lupine. The results of this rating are to be found in Table 1. The values given in Table 1 are the molar activity in relation to 2,4-D which was taken as 100. These values are in general accord with many published values given in the literature.

It is noted in this table that there is a very low order of activity for those compounds substituted in the 2 and 6 position, which observation was reported by Muir and Hansch (22), Leaper and Bishop (18), and Osborne *et al.* (26). It was suggested by Muir *et al.* (23) that the 2,6-disubstituted compounds were relatively inactive because of the requirement for free *ortho* position with which to react with a substrate molecule. Although it has been suggested several times that the phenoxyacetic acids probably reacted with the protein to form a new chemical species, the failure to find such a species by means of C^{14}-labeled 2,4-D or a specific effect with any of the intermediate Krebs cycle substrates (27) would appear to argue against specific chemical

Table 1. The percentage molar activity of some phenoxyacetic acids as measured in root-growth tests with corn and lupine.

Substituted Phenoxyacetic Acid	Molar Activity, Per Cent of 2,4-D
Parent Compound	0.05
2-Chloro-	9.4
3-Chloro-	15.0
4-Chloro-	53.0
2,4-Dichloro-	100.0
2,6-Dichloro-	11.0
3,4-Dichloro-	78.8
3,5-Dichloro-	32.7
2,4,5-Trichloro-	98.5
2,4,6-Trichloro-	35.0
2,3,5-Trichloro-	21.2

reactions. The suggestion of Veldstra (30) that the compounds may be acting at a protein-lipoid surface leads to speculation that the action of these compounds may be physical in nature. If the action of these compounds is physical, then there should be some measurement which would show obvious differences in the physical properties of the biologically active and the biologically inactive members of the series. The work of Wright (35) in correlating the biological activity of insect repellents to their infrared absorption spectra suggested that the infrared spectra of the chlorophenoxyacetic acids may reveal some relation to activity.

INFRARED AND ULTRAVIOLET SPECTRA OF CHLOROPHENOXYACETIC ACIDS

A series of chlorinated phenoxyacetic acids was examined qualitatively for infrared absorption, using 1 mg. of chemical to 300 mg. of potassium bromide in a pellet form. The spectra were run from about 2 μ out to 14 μ (Figure 1). The most obvious difference noted between an active (2,4-D) and a relatively inactive compound (2,6-D) was to be found in the absorption in the region of 12 μ (825 cm.$^{-1}$). The major difference noted in this region is that the biologically active compound had an intense absorption band at 794 cm.$^{-1}$, whereas the inactive compound had only a moderately strong absorption in this region, characterized by a missing Q branch. This fact is illustrated by

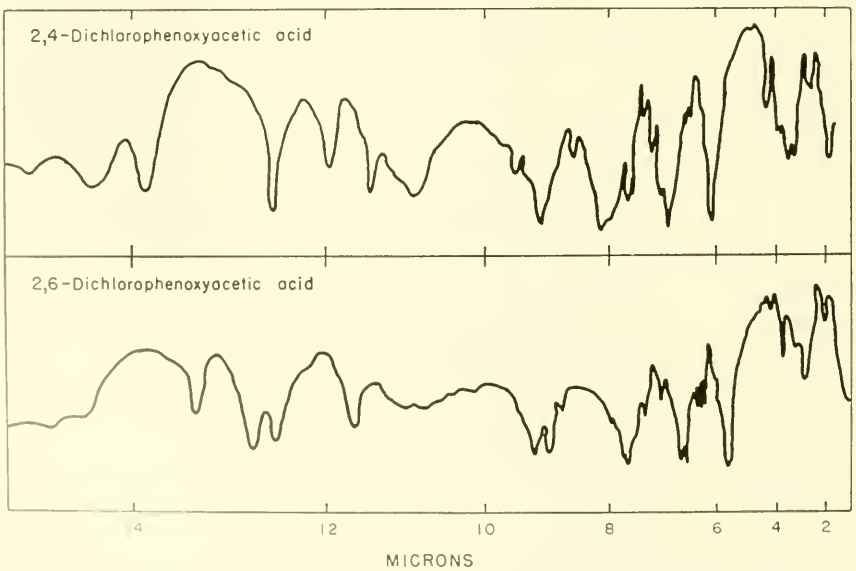

Fig. 1. Infrared absorption spectra of 2,4- and 2,6-dichlorophenoxyacetic acid.

Table 2. Infrared absorption of some phenoxyacetic acids and other growth substances.

Acid	Relative Absorption in 850 to 789 Cm.$^{-1}$ Region*	Presence or Absence of Q Branch
Phenoxyacetic	M	−
2-Chlorophenoxyacetic	W	
3-Chlorophenoxyacetic	M	+
4-Chlorophenoxyacetic	S	+
2,4-Dichlorophenoxyacetic	S	+
2,5-Dichlorophenoxyacetic	M	+
2,6-Dichlorophenoxyacetic	M	−
3,4-Dichlorophenoxyacetic	S	+
3,5-Dichlorophenoxyacetic	W	−
2,4,5-Dichlorophenoxyacetic	S	+
2,4,6-Dichlorophenoxyacetic	W	−
2,4-Dibromophenoxyacetic	M	+
Indole-3-acetic	M	+
1-Naphthaleneacetic	S	+

* S = strong; M = medium; W = weak absorption.

the absorption spectra for 2,4-D and 2,6-D. A series of such compounds was examined for infrared absorption in this region. The spectra were assigned a code number and were then examined and sorted into two groups, one being those thought to have appreciable biological activity and those having moderate or low biological activity. Following the sorting, the spectra were decoded and the absorption spectra and biological activity were found to give a positive correlation of 0.80.

A summary of the absorption characteristics of several of the chlorophenoxyacetic acids, 1-naphthalene acetic acid, and indole-3-acetic acid is given in Table 2.

The infrared absorption of substituted aromatic compounds in the 800 cm.$^{-1}$ region has been suggested by Bellamy (2) as being due to the in-plane, out-of-plane vibrations of the nuclear substituents. If this is indeed the case, the absorption spectra noted here might suggest that the biologically active compounds have a uniformly coupled plus-minus movement of the nuclear substituents, where the compounds of lesser biological activity have an alternate vibration pattern. In the former case, this behavior would make for a greater possibility of interaction of the ring with a hydrocarbon chain such as forms the skeleton of a protein molecule.

The apparent success in relating biological activity to infrared absorption spectra of the chlorophenoxyacetic acids raised a question as to whether there may be a similar relation between the electronic configuration of these molecules as indicated by their ultraviolet ab-

Table 3. Ultraviolet absorption and water solubility of some phenoxyacetic acids.

Substituted Phenoxyacetic Acid	mμ	Em × 10^{-3}	H_2O Solubility Moles/L
Parent Compound	269	1.4	
2-Chloro-	273	1.7	7.2×10^{-3}
3-Chloro-	273	1.5	11.6×10^{-3}
4-Chloro-	279	1.3	4.3×10^{-3}
2,4-Dichloro-	283	1.9	2.7×10^{-3}
2,5-Dichloro-	279	2.2	2.4×10^{-3}
2,6-Dichloro-	278	0.31	7.2×10^{-3}
3,4-Dichloro-	282	1.6	2.3×10^{-3}
3,5-Dichloro-	284	1.4	5.5×10^{-3}
2,4,5-Trichloro-	289	1.2	9.9×10^{-4}
2,4,6-Trichloro-	287	0.67	6.2×10^{-4}

sorption and their activity. Accordingly, the ultraviolet absorption spectra of a number of these compounds were determined with a Cary recording spectrophotometer and the molar extinction coefficient in the neighborhood of 280 μ calculated. The results of these determinations are given in Table 3. The results in Table 3 are not as unequivocal as was the case with the infrared absorption. It will be noted in the monosubstituted compounds that the molar extinction coefficient is actually less for the compound of greatest activity. However, the relationship between absorption spectra and biological activity holds very well for the di- and tri- substituted isomers studied. However, when these ultraviolet absorption data are studied in relation to the solubility of the compounds, a slightly different picture emerges. Thus, the molar solubility of the 2- and 3-chlorophenoxyacetic acids is approximately twice and three times that of the *p*-chlorophenoxyacetic acid, being $7.2 \times 10^{-3}M$, $11.6 \times 10^{-3}M$, and $4.3 \times 10^{-3}M$, respectively, for these compounds. In light of the relationship of water solubility to biological activity, it is reasonable to suppose that a higher molar dosage of the more soluble compounds would be required to achieve the same degree of biological activity, all other factors being equal (12). Table 4 represents an attempt to summarize the relation of the physical properties to biological activity. It will be noted that a negative value for any of the properties is associated with a compound of low activity. Thus, the importance of these properties to activity is clearly demonstrated.

ACTION OF 2,4-D ON ROOTS AND MITOCHONDRIA

If, as previously suggested, the primary event in the action of these synthetic growth regulators is adsorption, it should be possible to demonstrate a ready reversibility of the action of these substances (9). Thus, it would seem that removal of a plant or an organism from a short exposure to the chemical should result in a recovery from

Table 4. The relationship of biological activity to absorption spectra and water solubility of phenoxyacetic acids.

Substituted Phenoxyacetic Acid	Relative Biological Activity	H_2O Solubility $>6 \times 10^{-4}$ and $<5 \times 10^{-3}$ Moles/L	IR Absorption With Q Present	UV Em 280 1.2×10^3
Parent Compound. .	.05	−	−	+
2-Chloro-	9.4	−		+
3-Chloro-	15.0	−	+	+
4-Chloro-	53.0	+	+	+
2,4-Dichloro-	100.0	+	+	+
2,5-Dichloro-	89.0	+	+	+
2,6-Dichloro-	11.0	−	−	−
3,4-Dichloro-	78.8	+	+	+
3,5-Dichloro-	32.7	−	−	+
2,4,5-Trichloro-	98.5	+	+	+
2,4,6-Trichloro-	21.2	−	−	−

the effects of the chemical. The exposure period would have to be of sufficiently long duration to assure that adsorption was not a limiting factor; however, this was demonstrated by Blackman (7) and Osborne (25) to be a relatively short period. Such an experiment was performed with corn seedlings, exposing them to a concentration of $6.7 \times 10^{-7}M$ of chemical per seedling for varying lengths of time up to 48 hrs. Removal of the seedling from an exposure up to 24 hrs. resulted in a complete recovery, indicating that up to this period of time the action of the chemical was reversible. Measurement of the response was made 48 hrs. after the beginning of the exposure.

A more definitive experiment of this type would be an exposure of subcellular particles from the plant to a chemical such as 2,4-D, removal from exposure, washing of the particles, and a measurement of enzymatic activity. Mitochondria would seem to be the fraction of choice since isolation of these particulate fractions from plants and measurement of their activity is now a well-established practice. Mitochondria were isolated from cabbage in the usual manner, one aliquot of the preparation being held as a control; another incubated with 2,4-D, followed by washing with the suspending media to remove excess 2,4-D; a third aliquot was washed with the suspending medium to serve as the washed control. Previous experience had demonstrated that a 10 min. exposure to 2,4-D resulted in equally good inhibition as longer exposures. In order to assure complete saturation of the mitochondria with 2,4-D, they were incubated for 30 min. before removing by centrifugation and washing. Samples of the control, the washed control, and the 2,4-D treated mitochondria were tested for enzymatic activity in a Warburg apparatus. To aliquots of the latter

Table 5. Washing as a factor in the reversal of 2,4-D inhibition of oxygen uptake by mitochondria.

Treatment	O_2 Uptake, $\mu l/Hr$		
	No 2,4-D	+ 2,4-D	Per Cent Inhibition
Control	533		
2,4-D washed	515	188	64
Washed control	468	192	58

two 2,4-D was added to assure that the activity was still 2,4-D sensitive. The results of this study are to be found in Table 5.

It will be noted that exposure of the mitochondria to 2,4-D followed by washing to remove the chemical, resulted in a complete recovery of the activity as measured by oxygen uptake. However, addition of 2,4-D to the Warburg vessel demonstrated that the mechanism responsible for oxygen uptake was still 2,4-D sensitive. A similar situation was to be found with the washed control. It should be noted in passing, however, that long exposure of the mitochondria to 2,4-D, 40 min. or more, resulted in an irreversible loss of their oxidative capacity.

The behavior noted with these mitochondria strongly supports the theory that the primary event in the action of these chemicals is adsorption. It is obvious that had this chemical reacted with constituents of the mitochondria to form a chemical complex or compound, simple washing would not have removed a sufficient amount of the chemical to restore the full oxidative capacity of the mitochondria. On the other hand, if this chemical were simply adsorbed by physical forces on the surface of the enzyme, one would expect that washing would remove substantially all of the 2,4-D, thus permitting recovery of the oxidative ability. This apparent adsorption on the surface of protein also affords a possible explanation of why 2,4-D gives a rather general nonspecific inhibition of oxygen uptake of mitochondria using different members of the Krebs cycle intermediates. In seeking further evidence in support of the theory that 2,4-D is adsorbed by proteins, a study of 2,4-D adsorption by proteins was made by equilibrium dialysis. This study clearly demonstrated the adsorption involving four molecules of chemical per molecule of protein.

One of the characteristics of an adsorbing system is that less of the solute species will be adsorbed by a surface as temperature increases (9). Therefore, it should be possible to ascertain whether or

Table 6. Effect of temperature on the 2,4-D inhibition of oxygen uptake by mitochondria.

	O_2 Uptake, μl/Hr	
	25° C.	30° C.
Mitochondria	1068 ± 45	1078 ± 72
Mitochondria + 2,4-D, $4.24 \times 10^{-3}M$	285 ± 6	394 ± 5
Per cent inhibition	73	63.4

not the chemical was being adsorbed by the protein of the mitochondria by measuring the oxygen uptake at two different temperatures. If the 2,4-D is adsorbed, one would expect less inhibition of oxygen uptake at a higher temperature. This proposition was tested by using a mitochondrial preparation from cabbage, one aliquot of which was run at 25° C., the other at 30° C. In each case a control was compared with 2,4-D treated samples. The results of this study are reported in Table 6.

The data in Table 6, which represents an average of triplicate runs, clearly demonstrate that the rate of inhibition decreases with temperature. It is interesting to note that the decrease in activity, which is 13.7 per cent, is in good agreement with the increase in water solubility of 2,4-D over this same temperature range (18.5 per cent). The increase in the solubility again would make for decreased adsorption by virtue of the change of chemical potential of the solute species. These findings afford further support of the theory that the primary event involved in the action of growth regulators is one of adsorption.

ACTION OF 2,4-D ON CRYSTALLINE ENZYMES

Since it would appear from the foregoing data that physical adsorption of the chemical by an enzyme surface is involved in the biological action of these chemicals, the question arises as to the consequence to the protein of this adsorption. While many of the more common cases of enzyme inhibition come about by the chemical reacting with a particular functional group of the protein or competing with the substrate for a specific site, cases of inhibition by adsorption are less well known. While the adsorption probably occurs at sites adaptive to the structure of the compound and is therefore specific, these sites are not those occupied by the substrate. This is borne out by the noncompetitive nature of the inhibition of

peroxidase found in this laboratory. It would appear rather that there may be one of two alternative consequences of this adsorption: (a) the adsorption restricts energy transfer to the protein molecule, thus modifying the rate of reaction, or (b) the adsorption of the solute species results in a modification of the structure of the protein such that the kinetics of the reaction it catalyzes is changed (29, 32).

It has been suggested that inasmuch as many proteins contain fluorescent centers, the fluorescence intensity of such a species might correlate with enzymatic activity. Indeed such has been shown to be the case (21, 31). It has been suggested by Karreman *et al.* (16) that the fluorescent emission is the mechanism by which energy may be transferred from an enzyme to its substrate molecule. It would appear, therefore, that if 2,4-D reduced the fluorescence intensity of the enzyme, this would indicate interference with energy transfer. Also, if the enzymatic activity of the protein were affected by the concentration of 2,4-D, which reduces fluorescence intensity, this would indicate that adsorption interferes with energy transfer. Upon testing this theory with glyceraldehyde phosphate dehydrogenase and α-amylase, no discernible effect on enzymatic activity was found at a concentration of 2,4-D at which fluorescence intensity was markedly reduced. This measurement was made using the Aminco-Bowman spectrophotofluorometer.

Inasmuch as reduction in fluorescence intensity did not correlate with the change in enzymatic activity, it was felt that the effect of the chemical in modifying the enzymatic activity of the protein was not due to restriction or modification of energy transfer within the protein. From these considerations the assumption was made that the chemical exerts its influence by modification of the structure of the enzyme molecule. It is a well-known phenomenon that the modification of a catalyst's surface materially changes the property of the surface as a catalyst. In order to study this problem, attention was then turned to finding an enzyme, the activity of which was known to be a function of the structural integrity of the protein molecule. Peroxidase (horse-radish) has been cited as an example of a protein capable of undergoing reversible denaturation with heat. Thus, the degree of reversible denaturation of peroxidase may be followed by measurement of its enzymatic activity as a function of temperature. It was reasoned that if the adsorbed 2,4-D brought about a change in the structure of the molecule, this should facilitate the heat denaturation of peroxidase. Accordingly a study of the rate of change of horseradish peroxidase activity with increasing temperature with and without 2,4-D was followed. The results of this study are shown in Figure 2.

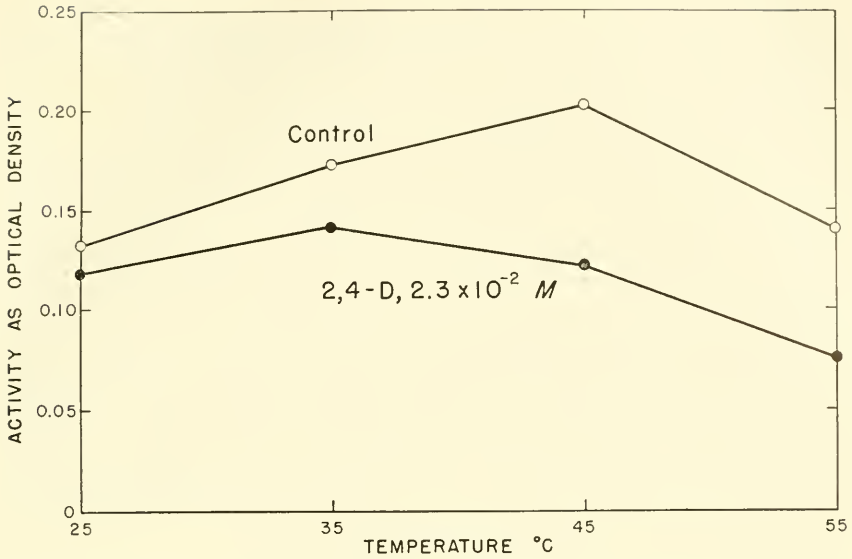

Fig. 2. The effect of 2,4-D on the enzymatic activity of peroxidase as a function of temperature.

It is immediately apparent from this graph that not only has 2,4-D accelerated the loss of activity of the peroxidase, but also the optimal activity of the peroxidase has been shifted from 45° C. down to 35° C. in the presence of 2,4-D. These data lend support to the assumption that the mechanism of action of 2,4-D in modifying enzymatic activity of a protein is a result of the change of structure of the enzyme molecule.

Consideration of these data raises the speculation as to how 2,4-D and other synthetic auxins may at one concentration cause growth stimulation and at higher concentrations inhibition. Reports in the literature, indicating that low concentrations of 2,4-D or other synthetic growth regulators may stimulate the activity of a certain enzyme and at higher concentrations cause inhibition of this enzyme, reinforce interest in this speculation. It is postulated that the enzymatic stimulation associated with low concentrations of 2,4-D could come about through a slight reversible modification of the enzyme structure which would make the enzyme a more efficient catalyst, but that as additional molecules of 2,4-D are added to this surface the modification of the structure becomes increasingly severe with a consequent loss of catalytic property.

In order to ascertain whether or not 2,4-D is capable of both stimulation and inhibition of an enzyme by variation in concentra-

tion, a number of crystalline enzymes were tested for activity in varying concentrations of 2,4-D. In the case of glyceraldehyde-3-phosphate dehydrogenase, a marked stimulation in activity was obtained with a concentration of about 100 p.p.m. of 2,4-D, and a very marked inhibition of enzymatic activity was noted at 1,000 p.p.m. of 2,4-D. In the case of glucose-6-phosphate dehydrogenase, a 40 p.p.m. 2,4-D solution resulted in a 22 per cent increase in activity, whereas a 1,000 p.p.m. concentration resulted in an appreciable loss of enzymatic activity. A similar situation was found to prevail with isocitric dehydrogenase. Little stimulation was found with the peroxidase, but inhibition by 2,4-D was appreciable above 500 p.p.m. While these concentrations of chemical appear quite high in considering the small amounts of 2,4-D required to bring about an auxin-like effect, it should be remembered that the media in which the enzymatic assays are made are not those of physiological conditions. Thus, in general the pH tends to be higher than would be found in the cell, reducing the efficiency of the 2,4-D because of increased ionization.

SUMMARY

The mechanism of action of a synthetic growth substance and the structural relations to activity appear to be two very closely interrelated problems. Seemingly, the solution to one affords at least some indication to the solution of the other. The data presented in this paper would appear to indicate that the primary event in the mechanism of action of the chlorophenoxyacetic acids is that of adsorption on a protein surface, and that as a consequence of this adsorption, the structure of the protein is modified with a consequent change in its enzymatic activity. This theory of the molecular level mechanism of action of the synthetic growth substances affords an explanation of how the same molecule may both stimulate growth at a low concentration and bring about inhibition at a higher concentration. The lines of evidence on which this postulate is based are as follows:

(1) The finding that the growth regulators can reduce the viscosity of cytoplasm and can stimulate streaming of the cytoplasm.

(2) The surface activity and behavior of growth substances as shown by Veldstra (30), Brian and Rideal (8), and Linser (19).

(3) The finding of Marinos (20) that exposure at high concentrations of growth substances results in the shrinkage of the cytoplasm and a leakage of the cell constituents.

(4) Examination of the ultraviolet and infrared absorption spectra suggests that the geometry of the molecule is important and that interpretation of these data suggests a plane surface per-

mitting the establishment of dipole or Van der Waal forces where adsorption may be involved.

(5) Water solubility appears to provide a natural limit to the behavior of these compounds. The demonstration of the adsorption of these compounds by proteins and by mitochondria.

(6) The change in structure of the peroxidase molecule.

These observations suggest that adsorption and consequent modification of protein structure may be the basic action of plant growth regulating phenoxyacetic acids. It may be inferred that not all enzymes or proteins are going to adsorb the chemical with equal facility and, therefore, there will be a marked difference among enzymes as to the response to the chemical. Likewise, the same enzyme derived from different sources would quite likely have different affinities for the chemical and, therefore, would show a different response in terms of change of enzymatic activity. This in part may help to account for the selectivity of these chemicals.

ACKNOWLEDGMENTS

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DISCUSSION

Dr. Wain: I would like to point out the danger of assuming that the reason phenoxyacetic acid is held to a protein and is unable to pass through a membrane in dialysis, is due to the fact that the ring system has been associated with the protein. There may be an association of poles of opposite charge. There could be, for instance, a protein with a free NH_2 group that could very readily associate with the carboxyl group of the phenoxyacetic acid to give an association of the molecule with the protein which has nothing to do with the ring system. Nevertheless, I fully agree that the ring system is all important, and what is more, the ring system must be an unsaturated ring system. The unsaturated benzene ring is flat, and it is well known that flat surfaces can readily attach themselves to other surfaces by Van der Waal's forces. Benzene itself is capable of forming molecular compounds with mercuric cyanide and platinum chloride, for example, and presumably is providing one coordinate valency. It may well be, therefore, that there is something of this kind taking place with the benzene ring to give a free pair of electrons. I'm not suggesting that that known pair is donated to form a rigid coordinate bond, but the electron density outer surface may very well be slightly modified by this kind of tendency so as to give an association with the benzene ring, which after all is perhaps significant in view of the fact that there must be unsaturation in the ring. Many people working in this field of auxinology are closing their eyes and are ignoring the fact that ethylene, carbon monoxide, propylene, etc., are active as plant growth regulators.

Dr. van Overbeek: I agree with Dr. Freed on the emphasis he has

placed on a physical mode of primary action. I can imagine that the effect of 2,4-D on mitochondria could be due to swelling of the mitochondrial membranes. I would like to ask how 2,6-D and the other homologues behave.

Dr. Freed: We have measured the effect of 2,6-D, 2,4,6-T, and a number of other compounds on the oxygen uptake of mitochondria. We do find that many of these compounds have an effect. This is as would be postulated since most of them are capable of being adsorbed to a greater or lesser degree. This has a parallel in that as a homologous series is examined for biological activity or activity on mitochondria, a few members may be found that are innocuous at quite a range of concentrations, but most of them will have measurable activity with a few having outstanding activity. This suggests that the structural requirement for activity is not absolute, which is consonant with an adsorption phenomena. Hence the moderate activity of 2,4,6-T is not unexpected.

Dr. Wain, we are fully aware of the possible implication of the carboxyl group in the absorption phenomenon. When I say absorption I'm thinking in rather broad terms. The ultraviolet spectra, of course, also implicate electron density because of the electronic configuration of the ring that has been modified both by the carboxyl group and the chlorine substituents.

Dr. van Overbeek: That's why I asked the question, because absorption is necessary but not the timely point.

Dr. Freed: It appears from these studies that adsorption is the first event in the action of these compounds, but the subsequent effect may well be the crucial mechanism in their action.

Dr. Bonner: Well, I know it's very attractive to think about how auxin molecules can be adsorbed, presumably by 2-point attachment, to enzyme molecules and cause them to be changed and to do something different than they otherwise would do. It's attractive to think that auxins might do their work by affecting, for example, the mitochondria which we know are very important, and the powerhouses of the cell, and I yield to no one in my admiration for mitochondria. One of the most fascinating and indeed useful aspects of auxins is, however, that they don't work on all kinds of organisms. We saw, for example, that auxins bind to bovine serum albumen, but it is a well-known fact that auxins do not work on cows. Now, it is a very remarkable fact, I think, that auxins work on such a small part of the spectrum of living organisms. They work on higher plants and a few algae but they don't work on all organisms. It seems to me quite clear, therefore, that when we try to find out how auxin does its work we have to think about processes that go on in and are unique to plants

and to algae and a few other organisms and not about processes that are common to all living things. For example, one of the lessons of comparative biochemistry is that mitochondria are very much the same in all living creatures. We all have the same kind of mitochondria, and I would think that, for example, if 2,4-D or IAA did its work on mitochondria, it ought then to work on all kinds of organisms. And it seems to me that the hypothesis that auxins exert their effects by general and nonspecific adsorption to enzymes breaks down in the face of the facts of comparative biochemistry.

Dr. Freed: Your points are well taken, Dr. Bonner. Now I call your attention to the following:

(A) It is known that a given enzyme isolated from different tissues will have slightly different substrate specificities yet catalyze the same essential reaction. This indicates that we are dealing with a population of molecules, some of which may give a remarkable response to a given chemical and still others of the same population give only a slight response. Thus the problem is one of degree rather than an all or none effect. This appears to arise from the differences in the structure of the protein. Such variation at the molecular level may account in part for the variation among different organisms in their response to IAA. However, one of the consequences of this postulate is that many organisms may be responding to IAA but either so slightly or in such a manner that we have not yet observed the response.

(B) In support of the foregoing it must be remarked that the first demonstration of the ability of 2,4-D to inhibit oxygen uptake by mitochondria was performed with mitochondria isolated from animal tissue rather than from plants.

(C) Finally, it should be noted that IAA has recently been found to have a marked effect on certain functions of mammalian metabolism. Thus, Mirsky and his coworkers (Endocrinol. 59: 369. 1956) have found IAA to function as an antidiabetic agent in mammals.

It thus appears that compounds may affect a wider range of organisms than previously suspected. This would seem to be compatible with the theory advanced.

Dr. Leopold: It is very exciting to think that adsorptive features of auxins might be relevant to their activities in biological systems. We have, like Dr. Freed, been very much taken by this possibility and have done some measurements of the influences of molecular structure on adsorption of auxins onto charcoal. This system suffers from its lack of biological specificity, to be sure, but it might tell us something about the influence of molecular structure on a simple adsorptive function. We found that compounds showing the greatest activity as auxins also showed the greatest adsorption onto char-

coal. IAA was very good, 2,4-D and 2,4,5-T were excellent, but when we examined a whole series of chlorinated phenoxy acids the correlation with growth activity was not good because the more you chlorinate the more adsorption you get regardless of where the chlorine is placed in the ring. Thus, 2,4-D and 3,5-D are of equal activity in this adsorption system, 2,6-D shows a little less adsorption, but 2,6-D is very much in the same class as 2,4-D. 2,4,6-T, and 2,3,6-T are less adsorbed than the other trichlorinated ones but they were better than 2,4-D. I think that it may have implications in some aspects of auxin function in cells, but there's not a good correlation between auxin activity and adsorption, in our system.

Dr. Wain: I think it's a very important point that Dr. Leopold raised. The compound with a ring system with more chlorine atoms can in fact be absorbed and can act as a competitive antagonist. But it's not necessarily a growth substance because other features of the molecule are important.

JAMES BONNER
California Institute of Technology

On the Mechanics of Auxin-induced Growth[1]

It is a cornerstone of growth-substance lore that auxins cause an increased rate of plant cell elongation. The question of how auxins bring about such auxin-induced cell elongation constitutes a classical problem of auxinology. We shall here consider the state of knowledge on this subject. The present discussion is restricted to information concerning but a single plant tissue, the *Avena* coleoptile section, about which much is known. The elongation of the *Avena* coleoptile is normally controlled by auxin produced in the apex of the organ. The native hormone, which appears to be chemically identical with indole-3-acetic acid (IAA) (29), moves by polar transport to the lower regions of the coleoptile and there promotes elongation. Excised sections of the coleoptile, floated in solution, respond to added IAA by increased growth rate, and such sections constitute a convenient system for the study of auxin-induced growth.

Two characteristics of the response of *Avena* coleoptile sections to IAA deserve particular note. The first, as illustrated in Figure 1, is that the response is large. The rate of elongation of the section in the presence of added IAA (at optimal concentration) is seven- to tenfold greater than the rate of elongation in the absence of the growth substance. The second noteworthy characteristic is that elongation in the absence of added IAA is not controlled by endogenous growth substance. This conclusion is based upon the fact that the rate of elongation of sections in the absence of added IAA is not slowed by added competitive inhibitors of auxin action. In the response of the *Avena*

[1] Much of the work here summarized has been made possible by the continued and generous support of the Herman Frasch Foundation. The present discussion is primarily a summary of work done with this support and is not intended as a general review of the entire field.

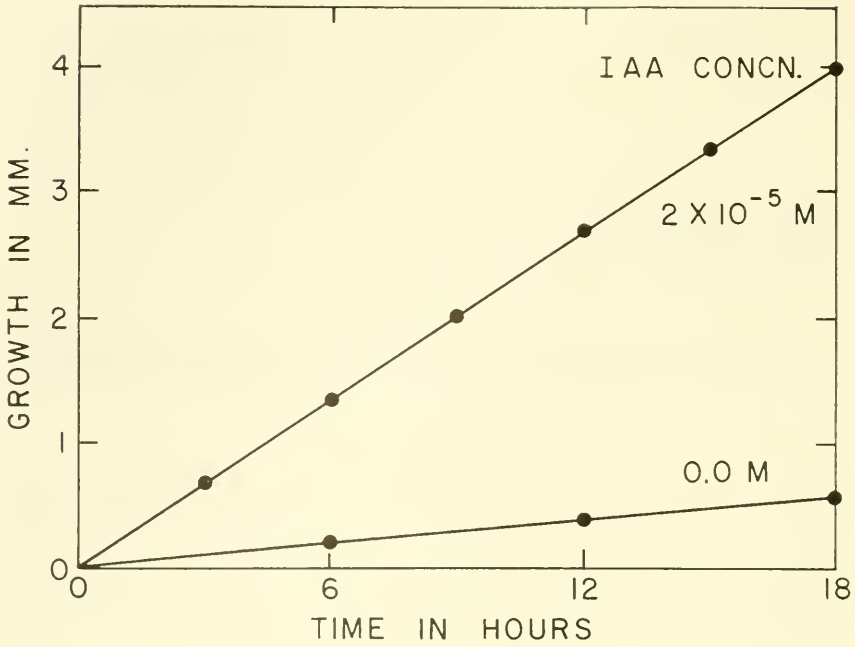

Fig. 1. Progress curves for growth of *Avena* coleoptile sections. Basal medium of sucrose, 0.09*M* and K-maleate buffer, 0.0025*M*, pH 4.5. IAA as indicated. Temp. 25° C. Initial length of sections 5.0 mm.

coleoptile section to added IAA we have, then, not only a large response but also one that is pure — uncontaminated by residual native growth substance.

THE DRIVING FORCE OF CELL ELONGATION

We will first ask, What powers the elongation of plant tissue? This is a general question and one relevant to all cell extension whether auxin-controlled or not. In the case of the *Avena* coleoptile section, the driving force of cell elongation is the osmotic pressure of the vacuolar contents of the individual cells. This is clear from the information in Figure 2. Sections placed in solutions isotonic (0.42*M*) with themselves essentially fail to elongate, either in the presence or absence of IAA. Sections placed in solutions containing an osmotically active solute (mannitol) in concentrations lower than isotonic grow at reduced rates. The rate of section elongation is, in fact, essentially a hyperbolic function of concentration of external solute.

Classical osmotic lore tells us that:

$$\text{Net DPD}_{\text{Tissue}} = \text{O.P.}_{\text{Tissue}} - \text{W.P.} - \text{O.P.}_{\text{External}}$$

where DPD designates diffusion pressure deficit, O.P., osmotic pressure, and W.P., wall pressure.

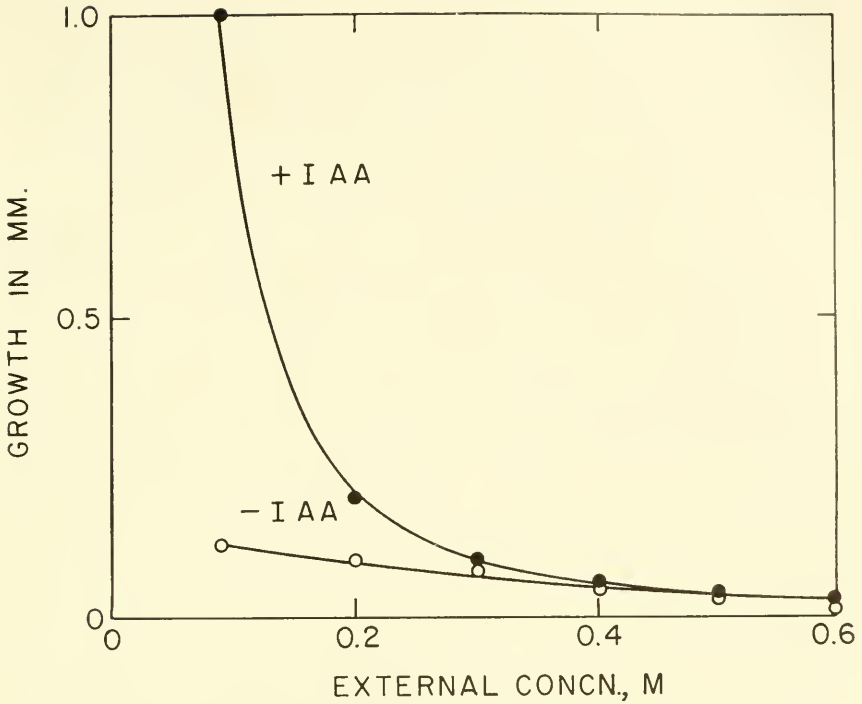

Fig. 2. Initial growth rate of *Avena* coleoptile sections as a function of external osmotic concentration. Growth in mm/section/4 hrs. Osmotic solutes made up of sucrose, 0.09*M* plus varied concentrations of mannitol. After Ordin, Applewhite, and Bonner (20).

The DPD of *Avena* coleoptile sections growing in solutions over the concentration range of Figure 2 has been shown to be null, within the precision of measurement (Ordin *et al.,* 20). The growing sections are therefore in osmotic equilibrium with the solution in all cases. In addition $O.P._{Tissue}$ is identical, or nearly so, for sections in solutions of different $O.P._{External}$. We may conclude therefore that it is the tension to which the cell walls of the tissue are subject which is altered by changing $O.P._{External}$

$$W.P. = O.P._{Tissue} - O.P._{External}$$

and that rate of section growth as a function of $O.P._{External}$ is in fact a measure of the rate of cell wall deformation as a function of cell wall tension. Our first conclusion is therefore that the rate of cell extension depends on the tension to which the wall is subjected by the osmotic pressure of the cell contents.

One further conclusion can be drawn from the information in Figure 2. This concerns the difference in growth rate between IAA treated and control sections. Since tissue osmotic concentration is not

altered by IAA treatment, the wall tension developed by IAA treated sections must be identical with that developed in control sections. The more rapid growth of IAA treated sections must therefore be due to more rapid yielding of the wall. Our second conclusion is therefore that IAA treatment increases the deformability of the cell wall.

ROLE OF OSMOTIC CONCENTRATION

The factors which immediately control the rate of *Avena* coleoptile section growth are then the osmotically induced load or tension on the cell wall and the resistance of the wall to deformation under load. If growth rate of the section is to remain constant with time, as is often desirable in growth studies, it is evidently essential to arrange circumstances under which both of these factors remain constant. Thus the growth rate of sections which elongate in water (containing IAA) steadily decreases with time as shown in Figure 3. This is due to the fact that as the section takes up water and elongates, its osmotically active solutes are progressively diluted, as is indicated in Figure 3. The tension to which the cell wall is subjected therefore

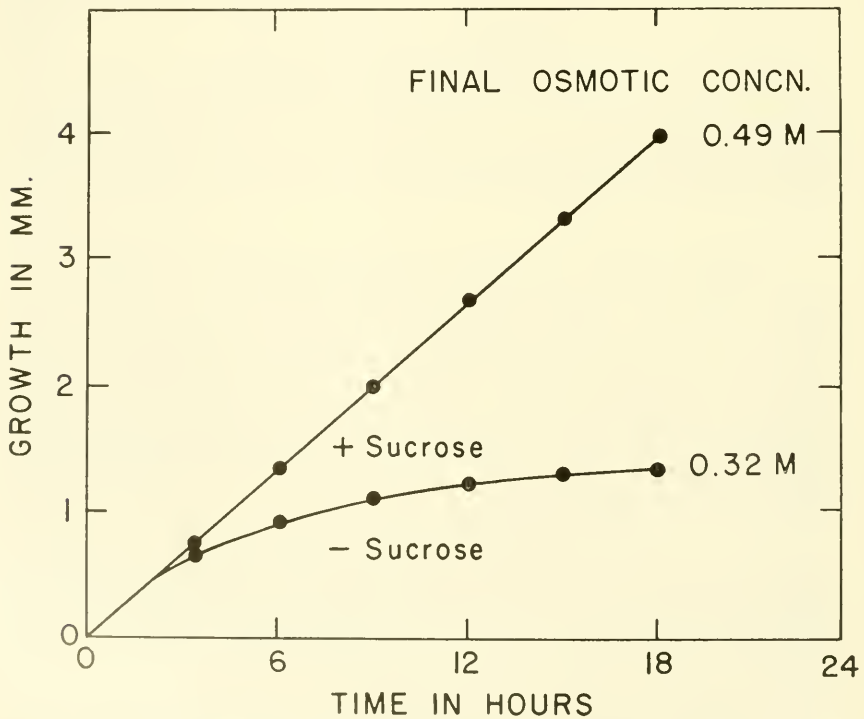

Fig. 3. Progress curves for growth of *Avena* coleoptile sections in IAA, $2 \times 10^{-5}M$, in presence or absence of sucrose, $0.08M$. Initial osmotic concentration, $0.42M$.

decreases correspondingly. The maintenance of constant growth rate requires the addition to the external medium of an absorbable solute which is then taken up by the tissue and contributes to tissue osmotic concentration. By appropriate choice of solute and concentration, conditions may be arranged so that the uptake of solute balances the growth-induced dilution of cell contents to maintain constant tissue osmotic concentration. In the experiment of Figure 3 this has been achieved by the addition to the medium of 0.08*M* sucrose, which in fact constitutes the standard medium for the growth of *Avena* coleoptile sections. A wide range of absorbable solutes can, however, replace sucrose in this function (Ordin *et al.,* 20). It is important that the role of tissue osmotic concentration in determination of section growth rate be generally understood since lack of such understanding in the past has led to acrimonious dispute (Bennet-Clark and Kefford, 2; Bonner and Foster, 7; Marinos, 18).

ROLE OF CELL WALL RIGIDITY

The effect of IAA in increasing the rate of coleoptile section elongation is due, in last analysis, to the effect of IAA in decreasing cell wall resistance to deformation under load as has been demonstrated above. There are, however, additional reagents which may be used to experimentally alter cell wall deformability. The chief of these is the calcium ion. The IAA-induced growth rate of sections is decreased in the presence of calcium ions (Thimann and Schneider, 26) as is illustrated in Figure 4. The relation of steady state growth rate to calcium ion concentration is a hyperbolic one, and the K_S or calcium ion concentration required to elicit half-maximal inhibition is *ca.* 3×10^{-3} equiv. That the effect of calcium ion is upon cell wall resistance to deformation is demonstrated by the fact that sections in solutions of varied calcium ion concentration are all in osmotic equilibrium with the solution (DPD = O) and that the initial osmotic concentration of the tissue is unaffected by calcium. Wall pressure therefore equals osmotic pressure in all cases. Since the force exerted on the wall is then independent of calcium ion concentration, it follows that the reduced rate of extension of sections in calcium-containing solutions is due to reduced rate of deformation of the walls in response to this constant force.

The effect of the calcium ion in increasing cell wall resistance to load is shared by the magnesium ion which is, however, less effective. Monovalent cations such as Na^+ and K^+ are essentially without effect on growth rate, at least in concentrations of 1 to 10 mequiv/l. Potassium ions do, however, act as an antidote against the inhibitory effect

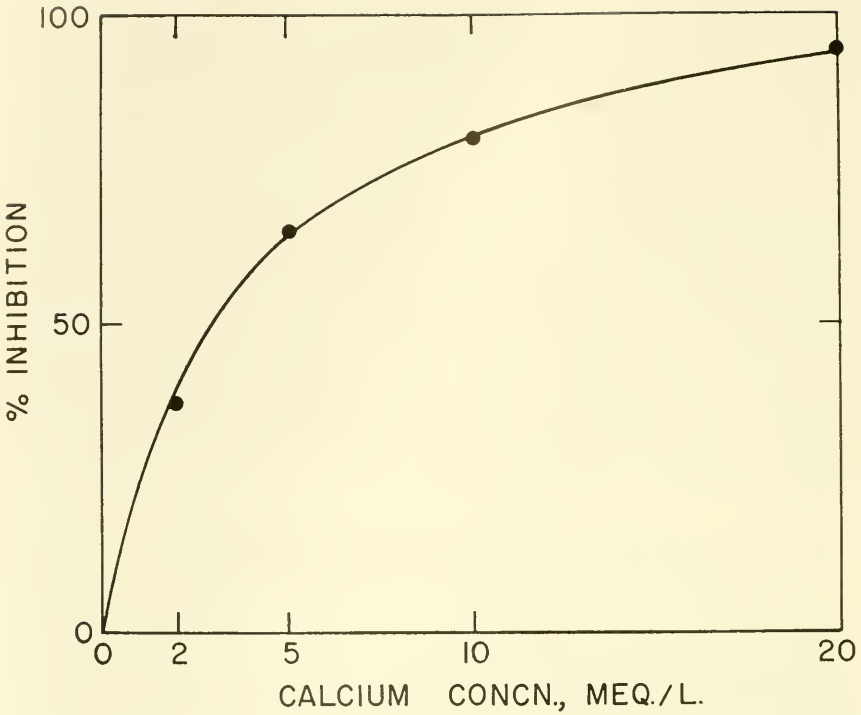

Fig. 4. Inhibition of IAA-induced growth of *Avena* coleoptile sections by $CaCl_2$. Based on steady state rates over a 6 hr. growth period. Basal medium contained sucrose, 0.09*M*. After Cooil and Bonner (12).

of calcium ions on section growth rate. The experiment of Figure 5 summarizes the growth interrelations of Ca^{++} and K^+ ions. Sections growing in the absence of either ion, when transferred to calcium-containing solution, quickly assume a new and slower steady state growth rate. If such sections are transferred to water, their growth continues at the rate characteristic of the calcium-containing solution and only slowly increases. Transfer of the sections to K^+ ion-containing solution results in immediate reversal of the calcium inhibition. We may summarize the information of Figure 5 by saying that growth inhibition by calcium ions behaves as though it were mediated by exchangeably bound ions.

MECHANICAL PROPERTIES OF COLEOPTILES UNDER EXTERNAL LOAD

The effects of IAA and of inorganic ions on the cell walls of coleoptile sections can be readily and rigorously demonstrated by methods which measure their deformability under artificially im-

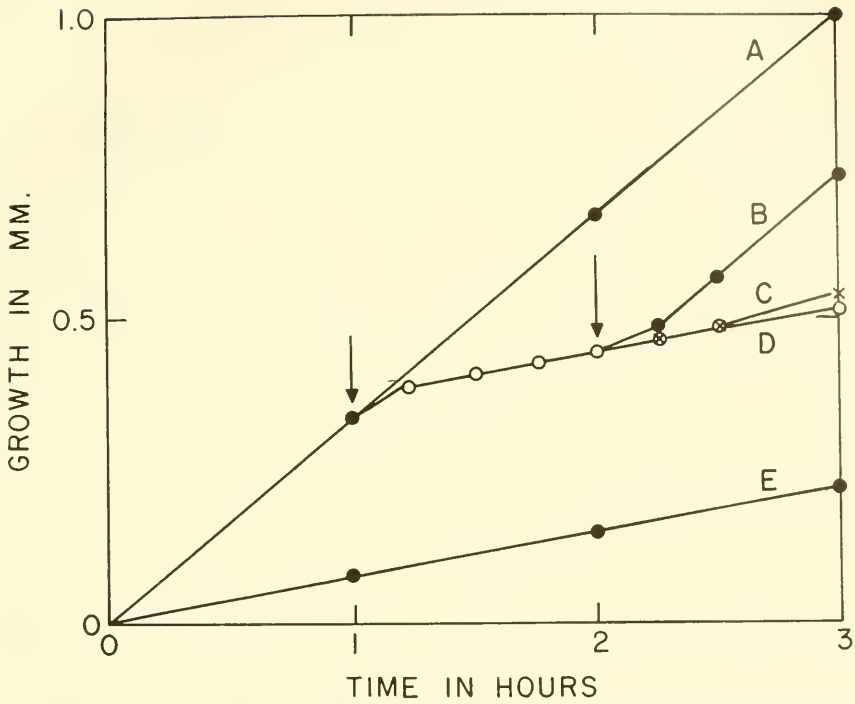

Fig. 5. Progress curves of IAA-induced *Avena* coleoptile section — growth as influenced by addition of calcium ions (10 mequiv/l) as well as by subsequent withdrawal of calcium. Basal medium contained sucrose 0.09*M* and IAA $2 \times 10^{-5}M$. A. Sections in basal medium throughout. B. Sections transferred to basal medium plus calcium at first arrow and thence to KCl (1 mequiv/l) at second arrow. C. Sections transferred to basal medium plus calcium at first arrow and thence to basal medium minus salt at second arrow. D. Sections transferred to basal medium plus calcium at first arrow and left in this solution. E. Sections in basal medium plus calcium throughout. Modified after Cooil and Bonner (12).

posed external load. The results of such measurements confirm and extend the conclusions reached above on the basis of growth rate studies, namely, that IAA increases cell wall deformability while calcium ions decrease it. A convenient measurement is that of the rate of bending of a coleoptile which is rigidly supported at one end and loaded with a weight on the other. The mechanical analysis of this method, which was first used by Heyn (15), is as follows: The turgid cylindrical section of plant tissue, composed of cells, may be thought of as a structure composed of bags of water, surrounded by interconnected walls. When the section is subjected to a force normal to its long axis, one side of the structure is placed in compression, the other in tension. The side which is under compression cannot, as a first approximation, compress. It is composed of incompressible liquid. The

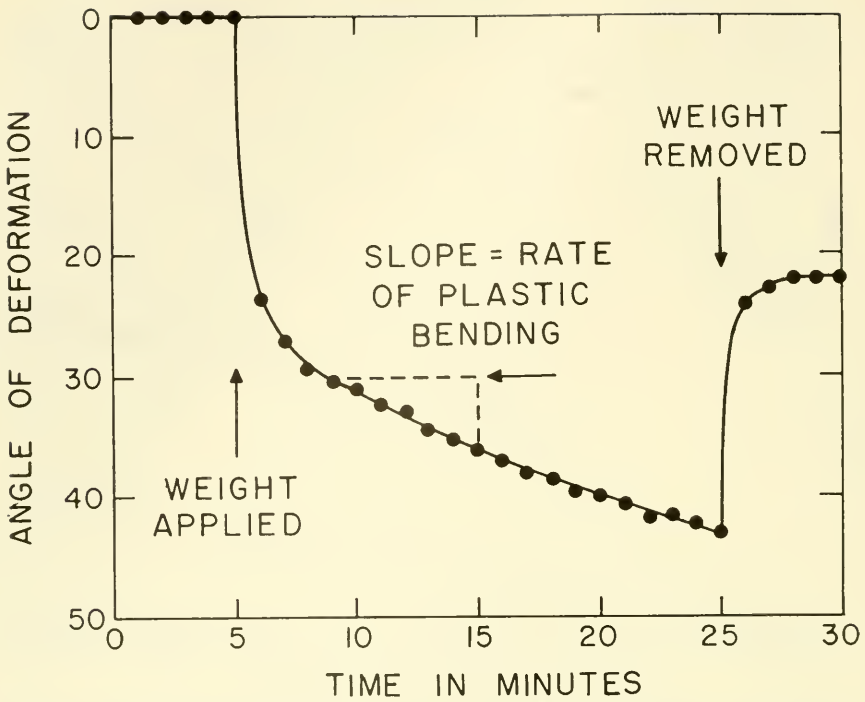

Fig. 6. Progress curve of deformation of a 2 cm. *Avena* coleoptile section in response to an applied force. The section is held rigidly at one end, a constant bending force applied to the other. Angle of deformation is plotted as a function of time. After Tagawa and Bonner (24).

side which is in tension can, however, yield to this tension since the supporting cell walls can stretch. The property measured in such a bending experiment is therefore the stretchability of the cell walls of the section. The principal complication is the possibility of pressure-induced water flow through the tissue from the compression to the tension side. Such flow may be expected to occur but to become significant only over time periods longer than those required (*ca.* 5 min.) for the measurement of rate of deformation of the wall.

Figure 6 presents data on the time course of deformation under load of an *Avena* coleoptile section. The initial rapid elastic deformation is followed by a period of steady plastic deformation. The rate of such plastic deformation under load is influenced both by IAA and by inorganic ions. Figure 7 presents data on rate of plastic deformation, at constant external load for sections equilibrated with varying concentrations of IAA. It is evident that the IAA concentration dependence for cell wall deformability closely resembles that for IAA-induced section growth and that, in fact, the concentrations

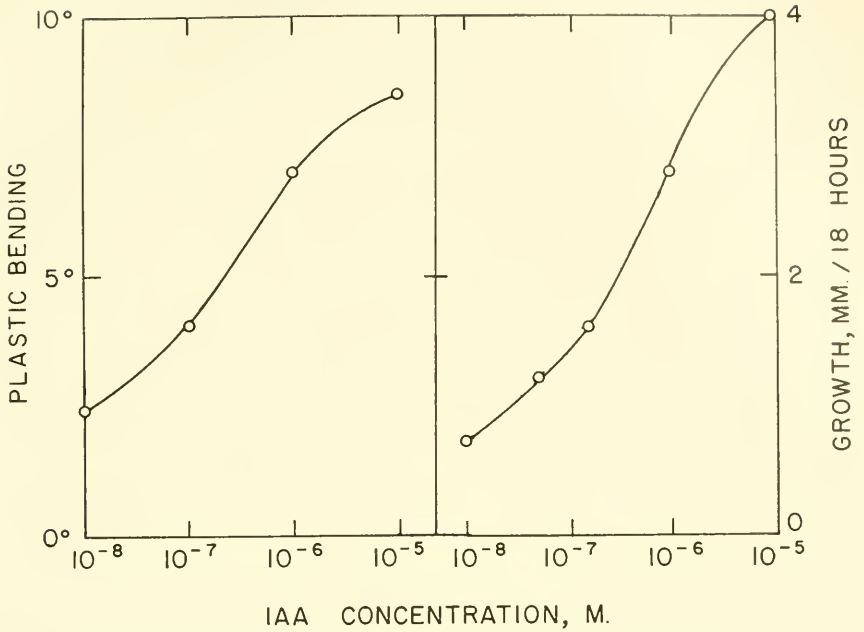

Fig. 7. IAA concentration dependence of (left) plastic deformation and of (right) growth of *Avena* coleoptile sections. In plastic deformation experiment, sections were equilibrated with IAA for 60 min. and plastic deformation then measured for 4 min. In growth experiment, sections were incubated in sucrose 0.09*M*, K-maleate buffer 0.0025*M*, pH 4.5, and IAA for 18 hrs.

of IAA which elicit half maximal response are identical within the errors of measurement.

That calcium ions increase the resistance of section cell walls to deformation under external load is shown in Figure 8. The calcium ions responsible for this effect appear to be exchangeably bound, as is shown by the fact that the cell wall stiffening effect persists when the tissue is transferred to water but is discharged when the tissue is transferred to K^+ ion-containing solution. The effects of calcium and of potassium on cell wall deformability as measured by rate of deformation under external load are then similar to the effects of the same ions upon cell wall deformability as measured by rate of cell extension in response to internal (osmotically induced) load.

CHEMICAL BASIS OF CELL WALL PROPERTIES

The facts adduced above lead to the conclusion that IAA increases cell wall deformability and that cell wall deformability is decreased in the presence of calcium ions. IAA increases cell wall deformability even in cell walls made stiff by the presence of calcium ions. In such

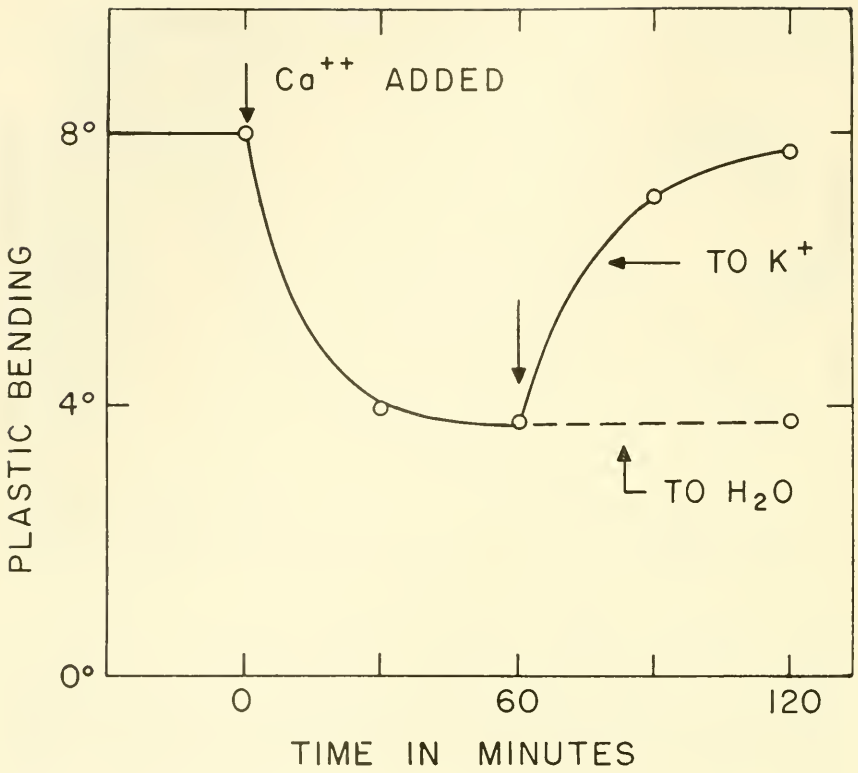

Fig. 8. Time course of inhibition of plastic deformability of *Avena* coleoptile sections by added calcium ions (20 mequiv/l) and of reversal of the inhibition by potassium ions (20 mequiv/l). Sections equilibrated in solutions for varied times as indicated and plastic deformation then measured over a 4 min. period.

walls the material which interacts with calcium limits deformability. That IAA does its work upon the same material which interacts with calcium is therefore implied although not rigorously demonstrated. It will now be shown by independent, chemical, methods that IAA and calcium ions exert their effects upon the same cell wall constituent, namely, the pectic material.

The nature of the material within the coleoptile which interacts with calcium ions to cause cell wall stiffening can be attacked in a straightforward manner. It has been shown above that the responsible ions behave as though exchangeably bound to the tissue. Living *Avena* coleoptile sections possess a readily measurable cation exchange capacity. The characteristics of such binding closely resemble those of the binding which causes cell wall stiffening, as is summarized in Table 1. The data of Table 2 establish that the capacity of such sections to bind calcium ions exchangeably is entirely due to the free

Table 1. Comparison of parameters which characterize interaction of calcium ions and *Avena* coleoptile sections with respect to cell wall stiffening and exchangeable binding.

Parameter	Cell Wall Stiffening	Exchangeable Binding
Ca^{++} concn. required to produce 0.5 max. effect	3×10^{-3} *N*	$2–3 \times 10^{-3}$ *N*
Time required for 0.5 equilibration	Ca. 10 min.	Ca. 10 min.

(nonesterified) pectic carboxyl groups of the cell wall. It may be concluded therefore that, in the presence of calcium ions at least, it is the cell wall pectic material which limits cell wall deformability. Whether this is also true in the absence of added calcium ions cannot be deduced. It is of interest, however, that the cell walls of sections taken from *Avena* seedlings germinated in distilled water contain measurable calcium and, in fact, a quantity sufficient to bind with approximately one-fifth of the free pectic carboxyl groups of the cell wall.

The pectic material of the *Avena* coleoptile makes up but 5 per cent of the total weight of cell wall substance (Jansen *et al.,* 16). Cellulose constitutes another 25 per cent (Bishop *et al.,* 3), while polysaccharides which yield on hydrolysis xylose, arabinose, glucose, and galactose make up the bulk of the remainder. It appears remarkable that a minor constituent such as pectic material should play a major role in determining cell wall mechanical properties. It is indeed clear that we have inadequate knowledge of the structural arrangements of cell wall components other than cellulose, and such knowledge will be needed before detailed discussions of cell wall properties can be fruitfully undertaken.

INFLUENCE OF IAA ON PECTIC METABOLISM

Since IAA increases rate of cell extension by causing increased cell wall deformability, it is evident that IAA in some way alters cell

Table 2. Demonstration that the exchangeably bound calcium of *Avena* coleoptile sections is held principally by free (nonesterified) pectic carboxyl groups of the cell wall. After Jansen *et al.* (16).

Cation exchange capacity of living coleoptile sections	3.1×10^{-3} Mequiv/g fresh wt.
Cation exchange capacity of cell walls	0.18 Mequiv/g dry wt.
Cation exchange capacity of cell walls calculated on basis of free pectic carboxyl groups	0.165 Mequiv/g dry wt.
Cation exchange capacity of living sections predicted on basis of 2	3.6×10^{-3} Mequiv/g fresh wt.
Cation exchange capacity of living sections predicted on basis of 3	3.3×10^{-3} Mequiv/g fresh wt.

wall chemistry. Such alterations have been sought since the beginnings of auxinology. Only in recent years, however, and after the advent of appropriate methodology have they been found.

With such methodology it has been shown that the application of IAA to *Avena* coleoptile sections results in an increased rate of pectic synthesis by the tissue. That IAA influences pectic metabolism was first detected by the use of C^{14} methyl-labeled methionine. The methyl group of methionine serves as donor of the methyl ester groups of pectic substances (Ordin *et al.,* 21; Sato, 23). The application of IAA to coleoptile sections in the presence of C^{14} methyl-labeled methionine (or of other methyl donors, as formaldehyde), increases the rate of appearance of labeled pectic methyl ester groups (Ordin *et al.,* 21, 22; Jansen *et al.,* 16). It was subsequently found that the increased rate of pectic methyl ester formation in the presence of IAA is paralleled by increased rate of polygalacturonic acid formation (Albersheim and Bonner, 1). IAA therefore increases the rate of formation of pectic material. The effect of IAA upon pectic synthesis possesses all of the earmarks of an authentic auxin-controlled reaction, with characteristics similar to those of IAA-induced growth. Thus the effect of IAA upon rate of pectic synthesis is a rapid one, manifest within 15 to 30 min. after application of the auxin. It is inhibited by antiauxins such as 2,4,6-trichlorophenoxyacetic acid. It takes place only under aerobic conditions (Cleland, 10). It is inhibited, as is growth, by ethionine. It occurs in sections which are restrained from growing by the presence of isotonic mannitol solution, a condition under which auxin-induced cell wall plasticization continues to occur. It would appear therefore that IAA-induced cell wall plasticization and IAA-induced alteration in pectin synthesis are similar and possibly identical reactions, although there is as yet no rigorous proof that this is so.

Further analysis of the effects of IAA on pectic metabolism requires a knowledge that the pectic material of *Avena* coleoptile sections includes three principal forms (Jansen *et al.,* 16). The first is the so-called hot water soluble pectin (extracted from the cell wall by hot water). This fraction is, as summarized in Table 3, highly esterified. A second pectic fraction is the cold water soluble, 70 per cent alcohol precipitatable material. This fraction, which constitutes approximately 5 per cent of the total pectic substance, is presumably removed from the cell wall during the preparation of the latter but can be recovered from the cold water washings by alcohol precipitation. The third pectic fraction — that which remains in the wall after cold and hot water washing — is known as the residual pectin.

Table 3. Characteristics of the pectic materials of the cell walls of *Avena* coleoptile sections. After Jansen *et al.* (16).

Fraction	Per Cent AUA* in Fraction	Per Cent of Total AUA	Per Cent Methyl Esterified
Whole cell walls	5.3	91	40
Cold H_2O soluble, 70 per cent EtOH insol.	4.0	5	*ca* 100
Hot H_2O soluble	23	14	90
Residual	43	78	31

* AUA = Anhydrouronic (galacturonic) acid.

It makes up some 80 per cent of the total, is roughly 30 per cent esterified, and contains essentially all of the free pectic carboxyl groups which contribute to the cation exchange capacity of the wall. This residual pectin may be solubilized by hot water in the presence of the chelating agent ethylenediaminetetraacetic acid (EDTA).

The effects of IAA, so far as pectic material is concerned, are concentrated in the hot and cold water soluble pectic fractions. Information bearing on this point is summarized in Table 4 for two types of experiments. In the one type of experiment, sections were merely incubated for 15 hrs. in the presence of glucose and in the presence or

Table 4. Cell wall pectic synthesis during the growth of *Avena* coleoptile sections. Summarized after Albersheim and Bonner (1).

Pectic Fraction		Analytical Increase in AUA* Mg/100 Mg Cell Wall Per 15 Hrs.	Ratio IAA/ Control	Radioactivity of AUA** CPM/100 Mg Cell Wall	Ratio IAA/ Control
Total wall	Control	0.32	1.5	11,850	1.2
	IAA	0.49		14,300	
Cold H_2O Soluble	Control	0.055	3.8	650	2.3
	IAA	0.145		1,500	
Hot H_2O Soluble	Control	0.102	1.6	2,500	1.6
	IAA	0.166		4,030	
Residual	Control	0.17	1.1	8,700	1.0
	IAA	0.18		8,800	

* AUA = Anhydrouronic (galacturonic) acid.

** Sections incubated 5 hrs. in uniformly labeled C^{14}-glucose, $2.5 \times 10^{-4}M$, 1.9 mc/millimole.

absence of IAA, and analytical determinations were made by an appropriate, sensitive, and specific method (Albersheim and Bonner, 1) of the initial and final amount of pectic material in each cell wall fraction. In the second type of experiment, sections were incubated for 5 hrs. in C^{14}-labeled glucose, and the amount and specific radioactivity of the pectic galacturonic acid of each fraction were determined. It is clear that in the presence of IAA the rate of formation of cold water soluble pectin is increased twofold or more and that of hot water soluble pectin 60 per cent, while the rate of synthesis of residual pectin is but little influenced.

The effect of IAA on pectic metabolism is, then, to increase rate of production of the more soluble pectic molecules. One can feel intuitively that this should in some way increase cell wall plasticity but as yet no rigorous demonstration of how it does so has been achieved.

EFFECT OF AUXIN ON INTERACTION OF CELL WALL COMPONENTS

The walls of the parenchymatous cells of the *Avena* coleoptile possess, in common with the primary walls of other cylindrical plant cells, a pattern of arrangement of cellulose microfibrils known as tube structure. The cellulose microfibrils are disposed in a manner predominantly normal to the long axis of the cell and thus in a "barrel-hoop" fashion. That this is so may be readily observed by electron microscopy (Mühlethaler, 19), although it was first deduced on the basis of birefringence measurements (Bonner, 5). Such measurements enable one to draw conclusions as to the orientation of cellulose microfibrils within the wall since the larger index of refraction of cellulose lies parallel to the long axis of the microfibril. It is characteristic of the microfibrillar network that, when stretched, the microfibrils tend to align themselves in the direction of stretch. The amount of stretching required to elicit a given degree of reorientation is a measure of the interaction between the units of the network as has been shown for model systems (Bonner, 5). It is of interest that IAA treatment exerts a profound effect on the tendency of the cell wall microfibrils of *Avena* coleoptiles to reorient in response to mechanical shear. This has been demonstrated by the following general technique: coleoptile sections (from which the epidermis had been stripped) were plasmolyzed in glycerine, clamped at the two ends, and stretched longitudinally under a polarizing microscope. As the tissue is stretched, the initial negative anisotropy (microfibrils statistically at right angles to the shear axis) diminishes, becomes null (statistical isotropy), and finally becomes positive (microfibrils statistically parallel to the shear axis). The course of microfibrillar reorientation with increasing stretch is

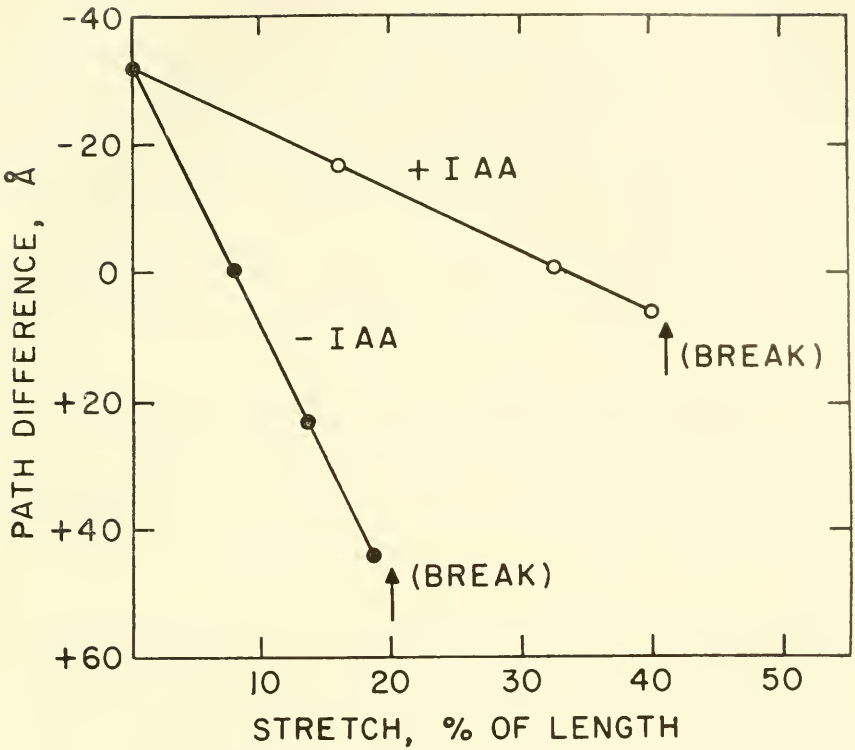

Fig. 9. Reorientation of cellulose microfibrils of *Avena* coleoptiles (one-half coleoptiles, epidermis removed) during longitudinal stretching as a function of prior (2 hrs.) IAA treatment. Path difference under polarized light measured with Sénarmont compensator. After Bonner (5).

shown in Figure 9, which also contrasts the behavior of IAA-treated and nontreated sections. It is clear that the reorientation of the wall microfibrils in response to shear is dramatically decreased by the presence of IAA. This effect is not an artifact of IAA-induced growth since it occurs equally strikingly in IAA-treated but nongrowing sections.

How are we to understand the effect of IAA treatment upon the response of the wall microfibrillar network to shear? Evidently the microfibrils reorient in the direction of shear because they interact, stick to one another here and there. In IAA-treated tissue this interaction is decreased and ability of the microfibrils to slide past one another correspondingly increased. IAA treatment of *Avena* coleoptile sections results in but slight effects on cellulose (Boroughs and Bonner, 9; Ordin *et al.*, 21, 22). However, the facts available suggest that the pectic material of the wall may constitute the glue through which the microfibrils interact.

ACTIVE AND PASSIVE ASPECTS OF IAA-INDUCED GROWTH

It has already been shown that cell extension in final analysis is stretching of the cell wall due to osmotically controlled water uptake. In this sense cell extension itself is a passive process. Cell extension in the *Avena* coleoptile section, however, is accompanied and in fact controlled by at least three distinguishable, metabolically powered and hence active processes. The first is the active accumulation of solute molecules or ions which, by maintaining the osmotic concentration of the cell contents, maintain the turgor-induced load on the cell wall. The second is the deposition of new cell wall material which normally keeps pace with cell extension. The third is the auxin-induced plasticization of the wall. That the plasticization process may be experimentally separated in time from actual cell extension has been shown over the years by Heyn (15), Thimann (25), and by Cleland and Bonner (11). In this type of experiment, sections are supplied with IAA but restrained from growing by the presence of a suitably high external concentration of nonabsorbable solute. The sections are then allowed to expand in water under conditions in which the action of auxin is blocked by the presence of a suitable inhibitor. An effect of the auxin pretreatment is manifested by growth of the auxin-treated sections greater than that of nonauxin-treated control sections. Alternatively, the cell wall deformability under external load of auxin-treated (but nongrowing) sections may be measured directly (Cleland, 10).

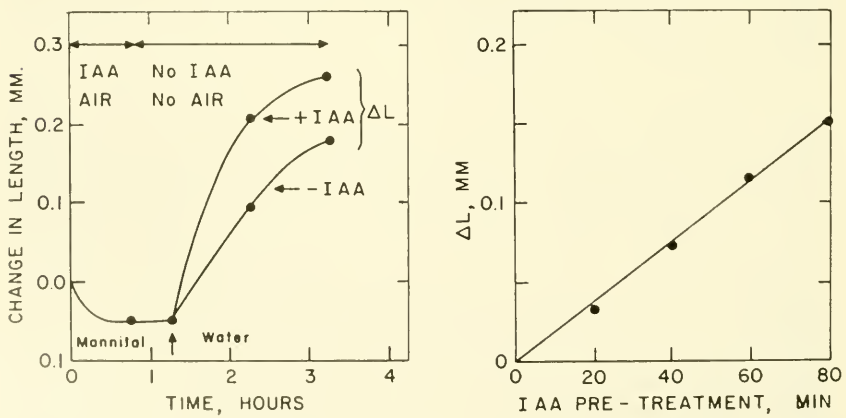

Fig. 10. Experimental separation of IAA-induced wall softening from the act of turgor-controlled cell extension. **Left**, *Avena* coleoptile sections placed in 0.3*M* mannitol with or without IAA for an initial pretreatment under aerobic conditions and then transferred (with an intermediate treatment) to water under anaerobic conditions. **Right**, residual auxin effect showing increase in length directly proportional to length of treatment. After Cleland and Bonner (11).

Experiments of this type have shown that IAA-induced cell wall plasticization takes place even though the section is not elongating. This plasticization is, however, of a special kind in that it is made and stored in an amount which is proportional to the length of time during which the tissue is treated with auxin. This relation is shown in Figure 10. A brief auxin pretreatment makes it possible for a specified amount of auxin-induced residual growth to take place in the second phase of the experiment. Twice as long an auxin pretreatment permits twice as much residual growth. Clearly, the action of IAA is not to bring about a general decrease in wall rigidity but rather to change the wall in such a manner that it can yield by a specified amount.

Further characteristics of auxin-induced wall softening include the facts that the process is aerobic, inhibited by varied respiratory inhibitors, and suppressed by ethionine. The act of elongation itself, on the contrary, is not inhibited by anaerobiosis and is relatively less sensitive to metabolic inhibitors.

ROLE OF RESPIRATION

It has been known for many years that the application of auxins, including IAA to plant tissue, including *Avena* coleoptile sections, results in rapid and considerable increases in respiratory rate (Bonner, 4, 6). It has been natural, therefore, to seek an understanding of auxin-induced growth in terms of the respiratory response. It appears, however, that such search is fruitless. IAA-induced increase in respiratory rate, in the *Avena* coleoptile at least, does not accompany IAA-induced cell wall softening in nonelongating sections (0.3*M* mannitol) (Ordin *et al.,* 20). The respiratory increase which accompanies IAA-induced growth is therefore an artifact of extension rather than a direct effect of IAA. It is also clear, however, that IAA-induced cell wall softening requires respiratory energy, supplied perhaps in the form of ATP since the phosphorylative uncoupling agent, 2,4-dinitrophenol, blocks IAA action.

INTERACTION OF CELL WALL AND CYTOPLASM

Since the ultimate effect of IAA treatment is upon the cell wall and since IAA-induced cell wall softening requires the participation of mitochondrial respiration, it is perhaps obvious that wall and cytoplasm interact in auxin-induced growth. It is nonetheless of interest that auxin-induced cell wall softening does not occur in plasmolyzed sections in which cytoplasm and wall are in only tenuous contact (Cleland, 10). Such sections are not injured by the treatment since they respond to IAA upon deplasmolysis.

ROLE OF PECTIN ESTERASE

There has been extensive discussion of the possible role of pectin esterase in auxin-induced growth. Glasziou (13) and Glasziou and Inglis (14) in particular have suggested, on the basis of experiments with tobacco pith and Jerusalem artichoke tuber tissue, that auxins function by binding and thus inactivating pectin esterase. *Avena* coleoptile sections do indeed contain readily detectable amounts of pectin esterase (Jansen *et al.*, 17), essentially all of which is bound to cell wall. IAA is, however, without direct effect either on this binding or on the activity of the enzyme in *Avena* coleoptile sections (17). It has already been shown above that the role of IAA in pectic metabolism lies in an earlier step than that mediated by pectin esterase.

SUMMARY

There appear to be two general approaches to the study of auxin action. The first is to determine with what material added auxin interacts within the cell, find out what the interaction product does, and so step by step, trace the sequence by which cell wall softening is ultimately brought about. The second approach is to start at the opposite end of the chain, namely with the final result of wall softening, discover what chemical changes bring about this effect, and step by step trace the sequence forward to the initial interaction of auxin with plant. Both approaches have been used but we are as yet far from linking them. On the one hand, it appears that auxin does interact within the plant with a specific receptor entity and that this interaction involves two point-combination of auxin and receptor (Bonner and Foster, 8). Identification of the auxin-receptor complex by the use of labeled auxin has so far failed, and we conclude only that the complex is present in the plant tissue at very low concentration, of the order of 1 part in 100,000,000 or less. The approach from analysis of cell extension itself has, however, been, as shown above, appreciably fruitful. It is clear that auxin-induced growth is the result of auxin-induced cell wall softening and that this is in turn associated with auxin-induced alteration in the synthesis of cell wall pectic material. Elucidation of the way in which auxin influences pectin synthesis requires that the enzymology of pectic synthesis first be understood, which it is not. This then is the present state of the study of the mechanism of auxin action in the *Avena* coleoptile section.

The facts presented above are numerous and complex. It may be perhaps of passing value to summarize them in terms of a model which, although it may very well be incorrect, will nonetheless serve to help us remember some of the facts. This model, which concerns the parenchymatous cells of coleoptile section and disregards the epi-

dermal cells (which serve merely to slow the growth of the section) (Bonner, 5), starts with the observations of Mühlethaler (19) and of Wardrop (27, 28) that during elongation of coleoptile parenchyma cell walls the constituent cellulose microfibrils are steadily separated from one another and dispersed from their initially transverse orientation. In addition, however, new, transversely oriented microfibrils are steadily added on the inner surface of the wall. Evidently as the wall is stretched during elongation, the microfibrils are pulled apart into a more disperse network and reoriented in the direction of stretch just as in inanimate model systems. We may imagine then that as the wall is stretched, as junction after junction yields and breaks under tension, it becomes progressively weaker. The mechanical strength of the wall is maintained only because of the constant addition of new material to its inner surface. The present model then assumes that the mechanical strength of the cell wall is primarily determined by the most recently deposited material.

Further questions with which the model concerns itself are: What properties of the wall determine how tightly the cellulose microfibrils are linked together? What determines how readily they may be pulled apart? The model nominates the pectic molecules for this function. The long random coils of pectic material intertwine the microfibrils as fungal hyphae intertwine the clay particles in a soil, binding the whole into an interconnected network. And in this function long pectic chains will evidently be more effective than short ones. The addition of auxin to the tissue encourages the production of short pectic chains. This model has many attractive features since, as consideration will reveal, many aspects of cell wall softening can be interpreted within this one framework. The particularly unattractive feature of the model lies, however, in the fact that it would seem to be most difficult to discover whether it corresponds to reality.

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DISCUSSION

Dr. Bennet-Clark: The suggestion that has just been made that growth is only enlargement and that division in two is not growth seems to me quite incorrect. Cell division involves complex differentiation. Cell enlargement can be an osmotic intake of water involving no differentiation, as for example, recovery from wilting.

The growth processes that we have been discussing are those promoted by so-called auxins or growth hormones, the very existence of which was first revealed by studies of tropisms. I would like to refer to the behavior of two geotropic mechanisms which seem to be remarkably different. The first is that of the first internode or mesocotyl of *Zea mays* which is a desirable research object as on geotropic stimulation it bends very sharply with small radius of curvature, which means that the length and volume of cells on the convex side are markedly larger (some 30 to 40 per cent) than those on the concave side and consequently analysis of the differences between them is facilitated. This bending is caused by different growth rates of the two sides of the mesocotyl.

The other quite distinct behavior is that of a grass node. When placed on its side, the lower side expands and the node becomes sharply bent upwards because these lower-side cells expand about 200 to 300 per cent in length and volume. Is this to be called growth? They do not have any normal capability of growth like mesocotyl cells, and expand only in response to the gravitational stimulus.

Some of the earlier workers in the last decade of the nineteenth century showed that a geotropic stimulation of stems (inflorescence axes) resulted in increase of extensibility of the tissue on the lower side. A given bending moment caused greater deflection away from the lower side than that away from the upper side. With a grass node this situation is reversed: it bends more readily towards the upper than to the lower side. This is associated with the fact that cells on the lower side are more turgid than those of the upper side, and they are consequently more rigid. The increase in their osmotic pressure "blows them up" and causes the expansion and the rigidity.

Structurally these cells are very remarkable: The tissue has a concertina-like appearance and the cells have relatively thin walls at right angles to the long axis of the node and very thick walls oblique to this long axis. On expansion, which is sometimes as much as a fourfold extension, these thick oblique walls pull out like the oblique folds of a concertina. This can hardly be described as growth. It does, however, involve factors important in growth; the first is the increase

in the plasticity of the thick oblique walls, and the second is generation of the necessary osmotic pressure to maintain or increase turgor.

Both these processes only occur after the geotropic stimulus. I would not like to state that we have "hormones," one producing plasticity and another generating high osmotic pressure until a better and more extensive survey of growth substances separated by chromatography has been completed. (I remember my first chief's advice: "Beware of the devil because the devil always sends positive results first!")

The point I want to make is that in almost all of our discussion about auxin action and about the activity of substituted phenoxyacetic acids, indoles, and so forth, we assume that because our test process is an expansion in length, it is always the same molecular process that is involved in bringing about the expansion in length. In the node it is quite clear that there are at least two (possibly more) completely separate processes: one is wall softening and the other is build-up of osmotic pressure. In the node this osmotic pressure is quite certainly due to entry of or production of sugars in the lower-side cells. The data will be published soon.

In corn mesocotyls, however, we find that the sugar concentration decreases, whereas that of potassium ions increases very markedly on the lower side.

Growth initiated by so-called hormones involves this complex of processes and may thus really require a complex of hormones for its completion. The process initiated by a synthetic substance like 2,4-D may be a different process from any of those initiated by the natural gravitational hormone or by IAA, though each of these possibly different processes provides the same end result — namely cell extension.

I would like to refer to the views put forward by me earlier. Indole-3-acetic acid does affect wall extensibility, and I thought cross linkages between polysaccharide molecules, especially pectins, the most likely point of attack, and that the mechanism was through control of the methylation of pectin. This view was based on preliminary analyses of the methoxyl content, and we thought also that we had demonstrated a transmethylation accelerated by IAA.

Later work in our laboratory has shown that there is no difference in the degree of methylation of wall pectin in stems and mesocotyls as a consequence of increased extensibility caused by IAA. We had, of course, thought that increased methylation and consequent decreased calcium in the wall was the cause of increased extensibility.

We still have to find a molecular mechanism to explain this increased wall extensibility, and I now no longer favor the pectin-methylation hypothesis.

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The Role of Auxins in the Control of Leaf Senescence. Some Effects of Local Applications of 2,4-Dichlorophenoxyacetic Acid on Carbon and Nitrogen Metabolism

It has long been recognized that senescence in detached leaves is retarded when root initials are formed along the petiole. The green color is then retained, and net protein syntheses and the accumulation of metabolites occur as long as the blade remains attached to living roots. If the roots are removed, the processes of senescence again take place. It has now been shown that the aging processes in detached leaves can be arrested by treatment with certain chemicals and that the presence of roots is therefore not essential for preventing senescence of the blade. For example, Richmond and Lang (5) found that chlorophyll and protein breakdown in detached *Xanthium* leaves was retarded by treatment with kinetin. Person, Samborski, and Forsyth (3) showed similar effects in detached wheat leaves following treatments with benzimidazole, and Brian, Petty, and Richmond (1) reported that both autumnal yellowing and subsequent leaf fall could be retarded in a number of deciduous species by spraying gibberellic acid on the leafy branches. More recent work (2) has shown that suitable applications of both 2,4-dichloro- and 2,4,5-trichlorophenoxyacetic acids (2,4-D and 2,4,5-T) are also effective in delaying certain degradative processes of leaf aging, and it is clear that the role of auxins in the control of leaf senescence must also be considered.

The surface treatment of both attached and detached autumn leaves of *Prunus serrulata* (2) revealed that droplet applications of the butyl ester of 2,4-D (10 to 100 μg.) in ethanol, would result in a retention of green and photosynthetically active chlorophyll below the spot, while the rest of the blade became yellow and senescent. Under these conditions, no roots were formed and there was no apparent cell enlargement or cell division. Autoradiograms of leaves treated

with a radioactive butyl ester of 2,4-D labeled with C^{14} in the carboxyl group showed that radioactivity was initially concentrated below the treated area with only traces of activity in the remainder of the blade. In attached leaves, activity was eventually confined to the areas below the applied drops and corresponded to the areas of tissue which remained green. It had therefore been possible to arrest senescence in a specific group of cells by a relatively high dose of 2,4-D and to maintain these cells within an area of similar cells containing little or none of the acid or related C^{14}-containing compounds.

This method of applying 2,4-D offers a means of studying the effects of relatively high auxin concentrations upon the general metabolism of groups of cells in situ, and for studying differential senescence within a single leaf blade.

The present communication is concerned with some of the changes which occur in the carbon and nitrogen fractions in the leaf blades of *Euonymus japonica* following such local applications of the butyl ester of 2,4-D.

MATERIAL AND METHODS

The experiments were carried out during the summer months of 1959 on well-developed *Euonymus* bushes growing out of doors. Only leaves from the second year wood were used.

One spot of 2,4-D butyl ester in ethanol (26 μg/μl) was applied by micropipette to the lamina on one side of the main vein of the adaxial surface of the blade, so that each leaf received 50 μg. of the ester. Each drop spreads over an area approximately 1 to 1.5 cm. in diameter, and the outer margins of the spread were finely marked round with a nonwater soluble white ink. Control leaves receiving ethanol only were marked in a similar way.

Within six days the leaf tissue bordering on a treated spot became visibly lighter green and later became progressively more yellow until the twelfth day from treatment, when the 2,4-D-treated area appeared as a fresh green spot in an otherwise yellowing blade. At intervals of 1, 3.5, 6, 12, and 13 days from the initial treatment, the respiration of different portions of the blade was measured and the distribution of nitrogen within these parts was determined. The estimations were carried out on 1 cm. leaf discs cut from the blade, and each sample comprised six discs. From each 2,4-D-treated leaf, one disc was cut from within the area delimited by the spread of the spot, and a second from the untreated and opposite half of the leaf. One disc was cut from each control leaf.

Respiration Measurements

Oxygen consumption was measured by the Warburg technique. In the samples from the 2,4-D-treated areas it was essential that residual ester remaining on the surface of the discs should not penetrate the tissues through the cut edges. To avoid this, the six leaf discs were balanced on edge in an upright position on a thin layer of 2 per cent agar which covered the bottom of the flask. Water was added to the side arm and potassium hydroxide to the center well. Each flask was wrapped in a black bag and equilibrated at 20° C. for one hr.; measurements of oxygen consumption were made at half-hour intervals up to six hrs. The discs were removed from the flasks and stored in 80 per cent ethanol and later used for the estimation of the nitrogen fractions.

Nitrogen Measurements

Total nitrogen, alcohol-insoluble and alcohol-soluble nitrogen were estimated separately. Each sample of six discs was macerated in 80 per cent (v/v) ethanol in a VirTis homogenizer and the suspension made up to volume. After removal of an aliquot of the suspension for the estimation of total nitrogen, the remaining suspension was centrifuged and an aliquot of the supernatant liquid used for the determination of alcohol-soluble nitrogen and the whole of the remaining precipitate used for the determination of the alcohol-insoluble fraction. Each sample was digested with selenium catalyst, made alkaline with alkaline metaborate, and the ammonia distilled off in vacuo into borate buffer which was titrated against HCl in the usual way.

RESULTS

There is a measurable effect of 2,4-D on attached *Euonymus* leaves as shown, within 24 hrs., by an increase in the rate of oxygen consumption in discs of leaf blade cut from the treated areas (Figure 1). The values for the rate of oxygen uptake of subsequent samples continue to rise until the thirteenth day after treatment, when the 2,4-D-treated tissues are green and the remainder of the leaf is yellowing. Discs from the treated spots are then respiring oxygen over three times faster than discs from the control leaves. The rate of oxygen consumption of discs from the untreated halves of 2,4-D-treated leaves shows an initial, but less marked stimulation, but this rate falls off again after the sixth day and then remains 30 per cent higher than that of the controls.

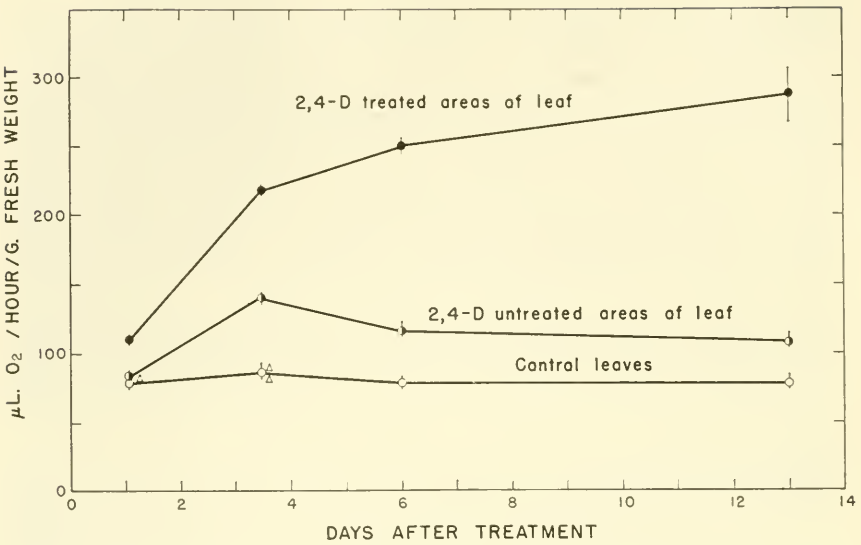

Fig. 1. Oxygen consumption by leaf discs cut from 2,4-D-treated *Euonymus* leaves and from control leaves at various time intervals after treatment. Values indicated by triangles at 1 and 3.5 days are for ethanol-treated control leaves. Duplicate values are indicated by vertical lines.

The nitrogen values given in Figure 2 show that after 3.5 days no measurable changes had occurred in any of the nitrogen fractions; but by the sixth day a significant fall in both total and alcohol-insoluble nitrogen had taken place in the untreated areas of the 2,4-D-treated leaves. This coincided with the first visible signs of yellowing in the untreated parts of the blade. At no time was there a net loss of total nitrogen or of protein in the 2,4-D-treated areas. The evidence suggests that there is an actual increase in both these fractions and it seemed likely that some of the nitrogen lost by the untreated parts of the leaf might be accumulated within the 2,4-D-treated tissues. This supposition is supported by the fact that yellowing occurs first in the tissues immediately surrounding the treated spot; the premature senescence of these surrounding tissues might well be due to a migration of readily respirable substrates from the areas of low auxin concentration towards those of high auxin concentration in which there is a high rate of oxygen consumption. Evidence for the accumulation of nitrogen compounds and possibly also for carbon compounds by the 2,4-D-treated tissues was obtained from the following experiments.

Evidence for the Accumulation of Carbon Compounds

The leaves of a small branch of an outdoor *Euonymus* bush were labeled with C^{14} in the following way: the branch was enclosed in a

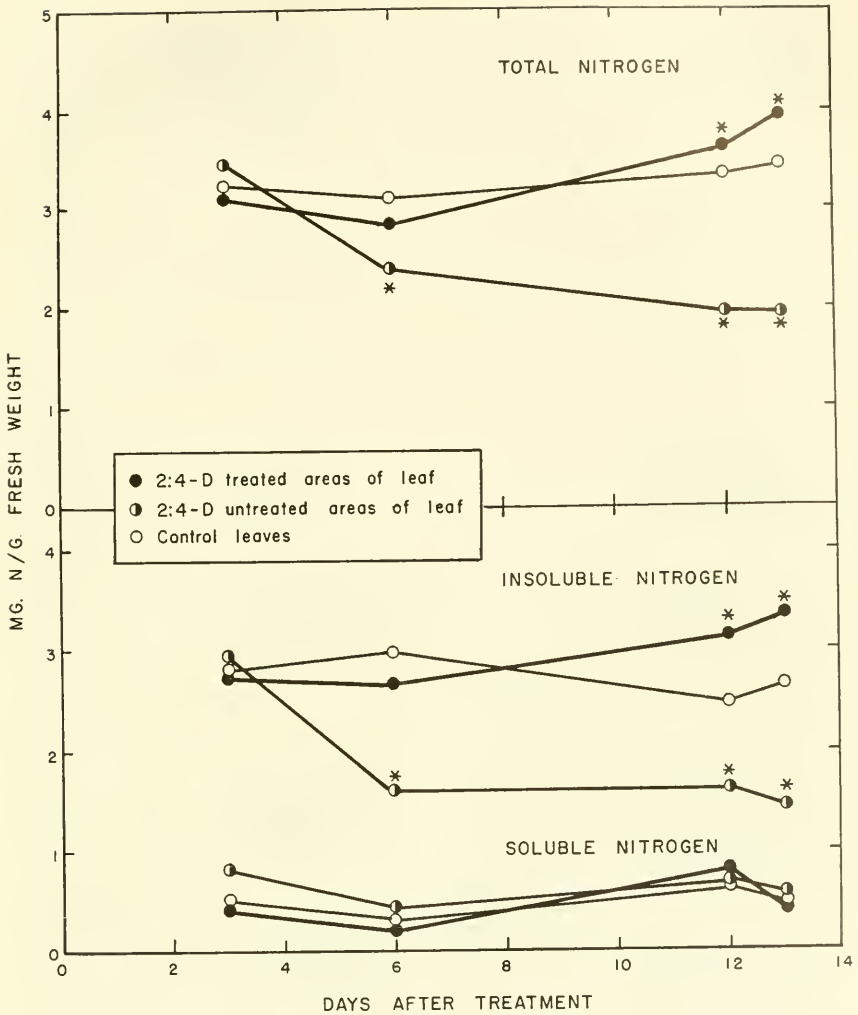

Fig. 2. Nitrogen content of discs cut from 2,4-D-treated *Euonymus* leaves and from control leaves at various time intervals after treatment. Values which are significantly different ($P = 0.65$) from the respective control at any one time-point are marked with an asterisk.

polyethylene bag attached to a flask containing $BaC^{14}O_3$. Carbon dioxide was liberated by injecting an excess of perchloric acid through a vial closure to give an internal concentration of approximately 2 per cent CO_2 and a total of 50 microcuries of C^{14} as $C^{14}O_2$. Four hours later the bag was removed, and drops of 2,4-D ester in ethanol, or ethanol only, were applied to the leaves in the manner previously described. At intervals of 2.5, 6.5, and 12 days from the application of 2,4-D,

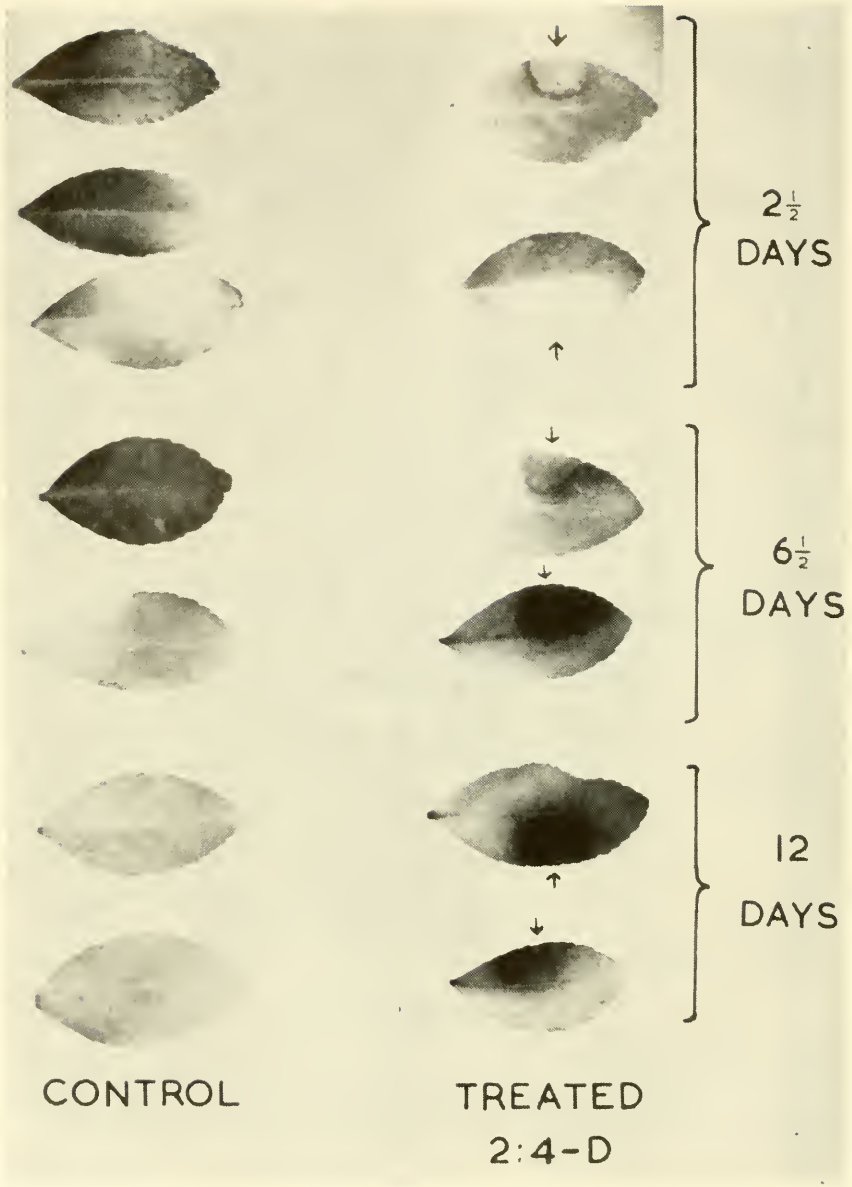

Fig. 3. Autoradiograms of *Euonymus* leaves exposed to $C^{14}O_2$ for 4 hours. Left. Leaves subsequently treated with drop of ethanol. Right. Leaves subsequently treated with a drop of ethanol containing the butyl ester of 2,4-D. Leaves sampled 2½, 6½, and 12 days after treatment.

sample leaves were removed from the branch and subjected to the usual processes of autoradiography.

Reference to the autoradiogram photographs reproduced in Figure 3 indicate that there was an appreciable fixation of $C^{14}O_2$ by all the leaves. Two and one-half days after treatment with 2,4-D some of the leaves showed considerable darkening near the boundary of the treated area indicating either an accumulation or a greater retention of C^{14} material. This is clearly visible (Figure 3, top) in several of the leaf veins leading to the treated spot. The center of the spot appears paler than the surrounding leaf after 2.5 days, possibly as a result of the more rapid loss of C^{14} as $C^{14}O_2$ due to the relatively higher rate of respiration in these areas. By 6.5 days and later, there is a marked concentration of C^{14} in all the 2,4-D-treated spots.

Evidence for the Accumulation of Nitrogen Compounds

It should be noted that control nitrogen values for attached leaves (Figure 2) show daily variation. This variability from occasion to occasion in outdoor control plants is not surprising and has been discussed by Steward *et al.* (6) in considerable detail. He has shown that a wide variety of environmental conditions can produce marked effects upon the nitrogen composition of plants. Although the data in Figure 2 show an increase in both total and protein nitrogen in the 2,4-D-treated spots, a more critical investigation was made using detached leaves so that the majority of the original nitrogen should remain within the leaf and none could be transported away from the blade to other parts of the plant.

The detached *Euonymus* leaves were kept in a relatively damp atmosphere in the following way. The cut end of each petiole was placed in a small tube containing 0.5 ml. of distilled water. To prevent drying out the base of each tube was embedded in 3 per cent agar in the bottom of a larger specimen tube which was plugged with cotton wool. The leaves were treated with a spot of ethanol containing 50 μg. 2,4-D or with ethanol only, and were stored out of doors away from direct sunlight. Nineteen days later, the 2,4-D-treated spots were still green, but the remainder of the blade was yellowing. Respiration and nitrogen determinations were made on discs cut from the leaves, and the results are listed in Table 1. It is seen that there is again a higher rate of oxygen consumption in the 2,4-D-treated tissues than in the untreated parts of the leaf, although both tissues are respiring faster than the controls. There is a loss of both total and alcohol-insoluble nitrogen in the untreated and yellowing areas of the 2,4-D-treated leaves and a statistically significant increase in the total nitro-

Table 1. Values for the respiration rate and for the nitrogen fractions of detached *Euonymus* leaves 19 days after treatment with 2,4-D or ethanol.

Treatment	Respiration μl. O_2/Hr/G Fresh Wt.	Nitrogen Fractions as Mg. N G Fresh Wt.		
		Total	Alcohol insoluble	Alcohol soluble
0 days				
Control leaves	...	3.72	3.21	0.43
Detached for 19 days				
Ethanol-treated area of control leaves	108	3.81	3.22	0.73
2,4-D-treated area of leaves (green)	195	4.38	3.33	0.99
2,4-D-untreated area of leaves (yellow)	164	2.83	2.11	0.49
Sign. diff. of nitrogen values P = 0.05		0.29	0.38	0.20

gen of the treated spots. There is no evidence of a net hydrolysis of protein in the 2,4-D-treated spots, and the increase in total nitrogen in these areas can be accounted for by an increase in soluble nitrogen which must have been accumulated from the untreated parts of the blade. This confirms previous results obtained with our detached cherry leaves (2).

DISCUSSION

It is tempting to speculate if the maintenance of differential rates of metabolism within a leaf by local variations in the auxin concentration could be a controlling factor in determining the movement of metabolites within the blade and thereby determining the differential states of senescence of the cells. The roles of kinetin, benzimidazole, and gibberellin in controlling leaf senescence might also be due, in part, to an effect upon the accumulation of metabolites in treated parts of the blade.

The mechanism for the stimulation of oxygen uptake in the 2,4-D-treated tissues remains to be investigated more fully. Since these tissues retain a photosynthetically active chlorophyll (2) and lose neither total nor protein nitrogen, in spite of the considerable changes which occur in these constituents in the surrounding cells, it might be that 2,4-D is acting in a manner analogous to that suggested for the thyroid hormone in animal tissues (4) by a stimulation of the basic metabolic rate of the cells.

These experiments are still in their early stages. The method of applying 2,4-D to a leaf is providing us with a very valuable way of studying the effects of 2,4-D on groups of cells in situ, where they are surrounded by tissues in which the applied auxin is either absent or in a relatively very low concentration.

SUMMARY

There seems little doubt that the presence of a relatively high concentration of 2,4-D in isolated groups of cells in *Euonymus* leaf tissue can maintain within these cells an abnormally high rate of respiration. The evidence from autoradiograms of C^{14}-labeled leaves has shown that carbon compounds either move into or are preferentially retained in the areas of high oxygen consumption below the spot of 2,4-D. The values from the nitrogen determinations indicate that nitrogenous compounds are accumulated within the 2,4-D-treated areas. These treated cells therefore comprise a specialized part of the leaf in which there is a high rate of metabolism and no net protein breakdown; they seem to act as metabolic sinks to which nitrogen and possibly carbon materials are drawn from the surrounding cells, with the result that there is a premature senescence in the untreated parts of the leaf which contain only a relatively low concentration of the applied auxin. The maintenance of differential metabolic rates within a leaf by local variations in the auxin concentration could be a controlling factor in determining the movement of metabolites within the blade and thereby determining the differential states of senescence of the cells. The roles of kinetin, benzimidazole, and gibberellin in controlling leaf senescence might also be due in part to an effect upon the accumulation of metabolites in treated parts of the blade.

ACKNOWLEDGMENTS

We are indebted to Mr. R. G. Powell of the Agricultural Research Council Unit of Experimental Agronomy, Oxford, for devising the simple method for supplying the attached *Euonymus* leaves with $C^{14}O_2$ and to Dr. D. C. Smith for his advice with nitrogen determinations. We also wish to thank Prof. G. E. Blackman for his constant interest and encouragement.

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DISCUSSION

Dr. Galston: I remember some recent papers by Dr. Reinhold who found that the application of auxin to sunflower hypocotyl sections causes a rapid egress of ninhydrin-positive nitrogenous materials, largely ammonia, I believe. Yet, in your experiments, there was, if anything, a competitive advantage conferred by the feeding of auxin to the treated cells. I wonder if you can resolve this apparent discrepancy?

Dr. Osborne: I can only give you a suggestion. In Dr. Reinhold's tissue there was no available surplus of substrates upon which the rapidly metabolizing tissues could draw, whereas in these leaf tissues we have substrates in the surrounding area of untreated leaf as a source of supply for the 2,4-D-treated cells. The auxin-treated area does not then have to break down nitrogenous materials inside its own piece of tissue to keep pace with the high metabolic rate. In Dr. Reinhold's experiments the loss of nitrogenous materials to the external solution was depressed by the addition of sucrose or succinate.

Dr. Galston: Do you imply that there is an increased senescence of the neighboring cells caused by a local application of 2,4-D?

Dr. Osborne: Yes, and may I add just a few comments on some work I did in the tropics on leaves of *Combretum* (Jour. Trop. Agric. 35: 145. 1958). Spot applications of 2,4-D were made on leaves and within 7 days the blade had yellowed, leaving the 2,4-D-treated area as a green spot on a yellow background. The leaves then abscised. If one collected leaves on the first two days after treatment and diffused the petioles into blocks of agar and tested the blocks in an abscission test, the petiolar diffusate was abscission-retarding. This might be expected following application of a substance such as 2,4-D. After about 3 days, when yellowing of the leaves was apparent, the petiolar diffusate had little or no activity in the abscission test. After 4, 5, and 6 days, the leaves became increasingly yellow and petiolar diffusate became increasingly abscission-accelerating. Since petiolar diffusates from naturally senescing leaves are abscission-accelerating, I suggest that these results give a further indication that

one is, in fact, getting increased senescence in the surrounding tissues following a local 2,4-D treatment.

Dr. Wareing: We have been doing some experiments which bear on Dr. Osborne's results. We are primarily interested in the possible effect of auxin on translocation, and have been feeding labeled sugar to older leaves of bean plants in the basal region of the plant and tracing the movement of the sugar without any applied IAA. As many people have found, we got movement from the applied mature leaf toward the young growing leaf. There is no appreciable movement into already mature leaves between the applied leaf and the shoot apex. If IAA is applied to one of these mature leaves, then sucrose moves into that leaf instead of into the young growing leaves. So, here again, we have movement of labeled nutrients toward a region where hormone is applied.

Dr. Crafts: We have found, using labeled urea, that we get an extremely rapid splitting of the urea and synthesis of the labeled carbon dioxide into sugar. The sugar moved in a perfectly normal fashion, indicating that this labeled urea may be a much handier tool than the sugar — cheaper, much more readily absorbed by the plant, and apparently perfectly normal in its distribution.

Dr. Bach: A minor technical point: Are you sure that the labeling you observed after the application of radioactive 2,4-D is actually in the tissue, not on top of it, or perhaps dissolved in the waxy layers?

Dr. Osborne: As Dr. Crafts has just said, perhaps the tissues would not respond unless some of the 2,4-D entered the leaf. The green areas correspond closely to the areas of radioactivity, but I have not extracted the leaves to determine the amount of activity actually within the tissue.

Dr. Freed: In Dr. Osborne's discussion here I was reminded of a famous name in auxinology, that of Dr. Ezra J. Kraus. He retired to Corvallis, Oregon, and shortly after his retirement suggested an experiment which consisted of treating a plant with IAA and 2,4-D and then measuring the amount of sugar and phosphorus accumulating in the various areas of the leaf. Now this was not with exogenously applied sugar or phosphate but with only the normal metabolic sugars, etc. Results showed an accumulation in the hypocotyl of both the sugar and phosphate in the plant. Radioautograms and chromatographic studies showed that the 2,4-D accumulated in the bean hypocotyl, bearing out Dr. Osborne's findings.

Dr. Osborne: We have done a few experiments in which we fed the plants labeled phosphate and then put on the 2,4-D spots afterwards, but we found no evidence of an accumulation of phosphate in

the 2,4-D-treated areas after 11 days. The leaf seems to be uniformly labeled.

Dr. Freed: Well, 2,4-D applied to a plant apparently inhibits phosphate uptake, but the endogenous phosphate accumulates in the areas with 2,4-D largely because of the stimulated respiration.

Dr. Wittwer: A number of years ago, we studied the effect of diethyl ether on the movement of calcium in the bean plant, then followed this with work on the effects of growth substances that suspended polarity. We found that in line with the results just mentioned by Dr. Osborne, there are a number of mineral nutrients that are altered in their transport and distribution in the plant following the application of such growth substances as 2,3,5-triiodobenzoic acid, l-naphthaleneacetic acid, and maleic hydrazide. Samish (Plant Analysis and Fertilizer Problems, pp. 156–165 [Institute de Recherches pour les Huiles et Oléagineaux, Paris, France, 263 pp.], 1954) and Kessler and Moscicki (Plant Physiol., 33: 70. 1958) both reported a greening up of chlorotic foliage following treatment with growth substances. Maleic hydrazide, l-naphthaleneacetic acid, and 2,3,5-triiodobenzoic acid have promoted the movement and transport of iron and calcium in tissues where otherwise it would not occur.

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Oxidants, Antioxidants, and Growth Regulation

The re-establishment of interest in oxygen toxicity (6, 8, 9, 10, 11, 25) has provided a new orientation in oxygen biochemistry. Recognition of the dual role of oxygen in life process opens the way to a more fundamental understanding of interrelationships involving the physical organic chemistry of oxidation, the metabolic machinery of the cell, and processes of growth and differentiation.

Interest in oxygen poisoning has led to the discovery that many radiation protectant-reducing compounds often active in catalytic quantities also provide a biological defense against oxygen (8, 11, 25). Such an overlap of protective agents is a logical consequence of the well-established interdependence between oxygen and ionizing radiation in inflicting cellular damage (8, 16). It has been suggested that damage produced by these agents is no more than an intensification of activity along already-existing radical-mediated (that is, one-electron) pathways of oxidation-reduction (25, 28), and that deteriorative changes so effected, even by oxygen alone, differ little from the terminal processes of senescence as they ordinarily take place. Thus, it has been concluded (29) that "The life-shortening action of radiation involves the induction of . . . . degenerative changes and stimulates in many respects acceleration of the natural aging process." The demonstration in the early 1950's of oxygen and antioxidant effects in heat damage led similarly to a concept linking tissue damage and accelerated aging processes (2).

These and other considerations, together with the recognition of antioxidant activity as a function of electron mobility (26, 27), have led to the formulation of the Regulator-Antioxidant Hypothesis, which seeks to relate regulation of growth, development, and aging

to the balance of oxidants (electron acceptors) and antioxidants (electron donors) in the cell tissue or organism:

A. Definition and properties of antioxidants.
   1. Any electron donor which inhibits the oxidation of suitable labile substrates with high stoichiometric efficiency is an antioxidant.
   2. Antioxidants may operate by:
      a. Reaction with an intermediate which is the electron-deficient member of an oxidation-reduction equilibrium.
      b. Trapping or deactivation of radicals or ions.
      c. Reaction with an oxidant which would otherwise attack a labile substrate.
      d. Reaction or interaction with the electron-deficient form of an oxidation (electron-transporting) catalyst. Both electron-transfer and π-complex formation are included.
   3. Antioxidant efficiency derives from the following mechanisms:
      a. Participation in a cycle wherein a suitable electron source can regenerate the antioxidant from its oxidized form.
      b. Trapping of radical or ionic initiators of chain reactions.
      c. Reducing the efficiency of an electron-transport catalyst with which it interacts.

B. Biochemical activities of antioxidants derive from the following:
   1. Antioxidants protect labile cellular components against attack by primary oxidants such as O_2 or derivative oxidants including H_2O_2 and other peroxides, oxidizing radicals, ions, or such moieties when bound in macromolecules.
      a. Non-specific protection entails a general shift of oxidation-reduction equilibria toward the reduced conditions.
      b. Specific protection may be conferred by thermodynamic and geometric restrictions which fix the antioxidant in electron transfer chains or localize it on selectively adsorbing surface sites of macromolecules or membranes.
   2. Antioxidants may influence or regulate specific metabolic pathways:
      a. By reductive activation of the inactive oxidized forms of enzymes (e.g., $\text{Enz - SS - R} + e^- + H^+ \rightarrow \text{Enz - SH} + \cdot\text{S - R}$).
      b. By blocking electron-transport catalysts, antioxidants may "shut down" specific metabolic pathways (e.g., inhibition of flavoproteins by formation of a π-complex between antioxidant and riboflavin).
      c. As mobile electronic systems by serving themselves as electron carriers or carrier moieties of oxidizing enzymes.

C. Oxidant toxicity — the physiological consequences of antioxidant action.
   1. Their essential role in metabolism notwithstanding, oxygen and other oxidants attack essential cellular components, and bring about a cumulative loss in essential functions.
   2. Ontogeny (the developmental sequence of the individual) begins with an excess of antioxidants, a relatively low oxygen tension, and frequently, a low oxygen demand.
   3. As development proceeds, oxidized substances accumulate. The rate of oxidant accumulation will vary with the environmental oxygen supply. Increase in environmental oxygen may be gradual, but frequently involves a discontinuity such as hatching or birth.
   4. As development proceeds further in continuous contact with oxygen, oxidized cellular components accumulate, increasing the ratio oxidant/antioxidant (or electron acceptor/electron donor). As the acceptor-donor balance shifts toward the oxidized state, cellular activities are altered:
      a. A loss in proliferative capacity — decreased mitotic activity — stabilized cell populations in tissues and organs. Differential oxidant tolerance results in unequal cessation of suppression of cell division.
      b. Certain cellular growth activities may persist in the more oxidized state, or may be favored at a higher acceptor-donor ratio than cell division. Cell enlargement, especially in plants, is indicated here.
      c. Further shifts in the acceptor-donor balance terminate all growth processes, but allow maintenance of the individual cell.
   5. Maintenance of the nongrowing cell, tissue, or organism characterizes the prematuration state.
      a. Prematurity is an unstable transition state. The acceptor-donor balance either attains a plateau or lies within range of values analogous to a range of maximum buffering capacity.
      b. Further maturation processes may entail chemical changes indicative of the progressive domination of the cell by oxidants and oxidation. Examples are melanization and lignification.
      c. Initiation of major secondary chemical changes may shorten the transition state leading to rapid loss of function.
   6. Beyond the efficient "buffer range," accumulation of oxidative

"faults" is an accelerated process leading to deterioration of structures and functions in nucleus and crytoplasm. Senescent degeneration is then under way.

7. Attainment of some large acceptor/donor ratio corresponds to major losses in maintenance and synthetic capacities (e.g. failure in protein synthesis) limiting survival of the cell, etc., to the lifetime of residual enzymes and other already formed species. The terminal phase of senescence is in progress.
8. Antioxidants which promote growth may do so by maintenance of a relatively youthful, reduced state. They enhance cellular abilities to resist oxidant damage.
9. Increased oxidant stress shifts the role of antioxidants from functions in quantitative growth control to functions in survival on an all-or-none basis.
10. Under reduced oxidant stress hormonal requirements will be lowered. Growth can be enhanced by increase in antioxidant or decrease in oxidant as long as their ratio lies within suitable limits. Further, maturation processes will be retarded.
11. Some low value for the acceptor/donor ratio should correspond to the chemically reduced state which favors proliferating cells. Electron mobility is characteristic of most estrogens, androgens, and carcinogens, and addition of such donors must lower the ratio into this critical region.

ANTIOXIDANT ACTIVITY AND GROWTH

General Considerations

The existence of a relationship between reducing agents and growth is established. In animal systems, the thiol-disulfide equilibrium has been implicated in cellular multiplication (1, 19). Thyroxine, a powerful antioxidant (23), is implicated directly in growth processes, possibly by way of its ability to shift the thiol-disulfide equilibrium toward the reduced state (13). Other substances which stimulate tissue growth or regeneration include phenols, polynuclear hydrocarbons, arylamines (10,11), carbazoles, stilbenes, and phenothiazine, and triphenylmethane dyes (1, 17, 19). Among these structures a common feature is their high level of electron availability. All of these molecular types contain mobile π-orbital electrons or coupled n(nonbonding)-π-systems if atoms such as N, S, or O are on or in the aromatic system.

Antioxidant or electron-donor properties may also be found among plant regulators, including indoles, aryloxy compounds, unsaturated hydrocarbon derivatives, coumarins, etc. (19, 23, 26, 27). Among the conventional reducing agents involved in growth processes are ascorbic acid and the thiols. In contrast with the growth-promoting inhibitors

of oxidation, more oxidized substances may be physiologically inactive or even inhibitory. Oxindoles are representative of the inactive state; quinones as thiol reagents and H-abstractors serve as harmful oxidants. Quinonoid substances have been implicated in normal cessation of growth and onset of differentiation in plants (18, 30). Quinones are known experimentally for their antimitotic and radiomimetic activities (19).

Experimental Studies

Methods employed in evaluation of antioxidants may be found in published or forthcoming accounts. The experimental oxidation systems used include iodide-H_2O_2 (26, 27); eugenol, peroxidase-H_2O_2 (22); eugenol, celery vascular tissue-H_2O_2 (21, 24); and to a lesser degree iodide, peroxidase-H_2O_2 and pyrogallol-O_2 (23). Measured oxidation products are, respectively, iodine; dimerization product; lignin; and in the last two systems iodine, once again, and a phenol polymer-polyquinone mixture.

The growth responses studied included germination of onion (*Allium cepa,* 'Yellow Globe'); elongation of turnip radicle (*Brassica rapa,* 'Purple Top White Globe'); elongation of cucumber hypocotyl sections (*Cucumis sativus,* 'Improved Long Green'); and elongation of flower stalk sections from *Taraxacum officinale.* All test materials were cultured in solutions buffered at pH 6.65 (0.066*M* phosphate) under 50 footcandles constant illumination (22, 27).

The chemical test systems have been used to demonstrate antioxidant activity in the following compounds: *Indoles,* including indole, indoline, methyl- and phenylindoles, IAA; other indolealkanoic acids, 5-hydroxy-IAA, tryptamine, tryptophane, serotonin, and carbazole; *Pyrroles,* including pyrrole, methyl- and phenylpyrroles, the bile pigments, and pyrrolidine; *Hydrazines,* including hydrazine and its salicylyl-, malonyl-, maleyl-, isonicotinyl-, aryl-, and alkyl- derivatives; *Diazines* and miscellaneous heterocyclic compounds, including purine, mercapto-purines, benzimidazole, pteridines, and benzthiophene; *Amines,* including alkylamines, phenylethylamines, and arylamines; *Aryloxy* compounds, including diphenylether, benzofuran, anisole, thyronine, and iodothyronines (thyroxine, for example); and various thiols, phenols, ascorbic acid, and Co^{+2} salts.

Activity-constitution relations have emerged in some instances (23, 27). Thus antioxidant efficiency among the indoles is reduced by electron-withdrawing groups, as in oxindole, oxindone, or indole aldehyde. Less marked reduction results from 3-substitution. Both deactivating effects are illustrated in the series indole$>$ skatole$>>$ indoleformic acid. Deactivation may also be effected by decreased

electron availability at the heteroatom, as in indole> benzthiophene> benzofuran>indene.

Other examples of chemically meaningful effects are deactivation of phenylhydrazine by the *p*-nitro group and enhancement of the activity of pyrrole by methylation at positions 2 and 5. Hydrogenation of pyrrole (yielding pyrrolidine) reduces its efficiency to the level of aliphatic amines which usually are weak reducing agents, whereas indole, after hydrogenation to indoline, retains its fundamental aromatic character (as an *N*-substituted aniline), i.e. remains highly active.

Of approximately 80 compounds studied, only one-fourth have been tested as growth regulators, although most are known to have some form of biological activity. Growth of the turnip radicle in $10^{-6}M$ test solutions was stimulated by indole, methylindoles; isoni-

Table 1. Growth promoting activity of antioxidants.

Compound	Concn. $M \times 10^6$	Elongation of 0.9 Mm. *Cucumis* Sections in 12 Hrs. Relative to:	
		Endogenous as 100	IAA as 100
Pyrrole	2.0	164	100
	5.0	130	100
Skatole	0.1	133	100
	1.0	175	120
Serotonin	2.0	160	94
Thyroxine	2.0	138	61
Hydrazines			
Methyl-	1.0	128	45
1,1-Dimethyl-	1.0	128	45
1,1-Diphenyl-	1.0	170	105
1-Naphthyl-	1.0	142	63
Isonicotinyl-	1.0	150	75
		Elongation of 1.2 Mm. *Taraxacum* Sections in 24 Hrs. Relative to:	
		Endogenous as 100	IAA as 100
Pyrrole	1.0	135	30
	10.0	147	31
Skatole	1.0	177	63
Thyroxine	2.0	138	32
Hydrazines			
Methyl-	1.0	122	18
1,1-Dimethyl-	1.0	122	18
1,1-Diphenyl-	1.0	133	27
1-Naphthyl-	1.0	133	27
p-Bromophenyl-	1.0	144	36
Isonicotinyl-	1.0	153	43

cotinyl hydrazine (isoniazid) and other hydrazines; and by tyramine and mescaline. The phenylethylamines promoted growth only 20 to 25 per cent, but the other compounds, all more efficient antioxidants, increased growth 90 to 150 per cent.

In the two section tests, extensive comparisons were made between IAA and a number of other antioxidants as elongation promoters (Table 1). *Cucumis* elongation is promoted to approximately the same degree by IAA, pyrrole, skatole, serotonin, and diphenylhydrazine. The other compounds listed show one-half to three-quarters of IAA activity. *Taraxacum* elongation is more specific for IAA, but even the weakest growth promoters were one-fifth as active as IAA.

When chemical and biological activities are considered jointly, it is apparent that substances of different constitution but high antioxidant efficiency, show qualitatively similar biological effects (Table 2). Percentage deviations from appropriate controls have been tabulated to show both the direction and magnitude of the effects.

Although some correlation may be found in comparing specific chemical and biological tests, experience has shown that this is not generally true. We must recognize that, ideally, antioxidant activity is a measure of electronic behavior whereas over-all biological effectiveness also depends upon thermodynamic and geometric properties which are the determinants of transport and localization.

Lignification is one of the processes which characterize maturation and cellular senescence in the green plant (21, 23). The experimental biosynthesis of lignin proceeds through a radical initiated chain mechanism and offers a model for the natural process. Experimentally, quinones accelerate lignin synthesis, a role consistent with their appearance in differentiating tissues. In contrast, various metabolites, especially DPN, are powerful inhibitors of lignin formation. Extensive lignification is thus indicative of cellular oxidation-reduction imbalance. It can be "forced" upon young cells experimentally but is most pronounced in xylem differentiation where it accompanies or is accompanied by protoplastic degeneration. IAA and other antioxidants both inhibit lignin synthesis and promote elongation. The growth-promoting activities of some biological agents acting as antioxidants in their proposed protective and metabolic roles may be reflected in their ability to delay the onset of lignification and attendant deteriorative oxidative processes.

OXIDANT-ANTIOXIDANT INTERACTIONS IN GROWTH

General Considerations

Application of reductants accelerates regeneration whereas callus formation is inhibited by oxygen and other oxidants (28). Cysteine, Co^{+2} salts, propyl gallate, and sympathomimetic amines have been

used successfully to protect ciliates, rodents, and vascular plants against oxygen poisoning. The culture of *Clostridium tetani,* an obligate anaerobe, in air can be accomplished in the presence of Co^{+2} salts (3). In this case, the antioxidant protects the organism against ordinary atmospheric levels of oxidant.

Both growth and enzyme levels in embryos of *Phaseolus vulgaris* ('Red Kidney') are reduced by incubation at elevated oxygen tensions (20, 23). Other signs of oxygen damage in a variety of plants suggest disturbance of the protoplast membrane and accelerated aging of leaf tissue (6). Pea root tissues damaged by incubation in pure O_2 show increased peroxide production and increased IAA destruction (7). The presence of Co^{+2} suppresses excessive oxidations and reduces visible injury. Mitochondria can be protected against harmful levels of oxygen by thyroxine (15). Enzymes whose activity is thiol-dependent are sometimes readily inactivated by oxygen, even at atmospheric level (14). Tyrosyl groups may be reasonably put forth as an additional oxidation-sensitive member of the peptide chain. Peroxides participate in the depolymerization of deoxyribonucleic acid and even more drastic chemical changes occur at elevated oxygen tensions (4, 12). Organic peroxides formed from cellular components can in turn attack other constituents such as thiols (5).

Experimental Studies

The study of oxygen and oxidant toxicity consists of simple germination and growth tests as previously described. Experimentally, responses of plant systems both to increased oxidant and to temporary removal of oxidant stress will be considered. The recognition of peroxides as probable intermediates in oxygen poisoning has led to their use in experiments.

Damage to lettuce seed (*Lactuca sativa,* 'Iceberg') incubated in hydrogen peroxide was reduced markedly in the presence of hydrazine, although the latter alone at the concentration used was also toxic (Table 3). A reciprocal relationship is indicated in the mutual cancellation of toxic effects of oxidant and antioxidant. A similar response is the removal of inhibitory effects of high levels of IAA by elevated oxygen tension. Organic peroxides, which are not decomposed by catalase, are generally more toxic than H_2O_2. Nevertheless, it is possible to obtain an appreciable amount of protection against their effects by the use of suitable antioxidants. For example, indole has been employed as a partial protectant against *p*-menthane hydroperoxide.

Table 2. Comparative effects of selected antioxidants on growth and oxidation processes.

System	Molar Concn. of Antioxidant	Percentage Change Produced by Antioxidant					
		IAA	Skatole	Oxindole	Thyroxine	Isoniazid	Pyrrole
Oxidation							
Iodide-H_2O_2	1×10^{-4}	− 24	− 65	− 10	− 50	−100	−100
Eugenol, peroxidase-H_2O_2	5×10^{-4}	− 53	− 25	0	− 40	− 97	− 28
Eugenol, vascular tissue-H_2O_2	5×10^{-5}	− 71	− 21	0	− 36	− 70	− 30
Growth							
Onion germination	2×10^{-6}	− 73	− 56	− 10	− 63	− 63	− 45
Turnip radicle	1×10^{-6}	+ 10	+121	0	+ 25	+200	+ 25
Cucumis hypocotyl	1×10^{-6}	+ 64	+ 75	0	+ 38	+ 50	+ 64
Taraxacum flower stalk	1×10^{-6}	+112	+ 77	+ 5	+ 38	+ 53	+ 37

Table 3. Interaction of peroxides and antioxidants in seed germination.

Oxidant and Concentration	Turnip Seed Germination (Per Cent) With Indole at:				
p-Menthane hydroperoxide, $M \times 10^{-5}$	0	0.05	0.50	1.0	$\times 10^{-5}M$
0	79	60	50	50	
2	44	52	55	58	
5	12	16	20	30	
50	0	6	6	10	
	Lettuce Seed Germination (Per Cent) With Hydrazine at:				
H_2O_2, $M \times 10^{-3}$	0	0.2	1.0		$\times 10^{-3}M$
0	96	80	60		
10	60	66	82		
20	8	14	36		
50	0	0	0		

Growth promotion under ordinary atmospheric conditions is common to a number of protectants against oxygen stress. Thus IAA and indole reduce damage by oxygen, ozone, and organic peroxide; mescaline and isoniazid protect against ozone; Co^{+2} protects against oxygen, hydrogen peroxide, and organic peroxides.

One example will illustrate the effects of simultaneous treatment with several protectants: lettuce seed in $5 \times 10^{-4}M$ *p*-menthane hydroperoxide failed to germinate even after 100 hrs. at 25°C. With $10^{-5}M$ ascorbic acid, 4 seeds germinated in a population of 200; with $10^{-7}M$ IAA or $CoCl_2$, 10 germinated. Addition of ascorbic acid to IAA had no further effect, but mixtures of $CoCl_2$ either with ascorbic acid or IAA permitted germination of 14 seeds. Finally, all three protectants combined enabled 26 seeds to germinate.

If the antioxidant properties of hormones are functionally important, hormonal requirements should be sensitive to environmental oxidant level. This proposition was tested with elevated levels of oxygen and peroxides. Reduction in oxidant level should lower the hormone requirement. Elimination of the aerobic Co^{+2} requirement for *C. tetani* by removal of oxygen provides a partial test of the proposed relationship. When 2-week-old cucumber seedlings are held under anaerobic condition (argon) for a limited period, the subsequent growth of hypocotyl sections is enhanced, and sensitivity to

Table 4. The effect of anaerobic pre-conditioning on growth of cucumber hypocotyls.

Treatment	Elongation in 12 Hrs., Mm.		
	In Buffer	In $1.5 \times 10^{-6} M$ IAA	IAA Buffer (per cent)
Air control	1.0	2.1	210
Argon: 1.5 hrs. Air: 4.5 hrs.	1.0	2.0	200
3.0 hrs. 3.0 hrs.	1.4	2.2	154
6.0 hrs. 0.0 hrs.	2.8	2.0	71
Submerged in water 6.0 hrs.	2.1	2.1	100

IAA diminished (Table 4). The data show that a period of anaerobic pre-conditioning can replace the external auxin requirement, and even render moderately inhibitory an ordinarily optimal IAA level. Anaerobic pre-conditioning may be equivalent to addition of an antioxidant such as Co^{+2}, which increases growth by preventing IAA destruction, or may effect a more general increase in the reduction potential of the cell, delaying oxidation of many labile components.

SUMMARY

This paper calls attention to a new interrelationship between oxidation and growth. The antioxidant properties of known hormonal entities provide the link between these two processes. Geometrically varied growth promoters share in common the ability to inhibit lignin synthesis and other oxidations diagnostic of unbalanced oxidation-reduction states in maturing (aging) cells. The toxic effects of oxygen constitute a major contribution to degenerative changes in cell senescence. Accordingly hormonal substances with antioxidant properties have been assigned a protective role by buffering the cell against cumulative damage by oxidants. The dynamic and continuous relation between oxidants and antioxidants is illustrated by substances which promote growth under ordinary aerobic conditions, become survival factors at elevated oxidant levels, and yet lose their effectiveness, or inhibit, when the oxidant level is reduced. Three general functions are proposed for antioxidants as bioregulators. First, antioxidants serve as protectants for specific structures or through their general effects on oxidation-reduction balance. Second, antioxidants may serve as metabolic regulators by selectively blocking specific pathways. Finally, as mobile electronic systems, antioxidants may act as cofactors in electron transport.

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The Intracellular Locale of Auxin Action: An Effect of Auxin on the Physical State of Cytoplasmic Proteins[1]

Modern theories of the mechanism of auxin action emphasize the demonstrable effects of auxin in increasing the plastic extensibility of the cell wall (4). These effects, first noted by Heyn more than 25 years ago, are attractive in explaining the cell elongational aspects of auxin action, since it is clear that the wall must be plasticized if elongation is to occur. This type of theory is not completely satisfactory in explaining auxin action, however, in that auxin is also known to produce marked changes in the cytoplasm (5, 8), and to initiate mitotic activity in certain cells (7). These latter effects are difficult to account for in terms of changes in cell walls mediated by auxin.

In the course of investigations on the intracellular location of C^{14}-carboxyl-labeled 2,4-dichlorophenoxyacetic acid (2,4-D) fed to green and etiolated pea stem segments, we discovered fortuitously a marked effect of this compound and of other auxins on the physical state of the proteins in a centrifugal supernatant devoid of all cellular particulates. The effect noted was a marked decrease in the heat coagulability of the proteins in the auxin-treated tissues as compared with the control tissues. The magnitude of the effect is as great as the often cited effects of auxin on the cell wall, and is therefore to be considered as of possible significance in the growth reaction initiated by auxin. We wish to emphasize that the altered heat coagulability of the cytoplasmic proteins is probably not, *per se,* the important physical property possibly related to growth; it is, however, a possible

[1] Aided by grants from the National Science Foundation and the U.S. Public Health Service. We are indebted to Mary Lyons and Drs. S. Maheshwari and N. Maheshwari for assistance with the experiments on heat coagulability of the proteins.

indicator of some fundamental change in the configuration of the protein molecules, the exact nature of which remains to be delineated.

MATERIALS AND METHODS

'Alaska' peas were used throughout these investigations, being grown under conditions already described in another article in this volume (2). Etiolated and light-grown sub-apical stem sections were grown overnight in the presence or absence of an auxin. After measurement of growth, the sections were homogenized in ice-cold 0.25*M* sucrose + .001*M* ethylenediaminetetraacetic acid (EDTA) in a prechilled mortar and pestle to yield a suspension of 12.5 mg. fresh wt/ml. This suspension was repeatedly centrifuged at various speeds in an International Refrigerated Centrifuge (Model PR-2) to yield, successively, cell wall fragments and unbroken cells, chloroplasts (when present), mitochondria, microsomes, and a final centrifugal supernatant fraction containing the soluble proteins of the cell. The fate of the nuclei in such a fractionation is unknown, but they or their fragments are presumed to sediment along with the plastids or possibly mitochondria.

In certain of the experiments, C^{14}-carboxyl-labeled 2,4-D was used as the auxin. This was purchased from Nuclear Chicago Company, and had an activity of 950 microcuries per millimole. Aliquots from the fractionation were pipetted on to 1¼" × 3/32" stainless steel planchets, and were counted in a stream of Q-gas with a Nuclear Chicago model D-47 gas flow counter equipped with a micromil window, mounted in a model M-5 sample changer, and attached to a model 186 decade Scaler.

RESULTS

The fractionation scheme employed for tracing the intracellular localization of exogenously applied labeled 2,4-D is shown in Figure 1. With green sections, the 2,4-D concentration applied was the $3 \times 10^{-5}M$, which is approximately optimal for growth in the light and in the presence of 1 per cent sucrose. Etiolated sections were given this same concentration of 2,4-D, although it is approximately 100 times too high for their optimal growth in length, but about optimal for increase in fresh weight.

The distribution of radioactivity in the various fractions of green and etiolated cells is seen in Table 1. It should be noted that the final supernatant contains a major part of the activity, as does the first precipitate, P_1, consisting of unbroken cells and cell walls. When the P_1 fractions were reground and recentrifuged, considerable activity was lost from the precipitate to the wash medium, two such

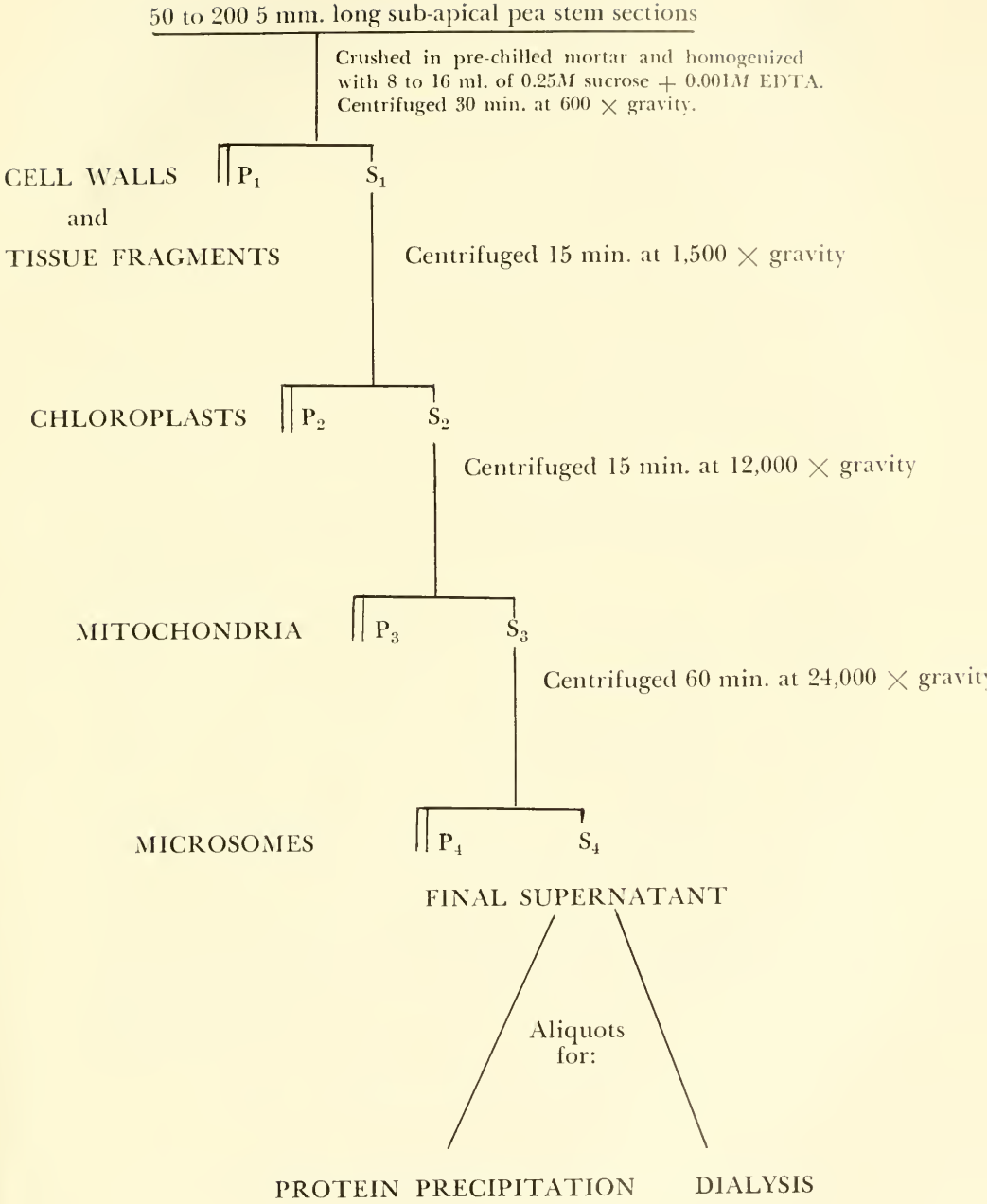

Fig. 1. Fractionation scheme for green pea stem sections.

Table 1. Localization of radioactivity in cell fractions of pea stem sections incubated overnight with $3 \times 10^{-5} M$ 2,4-D (950 μc/millimole, carboxyl-labelled).

Fraction	Description	Av. Corrected C.P.M.		Per Cent of Total Activity	
		Green	Etiolated	Green	Etiolated
P_1	Cell walls and tissue fragments	2,480	2,510	34	39
S_1	First supernatant	4,800	3,969	66	61
P_2	Chloroplasts	498	.....	7	..
P_3	Mitochondria	407	403	6	6
P_4	Microsomes	268	211	4	3
S_4	Final supernatant	3,640	2,175	50	34

Table 2. Effect of regrinding and washing on C^{14} content of cell wall fraction.

Fraction	Description	Av. Corrected C.P.M.		Per Cent of Original Counts Retained	
		Green	Etiolated	Green	Etiolated
P_1	Cell walls and tissue fragments	2,480	2,510	..	..
P_{1a}	P_1 ground, washed and centrifuged	500	740	20	29
P_{1b}	P_{1a} ground, washed, centrifuged	270	360	11	14

Table 3. Effect of washing in sucrose—EDTA on C^{14} content of various precipitate fractions of green sections.

Fraction	Description	Av. Corrected C.P.M.	Per Cent of Original Counts Retained
P_2	Chloroplasts	169	..
P_{2a}	Washed chloroplasts	10	6
P_3	Mitochondria	156	..
P_{3a}	Washed mitochondria	13	8
P_4	Microsomes	107	..
P_{4a}	Washed microsomes	10	9

operations sufficing to reduce the radioactivity in the precipitate to 11 to 14 per cent of the original level (Table 2). We thus consider that the wall fractions themselves contain little or no firmly bound 2,4-D. The same appears to be true of the other particulate fractions, in which a single washing in sucrose-EDTA reduces the radioactivity to under 10 per cent of the original value (Table 3). It is thus clear that the great bulk of the applied 2,4-D is not bound to any visible particulate in the cell, and must be assumed to be in the soluble phase.

Our next experiments were designed to test the possibility that the 2,4-D was bound to some macromolecule in the final supernatant (S_4) fraction, since several previous investigations had reported the presence or formation of auxin-protein complexes in plant cells (3, 6, 10). Dialysis of the final supernatant against the sucrose-EDTA medium used in preparing the homogenate revealed a ready outward passage of the label (Table 4). Two successive 16-hr. dialyses at 2° C. against 100 volumes of medium reduced the residual counts to about 3 per cent of the original value. Since this did not differ significantly from the behavior of 2,4-D added in vitro to control supernatant protein (from sections not treated with auxin during the overnight growth test), this experiment offers no evidence for the binding of applied 2,4-D to any non-dialyzable component of the final supernatant.

Attempts were next made to precipitate proteins by 0.5*M* trichloroacetic acid, by saturated $(NH_4)_2SO_4$ (pH 6.1), and by boiling (100° C. for 8 min.), and to test the precipitates for C^{14} content. The first two methods yielded only very weak activity in the washed precipitates; however, the boiling experiment, while providing no direct evidence for protein-bound 2,4-D, led to an observation of great interest. We noted that those homogenates derived from auxin-treated cells yielded little or no precipitate, the solution merely turning opalescent after immersion for 8 min. in a boiling water bath. The controls, on the other hand, invariably yielded a copious bulky-white precipitate. This experiment could be rendered quantitative by cen-

Table 4. Dialysis of final supernatant of green sections.

Fraction	Description	Average Corrected C.P.M.
S_4	Final supernatant	1,390
S_{4r}	Residue of S_4 dialysis	435
S_{4r1}	Residue of S_{4r} dialysis	45

Table 5. The effect of various concentrations of 2,4-D on the growth and protein coagulability of green and etiolated pea stem sections.

2,4-D Molarity	Green Sections			Etiolated Sections	
	Increase in fresh wt., per cent	Turbidity reading	Mg. dry wt. heat-coagulated protein	Increase in fresh wt., per cent	Mg. dry wt. heat-coagulated protein
0 (initial control)	. . .	113	37.7	. . .	65.3
0 (final control) . .	33	136	27.5	55	53.6
10^{-6}	67	122	28.7	114	40.3
10^{-5}	93	87	20.1	130	21.9
10^{-4}	86	56	17.6	124	18.4

trifuging down the precipitate, transferring it quantitatively to a tared weighing pan, drying overnight at 90° C., cooling in a desiccator, and weighing to the nearest 0.1 mg. Alternatively, in those instances where flocculent precipitates failed to form, the quantity of suspended material could be estimated turbidimetrically in a Klett-Summerson photoelectric colorimeter equipped with a #42 blue filter. Sample data from such measurements are presented in Table 5. It is clear that increasing concentrations of 2,4-D sharply reduce the quantity of heat coagulable proteins, whether measured gravimetrically or turbidimetrically.

Other experiments (1) have revealed that indole-3-acetic acid (IAA) is approximately as active as 2,4-D in decreasing the heat coagulability of the proteins; the weak auxins phenylacetic acid and 2,3,5-triiodobenzoic acid are slightly active, and the antiauxin *p*-chlorophenoxyisobutyric acid is completely inactive. The effect appears not to be produced in vitro by a mixing of control protein with the auxin. There also appears to be no effect of auxin on total protein, since the precipitates deposited in 0.5*M* trichloroacetic acid are equal in control and auxin-treated fractions. The effect is thus probably to be interpreted as an auxin-induced alteration of the physical state of the cytoplasmic proteins.

DISCUSSION

While the auxin effect we have described in this paper is new to plant physiology, there are certain previous references in the literature which appear to be closely related to it. For example, Thimann and Sweeney (8), in an elegant series of papers, showed that IAA applied to *Avena* coleoptile cells markedly increased the rate of cytoplasmic streaming. The effect was apparent within minutes, and the dose-response relationships and the inhibition by various agents par-

alleled closely the results obtained in growth experiments. In a somewhat similar series of experiments, Northen (5) applied auxins in lanolin paste to one side of 'Navy' bean petioles, and after a suitable incubation period, centrifuged the petioles at right angles to their long axis. Cells on the auxin-treated side showed predominantly displaced contents, while those on the control side did not. Northen interpreted these results as indicating an auxin-mediated decrease in cytoplasmic viscosity. This interpretation would harmonize well with the Thimann-Sweeney experiments, since the rate of cytoplasmic streaming must be assumed to be inversely proportional to the viscosity of the cell contents. Similarly, our results can best be interpreted as an alteration of the physical state of the cytoplasmic proteins, which could be reflected in altered heat coagulability patterns, as well as in viscosity and other physical properties.

Various workers, on the basis of structure-activity studies with various auxin analogues, have proposed attachment of auxin to a surface of some colloidal or polyphasic system, with a resultant swelling (hydrophily) of this system (9). Our results are also consistent with these observations.

The significance of these findings is as yet difficult to assess. At most, they may provide an explanation of how auxin acts in promoting cell growth; at the least, they provide an alternative to the current cell-wall theories of auxin action which, it seems to us, are inherently incapable of accounting for all aspects of auxin action.

SUMMARY

C^{14}-carboxyl-labeled 2,4-D was applied to etiolated and green pea epicotyl sections at concentrations promoting growth. The sections were subsequently homogenized in 0.25*M* sucrose + .001*M* EDTA and the various particulates separated by centrifugation and counted for C^{14}. No evidence could be found for the attachment of the 2,4-D to anything in the cell, the great preponderance of it remaining in the final centrifugal supernatant fraction. Attempts at heat coagulation of the proteins of this fraction revealed that 2,4-D greatly decreased the amount of protein so deposited without affecting total protein content. This effect of auxin on the physical state of the cytoplasmic proteins appears to be correlated with auxin-induced growth.

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Interrelationships Between Metallic Ions and Auxin Action, and the Growth Promoting Action of Chelating Agents

The part played by metallic ions in growth processes has gradually become more prominent in recent years. It is now clear that the ions of calcium, potassium, manganese, iron, and cobalt greatly modify the growth of plant sections. In addition it has been reported by Heath and Clark (10) that ethylenediaminetetraacetic acid (EDTA), 8-hydroxyquinoline, and other chelating agents have a small but definite growth promoting effect on wheat *(Triticum)* coleoptile sections and a growth inhibiting effect on wheat roots. The parallelism between the action of indole-3-acetic acid (IAA) and EDTA suggested to them that the two substances might act in a similar way, though it was stressed that their actions could not be identical. Since subsequent workers could find no real effect of EDTA on roots, all of what follows is restricted to shoot tissue.

As an explanation of the effect of chelating agents on growth, it was proposed (10) that growth is normally restrained in some way by a metal, and that growth promoting substances in general act by chelating this metal. In the case of IAA, a comparison was drawn between the nitrogen atom adjacent to the 6-membered ring and that in the chelating agent 8-hydroxyquinoline. However, this explanation is evidently most improbable, for several auxins, such as l-naphthaleneacetic and 2,3,6-trichlorobenzoic acids, could have only very weak chelating ability, yet their growth promoting action is very strong and on some plant material stronger than that of IAA; while on the other hand EDTA and 8-hydroxyquinoline are extremely powerful chelators, yet their growth promoting activities are relatively small. In Heath and Clark's 10 mm. wheat coleoptile sections

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the growth promotion caused by IAA was also very small and no larger than that caused by EDTA. But with lupine *(Lupinus)* hypocotyls, Weinstein *et al.* (25) found growth increments of 30 to 50 per cent with IAA, and EDTA was just about as effective. Under appropriate conditions elongation of 100 per cent is easily produced in oat (*Avena*) coleoptile sections, while it will be shown below that EDTA causes in them only a relatively small elongation. It is true that Cohen *et al.* (5) have demonstrated some chelation of both IAA and NAA with Cu^{+2} ions, but this took place in 50 per cent ethanol and in 0.02 *M* solution, and they found no chelation with Ca^{+2} or Mg^{+2}. When Recaldin and Heath (17) examined the reaction with ferric ions, what they observed was a slow development of color accompanied by O_2 uptake and IAA breakdown, in other words, a chemical reaction akin to the Salkowski reaction. Nakazawa (13) could find no evidence that IAA chelates with iron in pea seedlings. Thus, evidence that appreciable real chelation takes place under physiological conditions is lacking, and even if it did take place, there are as yet no indications of a causal relationship to growth.

However, in favor of some relationship between growth and chelation is the increasing evidence mentioned above that metallic ions strongly influence growth. Recently it has been suggested, indeed, that calcium specifically controls growth through its linkage to pectic acid groups in the cell wall (1), and it has been shown that calcium raises the osmotic content of *Avena* coleoptile cells while at the same time lowering the suction pressure — an effect interpreted as due to "tightening" of the cell walls (6). Experiments relating auxin action to pectin methylesterase (3, 7, 8, 16) are interconnected with this concept, though the effect does not appear to be a simple one. In any event, chelating agents might indeed promote growth by removing some of the hypothetical wall-bound calcium. The fact that the ferric-EDTA complex does not promote growth, under conditions where free EDTA does, supports this idea (25).

The whole phenomenon seemed to warrant restudy of the action both of chelating agents and of some metal ions on growth.

MATERIALS AND METHODS

The EDTA used was a three times recrystallized sample of the sodium salt sold as Sequestrene AAA, and kindly supplied by the Alrose Company of Providence, R.I. The stock solution was adjusted to pH 5.6. The 8-hydroxyquinoline (8-HOQ), and other chelating agents were CP chemicals. *Avena* coleoptile sections 10 mm. long were cut in the usual manner about 3 mm. below the tip from seedlings of 'Victory' oats 74 hrs. old, grown in water at 25° C. after receiving

5 hrs. of red light administered from the third hour after soaking. The first internodes (mesocotyls) from similar plants grown in complete darkness were sectioned in weak green light (15, 18), 4 mm. sections cut 2 mm. below the coleoptilar node being used. Both types of sections were allowed to grow in the solutions in complete darkness. All section elongation was measured after 24 hrs. at 25° C. unless otherwise indicated. Potato discs used had a 9 mm. diameter and were 1 mm. thick. They were cut from 'Katahdin' tubers of selected large size and washed 24 hrs. in water before use (9). The growth media contained 2 per cent sucrose, unless its absence is specified, together with either $5 \times 10^{-3}M$ KCl, $CaCl_2$ at $5 \times 10^{-3}M$ or lower, or both KCl and $CaCl_2$, or more generally potassium phosphate buffer, $0.01M$, pH 5.5.

The Action of Some Metallic Ions on Growth

In the first place the powerful growth inhibiting action of calcium must be emphasized. Since this inhibition was first reported on coleoptile sections in 1938 (23) it has been confirmed by many workers; it was shown very strikingly in pea stem sections, which show no similar inhibition by magnesium (24). Unlike other ions, calcium does not promote growth at low concentrations (24). On roots the effect is one of growth promotion, as has long been known, and in recent years studied especially by Burström.

Other ions, however, notably potassium (23), manganese (2), ferrous iron (19), and cobalt (12), promote growth. Some of the effects of buffers are largely due to their potassium content rather than to their anions —a fact often lost sight of. The action of Fe^{+2} has been ascribed to two different effects (19) (see Discussion). Cobalt is particularly powerful, being nearly 100 times as effective as manganese on coleoptile sections. It also strongly promotes growth of pea stem sections which show very little response to manganese (21).

Two observations suggest that cobalt operates by way of the organic acid metabolism: (a) when acetate is added growth is no longer promoted, but is strongly inhibited; (b) in presence of cobalt the normal excretion of hydrogen ions is prevented (21). On the other hand, Busse (4), from the facts that cobalt causes the growth of coleoptile sections to continue for a longer time than normal and that their respiration is not promoted but slightly inhibited, has concluded that cobalt inhibits a process of cell-wall deposition which otherwise would have made growth slow down.

Recently Dr. Kenneth Wright in my laboratory has extended the cobalt effect to potato tuber slices (Figure 1). Preliminary experiments with this tissue showed that the optimum concentration, about

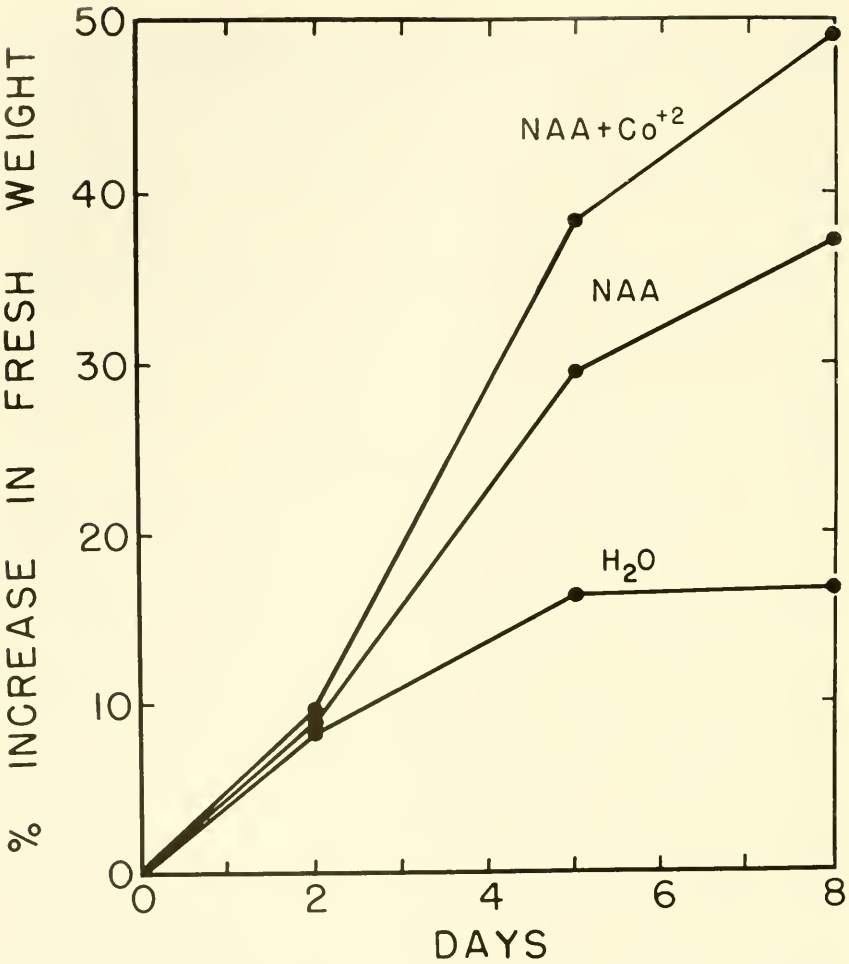

Fig. 1. Increase in fresh weight of potato tuber disks in water, in 1-naphthaleneacetic acid, 1 mg/l, or in 1-naphthaleneacetic acid, 1 mg/l with $10^{-5}M$ $CoCl_2$. Data courtesy Kenneth Wright.

$10^{-5}M$, is the same as with *Avena*. The promotive effect, though not quite as large as with *Avena* and *Pisum*, is clear enough. It will be noted that promotion of growth by cobalt appears as soon as promotion by auxin does, and that its effect is exerted during the period of most rapid growth. Thus, there is no indication here of a delayed effect like that just mentioned. Also, in our experiments with galactose, cobalt was found not to interfere with the conversion of this sugar to wall material (22).

Even with the *Avena* coleoptile our results do not indicate a delay of more than 12 hours in the onset of the cobalt effect (Figure 2).

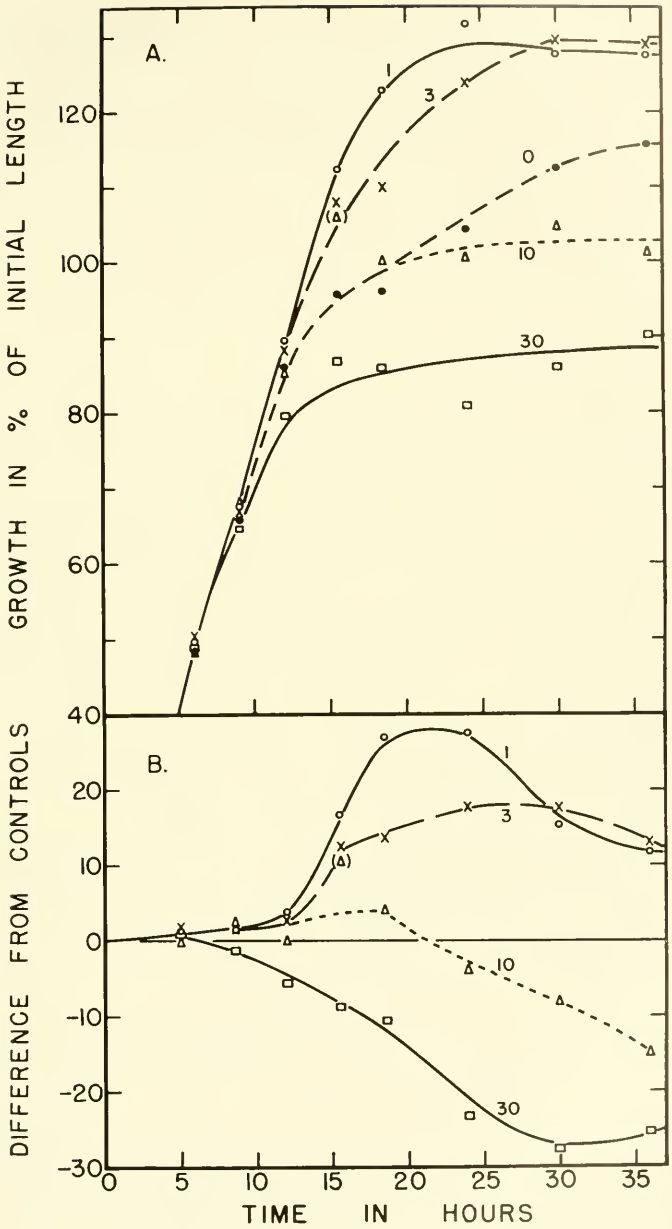

Fig. 2. **A.** Time course of the effect of cobalt chloride on the elongation of 10 mm. *Avena* coleoptile sections. All media contain sucrose 10 g/l, KCl 0.001*M* and IAA 1 mg/l (=$6.10^{-6}M$); all concentrations of cobalt indicated on the curves are $\times 10^{-5}M$; mean of 2 complete experiments, each including all 4 cobalt concentrations and the control, and of 3 experiments at the 18½ hour point. **B.** Time course of the positive or negative increments due to cobalt, derived from the above.

The inhibiting action of $3 \times 10^{-4}M$ Co^{+2} is indeed detectable at 9 hrs. (Figure 2B), and the promotion by $10^{-5}M$ and $3 \times 10^{-5}M$ Co^{+2} at almost the same time. However, it is true that the increments only become really large after 12 hours' growth.

Another difficulty in regard to Busse's interpretation is that if the promotion of growth by low concentrations of Co^{+2} is due to inhibition of the deposit of cell-wall, no explanation is offered for the inhibition of growth by higher concentrations. That $3 \times 10^{-4}M$ Co^{+2} should strongly promote a process which $10^{-5}M$ Co^{+2} equally strongly inhibits seems most unlikely. For these reasons the conclusion of Busse can only be accepted with reserve at present.

The Action of Chelating Agents

The essential results of Heath and Clark (10) could readily be confirmed. For *Avena* coleoptile sections the optimum concentration of EDTA was found to fall somewhat below $10^{-4}M$, and $10^{-3}M$ was generally inhibitory. The increment caused by EDTA occurred in all growth media tested; in $CaCl_2$ its value was not significantly different from that in KCl, buffer, or water. In the absence of sucrose it averaged about 10 per cent, in 2 per cent sucrose about 5 per cent. Since these increments are small differences between two fairly large numbers, they are subject to fairly wide variation; nevertheless the data agree rather well.

Other chelating agents have been studied less thoroughly. With 8-hydroxyquinoline (8-HOQ) the optimum concentration lay near $3 \times 10^{-5}M$, i.e., about one-third of that with EDTA. A representative group of three experiments is shown in Table 1; the increments (in 2 per cent sucrose) are comparable to those obtained with EDTA. A few trials with 9,10-phenanthroline, on the other hand, did not show significant growth promotion.

In the hope of improving the magnitude of the effect, sections

Table 1. Effect of 8-hydroxyquinoline on elongation of *Avena* coleoptile sections.*

Concn. of 8-HOQ $\times 10^{-5}M$	Elongation in Per Cent			Average	Mean Increase Over Controls, Per Cent
	I	II	III		
0	42	44	40	42.0	...
1	46	49	48	47.7	5.7
3	49	45	46	46.7	4.7
10	40	46	43	43.0	1.0
30	44	36	34	38.0	−4.0
100	24	..	..	24	...

* Medium consisted of 2 per cent sucrose plus $0.01M$ phosphate buffer at pH 5.0.

of *Avena* first internodes were used, since these have been found more responsive to very low auxin concentrations (15, 18). However, to our surprise, first internode sections showed no marked response, either to EDTA or 8-HOQ, over the whole range of concentrations up to $10^{-3}M$. They showed excellent response to IAA, however, at a concentration which showed only a small growth promotion in coleoptiles.

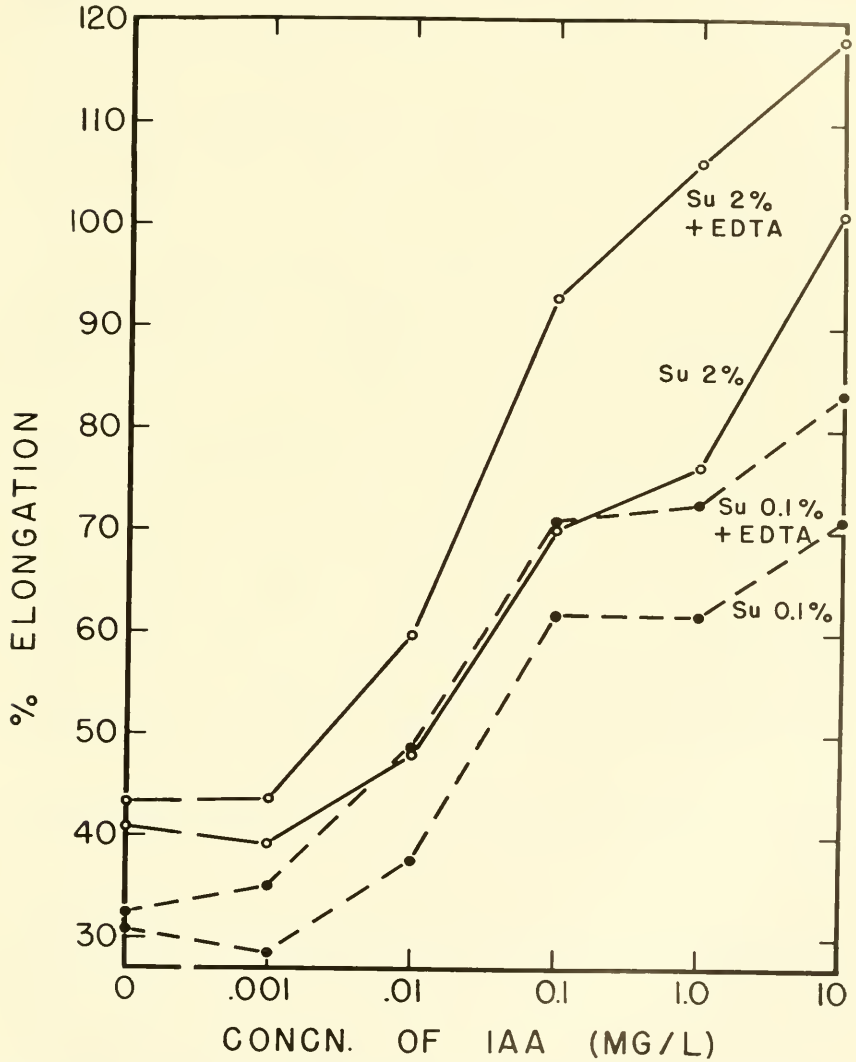

Fig. 3. Effect of EDTA on promoting the elongation of coleoptile sections, as a function of IAA concentration and presence of sucrose (Su). All experiments in $0.01M$ phosphate, pH 5.5.

The failure of the first internode to respond suggested that the chelating agents are not acting as weak auxins but in some other way. This was tested by studying their action in presence of a strong auxin, namely IAA. It was then found that in presence of IAA, EDTA at $2.5 \times 10^{-4}M$ could increase growth *by up to 50 per cent* (Figure 3). The action could be exerted in all growth solutions and in presence or absence of sucrose, though without sucrose the increment is only 5 to 10 per cent. At optimum sucrose, the increment caused by EDTA is largest when the IAA concentration is from 0.1 to 1 p.p.m., but at low sucrose it is best at from 1 to 10 p.p.m. It is clear, therefore, that the action is not simply that of a weak auxin. Comparable results were obtained with 8-HOQ, though the increment was smaller; for example, in 3 experiments with IAA 0.1 p.p.m. 8-HOQ at the optimum level ($10^{-4}M$) gave an average increment of 13.0 per cent.

With this knowledge a return was made to the first internode sections and it was found that in presence of IAA these sections did respond to EDTA. In Table 2 representative data are compared with those from coleoptile sections in the same experiment. Though more sensitive to low IAA concentrations than the coleoptile, the first internode gives a response to EDTA only one-quarter as great.

For further comparison, internode sections from etiolated 'Alaska' peas were used. Again the effect of EDTA was best in presence of auxin, but even at the optimal IAA level, namely 1 mg/l, the increment due to EDTA was only an additional 11 per cent. Addition of sucrose could increase the EDTA effect somewhat, but the response of these sections is still considerably smaller than that of coleoptiles. The effectiveness of EDTA, therefore, is a function of the tissue used, as well as of the amount of auxin present.

Although the action of EDTA is thus exerted mainly in combination with IAA, this is not a general interaction with auxins since,

Table 2. Effects of EDTA on sections from *Avena* coleoptile and first internode under the same conditions. All solutions contain sucrose 2 per cent and KCl $0.005M$; 24 hours.

Solution		Elongation in Per Cent of the Initial Length	
IAA $\times 10^{-7}M$	EDTA $\times 10^{-5}M$	Coleoptile	First internode
0	0	28.8	21.5
6	0	46.5	57.5
6	6	90.5	67.9
Increment due to EDTA		44.0	10.4

surprisingly enough, it is not shown in combination with NAA or with 2,4-D. Table 3 shows representative figures, the mean of three complete experiments with each of these auxins. IAA at one or two concentrations was included for comparison in each series; the contrast between the clear, positive effect of EDTA with IAA and its slight negative effect with the other auxins is evident.

Evidently, therefore, the action of the chelator is specifically concerned with that of IAA. It is reasonable to suggest that its very small but repeatable effect in the absence of added auxin is exerted by potentiating the endogenous IAA.

The Interaction Between EDTA and Calcium

As stated at the outset the promotion of growth by chelators might be due to their removing an endogenous growth-restraining concentration of calcium. It follows that the chelator should also be able to remove a growth restraint caused by externally added calcium. In other words, growth inhibition caused by added calcium should be antagonized by EDTA. Experiments to test this can be carried out in two ways: the calcium can be added directly to the growth solution or it can be taken up by the plants beforehand. The former method has the obvious disadvantage that chelation of the calcium can occur in the solution so that the effect on the plant is masked. The second is in some respects more problematical but it is freer from objection. While both methods have been used, and lead to the same conclusion, only the second will therefore be described here.

Avena seeds were soaked in $CaCl_2$ solutions of various strengths from 0.02*M* to 0.001*M*, and after 3 hrs. were laid out on filter paper in the usual way. Sections were cut from the coleoptiles 72 hrs. later and their growth compared with that of control sections (from seeds that had been soaked in water), both being tested on the same day. With low $CaCl_2$ concentrations the differences were not always statistically signficant, but with 0.02*M* they were real. As a mean of 10 experiments in sucrose-KCl the control sections grew 38.9 $\pm$ 2.3 per cent, the sections from Ca-soaked seeds 30.5 $\pm$ 1.5 per cent.

In order to be sure that this decrease in growth was due to calcium taken up into the coleoptile, the procedure was repeated using Ca^{45}. After 76 hours' growth the plants were dissected and the radioactivity counted. The activity expressed as percentage of the total was distributed as follows (mean of 10 plants individually sectioned and counted): roots 24, seed 39, coleoptile 32, leaf 5. Thus one-third of the Ca^{45} taken up was present in the coleoptile.

Table 3. Ineffectiveness of EDTA with NAA (Series I) or 2,4-D (Series II). *Avena* coleoptiles, ten 10-mm. sections in 4.0 ml. sucrose, 2 per cent phosphate buffer pH 5.5 0.01*M*.

Treatment	Percentage Elongation at Auxin Concentrations in Mg/L							
	0	0.001	0.01	0.1	(0.1 IAA)	1.0	(1.0 IAA)	10.0*
Series I: NAA Control	59.5	58.1	61.3	97.3	(115.8)†	126.3	(96.8)	133.2
Plus EDTA, $5.10^{-6}M$	60.7	57.8	61.3	95.3	(127.5)	107.4	(124.3)	115.3
Increment due to EDTA	+ 1.2	− 0.3	0.0	− 2.0	(+ 11.7)	− 18.9	(+ 27.5)	− 17.9
Series II: 2,4-D Control	64.3		67.2	96.9		115.1	(95.0)	113.4
Plus EDTA, $5.10^{-6}M$	65.4		67.1	96.6		113.2	(118.6)	113.1
Increment due to EDTA	+ 1.1		− 0.1	− 0.3		− 1.9	(+ 23.6)	− 0.3

* For 2,4-D the highest concentration was 5.0 instead of 10.0 mg/l.

† Data in parentheses refer to the corresponding concentrations of IAA which are included for comparison; in each case these were done on the same day as the other auxin.

Table 4. Effect of soaking *Avena* seeds in calcium chloride on subsequent sensitivity of the coleoptile sections to EDTA. All figures are elongation in per cent of the initial length. Mean of four separate experiments.

Soaking Treatment	Elongation of Sections		Increment Due to EDTA
	Control	Plus EDTA	
	Growth in Sucrose-KCl		
Water	40.8	43.9	3.1
$CaCl_2$, 0.02*M*	36.3	39.5	3.2
	Growth in Sucrose-KCl Plus IAA		
Water	66.5	102.7	36.2
$CaCl_2$, 0.02*M*	59.7	95.3	35.6

Sections from the plants of calcium-soaked seeds were now tested for their reaction to EDTA, in parallel and on the same day with sections from control plants. The results of four such experiments (all done in sucrose plus KCl plus IAA 0.1 mg/l) are averaged in Table 4. Without auxin the increments are very small and show no difference. In presence of IAA the increment due to EDTA in the control sections averaged 36.2 per cent of the length. In sections made from plants pretreated with Ca^{+2} the increment averaged 35.6 per cent of the length. The agreement is good and the conclusion clear; the growth promotion caused by EDTA in the sections enriched in Ca^{+2} is no greater than in the controls.

In these experiments the growth inhibition is known to be due to Ca^{+2}, which has been proved to be taken up by the tissue, yet EDTA does not overcome it in the least. The growth promoting effect of EDTA cannot therefore be due to chelation with calcium.

In two papers which appeared after this work was done, Ng and Carr (14) have shown that EDTA does not remove as much calcium from intact or homogenized coleoptiles, or from filter paper, as does citrate-phosphate buffer, and although their data show very small and unsatisfactory growth promotion by EDTA (of the order of 5 per cent greater than controls) their conclusion that EDTA could hardly act by chelating wall-bound calcium agrees with the above.

The complementary approach to the same problem would be to measure the actual loss of calcium from the sections during growth. For this purpose seeds were soaked in $Ca^{45}Cl_2$ as before and sections cut for growth experiments from the resulting coleoptiles. After 24 hours' elongation the radioactivity both of the solution and of the dried and powdered sections was determined. Unfortunately these

experiments, though extensive, have yielded no conclusive result. It was at first found that while EDTA alone had no effect, yet in the presence of both IAA and EDTA a significant amount of Ca^{45} was lost from the sections and recovered in the growth solution. Subsequent more extensive tests have revealed a degree of variation between experiments which makes this result doubtful. More extensive washing of the sections has not greatly reduced the variation. It must be concluded that if EDTA does remove any calcium it does not do so when added alone, and even the combination of EDTA with IAA removes so little, if any, that it is at the borderline of significance even with the highly sensitive radioactivity method.

The chemical and photometric calcium determinations of Ng and Carr (14) lead to the same conclusion.

DISCUSSION

One possible explanation of the large growth promotion which EDTA causes in presence of auxin might be merely that its action is proportional to the amount of elongation occurring. This is negated not only in Figure 3, but also by the data of Table 2 (and of numerous similar experiments) which establish that first internode sections in low auxin concentrations elongate more than coleoptile sections, yet show a smaller EDTA effect.

The failure of EDTA to promote growth appreciably in presence of NAA or 2,4-D suggests that EDTA in some way protects IAA from destruction within the tissues. While this is not impossible it is not wholly satisfying for the following reasons: (a) destruction of IAA in coleoptile sections in vivo is not very great, as witness the high recovery of C^{14} from carboxyl-labeled IAA in transport experiments; (b) Figure 3 shows that at the lowest IAA level, where presumably destruction has its largest effect, EDTA is inactive; (c) in *Pisum* sections, where IAA destruction is known to occur actively, the effect of EDTA is relatively small. On the other hand, the strong promoting effect of manganese on the IAA oxidizing system would certainly give a basis for inhibition by a chelating agent.

It seems more probable, however, that it is some other reaction of IAA, perhaps its conjugation, that is metal-promoted and therefore sensitive to EDTA. It would be of interest to investigate the several side reactions which remove IAA in vivo.

At this point we return to the actions of specific metal ions mentioned at the outset.

Recently, Shibaoka and Yamaki (19) have found that ferrous ions promote growth of *Avena* coleoptile sections in IAA but not in NAA.

and conclude that the action is due to inhibition of IAA destruction. However, they also showed that the translocation of *both* IAA and NAA was promoted, and conclude that Fe^{+2} has two different effects — to inhibit destruction and to promote transport. The parallelism with the action of EDTA is suggestive. The several parallels with the action of cobalt are also notable, for cobalt, like EDTA, shows little or no effect in the absence of auxin, but causes up to 50 per cent increment in presence of optimal auxin; both cobalt and EDTA are somewhat more effective on coleoptiles than on pea stems, and both are considerably more effective in presence of sucrose than in its absence (21). Cobalt differs from EDTA in that it promotes growth in solution equally well with NAA and IAA, but again there is a suggestion of similarity, since when acetate is present cobalt becomes strongly inhibitory but this occurs only with NAA and not with IAA.

It is worth noting that it is not impossible for a metal ion and a chelating agent to act in the same way, for, if a particular catalyst owes its activity to a metal complex, then an exogenous metallic ion could compete at its site of action, while the chelating agent, by forming a chelate in situ, could prevent the metal and its site of action from coming together.

Such an action need not be in the wall and indeed the possibility should be considered that it occurs in the mitochondria. It is known that the activity of these bodies is strongly influenced by their Ca^{+2} (11) and since the energy for growth of coleoptiles, pea stems, and freshly-cut potato sections is supplied via cytochrome oxidase, which is in the mitochondria, a close relation between metal ions and growth could be suspected here. Further, EDTA, as well as manganese, is known to prevent the swelling of mitochondria which occurs in isotonic solutions (20).[2]

It seems a safe prediction that the mode of action of auxin will not be fully understood until the role of the several metal-dependent reactions has been elucidated.

ACKNOWLEDGMENTS

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[2] The subsequent finding of Whitehouse, Staple, and Kritchevsky (Arch. Biochem. Biophys. 87: 193. 1960) that cobalt activates the oxidation of cholesterol, pyruvate, and octanoate by rat liver mitochondria supports this line of reasoning.

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DISCUSSION

Dr. Nitsch: At the time Dr. Thimann reported the very large effects he obtained with cobalt as an IAA synergist on *Avena* coleoptiles (Amer. Jour. Bot. 43: 241. 1956), we tried the effect of cobalt on oat first internodes. To our great surprise, we got little, if any, stimulation (J. P. Nitsch and C. Nitsch. Plant Physiol. 31: 94. 1956). We generally got a small cobalt effect with coleoptiles. We repeated these experiments several times but never obtained the large cobalt promotion which has been reported, although manganese gave results which agreed with those of other workers. The explanation for the discrepancy between Dr. Thimann's and our results came only this year when Busse's article appeared (Planta. 53: 25. 1959). Busse has shown that the cobalt effect is large under two conditions: (1) that the sections be long, and (2) that the test last for more than 24 hrs. We used 4 mm. sections, taken 3 mm. below the tip of the coleoptile, or 2 mm. below the node (in the case of first internodes), whereas Dr. Thimann used 10 mm. sections. In addition, we always measured elongation 20 to 24 hrs. after the start of the treatment whereas Dr. Thimann measured elongation after 48 hrs. These differences in technique, differences in the oat variety (we used 'Brighton'), and the fact that we added a citrate-phosphate buffer to the sucrose solution, explain the discrepancies observed in the results. They indicate that cobalt may retard a sort of aging process which allows IAA to act longer on older parts of the coleoptile.

Dr. Thimann: I have never done any large number of experiments with cobalt using 4 mm. sections. We have found perfectly good cobalt effects in 24 hrs. with both *Avena* coleoptiles and either 'Alaska' or 'Laxton's Progress' pea stem sections, although it is true that we have observed greater responses in 48 hrs. Busse's data on coleoptile sections show cobalt promotions from about 6 hrs. on. His conclusions are essentially that the effect of cobalt appears when the

growth of the controls is slowing down and that this slowing down is due to the deposition of cell wall material which cobalt prevents. According to Busse, the main effect of cobalt is to allow stretching for a longer period of time. I am not personally convinced that this is the whole explanation, although I have not made extensive time studies. As you saw, cobalt exerts its effect on potato slices while the control slices are still growing well. It is true that if you measure it over a very short time, 3 to 4 hrs., there is only a very small cobalt effect, so that while perhaps the effective times may vary with the experimental conditions, there is an increasing effect of cobalt with time. I found two effects of cobalt linked to organic acid metabolism; namely, that in the presence of acetate, the cobalt exerts only an inhibition and that cobalt prevents the normal excretion of organic acids by coleoptiles and pea sections. I think that the cobalt effect is probably quite complex.

Dr. Bonner: It might be appropriate to mention some further work which bears on the same final question. It is not otherwise similar to Prof. Thimann's work. We know that calcium is inhibitory to growth of isolated tissue sections. The question is, How does the calcium do it? What does it combine with to bring about this drastic inhibitory effect? Experiments that have been published for some years show that one characteristic of calcium-induced growth inhibition of *Avena* section growth is that the calcium ions that exert the inhibition are bound exchangeably. The same is true of the bound calcium ions which decrease the mechanical deformability of coleoptile tissue.

Apparently the calcium ions that make the tissue stiff and not grow rapidly or not bend rapidly are ions that go into the tissue and combine with something; then they stay there and don't come out in water but have to be encouraged out by some other kind of ion such as sodium or potassium. One can measure things about these exchangeably bound ions, the concentration needed to obtain half maximal inhibition of bendability, and the time constants for equilibration of the tissue with the ion. We can also find out, by classical chemical methods, whether the tissue can bind calcium ions exchangeably. We can take tissue and put it in some labeled calcium solution, find out the amount of ion bound by the tissue in such a form that it will not leak out in water but can be exchanged by unlabeled calcium, potassium, or some other cation. In this way it is possible to determine that *Avena* coleoptile sections, for example, do bind calcium ions exchangeably and they have a certain cation binding capacity that we can calculate in milliequivalents per gram of sections just as if one were a soil scientist. We can also determine

the kinetic constants for this binding. We can determine what concentration of calcium is needed to get half maximal binding of the exchangeable bound calcium, and we can measure the time constant for the binding. Now, the happy coincidence is that both the concentration of calcium needed for half-maximal effect and the time constant for the binding is about the same for the exchangeable binding of calcium as it is for the binding of the calcium which causes inhibition of growth rate or inhibition of bendability. Therefore, I conclude that it really is exchangeably-bound calcium that goes into the tissue and gets bound somewhere that exerts these effects. The only remaining question then is to discover what is in the tissue that binds the calcium. This is not so hard to do as it might seem. If we drop our tissue in boiling water and kill it, it still retains its cation binding capacity. If we take tissue and make a cell wall preparation from it, we find that this portion has essentially all of the cation-binding capacity of the intact tissue. So, I think the calcium ions that inhibit the growth rate and are exchangeably bound are bound in the cell wall.

The final question is, What is it in the cell wall that binds the calcium? There are free carboxyl groups in the cell wall and, as has been shown by Peter Ray and by our own group during the past two years, there are free pectic carboxyl groups in the cell wall and these account quantitatively for the cation-binding capacity of the wall. It seems to us that it is these free pectic carboxyl groups that bind the calcium in the cell wall which are then the calcium ions that cause inhibition of growth rate and inhibition of cell wall bendability.

Just one last comment. In these same experiments it has been shown that if we grow *Avena* seedlings in distilled water and then harvest the coleoptile sections and make cell wall preparations from them, then these cell wall preparations contain calcium to such an extent that there is enough calcium to occupy about one-fifth of all the free carboxyl groups in the cell wall. We propose, therefore, that perhaps one way that EDTA works in causing increases in growth rate is to pry out some of this calcium which is endogenously present in the cell wall, calcium which is contributed to the cell wall from calcium which the seed contains. Perhaps here again we're dealing with rigidity of cell wall induced by calcium.

Dr. Fawcett: The discovery by Cohen, Ginzburg, and Heitner-Wirguin (Nature. 181: 686. 1958) that the ultraviolet absorption spectra of IAA and of NAA are profoundly altered by the presence of cupric, but not calcium or magnesium ions, led them to postulate that the cupric ion reacted with these acids to form chelate complexes.

However, it is not necessary to use a chelation mechanism to interpret these absorption spectra.

By measuring the pH, Cohen *et al.* also confirmed that cupric nitrate reacted differently from calcium and magnesium nitrates when added to solutions of IAA and NAA. From a study of several closely related acids comprising active and inactive growth regulators, I found that the pH changes, observed upon addition of cupric ions, do not correlate with growth regulating activity and, moreover, are explicable by mechanisms not involving chelation (Nature. 194: 796. 1959).

We have recently compared a range of chelating agents with IAA, 2,4-D, and NAA in four different biological tests. The IAA, 2,4-D, and NAA were highly active in all tests. Of the forty chelating agents tested most were inactive, though weak activity was shown by nine of them, namely, ethylenediaminetetraacetic, diethylenetriaminepentaacetic, 1,2-diamino*cyclo*hexanetetraacetic, citric, tartaric, gluconic, nicotinic, succinic, and nitrilotriacetic acids. This slight but significant activity was shown only in the wheat coleoptile test. The remaining chelating agents were inactive in the wheat coleoptile, the pea segment, the slit pea curvature, and tomato epinasty tests. Thus, the results obtained in these experiments provide strong evidence against the idea that plant growth regulating activity is explicable in terms of chelation.

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Problems in the Biophysics of Cell Growth

It has become accepted that growth of plant cells by enlargement must depend upon phenomena occurring in the primary cell wall which result in stretching under the force imposed upon it by turgor pressure, and consequently, that the stimulation of growth by auxin must involve an effect on the properties of the cell wall. It is usual to refer to this effect as a softening or plasticizing of the cell wall and to think of growth as a plastic stretching of the cell. It must be remembered that this need not be a direct effect; it may be exerted via metabolic processes in the protoplasm.

The present remarks are made to help clarify our thinking about cell wall growth by pointing out that at least two fundamentally different mechanisms of growth may be confused under the idea of plastic stretching. It is convenient to illustrate these mechanisms with the pectate theory of cell wall growth proposed by Bennet-Clark (1) and Ordin, Cleland, and Bonner (5, 6). This does not imply a belief on the author's part that the pectate theory is necessarily a factual description of the important events involved in cell wall growth; it serves well, however, to illustrate some molecular principles which appear to be generally applicable whatever the actual chemistry of the growth process.

It must be assumed that the resistance of the cell wall to stretching by the force of turgor is due to some type of bonding between the molecular units of the cell wall, the polysaccharides composing the microfibrillar material and the matrix in which this is embedded. Growth, then, must involve the breaking of certain of these bonds in some manner. According to the pectate theory, the critical bonding forces are visualized as being electrostatic cross links between polyuronide chains, whose free carboxyl groups have formed salts with Ca^{+2} (Figure 1A).

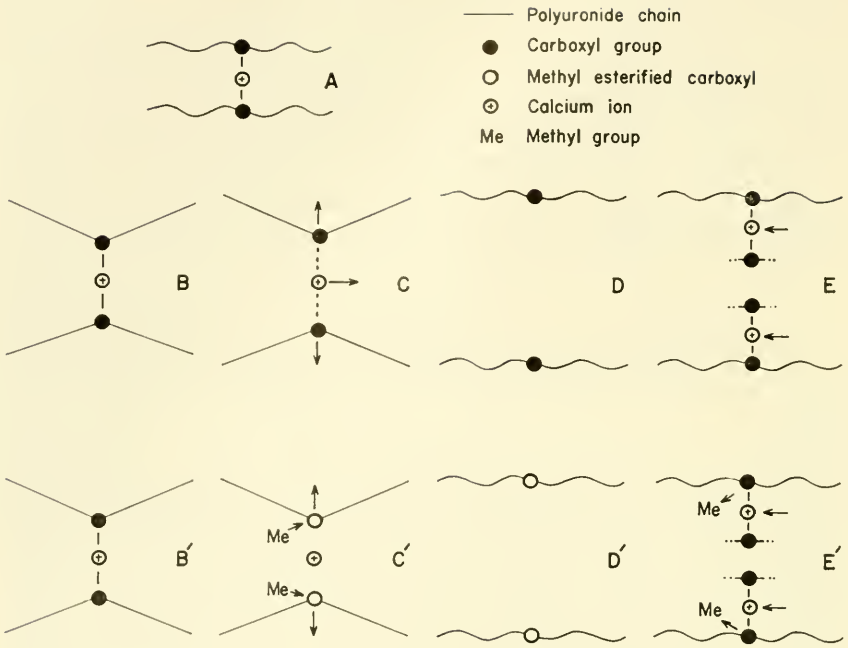

Fig. 1. Illustration of the plastic and molecular mosaic mechanisms of growth based on the pectate theory. **A** shows cross link in unstrained wall. **B** shows elastic expansion under turgor. In **C** the cross link is being broken by forcible separation; the polyuronide chains now assume relaxed configurations (**D**), and the carboxyl groups enter into salt linkage with other adjacent carboxyls (**E**). **B′** shows again a bond under turgor stress; in **C′**, bond is broken by methylation of the carboxyls. After the released chains reach relaxed positions (**D′**), methyl ester groups are removed and carboxyls enter into salt linkage with other adjacent carboxyls (**E′**).

Under the force of turgor these critical bonds are put under stress, as shown in Figure 1B. This will involve an increase in the bond length from the rest length, as well as elastic strain elsewhere in the cell wall structure. One way in which the structure could expand irreversibly would be that at some point the strain on the electrostatic bond becomes great enough to break the bond — i.e., further increase in bond length does not increase the restoring force between the bonded groups. The two polyuronide chains will then spring apart, taking up more relaxed configurations, and the carboxyls will enter into salt linkages with other carboxyls to which they now find themselves adjacent. This process will be repeated after similar bond breaking elsewhere in the structure has reimposed strain on the regions in which the two original carboxyl groups are located.

This process amounts to a passive distortion of the cell wall struc-

ture under force and can be called a plastic extension. The rate of extension will be greater, the greater the turgor pressure, since more bonds will be under a strain great enough to break them. To make the structure more plastic and to stimulate growth at constant turgor pressure (as auxin does), it is necessary to reduce the total number of bonds present. In terms of the pectate theory this requires an increase in the methyl ester content, so that fewer carboxyl groups are left unesterified and capable of forming salt linkages.

Another way in which the cell wall structure under strain might grow may be recognized by supposing that, in fact, turgor force is not great enough to break critical bonds in the structure – salt linkages in the pectate theory. Through metabolic machinery the cell, however, may be capable of breaking these bonds by insertion of a methyl ester group on one or both carboxyl groups (Figure 1C′). When this happens, the no-longer linked polyuronide chains will move apart to relaxed configurations, just as in the previous example, and subsequently they may enter into bonding with adjacent carboxyl groups after metabolic removal of the methyl groups (Figure 1E′). The cell wall will extend as these events take place successively in all parts of the structure.

This kind of growth is not a passive process as every unit of enlargement depends upon an act of metabolism; it cannot be considered a passive plastic stretching. Nevertheless, it is dependent upon turgor, because in the absence of strain in the structure, the bonded uronide chains will not separate when the bond between them is broken by methylation, but instead they will merely resume bonding subsequently. Also, note that the amount of growth achieved per active growth event will be greater, the greater the strain on the units acted upon, so the growth rate will increase with increasing turgor pressure, as was the case with pure plastic stretching. So an increase in the growth rate at constant turgor pressure is not to be looked for in a change in the number of bonds present but in an increased rate of bond breaking – of methylation in the pectate theory. One can see that an increased growth rate could be obtained in the presence of auxin, without any change in methyl ester content, provided there is an increased rate of metabolic turnover of uronide methyl ester groups.

This second picture of how the cell wall might grow is really an extension, to molecular dimensions, of the concept of mosaic growth advanced by Frey-Wyssling and co-workers (3,4). This conceives of extension as resulting from localized transformations of elastic strain into fixed deformation through some loosening process carried on by the protoplasm. The areas of loosening (as interpreted from electron micrographs) were much larger than the bond distances

which would be involved in the mechanism discussed here. We shall call the active process of bond breaking pictured here the molecular mosaic growth mechanism in contrast to the passive plastic type of deformation discussed earlier. The basic difference between them is, in sum, whether it is the force of turgor or an act of the cell which breaks the bonds as the cell grows.

These contrasting mechanisms provide some interesting problems. Many of the characteristics of the growth process could result from operation of either mechanism — this was illustrated above by the dependence upon turgor pressure. It is important to note that in the measurement of cell wall plasticity (7) one may be dealing with either mechanism of enlargement and therefore not necessarily with strict plasticity at all. On the other hand, a clearer view of the problem may suggest experimental approaches useful in revealing the actual mechanics of cell enlargement. As indicated above, one distinction can be made between the number of bonds present and the rate at which bonds are broken. It should be emphasized again that these bonds may not in fact be calcium salt bridges between uronide chains; the distinction will apply to whatever critical bonds are actually involved in cell enlargement.

It is not, of course, necessarily the case that cell growth can be described by any single type of molecular event, and it seems possible that both plastic and molecular mosaic types of extension may contribute to over-all growth. This does not diminish the importance of the distinction. It also can serve as a starting point for considering more complex forms of bonding which may be involved in cell growth. One example will be given: Suppose that the basis for rigidity of the cell wall matrix is interlocking of polysaccharide chains which run randomly and occasionally happen to be caught together in loops, as in a knitted fabric. Extension of such a structure can be brought about only by severing one or both chains in the vicinity of a loop (a molecular mosaic, since splitting of the covalent bond presumably would occur by a metabolic process). Expansion of the structure would soon result in other chains, which were previously not in contact, becoming entangled, and thus assuming the stress on the wall and restoring rigidity. In this model, new forces tending to stiffen the wall arise automatically upon extension, and auxin treatment of cells under no turgor could result in a subsequent limited extension of the wall, as has been observed in some experiments (2, 8).

SUMMARY

There are a number of problems in the biophysics of cell wall growth concealed under the vague concept of plasticity. The possible

mechanics of growth range from a viscosity problem to questions of macromolecular metabolism. It will be important to obtain further experimental evidence as to what types of bonding are important in the cell wall's mechanical properties and in its ability to expand irreversibly.

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The Effects of Decapitation and Growth Regulators on the Movement of Calcium in Apricot Trees[1]

The movement of calcium in plants is very limited or negligible (1, 4) once it has reached certain organs, particularly leaves and fruits. Calcium is not withdrawn from leaves prior to leaf fall in the autumn, and is regarded as being immobile in the phloem. Bledsoe *et al.* (2) showed that Ca^{45}, supplied to the root environment of peanut plants, moved directly to the stems and foliage, but could not move through the phloem to reach the developing fruits in any significant quantity. The latter findings have been confirmed by several authors (8), based on the immobility of radiocalcium applied as a foliar spray (4, 12, 19).

Bukovac *et al.* (4) reported that there was little movement of radiocalcium in the plants, but that after the plants had been anesthetized with diethyl ether, considerable quantities of Ca^{45} moved from the site of application to other parts of the plants. A similar effect upon the movement of Ca^{45} was obtained with triiodobenzoic acid (TIBA) (12), which interferes with auxin transport (14, 15, 16). The effect of TIBA upon the movement of metal ions, in addition to being a problem of translocation, became of interest in the light of suggestions that the activity of plant growth regulators might, in part, be due to their metal-chelating ability (6). This paper describes the effect upon downward translocation of radiocalcium of several growth regulators.

MATERIALS AND METHODS

One-year-old apricot seedlings and 6-year-old apricot trees were used for these experiments. Trees of similar performance were selected and each experiment was carried out in quadruplicate. The

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following growth inhibitors were tested: triiodobenzoic acid (TIBA), maleic hydrazide (MH), coumarin (CM), dichloroanisole (DCA), and *m*-tolylphthalamic acid (MTPA) (18). Decapitation of the terminal end of the stem was introduced as an additional treatment and was carried out just before the start of the experiments.

Each tree was sprayed with solutions containing 100 p.p.m. of one or another of the above substances. One terminal leaf of each plant was then painted with 0.1 ml. of Ca^{45} (0.8 μc/ml). The same amount of radiocalcium was applied to control plants which had been sprayed with water as pretreatment. For analysis the fifth leaf below the treated one was collected, dried, and weighed. This leaf was washed and the radioactivity counted. Both a thin end-window Geiger-Mueller tube and a gas flow counter fitted with a micromil window (Nuclear-Chicago) in an atmosphere of Q-gas were used. All counts are corrected for background and self absorption to infinite thinness. The results are expressed as total counts per minute (c.p.m.) per g. dry matter.

For measurements of the time effect, six similar branches on each tree were treated as above, and at intervals after the application of Ca^{45}, samples were collected from these six branches. Thus, any change in the movement of Ca could be detected.

In another series of experiments the effect of different concentrations of TIBA was tested. Concentrations of from 0 to 100 p.p.m. of

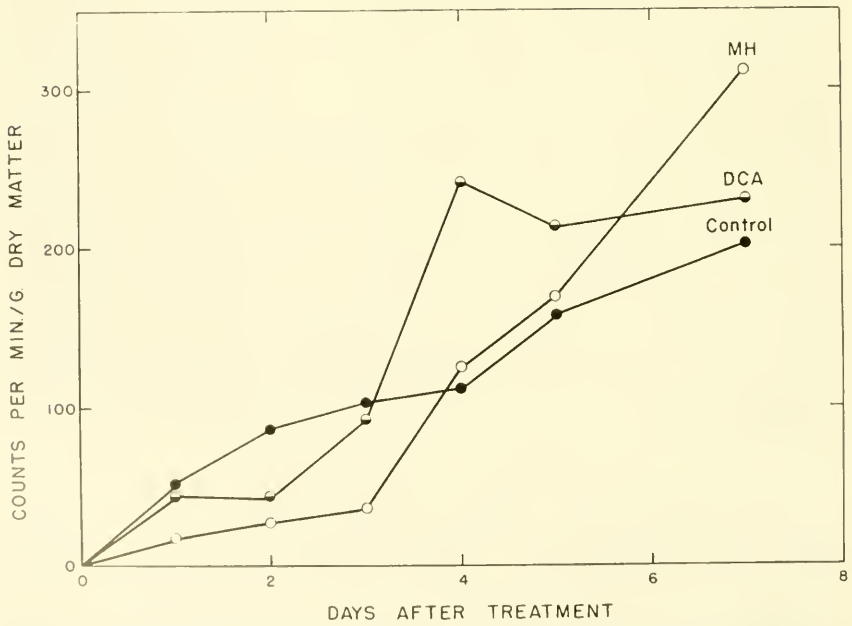

Fig. 1. The movement of foliar applied Ca^{45} in 1-year-old apricot trees and the effect of MH and dichloroanisole upon this transport.

TIBA were applied prior to the application of Ca^{45}. Leaves were collected for analysis 9 days after treatment. All treatments and replicates were completely randomized. For all tests with the young trees the same control experiment (shown in Figure 1) was used.

RESULTS

It can be seen from Figure 1 that in young apricot trees there exists a certain movement of foliarly-applied calcium. The radioactivity reached 241 c.p.m. 7 days after treatment, in contrast to that obtained with one-year-old apple trees (12). The effect of DCA was only noted after 3 days, after which a rise in the counts became apparent. MH, on the other hand, inhibited movement of Ca^{45} during the first 3 days and later caused it to increase. TIBA was particularly effective (Figure 2). This substance completely inhibited the movement of Ca^{45} in the first 4 days after treatment, but later caused the movement of Ca to increase to give 28,000 c.p.m.

Decapitation, coumarin, and MTPA produced rather unexpected results (Figure 3). Decapitation and MTPA increased calcium movements during the first 4 days while coumarin was ineffective. But in all three cases the radioactivity dropped again after the initial increase, reaching a low of about 50 c.p.m. on the 7th day, as against

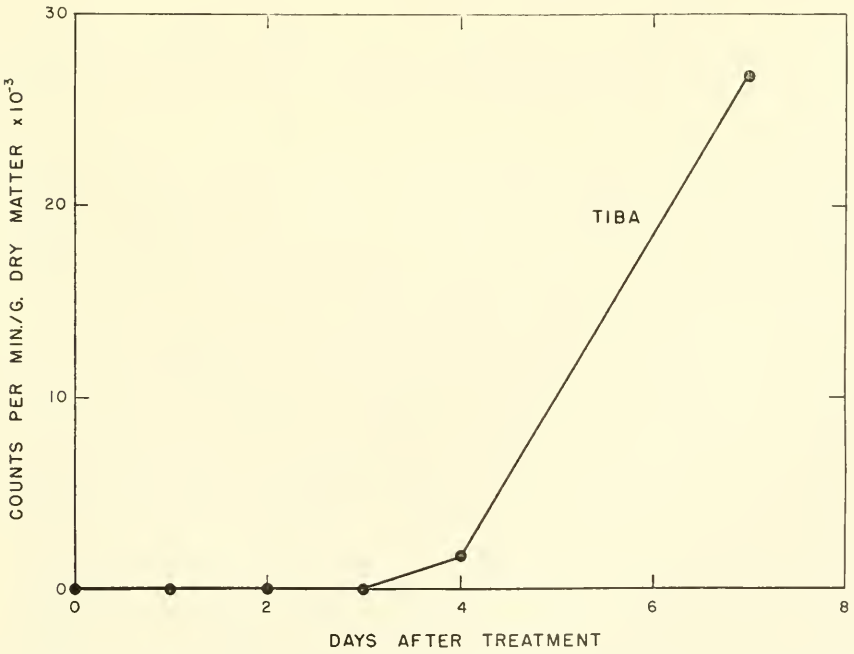

Fig. 2. The effect of TIBA upon the basipetal transport of Ca^{45} in 1-year-old apricot trees.

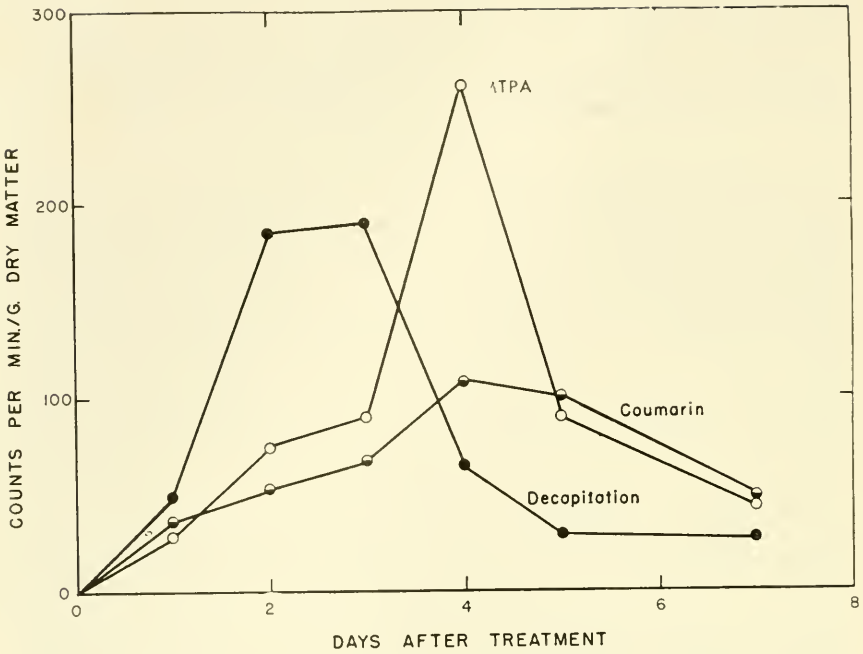

Fig. 3. The effect of decapitation, coumarin, and MTPA upon the basipetal transport of Ca^{45} in 1-year-old apricot trees.

241 c.p.m. for the control (Figure 1). This drop in radioactivity must be interpreted as being caused by a stop in the supply of Ca^{45} from the treated terminal leaf, while Ca^{45} continued to move out from the counted leaves.

If we compare the effect of these treatments upon the movement of Ca^{45} in one-year-old trees to that in 6-year-old trees, similar trends are obtained. From Table 1 it can be seen that in 6-year-old apricot trees, there was a considerably greater movement of foliar applied calcium than in younger trees. In the old control trees the radioactivity reached 15,560 c.p.m. on the 7th day after the treatment, as against 241 c.p.m. in the young controls (Figure 1). TIBA again showed a considerable promoting effect, while the radioactivity in MTPA-treated leaves dropped as before, after an initial stimulative effect. It is of particular interest that in this case decapitation strongly inhibited the downward movement of Ca^{45}. As previously stated, we must also assume that in older trees MTPA stops the movement of Ca^{45} from the terminal treated leaf after a time while Ca^{45} continues to move out from the counted leaf. Preliminary experiments in which the radioactivity of various leaves along the shoots was counted showed this assumption to be apparently true (Table 2).

Table 1. The effect of various growth inhibitors and decapitation upon the translocation of foliarly-applied Ca^{45} from the terminal to the fifth leaf in 6-year-old apricot trees.

Treatment	Counts Per Minute, 2, 4, and 7 Days After Treatment		
	2	4	7
Control	5,055	12,345	15,560
TIBA	20,307	27,683	48,775
MTPA	7,191	21,888	3,830
Decapitation	799	848	695

Table 2. Time course of the distribution of radioactivity from Ca^{45} along MTPA-treated branches of 6-year-old apricot trees.

No. of Leaf Below Treated Terminal	Counts Per Minute, 2, 4, and 7 Days After Treatment		
	2	4	7
3	45,529	30,740	31,078
5	7,191	21,888	3,830
7	133	4,533	15,683
9	158	585	6,346

In view of the activity of TIBA on the movement of calcium, the effect of different concentrations of this substance was tested. From Figure 4 it can be seen that the optimal concentration of TIBA was about 50 p.p.m. while higher concentrations were less effective.

DISCUSSION

The experimental results reported in this paper suggest that calcium is able to move in a downward direction in apricot trees, to some extent. The translocation of calcium in white pine trees has already been demonstrated by Ferrell and Johnson (7). But this movement is very different in various tree species and in trees of different ages of the same variety. It was previously found that there is no downward movement of calcium in one-year-old apple trees (12), a finding which is in conformity with the generally held opinion that little or no basipetal movement of calcium occurs within the plant. Comparing the movements of calcium in apple and apricot trees, it

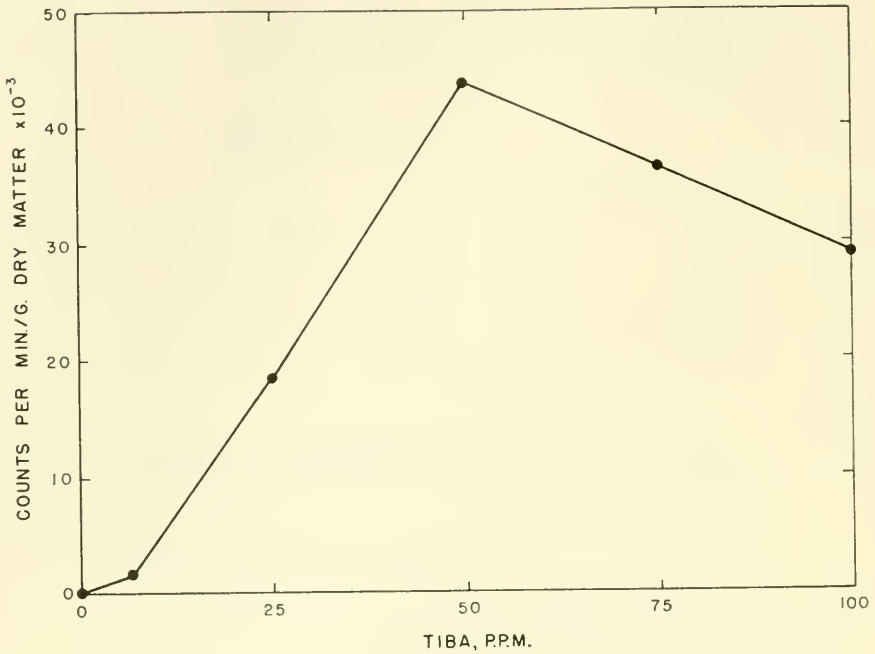

Fig. 4. The effect of different concentrations of TIBA upon the basipetal movement of Ca^{45} in 1-year-old apricot trees.

is of interest that apricot trees branch more easily than do apple trees and so do older trees in comparison to young ones. The phenomena are usually attributed to a stronger polarity (or apical dominance) inherent in the apple trees, as well as in young trees in general. This difference between apple and apricot trees and between young and old apricot trees may be reflected in the movement of foliarly-applied calcium (12), since in apricot trees there is some translocation. The relative translocation of calcium in young apricot trees is lower than in older trees. Thus, it appears that the translocation of Ca^{45} might be related to polarity. This suggestion has been made by Bukovac *et al.* (4), who were led to this hypothesis by the effects of an anesthetizing agent (diethyl ether) upon the movement of calcium. These investigators also found little or no transport of Ca^{45} through graft union involving reversed polarity (3). The inverse relation between the degree of apical dominance and the translocation of Ca^{45} raises the possibility that the movement of calcium might be somehow connected with the transport of auxin. The assumption that the movement of calcium is related to the transport of auxin is supported by the fact that 2,4-dinitrophenol, which alters the polar transport of auxin (14, 15), also stimulated the uptake of calcium (5).

In the course of other studies we recently obtained results which also might have some bearing on the mode of action of growth regulators on the translocation of metals in plants. It was found that calcium accumulates in tissues which are carrying on net ribonucleic acid (RNA) synthesis (9). It is possible that calcium is bound to ribonucleoproteins, which have been suggested to act as ion carriers (10, 13, 17), in an immobile form. In such a way the movement of calcium would be limited. Hence, it would accumulate in tissues where an intensive RNA synthesis takes place. A similar situation was found in our results, and in that case, one would expect that any action of ribonuclease (RNase) on RNA should considerably affect the translocation of calcium. This point is now under experimental test. To date, we have indications that TIBA influences the activity of RNase (11) in vitro, a result which gives some support to our hypothesis assuming some connection between RNA and the movement of calcium. Typical results of the effect of TIBA on RNase activity are presented in Figure 5. It can be seen that TIBA has a marked effect upon RNase activity but that this effect is somehow dependent on the level of RNase in the reaction mixture. At the low RNase level (1 μg/ml) there is an increasing inhibition of RNase activity with increasing TIBA concentrations, up to about 50 μg/ml. At higher TIBA con-

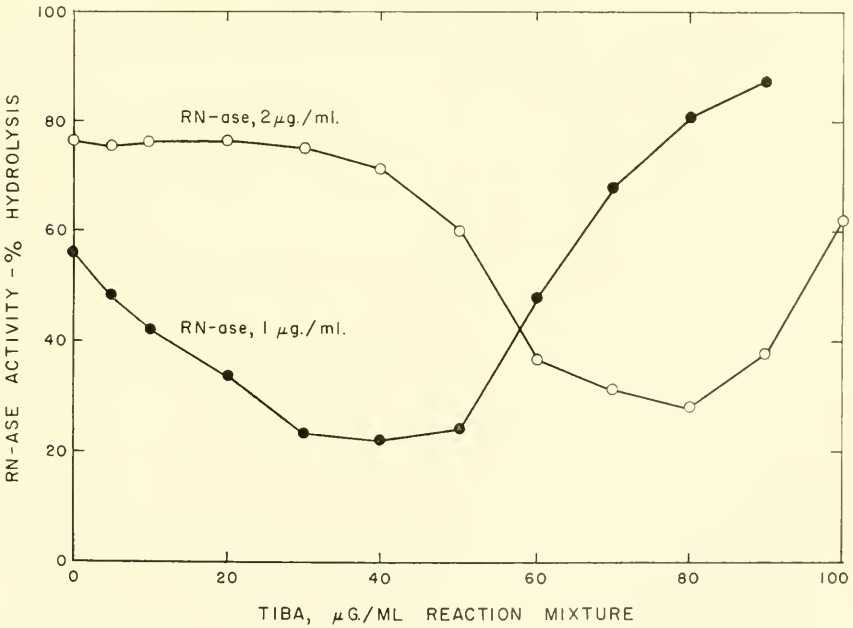

Fig. 5. The effect of TIBA upon the activity of RNase in vitro.

centrations, the activity of RNase was stimulated. Similar trends, but at different ranges of TIBA, were found at the higher RNase level (2 μg/ml). In this case, low TIBA concentrations had no effect. Above 30 μg/ml TIBA became inhibitory while at 80 μg/ml it became promotive. It is seen that TIBA has a dual effect on the activity of RNase, but that the effective TIBA concentrations change with the level of the enzyme.

If we now assume that TIBA has a similar effect in vivo on RNase activity as found in our in vitro experiments, we might consider the following possibility. TIBA, when applied to a plant at the concentrations employed, will stimulate RNase activity, causing a temporary increase in the destruction of RNA which may free RNA-bound calcium.

An hypothesis has been put forward that growth regulators possibly function directly through their chelating action on metals, thus acting as carriers, removing functional metal ions, etc. (6). From our results, it seems that even if there are functional relations between growth regulators and metals, the growth regulators might not act simply as chelating agents but through more complex relations, such as those which possibly exist between TIBA, RNase, RNA, and calcium translocation.

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The Polar Movement of Auxin in the Shoots of Higher Plants: Its Occurrence and Physiological Significance

The manifestations of polarity during development and regeneration are among the most striking and widespread phenomena in biology. In vascular plants, to cite a few examples, polarity is evident in the regeneration of roots on stem cuttings, in the basipetal reactivation of cambial cell divisions in the spring, in the basipetal movement of the phototropic curve in grass seedlings, and in the basipetal regeneration reported for xylem cells. These and other polar phenomena during development have been known since the turn of the century.

Hence, it was with the greatest interest that the work of Frits Went was received in 1928. In an extensive and elegant paper (22), Went reported the first separation of a plant hormone (later called auxin) outside the plant, described a quantitative bioassay for the hormone, elucidated many aspects of the role of auxin in the phototropic curvature of oat seedlings, and — of particular interest for this paper — provided evidence that auxin could move only from the apex toward the base in sections isolated from the seedling.

The significance of the polarity of auxin movement in explaining the various developmental manifestations of polarity was quickly realized; and all the polar phenomena mentioned above — plus many more — have in the intervening years been attributed to the action of polarly-moving auxin.

That a substance having such manifold effects should be the only hormone known to us which has such strictly controlled polar movement within plant tissue makes that polarity of great interest. Accordingly, this paper will attempt to review critically the present

status of our knowledge of auxin movement in shoots of higher plants.[1] Our emphasis will be on evidence concerning auxin movement as it relates to the *normal* physiology of the plant. We will eschew pharmacological studies, the movement of substances not known to occur as native auxins, and papers which infer auxin movement from some other effect presumed to be controlled by auxin.

The literature can be divided quite sharply as follows: (1) papers published before 1940, which present the general view that the normal movement of auxin is strictly basipetal and is remarkably uninfluenced by any sort of external factor; (2) more recent papers, in which auxin movement has been found to vary with temperature, ontogeny, type of tissue, and physiological condition, and in which even substantial acropetal movement of physiological significance has been found.

The first 10 years of research on auxin movement gave results which are well summarized in Went and Thimann's monograph (24). The general view was that auxin moved *only* from the apex toward the base — there was no acropetal movement even when an external supply of auxin was added to the basal end of the isolated section. The amount of auxin moved basipetally was reported to be unaffected by gravity, light, or an externally applied electrical gradient. The calculated rate of movement was 7 to 15 mm/hr and was considered not to be affected by temperature (20). Also, as one added more and more auxin to the top of the sections, more and more auxin was transported — that is, there apparently was no saturation of the transport-system (20, 23). Since a phototropic curve in the intact seedling moved strictly apex-toward-base, too, and at a rate corresponding to that reported by van der Weij for auxin, the results with isolated sections were taken as applying to the intact plant.

Before we turn to more recent work, there are a few points we should emphasize. First, note that most of the detailed papers on auxin movement dealt only with the organs of seedlings — particularly with coleoptiles of *Avena* and hypocotyls of legumes. Second, the papers of van der Weij (20, 21) are the main source of information on rate of movement, amount transported relative to amount added, temperature independence, etc. This is unfortunate: anyone who critically reads van der Weij's 1932 paper is bound to be awed at the number of exact, quantitative conclusions derived by careless procedures from the inexact data. For instance, in 12 of the 14 experiments in which acropetal movement was looked for, auxin was

[1] Auxin movement in roots is not covered for a variety of reasons, among them being the paucity of critical work, the probably more complex auxin metabolism in roots, and in particular the reviewer's lack of first-hand experience with auxin relations in roots.

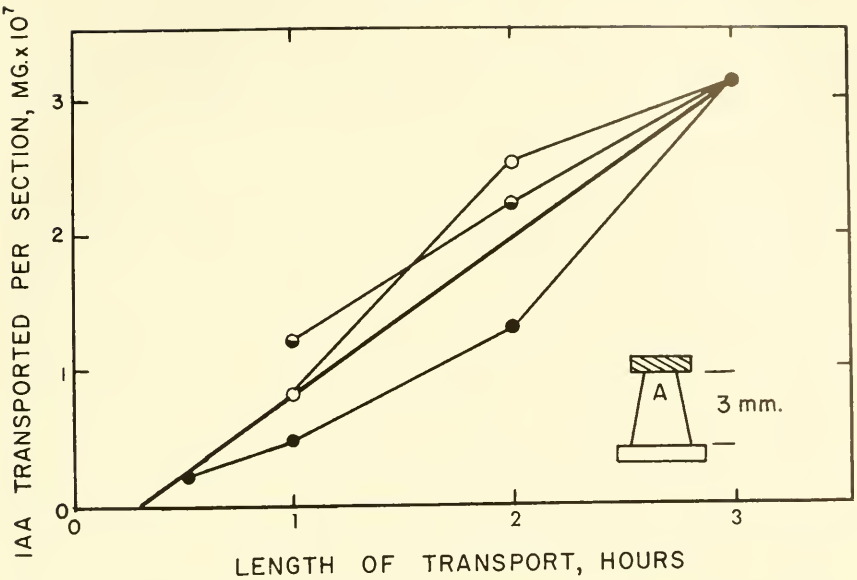


Fig. 1. Determination of the rate of transport of indole-3-acetic acid (IAA) through the intercalary meristem of the peanut gynophore as being 10 mm/hr. Data on each of the 3 individual experiments are from Jacobs (10); but the heavy line is the straight line which gives the best fit newly calculated by least squares. The calculated intercept is at 0.30 hr. (as contrasted to the less accurate graphical determination of 0.33 hr. in the original paper).

found in the apical collecting block — yet van der Weij concluded that auxin is not transported base toward apex. Again, to determine each rate of movement, van der Weij needed to find the intercept of a straight line (as in Figure 1): in 22 cases he used only 2 points to determine the straight line. And in one of these cases, the line actually drawn is at an 11° angle to the line determined by the two points. These are serious matters, since the actual position of these arbitrarily drawn lines determines his conclusions about both the speed and intensity of auxin movement.

The third point to emphasize is a source of error brought out by Went and White (25). They reinvestigated auxin movement in the *Avena* coleoptile, and found that when auxin was added to isolated sections kept at the usual high humidity, auxin would move in the film of water which formed on the surface of the sections. A substantial amount of van der Weij's difficulties was presumably due to this artifact. Similarly, an unspecified amount of Went's (23) results were also attributed to surface leakage.[2]

[2] The literature is critically discussed in terms of this and other sources of error (12).

During the last 15 years, I have been investigating the movement of auxin in plants and organs other than the *Avena* coleoptile — with the aim of checking just how generally applicable were the results obtained from this histologically simple, highly specialized structure of determinate growth.

A sharp gradient in the amount of added auxin transported basipetally was found along the axis of a nearly mature 8-day bean hypocotyl (Figure 2). At the level of the transition from stem to root structure, none of the added auxin was transported. No acropetal transport of added auxin was found at any level of the hypocotyl (8). Went suggested that less auxin might be getting through the more basal sections because of a mechanical blocking by more end walls. However, quite the reverse was found (Figure 3): There was a clear positive relation between the number of cells in a 5 mm. section and the amount of auxin that the section transported. A similar steep basipetal gradient of auxin transport can be seen in the data for the *Avena* coleoptile (20, 25), but the authors minimize the difference. Graphing the data of Went and White with cell-length

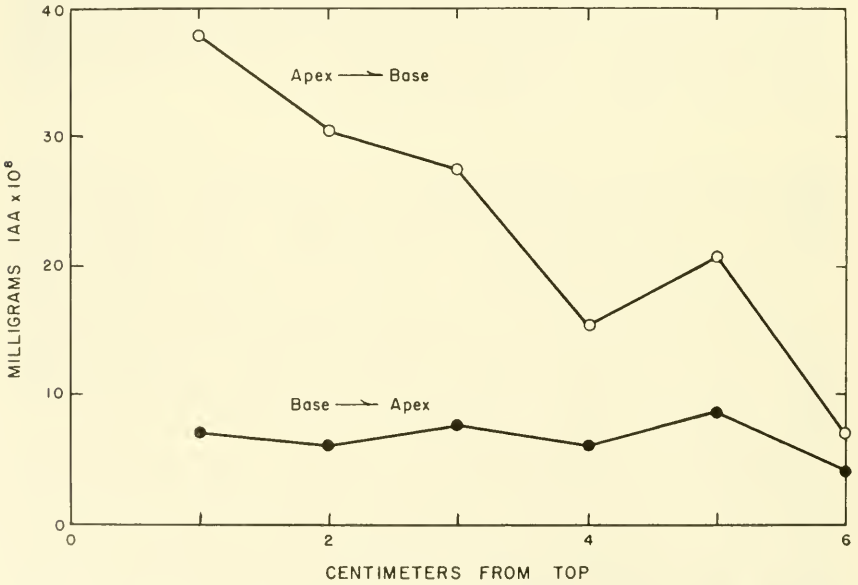


Fig. 2. Gradient in basipetal auxin transport along the 8-day bean hypocotyl [figure is from Jacobs (8)]. Data give the amount of auxin, calculated as IAA, collected in 3 hrs. at one end of 5 mm. sections when IAA at 2 mg/l of agar was applied at the opposite end. Parallel collections of the native auxin gave amounts at each end which were equivalent to that shown in this figure for base toward apex transport plus diffusion. Hence, there was no detectable acropetal transport.

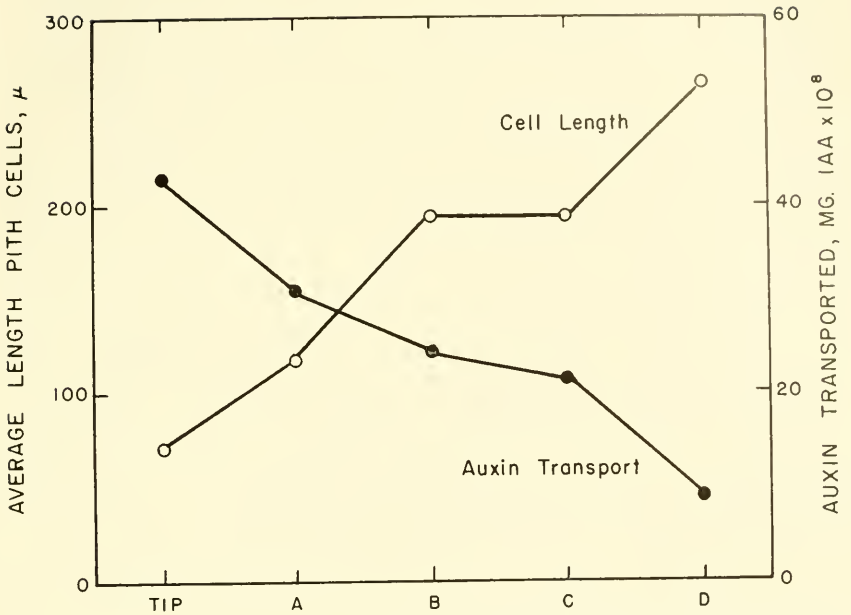


Fig. 3. Relation between auxin transport and mean cell length in 8-day-old bean hypocotyl. Data from Jacobs (8).

data derived from Avery and Burkholder (1) indicates that in the *Avena* coleoptile a similar relation exists to that in the bean hypocotyl — more cells correlate with more transport (Figure 4).

The development of auxin transport through the tip of young hypocotyls 2 to 8 days old was then studied. No auxin was transported acropetally in any stage. Basipetal transport first appeared in the 5-day tip and steadily increased in amount between the 5- and 8-day stages (Figure 5).

But does this gradual development of auxin transport, found in excised sections, have any significance in the normal development of the plant? We found evidence that it does (9). There are two regions of maximum elongation in the hypocotyl: the first occurs in the tip in the very early stages (2 to 3 days after germination); the second develops just below, and becomes the major zone of growth by 6 to 7 days. Studies on excised sections show that growth in this second zone is limited primarily by auxin, and yet this zone does not produce any significant amount of auxin itself — it gets auxin from the upper parts of the plant. But it cannot get the auxin it needs for growth until the tip region, standing between it and the auxin

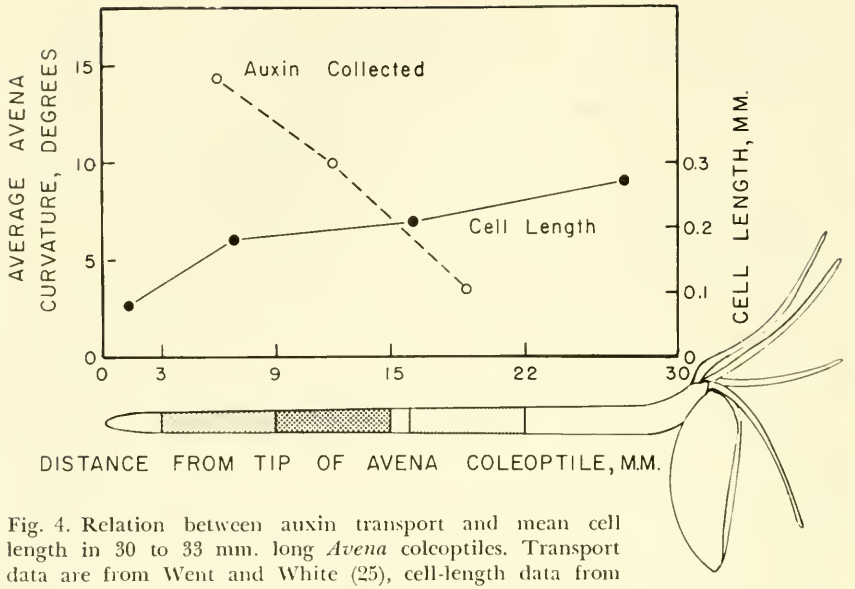


Fig. 4. Relation between auxin transport and mean cell length in 30 to 33 mm. long *Avena* coleoptiles. Transport data are from Went and White (25), cell-length data from Avery and Burkholder (1).

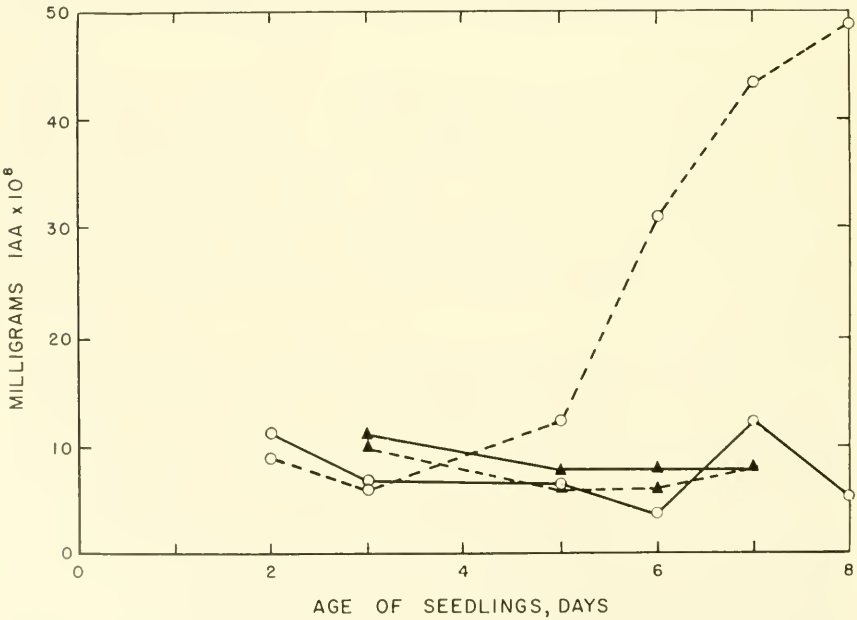


Fig. 5. The gradual development of basipetal auxin transport in the tip of young bean hypocotyls. The solid triangles represent native auxin; open circles represent native auxins plus added IAA. Solid lines represent auxin in base to apex collections; dashed lines represent auxins in apex to base collections. Data from Jacobs (8).

supply, differentiates a system for transporting auxin. Confirming this interpretation is the high and statistically significant correlation between the growth of the hypocotyl and the amount of added auxin transported basipetally through the tip.

In other words, the ability of the tip to transport auxin apparently controls the growth pattern of the 3- to 8-day hypocotyl (9).

The rate of auxin transport in the sections from *Avena* coleoptile was shown to be 9 to 12 mm/hr in the critical paper by Went and White (25). The old and unresolved question as to whether auxin moved faster through the vascular strands of the coleoptile cylinders than through the other cells was answered by them in the negative — although their published figures seem to show a slight increase in transport when the added auxin was over the vascular strand.

Since the coleoptile is famous for being nonmeristematic, I thought it would be interesting to study the transport properties of a meristem. Accordingly, the intercalary meristem of the gynophore of *Arachis* was investigated (7, 10). Auxin transport was strictly and basipetally polar, and the rate of transport was 10 mm/hr (Figure 1). In view of the results mentioned below, it should be noted that vascular tissues are maintained intact across the elongating meristem (7).

Other determinations of rate are few. Guttenberg and Zetsche (5), using a somewhat different method, provided evidence that the rate of auxin transport in sections cut from the hypocotyls of *Helianthus annuus* was also 10 to 12 mm/hr. Using a third method, Gregory and Hancock (4) estimated the rate at 5 mm/hr in sections from apple stems; however, a critical examination of their paper makes clear that this is a minimum rate — the maximum is 10 mm/hr, the same as for the other two organs studied. This uniformity is quite remarkable, considering the differences in the material used.

Gregory and Hancock had also been impressed by the flimsy basis of van der Weij's conclusions about temperature effects on auxin transport, so they examined the point with apple stems. Contrary to van der Weij's conclusions, though not his data, they found that increasing temperature resulted in increasing rate of transport.

Although the earlier work on coleoptiles left the impression that strictly polar transport was a characteristic of all cells, we had reason to think otherwise. During the period that auxin transport was gradually appearing in the tip of the bean hypocotyl, the only obvious histological change was the differentiation of vascular tissue. Similarly, the course of xylem regeneration in *Coleus* would be easier to understand if auxin were normally moving in the vascular tissue rather than through all the stem tissues. Accordingly, transport tests were run in which various areas of the stem were excised. No transport

was found in the pith alone, but as much transport as in the complete controls was found once the vascular area was included in the transport section (13). The most obvious conclusion is that the normal, high-speed transport of auxin goes on in the vascular tissues of stems.

Added auxin moves only basipetally in sections from the *Avena* coleoptile so long as surface leakage is forestalled and so long as more or less physiological concentrations of IAA (indole-3-acetic acid) are added — this was shown by Went and White in 1939 (25) and confirmed by Söding and Raadts in 1952 (19). If one doubted the universality of strictly basipetal auxin movement, where should one look for acropetal transport? Certainly not in seedlings, and probably not in determinate organs. A portion of stem from an adult plant, histologically much more complex than the coleoptile and so located that one would expect upward movement of metabolites, would be a likely guess. Internode 2 of adult *Coleus* satisfies these requirements (11). In addition, *Coleus* is from a large family (Labiatae) hitherto never critically examined in its auxin relations.

When the auxin relations of a rigorously standardized clone of *Coleus blumei* Benth. were investigated, substantial acropetal transport of added auxin was found in internode 2 in addition to the expected basipetal movement (Figure 6). Exhaustive refinements of technique to prevent surface leakage — including lowered humidity

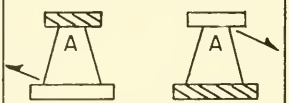
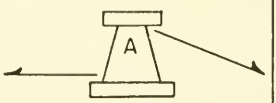
No.	Apex → Base		Relation of blocks to sections	Base → Apex		No.
	IAA	Curvature		Curvature	IAA	
11	157	17.5 ± 1.2		5.5 ± 1.0	49	10
10	8	0.9 ± 0.4		0.6 ± 0.3	5	8
	149	16.6	Auxin transported	4.9	44	

Fig. 6. Determination of acropetal and basipetal movement of auxin in sections cut from young internodes of *Coleus* when 2 mg/l of indole-3-acetic acid in agar is added (shaded blocks). The apical end of the section is designated A, IAA represents the collected auxin calculated as though it were all indole-3-acetic acid. From Jacobs (11).

and vaseline rings around all sections — did not affect the acropetal movement of auxin (11, 12). With 2 mg/l of IAA in agar added at each end, the ratio of basipetal to acropetal auxin movement was 3:1.

What reason was there to think that this acropetal transport in isolated stem sections was of significance in the more intact plant? Evidence was obtained from parallel studies of xylem regeneration in this same standardized internode. The leaves are the major source of auxin in *Coleus,* and excising the leaves causes a decrease in the number of xylem cells regenerated, the decrease being exactly proportional to the amount of diffusible auxin produced by the leaf. When synthetic IAA is substituted for the leaves so as to give exactly the same amount of auxin as they produce, the IAA exactly replaces the xylogenic effect of the leaves (11, 14). Thus we have detailed evidence that auxin from the leaves is the factor normally limiting xylem regeneration. Now, when regeneration occurs in plants with all their leaves left on, their course of regeneration parallels the observed 3:1 ratio of auxin transport — i.e., regeneration is mostly basipetal, but with a significant amount of acropetal regeneration occurring, too. And Figure 7 shows more quantitative data: if we

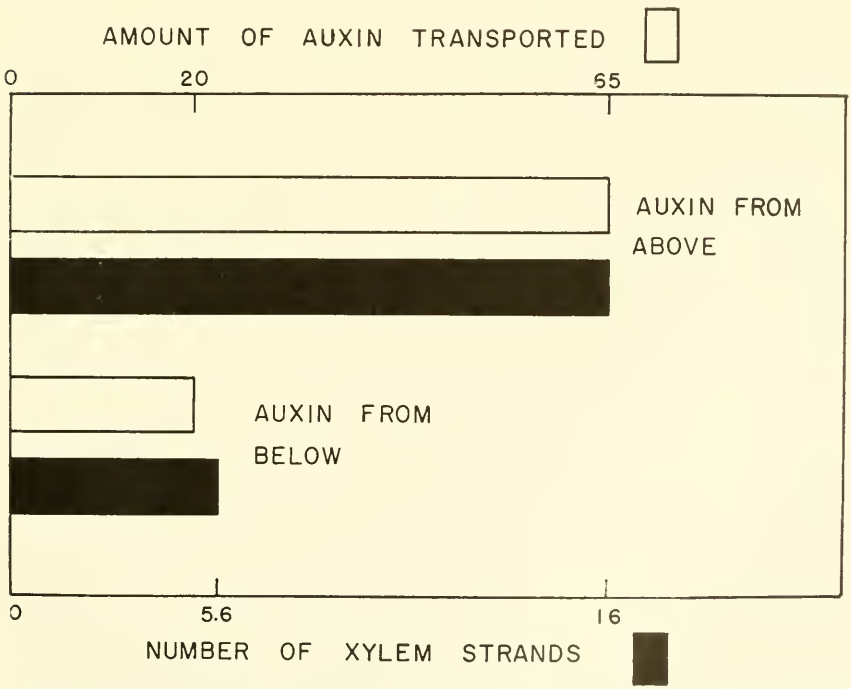


Fig. 7. Relation between amount of auxin transported through excised sections of *Coleus* and the number of xylem strands regenerated in the plant when leaves above and below are excised. From Jacobs (12).

compare regeneration in plants in which only distal sources of auxin are left on (i.e., leaves 1, 2, and the apical bud) with plants with only proximal sources of auxin (leaves 3 to 8), we see that the number of xylem cells regenerated is nicely proportional to the observed auxin transport (12).

Our conclusion is that the acropetal auxin transport is of real physiological significance.[3]

Another idea is suggested by musing over the results with the bean hypocotyl and *Coleus* internode 2. In both cases IAA was added at a concentration of 2 mg/l of agar. And in both cases, very exact relations could be observed between the amount of auxin transported under these circumstances and the amount of growth or of xylem regeneration. This suggests that, contrary to what one might expect from the reports of van der Weij (20) or Went (23), there is *not* a steady increase in auxin transported as one increases the amount added, but rather a plateau – and that 2 mg/l is *on* the plateau.

We are currently checking the hypothesis in two ways: by direct assay with the *Avena* curvature test of transport through isolated sections ringed with vaseline (Figure 8), and by indirect test using xylem regeneration in *Coleus* again as a measure of how much auxin is getting into the more intact plant. Xylem regenerated under the influence of the shoot tip could be completely replaced by 1 per cent IAA in lanolin. And as evidence of saturation was the fact that 10 per cent IAA in lanolin gave no further increase in the number of xylem strands which regenerated [Figure 5 in Jacobs *et al.* (15)]. The *Avena* curvature results are not definitive, since they do not yet include dilutions of the collected auxin. (This means that there is a possibility that the *Avena* curvatures for the transport of 1.25+ mg/l are above the proportionality range of the *Avena* bioassay. We are, of course, checking this possible artifact now.) But, so far as they are valid, the two tests confirm the hypothesis and each other. Auxin transport in *Coleus* seems to show a saturation effect at concentrations of 1+ mg/l of agar.

Experiments already described, plus others described by Jacobs in 1954 (12), provided evidence that the transport capacity at saturation normally limits how much auxin is available in the plant. Although the leaves normally produce auxin at a level matching the saturation capacity of the stem (12), their level of production is subject to fluctuation as the environment changes. The potential im-

[3] The appearance, with flowering, of acropetal auxin transport in stems of *Coleus* has been reported (16), the report criticized on grounds of inadequate technique (12), and found unconfirmable by Haupt (6). Professor Leopold has told me that his attempts to repeat the experiments have given inconsistent results.

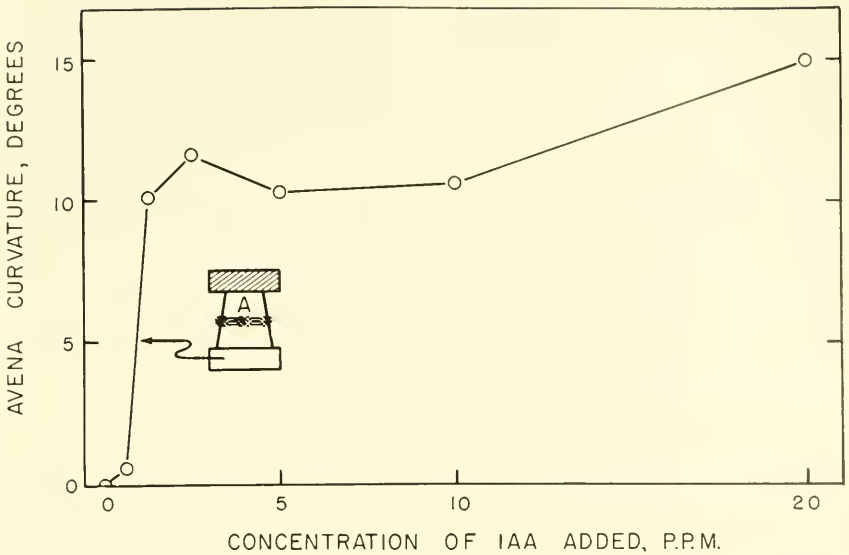


Fig. 8. Evidence of saturation in auxin transport in young internode sections of *Coleus*. The sections were prediffused for 2 hrs., ringed with vaseline (to obviate surface leakage), then 5 mm. sections were cut from the slightly longer prediffused sections. Transport time was 2.5 hrs.

portance of the saturation capacity in the physiology of the plant is thus obvious: it could act as a controlling valve which prevents excess amounts of auxin from being transported around the plant and thereby disturbing the balanced coordination of normal development.

That such a saturation capacity is not limited to *Coleus* is indicated by the results in bean hypocotyl mentioned above and by transport tests on *Avena* coleoptiles (Goldsmith, Ph. D. thesis, Harvard).

From the earlier view of normal auxin transport as something unique, strictly one-way, and unchangeable by various environmental factors, a new view has thus developed since 1939. Auxin transport, in these later researches, appears as a function which varies with the physiological and histological state of the tissue, which is responsive to changes in the environment and which is *not* always strictly polar. In addition, it has been shown not only that auxin transport controls the relative polarity of various developmental phenomena, but also that organs have a maximum capacity in the physiological range of concentrations for transporting auxin, and that this set transport capacity serves an important regulatory function by buffering the plant from sudden increases in auxin level.

What of the future? We need to know how general are the results now known in detail for seedling organs and a few vegetative stems. We need critical direct experiments on auxin transport in roots,

with the results meaningfully related to the normal physiology. To better understand the role of the plateau capacity of auxin transport, we need to know how stable is this plateau level. From the current view of auxin transport it would be reasonable to expect that continued exposure to higher than normal auxin levels would cause gradual regulation in the plateau capacity. Knocking out auxin movement by the addition of chemicals (2, 3, 17, 18) may lead to an understanding of the biochemical basis of auxin transport. It would be particularly intriguing if a chemical could be found which would actually knock out the *polarity* — i.e., allow equally fast movement of auxin in both directions in a normally strictly polar organ like the coleoptile.

ACKNOWLEDGMENT

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Polar Transport of Three Auxins[1]

In understanding the polar system which transports auxin through plants, it is particularly relevant to know whether the transport system is specific to indole-3-acetic acid (IAA). There is evidence in the earlier literature that some other auxins may be carried in the transport system (10), but it is not clear whether these compounds may all be moved with the same polarity and the same velocity as indole-3-acetic acid, nor is it clear how effectively the transport system can distinguish between auxins of different molecular structure. Experiments on these questions were undertaken in an effort to characterize further the polar transport system in plants.

METHODS

The auxins were assayed in every case by means of the *Avena* curvature test, following the procedure described by Leopold (4).

The auxins were transported through stem sections taken from the epicotyl of sunflower seedlings grown in the greenhouse between December and April. The method is identical to that described by Niedergang-Kamien and Leopold (6). A donor block was placed at the apical end of a 5 mm. stem segment, and the auxin transported into a receptor block at the basal end was assayed after 120 min. of transport at 25° C. Unless otherwise indicated, all transport tests with indole-3-*n*-butyric acid continued for 180 min.

The experiments with transport inhibitors were carried out by the method of Niedergang-Kamien and Leopold (7). The inhibitor was applied in an agar block at the basal end of the sunflower stem section, and the auxin was simultaneously applied as a 5 μl. droplet at

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the apical end. After 30 min. the stem section was blotted and transferred to a fresh receptor block in which the transported auxin was collected for an additional 90 min. In these experiments, the controls were transferred from a plain agar block to a fresh receptor block at the same time that the inhibitor treatments were transferred. This represents the only modification of the earlier technique.

RESULTS

Preparatory to a comparison of the transport of several different auxins, five auxins were tested for activity in the *Avena* curvature test. Included in this test were indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), indole-3-*n*-butyric acid (IBA), indole-3-propionic acid (IPA), and 2-naphthoxyacetic acid (NOA). Characteristic *Avena* test data for these five auxins are shown in Figure 1. Of course the greatest curvature response was obtained with IAA. Normal curvature responses were also obtained with NAA and IBA, though less curvature was obtained with these per unit of auxin. IPA and NOA yielded essentially no curvatures.

The *Avena* curvature test is, therefore, a valid assay for the three auxins: IAA, NAA, and IBA.

The transport of these three auxins in both basipetal and acrope-

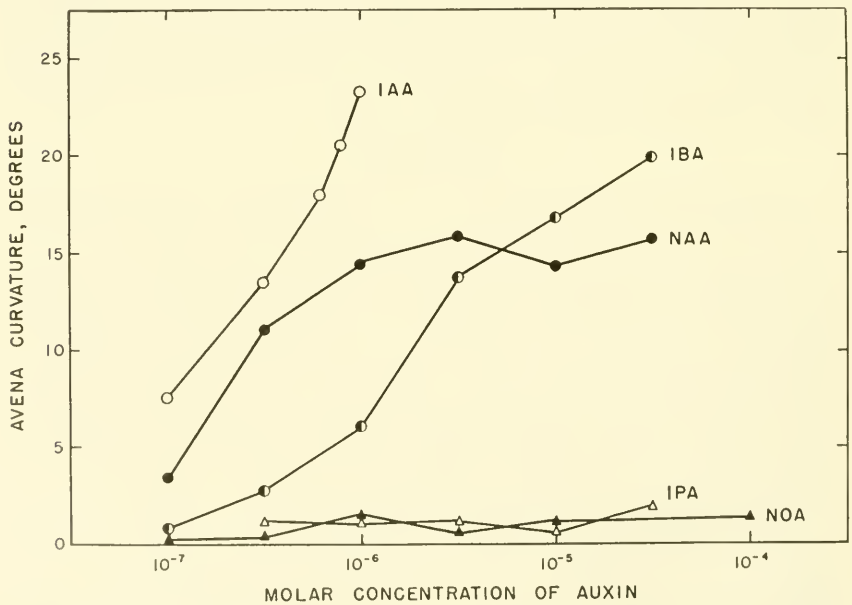

Fig. 1. Response curves for five auxins in the *Avena* curvature test: indole-3-acetic acid (IAA), indole-3-*n*-butyric acid (IBA), 1-naphthaleneacetic acid (NAA), indole 3-propionic acid (IPA), and 2-naphthoxyacetic acid (NOA).

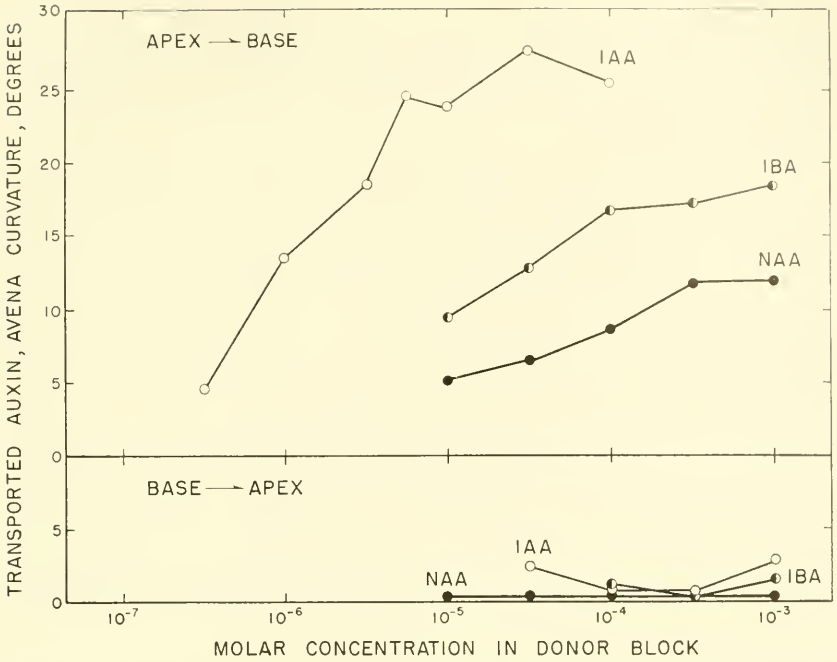

Fig. 2. The acropetal and basipetal transport of three auxins applied to sunflower stem sections in various concentrations. Symbols as in Figure 1. Transport time 120 min. for IAA and NAA, 180 min. for IBA.

tal directions was also measured. Each was presented in agar donor blocks to sunflower stem sections. Concentrations of the auxins were varied between $3 \times 10^{-7}M$ and $10^{-3}M$. Assays of the receptor blocks provide data such as those shown in Figure 2. Looking first at the basipetal transport, it can be seen that each of these three auxins is transported downward in the manner usual for IAA. The amount of auxin transported is presented simply as the *Avena* curvature without correction for differences in activities in the *Avena* curvature test or for length of transport period. It can be readily seen, however, that downward transport was obtained, and that NAA and IBA were apparently transported in somewhat smaller quantities than IAA. The tests of upward transport show that within the range of concentrations of donor blocks tested (10^{-5} to $10^{-3}M$) essentially no transport was obtained. We can, therefore, conclude that each of these three auxins is transported in a polar manner.

A further comparison of the transport of the three auxins can be made by measurements of the velocities of transport. Velocity of transport was measured in the manner originally used by van der Weij (9). Sunflower stem sections were provided with donor blocks

at the apical end for different periods of time. The donor blocks and the stem sections were removed from the receptor blocks which were then assayed for auxin. The concentration of IAA in the donor block was $10^{-5}M$, and the concentration of the NAA and IBA was $10^{-4}M$ in each case.

The results of velocity measurements are shown in Figure 3. It can be seen that 40 minutes of transport time were necessary before IAA could be detected in the receptor block. The stem section was 5 mm. long, and so the velocity can be estimated at 7.5 mm/hr. The first NAA detectable in the receptor block appeared after about 45 min., indicating a velocity of 6.7 mm/hr. The first IBA detectable appeared after about 95 min. indicating a transport velocity of 3.2 mm/hr. Each of these values was determined several times with separate experiments and the variation was not in excess of 1 mm/hr.

Another means of asking whether the three auxins are transported by similar or dissimilar physiological processes is to measure the sensitivity of each of them to inhibitors of transport. For these experiments the following inhibitors were selected: 2,4-dichlorophenoxyacetic acid (2,4-D), α-methoxyphenylacetic acid (MOPA), 2,3,5-triiodo-

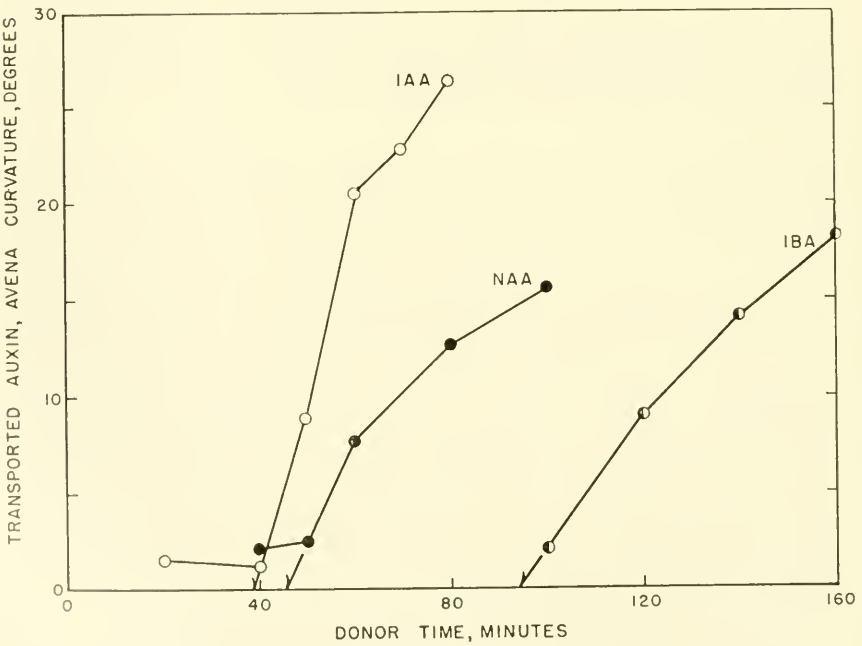

Fig. 3. Determinations of the velocity of transport of three different auxins. Sunflower sections transporting the auxins are applied to receptor blocks for increasing periods of time and the time of initial response in subsequent *Avena* test is indicated by the arrow. Symbols as in Figure 1.

Table 1. Inhibitions of transport of three auxins by various transport inhibitors. All of the data for a given auxin are taken from a single experiment. Transport time 120 min. for IAA and NAA, 180 min. for IBA, donor concentration $10^{-5}M$ for IAA, $10^{-4}M$ for NAA and IBA.

Auxin	Inhibitor none	2,4-D $10^{-4}M$	MOPA $10^{-4}M$	TIBA $10^{-5}M$	NOA $10^{-4}M$	IPA $10^{-4}M$
IAA curvature	17	9.5	8.1	2.7	6.1	10.1
Per cent inhibition		(44 per cent)	(52 per cent)	(84 per cent)	(64 per cent)	(41 per cent)
NAA curvature	15.7	11.8	11.0	1.8	13.3	11.5
Per cent inhibition		(25 per cent)	(30 per cent)	(88 per cent)	(15 per cent)	(27 per cent)
IBA curvature	13.4	3.4	4.3	1.0	2.9	4.5
Per cent inhibition		(75 per cent)	(68 per cent)	(93 per cent)	(78 per cent)	(66 per cent)

benzoic acid (TIBA), 2-naphthoxyacetic acid (NOA), and indole-3-propionic acid (IPA). Each of these except the MOPA and NOA has been reported as being an inhibitor of IAA transport, and these last two had been found to be inhibitors in experiments in this laboratory.

Results of typical inhibition experiments are presented in Table 1. The tests were conducted with each transported auxin in one test, so that the relative effectiveness of the various inhibitors as they influence one auxin is a more reliable comparison than as they influence the several auxins. The data show clearly that each of the inhibitors is effective against the transport of any of the three auxins. Within this array of transport inhibitors, then, there is no evident distinction between the auxins being transported. It is clear that TIBA is by far the most potent inhibitor of transport of each of the three auxins, causing more than 80 per cent inhibition of transport in each instance at a concentration of only $10^{-5}M$. The inhibitions obtained with the other inhibitors appear to be comparable to one another. It might be mentioned that the naphthalene containing inhibitor NOA was not apparently more effective against the transport of NAA than the other inhibitors. Similarly, IPA was not more effective against the transport of the indole auxins than the other inhibitors tested.

DISCUSSION

The experiments reported here provide a comparison of the transport characteristics of three different auxins: IAA, NAA, and IBA. It is clear that all three are polar in their movement, and they vary somewhat in velocities. They are all sensitive to the same transport inhibitors, and there does not appear to be a selectivity by transport inhibitors for the different auxins. The transport of the three auxins therefore appears to be very similar indeed, except for the differences in velocity.

While IAA and NAA appear to have very closely similar transport velocities, IBA is markedly slower. It has been established by Fawcett *et al.* (1) that IBA is metabolized in plant tissues giving rise to IAA, and while it is not certain that this conversion must occur before growth activity is obtained, it is reasonable to assume that there would be a gradual production of IAA in the tissues during transport tests. The delay in commencement of curvature responses in the time curves with IBA may well be due to the time required for the conversion of the IBA into IAA, which then would, of course, have the transport characteristics of that auxin.

A comparison of the transport velocities of these three auxins plus anthraceneacetic acid was made by Went and White (10) with some-

what different conclusions. They interpreted their experiments as indicating that IBA transport was 25 per cent slower than IAA. Our experiments indicate IBA to be about 50 per cent slower. They found NAA to be transported 58 per cent more slowly than IAA, and our experiments indicate the rate to be almost the same as for IAA. Examination of the method of determination of velocity used by Went and White suggests that precise calculations would be very difficult to make by their method. It involves the determination by photokymograph of the moment of initial curvature of *Avena* coleoptiles when the transporting coleoptile sections are placed unilaterally upon the tip and a donor agar block on top of that. The determination of the moment of initial curvature is complicated by nutational movements of the coleoptile, by initial negative curvatures of the coleoptile, and by uncertain rounded sections of the time curve which make definition of the unresponding and responding sections rather uncertain. Using the more precise method of van der Weij (9), we feel that the velocities reported here are quite exact within 1 mm. per hour variability at most. The possibility exists, of course, that the velocity of NAA transport in *Avena* coleoptiles may be slower than in sunflower stems. The strictness of polarity of transport of IAA reported by the earlier workers is confirmed here, and in addition the same is found for IBA and NAA. The report by Went and White (10) of lack of transport for indole-3-propionic acid is also confirmed here.

In view of the close similarities of the characteristics of transport of IAA, NAA, and IBA, it can be deduced that the transporting tissue cannot distinguish between these three auxins, except for the velocity difference for IBA as discussed above. Molecules with closely similar structures can be effectively differentiated against. Thus there is apparently no transport of indene-3-acetic acid (8), of indole-3-propionic acid, cis-cinnamic acid (10), the phenoxyacetic acids (5), or of 2-naphthoxyacetic acid. Indirect evidence may permit the inclusion of phenylacetic acid in this list (2). It appears, then, that the polar transport system is not specific for the indole ring, but it may be specific for a two-cyclic ring since none of the phenyl and phenoxy auxins seem to be transported in this system. The inhibition of apparent transport by two benzoic acid auxins (3) indicates that these monocyclic auxins may not be transported either.

It has been suggested by Niedergang-Kamien and Leopold (7) that an adsorptive phase may be involved in the polar transport system. Since the most likely site of adsorptive attachment of an auxin would be on the aromatic ring, it is reasonable to suppose that the specificity of the transport system for certain ring structures may be related to the adsorptive characteristics produced by the ring.

SUMMARY

The transport of indole-3-acetic, indole-3-*n*-butyric, and 1-naphthaleneacetic acid through sunflower stem sections has been examined. Evidence is provided that the transport of each of these auxins is polar in nature. The velocities of transport of indole-3-acetic acid and of 1-naphthaleneacetic acid are closely similar, while a slower apparent rate obtains for indole-3-*n*-butyric acid. The sensitivities of transport of the three auxins to transport inhibitors were also measured, and no striking differences between the three auxins were found. It is suggested that the three auxins move in the same transport system, and the system is not exclusively specific for the common plant growth hormone, indole-3-acetic acid.

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The Stimulation of Auxin Action by Lipides

Studies with the gibberellins have exposed a major paradox: intact dwarf peas respond markedly to treatment with gibberellic acid, whereas epicotyl sections cut for bioassay from similar plants respond hardly at all (1, 20). Thus a conventional straight growth section bioassay for growth substances is almost useless for the estimation of gibberellins. More importantly, this observation exposes a hiatus in our understanding of the mechanism of growth in these sections, and moreover, this gap seems particularly amenable to experimental analysis.

Our own measurements of the growth, under optimum conditions, of dwarf peas and of the sections obtained from them are summarized in Table 1 (18). It is apparent that, as has been reported by others, gibberellic acid (GA_3) itself has only a marginal influence on the sections; more significant is its further promotion of the effect of indole-3-acetic acid (IAA). Even so, the sections under these optimal conditions are not even two-thirds of the length they would have reached on the intact plant, and only one-third of the length they would have achieved with GA_3 treatment.

A likely explanation of this growth deficiency is that some factor required for gibberellin action is normally supplied to the section by the rest of the plant. In previous work (16) we found that a fat fraction could be isolated from peas which markedly promoted section growth, and it was natural to test this for its ability to restore part of this missing growth. This fat fraction was not a specific substance, however, as a wide variety of fatty acid esters could also increase the growth obtained.

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Table 1. Comparison of percentage increase in length in 24 hrs. of sections from, and of intact zones on, dwarf 'Laxton's Progress' peas. Plants and sections continuously exposed to medium by rotating on shaker within petri dishes. Data of two experiments (18), with standard deviations.

Percentage Increase in Length		
Medium	I	II
10 Mm. Sections		
Basal medium*	44.9 ± 5.6	39.9 ± 3.5
+ GA_3 (0.3 μM)	54.6 ± 5.3	39.9 ± 7.9
+ IAA (1.7 μM)	67.5 ± 7.0	65.1 ± 10.5
+ GA_3 + IAA	84.3 ± 6.4	70.1 ± 12.0
10 Mm. Zone on Intact Plant		
Basal medium*	132.4 ± 15.3	125.0 ± 16.8
+ GA_3 + IAA	201.1 ± 14.6	234.0 ± 24.1

* Sucrose, 1.25 per cent + 50 μM $CoCl_2$ + 5 mM KH_2PO_4 (pH 5.5).

Figure 1 shows the data obtained both with the dwarf 'Laxton's Progress' pea and the 'Alaska' pea. In each case, section elongation can nearly be doubled by an extract of pea glycerides or with pure methyl esters of certain fatty acids. The Tween detergents, which are also fatty acid esters, also showed growth-promoting activity. It is apparent that the differences between the dwarf and the 'Alaska' pea sections are small. In fact, although dwarf pea sections have been used predominantly in this work, each time results have been checked on 'Alaska' pea sections the response has been very similar.

When the new lipide factor is added to the tests of Table 1, using one of the most active compounds, methyl linoleate at 68 μM, the section growth is increased to 113.8 ± 7.3 in the first test and 107.4 ± 9.7 in the other. Even so, these increments still bring growth up to only four-fifths of that of untreated plants and to not quite half the growth anticipated in the presence of GA_3.

In the hope that some other lipide might futher stimulate the growth response of the sections, some thirty-odd pure substances have been examined. Table 2 summarizes these results, which indicate that many alkyl compounds with a chain length greater than twelve carbon atoms are capable of accentuating the growth of pea

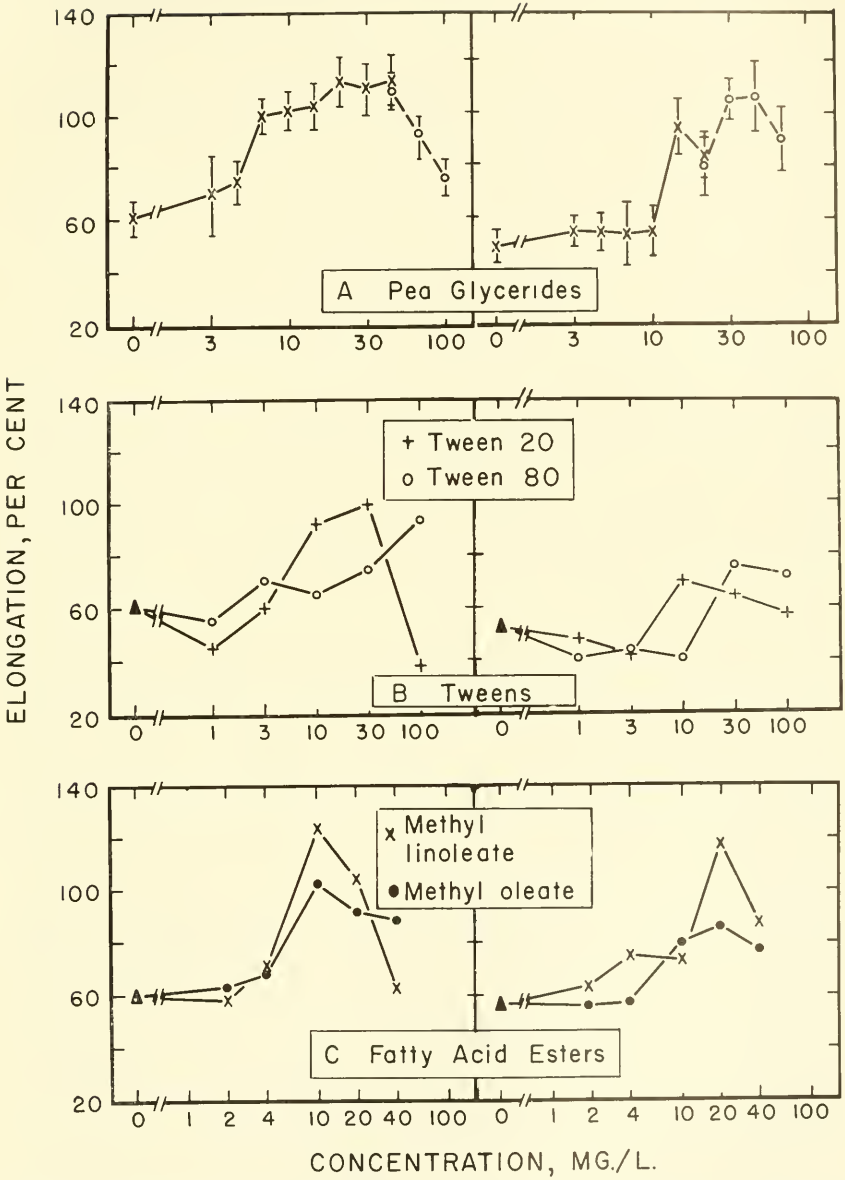

Fig. 1. Percentage elongation of 10 mm. pea epicotyl sections after 24 hrs. in 1.25 per cent sucrose + 50 μM $CoCl_2$ + 5 mM KH_2PO_4 (pH 5.5) + 1.7 μM IAA + 0.3 μM GA_3 plotted against a logarithmic scale for various lipides in mg/l. In (A) bars represent standard deviations; crosses and circles are different experiments. Reproduced from (16).

Table 2. Comparison of effectiveness of different classes of simple alkyl lipides in enhancing auxin-induced growth of 'Laxton's Progress' pea epicotyl sections. Full details are published elsewhere (18); the most active compounds are cited in text.

Alkyl Lipide Class	Efficacy
Compounds less than C_{12}	Many esters, glycerides, alcohols tested; none active.
Fatty acid methyl esters	All 9 tested (in C_{12} to C_{20} range) found active.
Triglycerides	All 4 tested (tri-palmitin, -stearin, -olein, and -linolein) active.
Fatty alcohols	4 (in C_{18} to C_{22} range) active, 2 (C_{14} and C_{16}) inactive.
Monoglycerides	2 (C_{16} and C_{18}) active, 4 (C_{14}, C_{18}, C_{20}, and C_{22}) inactive.
Free fatty acids	All 7 tested (in C_{12} to C_{22} range) found inactive.

sections. The free fatty acids are ineffective, despite the fact that their esters are among the most active compounds. The most active simple alkyl substances discovered to date are triolein, trilinolein, methyl myristate, and selachyl alcohol. All are of comparable activity, but none can bring section growth entirely back to normal — values like those reported above for methyl linoleate being typical of the group as a whole.

Thus it is likely that yet other factors are required for optimal growth, and in particular for the growth promotion caused by gibberellic acid. Lockhart's (13) and Galston and Warburg's (9) results may also be interpreted as indicating that the rest of the plant produces a factor which enhances gibberellin action on the sections. Nonetheless, attempts to extract such an additional factor have not been successful. It seems likely that the missing substance could be the caulocaline postulated some years ago by Went (24). It was deduced that this was synthesized in the roots and could only travel through living tissue. Went showed that it required pea tip auxin, but pea tips are now known also to produce gibberellin (13).

Alternatively, it may be that gibberellic acid and dihydrogibberellic acid are not the active form of gibberellin but are converted to it in some other part of the plant. This would fit in with the results of Phinney (15) reported at this conference, which indicate a biochemical pathway of gibberellin synthesis in maize.

Since in this study a technique had been adopted which made it possible to prepare stable aqueous emulsions of nearly any fat-soluble substance (18), it was thought desirable to investigate the effect of the hitherto inaccessible fat-soluble vitamins on the section bioassay. Tests of vitamins A, D_2, E, K_1, and β-carotene revealed that of these E and K_1 were as effective as the simple alkyl lipide compounds cited

earlier, and were active at even slightly lower concentrations (19). The absolute growth, however, remained no greater than before. The other substances were inactive.

Vitamins E and K_1 possess a bicyclic ring with an isoprenoid side chain. In animals the side chain is not essential for vitamin K activity and, accordingly, the side-chainless vitamin K_5 and menadione analogues were tested on pea sections (19). No growth promotion was noted, but when vitamin K_1's side chain, phytol, was tested by itself, growth promotion was found. A less exact analogue of the vitamin E side chain, farnesol, was ineffective. However, the activity of phytol makes it seem likely that the potency of both vitamin E and K_1 is due to their possession of a long chain hydrocarbon substituent.

Some common property of a wide variety of long-chain hydrocarbon compounds is thus responsible for the greater elongation of the pea stem sections. Tests of a number of components of lipide metabolism, such as coenzyme A, thioctic acid, acetate, pyruvate, cytidine, choline, etc., and of compounds involved in isoprenoid synthesis, such as mevalonic acid and its lactone, as well as adenine, niacinamide, and ribose, have not revealed any connection of this phenomenon with well-known biochemical pathways. Nor have physical factors such as surface action of these compounds or increased uptake of auxin been found to be implicated in any tests yet carried out. The details of these largely negative findings have therefore been left to other publications (18, 19).

In this connection, one of the most significant features of the lipide effect is the fact that the optimal amounts required are much too small to meet any major nutritive requirement of the sections. Table 3 shows that a 4 μM concentration of vitamin K_1 — scarcely more than twice the concentration of IAA used — suffices for a

Table 3. The dependence on auxin of the response to lipides of 10 mm. 'Laxton's Progress' pea epicotyl sections. Percentage increase in length with standard deviations after 24 hrs. (17).

Treatment	No Lipide	Plus Methyl Linoleate (50 μM)	Plus Vitamin K_1 (4 μM)	Plus Vitamin E (10 μM)
Basal medium*	39.1 ± 3.6	38.4 ± 3.2	40.6 ± 3.4	35.7 ± 5.9
+ GA_3 (0.3 μM)	41.8 ± 15.0	46.9 ± 5.8	51.2 ± 8.2	43.6 ± 9.4
+ IAA (1.7 μM)	55.7 ± 6.2	68.7 ± 6.5	62.9 ± 7.6	65.9 ± 4.6
+ GA_3 + IAA	67.4 ± 5.6	99.7 ± 5.0	104.3 ± 10.2	94.0 ± 6.2

* Sucrose, 1.25 per cent + 50 μM $CoCl_2$ + 5 mM KH_2PO_4 (pH 5.5) + 0.004 per cent Pluronic F-68.

considerable accentuation of section growth over that achieved with IAA plus GA_3 alone. The concentrations of vitamin E and methyl linoleate employed, although higher by 6 to 30 times that of IAA, still may be considered to lie within a hormonal range.

Further examination of the data of Table 3 reveals that the lipides are completely ineffective in the absence of an auxin (other tests have shown that 1-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid are also capable of potentiating the lipide response). A small promotion of gibberellic acid action is frequently found, but has never reached the level of statistical significance. The enhancement of auxin-induced growth is unmistakable, but it is in the presence of both auxin and gibberellic acid that the greatest margin of significance is attained. Hence it appears that the lipides act hormonally, as synergists of the action of auxins and, in the presence of an auxin, of gibberellic acid. Any explanation of the lipide effect thus must be linked to the actions of these hormones.

Although the evidence is still far from conclusive, we have ad-

Table 4. Comparison of effectiveness of different lipides in enhancing auxin-induced growth of sections of 'Laxton's Progress' pea epicotyls with their ability to restore cytochrome activity of isooctane extracted animal particulate preparations (17).

Lipide	Relative Effectiveness on Pea Sections*	Per Cent Restoration of Particulate Cytochrome Activity†
Natural lipide extract	+++	100
Vitamin E	+++	100
Vitamin K_1	+++	100
Phytol	+++	100
Methyl linoleate	+++	100
Methyl linolenate	+++	100
Triolein	+++	76
Trilinolein	+++	50
Ethyl palmitate	+++	50
Ethyl stearate	+	55
Monopalmitin	+	0
Tristearin	++	0
Tripalmitin	+	0
Menadione	0	50
Vitamin A	0	0
Lauric acid	0	0
Myristic acid	0	0
Palmitic acid	0	0
Stearic acid	0	0
Oleic acid	0	0

* Data of (16).
† Compiled from data in (5, 7, 23).

vanced the hypothesis that the effect of the lipides is due to their activation of the cytochrome system leading to a greater availability of energy for growth (17). The results which have been outlined above bear an amazing similarity to the data obtained by workers with animal systems of isooctane-extracted cell particles (5, 7, 23). In these systems the cytochrome activity of the particles can be restored by a number of lipide compounds which apparently facilitate the action of cytochrome-c reductase. Table 4 gives the results of a comparison of a number of compounds which have been tested in both the pea system and with the isooctane-extracted particulates. Of the 19 compounds only 4 show activity in one system and not in the other, and in each of these cases the activity that was observed was submaximal. The analogy probably does not hold in another case, since in a few tests with coenzyme Q_{10},[2] a lipide-soluble quinone which is found in plants (4) and which acts on mitochondrial electron transport, no convincing effect could be noted in our system. Furthermore, the inhibition of section growth caused by antimycin A was not reversed by vitamin K_1, but an irreversible effect of this inhibitor is also shown by some particulate systems (5).

Further support to the idea that the respiratory system is involved is given by the data of Figure 2 which show that both methyl myristate and vitamin E increase the respiration of the sections above the values obtained in auxin plus GA_3 alone. Although this promotion is not large, it is comparable to that produced by auxin over the controls in basal medium, which would be expected from the fact that the growth increments in each case are roughly the same.

Obviously, evidence of this kind cannot conclusively implicate the cytochromes. However, the work of Hackett and Schneiderman (11) makes it clear that the auxin-induced growth of pea sections is entirely mediated by cytochrome oxidase. Since this is the case, a limitation of growth imposed by a deficiency in cytochrome-c reduction is all the more plausible. Direct observations of cytochrome-c activation will have to be made to prove this hypothesis. This may be difficult without disruption of the tissue and a consequent loss of the linkage to cell elongation.

Additional speculation along these lines might also be made for the case of the growth-promoting effect of cobalt which requires sucrose (14, 22). The lipide effect is considerably accentuated by sucrose (18); cobalt has a smaller promotive role (19). In yeast, cobalt can induce respiratory deficiencies, probably due to an effect on

[2] Kindly supplied by Dr. F. L. Crane.

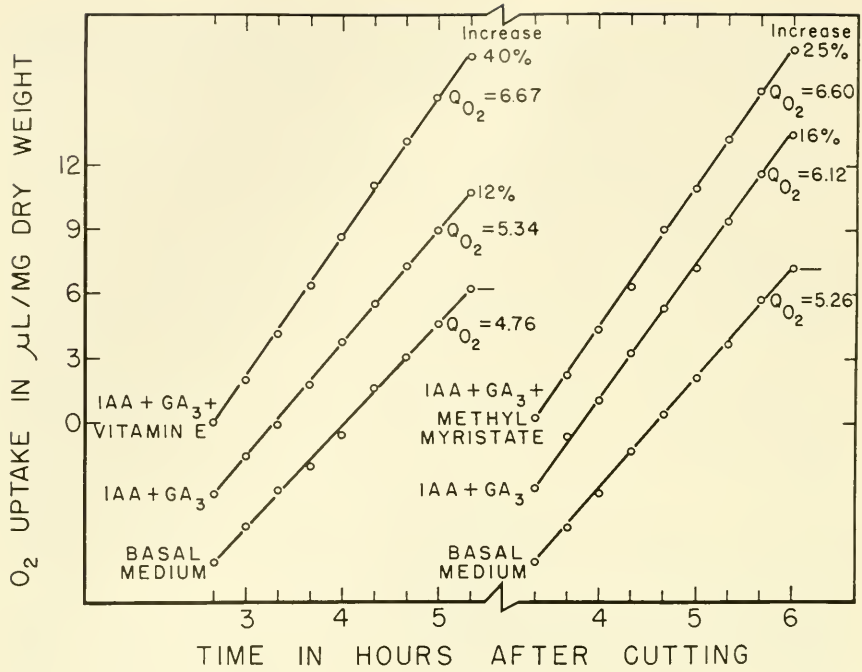

Fig. 2. Oxygen uptake of 10 mm. 'Laxton's Progress' pea epicotyl sections during log phase of growth in the presence of IAA 1.8 μM plus GA_3 0.3 μM and methyl myristate 40 μM plus vitamin E 10 μM. Basal medium 1.5 per cent sucrose + 50 μM $CoCl_2$ + 5 mM KH_2PO_4 (pH 5.5) + 0.002 per cent Pluronic F-68. Q = μl. O_2/mg dry wt/hr estimated from slope of best straight line through points. Compare Figure 1 in Christiansen and Thimann (3).

cytoplasmic particles (12), and in *Avena* coleoptiles it decreases respiration (2). Can it be then, that in peas cobalt suppresses an alternative pathway of respiration, channeling more of the energy derived from sucrose towards growth? As cytochrome-c is known to be the terminus of several respiratory pathways, this suggestion is particularly attractive in the light of the experimental results reported above.

Assessment of the merit of these speculations will have to await further experimentation. What has been firmly established is that trace quantities of lipide substances can play an important role in auxin action. But these lipides may not be limiting in all plant tissues, since in *Avena* coleoptiles they have not as yet been observed to enhance cell elongation (16).

This linkage of lipides to hormone action is, of course, not a new suggestion. Crosby and Vlitos (6) have presented evidence that a long-chain alcohol isolated from tobacco can be active in the *Avena* first internode bioassay. Struckmeyer and Roberts (21) have for some years also been working with a higher alcohol obtained from plant

tissues which they believe interacts with auxin. In pea sections themselves, Christiansen and Thimann (3) noted that lipide utilization paralleled auxin-induced growth, and that the same inhibitors which reduced the cell elongation also slowed the lipide decrease.

Antedating these reports is the work of English *et al.* (8) on wound hormones which has been further delineated by Haagen-Smit and Viglierchio (10). Although the wound hormones problem must have some relationship to the present work, inasmuch as in both cases trace quantities of lipides potentiate a hormonal response, there are some striking unexplained differences in the specificity of the two systems. To begin with, traumatic acid has no influence on pea section growth. Next, the most effective substances for wound tissue proliferation were found to include lauric, myristic, and linoleic acids, none of which stimulates epicotyl section elongation. Lastly, cytochrome-c, coenzyme A, and ascorbic acid all increase the wound hormone response whereas all had no influence on the peas (17). There is thus some fundamental divergence in the operation of the two hormonal systems. Unfortunately, none of the compounds now shown to be active in accelerating pea section growth was tested by the above workers on their material.

Lipoidal compounds have been linked to auxin action by several workers using several plant systems. Although the mechanisms behind these systems may not be identical, further analysis of these auxin-lipide interactions should prove fruitful for our understanding of hormonal regulation in plants.

ACKNOWLEDGMENT

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DISCUSSION

Dr. Jansen: At Beltsville we have been engaged in a long-term investigation of the effects of surfactants in herbicidal spray preparations. A number of surfactants have been encountered which, in the absence of herbicides, produce some rather striking growth stimulations in corn and soybeans. The individual surfactants do not always stimulate both species nor do they always produce stimulation at the same concentrations. Increases in growth up to 25 per cent

in height have been obtained in 2 weeks. Unpublished data show that 14 of 30 surfactants produced the most striking growth promotions in our evaluations. These surfactants are distributed throughout the four major classes, namely those of anionic, cationic, nonionic, and ampholytic natures. They also represent a number of the structural subclasses. In nearly all of the surfactants listed, long-chain aliphatic radicals from either acids or alcohols are integral components. I think this is a fact which we should probably bear in mind when we use any surfactant in our growth regulation studies.

Dr. Crosby: As we pointed out earlier, we found that the acidic inorganic esters of the long-chain alcohols, the 18-, 20-, and 22-carbon sulfates and phosphates, were all extremely active. We, too, feel that these surface-active effects are extremely important. In regard to Dr. Stowe's work, one brief comment for those who might become interested in working in this area. The compounds which may be purchased are all natural products. The benzyl alcohol that we used, and the esters which Dr. Stowe used, are initially impure. I think it is necessary to be sure that a rigorous purification is carried out on all these materials. I also notice that Dr. Stowe observed the same interesting concentration-activity relationships that we did, even though the compounds which he found to be active were as insoluble in the bioassay medium as were ours. I wonder if he would have any comment on how it is possible for there to be such a concentration-activity relationship.

Dr. Stowe: Well, it's possible, of course, that the concentration here is a bit misleading. These tests were carried out in a standard volume of solution, so we were always adding a known amount of lipide to the bioassay material. I want to emphasize that these are applied as a very good emulsion, and although you cannot, perhaps, speak of a true solution here, specific amounts of the substance were added to each dish, and then you find, as we showed, a nice linear relationship within the right concentration ranges.

Dr. Vlitos: Your Figure 1 and Tables 2 and 4 showed activity in the pea internode section test with fatty alcohols at different concentrations, and then Table 3 indicated that IAA was needed in your solutions to show activity. Now I'm not quite clear whether in the first cases you were dealing with endogenous auxins. Would you clear that up for me?

Dr. Stowe: In every case where you saw an effect, there was also added the optimum concentration of IAA and GA. What we were trying to do was to get maximal growth, and we added everything to the pea sections that we knew would promote growth, and then looked for still other growth-promoting factors.

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Electronic Effect of Substituents on the Activity of Phenoxyacetic Acids

In the two-point attachment mechanism of action which has been developed (14, 26, 27), it has been postulated that the plant growth regulators react with a protein substrate in plants by means of a carboxyl group and a ring position *ortho* to the attachment of the carboxyl group. Since the work of Osborne *et al.* (28, 29) on 2,6-disubstituted phenoxyacetic acids and that of Wain's group (30) on phenylacetic acids, it appears that, stereoelectronic conditions permitting, other points on the ring may function in place of an *ortho* position. Despite further evidence for the validity of this mechanism (6, 9, 10, 22, 23), considerable doubt still remains (37), and more evidence is necessary for complete understanding.

In attempting to correlate the effects of various substituents on the aromatic rings of the different growth regulators, there are three important factors connected with each substituent which must be considered: electronic, steric, and H/L factor (hydrophilic/lipophilic). It is relatively easy to compare molecules with respect to the first and third factors; however, the steric effect of substituents on reactivity is extremely difficult to assess since nothing is known of the geometry of the reaction site. Also, the nature of the side chains is such that assuming attachment by the side chain first, the ring with its attached groups could be presented in many ways to a second site. The purpose of this paper is to consider some monosubstituted phenoxyacetic acids which are simple enough that comparison of the electronic effects of the various groups is not too complicated.

First, a quick summary of the more important evidence favoring reaction vs. simple adsorption. Most important is the large amount of evidence indicating that the ring associated with growth regulators

must be aromatic in character. Despite a rather thorough search (8, 13, 19, 36, 39), no aliphatic compounds, with the possible exception of one or two thiocarbamates (12), have been found to be active in growth promotion. The only alicyclic compounds which have confirmed activity are 1-cyclohexenylacetic acid and its analogues (11, 19). It is not unlikely that these molecules could be dehydrogenated to the quite active phenylacetic acid or one of its derivatives. Such dehydrogenations are known to occur in biological systems (25). So far, our attempts to establish the occurrence of this dehydrogenation with *Avena* coleoptiles have been inconclusive. Thus, although there is slight evidence to the contrary, the overwhelming preponderance of evidence from thousands of carefully tested compounds indicates that an aromatic ring is necessary. Most effective are compounds which contain six-membered rings such as benzene, pyridine, and their homologues. However, compounds having the less aromatic five-membered rings, or compounds with one double bond which might easily be dehydrogenated to such, have been reported active (5, 11, 32).

If we proceed from the assumption that an aromatic ring is necessary, we must answer the question, What is it about such a ring that is necessary? Veldstra (37) and many others have taken the view that it is the lipophilic character of the ring that is important. Important as this character may be, it is of little help in explaining the enormous differences in activity obtained with the different substituted phenoxyacetic and benzoic acids. For example, phenoxyacetic acid is at best slightly active. The introduction of a fluorine atom in the 4 position increases the activity at least 200-fold. The fluorine atom would have very little effect on the lipophilic character and no steric hindrance; hence its effect must be electronic in nature. Again the differences between chlorine and methyl groups in size or in lipophilic character would be small, yet when these groups are compared in the 4 position of phenoxyacetic acid, the chlorine atom confers at least 100 times more activity. Another striking example results from the comparison of 3-methyl- and 3-trifluoromethylphenoxyacetic acids. Again, both groups have about the same size so that steric effects are ruled out. If anything, the trifluoromethyl group might make the molecule less lipophilic through inductive polarization of the ring, yet it is 100 times more active. Wain's group (7, 35), in a very careful analysis of the effect of substituents on phenoxyacetic acids, showed that substitution in both the 3 and 5 positions with either chlorine or methyl groups gave molecules which were inactive or of very low activity. That this was not due to a steric effect or lack of lipophilic

character was clearly shown by the fact that 2,3,4,5-tetrachlorophenoxyacetic acid was very active.

If simple lipophilic adsorption of the ring is not enough to explain its function, one might argue that adsorption of the ring through its *pi* electrons to give a weak *pi* complex would help rationalize the situation. Considering the nature of the phenoxy,

Table 1. Activity of phenoxyacetic acids compared to indole-3-acetic acid.*

Compound		Compound	
Derivative of phenoxyacetic acid	Relative Activity†	Derivative of phenoxyacetic acid	Relative Activity†
4-Fluoro-	6.00	3-Chloro-	2.00
4-Chloro-	5.00	3-Bromo-	2.50
4-Bromo-	1.50	3-Trifluoromethyl-	7.00
4-Methoxy-	0.03	2-Methoxy-	0.20
4-Methyl-	0.05	2-Chloro-	0.06
4-Hydrogen-	0.03	2-Bromo-	0.10
4-Iodo-	0.00	2-Iodo	0.10
4-Acetyl-	0.00	2-Methyl	0.20
3-Methoxy-	0.01	2-Ethyl-	0.00
3-Methyl-	0.07		

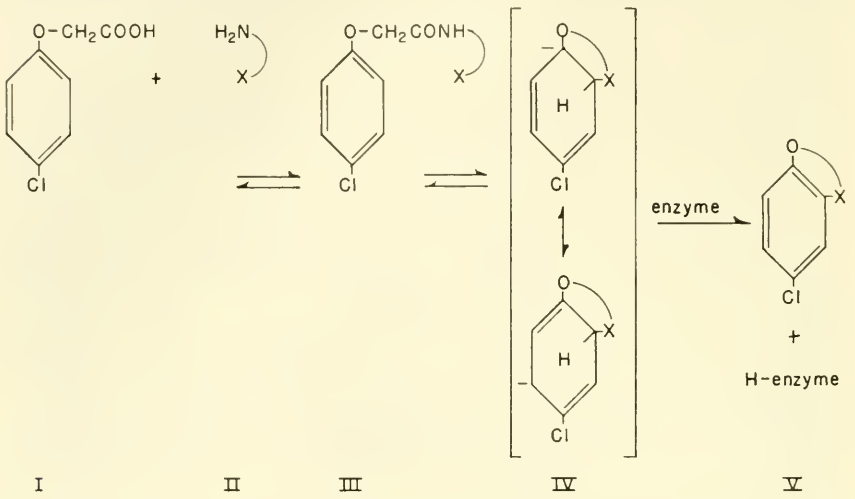
* All of the data reported in this table have been previously reported (26) except for 4-fluoro-, 4-acetyl-, and 3-trifluoromethylphenoxyacetic acids. 4-Hydrogenphenoxyacetic acid is simply phenoxyacetic acid.

† Indole-3-acetic acid = 100.

phenyl, naphthalene, and indole rings, adsorption to an electron-deficient site would seem more reasonable than the assumption of an electron-rich site. The relative activating effect of substituents in the 4-substituted phenoxyacetic acid as shown in Table 1 is exactly the opposite from what one might expect ($F > Cl > Br > CH_3O > I$) for such a mechanism. More difficult to rationalize in terms of a nonspecific *pi* complex is the great difference in effect of given substituents at various points on the ring. Thus, for example, 2,4-dichlorophenoxyacetic is very active, while the 3,5-dichloro-analogue is inert. As mentioned above, the difference in these two molecules cannot be rationalized as being due to steric factors (35). Many other such examples can easily be shown from data published on the phenoxyacetic acids.

If nonspecific adsorptions involving van der Waals forces or *pi* complex formation do not offer satisfactory solutions, adsorption or

reaction at a specific point seems to. Such a reaction could vary all the way from weak *pi* complex formation through *Sigma* complex to "normal" covalent bond formation at the particular ring carbon atom. For a working hypothesis we have visualized the following:



This scheme is drawn showing nucleophilic attack, and thus intermediate IV shows delocalization of a negative charge. Of course the same type of intermediate would result from electrophilic or radical attack and delocalization of a positive charge or single electron would be equally important for the stabilization of the intermediate. Intermediate III is shown as an amide, although most certainly simple salt formation could function to hold the auxin to the site for the much slower subsequent reaction, be it complex or covalent bond formation. Substance II is assumed to be protein in nature. A consideration of the available information has led us to believe that reaction must occur on one molecule (14).

There are three possible ways in which X in the above series of reactions could attack the aromatic ring: X+, X· or X:. In each case stabilization of an intermediate through delocalization of a positive charge or electrons will be very important in promoting the reaction. Such stabilization would not be possible in an isolated double bond, and therefore it is not surprising that such compounds have invariably been found to be inactive. In still another attempt to find an active aliphatic acid, we tested 4-pentynoic acid and found it to be inactive. In view of the relatively electron-rich character of most of the rings in the known auxins, one might expect that X+ would be the most likely group for two-point attachment. However, if this were true, then one would expect general activation by groups such as methyl and methoxy. That this is not so is readily seen from the

data in Table 1. Åberg (1, 2) in carefully controlled experiments has also observed that the substitution of hydrogen by methyl results in lowered activity in a variety of molecules. The deactivating effect of methyl and methoxy groups on phenylacetic acids is also clear in the data of Melnikov *et al.* (24). The fact that the activating ability of the halogens follows their increased inductive effect (—I effect) also points against attack by X+. Åberg (1) has observed the same order of activity for the 4-substituted halophenoxyacetic acids which we report in Table 1. He pointed out that the decrease in activity in going from F to I might be due to the increasing atomic radius of each substituent. This hypothesis would not explain the great difference in going from H to F in the 4 position, nor would this explain the difference between Cl or Br and CH_3, or CH_3 and CF_3. 3-Trifluoromethylphenoxyacetic acid is the most active biologically of all the monosubstituted phenoxyacetic acids which we have tested. Further evidence against attack by X+ has been found by Fukui *et al.* (10) for the benzoic acids. They have shown that there seems to be no correlation between *pi* electron distribution on the benzene ring and attack by an electrophilic reagent.

It should be noted that both in the phenylthioglycolic acids (17) and in the phenylacetic acids (24) the order for activity on substitution into the 4 position is $Cl > Br > CH_3 > I$, with the latter two substituents giving compounds of very low activity. The only groups which consistently increase activity in rings other than the benzene ring in the benzoic acids are F, Br, and Cl. (It seems very likely that CF_3 will be added to this group when more such derivatives are studied.) The nitro group sometimes gives weakly active compounds, but amino and hydroxyl groups invariably give low activity. This has usually been attributed to the fact that polar groups would greatly reduce the lipophilic character of the ring (37).

Since attack by electrophilic reagents seems unlikely, we have advocated attack by an electron-rich group. Of the two possibilities X: seems more likely than X·. In a recent critical review (3) of the nature of free radical attack on aromatic nuclei, Augood and Williams point out that almost all substitution on benzene results in an increased rate of attack by radicals at points *ortho* and *para* to the substituent. The only groups which do not have total rate factors greater than 1 are isopropyl, *tert*-butyl and trifluoromethyl. The latter has a value of 0.99, which means essentially no change in over-all reaction from benzene itself. Methyl and methoxyl groups are quite strongly activating. Again this is at odds with the biological activating effects of these groups in phenoxyacetic and phenylacetic acids. More interesting is the fact that the order for activation by halogen on benzene for radical attack is $I > Br > Cl > F$ (3). This of course is just opposite to the order found for the biological activating effect of the substi-

tuents in the auxins. In radical attack substituents have only slight effect on positions *meta* to them. In the unsubstituted acids one would expect phenoxyacetic acid to be more susceptible to radical attack than phenylacetic acid and therefore to be more active as an auxin if radical attack were involved. Again, just the reverse is found. Molecular orbital calculations (10) have also indicated that radical attack seems less likely for the benzoic acids than attack by an electron pair.

If radical attack and electrophilic attack on the ring do not seem to offer an explanation for the effect of substituents on auxin rings, what evidence is there to support nucleophilic attack? Our early work (14) with the phenylacetic, indoleacetic, and phenoxyacetic acids indicated that whenever both *ortho* positions were substituted with halogen, alkyl, or alkoxy groups, inactive molecules always resulted. This, plus the relative activating effect of substituents, led us to focus attention on reaction at an *ortho* position by an electron-rich reagent. This view was modified (27) when it was discovered that α-2,6-dichlorophenoxypropionic acids were active (28). It seems most likely that a *para* position must be involved in this reaction since introduction of a group in the *para* position always destroys the activity of α-2,6-dichlorophenoxypropionic acids in the *Avena* test. Some of the 2,4,6-trisubstituted phenoxyacetic acids show weak activity in the slit pea test. The phenylacetic acids seem to follow the same pattern (30), the 2,6-disubstituted acids being active and the 2,4,6-trisubstituted ones being inactive. A very interesting and significant difference is apparent in the requirements for activity with these two structures. 2,6-Dichlorophenylacetic acid is quite active, while 2,6-dichlorophenoxyacetic acid is at best very weakly active. If one assumes two-point reaction by means of the carboxyl and the 4 position of these molecules with a single substrate molecule, a logical explanation is possible. In the phenylacetic acids the two hydrogens on the methylene group interfere with the *ortho* halogens, locking the methylene group so that the carboxyl group may be held rather rigidly over the ring near the 4 position. In the phenoxyacetic acids, the relatively small oxygen atom holds the methylene group out far enough so that interference between the methylene hydrogens and the two *ortho* halogens is slight, and the whole side chain has freer motion. Introduction of the α-methyl group thus increases the biological activity because hydrogens on the methyl group would help lock the side chain so that the carboxyl group would be more rigidly held for two-point contact involving the 4 position. All of the data at hand, therefore, would indicate that a specific atom in the ring is involved in the two-point reaction.

If we assume then that normally an *ortho* position in the phenoxy-

acetic acids is the preferred point for 2-point reaction, a study of the substituents in the 3 and 4 positions should throw some light on the nature of this reaction. Substitution in the 2 position will be more difficult to evaluate because of steric interaction with the side chain. As mentioned earlier, there are three important variables connected with each substituent: steric, electronic, and H/L factor. The latter factor has two components which one must consider. The relative hydrophilic-lipophilic character of the molecule must be such that rapid penetration to the reaction site is possible. A second point which may or may not be of importance is the possibility that reaction may take place on a solid surface and adsorption might be governed by small H/L differences. To try to hold other variables constant so that the electronic factor could be evaluated, we chose to look at substitution in the 3 and 4 positions, and to consider groups relatively inert chemically which would have closely related H/L factors. In addition to the above points it must be borne in mind that if a substitution reaction occurred, it could be promoted by enzymatic stretching of a carbon-hydrogen bond.

A consideration of the data in Table 1 indicates that in general those groups which are placed so that their electronic effect is to reduce the electron density at the *ortho* positions, give an increase in activity over the unsubstituted phenoxyacetic acid. It is interesting to note that the activity of the 4-substituted acids falls off as the —I effect of the substituent on the *ortho* position decreases. The activity we have found for the 4-halo and 4-methylphenoxyacetic acids is essentially that reported by Åberg (1) except that he finds the 4-methyl substituent to be deactivating. Comparison of the 4-chloro and 4-methyl groups is particularly instructive since both groups have about the same size and effect on the H/L factor. The chlorine derivative is about 100 times more active. The fact that iodine in the 4 position gives an inactive molecule is significant and would lend support to Åberg's observation that the size of the group in the 4 position is particularly important. We have noted that 4-iodophenylacetic acid is inactive, and Kato has observed (17) that an iodine in the 4 position of phenylthioglycolic acid essentially destroys the activity of this molecule. Åberg has also pointed out that large alkyl groups in the 4 position also destroy activity. That 4-methoxy substitution gives an active molecule, although it is a bulky group, is probably due to the nonlinear carbon-oxygen-carbon bonding which permits the methyl group to assume a skewed position somewhat above the plane of the ring.

From Table 1 it is evident that 2 substitution has little effect. The introduction of halogen or methyl groups results in a slight increase in activity. No obvious correlation between activity and the three factors — electronic, steric, or H/L — appears. One might ex-

pect 2 substitution to cause some steric hindrance of resonance between the electrons of the ether group and those of the ring, with the result that the —I effect of the oxygen would be more important and activation should result. Although 2 substitution almost always results in an increase in activity, the effect is more apparent in the di- and tri-halo derivatives than with the mono. A very important consequence of the two-point attachment hypothesis, especially with the *phenoxyacetic* acids, is the nature of the spacial arrangement of the side chain with respect to the ring at the time of consummation of reaction at the second point (ring position). If the side chain is held so that electrons from the oxygen can effectively overlap with those of the aromatic ring, the electron density on the ring would be very much greater than if oxygen were twisted so as to prevent this overlap. It would seem most reasonable to assume that at the time of reaction at the second point, the side chain would usually be twisted so that only weak overlap could occur. The great sensitivity of the 2 position to steric factors is indicated by the fact that activity drops to zero in going from methyl or iodine to ethyl. When ethyl is in the 2 position, even introducing a chlorine atom in the 4 position does not restore activity. 2-Ethyl-4-chlorophenoxyacetic acid is completely inert.

A consideration of structure and activity in the phenoxyacetic acids is not complete without some consideration of the disubstituted derivatives. All of the 2,4-halo derivatives with the exception of iodine are quite active (35). The dramatic lack of activity of 3,5-dichlorophenoxyacetic acid was first shown by Leaper and Bishop (20) and then confirmed by others to hold for the 3,5-dimethyl derivative as well (26, 35). That this effect is not due to either steric or H/L factors is clearly shown by the discovery that 2,3,4,5-tetrachlorophenoxyacetic acid is quite active. The inactivity of the pentachloro derivative again points up the importance of an open *ortho* position (35). Both of these inactive 3,5-substituted molecules have groups which would relay electrons to the *ortho* positions via +M effects. Moreover, since there is no group in either *ortho* position, the oxygen atom of the ether linkage is able to exert maximum effect on the *ortho* position through +M action. The combined effect of three groups directing electrons to the *ortho* and *para* positions makes attack at these positions by an electron-rich reagent much less likely. Introduction of a chlorine atom in the 2 position should help lower the electron density at the *ortho* positions by providing a —I effect and some steric hindrance so that conjugation of the ether oxygen with the ring is not as effective. This is borne out by the fact that 2,3,5-trichlorophenoxyacetic acid is a moderately strong compound (35). Adding another chlorine atom with its —I effect to the 4 posi-

tion gives the biologically quite active tetrachlorophenoxyacetic acid. Fawcett *et al.* (7) have also shown that 2,3,5-trimethylphenoxyacetic acid has very slight activity. Here again, 2-substitution would inhibit the +M effect of the oxygen and enhance its —I effect on the *ortho* position. We have found that 3,5-dimethylphenylacetic acid, although less active than phenylacetic acid, is still very definitely active. Although the methyl groups would affect the *ortho* positions by hyperconjugation, the +M effect of the methylene group would be very weak compared to the oxygen atom of the phenoxyacetic acid series. A check on the above hypothesis can be made by testing the activity of phenoxyacetic acid substituted in both *meta* positions with trifluoromethyl groups. On the basis of information now available such a molecule would be expected to be highly active.

If a reaction does indeed occur at an *ortho* position or under special conditions at another ring position, one would expect that stabilization of the charged intermediates, such as IV shown on page 434, would be very important in determining the relative activities of closely related compounds. Differences in the activities of closely related compounds can, in certain instances, be explained in terms of one molecule forming an intermediate more ably than a closely related isomer to delocalize an electron pair. It has long been known that for chemical attack, the α position on naphthalene is much more reactive than the β position (16), be the attack by an electron-rich or electrophilic reagent (40). The well-known fact that 2-naphthoxyacetic acid is much more reactive than 1-naphthoxyacetic acid can be explained by assuming better resonance stabilization of the intermediate with the former than with the latter. With 2-naphthoxyacetic acid, reaction could occur at the 1 position to give an intermediate stabilized by seven relatively stable resonance structures, four of which would not disturb the benzenoid resonance of the nonreacting ring. In 1-naphthoxyacetic acid, reaction would occur at the 2 position, the intermediate of which would be stabilized by only six relatively stable structures, in only two of which would the benzenoid resonance in the nonreacting ring be preserved. The fact that reaction might occur at an α position by reaction at the 8 position in the case of 1-naphthoxyacetic acid must be considered, however. That the 8 position is much less favorably situated for reaction with an $-OCH_2COOH$ side chain is evident from the work of Toothill *et al.* (35). These workers have amply demonstrated that the introduction of another atom in the oxyacetic acid side chain destroys activity. Such would be the situation if two-point reaction in 1-naphthoxyacetic acid were required to occur by means of an 8 position. The unsuitability of the 8 position sterically is nicely illustrated by the inactivity of 2,4-dichloro-1-naphthoxyacetic acid (35, 36). An even

better illustration of the greater reactivity for two-point attachment of the α position in naphthalene over that of the β comes from the work of Luckwill and Woodcock (21). These workers confirmed the earlier report (15) that 1,3-dichloro-2-naphthoxyacetic acid is completely inactive, as would be expected. The superiority of the α position over the β position for two-point attachment was shown by comparing 1-chloro-2-naphthoxyacetic and 3-chloro-2-naphthoxyacetic acids. The latter isomer, where α attachment is possible, is highly active, while the former compound, where β attachment is demanded, is almost inert. The interesting observation that introduction of chlorine in the 8 position of 1-naphthaleneacetic acid greatly lowers the activity of this molecule (15), can also be interpreted in terms of resonance stabilization of a reaction intermediate. Thus 8 substitution blocks α reaction and forces reaction at the less active β (2) position. One would expect the 8 position in 1-naphthaleneacetic acid to be more favorably placed for reaction than the 2 position. The reasoning behind this is that if reaction occurs at the 8 position, one can consider the structure to be related to cinnamic acid, while if reaction occurs at the 2 position, one must consider the reaction with respect to the side chain to be more like that of phenylacetic acid. *Cis*-cinnamic acid has been shown to be at least five times as reactive as phenylacetic acid in the pea test (19). Similar reasoning can be used to rationalize the difference in activity between 1- and 2-naphthaleneacetic acids. Also the fact that partial hydrogenation of the naphthalene ring leads to lower activity (36) may be interpreted as decreasing the ability of the molecule to stabilize an intermediate through charge delocalization. The observation (31) that indole-2-acetic acid is much less active than the isomeric indole-3-acetic acid again lends support to the above hypothesis. Reaction at the 3 position would not permit as effective charge delocalization as would reaction at the 2 position. It is noteworthy that delocalization of a negative charge with reaction at the 3 position would be even less favorable than delocalization of a positive charge. That the 2 position in the indole series is more favorable for reaction than the 4, is indicated by the fact that 2-methylindole-3-acetic acid is less active than IAA, while 4-chloroindole-3-acetic acid is more active than IAA (26). Again such reasoning can be used to rationalize the difference in activity found with 2-thianaphtheneacetic and 3-thianaphtheneacetic acids (18).

Although the overwhelming preponderance of evidence supports our two-point attachment hypothesis, two important molecules which do not fit neatly into place are 2,4-dichloro-6-fluorophenoxyacetic acid and 3,5-dichloro-2-pyridoxyacetic acid (37). In the latter mole-

cule, one would have to postulate either displacement of a chlorine or reaction at a *meta* position. The ring nitrogen atom would activate the substance for attack by an electron-rich reagent at the *meta* position. Although it has not been shown in other systems that this is sterically favorable, this seems more reasonable than the displacement of chlorine. Although it has been postulated that in the 6-fluoro compound fluorine might be displaced, evidence is still lacking to support this point.

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DISCUSSION

Dr. Bonner: I think that everybody agrees that for a molecule to be active as an auxin, it has to have some appropriate geometry, presumably because it has to fit into particular kinds of holes, and it has to have two particular reactive groups. It seems clear to me that the reactivity of the aromatic nucleus has an important function in determining the activity of an auxin. Dr. Hansch referred to the strange case of the 2,6-dichlorophenoxyacetic acid and 2,6-dichloropropionic acid. At low concentrations these compounds behave as auxin antagonists, and at higher concentrations they are active and behave as auxins, but their activity has some very strange characteristics.

Dr. Osborne: If 2,6-dichlorophenoxyacetic acid is tested in the *Avena* straight growth test, one finds that even with relatively low concentrations there is an initial but small stimulation of growth lasting for a few hours followed by a retardation in growth. I am not sure how one would explain this on a 2-point attachment theory.

Dr. Bonner: The 2,6-dichlorophenoxyacetic and propionic acids are active, but their activity has the interesting feature that this activity is manifested for a very short time in contrast to 2,4-D or other auxins. It is a "short-term" auxin; it goes in and does something for only a short time.

Dr. Osborne: This appears true for 2,6-dichlorophenoxyacetic acid, but if one does similar experiments with the propionic derivative, the growth stimulation can be continuous for a period up to 24 hrs. One does not find the subsequent retardation of growth. The stimulation with this 2,6-substituted phenoxy compound is apparently there all the time.

Dr. Wain: In relation to the 2-point contact theory which we have been hearing so much about, the carboxyl group probably does react chemically in the manner you have suggested. The question is, what is happening in this second contact which you have postulated. It might be reacting with some essential or unessential thiol group in

a protein. The 2-point contact theory does not explain the specificity of stereoisomers. We have shown that for activity, the molecule should, in general, have at least one hydrogen attached to the carbon adjacent to the carboxyl group. This hydrogen may well be involved in the growth reaction. If you accept this point, then you are able not only to explain the specificity of stereoisomers but, as Dr. Wightman has shown, you can put competitive antagonism on a logical basis.

Dr. Hansch: I feel perfectly easy with respect to the 2-point attachment theory and stereoisomerism in the side chain. There are a lot of things that bother me much more than that. There are so many cases in organic and biochemistry where two asymmetric centers react. You can get an enormous difference in reactivity between D and L forms. This difference that you mentioned is very important. It is one of the things that reinforces our ideas about the fact that the carboxyl group does react to form an amide, covalent bond. If it were only an ionic bond, then both stereoisomers could react at the same point with little interference. When there are two optically-active centers forming a chemical bond, you get into such differences of activity with stereoisomers.

Dr. Thimann: With a colleague and student, Mr. William Porter, we have spent a great deal of time in reviewing the whole problem of structure and activity again, and I would like to make a new suggestion. It could not be dignified by the name of a fully developed theory yet. First let me say this owes a great deal to various earlier theories; I need not remind you that in the last 20 years a large number of ideas have been proposed, such as that the side chain is at an angle to the ring, that a free hydrogen is needed in the side chain, the concept of an essential distance between the carboxyl and the ring, the concept of lipophilia, and the more recent argument about reactivity at the *ortho* position. Of course, the idea of studying structure and activity is to get some clue as to what the receptor is like, and I will say nothing about the receptor except to remind you of the suggestion I made some time ago (Amer. Naturalist 90: 145. 1956), that the receptor may be a family of closely related bodies; even within one plant there would be a group of slightly different receptors — an idea which makes it easily possible to explain some of the phenomena of synergism.

Now, if we simply review the accumulated data, what can we deduce? Let me start with indoleacetic acid and its family of similar isosteric substances, in which instead of nitrogen there occur methylene, oxygen, or sulfur in the same position. It is appropriate to start with these because the compound with a carbon atom in place of ni-

trogen indene-3-acetic acid happens to be the first analogue of indoleacetic acid to have been studied. If we look at these four substances, the molecules are the same in general shape. Originally it was thought that they are all active because they all have the same shape. It is now clear that molecular shape is not nearly as simple a criterion as it sounds, for there are compounds like 2,3,6-trichlorobenzoic acid which are highly active, and of greatly differing shape from that of IAA. So we have to ask, What do these molecules have in common other than their shape? One answer is that there is in all four of them a strong fractional positive charge on the atom at the bottom of the 5-membered ring. In the case of nitrogen there is a lone pair of electrons which is drawn into the ring, leaving the nitrogen positively charged; oxygen and sulfur have similar lone pairs that are drawn in and are left positive. Furthermore, there is good evidence that cyclopentadiene and indene have exactly the same property on this methylene atom as the other cycles have on the hetero atom. The cyclopentadiene or indene ring is sensitive to acids; the hydrogen is replaceable by potassium and it combines with NO. Furthermore, the dipole moments of all these rings indicate a marked fractional positive charge. The first thing that is common to these four molecules, then, is the fractional positive charge in a characteristic position.

Now, let us look at the other end of the molecule. What is characteristic of all auxins is the carboxyl group, which can ionize, so that here we have in a characteristic position a potential negative charge. Now, of course, the idea is current that the carboxyl reacts chemically to form an amide type linkage or an acyl group of some sort, but I feel that the evidence points against that. Perhaps the strongest reason for thinking so is, as Dr. van Overbeek has pointed out, Veldstra's work with the tetrazoles. It is almost impossible to see how a tetrazole could form any kind of an acyl type linkage, and yet the one thing that the tetrazole does do is to dissociate an H^+ and thus produce a charge at this point. I suggest that there may be no true chemical reaction in the sense of covalent bonds, and that we have to deal rather with the approach to a receptor, based upon the electronic configuration of the molecule. The distance between these two charges, of course, varies — there is free rotation — but it centers around 5½ angstroms. Dr. Wain has presented evidence that long side chains are reactive only after *beta* oxidation. An exception is made in the case of propionic side chains which are apparently active in themselves. Correspondingly, in *p*-chlorophenoxypropionic acid, for instance, the distance between the positive charge at the *ortho* position and the carboxyl is not much greater than the desired order of magnitude. In *p*-chlorophenoxyacetic acid it is just right.

Suppose we introduce a second chlorine atom at one of the *ortho* positions. It will intensify this charge, and so we get one of our most active auxins. However, if chlorine is present at both *ortho* positions, we have 2,4,6-trichlorophenoxyacetic acid in which the positive site is occupied and the compound is, as might be predicted, inactive.

Recently we have conducted a study of the phenylacetic acids, which we obtained through the courtesy of Drs. Steward and Schantz at Cornell, who got them in turn from Dr. Brian of Imperial Chemical Industries in England. I will not discuss them in detail, because Dr. Wain has already mentioned something of their activity, but will make one point, namely that in phenylacetic acid, the side chain is shortened by 1 atom, so that in order to get the most favorable distance to the positive charge, we want it at the *meta* (instead of the *ortho)* position. It is interesting that in the phenylacetic acid series, the activities of differently substituted compounds do not vary as widely as they do in the benzoic acid series or in the phenoxyacetic acid series, but the activities are more nearly constant, which suggests that while the *meta* is the most favored position, still the compound, through its free rotation, is able to react at the *ortho* or the *para* position. Tests with the tri-substituted phenylacetic acids show this very nicely. Substitution in the 2,4,5 positions should force the compound to react at the 3 position, and this turns out to be the most active. Substitution at 3,4,5 forces reaction at the 2 or 6 positions, and this compound is less active. The same is true when the compound is forced to react at the *para* position.

The effect of methyl substituents was mentioned by Dr. Muir. Methyl substituents do enhance activity. Every English farmer who uses methoxone to kill his weeds knows very well that this is the case; 2-methyl-4-chlorophenoxyacetic acid is an extremely active substance. Now since methyl activates the ring, while chlorine deactivates it, one wonders how two oppositely effective groups can have essentially the same effect. The concept of the spatial distribution of charges explains that perfectly because both are *ortho-para* directive. That means that, although they do it to different degrees, they essentially act in the same general direction. In a series of substituted phenylglycines, which were reported by Takeda a few years ago, it was very characteristic that *only* the chloro and the methyl derivatives were appreciably active.

There are two or three outstanding special compounds; one is 2,6-dichlorobenzoic acid. This compound is blocked in the *ortho* position; therefore according to Muir and Hansch it should not be active, but of course in benzoic acids the side chain is shortened, and as Dr. Leopold and I pointed out in 1955 (The Hormones, Vol. III,

ed. G. Pincus and K. V. Thimann. Academic Press, New York. p. 13. 1955) one would not expect the approach to take place in the *ortho* position, but further down the molecule, so if a chlorine is present at the 2 position, positivity accumulates at the 4, and if a second one is present at the 6 position, the effect is reinforced. So 2,6-dichlorobenzoic acid would be expected to be very markedly active; 2,3,6- is also active and, as was pointed out a little earlier, 2,5- is active. 2,6-Dichlorophenoxyacetic acid is, of course, the comparable opposite case, for the positive charge is at the 4 position, which is rather far for the group to operate, and correspondingly it is only very weakly active (though it has real activity). Another rather striking fact, brought out by the French biochemist Julia, is that when there are two carboxyl groups in the molecule, activity disappears. Thus, 4-chloro-2-carboxymethylphenoxyacetic acid is like methoxone, 2-methyl-4-chlorophenoxyacetic acid, except that it has a second carboxyl. One might expect it to partake to some extent of the activity of its parent compound, but it does not at all; its activity is reported to be zero. Presumably an additional negative charge at the wrong point would prevent activity.

And lastly, the vexing question of the 3,5-disubstituted compounds. You remember that in the phenoxyacetic acids the optimum site for the positive charge at 5½ angstrom units from the negative is close to position 6. A chlorine atom in the 3 or 5 position has the opposite effect, conferring positivity only on the 1 position where it is impossible for it to react. So one could deduce that 3,5-dichlorination is doing nothing to increase the reactivity of the molecule. Correspondingly we find that it is totally inactive, like the parent compound. This is also true in the case of benzoic acid where again the 3,5-disubstituted molecule is just as inactive as is the parent benzoic acid. In the case of phenylacetic acid, however, the 3,5-disubstituted derivative has some activity, and again it is about the activity of the parent molecule, which in the case of phenylacetic acid is quite appreciable. Thus a number of the observed phenomena can be explained. Now if this view is correct, the impression it gives us of the site of reactivity of auxin is something like that outlined by Dr. Freed, that the auxin approaches a surface in which charges are placed in characteristic positions and the relatively flat benzene ring is borne down with van der Waals forces to rest upon the surface. There is a similarity here to the discussions about the mode of action of chymotrypsin.

Dr. Crosby: For some time, we've been arguing with Prof. Hansch and others about the importance of the *ortho* position in simple cases, such as *p*-chlorophenoxyacetic acid. We decided that perhaps

we might be able to help finish this argument by making a *p*-chlorophenoxyacetic acid which didn't have the usual type of *ortho* positions. We did this by putting nitrogens adjacent to the side chain. Considering that our bioassays were carried on in acid solution, we could conclude that these nitrogens should be positively charged to some degree. Consequently, they would have the tetrahedral configuration as do the carbon atoms, and so we would have, in effect, a *p*-chlorophenoxyacetic ester which had no *ortho* positions open to electrophyllic attack. We bioassayed this compound in four different tests and found that it was not only completely inactive as a stimulant of growth, but that it was a competitive inhibitor of *p*-chlorophenoxyacetic acid and ester.

Dr. Bonner: Are these systems pH sensitive?

Dr. Crosby: We carried these out actually only at two pH values, 4.5 and 6.0.

Dr. Åberg: I should like to raise a question which turned out to be a useful touchstone for various hypotheses on the relation between structure and activity of the auxins, at the conferences in Lund and at Wye. How does the present hypothesis explain the fairly strong activity of *d* (+)-*α*-phenoxypropionic acid as contrasted to the very weak activity of phenoxyacetic acid?

Dr. Thimann: Dr. Åberg raised a very interesting question. I am not prepared to explain it all, but this applies not only to phenoxyisopropionic acids. Frequently, alkyl substitution increases or modifies activity. I think a complete explanation would depend on the availablity of data on the effect of this on the charges of the atom. As you know, in the case of indole, two alkyl groups do not destroy activity, although in the case of phenoxy they do. I think all these points depend on a much more careful evaluation of the properties of the compound than I am now prepared to make.

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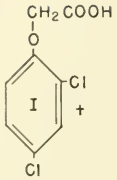
New Theory on the Primary Mode of Auxin Action

The search for the primary action of auxin has so far only given negative results. It appears unlikely that in the primary auxin reaction the molecule undergoes covalent bonding, and it appears also unlikely that the primary auxin reaction involves any one specific enzyme system.

Previously it had been assumed that auxin combines with some entity, perhaps a protein, both through an *ortho* position on the ring, and through the carboxyl group. This idea was developed with the phenoxyacetic acids in mind. The highly active ones, such as 2,4-D (Figure 1, I) all have at least one unsubstituted *ortho* position. When both *ortho* positions are substituted (II) activity is lost. However, at present we realize that this is not generally true. The di-*ortho* substituted phenylacetic acid (VI) is highly active, and so is 2,6-dichlorobenzoic acid (IV). On the other hand, 2,4-dichlorobenzoic acid (III) is inactive, while 2,4-dichlorophenylacetic acid (V) is rather weakly active (3, 4, 5). Even if one would suppose that the halogenated *ortho* position were reactive, this is made entirely unlikely by the fact that 2,6 methyl substituted acids (VII, VIII) show considerable activity (7). The chlorine atom is electro-negative, and the methyl group is electro-positive. For this reason, covalent bonding at the *ortho* position of these halogenated or methylated benzoic acids (IV, VII, VIII) would appear impossible.

As far as the carboxyl group is concerned, Veldstra *et al.* (7, 9, 10) have shown that this could be replaced by a number of other acidic groupings including tetrazole (X, XII). Therefore, it also seems impossible that the carboxyl group could be involved in covalent bonding in the auxin reaction. We are, therefore, forced to conclude that the



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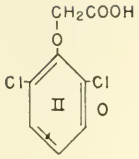
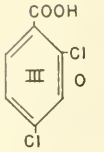


2,6-Dichloro-phenoxyacetic acid



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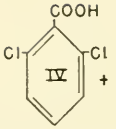
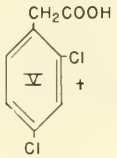
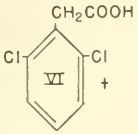


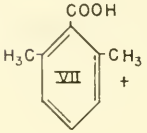
2,6-Dichloro-benzoic acid



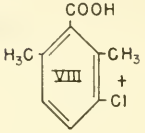
2,4-Dichloro-phenylacetic acid



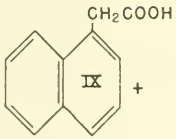
2,6-Dichloro-phenylacetic acid



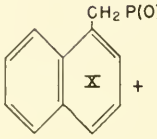
2,6-Dimethyl-benzoic acid



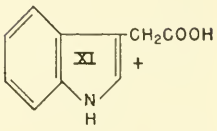
3-Chloro-2,6-Dimethyl-benzoic acid



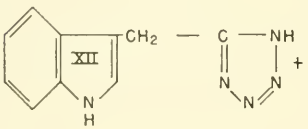
1-Naphthaleneacetic acid



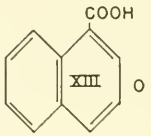
1-Naphthylmethyl-phosphonous acid



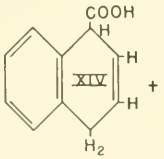
Indole-3-acetic acid



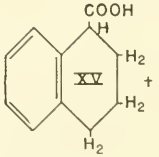
5-(3 Indolemethyl)tetrazole



1-Naphthoic acid



1,4-Dihydro-1-nophthoic acid



1,2,3,4-Tetrahydro-1-nophthoic acid

Fig. 1. Structural formulae and activity of auxins and related compounds.

mode of action of auxin must be of a physico-chemical rather than of a chemical nature.

All attempts to activate an enzyme system in vitro with auxin have failed, as far as is known. However, the activity of many enzyme systems is affected after a plant has been treated with auxin. This would lead one to conclude that the primary auxin reaction does not involve a single key enzyme, but that the auxin acts on a number of enzyme systems simultaneously — perhaps via the cytoskeleton, a membrane system upon which or in which enzymes are located (6). If one accepts this view, one will see that the auxin molecule is not a direct participant in the enzyme reactions affected by auxins. One might visualize this by imagining that sorption of an auxin molecule in the lipoprotein membrane of the cytoskeleton may lead, for instance, to a local change in the hydration of the membrane. Such a hydration, in turn, would change the relative distance between the enzyme components on or in the membrane. This would change the relative reaction rates between these enzymes, which ultimately would lead to a changed ratio of metabolites. Such a changed ratio of metabolites is the basis for a changed physiological pattern, as the researches of Skoog *et al.* have shown.

Let us assume that the auxin molecule moves with its ring into a cavity of the membrane of the cytoskeleton, and that the polar side-chain sticks out. This cavity, of course, is a temporary opening created by the thermal agitation of the molecules of the membrane. The penetration of the ring into it is simply an aspect of the phenomenon of solubilization in surface chemistry.

Our next problem is to imagine what the polar side-chain, sticking out of the surface, could accomplish. It has become known that on the surface of polymers, hydrogen bond systems can occur. Under some conditions these hydrogen bonds become coordinated in forming an oscillating system (2). This strengthens the H-bonding capacity of the system. Let us now postulate that the polar group of the auxin molecule becomes part of an H-bond system at the surface of the cytoskeleton, and that this polar group provides the missing link in the system and sets it to oscillating. Providing this missing link and making the H-bond network oscillate would then be the primary auxin function.

It is not hard to see that such a strengthening of the H-bond system would affect the structure of the membrane. It might contract it or, via hydration, expand it.

According to this picture then, the polar group of the auxin is the crucial part. In order for it to activate the H-bond network, this polar group must be placed just right to function as the missing link.

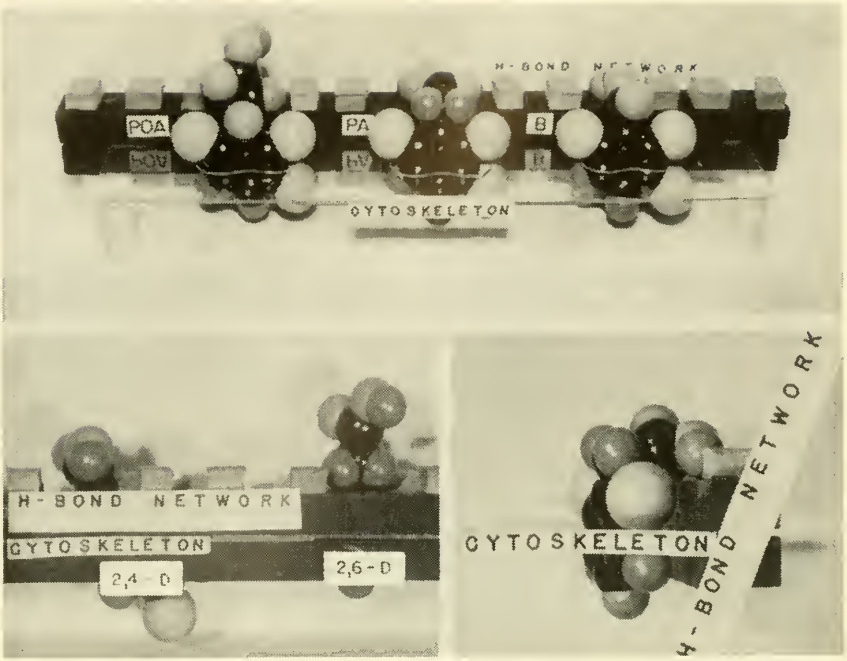


Fig. 2. **Upper:** Comparison between models of molecules of 2,3,6-trichloro substituted phenoxyacetic (POA), phenylacetic (PA), and benzoic (B) acids as they are envisioned to fit into the cytoskeleton and its accompanying layer of H-bonds. Because of the two bulky chlorine atoms on both *ortho* positions, the rings have penetrated only relatively shallowly. The chlorine in the 3 position (below the plastic) helps in anchoring the ring. This shallow penetration locates the carboxyl groups of PA and B just right for becoming part of an oscillating H-bond system at the surface of the cytoskeleton, indicated by the square pieces of foam rubber. PA and B are therefore active. The side-chain of POA is too long for such shallow penetration in the cytoskeleton; it protrudes too far and its carboxyl group is out of reach of the H-bond network. POA is therefore inactive. **Lower left:** Rear view of the cytoskeleton model showing 2,4-dichlorophenoxyacetic acid and 2,6-dichlorophenoxyacetic acid. 2,4-D has a slender ring configuration and passes deeper into the cytoskeleton than the bulky 2,6-D. The carboxyl group of 2,4-D makes contact with the H-bond network, while that of 2,6-D extends ineffectively above it. **Lower right:** Side view of model showing 2,6-dichlorophenylacetic acid. While the ring is anchored in the cytoskeleton, the side-chain sticks out laterally and its carboxyl group becomes part of the H-bond network.

It is the function of the auxin ring to anchor the molecule in the cytoskeleton and thereby hold the polar group in place. By means of a model (Figure 2) we will see how this explains many questions of auxin physiology that heretofore have remained unanswered.

(1) Before the auxin molecule can fall in place (solubilize) in the cytoskeleton, it must first arrive there. Since the membrane of the cytoskeleton is probably a lipoprotein, the auxins must have a partition coefficient favorable for partitioning into fats. It is well known

that all the highly active auxins do have such a partition coefficient (8). Ester and nitrile forms of auxins are more fat-soluble than the acid forms and subsequently they often have a higher auxin activity. Chlorination also improves fat solubility of molecules and this is undoubtedly one of the reasons (although a minor one) for the high activity of chlorinated auxins.

(2) Once the auxin molecule has arrived at the site of action, its ring must sink into the membrane of the cytoskeleton to such a depth that the polar group of the auxin molecule fits into the system of H-bonds at the membrane surface. If it sinks in too deeply or not far enough, the proper contact between the polar group of the auxin molecule and the H-bond network is not made (Figure 2). These considerations make it obvious that bulky groups on both *ortho* positions broaden the molecule to such an extent that the ring will not sink deeply into the cytoskeleton; thus with the phenoxyacetic acids the side-chain is relatively long, and in molecules with bulky groups on both *ortho* positions, the polar group will stick out above the H-bond network (Figure 2). This, then, is the reason for the inactivity of the 2,6-dichlorophenoxyacetic acid (II). On the other hand, the compounds with relatively short side-chains such as benzoic and phenylacetic acids benefit by the presence of two bulky *ortho* groups (1), as it locates the polar group exactly in the right position relative to the H-bond network. It is obvious that methyl groups in the *ortho* positions would be just as effective as chlorine atoms in preventing the molecule from sinking into the membrane too deeply.

(3) In addition to a vertical positioning of the polar groups, there is also a lateral positioning. It seems obvious that the H-bond network cannot block the hole into which the ring must slide, so it must be located to the side of it. This explains the well-known requirement for auxin activity that the side-chain must be perpendicular to the plane of the ring. It explains why 2,6-dichlorophenylacetic acid is a stronger auxin than 2,6-dichlorobenzoic acid. The phenylacetic side-chain sticks out farther laterally than the short carboxyl group of the benzoic acid.

(4) Since the polar group has to be held in place, it requires secure anchoring of the molecule into the cytoskeleton. This is a function of the ring which is held by van der Waals bonding to the cytoskeleton. Heavy atoms, such as chlorine, especially on the 3, 4, and 5 positions of the benzene ring, help this anchoring process materially as the strength of the van der Waals forces is a function of the atomic weight. This explains the high activity of 2,4-D and 2,4,5-T; further, why 4-chlorophenoxyacetic acid is highly active, while 4-methylphenoxyacetic acid is poorly active (3).

(5) Since the polar group of the auxin molecule has to become part of an oscillating H-bond system, this requires it to be undissociated and isolated electronically from the rest of the molecule. If a polar group such as carboxyl is in resonance with the benzene ring, electrons are withdrawn from the group and it becomes more highly dissociated. The insulation of the polar group is achieved in the phenyl and phenoxyacetic acids by the carbon atom between the carboxyl group and the rest of the molecule. The insulation of the polar group in the benzoic acids is achieved by forcing the carboxyl group out of the plane of the ring by the two bulky *ortho* substituents and thereby minimizing resonance interaction between the carboxyl group and the benzene ring. This simple reasoning also explains the interesting behavior of the naphthoic acids (1). 1-Naphthoic acid is like an unsubstituted benzoic acid. Its carboxyl group is in resonance with the ring; therefore, the acid is relatively strong and poorly fit for H-bonding. 1-Naphthoic acid, therefore, is poorly active as an auxin. Auxin activity is vastly increased by the simple expediency of saturating the bond next to the carboxyl group (XIV, XV). A tetrahedral structure is thereby achieved, whereby the carboxyl group is forced out of the plane of the ring, and thus removed from resonance interaction with the double bonds in the ring. It becomes a weaker acid and thus becomes more suitable for participation in H-bonding. In addition, of course, this lateral movement of the carboxyl group places it in a more favorable position to participate in the oscillating H-bond network (see number 3 above).

Let us examine an auxin molecule and try to explain, with the aid of Figure 2, why the molecule is active. Take 2,3,6-trichlorobenzoic acid. It is active because:

(1) Its chlorine atoms make it partition more into the fat of the membrane of the cytoskeleton than the unsubstituted benzoic acid;

(2) The chlorine atoms in the 2 and 6 positions force the carboxyl group out of the plane of the ring. This insulates the carboxyl electronically from the ring system and, in addition, places the carboxyl group in the lateral position needed for becoming a partner in the H-bond network;

(3) The chlorine atoms in the 2 and 6 positions prevent the ring from sinking into the cytoskeleton too far;

(4) The chlorine atom in the 3rd position helps in anchoring the ring more firmly by van der Waals forces. This makes the 2,3,6-substituted benzoic acid a more active auxin than the 2,6-substituted benzoic acid.

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DISCUSSION

Dr. Henderson: We have been studying the mechanism of 2,4-D action at concentrations of 10^{-3} to 10^{-5} *M*. We found that oxygen uptake of oat and pea sections during the time of IAA disappearance was correlated over a six hour period. When one added 2,4-D at 10^{-3} and 10^{-5} *M*, the oat showed a marked decrease in IAA disappearance and the pea showed a more rapid disappearance of auxin. This we called a sparing action of 2,4-D. Recently, a new phenomenon was found which turned out to be the effect of 2,4-D on phototropism in weak blue light. When 2,4-D was sprayed on coleoptiles, phototropic action ceased. When we dipped the coleoptiles in Tween (0.01 per cent) + 2,4-D the same effect was obtained. In 1957 and 1958 we screened some 30 compounds of six different groups. No single family was necessarily similar in structure and, therefore, there was no functional group that satisfied an explanation for this phenomenon. We found that out of some 30 compounds, 15 of these at 10^{-3} *M* negated phototropism. Only six of these, however, were effective at concentrations of 10^{-5} *M* or below: IAA, IAN, IBA, NAA, 1-naphthaleneacetonitrile, and 2,4-D. Since chemical and physical conditions also must influence this phenomenon, we have experimented with 2,4-D from 10^{-3} to 10^{-10} *M*, with the pH from 3 to 9 in buffer solutions.

My question, based on some of the statements that you have made is, Would this evidence fit into the pattern you have presented?

Dr. van Overbeek: Offhand, I would certainly think so. You raised two interesting points. One is the antagonism between 2,4-D and

Table 1. Activity of phenylacetic acid and some of its chloro- derivatives in three tests.

Derivative of Phenylacetic Acid	Response to Various Molar Concentrations														
	Wheat cylinder test					Pea segment test					Pea curvature test*				
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Parent compound	100	98	100	120§	142§	99	98	100	111§	130§	0	0	0	4	6
2-Chloro-	102	125§	148§	156§	150§	99	101	109§	131§	134§	0	0	4	6	5
3-Chloro-	104	120§	151§	147§	146§	102	105†	118§	134§	125§	0	2	6	6	3
4-Chloro-	100	98	118§	150§	136§	101	101	106‡	130§	125§	0	0	4	5	2
2,3-Dichloro-	135§	157§	160§	157§	140§	105†	118§	130§	130§	117§	1	4	6	6	2
2,4-Dichloro-	100	103	120§	145§	144§	99	106‡	119§	125§	114§	0	2	5	6	2
2,5-Dichloro-	101	102	137§	146§	136§	103	113§	121§	131§	119§	0	3	5	6	2
2,6-Dichloro-	103	113§	147§	154§	141§	101	114§	122§	132§	121§	0	3	6	6	3
3,4-Dichloro-	98	104	140§	136§	108†	99	110§	123§	128§	113§	0	4	6	6	2
3,5-Dichloro-	97	99	100	106	94	97	100	101	116§	109§	0	0	2	3	1
2,3,6-Trichloro-	112§	158§	160§	157§	146§	123§	133§	134§	131§	119§	4	6	6	6	2

* Activity in this test assessed on range 0 (inactive) to 6 (highly active).
† Results significantly different from the water controls at the 5 per cent level; ‡ at 1 per cent level; § at 0.1 per cent level.

IAA. That, of course, is very readily explained and doesn't differ in principle from the antagonism system of Dr. Bonner and his school. It is a question of materials competing for the same site. In the case of IAA versus 2,4-D, you have 2,4-D sitting here and IAA can't get in, and vice versa. So that is simply explained by this scheme of solubilization adsorption in the cytoskeleton. The other point you mentioned regarding nitriles and methyl esters — they, of course, are more fat soluble than the acids; therefore, they solubilize better into the lipophilic cytoskeleton so they concentrate there and, therefore, the chances of attaching to a specific auxin site are also increased. The materials are probably hydrolized before they act as auxins (Figure 3 A and B).

Dr. Wain: There are quite a number of points I would like to raise on this paper, but wish to deal with four. I want to point out that with mono- and dichlorophenoxyacetic acids you get activity in a number of cases. The 2,6-derivative is active, as has been demonstrated by numerous workers; much less activity is shown by the 3,5-compound; indeed, this substance is inactive in all our tests. With the mono- and dimethylphenoxyacetic acids you have inactivity both with the 3,5- and the 2,6-derivatives. Methyl derivatives, in general, are less active than the corresponding chloro-compounds. A full paper on the phenylacetic acids will appear shortly, but in the meantime here are some results obtained in three tests (Table 1). You will observe that phenylacetic acid itself is active, and so are all the chloro-derivatives we examined. Now, the point I want you to notice here is that there is particularly good activity in the 3-, the 2,3-, the 2,6-, and the 2,3,6- derivatives.

Bearing in mind what I've just said, I want to go back to Dr. van Overbeek's diagram of phenoxyacetic acid with 2,6 blocking; the molecule is sitting up there with the carboxyl group in the wrong position for activity. If you have 3,5 blocking, the molecule will be still further out of the so-called cytoskeleton. Dr. van Overbeek would say, "That's exactly what I would expect because the compound is inactive." But, in fact, if you make the 2,3,5- or the 3,4,5- compounds, you restore activity; yet the compound is still presumably in the same position as it was before. Secondly, we have the tetrachloro-derivatives; the 2,3,4,5-compound, again 3,5 blocked, is quite active, as Dr. Smith has shown.

Dr. van Overbeek: Let me answer the easy question as to why the methyl derivative is less active than the chloro compound: The methyl group, being lighter in weight, even though it has the same bulk, naturally sticks less rigidly to the cytoskeleton by van der Waals force. Dr. R. Brian, at the Wye College conference, showed that the behavior of the penetration of these auxin molecules into a mono-

film is not entirely predictable from their two dimensional structural formulae. After we have studied penetration into membranes, perhaps then we could answer that question. I am not proposing here a ready-made theory, but I am simply asking the question, "In which direction are we looking?" Are we going to continue to look for an answer to the primary mode of auxin action in the direction of covalent bonding, activation of a single enzyme system, water soluble systems, etc., or are we now going to try to look at it from the point of view of surface chemistry, membranes, etc., and in the direction of things such as Dr. Freed and I discussed?

Dr. Wain: Well, Dr. van Overbeek, I accept that. May I go on to my second point? This is in relation to the phenylacetic acids. Now, according to your concepts, the phenylacetic acids are active when you have 2,6 positions blocked because the 2,6-dichloro atoms sit on top of the cytoskeleton and leave the carboxyl group in precisely the right position. Reference to our results, however, shows that quite good activity is found in 2,3-dichlorophenylacetic acid and there, according to your idea, the compound should be able to penetrate well down into the cytoskeleton. Exactly the same principles apply to the benzoic acids, where 2,3-dichlorobenzoic acid is active. This compound, according to your concept, would penetrate deeply into the membrane, leaving the carboxyl group far too low to join up with the oscillating hydrogen bonding network.

Dr. van Overbeek: When one studies these problems with molecule models and especially when one tries to fit these into 3-dimensional holes, one finds that the 2,3-dichlorobenzoic and phenylacetic acids do, indeed, fit the theory very nicely, contrary to what one might expect from 2-dimensional reasoning with structural formulae. This type of 3-dimensional hole is shown in figure 3 and was suggested several years ago by Dr. Mullins (cf. van Overbeek, Bot. Rev. 25:300. 1959).

The phenylacetic acids have the added feature that the side chain sticks out of the plane of the ring in a very prominent fashion. This gives this type of molecule a tendency to hang itself up, thus favoring activity. Incidentally, the prominent perpendicular side chain may also be a reason why indole-3-propionic acid is highly active, as you have heard during the discussions on the first day. It, too, has a natural tendency, on account of the angular side chain, to hang itself up and, therefore, stabilize itself in the cytoskeleton system.

Not only does my view of looking at the primary mode of action of auxin as a solubilization phenomenon explain puzzles such as why the 2,3-dichlorobenzoic and phenylacetic acids possess activity, but it also gives a plausible explanation for the activity of the structurally totally unrelated 2-heptadecanol, which Dr. Crosby told us the first

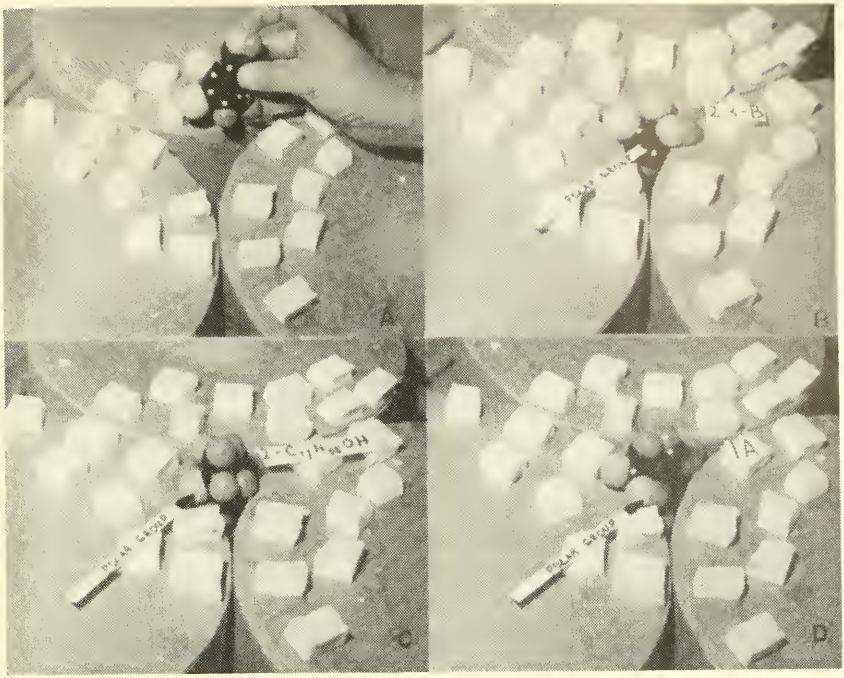

Fig. 3. **A.** Model of 2,3-dichlorobenzoic acid about to be lowered into a 3-dimensional hole. The 3 cylinders are imagined to be 3 molecules of the cytoskeleton. The white pieces of foam rubber indicate part of the hydrogen network on the surface of the cytoskeleton. **B.** Model of 2,3-dichlorobenzoic acid fits tightly into the hole of the cytoskeleton so that it anchors the polar group in position in the hydrogen bond network. **C.** Model of 2-heptadecanol fitting into the 3-dimensional hole of the cytoskeleton. Note how the –OH group is held into place for participation in the H-bond network. **D.** Model of indole-3-acetic acid fitting into a hole in the cytoskeleton. An arrangement of this sort, because of the uniformity of the cylinders (molecules that make up the cytoskeleton) may give the false impression that all holes are of uniform size. This is not intended as the holes into which the auxins fit must be specific (see F. H. Dickey, Jour. Phys. Chem. 59: 695. 1955).

day he has isolated from tobacco tissue. If, indeed, as I am proposing, the primary function of an auxin is the participation of the undissociated acid group in a hydrogen bond system, then there is no physicochemical reason why another polar group, such as the -OH of an alcohol, could not serve equally well. The discovery by Crosby and Vlitos of the alcohol auxin, therefore, supports my views. It will be noted that the -OH group of this alcohol is on the second carbon. Therefore, it is in a lateral position quite comparable to that of a hindered carboxyl group of a benzoic acid. The long hydrocarbon chain fits the auxin binding site in the cytoskeleton (Figure 3C) and is held in position by van der Waals forces.

Dr. Wain: My third point is in relation to the naphthoic acids. The 1-naphthoic acid has only weak activity; if you reduce the second ring then the 3,4-dihydro-derivative still has only weak activity. On the other hand, the 1,4-dihydro- is highly active; the 1,2-dihydro- is highly active, and so is 1,2,3,4-tetrahydro-1-naphthoic acid. The experimental evidence is not in question, but there are other possibilities in addition to those put forward by Dr. van Overbeek which explain the activity of these molecules. It will be noted that in all the active compounds there is a hydrogen atom attached to the carbon atom adjacent to the carboxyl group. As you all know, we, at Wye, have repeatedly stressed the importance of the *alpha*-hydrogen atoms in relation to activity, for I think this is the key to all considerations of this kind. I refer to the fact that in those growth substances in which the carbon atom adjacent to the carboxyl group is asymmetric, there is usually activity with one enantiomorph and not the other. To my mind this is one of the most important things of all, and I'm rather surprised that steric considerations of this kind have not been mentioned throughout the whole of this conference, for here, surely, is an important clue to mode of action. We have very definite ideas about this, but they are only ideas, and other views, of course, are possible and will be put forward. But, I do think that any proposed theory — and I do congratulate you, Dr. van Overbeek, on the thought you have put into your theory — must take steric considerations into account. I come back to what I said this morning, and that is that so complex is the growth response — your molecule must get in, must have the right physical properties to penetrate, to move in the tissues, it must have adequate stability, then it must have the structural requirements for activity at the site of action — that no simple theory on mode of action is likely to be satisfactory. But, let's not overlook this rather interesting clue to the whole situation — this specificity of stereoisomers which, incidentally, was first discovered by Dr. Smith and has been developed very considerably by the Swedish school since that time.

Dr. Bitancourt: I believe that the site of action of IAA is at the interface between the cytoplasm and the cell wall, and Dr. van Overbeek's theory seems to fit very well into this scheme. But if the hole in the cytoskeleton is so small that only the dichlorophenoxyacetic acid fits in there, how does the indole nucleus fit into this hole?

Dr. van Overbeek: Indeed, the indole nucleus does fit. One has to make the stereo models (Figure 3 D) in order to appreciate the 3-dimensional aspects.

Dr. Bitancourt: Well, this can be understood, but then once all the holes are occupied nothing ought to happen, and so you can't

explain the part of the action curve of IAA where there is inhibition. This can be explained by the two attachment point theory.

Dr. Ray: The crucial point of this theory is one of a specific site involving points at which the auxin combines and brings about activity. I do not feel that it is fundamentally different from what we have all been thinking about for some years concerning the nature of auxin activity: combination of the auxin molecule with a site of specific shape. I do not think it is at all a question, therefore, of surface chemistry or nonspecific adsorption, but as far as the data that this theory is attempting to explain, one primarily of specificity.

Dr. Fawcett: If one considers the 2,4-dichlorophenoxy structure, then the acetic acid homologue is a highly active compound, although β-(2,4-dichlorophenoxy)propionic acid, with its somewhat longer side chain, is inactive in, for example, the split pea curvature test. But, if one considers β-(2,3-dichlorophenoxy)propionic acid, this is more active than 2,3-dichlorophenoxyacetic acid (Fawcett *et al.*, Proc. Roy. Soc. 150 B: 95. 1959). Are these examples in line with the present hypothesis?

Dr. Osborne: One small point — if one of the hydrogens on the side chain is replaced with a methyl group in certain of the 2,6-substituted phenoxyacetic acids the biological activity is enhanced although, supposedly, the ring would still have difficulty fitting into the hole and the side chain would still be too high for activity.

Dr. van Overbeek: Yes, but introduction of methyl will improve its partitioning properties so you have more molecules near the site of action and thus a better chance of getting them into position. In addition, the methyl group causes steric hindrance so it brings the side chain over in the effective lateral position. Many things have to be taken into consideration. I disagree with Dr. Ray's comments. This theory is fundamentally different because I am no longer looking at a water-soluble system but at an oil-soluble membrane system; we are not looking at covalent bonding, we are looking at physico-chemical systems. The principle is that of hooking a small polar group into position. What I have tried to do is to show the direction in which we must look for an answer.

The Gibberellins

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The Early History of Gibberellin Research

Under the direction of Dr. Stowe, the initial workers with the gibberellins on three continents present the original difficulties and problems encountered when they first got into gibberellin work. They represent the Tokyo (Japanese) group, the I. C. I. (British) group, and the United States Department of Agriculture (American) group.

DISCUSSION

Dr. Stodola: The first suggestion that I could find in the literature that the bakanae effect might be due to the fungus, appeared in 1912 in a paper by the Japanese plant pathologist, Sawada (7). In this paper he said, "On microscopic examination the plant system is found to contain mycelium. It is thought that the plants grow taller due to some stimulation from the mycelium." In the early 1920's, a young graduate of Chiba Horticultural College — Eiichi Kurosawa — came to Formosa to work with Sawada at the Central Research Institute of the Formosa Department of Agriculture. His problem was to work on methods of controlling the bakanae disease which, at that time, was causing severe rice losses on the island. In the course of his investigations, Kurosawa became interested in the unusual symptom of hyperelongation which characterizes the disease, and he undertook to determine the nature of the responsible agent and, if possible, to isolate it. In the summer of 1925 Kurosawa started his experimental

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work on this phase of the problem, and by the following year he had published in Japanese his now classical paper, "Experimental studies on the secretion of *Fusarium heterosporum* on rice-plants" (5). Kurosawa showed that sterile filtrates from the bakanae fungus gave marked growth stimulation in rice and grass. In this paper appeared the first photograph found in the literature which illustrated the stimulatory effect of the fungus secretion.

It is to Kurosawa, then, that we are indebted for opening up this fertile gibberellin field. I felt that a pioneer such as this deserves to be better known than he is. Kurosawa was born in 1894 in the town of Itabashi in Ibaraki Prefecture, and he died in 1953 at the age of 59. After considerable effort Dr. Hayashi was able to find the informal picture shown on the facing page. There you see the man responsible for the work that we are so much interested in.

The publication of Kurosawa's paper at once stimulated other workers to take up the problem, of course, and in 1928 papers by Hemmi and Seto appeared from the Phytopathology Laboratory of Kyoto University (4, 8). In 1932 Shimada at Hokkaido University in northern Japan published the first paper on the chemical nature of the growth promoting principle (9). It was at this time that the University of Tokyo workers became interested in the problem.

Dr. Hayashi: In 1930 Dr. Yabuta, formerly professor of Agricultural Chemistry of Tokyo University, Dr. Kannbe and I studied the isolation of the growth stimulating substance. In 1934, contrary to what we expected at first, we obtained a growth deterring substance, fusaric acid, that is, 5-*n*-butylpicolinic acid, by extracting the cultured solution either with benzene or petroleum ether. It is reported that in some cases rice plants infected with bakanae organism show reduced growth instead of the usual elongation. Judging from this fact, fusaric acid might be responsible for the symptoms of retarded growth. In 1934 Kurosawa changed his position to Tokyo and, with his help, Dr. Yabuta and I again began isolation of the growth stimulating substance. Kurosawa furnished us with a fungus which produced the active substance abundantly and indicated suitable cultural conditions.

The isolation of the active substance from the culture filtrate was carried out as follows: The fungus was grown in a medium containing ammonium chloride, monopotassium phosphate, and glycerol by the culture method in flask. After about one month, the culture solution was filtered and the filtrate was treated with activated carbon. The carbon was then eluted with methanolic ammonia. The eluate was concentrated in vacuo. The resulting residue was dissolved in aqueous sodium bicarbonate solution and extracted with ether to

Dr. Eiichi Kurosawa, 1894–1953

remove neutral and phenolic substances. The bicarbonate solution was then acidified and again extracted with ether. After treating with lead acetate, the ether extract was evaporated, and then the active substance was obtained as a white powder. In 1935 Dr. Yabuta gave it the name of gibberellin on the basis of the scientific name of the fungus, *Gibberella fujikuroi* (Saw.) Wollenw. This was the first use of this term in the literature. Later, in 1938, Dr. Yabuta and Dr. Sumiki reported the isolation of crystalline gibberellin A and B (15). The studies on the chemical structure of gibberellin started at this time. By 1950 we had published 15 papers on the production of gibberellin and on its chemistry (cf. 10, 13, 14). It was at this time that work outside of Japan began.

Dr. Stodola: The first work on gibberellin outside of the Orient was done at the Chemical Corps Biological Laboratories at Camp Detrick, Maryland; in March, 1950, Dr. J. E. Mitchell reviewed this work in a talk before the American Phytopathological Society (6). That summer the Korean War started, and then gibberellin took on a military aspect as far as this country was concerned. A large scale production was needed to provide sufficient material for proper testing, and because of our experiences at Peoria with industrial fermentations, the problem was brought to us in August, 1951. Dr. Raper, the head of our culture collection section, was assigned to carry out the fermentation studies, and I was to work on the isolation and characterization of the gibberellin.

I was very fortunate at the start to be able to talk over the whole problem with Prof. Sumiki, who is the authority on the production and chemistry of gibberellin, while we were at the International Congress of Pure and Applied Chemistry in New York in early September, 1951, where Prof. Sumiki gave a talk on gibberellin. In getting started on work of this sort, one needs first cultures of the organism, a suitable medium for growing it, assay procedures for estimating the amount that is produced, and pure compounds for use as standards. We had trouble with all of these, as you will see from the following excerpts from letters that I picked out. For example, a letter from Dr. Mitchell to Dr. Raper, written in October, 1951, states, "I'm very sorry to hear that the cultures I brought to Peoria did not survive. I can't understand why that should have happened, inasmuch as I have kept them for much longer periods of time in the past on that medium without trouble." Another letter from Mitchell two months later, "I am sorry to hear that you are having difficulty with your assay procedure. I wish that I could give you details of an effective assay that would give the desired results. We likewise have not gotten the results that we had hoped for." A

letter from me to Professor Sumiki in February, 1952, goes in part like this, "In recent experiments we have failed to obtain even moderate growth using your medium containing glycerol, dihydrogen phosphate, and ammonium chloride. We have found, however, that when magnesium sulfate is added, in even small amounts, growth is greatly enhanced. We are wondering if you have found it necessary or desirable to add magnesium sulfate to your fermentations." Finally, a letter to me from Sumiki in March, 1952, "I am very surprised that the sample of gibberellin A sent to you by my assistant while I was in the United States showed little activity. The activity of that sample was not tested but this is the first time we have heard of crystalline gibberellin losing its activity so fast. We are now preparing a new sample to send to you."

By May, 1952, these difficulties had been straightened out, and in June we had a successful pilot plant run which yielded 12 g. of crystalline gibberellin. From this I was able to isolate by repeated crystallization a sample of pure gibberellic acid with a rotation of $+90°$ (12). Later, we developed a chromatographic method that would efficiently separate gibberellin A_1 and gibberellic acid (11). Our experience was passed on to a number of fermentation companies in this country, and before long there was enough gibberellin available here for everyone. All this time, of course, the British workers were carrying out their work on gibberellic acid.

Dr. Brian: We started work either in 1951 or late 1950. Undoubtedly, the stimulus to us was the sudden spate of abstracts of Japanese work which hadn't reached us during the war years. Looking back on things, I am very much more struck by stupendous pieces of luck that we had rather than by difficulties. I can just mention a few of these.

First of all, our strains of the fungus, *Gibberella fujikuroi,* were obtained originally simply by getting them from culture collections. By far the best that we found in the early days and by far the best that we still have was a strain which to my certain knowledge had been kept in culture collections for over 30 years, and I believe, in fact, was the type isolated by Sawada. We were very lucky indeed, I think, to come across so stable an organism to work with.

The second piece of luck we had was that in carrying out our preliminary fermentations, we completely ignored any previous work and used the kind of media that we had been used to using in our other work on fungal metabolic products. We immediately got yields of a gibberellin in far greater quantities than any previously-recorded yields – again I think purely by accident (1, 2). Very shortly after we got this material, one of my colleagues, Philip Curtis, said that this stuff

wasn't the same as the Japanese gibberellin A. We, I'm afraid, told him he was talking nonsense, that this was virtually impossible, that it should be something different; but in point of fact, he turned out to be right. We had, as a result of using the strain we did and the culture media we did, arrived straightaway at a pure gibberellin, a material now known as gibberellic acid or gibberellin A_3. A further piece of luck that we had was that almost immediately in our biological work we stumbled on this dwarf-tall relationship which had the effect of first making us think of the possibility of isolating natural gibberellins and also provided us in the very early days with a very convenient assay, the dwarf pea (3). Looking back on things, I feel that our early history was characterized by quite extraordinary and undeserved pieces of luck rather than the kind of difficulty that Dr. Stodola mentioned.

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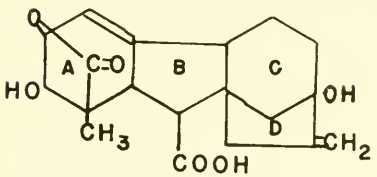
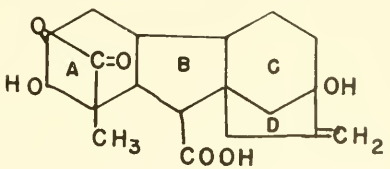
The Chemistry of Gibberellins From Flowering Plants[1]

The chemistry of the gibberellins derived from culture filtrates of the fungus *Gibberella fujikuroi* has been extensively investigated primarily by two groups of chemists — one at the College of Agriculture of the University of Tokyo and the other at the Akers Research Laboratories of the Imperial Chemical Industries, Limited in England. The results of the investigations of Grove *et al.* of the Akers research group have led to the following proposed structures for gibberellic acid (A_3) (2) and gibberellin A_1 (A_1) (3). [2]



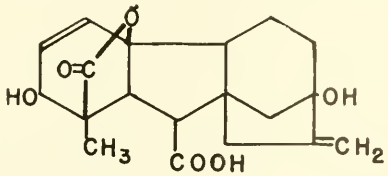
A_3



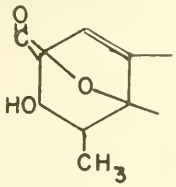
A_1

[1] This research was supported in part by grants from the National Science Foundation (G-3526) and Merck & Co., Inc.

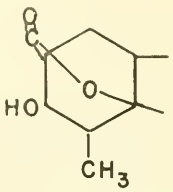
[2] After the preparation of this manuscript, the author was informed of a report to be published by B. E. Cross, J. F. Grove, J. MacMillan, J. S. Moffatt, T. P. C. Mulholland, and J. C. Seaton in which the proposed structure of gibberellic acid is revised to the following:



Sumiki and his collaborators at the University of Tokyo interpret their results to indicate the same structure for A_1 and A_3 as above, except for the position of the lactone ring in ring A (4, 8, 10). They report that lithium aluminum hydride reduction of the methyl ester of A_1 leads to a product which could not be oxidized by periodate. They also reported the isolation of a degradation product from the lithium aluminum hydride reduction product which was identified as a 1,3-dimethylfluorene derivative. In line with their findings they propose the A rings of A_1 and A_3 to have the following structures:

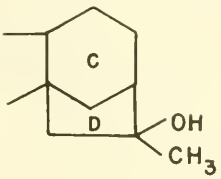


A_3

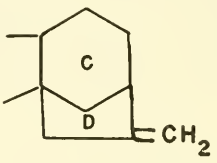


A_1

Sumiki and his co-workers have also proposed structures for gibberellin A_2 (A_2) and gibberellin A_4 (A_4) which they have isolated from the fungal filtrates. Both are thought to have the same A-B ring structures as A_1 but are modified in the C-D rings as follows:



A_2



A_4

There is now abundant evidence for the presence of substances with biological properties similar to those of the fungal gibberellins in flowering plant tissues. The obvious implication is that such substances act as natural regulators of growth and development in the plant. It is important, therefore, to isolate these substances and determine their structure if we are to approach intelligently questions regarding their role in controlling growth and development, the mode of their biosynthesis, and the mechanism of genetic control of these processes.

MacMillan and Suter (6) have reported the isolation of A_1 from immature bean seed (*Phaseolus multiflorus*). The identification was based on the identity of the infrared spectra of the free acid and methyl ester of the isolated specimen and those of authentic A_1 free acid and ester and the identity of the melting points and mixed melting points of the methyl esters. West and Phinney (11) have reported the isolation of two crystalline compounds with gibberellin-like biological properties, called bean factor I and bean factor II, from immature bean seed (*Phaseolus vulgaris*). These substances were incompletely characterized. Sumiki during this conference reported the isolation of A_1 from water sprouts of mandarin orange (*Citrus unshiu*) (9). These are the only reports to date of the isolation of gibberellins from flowering plants in a sufficient state of purity to allow a useful determination of physical and chemical properties.

The purpose of this paper shall be to review the properties of bean factor I and bean factor II and their implications for the structures of these materials.

EXPERIMENTAL PROCEDURE

Isolation

The procedure employed for the isolation of bean factors I and II has been described (11). The initial steps included extraction of approximately 25 kg. of immature bean seed with acetone-water (1:1), adsorption of the active substances onto charcoal from aqueous solution and re-elution with acetone (omitted in the second run) and extraction of the active substances from aqueous buffer at pH 2 with ethyl acetate. Further purification of this concentrate was achieved by column chromatography on charcoal, column chromatography on silicic acid, and countercurrent distribution. Crystallization was effected from ethyl acetate-petroleum ether solvent mixtures. The progress of purification was followed by bioassay on dwarf mutants of maize. In one run approximately 2 mg. of bean factor I and 2 mg. of bean factor II were recovered. In a second run approximately 1 mg. of bean factor I and 5 mg. of bean factor II were obtained.

Silicic acid chromatography has proved the most useful technique for fractionating bean factor II from bean factor I. In a typical column 40 g. of prewashed and oven-dried silicic acid is dry-packed in a column. The material to be chromatographed is adsorbed on a small amount (2 g.) of silicic acid by evaporation from an organic solvent and this mixture is packed on the top of the adsorbent column. The column is developed in succession with 400 ml. chloroform, 400 ml. of 20 per cent ethyl acetate in chloroform (by volume), 400 ml. of 40 per cent ethyl acetate in chloroform, 400 ml. of 60 per

cent ethyl acetate in chloroform, 400 ml. of 80 per cent ethyl acetate in chloroform, and 400 ml. of ethyl acetate. Forty ml. fractions are collected and aliquots are tested for activity. Bean factor II is eluted primarily in those fractions obtained with 40 per cent ethyl acetate in chloroform as the developing solvent and bean factor I is eluted by 60 per cent ethyl acetate in chloroform as the developing solvent. Under identical conditions A_1 and A_3 are eluted with the latter developing solvent. Such columns have been used to demonstrate the presence of gibberellin-like substances in crude extracts and also as a terminal step in purification.

Biological Properties

The biological properties of bean factor II are discussed more completely by Phinney elsewhere in this volume (7). In quantitative assays on dwarf mutants of maize, bean factor I and A_1 show the same activity on a weight basis. The growth response of maize mutants *dwarf-2, dwarf-3, dwarf-5,* and *anther-1* to bean factor II is equal to or greater than the response to an equivalent amount of A_3, the most active of the fungal gibberellins. However, bean factor II is less than 5 per cent as active as A_3 for the *dwarf-1* mutant. Thus, bean factor II is quite distinct from A_1, A_2, A_3, and A_4 in its biological properties.

Neutral Equivalent

A spectrophotometric micro-method for the determination of the neutral equivalent weight of carboxylic acids was developed. A known weight (about 0.05 microequivalent) of acid to be tested was dissolved in 3.50 ml. of a freshly prepared solution of the sodium salt of phenolsulfonphthalein (phenol red) (3 mg. per 100 ml. of boiled distilled water). Care was taken to exclude atmospheric carbon dioxide. The absorbancy at 550 mμ was measured in a Beckman B spectrophotometer for (1) a reagent blank (no acid added), (2) the solution of an unknown acid, and (3) the solution of a standard acid. The absorbancy of sodium phenolsulfonphthalein decreases at 550 mμ in the presence of an acid due to the conversion of the indicator from the base to the acid form. The magnitude of the decrease is a function of the equivalents of acid added and can be standardized by reference to a standard acid.

Determinations of the neutral equivalent of bean factor I by this technique with A_3 as standard[3] gave values of 370 and 340 (average = 355) and values of 380 and 340 (average = 360) were obtained for

[3] Reference samples used in these studies were kindly supplied as follows: A_1, A_2, and A_4 — Prof. Y. Sumiki, University of Tokyo, Tokyo, Japan, and A_1 and A_3 — Dr. Frank Stodola, Northern Regional Research Lab., USDA, Peoria, Ill.

Table 1. Paper chromatography of the gibberellins.

Gibberellin	R_{ga}* in Solvent System†		
	A	B	C
A_3	(1.0)	(1.0)	(1.0)
A_1	1.0	1.0	2
A_2	1.1	1.1	4
A_4	2.3	1.3	‡
Bean factor I	1.0	1.0	2
Bean factor II	2.0	1.2	9

* R_{ga} = Migration relative to A_3.

† Solvent systems: A = Upper phase of a mixture of *n*-butyl alcohol: 1.5*N* ammonium hydroxide (3:1). B = Upper phase of a mixture of *n*-amyl alcohol:pyridine:water (35:35:30). C = Upper phase of a mixture of benzene:acetic acid (glacial):water (4:1:2) (1).

‡ Migrated off the end of the chromatogram under the conditions employed.

bean factor II. Theoretical neutral equivalents for A_1 and A_3 are 348 and 346. Thus, bean factors I and II are clearly shown to have an acidic functional group with a neutral equivalent close to those of the fungal gibberellins.

Paper Chromatography

It can be seen by reference to Table 1 that A_1, A_2, and A_3 migrate to approximately the same extent on paper chromatograms in the three solvent systems shown, whereas A_4 moves considerably further from the origin. Bean factor I behaves as A_1 in these systems. Bean factor II is closer to A_4 in its behavior, though not identical with it. Since A_1, A_2, and A_3 each have two alcoholic hydroxyl groups and A_4 has only one, these results might be interpreted as presumptive evidence for the presence of one alcoholic hydroxyl group in bean factor II.

Determination of Ethylenic Double Bonds

A preliminary microhydrogenation experiment suggested that bean factor II has two ethylenic double bonds per acid equivalent as does A_3 and bean factor I has one as does A_1.

Confirmation of these conclusions came from the application of a microtechnique for the determination of such double bonds. A sample of 20 to 40 micrograms of gibberellin of known weight was dissolved in 3.5 ml. of freshly prepared potassium permanganate solution (3 mg. per 100 ml. solution in distilled water). After 15 min. the absorbancy of the solution was determined at 415 mμ in a Beckman

Table 2. Ethylenic double bonds from permanganate reduction studies.

Gibberellin	Double Bonds per Acid Equivalent	
	Found	Theory
A_3	2.0	2
A_1	1.3	1
A_2	0	0
A_4	1.3	1
Bean factor I	1.0	...
Bean factor II	1.9	...

B spectrophotometer. The absorbancy of an unknown relative to that of a standard (absorbancy of blank subtracted from each) was used to calculate the number of double bonds per unit weight. Table 2 shows the results for A_1, A_2, and A_4, bean factor I, and bean factor II with A_3 taken as a standard.

Determination of Exocyclic Methylene Groups

A procedure adapted from Lemieux and von Rudloff (5) was employed for the micro-estimation of terminal methylene groups of the type reported to be present in A_1, A_3, and A_4. The procedure is based on conversion of the methylene group to formaldehyde by means of a periodate-permanganate reagent followed by the colorimetric estimation of the formaldehyde produced with a chromotropic acid reagent. Table 3 summarizes the results obtained with this method for the fungal gibberellins and the bean factors. The yields are in the range of those found by Lemieux and von Rudloff. These results suggest the presence of exocyclic methylene groups in bean factor I and bean factor II. However, this interpretation is tentative since A_2 seems to yield formaldehyde even though it is thought not to have such a group.

Table 3. Determination of exocyclic methylene groups.

Gibberellin	Moles of Formaldehyde Per Acid Equivalent	
	Found	Theory
A_1	0.76	1
A_2	0.61	0
A_3	0.51	1
A_4	0.53	1
Bean factor I	0.83	...
Bean factor II	0.59	...

Infrared Absorption Spectra

The infrared absorption spectrum of bean factor I in KBr pellet is identical with that of a sample of A_1 supplied by Stodola.

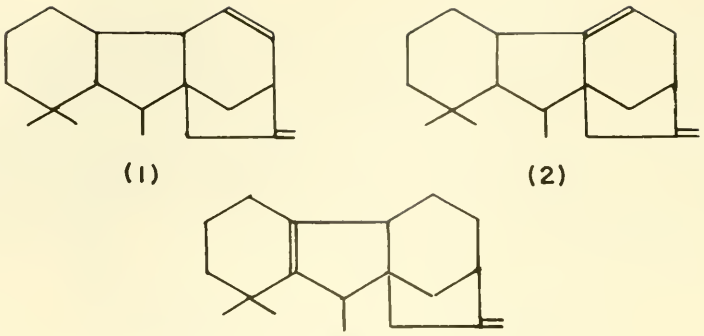
The infrared absorption spectrum of bean factor II in KBr pellet resembles in a general way those of the fungal gibberellins, but there are some distinct differences. Some absorption maxima for bean factor II and tentative structural assignments based on similar features of fungal gibberellins are as follows:

3400 cm^{-1}	alcoholic hydroxyl
1750 cm^{-1}	γ-lactone carbonyl
1720 cm^{-1}	carboxylic acid carbonyl
1650, 890 cm^{-1}	$>C{=}CH_2$
1620 cm^{-1}	ethylenic double bond

DISCUSSION

The identity of the infrared spectra of bean factor I and an authentic sample of A_1 leads to the conclusion that bean factor I and A_1 are the same compound. All the other properties determined are consistent with this conclusion. Thus, there are three species of flowering plants which have been shown to contain A_1 as a natural constituent in small amounts — immature seed of *Phaseolus multiflorus,* immature seed of *Phaseolus vulgaris,* and water sprouts of *Citrus unshiu.*

The properties of bean factor II indicate that it is structurally similar to the fungal gibberellins but not identical with any of those reported to date. The presence of a carboxylic acid group is clearly indicated, and the neutral equivalent is in the range of those of the fungal gibberellins. A γ-lactone group is also indicated by the infrared spectrum. The evidence also suggests the presence of one alcoholic hydroxyl group. Two ethylenic double bonds are present per acid equivalent. One of these is most likely bonded to a terminal methylene group. These two double bonds are not in conjugation with other sites of unsaturation since bean factor II does not show an ultraviolet absorption maximum above 220 mμ. If the seemingly reasonable assumption is made (for which there is no direct evidence) that bean factor II has the same carbon skeleton as the fungal gibberellins and the exocyclic methylene group is as in A_1, A_3, and A_4, then the following positions seem the most likely possibilities for the sites of unsaturation.



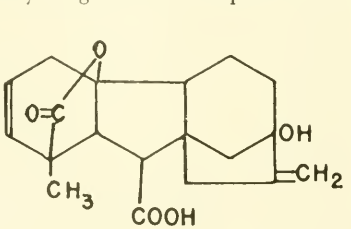
Structure 3 would seem less likely since bean factor II does not fluoresce when dissolved in sulfuric acid, whereas A_3, with a double bond in the A-ring, does.

There is no evidence to assist in the placement of a lactone, a carboxylic acid, or an alcoholic hydroxyl, although it would seem most likely they would be substituted in one of the positions occupied by such groups in A_1, A_2, A_3, or A_4.[4]

SUMMARY

Two crystalline substances, with gibberellin-like biological properties, called bean factor I and bean factor II, have been isolated from acetone-water extracts of immature seed of *Phaseolus vulgaris*. The infrared spectrum and other properties of bean factor I demonstrate that it is identical with gibberellin A_1 isolated from the fungus *Gibberella fujikuroi*. The biological and chemical properties of bean factor II show that it is not identical with the fungal gibberellins, A_1, A_2, A_3, or A_4. It has a carboxylic acid group and a neutral equivalent of approximately 360. Evidence is also presented for the

[4] After the preparation of this manuscript the author was supplied with a copy of a report to be published by J. MacMillan, J. C. Seaton, and P. J. Suter in which they describe the isolation of a substance (gibberellin A_6) from seed of *Phaseolus multiflorus*. They assign to this compound the structure:



A comparison of the infrared spectrum of this substance with that of bean factor II shows them to be identical.

presence of a lactone, an alcoholic hydroxyl, and two ethylenic double bonds not in conjugation with other sites of unsaturation. These properties are discussed in terms of possible structures for bean factor II.

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DISCUSSION

Dr. Galston: I don't need to remind you that I am not a chemist, but I had understood from previous reports that when the lactone ring was broken, the evidence indicated that the position of two hydroxyl groups was *ortho* to each other on the A-ring. This new structure proposed by the I.C.I. group would not permit this. Is that correct?

Dr. West: It is correct that when you hydrolyze gibberellic acid with base to open the lactone ring, the resulting product reduces periodate. Both the British and Japanese groups found this and it was one of the reasons why the British group suggested the structure given. However, the new position is this: when you hydrolyze the

lactone ring in the newly proposed structure, you obtain an alcoholic group allylic to a double bond. Such alcoholic groups migrate extremely easily and by such a rearrangement in this case you obtain a product with adjacent alcoholic hydroxyl groups which can reduce periodate as observed. (The Japanese had suggested that some rearrangement does occur during basic hydrolysis.) So there is a good chemical explanation consistent with the periodate data.

Dr. Crosby: Actually, I have only one comment to make. I think that those who are not familiar with complicated chemical structures ought to be reminded that this picture that we draw of gibberellic acid actually bears no resemblance to the way that gibberellic acid looks in space. Work is going on in a number of laboratories now to try to determine more closely how this material actually does look. I'm afraid that many of us may be misled if we continue to think of the chemistry of gibberellic acid and the other gibberellins in terms of symmetrical drawings that we can make on a blackboard or on a piece of paper, when actually the configurations are not like that at all. My point is, of course, that when we wish to relate chemistry to growth promoting activity, any previous ideas of these correlations which we have obtained with simple planar structures that we could write on a blackboard may not be adequate for spatially-complex substances such as gibberellic acid.

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Occurrence of Gibberellin A_1 in the Water Sprouts of Citrus

In 1951, the occurrence of a new phytohormone which greatly stimulated stem elongation in immature bean seeds was reported by Mitchell, Skaggs, and Anderson (3). It was the first description of the occurrence of a gibberellin-like substance in higher plants. West and Phinney (8) reported the occurrence of gibberellin-like substances from species of several different families of flowering plants. Since then a number of reports (4, 5, 6, 7) related to gibberellins or gibberellin-like substances have been published.

Last year, MacMillan and Suter (2) obtained gibberellin A_1 from the immature seeds of runner bean, *Phaseolus multiflorus,* which was the first successful isolation of one of the known gibberellins.

In our laboratory, the constituents of water sprouts of mandarin orange were examined, and gibberellin A_1 was obtained in pure crystalline form.

The material used in this experiment was a bud variation of *Citrus unshiu,* first found by K. Furusato (1) in Shizuoka Prefecture in 1949. In spring, many long twigs sprout from its apex like a witches'-broom of the cherry tree. The leaves are lighter green and smaller than those of the normal branches and flowering is not initiated. In this paper, the procedure of isolation and identification of gibberellin A_1 from the elongated water sprouts is described.

EXPERIMENTAL

The elongated water sprouts (ca. 1.8 kg.), harvested in November, 1957, were divided into leaves (0.6 kg.) and shoots (1.14 kg.), and the latter portion was cut, ground by the blender, immersed in 1.5 l. of 50 per cent aqueous acetone, and extracted overnight at room temperature. After the extraction was repeated, the eluates were combined

and evaporated to a small volume under reduced pressure, and a dense greenish liquid (pH 5.4) was obtained. It was adjusted to pH 8.0 with sodium bicarbonate, extracted five times with ethyl acetate (total volume 1.5 l.) to remove the nonacidic substances. The aqueous layer was then acidified to pH 3.0 with dilute sulfuric acid, and again extracted five times with ethyl acetate (total volume 1.5 l.). Upon evaporation of the solvent, a dark greenish syrup was obtained. This did not show any physiological activity in the gibberellin bioassay.

For the purification of gibberellin-like factor from this crude acidic substance, the countercurrent distribution method was applied [14 plates, ethyl acetate: 1*M* phosphate buffer (pH 5.2)]. After development, the buffer layer of each plate was acidified to pH 2.0 with sulfuric acid, extracted three times with an equal volume of ethyl acetate added to the upper layer, and dried with anhydrous sodium sulfate. The results of this method and subsequent bioassay indicated that the peak was near plate 8.

After the fractions of plates 6 to 10 were combined and countercurrent distribution was repeated, the small portion of plate 8 was spotted on Whatman No. 1 filter paper and developed by ascending method with the solvent system of isopropanol–28 per cent ammonia–water (10:1:1). At the same time, samples of gibberellins A_1 and A_3 were subjected to the same procedure. When the solvent front ascended 24 cm., the paper was dried, divided into twelve sections, and assayed. The physiologically active zone from the water sprouts coincided closely with that of gibberellin A_1 or A_3; the latter were detected also by spraying bromocresol green indicator.

Another collection (7.2 kg.) of water sprouts, harvested in October, 1958, was treated with the same procedure, i.e., aqueous acetone extraction, ethyl acetate extracion, and two countercurrent distributions. The active fraction was poured onto a column of cellulose powder (3 × 32 cm., Whatman No. 1), and eluted with the solvent system of isopropanol–28 per cent ammonia–water (10:1:1) at the flow rate of 1 drop per 2 sec. Ten ml. fractions of eluates were collected and the fractions 4 to 7 were combined and yielded 98 mg. of a colorless oily substance. The material was then spotted on sheets of Whatman No. 1 filter paper (four sheets, 8 × 40 cm.) and developed with the solvent system described above. The appropriate areas, detected by guide spots of authentic specimen on both sides of paper sheets, were cut out and eluted with methanol (5 ml. per area).

Then, in order to change the above ammonium salt solution to free acid, the eluate was passed through a short column (0.8 × 5 cm.) of Amberlite IR 120 (H^+ form). On evaporating the solvent under reduced pressure, 12 mg. of the colorless amorphous powder was ob-

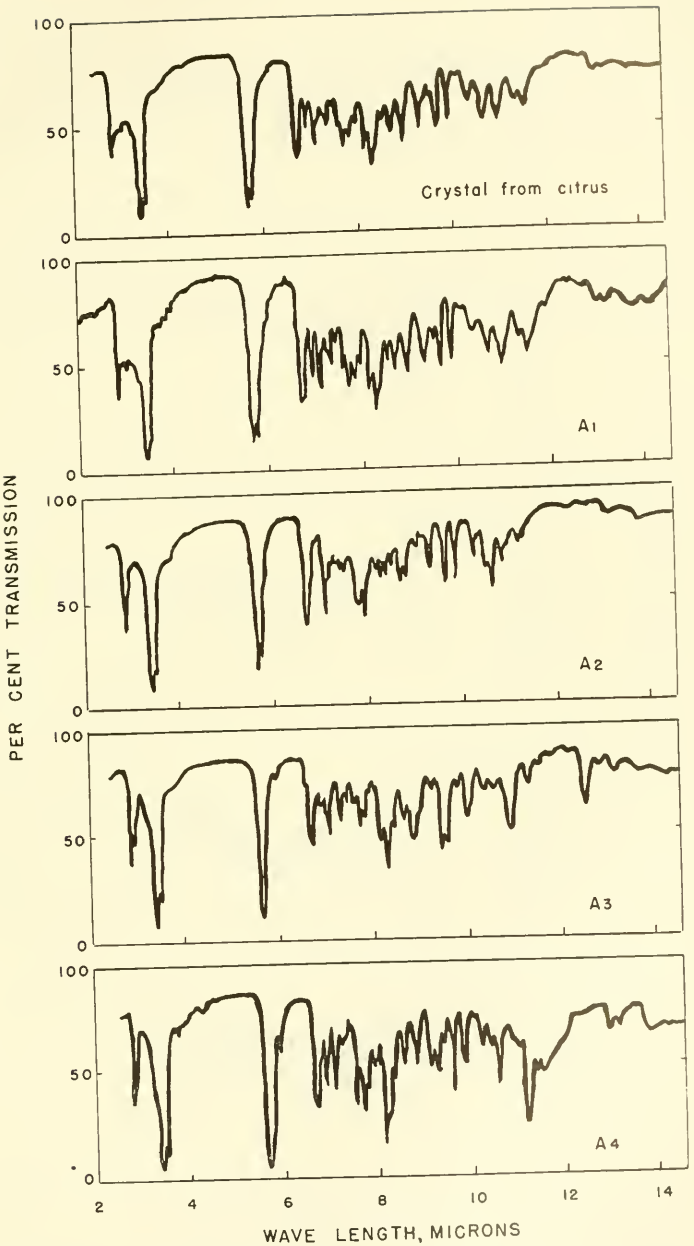

Fig. 1. Infrared spectra of the crystals from *Citrus unshiu* and of the known gibberellins.

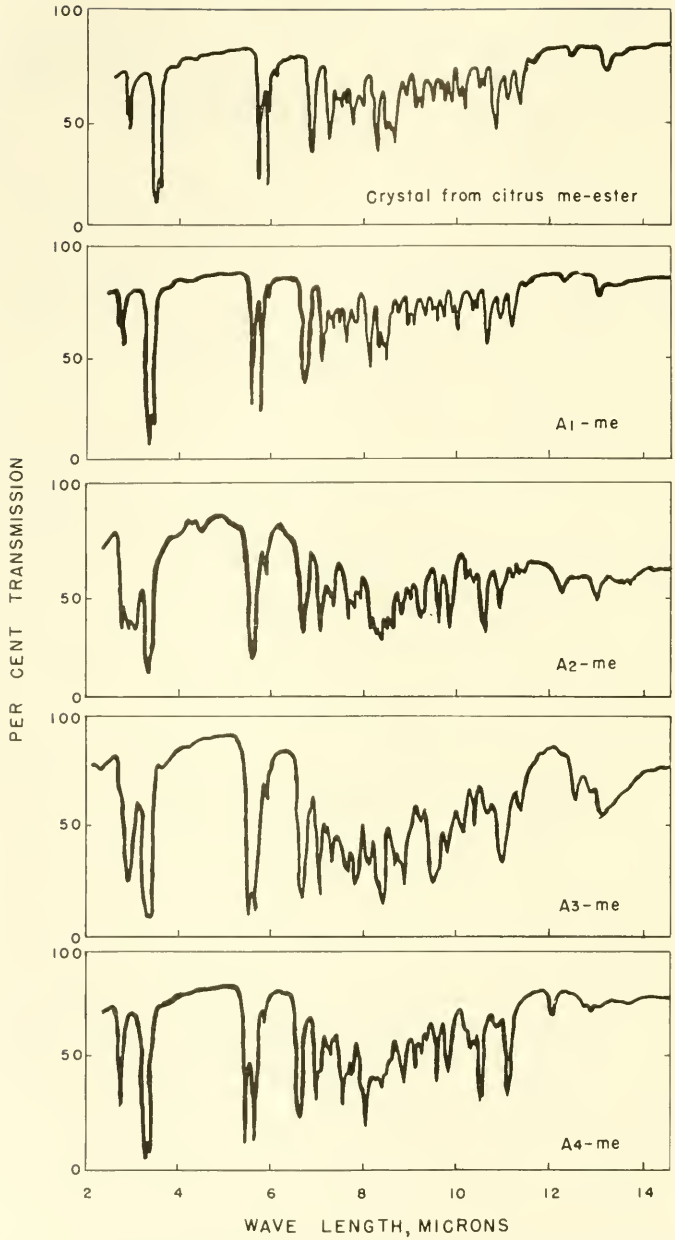

Fig. 2. Infrared spectra of the methyl esters of the crystals from *Citrus unshiu* and of the known gibberellins.

tained which, on crystallization from ethanol–ethyl acetate–ligroin, gave about 2 mg. of colorless prisms. These crystals melted at 230° to 236° C. (decomposition) on a Koffler block, and then infrared spectrum (Nujol) coincided with that of gibberellin A_1 (Figure 1). The infrared spectra of the methyl esters of these compounds again demonstrated the identity of the *Citrus* gibberellin with gibberellin A_1 (Figure 2).

SUMMARY

Gibberellin A_1 was isolated from the elongated water sprouts of a bud variation of *Citrus unshiu*. Its identity was established from infrared spectra and physiological properties.

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Dwarfing Genes in Zea mays *and Their Relation to the Gibberellins*[1]

Evidence has accumulated in the field of biochemical genetics to support the hypothesis that the gene acts as a physiological unit through the control of a single chemical reaction (4, 6). Thus, a mutant gene may determine a particular phenotype by interfering with a specific step in a sequence of chemical reactions leading to a particular product. This product may be any one of a number of substances necessary for normal growth. The accumulated evidence also suggests that nonallelic mutant genes concerned with the same growth substance control different steps in the reaction sequence leading to this substance.

In *Zea mays* L. there are more than twenty mutant genes[2], each of which results in the dwarf habit of growth (1, 2, 3). The nonallelism is well established for all but one of the ten dwarfing genes used in the studies reported here; nine of them are simple recessives, one is a simple dominant. The knowledge of the precise genetical basis for the dwarf habit of growth allows for the interesting speculation that the normal allele of each dwarfing gene in some way controls the presence, or at least the availability, of a substance necessary for normal growth. Studies on the physiology and biochemistry of these single gene mutants could lead to specific information on the biochemical mechanisms controlling growth. It is the purpose of this

[1] Certain aspects of the studies reported here were supported in part by grants from The University of California Reseach Committee, National Science Foundation (G-3526), and Merck & Co., Inc.

[2] The names and abbreviations for the nonallelic dwarf mutants of *Zea mays* used in this paper are: dwarf 1 (d_1); dwarf 2 (d_2); dwarf 3 (d_3); dwarf 5 (d_5); anther ear 1 (an_1); nana 1 (na_1); nana 2 (na_2); petite 1 (tiny 4963) (pe_1); and midget 2 (midget 8043) (mi_2). The linkage relationship of the mutant, dwarf 8 (dominant dwarf) (d_8), is as yet unknown.

paper to evaluate certain evidence which leads to an interpretation of the physiological action of the dwarfing genes d_1, d_2, d_3, d_5, and an_1 of *Zea mays*.

Both auxins and gibberellins[3] have been studied in their relation to the growth of several of the dwarf mutants of *Zea mays*. While the auxin level from certain of the dwarfs can be shown to be less than that from normals, these mutants, as well as other dwarfs of *Zea mays*, exhibit no growth response to added indole-auxins such as IAA, IAEE, IAN, IBA, or to the auxin NAA, or to kinetin and numerous individual amino acids (5, 7, 9, 14, 15). In contrast, the five mutants d_1, d_2, d_3, d_5, and an_1 respond by normal growth to microgram amounts of the gibberellin GA_3 (8, 9, 13) (Figures 1 and 2). It has been suggested that these GA_3-responding mutants might be controlling different steps in a biochemical pathway leading to the production of a naturally occurring gibberellin which is similar to GA_3 and necessary for the normal growth of *Zea mays* (10, 11). This native gibberellin would then be limiting in the mutants and its absence or presence in limiting amounts responsible for the dwarf habit of growth. Five other mutants, na_1, na_2, d_8, pe_1, and mi_2, give no growth response, or only a slight growth response in the early seedling stages, to added GA_3. It has been suggested that these five nonresponders could have blocks in a pathway subsequent to a GA_3-like compound; or they could be due to blocks in a biochemical pathway or pathways unrelated to the gibberellins (10, 11).

If the interpretation of the GA_3-responding mutants is correct, it is possible to make certain predictions and subject them to experimental tests. Some of the predictions that will be considered are as follows:

(1) It should be possible to find gibberellins producing differential growth responses for the five GA_3-responding mutants. Compounds from different steps in the presumed gibberellin pathway should either be active or inactive for a particular mutant, depending on the position of the mutant block in this pathway. Thus, there should be some gibberellins that produce a normal growth response to four of the five GA_3-responding mutants; others that produce a growth re-

[3] *Auxin* will refer to any native growth regulator found to be active in the *Avena* curvature test or the *Avena* straight growth test. The term gibberellin is used for substances active in the d_1, d_2, d_3, d_5, or an_1 bioassay of *Zea mays*. As used here, the term is further restricted to substances found to contain a fluorene ring system. The term *gibberellin-like* is used for substances having biological properties similar to the gibberellins but for which the chemical properties are unknown. The abbreviations used for certain growth factors considered in this paper are: IAA, indole-3-acetic acid; IAN, indole-3-acetonitrile; IAEE, ethyl indole-3-acetate; NAA, 2-naphthoxyacetic acid; IBA, 4-(indole-3-)-*n*-butyric acid; GA_1, GA_2, GA_3, and GA_4, gibberellin A_1, A_2, A_3, and A_4; and BF-II, the gibberellin, bean factor II.

Fig. 1. Growth response of the mutants, anther ear 1, dwarf 1, dwarf 2, dwarf 3, dwarf 5, and dwarf 8, to gibberellic acid; treated mutants above, controls below. Dominant dwarf is a nonresponder; the other five nonallelic mutants are GA_3 responders. GA_3 was added in aqueous solution to the uppermost unfolding leaves at two- to three-day intervals from the earliest seedling stage to maturity; dosages varied from 1 to 10 μg. per treatment; total amount applied was 300μg.

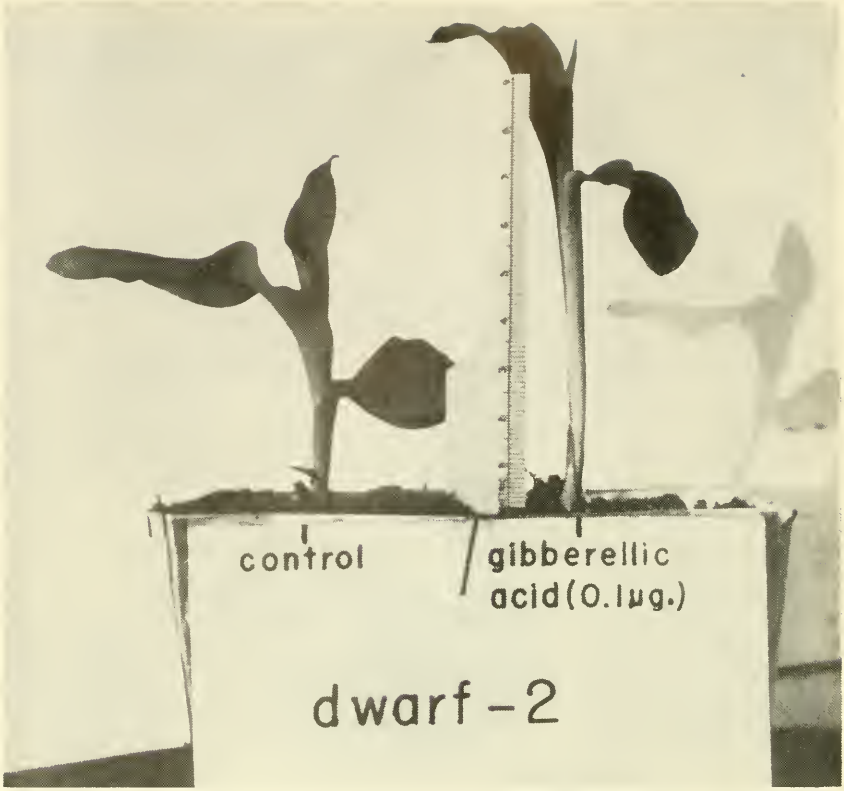

Fig. 2. Response of the first leaf sheath of the mutant, dwarf 2, to a single application of gibberellic acid. Plants photographed 5 days following treatment.

sponse to three of the five; still others that produce a growth response to two of the five, etc. In this way the order of the dwarfing genes in blocking intermediate steps in the gibberellin pathway would be established.

(2) Any gibberellin found to be active in promoting the growth of a dwarf mutant should produce less growth when applied to normal plants, if native gibberellins are the limiting factor distinguishing the two types of growth. Also, gibberellins producing a differential response for a mutant should not produce a differential response when applied to normal plants. This would be expected since the normal form of each dwarfing gene would presumably be able to carry out its function of converting a particular intermediate to the next step leading to a final gibberellin product necessary for growth.

(3) Gibberellins should be present in normal plants and absent, or present in reduced amounts, in the dwarf seedlings. However, the

total amount of gibberellins in normal seedlings may be difficult to evaluate if the active substances represent a mixture of different kinds of gibberellins, some of which are intermediates in the pathway controlled by the normal forms of the dwarfing genes. In this event, the choice of the mutant used for bioassay becomes critical. It is also possible that the dwarf mutants may be accumulating gibberellin intermediates which would be inactive when assayed on the accumulating mutant, yet active on one or more of the other GA_3-responding mutants. The dwarf controlled by the mutant gene farthest back in the presumptive pathway would be the best choice for determining total amount of gibberellins. In the final analysis, quantitative crossfeeding studies with all five GA_3-responding mutants are necessary for the proper evaluation of amounts and kinds of native gibberellins from normal and mutant seedlings.

Experimental tests of the above predictions require the use of a specific and quantitative assay for the detection of gibberellins and gibberellin-like substances, and for the estimation of their relative activities. The dwarf mutants of *Zea mays* have been used for such a purpose because of their specificity, sensitivity, and rapidity of response to the gibberellins (10, 11). For quantitative studies, standard procedures are used for growing, treating, and measuring the growth of the assay plants. Ten mutant seedlings are used for each dosage level of a particular compound or preparation to be tested for activity. Mutant seedlings are treated by placing 0.1 ml. of the material to be assayed into the first unfolding leaf as it emerges from the coleoptile. The test plants are grown at temperatures ranging from 25° C. to 35° C. for a period ranging from seven to ten days. The length of the first leaf sheath or the sum of the lengths of the first and second leaf sheaths is used as a measure of response. Dosage-response curves for GA_3 have been found to be linear over the range of 0.001 μg/plant to 1.0 μg/plant when the logarithm of the response is plotted against log dosage (Figure 3). The bioassay is used for quantitative studies only when the curves for the standard and unknown(s) are straight and parallel to each other. Estimations of relative activities are made from graphic analyses of the response curves. The statistical significance of differences in relative activities is determined from analyses of variance of the original response data. Differences in activity of 20 per cent can be shown to be statistically significant at the 5 per cent level. For the qualitative use of the bioassay, single plants are treated repeatedly with the unknown; growth responses 25 per cent over the largest dwarf control are considered as evidence for activity. All unknowns are run in triplicate. The variables of light, photoperiod, and temperature are minimized by running the standard and

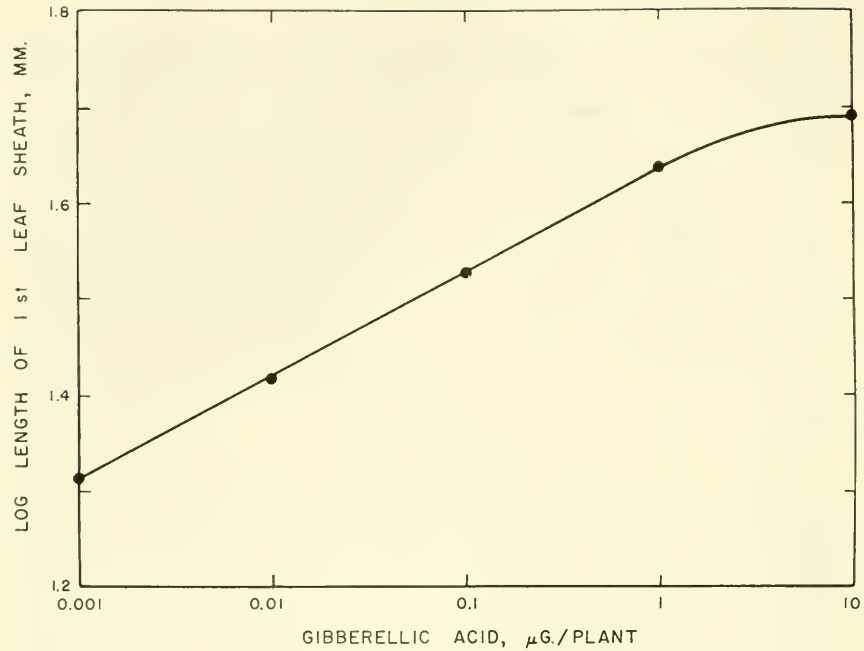

Fig. 3. Dosage response curve for gibberellic acid, using the mutant, dwarf 1, for bioassay. Measurements are for length of the first leaf sheath only. Each point represents the mean of 10 measurements. When the log of lengths of both the first and second sheaths is used, the dosage-response curve is linear over the range of 0.001 to 10.0 μg. per plant. Data from Neely (7).

unknown(s) simultaneously as a single bioassay. Surfactant effects have been found to be an important variable. Introduction of a wetting agent or organic solvent into the solvent solution (water) will increase the sensitivity of the bioassay fifty-fold (Figures 4 and 5). Because of this variable, all solutions to be assayed contain the wetting agent, Tween-20, at the concentration level of 0.05 per cent by volume. Organic solvents, as well as the wetting agent, Tween-20, have been found to give no growth response by themselves.

EXPERIMENTAL RESULTS

Relative Activities of the Gibberellins for Dwarf Mutants of *Zea mays*

The three gibberellins GA_1, GA_2, and GA_3 have been shown to be active in promoting shoot growth for the mutants d_1, d_2, d_3, d_5, and an_1 (9, 13). The relative activities of these three gibberellins and the gibberellin, BF-II, have been determined for the GA_3-responding mutants (Table 1). Each gibberellin was applied to sets of ten mutant seedlings at three dosage levels for the mutants d_1, d_2, d_3, and d_5 and

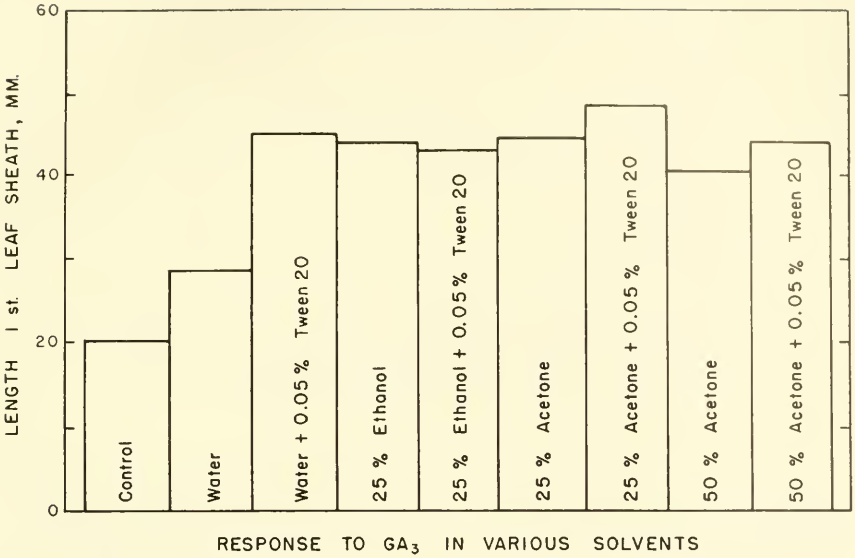

Fig. 4. Effect of solvents and of Tween-20 on the gibberellin response of the mutant, dwarf 1. Values are averages for the first leaf sheaths of ten seedlings. Data from Neely (7).

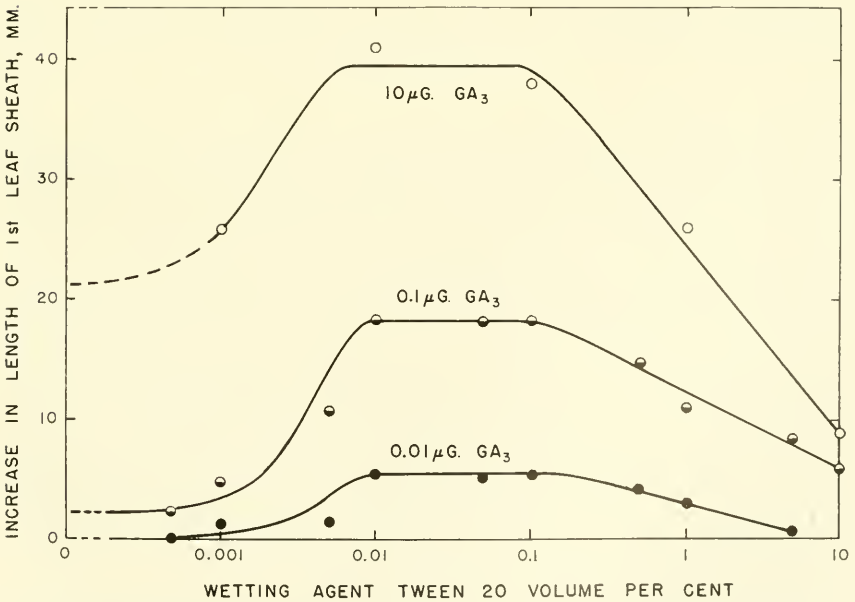

Fig. 5. The effect of various concentrations of Tween-20 on the gibberellin response. Data from Neely (7).

Table 1. Relative activities of the gibberellins, GA_1, GA_2, GA_3, and BF-II for the mutants dwarf 1, 2, 3, and 5 of *Zea mays*.

	Activity of Gibberellins Expressed as Per Cent of Activity of GA_3†		
Mutant*	GA_1	GA_2	BF-11
Dwarf 1 . . .	30	4	5
Dwarf 2 . . .	35	5	210
Dwarf 3 . . .	41	5	250
Dwarf 5 . . .	35	5	98

* From one bioassay run at one dosage level, the relative activities of the gibberellins for the mutant, *anther ear*-1, were very similar to those for the mutant, d_5.

† Each value is the average from four quantitative bioassays, each assay run at three dosage levels.

to sets of ten seedlings at one dosage level for the mutant an_1. Gibberellin A_1, GA_2, and GA_3 show an order of activity of $GA_3 > GA_1 > GA_2$ for all five mutants. Preliminary experiments suggest that GA_4 is intermediate in activity between GA_3 and GA_1. In contrast to the similar order of activities of these gibberellins for the GA_3-responding mutants, bean factor II is 5 per cent as active as GA_3 for the mutant d_1 (Figure 6); it is as active or more active than GA_3 for the mutants d_2, d_3, d_5, and an_1. At dosage levels of 0.1 μg/plant, BF-II produces no growth response for the mutant d_1, and a normal type growth response for the other four GA_3-responding mutants.

Relative Activities of the Gibberellins for Normal *Zea mays*

The relative activities of the gibberellins GA_1, GA_2, GA_3, and BF-II have been determined using normal *Zea mays* seedlings for bioassay. The seedlings were treated and measured in the same manner as described for the quantitative use of the dwarf bioassay. GA_3 and BF-II were applied at three dosage levels, GA_1 and GA_2 at only one dosage level. The activities of GA_3 and BF-II were found to be very similar to each other (Figure 6). Comparison of dosage-response curves suggests that the order of one hundred times as much GA_3 is necessary for an initial growth response for normal seedlings as compared to the GA_3-responding dwarf seedlings. Gibberellin A_1 and GA_2 were also found to produce a smaller growth response for normal seedlings than for the dwarf mutants. The order of activity for normal seedlings of *Zea mays* appears to be $GA_3 = \text{BF-II} > GA_1 > GA_2$.

Gibberellin-Like Substances From *Zea mays*

Gibberellin-like substances have previously been reported from immature normal kernels of *Zea mays* using the mutant d_1 for bio-

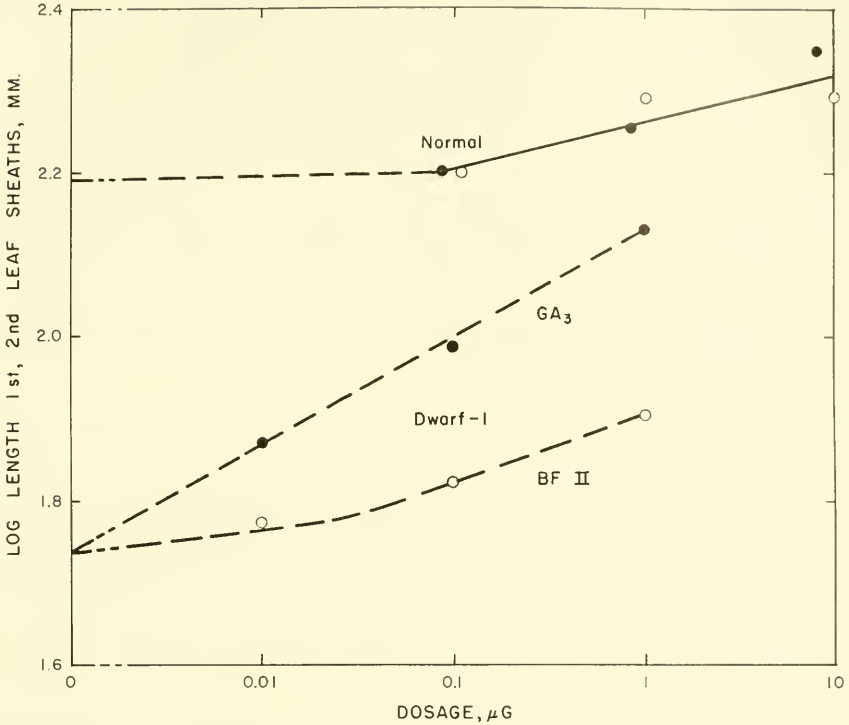

Fig. 6. Quantitative bioassays for gibberellic acid and bean factor II using normal and dwarf 1 seedlings. Each point represents the average of ten measurements of the sums of the first and second leaf sheaths.

assay (13). Experiments reported here with the mutant d_5 for bioassay show the presence of gibberellin-like substances from seedling shoots of normal *Zea mays,* their absence, or presence in reduced amounts, from the mutants d_1, d_2, d_3, d_5, and an_1.

Both normal and mutant seedlings were grown in the greenhouse for periods ranging from 2 to 6 weeks. Five hundred gram samples of the shoots were harvested and stored at — 20° C. Acetone-water extracts were obtained from duplicate samples of each type of material and purified according to methods developed by West (16, 17). Silicic acid chromatography (16) was used as the final step of purification for one sample, paper chromatography was used as the final step of purification for the second sample. Each fraction obtained from elution of the columns and from elution of the chromatograms was assayed for the presence of gibberellin-like substances. Evidence for activity was based on the qualitative use of the d_5 bioassay. As a measure of the total response from any one extract, the response data for all active fractions from any one extraction were added together.

The sums obtained from different extractions were then compared with each other as a crude estimate of relative activities.

Both methods of chromatography revealed two zones of gibberellin-like activity from extracts of normal seedlings. Activity from silicic acid chromatography appeared following elution with 20 per cent ethyl acetate in chloroform, and again following elution with 60 per cent ethyl acetate in chloroform; likewise two zones of activity were found by paper chromatography, these being at R_f values of 0.15 and 0.43. Gibberellin-like activity was obtained from as little as 100 grams of shoot tissue, fresh weight. Gibberellin A_3 controls showed activity from silicic acid chromatography in the fraction following elution with 20 per cent ethyl acetate in chloroform, and at an R_f value of 0.41 from paper chromatography.

No gibberellin-like activity was obtained from extracts of the mutants d_3, d_5, and an_1 either by paper chromatography or by silicic acid chromatography. Additional extractions from 1 kg. samples failed to reveal any evidence of activity. Extracts from the mutants d_1 and d_2, however, showed evidence of the presence of gibberellin-like substances from silicic acid chromatography and from paper chromatography. Two regions of activity were present which had positions indistinguishable from those obtained from normal seedlings. Comparisons of the total responses from the active fractions of d_1, d_2, and normal material suggest that these mutants contain less than half as much total gibberellin as do the normals. The responses were lower whether compared on a fresh weight, dry weight, or per plant basis.

Cross-Feeding Studies

Only preliminary studies have been made to test for the accumulation of unique gibberellin-like substances by the dwarf mutants of *Zea mays*. Qualitative bioassays show the gibberellin-like substances from the mutants d_1 and d_2 and from normal seedlings to be d_1 inactive and d_3, d_5, and an_1 active. All fractions obtained from extracts of the mutants d_3, d_5, and an_1 have been inactive when tested on the five GA_3-responding mutants. While the over-all activity obtained from d_1 and d_2 mutants is appreciably less than from normal plants, regardless of the mutant used for bioassay, it should be emphasized that more careful purification followed by the quantitative evaluation of each active fraction is necessary for a proper evaluation of the cross-feeding studies. The fractions obtained from extracts of normal seedlings and from the dwarf mutants have not been tested for activity on normal seedlings.

DISCUSSION AND CONCLUSIONS

Evidence is presented in this paper to support the interpretation that the five mutant genes d_1, d_2, d_3, d_5, and an_1 are responsible for

the dwarf habit of growth through the control of the amount of native gibberellins in *Zea mays*. It is suggested that these genes interfere with different steps in a gibberellin pathway leading to a product necessary for the normal growth of *Zea mays*.

The normal type growth response of the five mutants to the gibberellins, combined with their lack of response to other known plant growth regulators, implicates gibberellin as the limiting factor responsible for the dwarf habit of growth. The correlation of presence of gibberellin-like substances in normal plants, and the lowered amounts or absence in the mutants are further evidence for a causal relation between gibberellin and the dwarf habit of growth. The relatively small response to added gibberellin found for normal plants would be expected if native gibberellins were less limiting in normal plants than in the dwarf mutants.

The response of all five mutants to gibberellic acid suggests that GA_3 or a compound very similar to it occupies a position in a metabolic pathway in *Zea mays* subsequent to the steps controlled by the five dwarfing genes. While the gibberellins GA_1, GA_2, and GA_3 exhibit the order of activity of $GA_3 > GA_1 > GA_2$, this order is the same for all five mutants and for normal plants. Such similarities in relative activities would exclude any interrelationship between these gibberellins controlled by the five dwarfing genes. The similarity in the relatively high activities of GA_3 and BF-II on the four mutants d_2, d_3, d^5, and an_1, contrasted with the high activity of GA_3 and the low activity of BF-II for the mutant d_1, suggests a relationship between these two gibberellins controlled by the d_1 gene; bean factor II would be an intermediate the conversion of which to gibberellic acid is blocked by the d_1 gene. The other four genes could then control steps in the gibberellin pathway prior to compounds having properties similar to GA_3 and BF-II. Further support of this interpretation is given by the lack of a differential response of normal seedlings to GA_3 and BF-II.

The difference in auxin level between normal and dwarf seedlings of *Zea mays* can be attributed to an indirect effect of the dwarfing genes. A lower auxin level would then be the result of the reduced amounts of native gibberellins in the mutants. The lack of response of the dwarf mutants of *Zea mays* to a number of auxins is in agreement with this interpretation.

The accumulation of inhibitors of gibberellin-induced growth could be an obvious alternate explanation for the dwarf habit of growth in *Zea mays*. Added gibberellin would then be expected to overcome this inhibition effect, resulting in a normal growth response for the GA_3-responding mutants. Such an explanation would require a series of inhibitors, one specific for gibberellic acid, another specific

for bean factor II, etc. As yet there is no evidence to suggest such an alternate explanation.

The evidence presented in this paper suggests that the primary action of the mutant genes d_1, d_2, d_3, d_5, and an_1 is to control the amount of native gibberellins in *Zea mays* by interfering with different steps in a biochemical pathway leading to a gibberellin product necessary for normal growth. The present evidence would suggest that the gene d_1 is controlling a terminal step in this series. The lower auxin level found in certain dwarf mutants of *Zea mays* is attributed to an indirect effect of the dwarfing genes.

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DISCUSSION

Dr. Barton: Dr. Phinney has mentioned that genetic dwarfs are controlled by genes, as of course they are, but we have found that physiologic dwarfs can also be stimulated to elongate by the use of gibberellic acid. These physiologic dwarfs are those of peach, apple, crabapple, etc., dwarfed because the seeds were not given low temperature pre-treatment. Is this also controlled by genes?

Dr. Phinney: It cannot be said that a particular physiological property is controlled by a *single gene* until the inheritance pattern is known for this property.

Dr. Galston: I would like to ask a question based on the point that Dr. Barton raised, i.e., the whole problem of whether any of the overgrowths noted in pathology or in commensalisms can be explained in terms of abnormal production of GA or its analogs. Several years ago we found that when we applied GA to some dwarf bean plants, the number of nodules on the roots was depressed. This has been confirmed in various ways by Dr. Brian and by Dr. Kefford, the latter using sterile culture. Now, I wonder whether there is any possibility that nodule formation may be in some way related to the fact that the plant has a sub-optimal level of gibberellin, and that the bacterium causes a localized production of something like gibberellin, which gives to the cells in that region a selective growth advantage. The application of gibberellin to such a plant could remove that selective advantage, thus repressing nodule growth. My question should really be addressed either to Dr. Stodola or to Dr. Brian, who have worked with microbial fermentations. Is there any evidence that any organisms related to the nodule-forming forms produce anything like gibberellin, either in culture or in contact with plant cells?

Dr. Brian: There is not to my knowledge. So far as I know, there's just one *Gibberella fujikuroi*. There is a recent Russian claim of a yeast which produces GA. So far as I know, these are the only two microorganisms ever suspected of producing gibberellins.

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Relation Between Chemical Structure and Physiological Activity

For the purpose of elucidating the growth regulating activity of gibberellins, some 20 derivatives or degradation products were prepared and their physiological activity observed using rice seedlings.

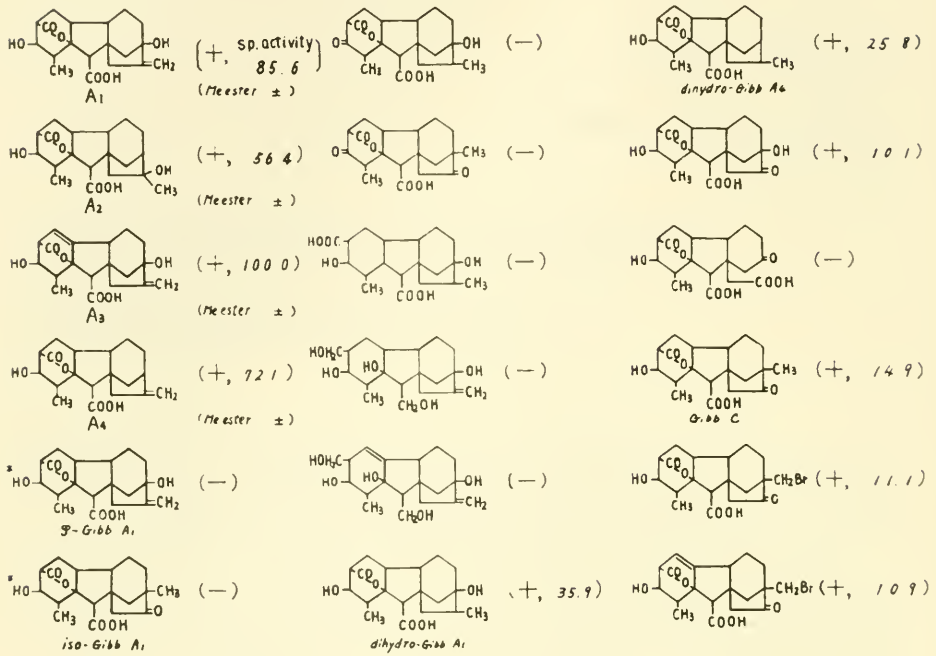
Gibberellins have recently come to be recognized as important in the growth regulating system of higher plants, and the chemical constitutions of gibberellins are almost established (1, 2, 3, 4, 5) though much remains to be elucidated about their stereochemistry. The authors believe it is of considerable interest to study the relation between the chemical constitution and physiological activity of gibberellins.

Moreover, the authors expect that the determination of the partial structures essential for showing growth response will be a guide for the synthesis of new gibberellin-like substances having more simplified structures than the native gibberellins.

The compounds, i.e., the four gibberellins and their derivatives or degradation products, were examined as to their purity by paper chromatography. The physiological activity was observed by measuring the length of the second leaf-sheath of rice seedlings on incubation at a concentration of 10 μg/ml as described in a previous paper.

The results are illustrated in Figure 1. The values indicate the relative activity of each compound when that of gibberellin A_3 is assumed to be 100. Data in Figure 1 may be summarized as follows: (1) On A-ring — δ-lactone ring and secondary hydroxyl group are essential, and by the inversion of stereochemical configuration of hydroxyl activity is lost. On B-ring — physiological activity is much reduced where the carboxyl group is masked by methylation. On C- and D-rings — (a) exocyclic methylene, or double bond, is not essential

Relation Between Chemical Structure and Physiological Activity



but activity is decreased on catalytic hydrogenation or ozonolysis; (b) also, tertiary hydroxyl (bridge head) is not important; (c) by opening D-ring, the activity disappears.

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Biological Evaluation of Gibberellins A_1, A_2, A_3, and A_4 and Some of Their Derivatives

Early difficulties experienced by American and British scientists in the production and isolation of gibberellin A,[2] as announced by Yabuta and Sumiki (20), eventually led to the characterization of several fungal gibberellins. Stodola *et al.* (14) announced the isolation of gibberellin A and a new gibberellin designated as gibberellin X. Concurrently, Curtis and Cross (7) isolated gibberellic acid, which was found (6) identical to Stodola's gibberellin X, but differed from the Japanese gibberellin A. The Japanese workers then re-examined their product and found a mixture of three gibberellins, which they termed gibberellins A_1, A_2, and A_3 (17). Gibberellin A_1 was identical to Stodola's A and gibberellin A_3 to gibberellin X and gibberellic acid. Takahashi *et al.* (18) next reported the isolation of gibberellin A_4. Henceforth, the four fungal gibberellins will be referred to as gibberellins A_1, A_2, A_3, and A_4.

Extensive field and greenhouse experiments have now been conducted and the results summarized (1, 15, 16, 19) for a wide variety of plant and crop responses produced with commercial gibberellin preparations consisting largely of A_3 or undetermined mixtures of A_1 and A_3. Meanwhile, little attention has been devoted to the biological effects of gibberellins A_2 and A_4. With the exception of some preliminary reports (3, 5, 9, 12), no critical studies of the effects of the four fungal gibberellins and their derivatives on diverse responses of higher plants have appeared.

[1] Journal article no. 2445 from the Michigan Agricultural Experiment Station.
[2] Initially named gibberellin B and changed to gibberellin A (20, 21).

COMPARATIVE BIOLOGICAL ACTIVITIES OF GIBBERELLINS A_1, A_2, A_3, AND A_4

Vegetative Extension

Epicotyl elongation in beans (Phaseolus vulgaris, 'Blue Lake'). Bean seedlings were germinated in quartz sand, and transferred to aerated solution cultures containing a complete nutrient solution when the primary leaves were approximately 50 per cent expanded (4). After 24 hrs. 10 μl. of a 3×10^{-5}, 3×10^{-4}, or 3×10^{-3} *M* solution of gibberellins A_1, A_2, A_3, or A_4 (Table 1) were applied to the terminal bud (Figure 1) or to the upper surface near the base of one of the primary leaf blades. Epicotyl elongation was determined 48 or 96 hours following treatment.

Application of gibberellin A_1 or A_3 to the leaf blades resulted in plants with significantly longer epicotyls than plants similarly treated with A_2 or A_4 (Figure 2 and Table 2). Length of epicotyls of plants treated with gibberellin A_2 on the leaf blades did not differ significantly from the controls. All gibberellins when applied to the terminal bud resulted in plants with epicotyls that were significantly longer than those on the controls. Gibberellins A_1, A_3, and A_4, however, were slightly more effective than A_2 (Table 2). This same relationship held for all three concentrations of the various gibberellin solutions.

Petiole elongation in celery (Apium graveolens, 'Utah 10-B'). Seedlings were started in sand, selected for uniformity of fresh weight and petiole length, and transferred to solution cultures at the three to four true leaf stage. Ten μl. of a 3×10^{-4} or 3×10^{-3} *M* solution of gibberellins A_1, A_2, A_3, or A_4 were applied to the youngest unfolding leaf 96 hrs. after transfer of the plants to solution cultures. The length of the second outer petiole was recorded 14 days later. Treatment

Table 1. Characteristics of the gibberellins assayed.

Gibberellin	Empirical Formula	Melting Point* (degrees C.)	Optical Rotation (degrees)
A_1	$C_{19}H_{24}O_6$	232–35	$[\alpha]_D^{28}$ + 42.3
A_2	$C_{19}H_{26}O_6$	235–37	$[\alpha]_D^{15}$ + 11.7
A_3	$C_{19}H_{22}O_6$	232–35	$[\alpha]_D^{20}$ + 92.0
A_4	$C_{19}H_{24}O_5$	222	$[\alpha]_D^{20}$ − 20.8

* Decomposition.

Fig. 1. Placement of solution on the vegetative apex for assay of gibberellin in the bean epicotyl elongation test.

Table 2. Comparative biological activities of gibberellins A_1, A_2, A_3, and A_4 (expressed as per cent of control).

Assay	Microliters Applied ($3 \times 10^{-3} M$)	A_1	A_2	A_3	A_4	Least Significant Differences (P = .01)
Epicotyl elongation in beans						
Leaf blade application	10	340.0	120.0	390.0	170.0	70.0
Terminal bud application	10	480.0	370.0	500.0	450.0	70.8
Petiole elongation in celery	10	219.6	160.9	217.4	187.0	17.4
Stem elongation in cucumbers	10	153.8	117.5	132.9	279.1	11.5
Acceleration of flowering in lettuce						
Heading type ('Great Lakes')	30	77.6	93.2	72.7	95.6	4.3
Leaf lettuce ('Grand Rapids')	30	84.8	96.2	80.3	96.2	3.8
Growth of tomato ovaries	10	179.0	183.0	166.0	179.0	37.0
Overcoming photo-induced dormancy in *Weigela*	40	14.0	0.0	38.0	1.0	3.4
Stimulation of lettuce seed germination	*	455.8	402.1	481.1	465.3	7.2

* Two ml. of a 100 p.p.m. solution per 6-cm. petri dish.

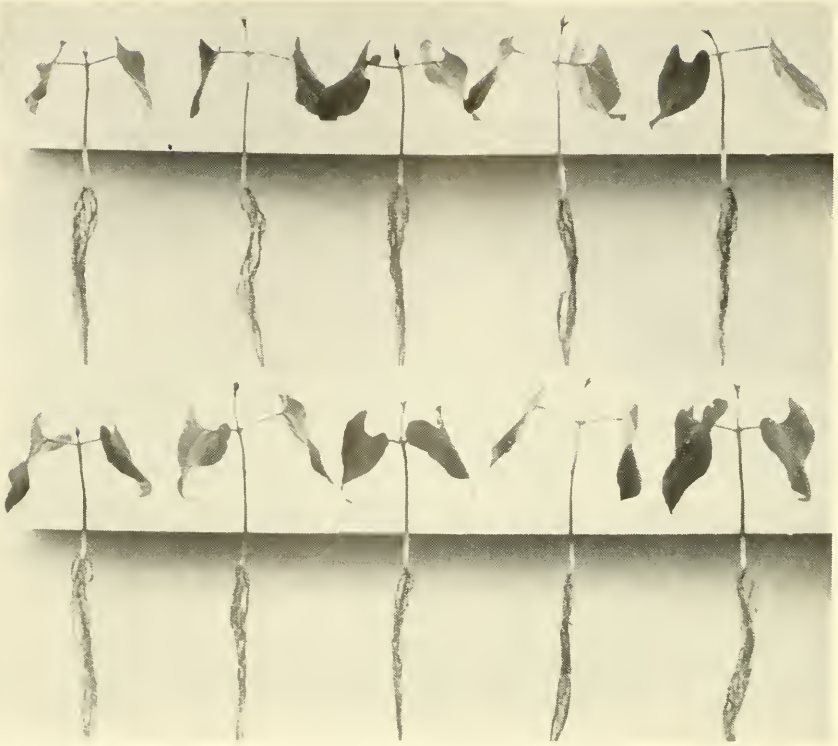

Fig. 2. Growth of bean epicotyls following application of 10 μl. of 3×10^{-3} *M* solution of gibberellins A_1, A_2, A_3, or A_4 to one of the primary leaf blades (**top**), or to the terminal bud (**bottom**). **Left to right:** control, A_1, A_2, A_3, and A_4 (photographed 96 hrs. after treatment).

with 3×10^{-4} *M* of A_1, A_3, or A_4 produced plants with significantly longer petioles than nontreated controls. Plants treated with A_2 did not differ from the controls. Petiole elongation was equally stimulated by A_1 and A_3 at 3×10^{-3} *M*, and significantly more so than with A_2 and A_4 (Table 2).

Stem elongation in cucumber (*Cucumis sativus, 'Burpee Hybrid'*). Cucumber seeds were germinated in vermiculite and transplanted into soil after the cotyledons had emerged. Twenty-four hours later, a ten μl. aliquot of a 3×10^{-5}, 3×10^{-4}, or 3×10^{-3} *M* solution of gibberellin A_1, A_2, A_3, or A_4 was applied to the terminal bud. At all concentrations, A_4 was strikingly more effective than A_1, A_2, or A_3 in stimulating stem elongation (Table 2). The comparative stem elongation of the 'Burpee Hybrid' cucumber and 'Blue Lake' bean following application of various amounts of gibberellins A_3 and A_4 to the terminal bud is illustrated in Figure 3.

Fig. 3. Stem elongation of the 'Burpee Hybrid' cucumber and the 'Blue Lake' bean, 120 hrs. subsequent to terminal bud applications of gibberellins A_3 (**left**) and A_4 (**right**). **Top to bottom:** 10 μl. per plant of 3×10^{-3}, 3×10^{-4}, 3×10^{-5} *M*, and water (control).

Flowering in lettuce (Lactuca sativa, 'Great Lakes' and 'Grand Rapids'). Plants were grown in pot cultures in a greenhouse under the prevailing winter photoperiod (9 to 11 hrs.) and at a night temperature of 18° C. After six to eight true leaves had developed, 3×10^{-3} *M* solutions of the various gibberellins were applied to the stem apices, and the treatment repeated after two and four weeks.

Gibberellins A_1 and A_3 were considerably more active than A_2 and A_4, both in accelerating flowering (Table 2) and stimulating seedstalk elongation (Figure 4). While no significant differences were noted

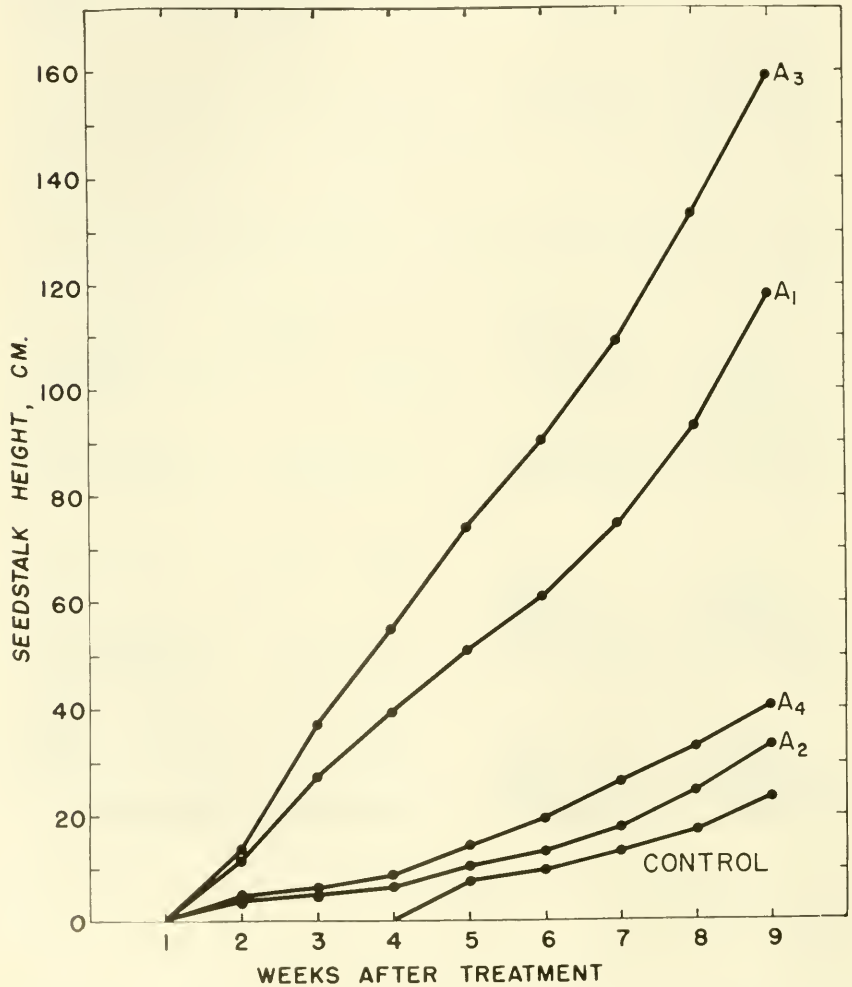

Fig. 1. Rate of seedstalk elongation in 'Grand Rapids' leaf lettuce following application of gibberellins A_1, A_2, A_3, and A_4 to the youngest unfolding leaf. (Three applications of 10 μl. of 3×10^{-3} *M* solutions at 2-week intervals.)

between A_2 and A_4, gibberellin A_3 was slightly more active than A_1. All plants of both varieties treated with any of the gibberellins flowered in fewer days (Table 2) than the controls.

'Great Lakes' lettuce plants treated with A_1 or A_3 did not head and seedstalks commenced to elongate within ten days (Figure 5). Plants treated with A_2 and A_4 headed, as did the controls, and the seedstalks, as with controls, later emerged through the heads. At the time of first visible flower primordia, seedstalk heights for plants treated with A_1, A_2, A_3, and A_4 were 41, 28, 84, and 29 cm., respectively, and 30 cm. for controls.

The effects of different levels (two applications of 1, 10, 50, 100, and 200 μg. per plant) of each of the gibberellins on flowering of lettuce were next determined. Increasingly greater amounts of A_1 or A_3 resulted in progressively taller seedstalks, and hastened flowering in

Fig. 5. Response of 'Great Lakes' head lettuce to treatment with gibberellins. **Left to right:** control, A_1, A_2, A_3, and A_4. Note the absence of heading in A_1 and A_3 treated plants. (Three applications of 10 μl. of 3×10^{-3} *M* solutions at 2-week intervals.)

both varieties. Increasingly greater amounts, however, of A_2 or A_4 did not accelerate flowering, but the repeated dosages of 100 and 200 μg. per plant resulted in seedstalks that were slightly taller than the controls. 'Great Lakes' head lettuce plants treated with gibberellins A_2 or A_4, up to total dosages of 400 μg. per plant subsequently produced heads, as did the controls, and seedstalks later emerged through the heads. By contrast, as little as 20 μg. of A_1 or A_3 resulted in immediate bolting, and head formation was bypassed.

Parthenocarpic fruit growth of the tomato (Lycopersicon esculentum, 'Michigan-Ohio Hybrid'). Tomato ovaries, were emasculated approximately 24 hrs. before anthesis, and treated with ten μl. of a 3×10^{-5}, 3×10^{-4}, or 3×10^{-3} *M* solution of each gibberellin. As controls for comparison, ovaries were also treated with indole-3-acetic acid (IAA) and *p*-chlorophenoxyacetic acid (ClPA). Additional controls consisted of emasculated and non-pollinated, as well as pollinated ovaries. Three single plant replicates, each with 3 ovaries on the first flower cluster comprised a treatment. The diameter of the ovaries was measured after six days.

Ovaries treated with the 3×10^{-5} *M* concentration showed greater growth with gibberellins A_1, A_3, or A_4 than with IAA or ClPA, but significantly less than the pollinated control. While gibberellin A_2 was not active at 3×10^{-5} *M*, it was as effective as any of the gibberellins at 3×10^{-3} *M*. At the latter concentration all gibberellins produced ovaries of approximately the same size as the pollinated controls and IAA, but smaller than those treated with ClPA (Table 2).

Dormancy

Dark-induced dormancy in seed of lettuce (Lactuca sativa, 'Grand Rapids'). 2 ml. of a 100 p.p.m. solution of gibberellins A_1, A_2, A_3, A_4, or distilled water were added to 6-cm. petri dishes containing 50 seeds on Whatman No. 1 filter paper. Seeds were germinated in the dark at 26° C. for 96 hrs. All four gibberellins effectively replaced the light requirement for germination (Table 2).

Photo-induced dormancy of Weigela ('Vanicek'). Dormancy was induced in *Weigela* plants by exposure to a short (9 hr.) photoperiod. Ten μl. (3×10^{-3} *M*) of gibberellins A_1, A_2, A_3, or A_4 were then applied to the dormant terminal buds at weekly intervals for four weeks. Dormancy was partially broken by gibberellins A_1 and A_3 but not with A_2 or A_4 (Table 2).

EVALUATION OF SEVERAL ESTERS OF GIBBERELLIN A_3

A homologous series of *n*-alkyl esters of gibberellin A_3 (13) was prepared with the appropriate alkyl iodide (10). Physiological activity

Table 3. Characterization of several *n*-alkyl esters of gibberellin A_3 and their effect on lettuce seed germination in the dark (13).

Ester	Empirical Formula	Melting Point, Degrees Centigrade	Germination of Lettuce Seed, Per Cent
Control	.....	.....	41.2*
H (gibberellin A_3)	$C_{19}H_{22}O_6$	232–35	80.6
Methyl	$C_{20}H_{24}O_6$	202	63.5
Ethyl	$C_{21}H_{26}O_6$	155	78.4
n-Propyl	$C_{22}H_{28}O_6$	138	68.9
n-Butyl	$C_{23}H_{30}O_6$	145	83.7
n-Amyl	$C_{24}H_{32}O_6$	165–66	79.1
n-Hexyl	$C_{25}H_{34}O_6$	188–89	56.8
n-Heptyl	$C_{26}H_{36}O_6$	181–82	57.2
n-Octyl	$C_{27}H_{38}O_6$	157–58	48.0
n-Nonyl	$C_{28}H_{40}O_6$	131–32	46.7
n-Decyl	$C_{29}H_{42}O_6$	102.5–108.5	40.0

* L.S.D. at P .05: 11.7; L.S.D. at P .01: 16.4.

was then assayed in terms of the promotion of lettuce seed germination in the dark, stimulation of bean epicotyl elongation, and growth of tomato ovaries.

Germination was significantly enhanced by the methyl, ethyl, *n*-propyl, *n*-butyl, *n*-amyl, *n*-hexyl, and *n*-heptyl gibberellates at 3×10^{-5} *M* (Table 3). The *n*-octyl, *n*-nonyl, or *n*-decyl gibberellates were inactive. Germination from the methyl, ethyl, *n*-propyl, *n*-butyl, and *n*-amyl gibberellates equalled or approached the response from the free acid. By contrast, none of the esters promoted the elongation of bean epicotyls or, over a wide range of concentrations in lanolin (3×10^{-6} to 3×10^{-3} *M*), the growth of tomato ovaries.

In separate experiments, however, the butyl cellosolve ester of A_3 (Merck & Co., Inc.) was found equally as effective as the free acid in promoting lettuce seed germination, tomato ovary growth, and elongation of the bean epicotyl.

COMPARATIVE ACTIVITY OF SEVERAL DERIVATIVES OF GIBBERELLINS A_1, A_3, AND A_4

Several derivatives of gibberellins A_1, A_3, and A_4, procured from Dr. Y. Sumiki, were next assayed for their promotion of epicotyl elongation in bean seedlings and growth of tomato ovaries.

The relative activities of the respective derivatives as related to the parent compounds are given in Figures 6, 7, and 8. Dihydro-gibberellin A_1 (Figure 6, II) and the keto product of dihydro-gibberellin A_1 (Figure 6, III) were slightly active. Compound IV, in which the

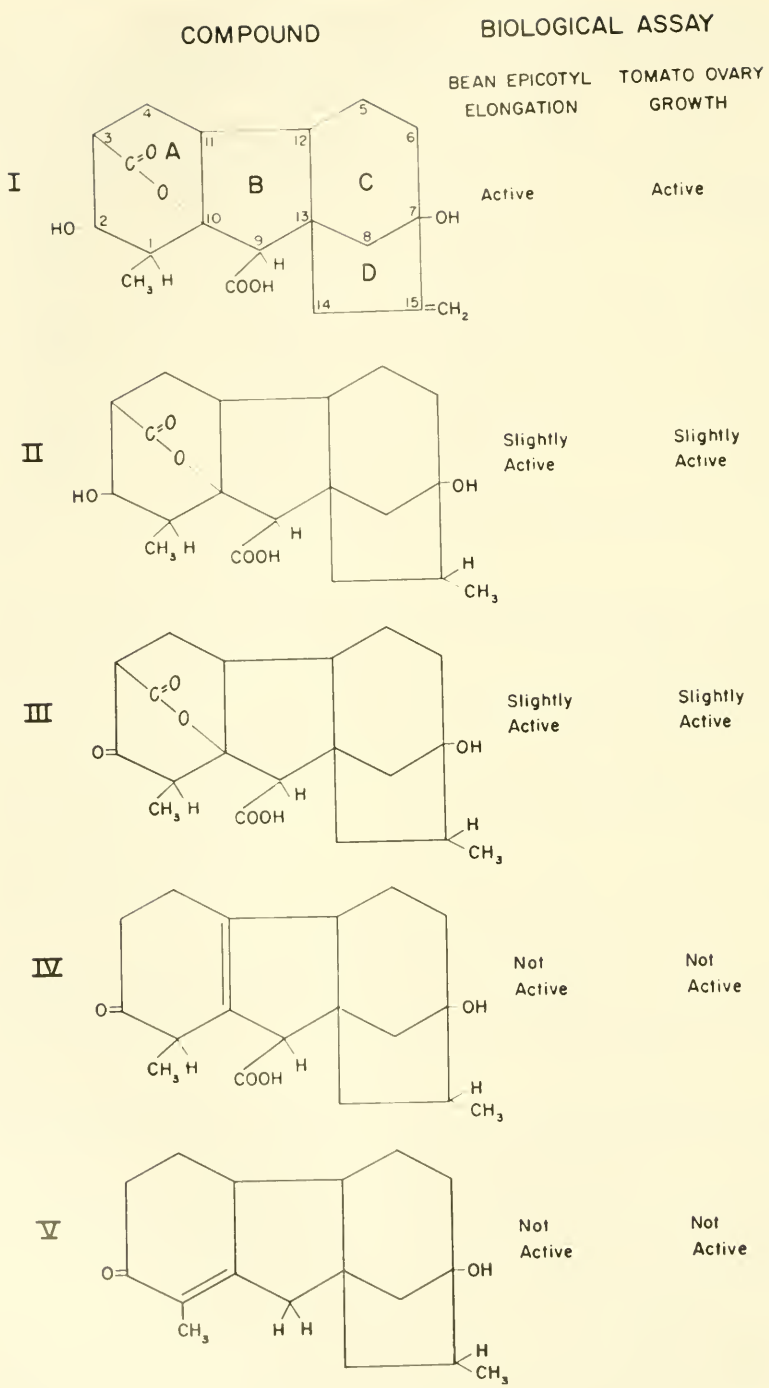

Fig. 6. Relative activity of gibberellin A_1 derivatives in elongation of the bean epicotyl and growth of tomato ovaries.

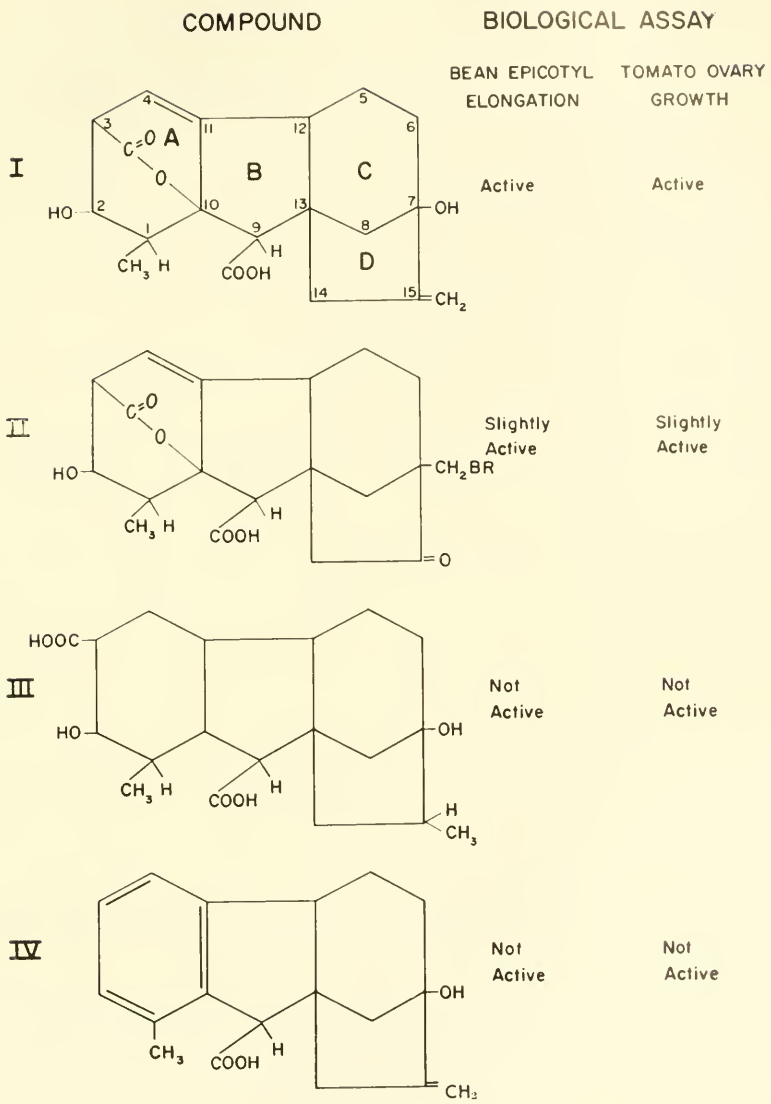

Fig. 7. Relative activity of gibberellin A_3 derivatives in elongation of the bean epicotyl and growth of tomato ovaries.

lactone group on ring A was removed, and compound V, in which the lactone group was removed and the product decarboxylated, were not active. The Wagner-Meerwein derivative of A_3 (Figure 7, II) was slightly active (less than 20 per cent of A_3), while products III and IV (*allo*gibberic acid) were not active. Dihydro-gibberellin A_4 (Figure 8, II) was about 50 per cent as active as A_4. When the D ring

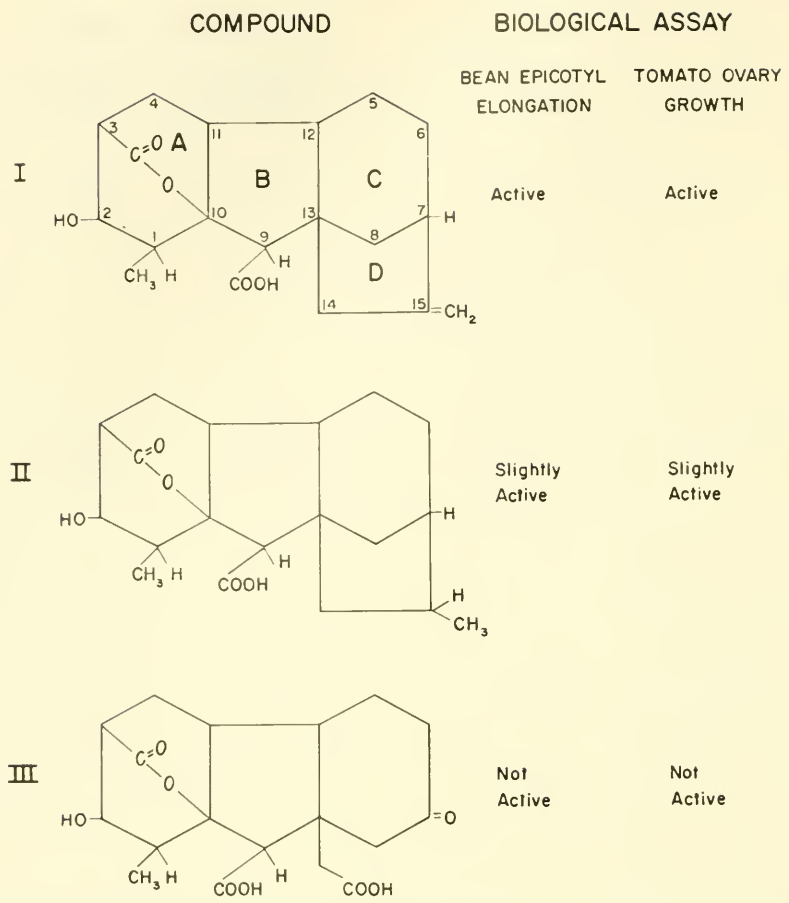

Fig. 8. Relative activity of gibberellin A_1 derivatives in elongation of the bean epicotyl and growth of tomato ovaries.

was ruptured and a keto group substituted for the hydrogen (carbon 7) the compound was inactive (Figure 8, III).

DISCUSSION

The diversity of alterable responses following application of the gibberellins and their derivatives to the intact plant presents unlimited possibilities for assessing comparative biological activity. Only a few representative areas of plant behavior — vegetative extension, flowering, dormancy, and fruit growth — were selected for assay in these studies. As might be anticipated, the biological activities of the four fungal gibberellins and their derivatives varied with the assay, the site of treatment, and the quantity applied.

With stem elongation in the bean, terminal bud applications (10

μl. of 3×10^{-3} *M*) of all gibberellins resulted in comparable activities with epicotyls significantly longer than nontreated controls. The first measurable response on bean epicotyl elongation was observed with ten μl. of gibberellin A_3 at 3×10^{-6} *M*, for A_1 and A_4 at 3×10^{-5} *M*, and 3×10^{-4} *M* for A_2. Gibberellins A_1 and A_3, however, applied to the leaf blade induced significantly greater growth than A_2 or A_4. If the length of the epicotyl following application of each of the gibberellins to the terminal bud was divided by the length of the epicotyl following application to the leaf blade, the ratios for A_1 and A_3 approached unity (Table 2). Gibberellins A_2 and A_4, in contrast, were much more active when applied to the terminal bud than to the leaf blade, and growth from the former was 2 to 3 times greater. These data suggest possible limitations in the absorption and transport of A_2 and A_4 from the primary leaves, and that the rate of penetration and transport of exogenously applied gibberellins from the treatment site to the site of action may be an important consideration in assessing activity by a selected assay.

The strikingly greater effect of gibberellin A_4, in contrast to A_1, A_2, or A_3, on stem elongation of the cucumber is an intriguing departure from the usual response pattern. Such specificity among the gibberellins for any vegetative elongation response has not heretofore been recorded, and has not been duplicated in other bioassays employed. The response of the cucumber to gibberellin A_4 may be utilized as a bioassay for differentiation between naturally occurring A_4 and A_1, A_2, and A_3 in higher plants; and further serves as a guide for the study of the controlling mechanisms in flower sex expression of cucurbits (19).

Quantitative as well as qualitative differences were recorded among the four gibberellins. A_2 did not stimulate the growth of tomato ovaries at 3×10^{-5} *M*, but was highly effective at 3×10^{-3} *M*. In contrast, relatively large quantities (400 μg. per plant) of A_2 and A_4 failed to promote seedstalk elongation in 'Great Lakes' head lettuce, whereas 20 μg. of either A_1 or A_3 per plant induced bolting without heading. The presence of gibberellin A_1 in some higher plants (11, 12) may alter responses to exogenous applications of the fungal extracted gibberellins. Marked specificity among the latter has already been noted for vegetative extension, the most commonly observed gibberellin effect. As more species and responses are examined these relationships will undoubtedly be multiplied.

Esterification of the carboxyl group of gibberellin A_3 resulted in a complete loss of biological activity when elongation of bean epicotyls or growth of tomato ovaries constituted the assay. For promotion of germination of lettuce seed in the dark, however, the ethyl, *n*-butyl, and *n*-amyl esters equalled the activity of gibberellin A_3, while

the methyl, *n*-propyl, *n*-hexyl, and *n*-heptyl esters showed a significant increase above the controls. The pronounced activity of some of the gibberellates in lettuce seed germination may have resulted from their hydrolysis in the germinating medium and/or on the seed surface. In this regard the butyl cellosolve ester of A_3 was equally as active as gibberellin A_3 in promoting the elongation of bean epicotyls, growth of tomato ovaries, or lettuce seed germination, whereas *n*-butyl gibberellate was inactive except in promoting the germination of lettuce seed. Solubility and penetrating properties may have been favorably altered by the side chain of the butyl cellosolve ester of A_3.

Other derivatives of gibberellins A_1, A_3, and A_4 were biologically active. Compared with the parent compounds, however, the activity was less and seldom exceeded 60 per cent. Activity disappeared when the lactone group of ring A was removed. Likewise, activity was lost following rupture of the D ring. Intact A and D rings and a reactive carboxyl group appear to be essential for biological activity. The report of Brian *et al.* (2) that *allo*gibberic acid is not biologically active has been confirmed. The necessity of ring A for activity has also been confirmed in that gibberellenic acid is inactive (8).

SUMMARY

Gibberellins A_1, A_2, A_3, and A_4, the *n*-alkyl esters of A_3, and some derivatives of A_1, A_3, and A_4 were bioassayed utilizing several test systems with intact plants. In promotion of vegetative extension of bean epicotyl and in celery petiole elongation, A_3 was most active, followed by A_1, A_4, and A_2. Reduced epicotyl elongation following leaf blade applications of A_2 and A_4, as compared with A_1 or A_3, suggested limitations in the transport of A_2 and A_4 in the bean plant. A marked deviation from the usual order of growth extension activity among the four gibberellins occurred in stem elongation of the cucumber. Gibberellin A_4 was strikingly the most active followed by A_1, A_3, and A_2. Gibberellins A_3 and A_1 were more effective than A_2 or A_4 for accelerating flowering of both 'Great Lakes' head and 'Grand Rapids' leaf lettuce. Whereas gibberellin A_3 was more active than A_1, there were no differences between A_2 and A_4. Increasing the dosages of A_2 or A_4 twenty-fold above A_1 or A_3 did not compensate for the markedly greater acceleration of flowering resulting from the latter. Gibberellins A_1, A_3, and A_4 were more active than A_2 in promoting the growth of tomato ovaries at low dosages (3×10^{-4} or 3×10^{-5} *M*), while growth was comparable with all four gibberellins at 3×10^{-3} *M*. All gibberellins stimulated germination of lettuce seed in the dark. Photoinduced dormancy of *Weigela* was partially overcome by treatment with gibberellins A_1 and A_3, whereas A_2 and A_4 were inactive.

The *n*-alkyl esters (methyl to *n*-decyl) of gibberellin A_3 were not active in promoting growth of bean epicotyls or tomato ovaries. Lettuce seed germination, however, was enhanced by the methyl, ethyl, *n*-propyl, *n*-butyl, *n*-amyl, *n*-hexyl, and *n*-heptyl gibberellates.

The presence or absence of biological activity of several other derivatives of gibberellins A_1, A_3, and A_4 showed that intact A and D rings and a reactive carboxyl group were essential.

As of publication date, five additional gibberellins (A_5, A_6, A_7, A_8, and A_9) have been isolated and characterized — A_5, A_6, and A_8 from immature seeds of the 'Scarlet Runner' bean and A_7 and A_9 from the fungus *Gibberella fujikuroi*. No data are available on their comparative biological activity. Also, a revised structure for A_3 has been proposed in which the lactone of ring A is attached to carbons 1 and 11. (See Cross *et al.* Proc. Chem. Soc. p. 302. 1959.)

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Gibberellins, Cell Division, and Plant Flowering

Recent studies on the gibberellins indicate that there is an activation of cell division as well as an activation of cell elongation (4, 14, 23, 24, 34). The discovery of the primary effects of gibberellin on stem elongation led to the idea that the action of the gibberellins was similar to that of auxin. Since that time many other activation effects have been observed (6, 21, 22, 25, 26, 33). Some are concerned with the functioning of the growing point of the stem and particularly with a modification of the rate or the direction of cell division.

It is now clear that in some cases the effect of gibberellins (GA) on stem elongation is partly due to enhanced cell division activity. Figure 1 shows longitudinal sections of *Perilla* stems in which the dimensions of the control cells are approximately the same as those of the treated cells, while the internode length of the treated plants was 2.3 times that of the control. Sometimes the stem elongation is promoted more easily in the inflorescence than in the vegetative stem. With *Iberis amara,* for instance, we obtained very little length increase in the vegetative stem, but the length of the terminal inflorescence was markedly increased (Figure 2). Such specific effects have been observed in *Begonia* (14) and in strawberry (R. Lemaitre, personal communication). Enhanced cell division plays an important role in these effects. The activated cells are those in the zone immediately under the apical meristem, as noted by Sachs and Lang (34) in vegetative plants of *Hyoscyamus niger.*

Modifications of leaf form and size induced by GA have often been observed. Two very characteristic cases are those of *Statice sinuata* and *Lepidium ruderale* (Figure 2). The continual application

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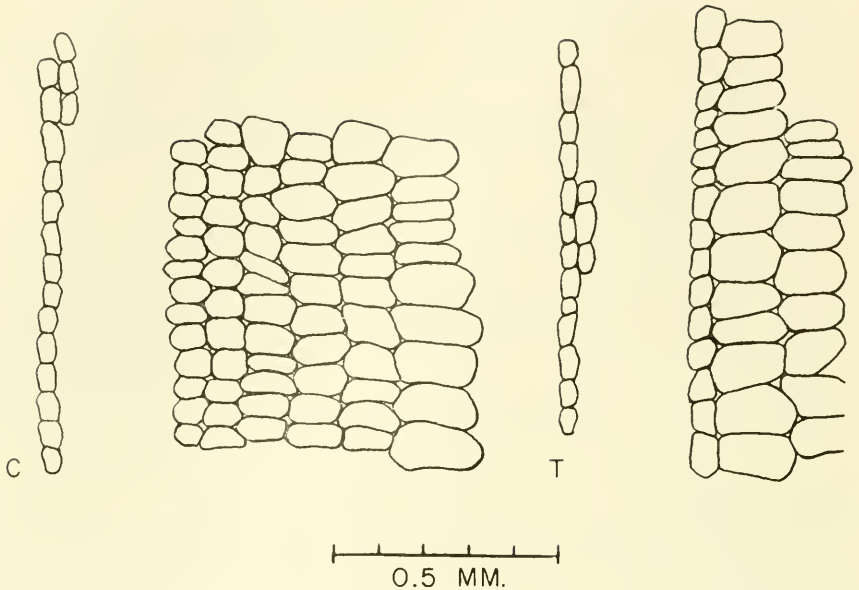

Fig. 1. Longitudinal sections of the external (**left**) and central (**right**) parenchyma of stems of *Perilla nankinensis* **C**, control; **T**, treated with 100 p.p.m. GA.

of GA to the growing point does not promote the growth of the stem, but the shape of the leaves is very strongly modified. In these two cases the leaves are larger in the treated plants but their form is more simple. This can only be explained by postulating a modification of mitotic activity within the leaf initials.

The action of GA on flowering of long-day (LD) plants grown in short days is also an effect on cell divisions in the stem apex (24). In short days the functioning of the apex is normally restricted to the formation of leaf initials. The application of GA enhances cell division in such a way that the whole meristematic region is activated, giving rise to the *"manteau de Gregoire"* (12) from which the flower primordium is formed.

The occurrence of these different effects fits relatively well with the anatomical and cytological description of distinct zones inside the meristem as proposed by Buvat (7). We can visualize that, depending on the species and upon the circumstances, GA acts selectively on one or another meristematic zone of the stem and on the young tissues initiated by the activity of the meristem. In stem elongation, the *"méristème médullaire"* and the zone situated immediately under it would be activated. In modifications of leaf form, the activation would affect the *"anneau initial"* and the leaf initials. The formation of the flower would correspond to a more complete activation of the

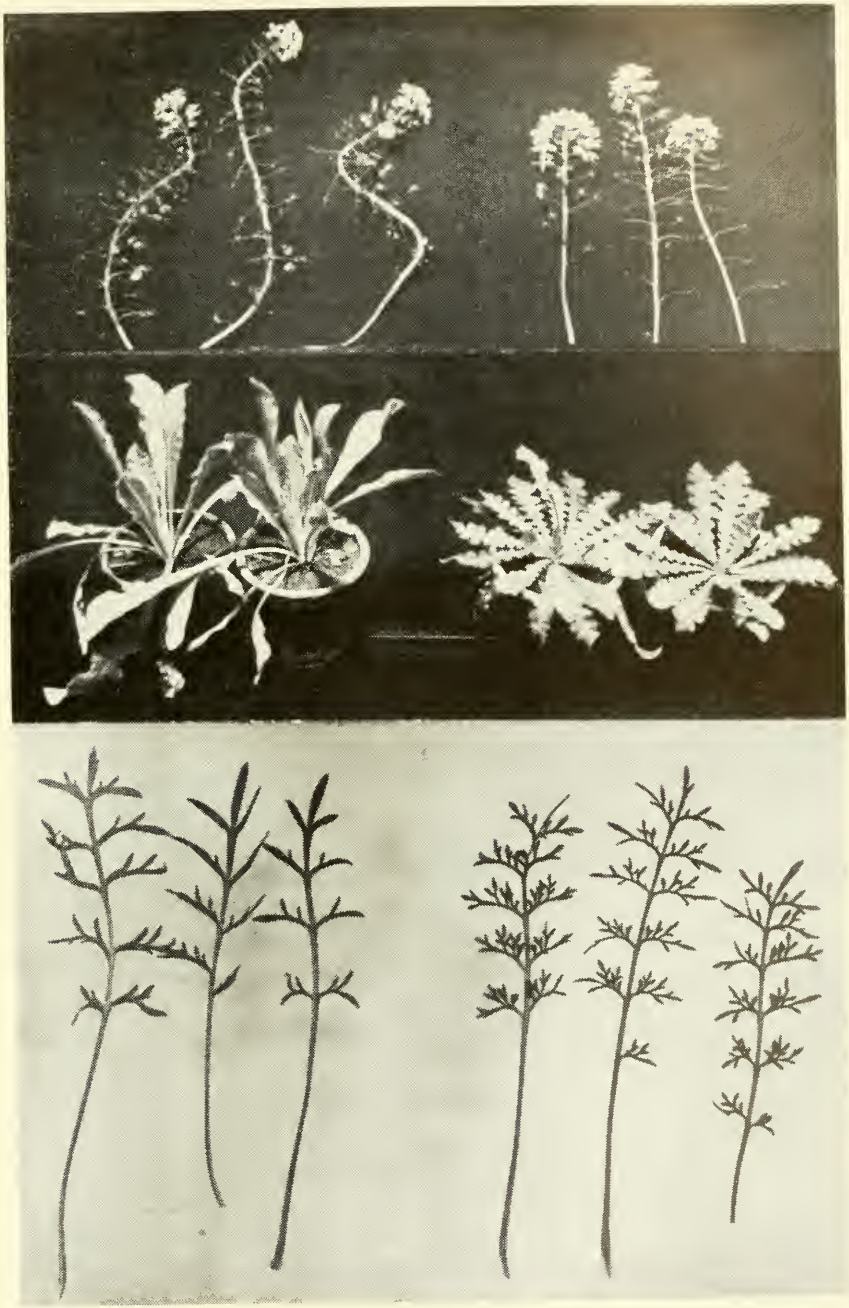

Fig. 2. Effect of gibberellic acid on morphogenesis. Elongation of the inflorescence of *Iberis amara* (***top***), modification of leaf shape in *Statice sinuata* (***middle***), and *Lepidium ruderale* (**lower**). Plants at right are controls; plants at left were treated with 100 p.p.m. GA.

mitotic capacity of the meristem as a whole including the "*meristeme d'attente*." These three effects could coincide in time.

To distinguish between the different meristematic activities of the growing point of the stem, one can compare the differential action of GA on LD and SD plants. When GA is applied to SD plants grown under long days, it cannot induce flowering but acts only on stem elongation. When applied to LD plants grown in short days, however, GA promotes both stem elongation and flowering. Using Buvat's concept, this means that the "*meristeme d'attente*" cannot be activated by GA in SD plants, while it can be activated in LD plants. Tschailachjan (42) has proposed a theory that accounts for this. He postulates that florigen is composed of two hormones, GA and anthesin. When the two hormones are both present, flowering is promoted, as evidenced by the flowering behavior of SD plants under short days and LD plants under long days. Following Tschailachjan, LD plants synthesize anthesin in short days, and the addition of GA results in the formation of florigen (GA + anthesin). On the other hand, SD plants synthesize GA in long days, and a further addition of GA has no effect on flowering because anthesin is lacking. It is clear that these differences in reaction when GA is applied indicate that an SD plant grown under long days is not identical to an LD plant in short days. There is a sort of dissymmetry which Tschailachjan's proposal attempts to interpret.

But this does not change the principal open question: How does GA activate cell division in the young tissues of the stem?

BIOCHEMICAL APPROACH TO THE ACTIVATION OF CELL DIVISION BY GA

Biochemically speaking, cell division is a very complicated phenomenon involving the synthesis of protein for which several biochemical conditions must be met. The role of ribonucleic acids in protein synthesis has been shown in animals as well as in plants. Protein synthesis also depends on the availability of a sufficient source of energy with the resulting adenosine triphosphate (ATP) playing a prominent role (1, 3, 8). This ATP may be synthesized in both respiration and in photosynthesis (16, 43). It is likely that some of these conditions are absent in meristems, particularly in the "*meristeme d'attente*" of an LD plant grown in short days or in that of an SD plant grown in long days. Protein synthesis would, therefore, be at a level insufficient for accelerated cell division. The type of block may well be different for the two groups of plants.

It is highly probable that GA can modify the rate of protein synthesis in the cells of the growing point of the stem. Further promotion of respiration (2, 19), action on several enzyme systems (40, 41), modifi-

cation of sugar content (5, 30, 40), reduced nicotine content in tobacco (45), increased ascorbic acid levels in clover (30), action on chloroplast pigments (5, 14, 30), etc., have been reported to occur after treatment by GA. In many species the effect on the pigments is grossly evident, but it is rather complex. Without a supplementary supply of mineral nutrients, as in a normal garden soil, there is generally a lowering of the pigment content. Table 1 lists nine species we have studied. In some cases the anthocyanin content is also modified. When mineral fertilizers are added in the presence of GA, the chlorophyll content does not drop much or does not drop at all. However, the drop remains evident when the treated plant flowers (30). As shown by Mosolov and Mosolova (30), redox processes are strongly enhanced in the leaves of GA-treated clover plants, and the sugar content of the leaves increases. The assimilation of mineral nutrients also increases.

All these facts show that GA profoundly affects the metabolism of plants. In spite of the fragmentary data, some of these facts clearly indicate that under adequate cultural conditions in which mineral nutrition is not limiting, GA enhances certain essential metabolic processes and increases the availability of some important metabolites. This is likely to be very favorable for protein synthesis inside the meristem and in the young tissues of the treated plants.

Another argument supports this conclusion. Photoperiodic induction of flowering, which can be replaced by the application of GA to LD plants grown in short days, seems to induce an immediate change in the capacity of meristematic cells to synthesize proteins. This appears from the following facts:

(a) Metzner (27, 28) reported that the proportion of amino acids in the protein fraction of the meristems of *Kalanchöe blossfeldiana*

Table 1. Effect of 100 p.p.m. of gibberellic acid on pigment content of plants.

Species Tested	Direction of Change in Chlorophyll Content of Leaves	Anthocyanosides	
		Direction of change in anthocyanoside content	Locus of effect
Statice sinuata	−	0	
Draba aizoides	−	0	
Capsella bursa-pastoris	0	0	
Iberis amara	−	−	Stem
Lepidium ruderale	−	0	
Beta vulgaris	−	−	Petioles
Bellis perennis	−	+	Stem
Perilla nankinensis	0	−	Leaves
Cheirantus cheiri	−	0	
Salvia splendens	−	0	
Ageratum mexicanum	0	0	
Arabidopsis arenosa	−	0	

grown in long days undergoes a rapid modification following several short days. Moreover, modifications in the nucleic acid fraction of the meristems occur during this SD induction.

(b) It is well known that photoperiodic induction rapidly changes the type of the gas exchange between the plant (in particular its leaves) and the environment (13, 35). Respiration measurements of very young isolated leaves (including the meristem) of an LD strain of *Salvia splendens* showed that during the induction phase the respiration is significantly higher under long-day conditions than under short-day conditions (10).

(c) Studying the total hematin content of leaves of LD and SD plants of *Perilla nankinensis* (SD), *Cannabis sativa* (SD), *Sinapis alba* (LD), and *Salvia splendens* (LD), we found (unpublished) that induction always causes a decrease of the molar ratio chlorophyll:hematin of the leaves. This decrease is most evident in young leaves. The modification is very rapid and is measurable a few days after the beginning of induction. We always observed that in the very young leaves the chlorophyll accumulation becomes slower upon induction, while hematin accumulates more rapidly.

(d) In flowering *Fragaria vesca* the vitamin E content of the young leaves is approximately proportional to the day length. In field experiments a maximum is found in June to July, coinciding with the increase of flower initiation (38). As shown by Nason and Lehman (32), vitamin E acts in vitro as an activator of cytochrome *c* reductase.

Points c and d directly relate to chlorophyll metabolism which is controlled by day length, although the exact site of the photoperiodic control is not yet known (9, 11, 29, 36, 37). Points b, c, and d suggest some inductive change in enzyme systems of the young tissues, a possibility which is very consistent with point a. Taken together, the four classes of facts support the following hypothesis:

In affecting chlorophyll metabolism, photoperiodic induction acts on several important metabolic processes; it enhances the respiration of the young tissues of the stem and it provides them with an improved system of hydrogen carriers passing through the series of cytochromes [the cytochrome carriers are known to be regularly associated in higher plants with meristematic activity (15)]. It therefore increases the ATP supply which is necessary for the changes in the protein fraction (27, 28) as well as for increased cell division and flowering. It would be very interesting to see if the activation of cell divisions by GA follows a scheme of this type.

ON A POSSIBLE DIFFERENCE BETWEEN SD AND LD PLANTS

Finally, we may ask why GA induces flowering of LD plants grown in short days but is ineffective in SD plants grown in long days. In

other words, why does GA activate cell divisions of the whole meristem in the first case and not in the second?

Many hypotheses are possible. We can suppose, as Tschailachjan does, that in SD plants an activator other than GA is necessary and that this activator is lacking in SD plants under long days. We can also suppose that the action of GA on metabolism is not exactly the same for LD and for SD plants, or that the necessary level of activation must be higher in SD plants than in LD plants and cannot be achieved through GA application. But there is another possibility which cannot be neglected. It is known that chloroplast structure is very delicate and that it is very rich in many enzyme systems. Within the plastid, chlorophyll is not distributed at random but is in close association with protein and lipide, the spatial organization of which is now under study in some laboratories (44). To some extent the organization protects the chlorophyll from photodestruction. The degree of protection varies from one species to another, or in the same species in accordance with the conditions of its culture. This appears evident when one studies photooxidative effects. We have found that *Chlorella pyrenoidosa* (Kandler's strain K) was relatively resistant to photooxidation, while *Chlorella vulgaris* (Pirson's strain P) was much more sensitive (39). In some mutants, photooxidation is very easy (17), but chlorophyll destruction appears to be only the final consequence of photooxidation. Long before it occurs, photosynthesis has completely ceased in high-intensity light (18, 20), phosphorylations are inhibited (18), and oxygen consumption rises probably with attendant peroxide formation (18, 20, 31). A general poisoning of metabolism occurs. Crawford (unpublished) has studied the sensitivity of the LD plant *Salvia splendens* to photooxidation by intense light. He found that photooxidation (as measured by the inhibition of photosynthesis in white light) is much more marked in the leaves of *Salvia splendens* grown in short days; the plants grown in long days are evidently more resistant to photooxidation. The high photosensitivity of *Salvia* grown in short days may be due to an insufficient protection of chlorophyll inside the chloroplasts, possibly resulting from an abnormal structure of the plastids themselves. Indeed, under short days the chloroplasts of *Salvia* do not accumulate their pigments in a normal fashion.

In practice this means that, during a given short day with light of sufficient intensity, the metabolism of an LD plant grown in short days can be partially inhibited through photooxidative processes. It can therefore be concluded that short days do not permit flowering of LD plants for two interrelated reasons: (1) suitable metabolic conditions (of the kind described above) for increased cell divisions in the meristem are lacking, and (2) photooxidation products poison metabolism during the light period.

It would be very useful to know if such a poisoning also occurs in SD plants grown in long days. In *Kalanchöe blossfeldiana,* for instance, the chlorophyll metabolism is undoubtedly different in short or long days (37). If this corresponds to a decreased level of protection, a long day with relatively intense light is likely to produce a drastic photooxidation proportional to the length of the photoperiod. Perhaps the explanation of the dissymmetry revealed by GA between the behavior of LD and SD plants is to be found here. During long days SD plants could withstand more severe metabolic inhibition of a photooxidative nature than could LD plants during short days. GA would be able to overcome this inhibition in the last case but not in the first.

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Effect of Gibberellins and Derivatives of Nucleic Acid Metabolism on Plant Growth and Flowering[1]

The discovery of gibberellins (GA) is an outstanding achievement in research on plant ontogeny, as mankind has now received a new powerful tool for controlling the growth and development of plants (2, 28). On the other hand, it may serve as a crucial test of the correctness of those hypotheses and of theoretical generalizations which at different times have been made regarding the inner causes of plant flowering since, as is usually the case, the appearance of new facts of outstanding importance revolutionize our theoretical conceptions and excite new ideas.

It is quite evident that the discovery of gibberellins is directly related to the concept of flowering hormones in plants or, as it was called, florigen, which we proposed over 20 years ago (8). This is especially true if it be noted that a comparison of data on the effect of GA on plants and the results of previous grafting experiments show that GA is not a flowering hormone which is essentially the same for long-day and short-day species.

As may be recalled, the concept of flowering hormones, among others, was based on grafting experiments in which it was shown that flowering of short-day plants under long-day conditions can be induced by substances produced in the leaves of long-day species, and conversely that long-day plants flower under short-day conditions in the presence of substances from the short-day components of the grafts (7, 23, 24, 25, and others). This was the basis for suggesting that in long-day, short-day, and neutral species flowering hormones (florigen) are of the same type.

[1] Read at the Conference by Dr. Clark A. Porter, Boyce Thompson Institute.

On the other hand, consistent results have been obtained by many authors and have indicated that GA accelerates flowering in many long-day plants, including some winter forms and seedlings of biennials, but does not affect the flowering of short-day species (3, 4, 9, 10, 11, 14, 16, 17, 18, 20, 22, 30).

Thus, an important problem was to clarify the relation between GA and florigen. To this purpose we carried out some experiments in 1957, together with L. I. Khlopenkova and T. N. Konstantinova at the Institute of Plant Physiology of the USSR Academy of Sciences. The influence of GA on the long-day species of rudbeckia (*Rudbeckia bicolor*) and tobacco (*Nicotiana silvestris*), and on the short-day species of red perilla *(Perilla nankinensis),* 'Mammoth' tobacco *(Nicotiana tobacum),* and winter rape *(Brassica napus* var. *oleifera)* was studied.

The results of these experiments led us to the conclusion (11) that two sets of substances compose the flowering hormones, or florigen, which are common to all plants. These are, on the one hand, GA, which is necessary for the formation and growth of stems and, on the other hand, some substances that are necessary for the formation of flowers and which have tentatively been called *anthesins.* From this standpoint the absence of flowering of long-day species under short-day conditions is explained by a lack of GA, whereas the absence of flowering of short-day species under long-day conditions is ascribed to anthesin insufficiency. Absence of flowering in winter forms and in seedlings of biennials is due to gibberellin deficiency under long-day conditions and to gibberellin and anthesin deficiency under short-day conditions (Figure 1).

This assumption, which is based on new facts concerning the influence of GA on the growth and development of plants and also on data from grafting experiments, requires, of course, further experimental and theoretical study.

One proof of this hypothesis would be to extract GA-like substances from the leaves of short-day plants located under long-day conditions and to induce, with the aid of these substances, flowering in long-day plants located under short-day conditions. A circumstance which facilitated the solution of this problem was that these substances have been isolated from the seeds and unripe fruits of a number of plants (5, 19, 21, 26).

Together with V. N. Lozhnikova we carried out an experiment (13) for extraction of GA-like substances from leaves of short-day plants ('Mammoth' tobacco and *Perilla*), as well as of long-day plants (*Rudbeckia*). The plants were cultivated under long (L) and short (S: 9-hr.) days, and after buds began to form under a day length suit-

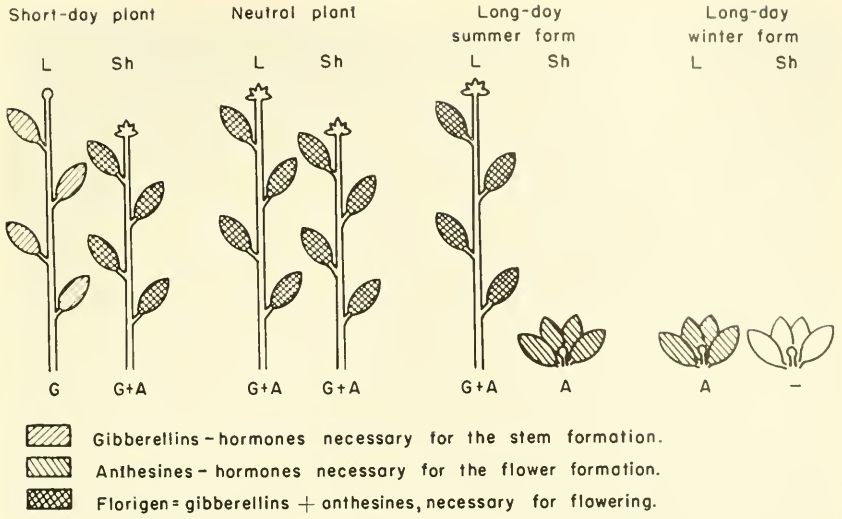

Fig. 1. Scheme of formation of flowering hormones in various plant species.

able for development, leaf samples were prepared with the aim of extracting GA-like substances. Acetone was employed, and after evaporation the dry residue was dissolved in distilled water.

Determination of GA-like substances by the growth reaction of maize seedlings was performed by the method developed by Boyarkin and Dmitrieva (1) in our laboratory. It was found that GA-like substances were present in all tested extracts, there being more of them in extracts from plants kept under long-day conditions than in extracts from the leaves of plants kept under short-day conditions.

The action of GA-like substances on the growth and flowering of *Rudbeckia* was verified by the drop method for rosette plants sown in the spring and kept under conditions of a short (9-hr.) day. A drop of the tested extract, GA, or water was daily applied to the center of the rosette of each plant. The experiment was carried out according to the following scheme: (1) 0.02 per cent GA_3; (2) an extract from the leaves of long-day (L) 'Mammoth' tobacco; (3) the same for a short-day plant (S); (4) an extract from the leaves of long-day red perilla (L); (5) the same for a short-day plant (S); (6) an extract from the leaves of long-day *Rudbeckia* (L); (7) the same for a short-day plant (S); (8) the control, water. Four plants were used in each experiment. The plants were treated during three months from December 9, 1958 to March 8, 1959. The experiment was completed on April 4.

As usual in plants treated with gibberellin, a fast reaction was observed. They rapidly began to bud and flower, whereas up to the

end of the experiments the control plants remained in the rosette stage. Two of the experimental plants that were treated with an extract from the leaves of long-day 'Mammoth' tobacco developed at an especially high rate, and one of them budded and flowered almost at the same time as the plants treated with gibberellin. The plant treated with an extract from the short-day 'Mammoth' tobacco developed more slowly (Figure 2).

Later, growth of the stems of plants treated with an extract from long-day 'Mammoth' tobacco was quite intense, and at the end of April many flowers had formed on the thick stem of one of the plants. The stem of the plant treated with a short-day 'Mammoth' tobacco extract was also thick, but much shorter, and had only one terminal flower (Figure 3).

Two plants treated with an extract from leaves of long-day *Perilla* only at the end of April formed small buds on short shoots and did not flower. Plants treated with short-day *Perilla* extracts did not bud at all. A plant treated with an extract from long-day *Rudbeckia* budded and began to flower earlier than that treated with an extract from the leaves of short-day *Rudbeckia* (Figure 4).

Fig. 2. Effect of extracts from 'Mammoth' tobacco leaves on the growth of *Rudbeckia*. **A**, 0.02 per cent GA; **B**, extracts from leaves of long-day plants; **C**, extract from leaves of short-day plants; **D**, control, water.

Fig. 3. Effect of extracts from 'Mammoth' tobacco leaves on the growth and flowering of *Rudbeckia*. **A**, extract from leaves of long-day plants; **B**, extract from leaves of short-day plants; **C** control, water.

Fig. 4. Effect of extracts from *Rudbeckia* leaves on growth and flowering of *Rudbeckia*. **A**, extract from leaves of long-day plants; **B**, extract from leaves of short-day plants; **C**, control, water.

Table 1. Effect of gibberellin-like substances from plant leaves on the growth and flowering of *Rudbeckia* under short-day conditions.

Treatment	Photo-period	Date of Shoot Formation	Date of Bud Formation	Date of Flowering	Growth of Stem (cm.)	
					Length	Diameter
GA (0.2%)	..	Dec. 22, '58	Feb. 16, '59	Mar. 5, '59	101	0.7
Extract from tobacco leaves	L†	Jan. 6, '59	Feb. 12, '59	Mar. 8, '59	48	1.7
			Apr. 30, '59			1.6
	S*	Jan. 20, '59	Mar. 7, '59	Mar. 26, '59	30	2.0
Extract from *Perilla* leaves	L†	Jan. 6, '59	Apr. 12, '59	..	4	1.6
			Apr. 30, '59		1	1.5
	S*	Dec. 3, '58	....	....	2	1.7
Extract from *Rudbeckia* leaves	L*	Dec. 23, '58	Mar. 10, '59	Apr. 10, '59	9	1.8
	S*	Dec. 27, '58	Mar. 12, '59	May 4, '59	2	1.8
Water		....	....	..		

* The data refer to a single plant in each separate treatment.
† The budding, flowering, and growth data refer to two plants in each separate treatment.

Results of observation of development of plants and also data on stem growth are presented in Table 1.

The data of the table show that all tested extracts contained GA-like substances, the amount in extracts from long-day plants being larger than that in short-day plant extracts, which is in agreement with the results of determination of GA-like substances in maize seedlings.

The experiment shows that *Rudbeckia* can be made to flower under short-day conditions by treating it with extracts from leaves of various plants containing GA-like substances. It is especially significant that flowering of a vegetating plant of the long-day type *(Rudbeckia)* under short-day conditions could be attained by treating it with an extract from the leaves of the short-day species of 'Mammoth' tobacco which vegetated under long-day conditions.

Another way of confirming our hypothesis would be to isolate anthesin, which is of a nitrogenous nature, from the leaves of long-day plants growing under short-day conditions, and to use it to induce flowering in short-day plants growing under long-day conditions. Substances of this type have not yet been isolated, but some data obtained by us indicate to a certain extent the nature of the substances which induce flowering in short-day plants located under long-day conditions.

The point here is that recently some data have been presented which indicate a relation between the nature of the photoperiodic reaction in plants and the peculiarities of nucleic acid metabolism (6, 29), and also an inverse relationship of flowering of the short-day plant cocklebur on substances of the 5-fluorouracil type, which is an antinucleoside (27). Correspondingly, we undertook some experiments to study the effect of adenine, kinetin, and some physiologically active substances on differentiation of flower buds of plants under conditions of cultivation of isolated tips by the technique proposed by Butenko (12).

For this purpose, terminal buds 3 to 4 mm. in size were taken from the main or upper lateral shoots of vegetating red *Perilla* plants grown under long-day conditions. After sterilization the buds were planted, under sterile conditions, in test tubes containing a White's agar medium (31), to which were added microelements according to Heller (15) and 2 per cent sucrose.

The test tubes were then placed in a greenhouse under the following illumination conditions: (1) short, 9-hr. day; (2) long natural day with addition of fluorescent light to daylight; and (3) continuous darkness. The experiments were carried out according to the following scheme: (1) control, stock White's medium; (2) adenine (0.0001

g/l); (3) kinetin (0.001 g/l); (4) heteroauxin (0.00001 g/l); and (5) GA_3 (0.0005 g/l). Altogether, three sets of experiments were carried out. The results of the third set, which was started January 14, 1959, are mainly given in the present paper.

Two or three days after the isolated tips were transferred to the artificial nutritional medium, growth of leaves began, and during the next 7 to 10 days growth of shoots and formation of calluses were observed. Root formation commenced from the 15th to the 20th days. Those plants in which roots were formed had a rapidly growing main shoot, whereas in the absence of roots, growth of shoots was much slower. *Perilla* leaves which developed before the appearance of roots were usually red, whereas those on the rapidly growing shoot formed after the appearance of the roots were green (Figure 5).

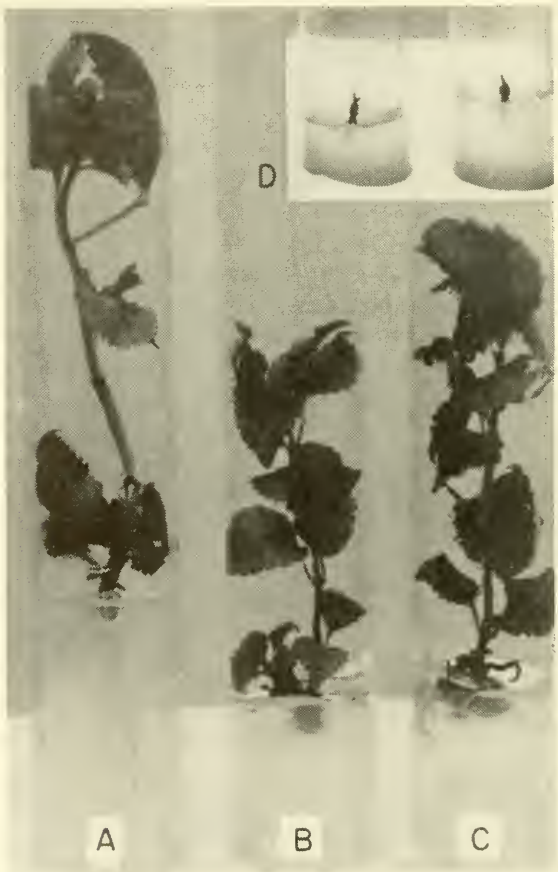

Fig. 5. Growth of young *Perilla nankinensis* plants cultivated in test tubes in White's medium under long-day conditions. **A**, control; **B**, adenine (0.001 g/l); **C**, kinetin (0.0001 g/l). **D**, The terminal buds of *Perilla* during the first day of planting are shown in the upper right part of the figure.

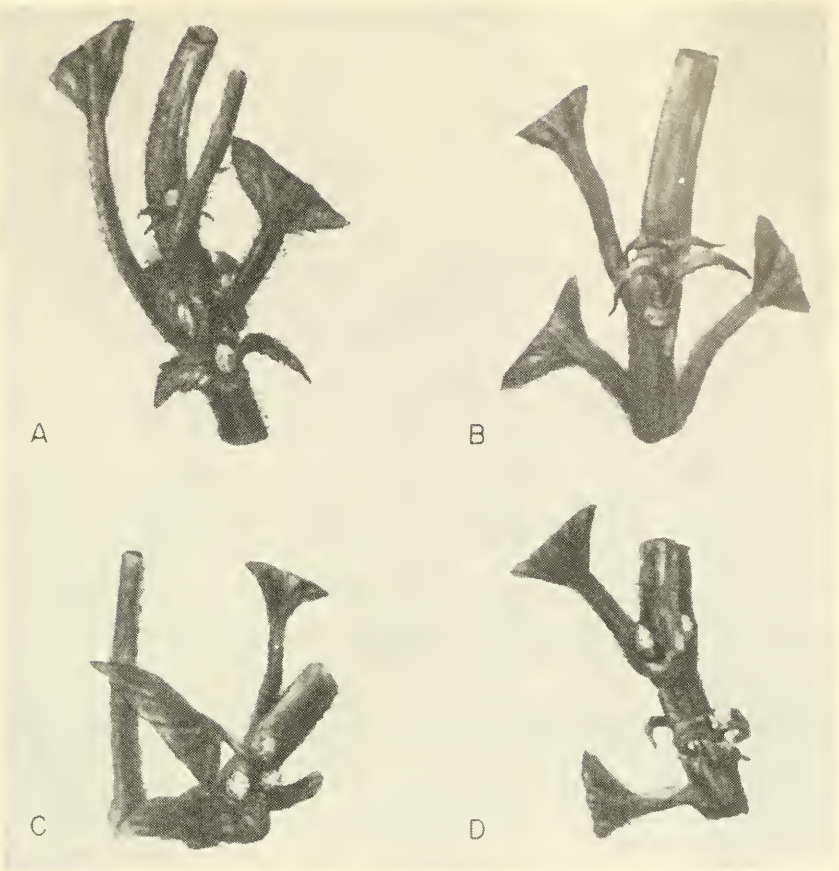

Fig. 6. **A**, Lower part of *Perilla* plant under short-day conditions. Flower buds in the axils of the lower leaves. **B**, Lower parts of control *Perilla* plant under long-day conditions. Small leaves of the growth shoots are visible. No flower buds are present. **C**, Lower part of *Perilla* plant under long-day conditions in White's medium with adenine. Flower buds in the axils of the lower leaves. **D**, Lower part of *Perilla* plant under long-day conditions in White's medium containing kinetin. Flower buds in the axils of the lower leaves. Magnification × 10.

Under short-day conditions, which were favorable for generative development of the *Perilla* plants, their growth was quite rapid; addition of GA and kinetin to the medium retarded root formation and, as a result, growth of aerial parts of the plants was also slower. In all experimental plants, except those treated with kinetin, (in which growth was strongly retarded) differentiation of flower buds was observed 25 to 30 days later, mainly in the axillary shoots of the lower leaves formed before the appearance of roots (Figure 6,A).

When kept continuously in the dark the plants formed elongated, etiolated shoots consisting of one internode and terminating in two

reduced etiolated leaves. Addition of GA and kinetin to the medium led to complete inhibition of root formation and, as a result, to slowing down of shoot growth. In all experiments, excluding those with kinetin, differentiation of flower buds on the tips of the etiolated shoots was observed 25 to 30 days after planting.

Plant growth was more intense under long-day than under short-day conditions. The growth was somewhat weaker in the heteroauxin, adenine, and kinetin experiments and especially in the GA experiments. Differentiation of flower buds under long-day conditions occurred only in experiments in which adenine and kinetin were added 25 to 30 days after planting. With growth of the differentiated buds they became distinctly visible.

The data obtained show that when adenine and kinetin are introduced into the medium, *Perilla* plants grown from isolated buds under long-day conditions form flower buds. This permits one to suggest that nucleic acid metabolism as a whole, and some derivatives of this metabolism, are related to processes responsible for the initiation of flower organs in short-day species.

The experimental data presented here do not, of course, solve the problem of elucidating the nature of the flowering hormones. Nevertheless, it seems to us that they confirm the main idea that flowering of very different types of plants is the result of interaction of two groups of substances which compose florigen, or the flowering hormone complex, which is the same for all plants.

SUMMARY

The elucidation of the problem on the interrelation between gibberellins and florigen led to the idea that flowering hormones common to all plants (florigen complex) consist of two groups of substances: gibberellins, necessary for stem formation and growth, and anthesins, required for flower formation.

This idea finds support in the induction of flowering in a long-day species, *Rudbeckia,* under short-day conditions by means of gibberellin-like substances. These latter are present in acetone extracts from the leaves of short-day species, 'Mammoth' tobacco, which vegetates under long-day conditions. Another proof is given by the induction of flower bud formation in plants of a short-day species, red *Perilla,* reared from isolated tops under long-day conditions under the effect of adenine and kinetin.

These data prove the idea that flowering of various plants is a result of the interaction of two groups of substances which comprise the flowering hormone complex, which is the same for all plants.

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The Hormonal Mechanism of Growth Inhibition by Visible Radiation[2]

A fundamental problem of plant physiology is to gain an understanding of the mechanisms by which plants respond to their environment. The response may be any one of three general types: metabolic, tropic, or morphogenetic. Each of these responses is the resultant of an interaction between the environment and the genetic and ontogenetic potential of a given organism. Hormones may participate in the regulation of any of these responses, but they are probably most significant in the last two.

We are already familiar with the nature of several plant hormones and can infer the existence of others. This paper is concerned specifically with the control of stem growth. Two hormones (or types of hormones) are known to participate in the control of stem growth. The first is auxin, which now appears to function primarily in the tropic responses. Second is gibberellin, which apparently acts as a controlling mechanism in many morphogenetic responses. A third hormone, caulocaline, produced in roots, is also required for normal stem growth, at least in many species (40). It has not yet been possible to isolate or characterize this hormone.

The present paper will consider evidence purporting to show that gibberellin is, in fact, the controlling factor in regulation of stem growth by visible radiation.

MORPHOLOGICAL EFFECTS OF VISIBLE RADIATION

Visible radiation has two distinct morphological effects on stem growth. Irradiation reduces total length of stem and generally causes it to become thicker and stiffer as well. This is the most noticeable effect of high irradiances, although measurable effects are often ob-

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served at very low energy levels as well. The most obvious effect of low intensity or brief high intensity irradiation is usually morphological. Internodes which are elongating at the time of irradiation are markedly inhibited but growth of subsequent nodes may be promoted proportionally. In many cases, then, low radiation energies may reduce the length of the first internodes but have little or no effect on total growth rate. This appears to be essentially a problem in rate of node formation, since irradiated plants have more nodes, but the same total stem length as dark-grown plants. Essentially nothing is known as yet about the mechanism which controls node formation, but considerable information is available on the mechanism of control of total stem elongation.

THE NATURE OF THE VISIBLE RADIATION AFFECTING STEM GROWTH

Several distinct effects of visible radiation on stem growth have been observed. Radiation energy level and apparently species as well determine the response of plants to radiation. Early workers utilized filters to isolate broad spectral regions of high-intensity solar radiation (cf. 8). They found, generally, that blue radiation produced short, thick stems while green and red radiation yielded relatively tall, slender plants. These responses have been confirmed with high-intensity fluorescent lamps (25, 37, 39). At low energy levels, on the other hand, red radiation is the most inhibitory to stem growth. As radiation energies are increased, a cross-over point is reached at which red is no longer more effective than blue. At irradiances above the cross-over point, blue radiation is most effective. Thus, red radiation is more inhibitory at low intensities, but maximum inhibition is attained with high-energy blue radiation. Whether or not different mechanisms are involved is not known. The red far-red pigment is the photoreceptor for the low-energy red response (2, 5, 29). Its action may also be manifest in a somewhat different manner. If plants are grown in white light for 8 hrs. a day, their growth takes place largely during the 16-hr. dark period (33). Wassink and Stolwijk (39) found that the extent of growth during the dark period was controlled by the spectral distribution of radiation received immediately preceding the dark period. This radiation may be of high or low intensity; growth depends only on the ratio of red to far-red radiation (6). Thus the red far-red pigment system acts to modify growth even when plants are grown in high-intensity artificial or solar radiation. The only results available (33) suggest that the growth inhibition by high-intensity blue radiation is not reversed by far-red irradiation at the beginning of the dark period.

A specific high-energy radiation effect on stem growth has been reported by Mohr (26). Stem growth of *Sinapis alba* was found to be unaffected by the low-energy red far-red pigment system; thus, the action spectrum for a high-intensity growth inhibition could be determined unambiguously for this species. Two spectral regions of maximum effectiveness were found. The most effective region includes the red far-red region, from about 660 to 740 mμ. The second region, in the blue, shows a maximum at about 455 mμ. This effect is presumably comparable to the high energy effects observed in other plants. If so, these results would represent a "composite" action spectrum near the cross-over point. The cross-over point would be at unusually low intensities in this species, because of its relative insensitivity to red.

Recently, Mohr (27) has redetermined the blue far-red action spectrum. One peak is found in the blue (ca. 440 mμ) and a much higher peak in the far-red (ca. 730 mμ), with a shoulder extending through the red.

Ultraviolet radiation (254 mμ) may also inhibit stem growth, but it does so only at relatively high irradiances. The mechanism of growth inhibition is different from that of visible radiation (see below).

INTRACELLULAR MECHANISM OF RADIATION INHIBITION

Visible radiation inhibits stem growth primarily through a decrease in cell elongation. This conclusion is supported by cell counts (33, 35) and by findings that growth inhibition occurs in the morphological region of cell elongation (14; Lockhart, unpubl.). The only known exception to this generalization is in Gramineae (i.e., *Avena*) where radiation-inhibition of the first internode is due to a reduction of cell division as well as of cell elongation (1).

Cell elongation and, thus, to a substantial extent, stem elongation are controlled by the rate and extent of plasticization of the primary cell walls. The cell walls become more plastic while the internal osmotic pressure remains constant. Thus, more water moves into the cell, resulting in an increase in cell volume. In an elongating stem this increase is mostly an increase in cell length. The osmotic pressure in the cell is subsequently restored to its original volume by uptake of solutes (or hydrolysis of existing substrates). A further increase in plasticity results in a second increment of growth, etc. (28, 34).

What phase of this growth process is affected by irradiation? The relation of radiation to various individual factors which might con-

trol growth has been investigated from time to time (e.g., 7, 10, 11, 32). However, the results obtained represented only correlations between these various factors and the radiation regime.

In more recent investigations, however, all factors known to influence growth rate have been examined in relation to both radiation regime and growth rate (19). Of the various factors which might affect growth in pea seedlings, only one, cell wall plasticity, shows changes which parallel changes in growth rate in response to irradiation. Within two hrs. after the onset of irradiation, when growth rate has been reduced 50 to 70 per cent, cell wall plasticity has decreased by about 75 per cent as compared to dark-grown plants.

Plasticity is here defined as the attribute of a tissue which is measured by irreversible bending or stretching. It was measured as the amount of residual bending (that left after removal of the load) after the tissues had been subjected to a standard transverse load for a standard time (Figure 1). Plasmolysis of these curved sections did

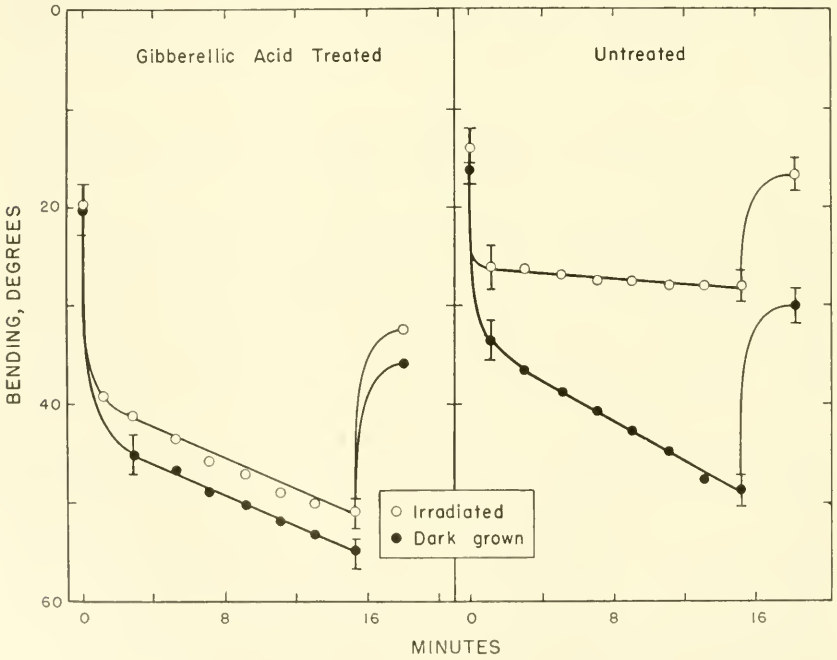

Fig. 1. Plasticity of the elongating region of stems of 'Alaska' peas, dark-grown or after 3 hrs. red irradiation. Plants treated with gibberellic acid (1 μg. per plant) 3 hrs. prior to time of irradiation. Plasticity is measured either as the slope of linear bending or as the amount of residual bending after the load has been removed. Illustrated are the standard deviations of the means at representative points. Seven plants per treatment.

not change the relative curvatures. Plasticity has also been measured as the irreversible stretching of freeze-killed tissue under a longitudinal force. After 2 hrs. irradiation a decrease in plasticity of about 75 per cent was found by this method also.

Gibberellic acid applied to plants shortly before irradiation completely prevents the decrease in plasticity just as it prevents inhibition of growth. It has no effect on the plasticity of dark-grown plants (Figure 1). The participation of gibberellin in radiation inhibition of growth is discussed below.

To demonstrate that the change in plasticity is a cause rather than an effect of growth inhibition, the various other factors which could affect growth rate were investigated. No change in any of these factors was found, even after growth inhibition was complete. The osmotic pressure of elongating stem cells was measured by a turgor method developed for the purpose (21) and also by determining the osmotic pressure of expressed sap. No difference was found in the osmotic pressure of dark-grown and irradiated plants by either method. Water permeability of the cells was determined by measuring the rate of opening of plasmolyzed, split stem sections when placed in distilled water. No effect of irradiation was found. Rate of water movement through growing tissue was measured as the rate of plasmolysis of stem tissue sections. No effect of irradiation was found. A possible water deficit in irradiated plants was investigated by determining the rate of water loss from stems of dark-grown and previously irradiated plants. No difference was found. The possibility that a water deficiency might cause growth inhibition of irradiated plants was further tested by measuring growth inhibition in a water-saturated atmosphere. No effect of relative humidity was found on radiation-inhibition of growth.

MORPHOLOGICAL REGION OF RADIATION SENSITIVITY

Idle (14) has compared the radiation sensitivity of the stem tip with that of the elongating region of *Vicia faba*. Either the tip or growing region was briefly irradiated and the time-course of the growth response (of the elongating region only) was followed. Substantial growth inhibition was observed in both cases but irradiation of the tip was most effective. When the tip was irradiated, inhibition was rapid and substantial, and growth of the elongating region never recovered. When the elongating region itself was irradiated, inhibition was equally rapid but somewhat less complete. The most striking effect, as a result of irradiation of the elongating region, was the observation that growth of the irradiated region recovered almost completely after 4 to 5 hrs.

The stem apex is the site of gibberellin production in *Pisum,* a closely related species (17). Thus irradiation of the elongating region probably inactivates gibberellin in situ. Recovery would occur when a new supply of gibberellin is received from the nonirradiated stem apex. Irradiation of the apex would inactivate gibberellin in a region of relatively high gibberellin accumulation and would result in a greater and longer lasting effect. Still unexplained is the rapidity of transmission of inhibiting stimulus from tip to elongating region compared to the slow recovery on direct irradiation of the growing region.

THE ROLE OF GROWTH HORMONES IN THE EFFECTS OF RADIATION ON GROWTH

Gibberellins

An interaction between gibberellin and visible radiation was suggested in some of the earliest work on the effects of gibberellin on higher plants. Treatment of plants with large doses of gibberellin results in elongated internodes and long, narrow leaves in monocots and extensive stem elongation in dicotyledonous plants. Decreases in leaf area have also been reported (42), although this is less common (3, 41, 43).

Direct evidence that application of gibberellin reverses growth inhibition caused by visible radiation has been reported for etiolated plants by Lockhart (16) and for light-grown plants as well by Lona and Bocchi (23).

Inhibition of light-grown *Perilla* was accomplished by following the daily light period with exposure to red radiation, while relatively noninhibited plants were produced by following each light period with far-red radiation. Gibberellic acid treatments completely prevented the inhibition of growth due to red radiation. It resulted in equal growth in red or far-red irradiated plants.

With a tall variety of *Pisum sativum,* 'Alaska,' normal stem growth in complete darkness is unaffected by gibberellin treatment. However, when gibberellin is given immediately prior to exposing plants to continuous visible radiation, growth inhibition is completely prevented. Dwarf varieties of *Pisum* respond to gibberellin treatment even when grown in complete darkness. At saturating doses of gibberellin, however, the growth rate of treated plants is identical, whether irradiated or dark-grown (Table 1).

In *Pisum,* application of gibberellic acid will prevent inhibition of stem growth at continuous irradiances as high as 2,000 ergs· $cm.^{-2}$ ·$sec.^{-1}$, both red and blue (18, 38). These are the highest intensities so far tested and are of the same order of magnitude as the high-energy blue far-red inhibition of stem growth reported by Mohr (26).

On the other hand, inhibition of stem growth by ultraviolet radia-

Table 1. Response of *Pisum sativum*, 'Alaska' to red radiation and gibberellic acid treatment.

	Dark*	Irradiated†
Control	18.5 ± 0.30 cm.	11.9 ± 0.30 cm.
Gibberellic Acid Treated‡	19.3 ± 0.30 cm.	19.8 ± 0.30 cm.

* Both dark and irradiated plants were kept in rooms in which the air was filtered through activated carbon. The dark plants were kept in a chamber in which the air was also bubbled through alkaline potassium permanganate.

† Irradiation consisted of filtered red fluorescent radiation (ca. 150 ergs·cm.$^{-2}$·sec.$^{-1}$) for the duration of the experiment.

‡ Gibberellic acid was applied as a 4 μl. ethanolic drop giving a dose of 1 μg. per plant. Limits are standard deviations of the means.

tion (254 mμ) is completely unaffected by gibberellic acid (and auxin) treatments (18).

It is possible to measure the level of the nongibberellin factors controlling stem growth. This is done by saturating the growth system with gibberellic acid. Growth, then, will be limited by the sum of the other growth factors. In this manner, the level of nongibberellin growth factors may be compared in irradiated and dark-grown plants.

In *Pisum*, saturation with gibberellin results in equal growth of irradiated and dark-grown plants. Thus the level of nongibberellin growth factors is unchanged by irradiation.

Where N is the effective sum of the nongibberellin growth factors and g = growth, or growth rate,

In dark: $N_d \cdot \text{natural } GA_d = g_d$.

In light: $N_l \cdot \text{natural } GA_l = g_l$,

and $g_l < g_d$.

Since $g_l < g_d$, then $N_l < N_d$ or $GA_l < GA_d$ (or both).

Now, saturate the plants with gibberellic acid.

Growth is now equal $\therefore g_l = g_d$.

Since gibberellic acid is saturating: $GA_l = GA_d$.

Therefore N_d must be equal to N_l; and the change in growth due to irradiation must be due to a change in endogenous gibberellin.

The next question is, then, how irradiation affects the endogenous gibberellin system. In general terms, it could affect (1) synthesis, (2) effective endogenous level of the hormone, or (3) response of the plant to a given level of hormone. Two lines of evidence make possible a partial answer to this question.

It has already been pointed out that dwarf peas respond to gibberellic acid treatment even when grown in darkness. It is possible, then, to compare the responsiveness of irradiated and dark-grown plants to various concentrations of added gibberellic acid. When this was done (20) it was found that the responsiveness under the two

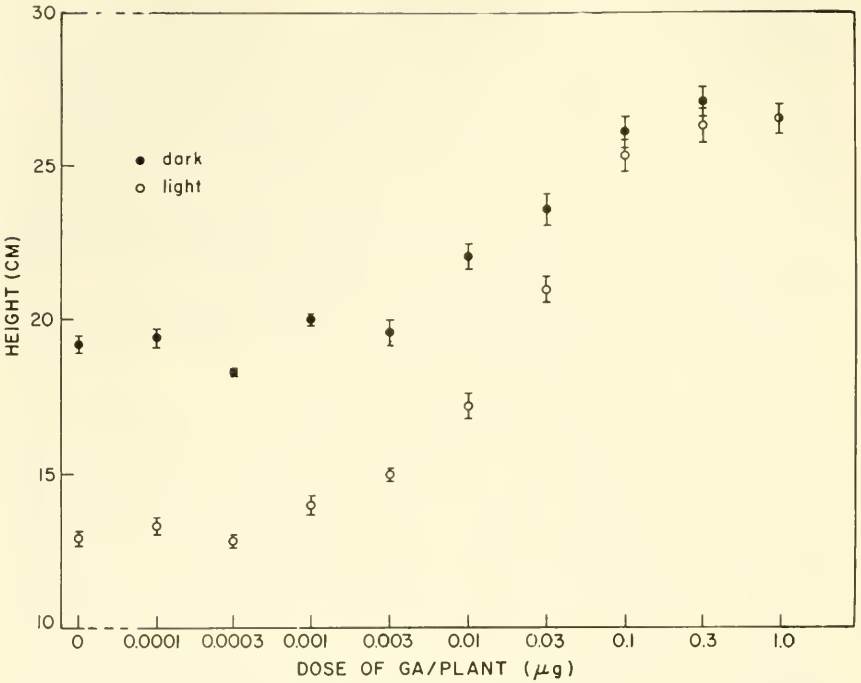

Fig. 2. The height of dwarf *Pisum* ('Morse's Progress #9') 4 days after treatment with gibberellic acid (at the doses indicated) and transfer of certain plants to continuous red radiation (150 ergs·cm^{-2}·sec^{-1}). Plants 6 days old at time of treatment (ca. 4 cm. above soil level). Also indicated are the standard deviation of the means. Twelve plants per treatment.

conditions was equal. That is, the amount of gibberellic acid (or gibberellic A_1) required to give a threshold response, a half-maximal response, or a saturating response is equal in irradiated and dark-grown plants (Figure 2). This indicates the effective level of endogenous gibberellin is reduced by irradiation, while responsiveness of the plant to gibberellin remains unchanged. Thus, radiation either reduces synthesis, or diverts or destroys existing gibberellin. Phillips *et al.* (30) have recently shown that the level of extractable, endogenous growth substances in the 'Alaska' pea is reduced by irradiation. Gibberellins and auxins were not completely distinguished, but all acidic growth factors showed a general correlation with growth rate. These analyses provide further support for the conclusion that visible radiation acts on stem growth through a decrease in endogenous gibberellin.

Cell Wall Plasticity

It was pointed out above that inhibition of growth by radiation acts through a reduction in the effective level of endogenous gibberellin. Furthermore, it was shown (p. 545) that cell elongation of irradi-

ated plants is reduced as a result of a decrease in cell wall plasticity. This must mean that a decrease in endogenous gibberellin causes a reduction in plasticity. Gibberellin, then, is necessary for the maintenance of normal (maximum) cell wall plasticity. Experimental measurements have demonstrated that treatment of irradiated plants with gibberellic acid will result in high cell wall plasticity, equal to that of dark-grown plants.

Relation of Gibberellin to the Photochemical Reaction

The same red far-red photochemical process affects various morphological responses of plants in addition to stem length. In the same plants, i.e., *Pisum* seedlings, irradiation affects stem length, leaf development, epicotyl hook opening, and rate of node formation. Gibberellin treatments will completely reverse the effect of radiation on stem growth, but it has no effect on these other photomorphogenic responses. Therefore, applied gibberellin does not act directly on the initial photochemical act, or even on subsequent thermochemical processes which are common to all these reactions. Rather, gibberellin must act on the terminal reactions controlling stem growth. It clearly does not influence any reaction common to all these photomorphogenic processes (16).

Gibberellin Reversal of Stem-Growth Inhibition in Various Species

Interactions between gibberellic acid and radiation-inhibition of stem growth have been examined in various other species in addition to *Pisum* (18). Responses of plant species so far examined appear to fall into four groups, as follows:

(1) Gibberellin results in complete reversal of radiation inhibition. Examples: *Pisum sativum* 'Alaska' (tall) and 'Morse's Progress No. 9' (dwarf), *Helianthus annuus, Hordeum vulgare.*

(2) Gibberellin results in partial reversal of radiation-inhibition. Examples: *Cucurbita pepo, Cucumis sativus.*

(3) Gibberellin elicits no growth response in either irradiated or dark-grown plants. Example: *Sinapis alba.*

(4) Gibberellin gives no growth promotion in darkness, but promotes growth markedly when plants are irradiated. Examples: *Phaseolus vulgaris,* various dwarf and normal clones.

It is not yet known, then, just how widespread is the interaction between gibberellic acid and visible radiation or in how many species radiation acts through the endogenous gibberellin system. Studies on the mechanism of interaction have been pursued with *Pisum,* since the interaction is complete in this species, and since it is a desirable experimental plant in other respects. Just how far the results obtained with *Pisum* may be extrapolated to other species remains to be seen.

Participation of the endogenous gibberellin hormone in control of stem growth by radiation cannot be ruled out in any of the cases listed above (see discussion in reference 18). It may be suggested here that the gibberellin hormone in various plants differs chemically (31).

Interaction Between Light and Auxin

Meijer (24) showed that when young tomato plants are exposed to far-red radiation a strong epinastic response occurs, just as when the plants are treated with auxin. Furthermore, the radiation-induced epinasty can be prevented by anti-auxin treatment. It must be kept in mind that responses such as this may well be due to a balance between factors. Thus, de Zeeuw and Leopold (44) showed that the epinastic response of tomato to auxin can be prevented by ultraviolet irradiation. Since auxin (1-naphthaleneacetic acid) was added after irradiation, radiation must reduce the response of the plant to auxin. Perhaps far-red radiation increases the responsiveness of petioles to endogenous auxin. Then epinasty would occur as a result of irradiation and could be prevented by added anti-auxin.

One of the few reports of an actual promotion of stem growth in intact plants as a result of added auxin is due to Meijer (24). He found a 60 per cent promotion of growth of irradiated gherkin (*Cucumis*) seedlings as a result of tryptophol treatment as well as a somewhat lesser response to IAA (indole-3-acetic acid). However, the growth elicited by tryptophol was only a small fraction (< 40 per cent) of the growth of these seedlings in darkness. In any case, these results are for hypocotyl length only. Thus, it is impossible to distinguish between true effects on stem elongation and hypocotyl inhibition due to an increase in epicotyl growth. Lockhart (18) found no effect of added IAA on another variety of gherkin, either with or without simultaneous gibberellin treatment.

van Overbeek (36) studied the relation of auxin to inhibition of stem elongation by red radiation in *Raphanus*. Added auxin would partially restore growth of irradiated stems, but a large proportion of the inhibition had to be attributed to "the responsiveness of the cells to auxin," presumably gibberellin (?).

Galston and co-workers have observed many correlations between irradiation and auxin metabolism, but have found no evidence for a causal relationship between auxin and the effects of radiation on stem growth in intact plants (9, 10, 13).

Other Hormonal Factors

Brian and Hemming (4) have suggested that gibberellin acts to promote stem growth by counteracting an inhibitor from some other part of the plant. They reach this conclusion by demonstrating that

excised internode sections of a dwarf pea (treated with IAA) grow much more than the same internode on the intact plant (even if IAA is added to the plant). Gibberellic acid added to excised internodes had only a relatively small effect. (If the intact plant is treated with gibberellic acid, the growth of the internode is 6 times as great as maximum section growth.)

The critical question here is: How can the excised internode grow more than it does on the plant, when no gibberellic acid is added? Their conclusion is that an inhibitor, formed in some other part of the plant, prevents elongation in the intact plant, while excision of the internode removes the source of this inhibitor. Clearly this internode is capable of greater extension, even without added gibberellic acid, than normally takes place in the plant. Thus, some form of growth-limiting effect from another part of the plant must act on the intact internodes.

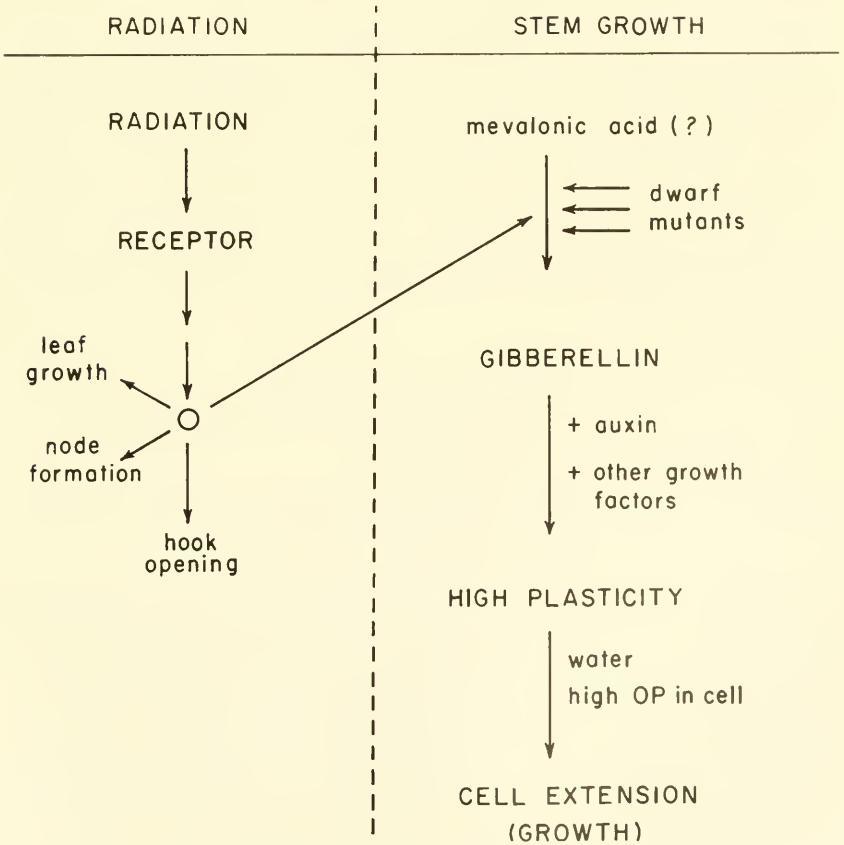

Fig. 3. Suggested mechanism by which visible radiation limits stem elongation.

This inhibitor concept is not, of course, irreconcilable with the function of gibberellin discussed above. Incorporating the inhibitor, radiation would either decrease the gibberellin, resulting in a lower gibberellin-inhibitor ratio, or increase the inhibitor with the same effect on the ratio and, thus, the same effect on growth.

The mechanism by which visible radiation may limit stem elongation is shown in Figure 3.

SUMMARY

The mechanism by which visible radiation limits stem elongation as we understand it today may be outlined as follows.

Radiant energy is intercepted by the receptor pigment(s). The activated pigment transfers (and amplifies) the signal through one or more thermochemical steps common to all photomorphogenetic processes. From one or more "master reactions" the signal is divided and passed eventually to some step in the developmental sequence of each of the processes under its control.

One of the processes affected by visible radiation is stem elongation. Stem elongation normally proceeds by an increase in plasticity of the young cell walls. For maximum increases in plasticity, evidently, many growth factors are required. Among the growth factors recognized to be necessary for increased plasticity are auxin, gibberellin, and probably caulocaline. Visible radiation, through an unknown sequence of reactions, acts to reduce the amount of available gibberellin. Thus, plasticity of the cell walls is decreased and growth is reduced.

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DISCUSSION

Dr. Burström: I would like to ask Dr. Lockhart about his results on the influence of gibberellic acid on cell walls. This is a most interesting point, of course, and most important, because if you have a change of growth you should have some direct or indirect effect upon the cell wall. I would like to ask if you know any more about what is happening here. You measure plasticity by the bending test, and there are two points which surprise me. Firstly, that this plastic bending is going on at a very slow rate and apparently does not come to an end. That would not be expected from a real plastic bending; it ought to be a fairly rapid change. Secondly, you determine, if I am right, the plastic bending after subtracting the reversible elastic bending which obviously is the same at the beginning and at the end of the tests. You have no change in the elastic tension. This is surprising. If you have a plastic change of this kind, you should have a change in the elastic bending because that's also a function of the

changes which take place during the plastic deformation of the cell walls. You shouldn't have a constant elastic tension before and after if the gibberellic acid has not changed elasticity. I don't know what this means quantitatively, but this general point of view should not be overlooked.

Dr. Lockhart: I can partially answer this by adding that I have also measured plasticity by imposing a longitudinal load on freeze-killed stem sections. The effect of radiation on plasticity was almost identical as measured by the two methods. No significant change in elasticity was observed during the bending procedure. It's not clear to me whether a substantial change in elasticity would be expected, a priori, or not. The deformation of the tissue is, after all, relatively small.

Dr. van Overbeek: May I answer that question about elasticity and plasticity? The earliest research I did in the field of auxin research was to work together with Heyn on elasticity and plasticity of coleoptiles under influence of the auxins. (A. N. J. Heyn and J. van Overbeek. Proc. Kon. Akad. Wetensch. Amsterdam 34: 1. 1931.) One of the things we found invariably, by the stretching technique of plasmolized material and by the bending technique, was that the plasticity always increased, whereas the elasticity was not changed at all.

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Acceleration and Retardation of Abscission by Gibberellic Acid

Although there is a voluminous literature on the growth-promoting effects of gibberellic acid, there are very few publications on its abscission effects. The first two investigations found no effect of gibberellic acid on excised leaf abscission zones of *Coleus* (5) and none with corollas of *Papaver* and *Origanum* (8). However, gibberellic acid when applied to flowers or young fruit increased fruit-set in tomatoes (9, 12), and increased fruit-set in cotton. Gibberellic acid sprayed on branches of deciduous trees in late summer had little or no effect on some species, but retarded leaf abscission in other species and accelerated branchlet abscission in *Taxodium* (6).

In view of the now considerable knowledge of the effects of auxins on abscission (1) and the recent discovery of an abscission accelerating hormone (7), and in view of the differences among the results of the abscission experiments with gibberellic acid, further investigation of the role of gibberellic acid in abscission and of its interrelations with the above hormones appears well justified. This paper reports an investigation in which both accelerated and retarded abscission resulted from applications of gibberellic acid to cotton explants (excised abscission zones) and discusses the seeming contradictions in the literature.

MATERIALS AND METHODS

Seedlings of cotton (*Gossypium hirsutum,* 'Acala 4–42' and 'M8948') were grown in the greenhouse or under fluorescent lamps. No differences in response were noted between the two cultivars. The planting medium was Sponge Rok, a white volcanic ash. The seedlings were watered with tap water fortified with ferric salts to prevent chlo-

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rosis. Explants were cut when the seedlings were 2 to 3 weeks old. In most experiments the cotyledons were debladed 24 hrs. before the explants were cut.

The explant technique was a modification of that described by Addicott *et al.* (3). The explants consisted of 5 mm. stumps of cotyledonary petioles and stem, with 10 mm. of the hypocotyl. Explants were placed in plastic or stainless steel holders in petri dishes containing a sheet of moistened filter paper and kept in the dark at 25° C. Gibberellic acid was applied in agar blocks of the size used in the standard *Avena* coleoptile assay (2.7 × 2.7 × 1.0 mm.). Application was either *distal* (to cotyledon petiole stumps) or *proximal* (to stem stump). The blocks were removed after 24 hrs. to facilitate testing for abscission. Abscission was determined by means of an abscissor (a spring instrument) calibrated to deliver a force of 5 g.

The gibberellic acid used was provided by Merck and Company as samples No. 57-RTS-931 and No. 57-RTS-1000.

EXPERIMENTS AND RESULTS

In the first experiments, gibberellic acid was applied distally to explants from debladed seedlings and in concentrations of 0.01, 0.1, 1.0, and 10 μg. per block; blank agar blocks were applied proximally. In the controls, blank agar blocks were applied both distally and

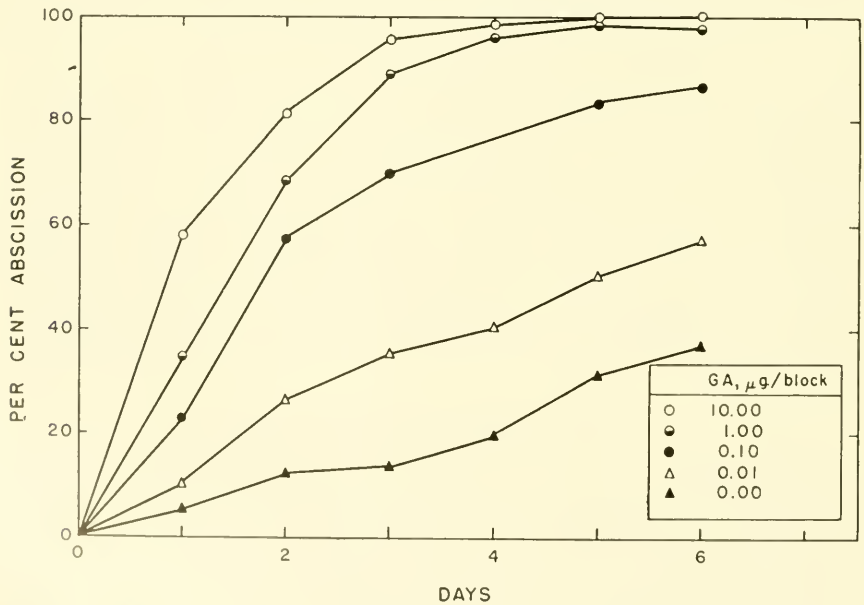

Fig. 1. Acceleration of abscission by *distal* application of gibberellic acid. Each line is the average of three experiments totaling 100 abscission zones.

proximally. Results are summarized in Figure 1. With the lowest concentrations of gibberellic acid, 0.01 μg. per block, abscission was appreciably faster than in the controls, and the rate increased with increasing gibberellic acid. In similar experiments higher concentrations of gibberellic acid were used; 100 μg. per block (the highest concentration which would remain in solution) produced somewhat more rapid abscission than 10 μg.

The second group of experiments was similar to the first except that lower concentrations were applied: 0.0001, 0.0005, 0.001, and 0.005 μg. per block. Results are summarized in Figure 2. Slightly increased abscission followed the 0.005 μg. application. Results from the other applications were very close to the controls.

In the third group of experiments gibberellic acid was applied proximally and blank agar blocks distally; other conditions were identical with those of the first two groups of experiments. The results (Figure 3) were closely similar to those of the first group of experiments. The rate of abscission increased with increasing concentrations of gibberellic acid.

In a few experiments the proximal applications included 100 μg. of gibberellic acid. As with distal applications these produced somewhat more rapid abscission than 10 μg. However, another quite unexpected result also occurred: The stem stump abscised in some

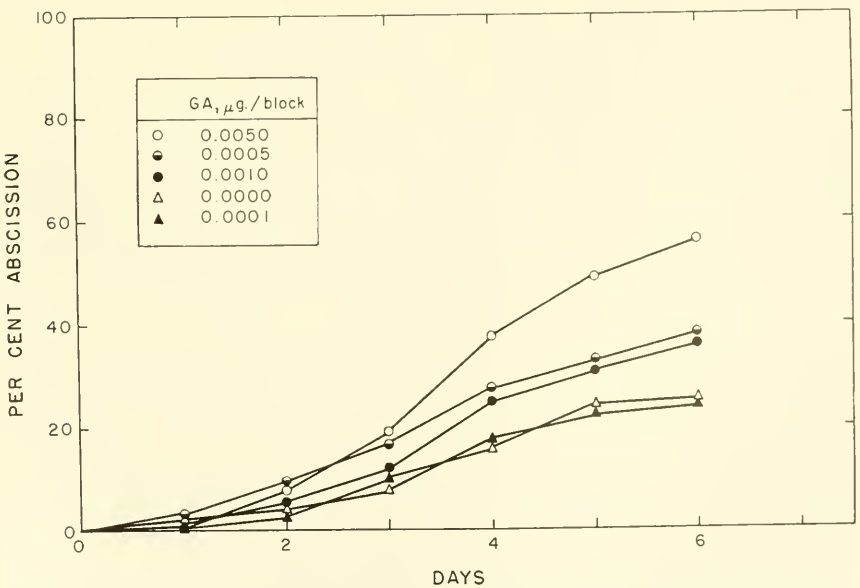

Fig. 2. Abscission following *distal* application of small amounts of gibberellic acid. Each line is the average of five experiments totaling 200 abscission zones.

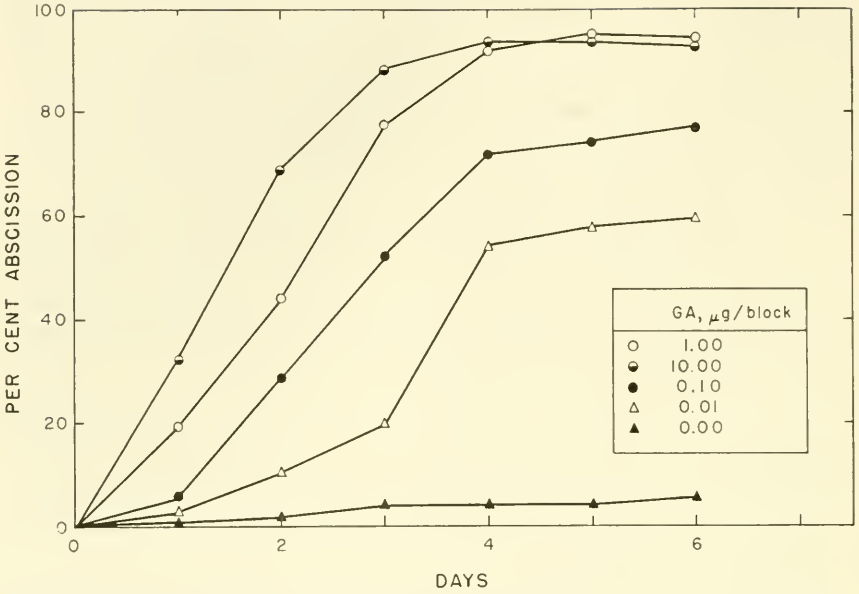

Fig. 3. Acceleration of abscission by *proximal* application of gibberellic acid. Each line is the average of four experiments totaling 160 abscission zones.

(but not all) of the explants. The anatomical changes in this stem abscission were found to be identical with the changes which normally occur in the abscission zones of cotyledonary petioles.

In the fourth group of experiments the seedlings were not debladed prior to cutting the explants; gibberellic acid was applied proximally in low concentrations. In these experiments (Figure 4) gibberellic acid retarded abscission. The highest concentrations of gibberellic acid, 0.005 μg. per block, retarded the rate only slightly, but from 0.001 down, retardation was appreciable. The rate of abscission decreased with decreasing concentrations of gibberellic acid.

DISCUSSION

These experiments show that gibberellic acid can affect abscission when applied close to the abscission zone, and can either accelerate or retard abscission depending on concentration and site of application.

Under the conditions of these experiments, gibberellic acid probably reaches the abscission zone and directly influences the process of abscission. Under other conditions, the effects of gibberellic acid on abscission appear to be indirect. For example, the retarded leaf abscission of sprayed branches (6) and increased fruit-set (9, 12) were probably indirect effects resulting from increased vigor or growth

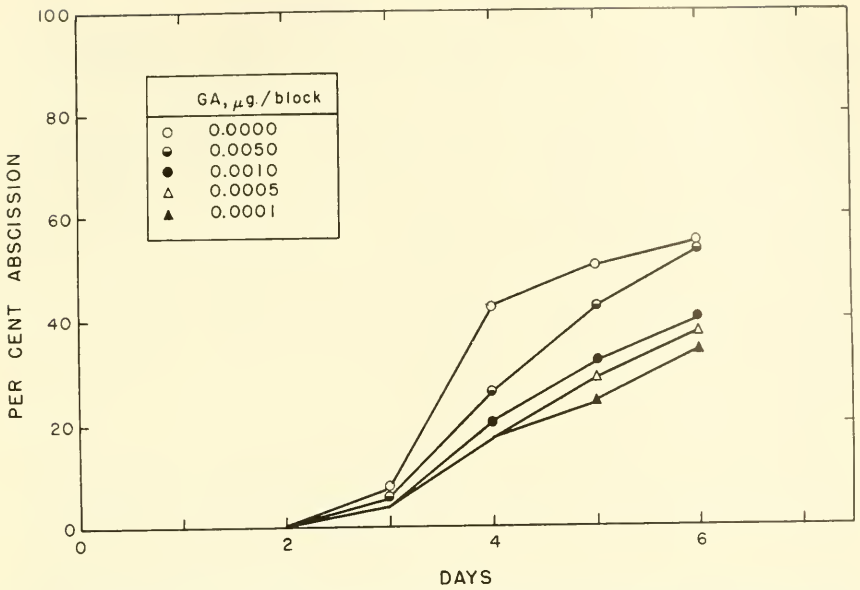

Fig. 4. Retardation of abscission by *proximal* application of small amounts of gibberellic acid. Each line is the average of three experiments totaling 120 abscission zones.

of the branch or young fruit, rather than direct effects of gibberellic acid on the process of abscission.

In other investigations where no effect of gibberellic acid was found, the results appear due, in some cases at least, to the limitations of the experimental material. For example, the *Coleus* petiole explants used by Brian *et al.* (5) abscised so rapidly that both untreated and treated petioles fell before the first observation was made. Under such conditions, of course, accelerated abscission was not detected, although it could well have occurred. For exploratory experiments on abscission, the plant material should be so grown or so selected that the controls show a moderate rate of abscission, thus permitting the detection of both acceleration and retardation.

The anomolous stem abscission following proximal application of 100 μg. of gibberellic acid is of considerable interest in that abscission of stem stumps has not been previously reported in cotton. (However, abscission of weakened or injured branches is characteristic of a few plant species.) The stem abscission observed here was somewhat similar to the anomolous abscission of bean stems following application of triiodobenzoic acid to the apical bud, described by Whiting and Murray (11). These unusual types of abscission deserve further physiological investigation.

Although our knowledge of the role of gibberellic acid in abscission is still meager, it is consistent with the concept emerging from other fields of research: that gibberellic acid functions through interaction with auxin (4). The evidence on auxin and abscission accumulated since 1935 indicates that auxin is the principal endogenous regulator of abscission (2). Now it is apparent that gibberellic acid sometimes may be similarly involved in the regulation of abscission (although the frequency with which it is a critical or deciding factor under natural conditions is still obscure). Considered together, all the evidence now available suggests a new hypothesis: that three hormones — auxin, gibberellic acid, and the abscission-accelerating hormone — interact in a common mechanism which regulates the process of abscission.

Since knowledge of the physiology of endogenous gibberellic acid, as well as of gibberellic acid's interactions with auxin, is still very fragmentary, speculation on the nature of their interaction in abscission is not yet justified. Thus the recent suggestion that gibberellic acid and auxin counteract each other in the regulation of young fruit abscission — gibberellic acid preventing and auxin promoting the abscission (10) — appears premature; and further, it is unsatisfactory in failing to account for the numerous investigations which show auxin to be involved in the prevention of young fruit abscission (1, 2).

Further experiments are being directed to the definition of the role of gibberellic acid in abscission and to the understanding of its physiological and biochemical interactions with auxin and the abscission-accelerating hormone.

SUMMARY

Gibberellic acid accelerated abscission in excised cotyledonary nodes of cotton when applied in relatively high concentrations either proximal or distal to the abscission zone. Gibberellic acid retarded abscission when applied in relatively low concentrations proximal to the abscission zone.

In these experiments gibberellic acid was probably directly influencing the process of abscission. In some experiments, reported by others, gibberellic acid effects were probably indirect; e.g., increased fruit-set following gibberellic acid application appears to be an indirect effect on abscission resulting from stimulation of fruit development. Failure to obtain abscission responses from gibberellic acid applications appears due in some cases to limitations of the experimental materials. The hypothesis is advanced that the three hormones — auxin, gibberellic acid, and the abscission-accelerating hormone — interact in a common mechanism which regulates the process of abscission.

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Shoot Histogenesis and the Subapical Meristem: the Action of Gibberellic Acid, Amo-1618, and Maleic Hydrazide

Until the late 1950's most discussions of shoot growth were concerned primarily with cell division at the apical meristem and cell elongation in the more distal regions of the stem. The few notable exceptions in which evidence was presented for cell division below the apical meristem and its contribution to shoot development were ignored (1, 9, 17). With the discovery of the action of the gibberellins, the quaternary ammonium carbamates and maleic hydrazide on stem growth, subapical meristematic activity assumed new importance. Thus, the experiments relating to the re-evaluation of subapical cell division represent, at the same time, an account of one mode of action of three plant growth regulators which have been the subject of intensive study.

GIBBERELLIN-INDUCED STEM ELONGATION IN ROSETTE PLANTS

Cytological examination of *Samolus parviflorus,* a long-day rosette plant, revealed a great increase in mitotic figures in the regions immediately below the apex within 24 hrs. after the application of gibberellic acid (gibberellin A_3, abbrev. GA) (11). Similar evidence is available for other rosette plants [Table 2 in (18)], but only the data for *Samolus,* which has been studied in greater detail, will be presented. As the influence of GA continues, the zone of cell division increases in length, exactly equalling the growth in length of the stem (Figure 1). This new zone of division, comprising the cortical, vascular, and pith tissues, can be considered as a virtual subapical meristem. Another important feature of GA action is illustrated in Table 1. There is no increase in cell length for 72 hrs. following GA

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Table 1. Length of pith cells of *Samolus* following application of gibberellic acid.

Hrs. After Application of GA	Distance Below Apical Meristem in Microns					
	0 to 200	200 to 400	400 to 600	600 to 800	800 to 1000	1000 to 1200
Controls	15 (8–25) *	23 (20–30)	32 (25–40)	40 (30–50)	53 (45–65)	64 (55–75)
24 hrs.	12 (5–18)	22 (15–30)	31 (25–35)	40 (35–45)	45 (35–50)	64 (50–80)
48 hrs.	11 (5–15)	11 (10–13)	19 (10–25)	23 (15–30)	21 (15–25)	31 (25–35)
72 hrs.	11 (5–18)	15 (10–20)	14 (10–25)	18 (13–25)	18 (10–25)	28 (15–40)

* Numbers in parentheses indicate range in cell length for each zone.

application. It is clear then that in the initial stages of stem elongation GA causes only an increase in cell number. Equally noteworthy is the fact that more than 80 per cent of the induced cell divisions are transverse; i.e., they are oriented to contribute to stem elongation (18, 20).

What are the quantitative aspects of GA-induced subapical meristematic activity? In a few experiments with *Samolus* in which the dose was varied, a minimum cell division response was obtained with 0.5 μg. GA per plant, but for several reasons discussed elsewhere (19) it is difficult to establish the precise relationship between GA

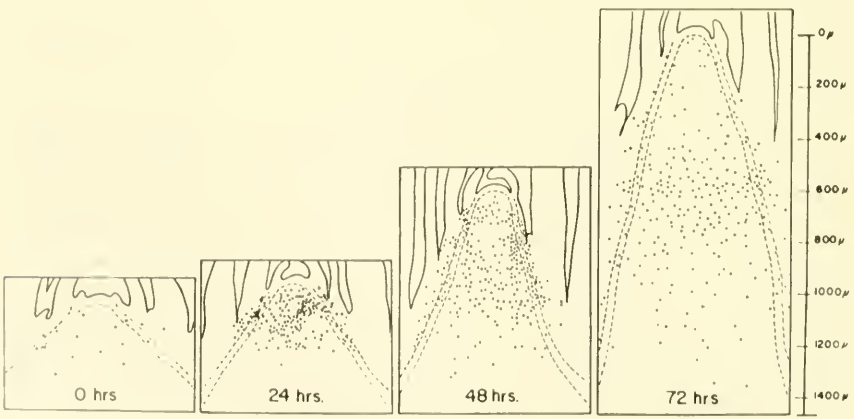

Fig. 1. The effect of GA upon stem elongation and cell division in *Samolus*. Number and position of mitotic figures per 48 μ median longitudinal section at 26° C. There were six plants per group collected at the indicated times, and the diagrams are composites of six median sections (each 8 μ thick) taken from one plant. Each dot represents a mitotic figure. The boundaries of the vascular tissue and apical meristem are indicated by dashed lines.

dose and mitotic activity. We have, however, studied in some detail the nature of the cell division response in *Samolus* at saturating doses of GA (in excess of 10 μg. per plant daily). With these relatively large doses, periodic fluctuations in the number of mitotic figures appearing in the pith tissue could be observed for at least 72 hrs. (Figure 2). These maxima are unrelated to the time of day or manner of application of GA (Figure 3). They are, however, related to the temperature at which the treated plants were maintained and the initial application of GA, always appearing at definite times after the initial application, this time depending on temperature (Figure 4). Thus, we concluded that the peaks in mitotic counts reflect a partial synchronization of cell division and that the average pith cell gener-

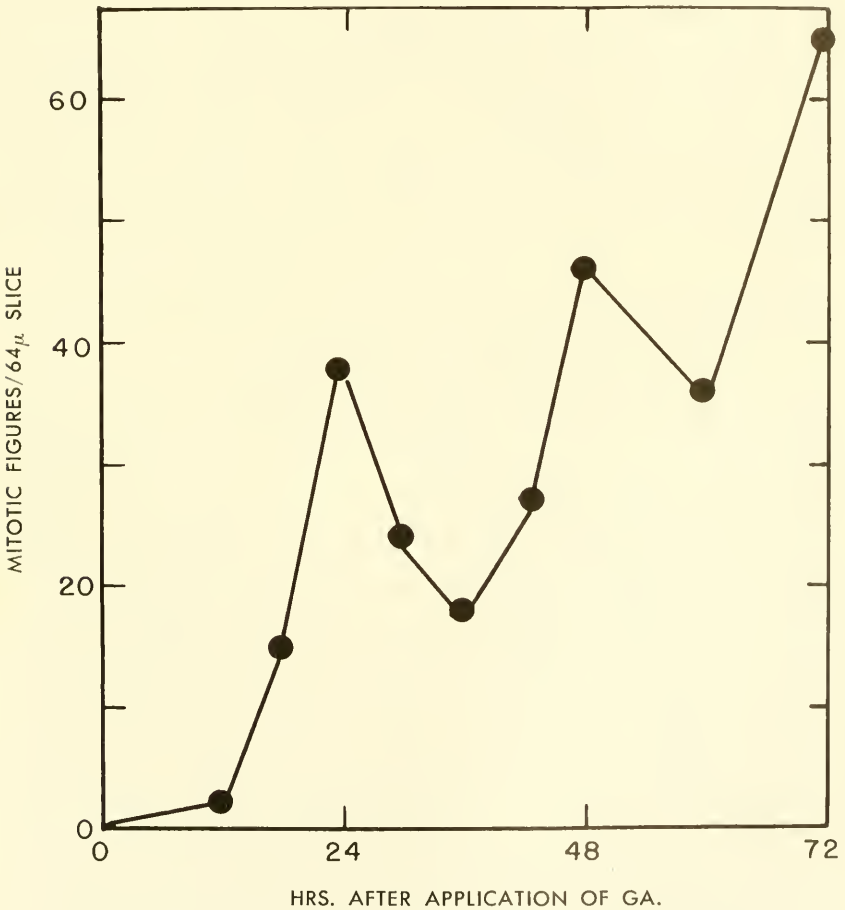

Fig. 2. Counts of transverse mitotic figures in the pith tissue at various times after the initial application of GA at 26° C. Each point is the average of counts made for six plants. The plants received 25 μg. of GA at 0, 24, and 48 hrs.

ation time in the subapical pith region of GA-treated *Samolus* plants is approximately 24 hrs. at 26° C., 32 hrs. at 20°, and 46 hrs. at 17° (Figure 4). The Q_{10} for mitosis, then, is equal to 2.1, a value similar to that found for the apical cells of pea roots (3).

Approximately 6,300 pith cells are produced by GA-induced subapical mitotic activity at 26° C. in the initial 72 hrs. (19). Assuming that the pith cell generation time is 24 hrs., then there are 3 cell generations in 72 hrs.; hence, in the pith region in the initial 24-hr. period, 900 cells, representing the first generation, divided in response to GA [for calculations see (19)]. Further calculations reveal that the average duration of karyokinesis (in our study equal to the time required for cells to progress from metaphase through telophase) is about 32 min., which is very close to the figure calculated by Brown (3) for the apical cells of pea roots, namely, 40 min.

The time required for GA to diffuse to the active sites in the pith cells of *Samolus* is less than 2 hrs. (19), which is within the limits of accuracy of our determination of cell generation time. Hence, the 24-hr. delay in the mitotic effect of GA is an inherent trait of the mechanism of action of this substance on cell division. Since the delay approaches 24 hrs. and since, in addition, the pith cell gener-

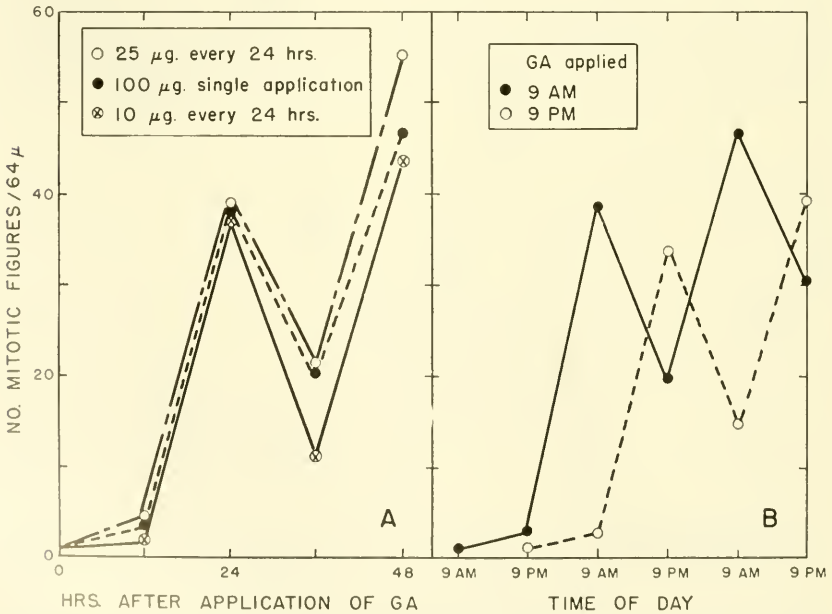

Fig. 3. Periodic fluctuations in mitotic activity in the pith tissue as related to the mode of application of GA (**A**) and time of day of the initial application (**B**). In the latter case, 25 μg. of GA were applied at 0 and 24 hrs. to both groups.

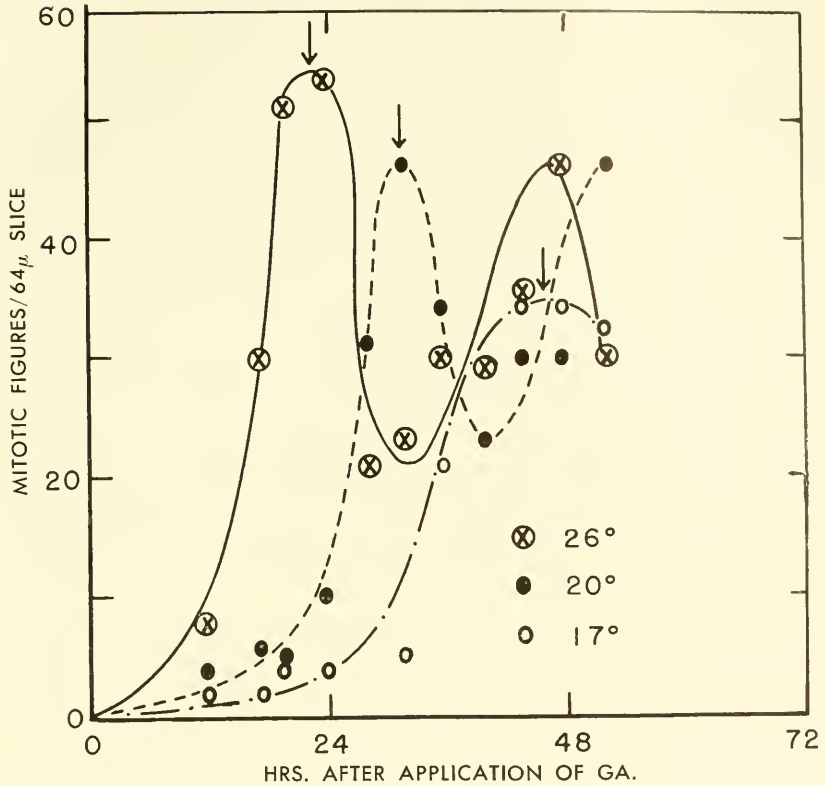

Fig. 4. The effect of temperature upon pith cell generation time. Each point is the average count made for six plants. The arrows indicate the point from which the generation times have been measured. All plants received 100 μg. GA at 0 hrs.

ation time is also 24 hrs., it appears that the stage at which GA exerts its first effect is early interphase (12, 19).

With regard to shoot growth in rosette plants, there is little doubt that the subapical meristem is responsible for the marked increase in stem elongation following GA treatment. In fact, the cell contribution from this region is so large that the direct contribution of the apical meristem to GA-induced stem growth in rosette plants may be disregarded (18, 19). Furthermore, at least two facts show that GA-induced stem elongation is closely related to the natural case in rosette plants: (A) A gibberellin-like substance extracted from the fruit of wild cucumber (15) initiates the same cytohistological development as GA (18) and, eventually, flowering (13). (B) In rosette plants in which stem elongation is induced by the proper environmental conditions, the subapical meristem develops in the same manner as in the GA-treated plants, although more slowly.

AMO-1618-INDUCED INHIBITION OF STEM ELONGATION IN CAULESCENT PLANTS

Extensive work in the U. S. Department of Agriculture on quaternary ammonium carbamates (5, 14, 16, 24) has shown that these substances, the most readily available of which is Amo-1618 [(5-hydroxycarvacryl)trimethylammonium chloride, 1-piperidinecarboxylate], cause a striking inhibition of stem elongation in many plants. In connection with our studies, it was of particular interest that GA reversed the inhibition induced by Amo-1618 in *Chrysanthemum* (6).

Microscopic examination of several caulescent plants (i.e., plants possessing elongate stems throughout their life), including *Chrysanthemum, Xanthium,* and tomato, revealed a subapical zone of cell division comparable in length to that of GA-treated rosette plants. The relatively great number of mitotic figures observed in the subapical regions suggested that in caulescent plants, too, the subapical meristem plays an important role in shoot development (18). Thus, it seemed a logical step to observe the action of Amo-1618 (and GA) on subapical cell division in *Chrysanthemum.*

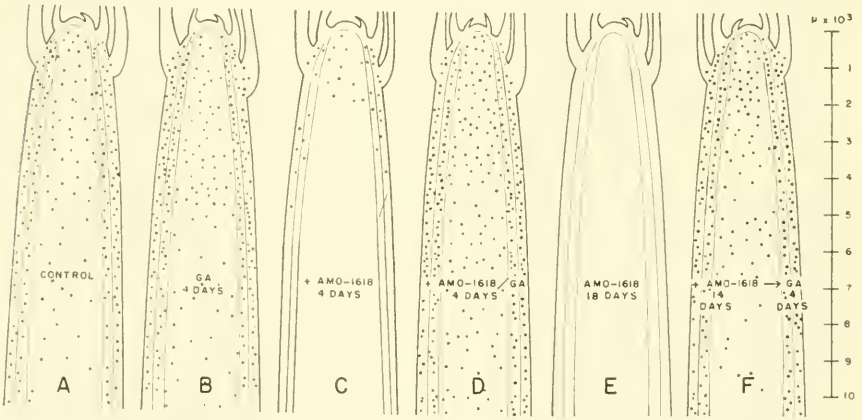

Fig. 5. Number and position of mitotic figures in the pith tissue of *Chrysanthemum* per 60 μ median longitudinal section. Rooted cuttings were immersed for 24 hrs. (continuous light) at 26° C. in one of the following solutions: **A**, water; **B**, GA (100 mg/l); **C**, Amo-1618 (5-hydroxycarvacryl)trimethylammonium chloride, 1-piperidinecarboxylate, 100 mg/l); **D**, mixture of Amo-1618 and GA (both at 100 mg/l). The plants were transferred to soil and placed in long-day greenhouse conditions, and 4 days later they were collected and examined. The plants of groups E and F were immersed in Amo-1618 (same as **C**) and transferred to long-day greenhouse conditions; 14 days later 200 ml. of water (**E**) or of GA (100 mg/l) (**F**) were added to the soil in two doses of 100 ml. each on two successive days. Four days after addition of water or GA to the soil, the plants were collected and examined. Each group contained four plants, and the diagrams are composites of six median longitudinal sections (10 μ per section) taken from one of the treated plants. Each dot represents a transverse mitotic figure. The boundaries of the vascular tissue and the lower limit of the apical meristem are indicated by solid lines.

Within 4 days after the application of Amo-1618 the number of mitotic figures and the length of the zone of subapical activity were substantially reduced (Figure 5); as the inhibition progressed mitotic activity practically disappeared in the subapical meristem (Figure 5, Amo-1618, 14 days) whereas the apical meristem remained relatively unaffected. If GA was added simultaneously with Amo-1618, there was no inhibition of cell division and, perhaps more significant, GA applied some time after Amo-1618 almost completely reversed the inhibition within a period of 24 to 96 hrs. (Figure 5, Amo-1618, 14 days → GA, 4 days). In every case, the activity of the subapical meristem could be correlated with stem elongation: The plants that received Amo-1618 alone assumed a rosette habit of growth; i.e., leaf initiation was normal, or almost so, but stem elongation ceased; plants receiving Amo-1618 and GA at the same time grew normally; plants receiving GA after Amo-1618 reverted from a rosette to a caulescent habit of growth. These results thus strongly support the contention that the subapical meristem is responsible for shoot histogenesis in caulescent plants no less than in rosette species. GA or GA-like substances appear to play an important part in the regulation of the subapical meristem. In rosette plants they seem to be the factors limiting its activity; in caulescent plants they are apparently present at levels insuring optimum mitotic activity, as application of GA to caulescent plants — at least those with which we have worked — does not markedly increase subapical cell division. By use of Amo-1618 to reduce subapical meristematic activity in these plants, we have been able to demonstrate that GA participates in the regulation of this activity and, thus, of shoot histogenesis, in caulescent as well as rosette plants (21).

MALEIC HYDRAZIDE INHIBITION OF SHOOT GROWTH

There have been several studies on the inhibition of stem growth by maleic hydrazide (MH) (7, 22, 25); one report on tomatoes (8) cited evidence for a reduction in cell number in the treated plants and suggested that this was the main reason for the inhibition of stem elongation by MH. In view of these experiments, we studied the effect of MH upon the subapical meristem in *Xanthium*. Our results show that MH, in appropriate doses (0.4 mg. in aqueous solution) completely prevents cell division not only in the subapical regions (Figure 6) but also in the apical meristem.

MH-treated plants, though no longer capable of stem elongation, do not assume a rosette habit of growth because leaf initiation is also prevented; hence, its action as a regulator of cell division is quite different from that of Amo-1618 or GA, lacking the selectivity of the latter two substances. Furthermore, GA (0.4 mg. in aqueous solution),

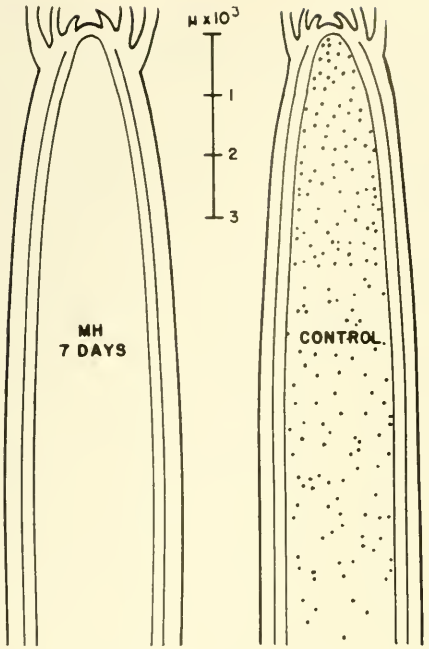

Fig. 6. Effect of 0.4 mg. maleic hydrazide on the number and position of mitotic figures in the pith tissue of *Xanthium* per 60 μ thick median longitudinal section. Each group contained four plants and the diagrams are composites of six median longitudinal sections, each 10 μ thick. Each dot represents a transverse mitotic figure. The boundaries of the vascular tissue and the lower limit of the apical meristem are indicated by solid lines. The MH was applied in aqueous solution to the shoot apex (three equal applications on consecutive days).

whether applied together with or after MH, does not prevent or reverse the inhibitory effect on cell division. There is some controversy in the literature on the interaction of MH and GA. Bukovac and Wittwer (4) reported that GA overcame the inhibitory effects of MH on the epicotyl growth of beans, and Kato (10) showed with cucumber seedlings that GA partially prevented the inhibition of shoot growth by MH. Brian and Hemming (2), working with a variety of peas not responding to GA, concluded that GA did not reverse MH-induced inhibition of stem growth and that MH probably interfered with the normal growth response at some stage before GA exerts its effect. They interpreted Bukovac and Wittwer's experiments as well as their own as showing that MH reduced or prevented the response of GA-sensitive plants to GA. GA does not reverse MH-induced inhibition of cell division, yet the story may be quite different where cell expansion is involved. For this reason it is still difficult to assess MH-GA interactions in the over-all growth of plants.

Table 2. Apical and subapical meristematic activity based on mitotic figures as affected by various treatments.

Species	Treatment	No. of Mitotic Figures Per 60 μ Pith Tissue Slice												
		Subapical												
		Distance below apical meristem ($\mu \times 10^3$)											Total	Apical
		1	2	3	4	5	6	7	8	9	10	11		
Xanthium pensylvanicum	Control	18	23	25	27	17	16	21	17	16	..	..	180	9
	MH	0	0	0	0								0	0
Chrysanthemum morifolium	Control	14	22	16	15	8	13	14	8	8	6	3	127	8
	Amo–1618	1	0	0	0	0							1	6
	Amo–1618 GA	3	25	15	8	4	11	9	3	2	4	1	85	6
Mathiola incana 'Avalanche'*	Control	7	11	6	3	0	0	0					27	7
	GA	8	10	14	8	7	3	2	1	0			53	8
Gerbera jamesonii (12 mm. stalk)	.....	9	12	16	16	22	16	24	22	17	19	7	180	
Hyoscyamus niger var. *biennis*†	Control	2	0	0	0								2	9
	Vern.	30	25	0	0								55	9
	GA	27	29	0	0								56	8
Samolus parviflorus‡	Control	2	0	0	0								2	8
	LD	4	9	9	3	0							25	8
	GA	17	23	22	6	1	0						69	9
Lilium longiflorum 'Croft'§	2 mo. 0–4° C.	14	3	14	23	9							63	

* Plants received weekly sprays of GA (100 mg/l), and both the controls and treated plants were collected 3 weeks later. At this time, the plants possessed approximately 20 fully expanded leaves.

† Controls—Plants growing in long days (18 hrs. artificial light) at 26° C. Vernalized—Plants received 6 weeks at 4 to 6° C. and then placed in long days at 26° C. Plants collected after 8 long days. GA—Plants received daily applications of 25 μg. GA and collected after 3 days at 26° C.

‡ Control—Plants growing at 26° C. in short days (8 hrs. artificial light). LD—Plants received 15 long days (18 hrs. artificial light daily) at 26° C. GA—Plants received daily applications of 25 μg. GA and were collected after 7 days at 26° C.

§ Bulbs stored for 2 months at 0 to 4° C. and then transferred to greenhouse conditions (natural day length, minimum night temperature 21° C.); apical pieces were collected 8 weeks later when the shoots were approximately 20 cm. long.

CONCLUSION

From examination of the data in Table 2, which is a summary of our investigations on mitotic activity in the subapical and apical regions of various plants, there can be little doubt that subapical meristematic activity is widespread and may far surpass the apical meristem in the number of cells contributed to stem elongation. The mitotic counts in Table 2 are for the pith tissue alone, and since the subapical meristem embraces the epidermis, cortex, and vascular tissues as well, they represent no more than 50 per cent of the total subapical cell divisions. *Gerbera* is a particularly interesting case because the flower is initiated while the shoot is still rudimentary, i.e., before stem elongation occurs. Since the apical meristem is completely occupied with the processes of flower differentiation, the cells for the flower stalk (or shoot) are generated in their entirety by the subapical meristem.

Thus, it appears that while shoot organization is mainly determined in the apical meristem (23), shoot histogenesis, i.e., the actual formation of the cells and tissues which constitute the mature stem, takes place in the subapical region which, for all purposes, may be considered as an intercalary meristem. Furthermore, the action of two growth regulators, GA and Amo-1618, is of fundamental importance in controlling cell division apparently specifically in this region.

SUMMARY

Gibberellic acid (GA) caused stem elongation in rosette plants by stimulating mitotic activity in the regions immediately below the apical (pro- or eu-) meristem. The cells produced in this zone, after elongation, constitute the tissues of the mature, elongate stem. In caulescent plants a subapical zone of mitotic activity was observed as much as 2 cm. in length, similar in activity to that in rosette plants which received prolonged treatment with GA. By treatment with Amo-1618 subapical (but not apical) cell divisions were considerably reduced and soon thereafter shoot elongation ceased. Since the apical meristem functioned normally or almost so, and leaf initiation was continuing, the treated plants assumed a dwarf or rosette habit of growth. GA prevents and reverses inhibition of subapical cell division induced by Amo-1618. Thus, in caulescent as well as in rosette plants, GA plays an important role in regulating subapical mitotic activity, thereby controlling shoot elongation. Maleic hydrazide is a powerful inhibitor of cell division in the subapical regions; however, in contrast to Amo-1618, it inhibited apical meristematic activity as well and GA did not counteract the inhibition.

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The Effect of Gibberellin Treatment on the Photosynthetic Activity of Plants

It has been generally known that plants increase their height, leaf area, and dry weight by treatment with gibberellin (GA). The increase in dry weight may result from an increase in photosynthetic activity, or in the efficiency of utilizing photosynthetic products. In order to make this point clear, studies have been made (2, 4) in which the fixation of radioactive $C^{14}O_2$ by detached leaves was determined. These results showed that GA does not distinctly affect CO_2-fixation activity.

It seemed desirable, however, to measure the CO_2-fixation activity using whole plants; also in reference to the activity per unit leaf area, it seemed preferable to use leaves attached to intact plants.

In this report, the effects of GA treatment on the photosynthetic activity of whole plants and of attached leaves, also on the content of some carbohydrates, are given. As test plants, tomato and rice plants were used.

EXPERIMENTAL METHODS

Tomato plants, 'Sekai-ichi,' cultured in Wagner pots, and about 25 cm. in height, were used. GA in 50 p.p.m. aqueous solution was sprayed onto the plants twice, at intervals of 3 days. In all experiments, crystalline GA from the Kyowa Fermentation Industry Co., Ltd. was used.

Rice plants, 'Aichi-Asahi,' were first grown in a seed bed, and were then transferred to Kasugai solutions when they were 20 cm. in height, with six expanded leaves. The culture vessels were 500 ml. or one l. glass jars, and they were placed in the greenhouse. GA was added to the culture solution at a concentration of 10 p.p.m.

The CO_2-fixation activity for whole plants was measured using a Yamada-Murata apparatus (7) in the open field. Potted plants were introduced into a chamber which consisted of a wooden frame covered with transparent sheets. Air was forced into this chamber by an electric blower. Both the air leaving the chamber and the air from outside were bubbled through aqueous NaOH solutions, and the amount of CO_2 fixed by the plants was calculated from the difference between the alkalinities of the two NaOH solutions.

For determination of fixation by attached leaves a tomato leaf attached to an intact plant was placed into a transparent chamber made of polyacryl resin. The CO_2-fixation activity was measured in the same manner as for the whole plants in the open field.

Leaf area was measured by weighing blue-print paper pieces trimmed accurately to the shape of the leaf.

Dry weight was determined by drying samples at 90° C. for one hr., then at 70° C. until constant weight was attained.

For the determination of sugar content, samples were pulverized to pass through an 80 mesh sieve. A 500 mg. portion of the powder was then extracted with 80 per cent ethanol, and the amount of reducing sugar in the extract was determined by the Somogyi method (6). Total sugar was determined after hydrolyzing the same extract with 2 per cent H_2SO_4 for 15 min. Starch was estimated in the residue from the ethanol extract by the method of Pucher *et al.* (5).

RESULTS

Effect of GA Treatment on Photosynthetic Activity of Whole Plant

Changes in the height of tomato plants treated with GA are shown in Figure 1. In both control and treated plants each circle represents the mean value of ten determinations.

Five tomato plants were introduced into the large chamber mentioned above, and their photosynthetic activity was determined in the open field. Two chambers with five plants each were used for the control, and two chambers with five plants each were used for treatment.

The curves showing the photosynthetic activity (the amounts of CO_2 fixed) versus the horizontal intensity of illumination for the plants 8 days after the start of the treatment are given in Figure 2. The changes in the photosynthetic activity during the 8 day period following the treatment at 20,000 lux and 60,000 lux are also given in the same figure. The photosynthetic activity of whole tomato plants was increased about 18 per cent by the GA treatment.

In rice plants, the increase in height, especially the elongation of the leaf sheath, was appreciable. Three rice plants were cultured in

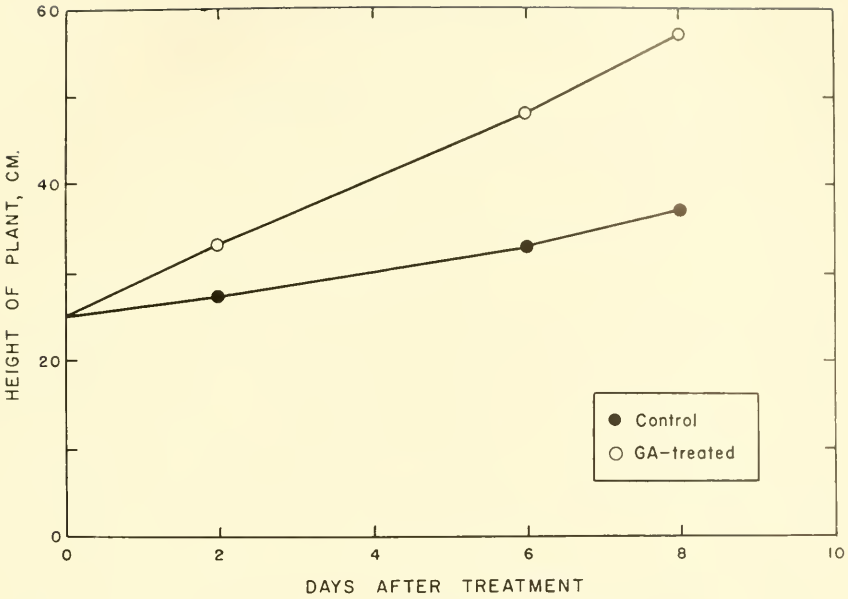

Fig. 1. Effect of treatment with gibberellin on the heights of tomato plants.

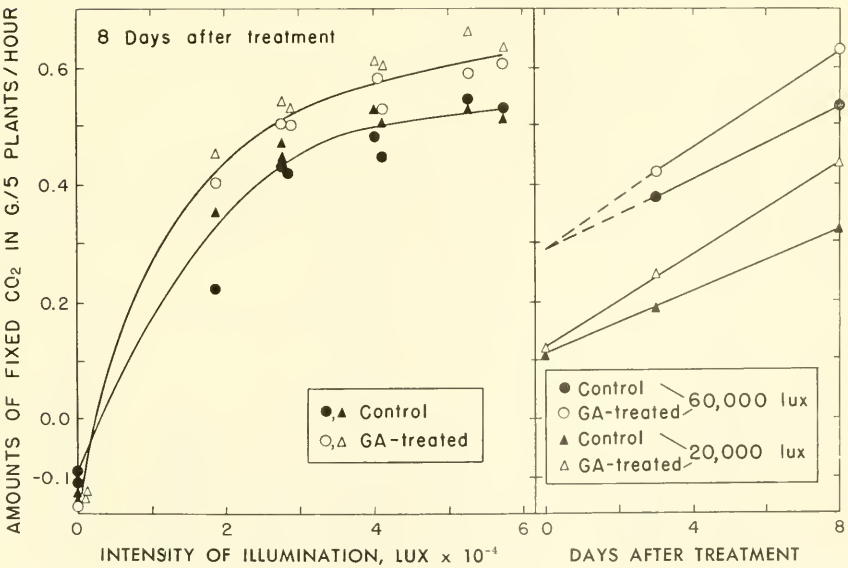

Fig. 2. Photosynthetic activity of tomato plants after treatment with gibberellin.

Table 1. Effect of treatment with gibberellic acid upon leaf area.

Treatment	Total Leaf Area in Cm.² Per Plant	
	Tomato	Rice
Control	776	140.8
GA treated	1,048	143.8

500 ml. glass jars by the water culture method. Ten jars were introduced into the large chamber for measurement of photosynthetic activity. As with tomato plants, two chambers were used for the control and treatment for each experiment. The curves of the CO_2-fixation activity versus the horizontal intensity of illumination for the plants 7 days after treatment are shown in Figure 3. The figure shows that the activity was increased about 10 per cent by the GA treatment.

The dry weight of both control and treated plants is shown in Figure 4. These are the data for the 8th day after the start of the treatment with the tomato plants, and for the 7th day with the rice plants. As a result of the GA treatment in the tomato plant, the dry weight of all parts increased; in the rice plant, the dry weights of the leaf, sheath, and stem increased, while the dry weight of the root decreased. Table 1 shows the effect of GA treatment upon leaf area.

Effect of GA Treatment on Photosynthetic Activity of Attached Leaves

The change in the photosynthetic activity of attached leaves was studied following the growth of the same leaves. The activity of young and adult leaves is shown in Table 2 in which the activity is expressed by the amounts of CO_2 fixed per unit leaf area. The young

Table 2. Effect of treatment with gibberellic acid on CO_2-fixation by attached leaves.

Days After Start of Treatment	CO_2 Fixed, Mg. Per Cm.² of Leaf Area Per Hr.			
	Control		GA treated	
	Young	Adult	Young	Adult
0	0.209	0.188	0.182	0.202
2	0.203	0.194	0.216	0.199
5	0.213	0.210	0.209	0.202
7	0.202	0.151	0.188	0.170
9	0.218	0.175	0.192	0.150

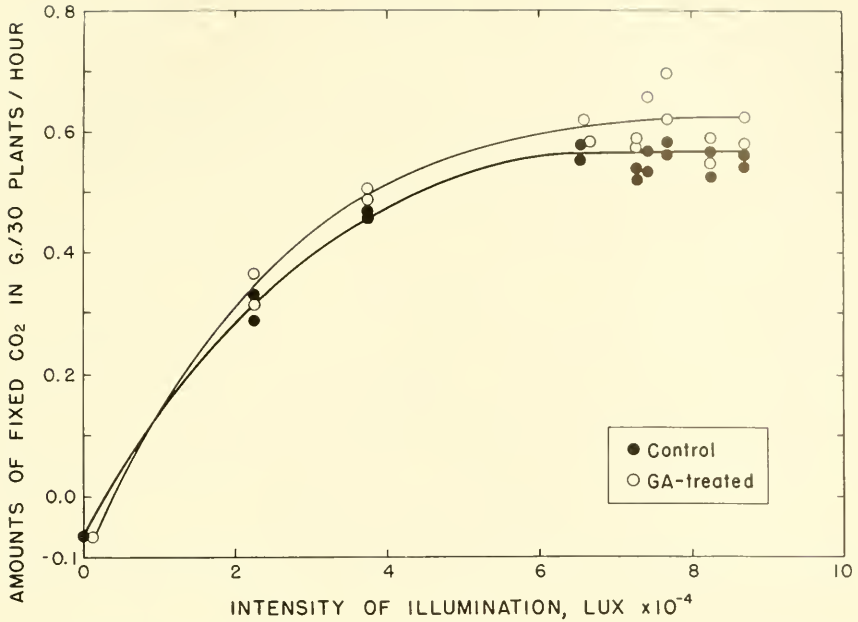

Fig. 3. Photosynthetic activity of rice plants 7 days after treatment with gibberellin.

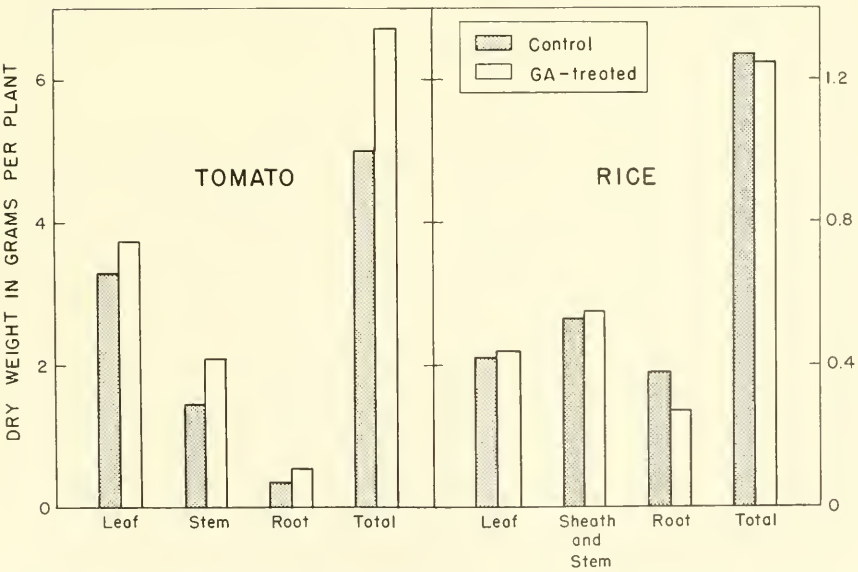

Fig. 4. Effect of treatment with gibberellin on the dry weights of tomato and rice plants.

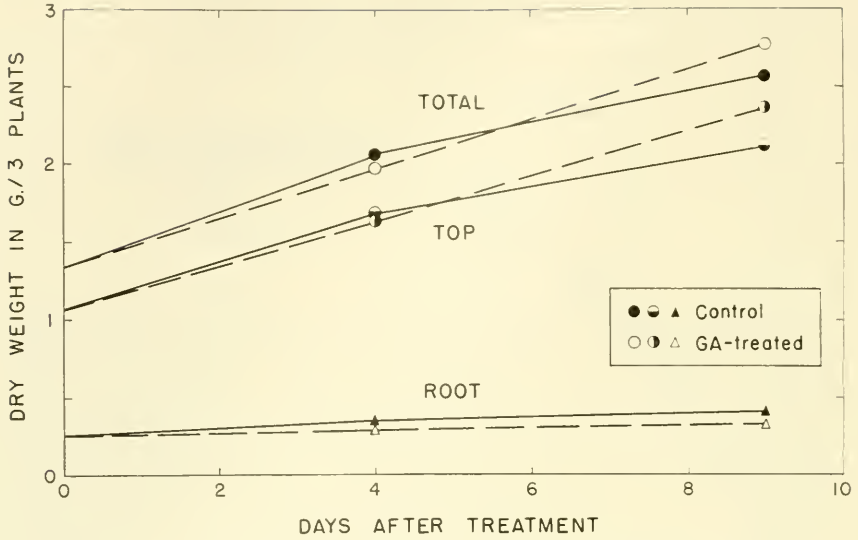

Fig. 5. Changes in dry weight after treatment with gibberellin.

leaves were those which were growing vigorously during the experiment, and the adult leaves were those which had already attained the maximum leaf area at the start of the experiment. The difference between young and adult leaves was practically two leaf ages. The table shows that there is no significant difference in the photosynthetic activity between the control and the treated plants. It seems, therefore, that GA does not affect the photosynthetic activity on a unit leaf area basis.

Changes in Content of Sugars and Starch by GA Treatment

Only rice plants were used for this experiment. Cultural conditions were the same as in the measurement of the photosynthetic activity. The effects of GA treatment on the morphology of rice plants are remarkable. For example, elongation of the leaf sheath of the youngest leaf during 9 days was 1.2 cm. in the control, while it was 16.5 cm. in the treated plants.

Changes in dry weight are shown in Figure 5. The dry weight of the top increased, but that of the root decreased, as the result of the GA treatment. In total, dry weight was increased by the treatment.

Changes in the content of sugars are shown in Figure 6. In this figure, the scale for the root is magnified five times relative to that for the top. At the start of the treatment, the sugar content was very low owing probably to the after-effect of transferring plants from soil culture to water culture. Four days after the treatment, the content

of total sugars in the top is the same in the control and the treated plants, but the content of reducing sugars is higher in the treated plants than in the control. In the root, the contents of both reducing and total sugars was decreased as a result of the GA treatment.

Figure 7 shows the changes in the starch content. It is noticeable that GA lowers the starch content in the top of the plants. In the root, the starch content was almost nil in both the control and the plants treated with GA.

DISCUSSION

The 10 to 18 per cent increase in photosynthetic activity of whole plants about one week after treatment (Figures 2 and 3) may be accounted for by any of the following possibilities: (1) photosynthetic activity per unit leaf area is increased; (2) the activity per unit leaf area remains unchanged, but, owing to the increase in leaf area by the treatment, the amount of carbon dioxide fixed by the whole plant increases; (3) the activity per unit leaf area decreases but the increase in leaf area compensates for the decrease in activity; (4) the surface area of organs other than the leaves, for instance, the leaf sheath in rice plants, is increased by the treatment and the increase in photosynthetic activity of these organs accounts for the increased activity of the whole plants; (5) the treatments affect the shapes of plants, in-

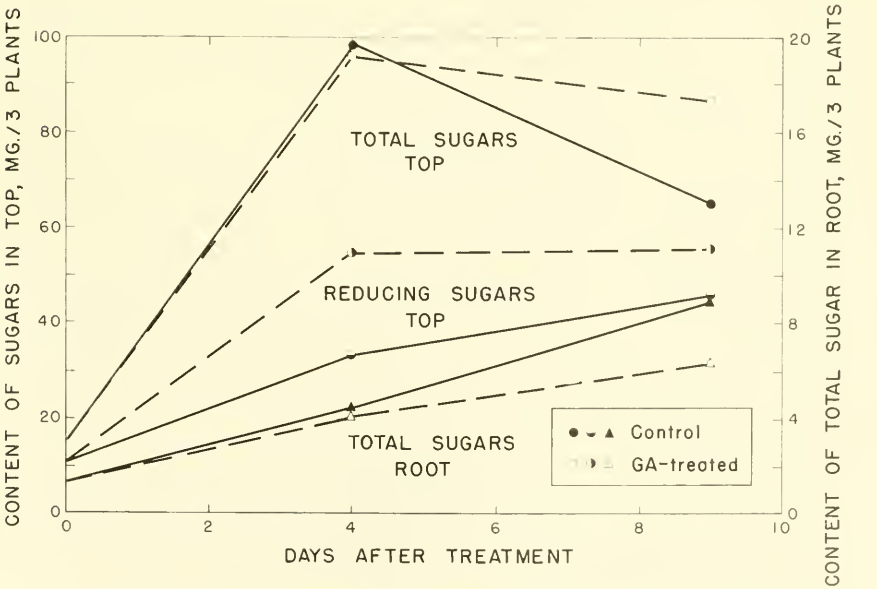

Fig. 6. Changes in the sugar content of rice plants after treatment with gibberellin.

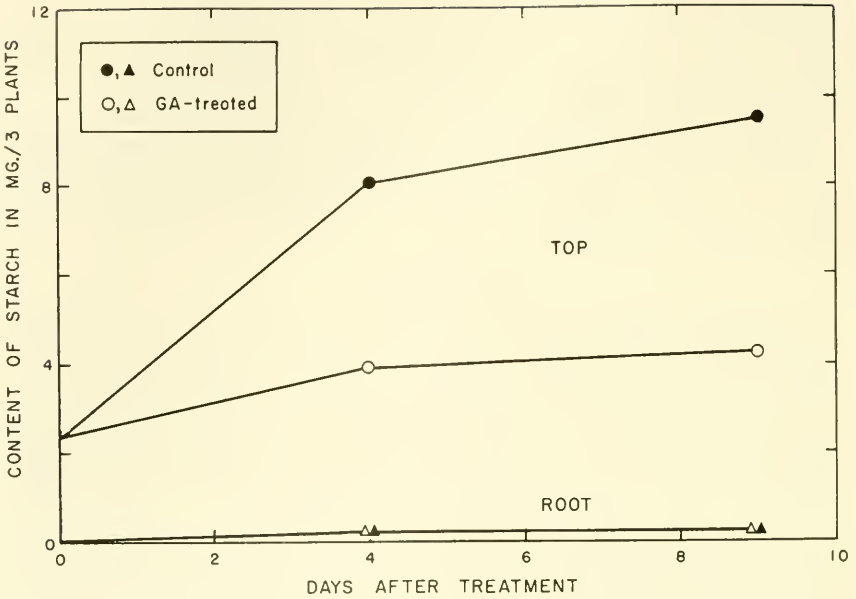

Fig. 7. Effect of treatment with gibberellin on the starch content of rice plants.

crease the light reception, and, therefore, increase carbon dioxide fixation.

Among these possibilities, the second seems to be most feasible in view of the data given in Figures 2 and 3 and Table 2. The photosynthetic activity per unit leaf area does not change as the result of the GA treatment; but owing to the increase in leaf area, the photosynthetic activity of the whole plant increases.

In these experiments, sufficient fertilizer was supplied. If the fertilizer supply is insufficient, the leaves may become yellowish by GA treatment, the photosynthetic activity per leaf area should then decrease and the activity per whole plant may also decrease.

In rice plants, the increase in the sheath area by GA treatment is often larger than the increase in the leaf area as in the example given in Figure 3. In this instance that may be important in increasing carbon dioxide fixation. Brian *et al.* (1) and others have previously referred to this possibility.

An increase in the efficiency of light reception due to the change in plant shape may also be working in these experiments. In plants which are grown in groups, and subjected to mutual overlapping in the field, an increase in the efficiency of light reception by GA treatment may be appreciable.

Next, referring to the carbohydrates produced, the dry weight in the top increases, while in the root it decreases by the GA treatment as given in Figures 4 and 5.

Brian also recognized the growth inhibition of roots when GA was added to the culture solution. He considers this to be due to the direct action on roots of GA in a high concentration.

Since the data given in Figures 6 and 7 represent only the steady state of the contents of sugars and starch, it is rather difficult to explain inclusively all these facts. Even if the photosynthetic activity does increase, the content of such carbohydrates does not necessarily increase. Any decrease in these carbohydrates may have resulted from the increase in the activity of the system utilizing the photosynthetic products.

The increase of reducing sugar in GA-treated plants corresponds with the increase in the invertase activity which has been described in a previous report (3). The decrease of starch, on the other hand, does not correspond with the decrease in the activity of amylase which has also been described in that report. More studies are required to clarify these observations.

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DISCUSSION

Dr. Tolbert: Dr. Haber and I have confirmed these results working with other plants. We found that radioactive CO_2 fixation per

unit fresh weight of leaves was not increased by gibberellin treatment (Plant Physiol. 32: 152. 1957).

Dr. Larsen: I should like to mention an additional point, namely the possible effect of gibberellin on the rate of respiration, an item that also enters the balance sheet for the production of dry matter. Does Dr. Hayashi have data on the output of carbon dioxide in the dark? It would be interesting to know whether leaf sheaths of rice can have a positive net rate of photosynthesis or whether their maximum rate of photosynthesis is just capable of counterbalancing the output of carbon dioxide by respiration. If there are stomata on the leaf sheaths of rice (as there are on those of *Avena),* the sheaths may contribute to the net increase in dry weight. The effect of gibberellin, not only on the leaf area but also on the sheath area, would thereby become important.

Dr. Hayashi: I haven't discussed the respiration rate, but I have determined it in these experiments. The respiration rate is from 10 to 20 per cent of the photosynthesis rate at saturated-light conditions, based upon CO_2 exhaustion and fixation, respectively.

Dr. Larsen: Would gibberellin influence the rate of respiration?

Dr. Hayashi: Yes, gibberellin increases the rate per plant, but the increase is small.

Professor Blackman: May I make a comment and a suggestion in relation to the analysis of the effects of gibberellin on growth? The value of growth analysis is admittedly a hobby horse of mine, but it seems to me that these concepts can help in elucidating how these changes in growth are brought about in the field. It can be shown that the relative growth rate (the rate of gain in dry matter per day) is the product of the net assimilation rate (rate of gain in dry matter per unit area of leaf) and the leaf area ratio (total leaf area to total plant weight). It follows that if gibberellin increases the growth rate but does not bring about much change in the rate of assimilation, then it must increase the leaf area ratio. I suggest that it would be worthwhile seeing how far gibberellin has altered this ratio. There is another point which needs to be taken into account, namely, that there may be self-shading of one leaf by another and in consequence the relationship between light intensity and photosynthesis will not be the same for individual leaves and whole plants. We have been working with *Salvinia natans* where there is no trouble of self-shading since all the leaves are flat on the surface of the water. Gibberellin has little effect on the net assimilation rate, but it depresses the relative growth rate by decreasing the leaf area ratio.

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Does Gibberellin Act Through an Auxin-mediated Mechanism?

Probably most studies of the chemical control of plant growth have dealt with the action of auxins. Recently, however, an increasing number of other growth regulators have claimed the attention of plant physiologists. These include the gibberellins (2), various substituted purines (13), and compounds obtained from endosperm tissue (12). Since the mechanisms of action of all such compounds, including auxins, are unknown, the question as to whether the gibberellins act through an auxin-mediated mechanism cannot be answered conclusively. The chief motive for this attempt is that, for various reasons, the working hypothesis implied by the question appears to have been somewhat uncritically accepted as established in a few papers. In our view, the evidence is largely negative, and such acceptance unjustified. Our first task will be to define the question more precisely.

DEFINING THE QUESTION

The term auxin will be used to mean indole-3-acetic acid (IAA) or closely related natural or synthetic compounds presumed to have similar physiological action. This is similar to the definition given by Tukey *et al.* (14) except that their definition could also be used to include the gibberellins, which would merely confuse the issue here. For gibberellin see the review by Brian (2). Gibberellic acid (GA) was used in the experiments reported.

An affirmative answer to the question would mean that the physiological action of gibberellin is due to a primary effect on a biochemical system participating directly in auxin synthesis, transport, action, or inactivation. The term auxin-mediated thus implies an intimate relation between gibberellin action and auxin, and the ques-

tion should not be paraphrased "Do the physiological effects of gibberellin and auxin interact?" Several possible mechanisms of auxin-mediated gibberellin action (cf. 2) can be summarized briefly as follows: (A) Gibberellin may protect native or exogenous auxin from inactivation within the tissue. (B) It may act by increasing the synthesis of native auxin, or its translocation or binding to active sites. (C) It may increase the number of sites available with which auxin molecules can react to cause growth.

Such proposals all envisage gibberellin as acting by somehow increasing net auxin activity. Actions in the reverse sense are not usually considered, in view of the many similarities between gibberellin and auxin activities, but would still constitute auxin-mediated actions.

Most of the evidence available is derived from growth experiments, while some comes from studies of the enzymatic activity or growth substance content of extracts. Our own work has been entirely of the former kind; it will be reviewed in the succeeding section and then considered as part of the total data available.

EXPERIMENTAL

The etiolated pea epicotyl section test was the experimental system chosen, since it responds to both GA and IAA. Sections were cut from developing third internodes of 7- to 8-day seedlings of *Pisum sativum,* 'Alaska,' grown in total darkness. They were incubated in darkness for the next 20 to 24 hrs. in a basic medium consisting of phosphate buffer plus 2 per cent sucrose (except as otherwise noted) and further supplemented with GA or IAA as desired. For details of the methods employed and of data described but not presented here see (8) and (9).

If sections are cut from various regions of the third internode and their elongation in buffer-sucrose medium compared, the more apical the section the greater the elongation. Thus S1 sections (S standing for short, i.e., 5 mm., and 1 standing for 1 mm. below the plumular hook) elongate more than S4 sections, which in turn elongate more than S7 sections. If IAA or GA is added to the medium, further differences are found. S1 sections under these conditions show a very low IAA optimum (about 10^{-7} *M*), and the additional elongation over the endogenous (buffer-sucrose only) caused by optimal IAA is quite small; higher levels of IAA are inhibitory. The lower sections show a higher IAA optimum (about 10^{-6} *M*), and the additional elongation caused by optimal IAA is greater than for S1 sections. The situation is quite different with respect to GA. There is no pronounced optimum for GA activity, and a plateau is reached at about 10^{-6} *M*. The additional elongation caused by GA is greater in S1 sections than in

S4, and greater in S4 than in S7 sections. In summary, the greatest response to GA appears in the more apical sections, while the greatest response to IAA is in the lower, more mature tissues (8).

When similar experiments are conducted in the absence of sucrose, the results are different. Under such conditions, the endogenous elongation of S1 sections is greatly reduced. The response to GA of all types of sections is so low as to be frequently undetectable; GA and sucrose are in fact synergistic in promoting elongation of S1 sections (9). The interaction between sucrose and IAA in the elongation of S1 sections is different and more complex; it has been described in detail elsewhere (7). In brief, concentrations of IAA ($> 10^{-6}$ *M*) which inhibit elongation in the presence of sucrose will promote elongation in its absence.

Since the object here was to measure the physiological effects of GA and IAA separately and together with a view to judging the degree to which these might be biochemically linked, the first noteworthy point is the apparent spatial separation between tissues most responsive to GA and those most responsive to IAA under the conditions employed. A number of workers (cf. 2) have noted the rough correlation between endogenous growth capacity and responsiveness to GA. It has been suggested that the more apical sections show a greater response to GA because of their higher endogenous auxin level, a factor which might also occasion their lower auxin response. A partial test of this hypothesis can be made by seeing whether the response of more basal sections to GA is increased in the presence of IAA. Results of all such experiments have been negative. A typical experiment is summarized in Figure 1, where it is evident that the increment of elongation caused by GA in S1 sections is greater than that in S5 sections; levels of IAA which increased S5 elongation to that of the endogenous S1 elongation failed to increase their GA response.

Numerous systems have been described in which the GA response is markedly dependent on exogenous IAA and in which synergisms between IAA and GA occur. We have not found any such relationships in etiolated pea epicotyl sections. Usually the elongation caused by one substance is approximately additive to that caused by the other under all conditions tried. This is true even if the test sections are starved by incubation in sucrose-free buffer for 24 hrs. before the test proper commences (9). It is also particularly significant that, in S1 sections, a marked and only slightly reduced response to GA is obtained even in the presence of highly inhibitory levels of IAA. An experiment showing this is presented in Figure 2.

Our further attempts to establish some relation between GA and IAA metabolism took the form of treatments which would affect the

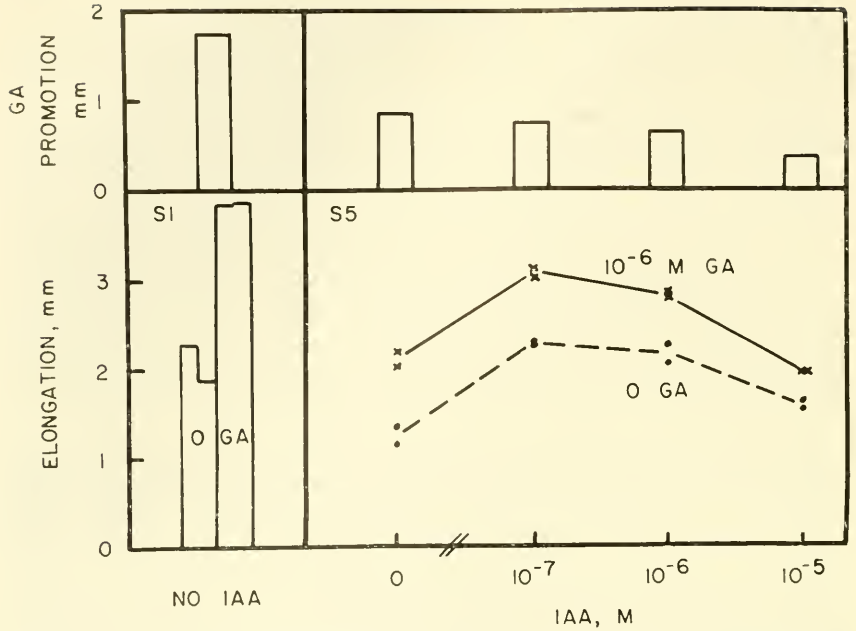

Fig. 1. Effect of distance from apex and of IAA on response of etiolated pea epicotyl sections to GA. Lower histogram: response of S1 sections (apical) to 10^{-6} *M* GA in the absence of IAA. Curves: response of S5 sections (subapical) to GA in the presence of various IAA concentrations. Upper histograms: magnitude of GA responses (GA-treated value minus corresponding control). Divisions of bars and pairs of points represent replicate lots of ten sections. Control medium: phosphate buffer, pH 6.1 plus 1 per cent sucrose. Incubation: 20 hrs. in darkness, about 30° C.

IAA responses, and thus inferentially the auxin relations of the tissues. Two such experiments are shown as Figures 3 and 4. Figure 3 shows the effects of adding α-(*p*-chlorophenoxy)isobutyric acid (PCIB) to the test medium on the control elongation and on the GA and IAA responses of S1 sections. PCIB has been described as an anti-auxin (3), but the interpretation of this experiment does not depend on the correctness of this view. It is evident from Figure 3 that a level of IAA which has essentially no effect in the absence of PCIB greatly promotes elongation in its presence, returning the PCIB-inhibited sections to (or occasionally above) the control. PCIB thus inhibits control elongation and brings about a greatly increased auxin response. The additional elongation conferred by GA, however, is essentially the same in the presence or absence of PCIB, whether or not IAA is present as well.

Very similar results are obtained by an entirely different treatment which also alters the auxin response of S1 sections. This treatment consists of decapitating the seedlings several hours before the

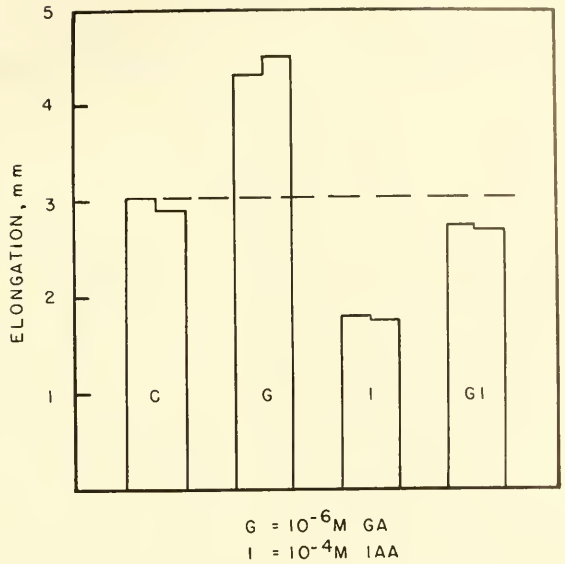

Fig. 2. Effect of inhibitory levels of IAA on response of S1 sections to GA.

test sections are cut from them. As shown in Figure 4, such treatment again markedly changes the IAA response. The control elongation of sections from decapitated plants is reduced, and a level of IAA formerly inhibitory now promotes elongation. Again, however, the additional elongation caused by GA is unaffected by such changes.

DISCUSSION

Because of space limitations this discussion will have to proceed in general terms; for detailed citations of the relevant literature see (2) and (9), and elsewhere in this volume.

Evidence Is Against Auxin-mediated Mechanisms

Some possible mechanisms of auxin-mediated gibberellin action were summarized earlier, and that outline will be followed here.

The possibility (A) that gibberellin acts by protecting native or exogenous auxin from inactivation in some manner has been raised by a number of workers who have interpreted gibberellin effects on peroxidase or IAA oxidase activities as evidence for this view. However, the literature taken as a whole is somewhat contradictory. Reports have been made of decreased, unchanged, or increased peroxidase activities in extracts from plants previously treated with gibberellin; of these, only the first kind would be consistent with an auxin-protecting or auxin-sparing role of gibberellin. IAA oxidase

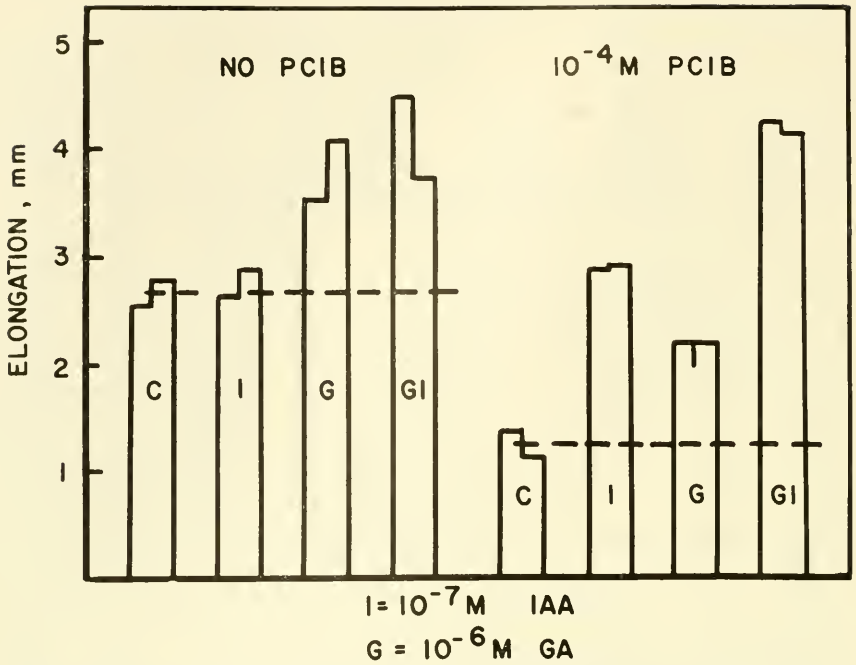

Fig. 3. Effect of α-(*p*-chlorophenoxy)isobutyric acid (PCIB) on responses of S1 sections to IAA and GA.

activity has been reported as decreased or as unaffected by gibberellin treatment, and gibberellin has also been found to inhibit the activity of IAA oxidase preparations in vitro. Whether or not such results are meaningful for the question at hand depends on whether IAA oxidase or other peroxidase-based systems known to destroy IAA in vitro are believed to do so in vivo. There is no compelling evidence for such a view at present (cf. 4); while there are frequent correlations between certain developmental phenomena and IAA oxidase or peroxidase activity, the body of data is not consistent.

The fact that GA can still promote pea section elongation in the presence of inhibitory auxin levels (Figure 2) argues strongly against any auxin-protecting mechanism of GA action. In addition, there are in the literature growth systems in which auxin and gibberellin appear to have opposite effects, and these are also inconsistent with such a mechanism.

The growth phenomena just mentioned are almost equally valid objections to the suggestion (B) that gibberellin acts by increasing the production or translocation of native auxin. Here again, reports on whether or not native auxin levels increase following gibberellin

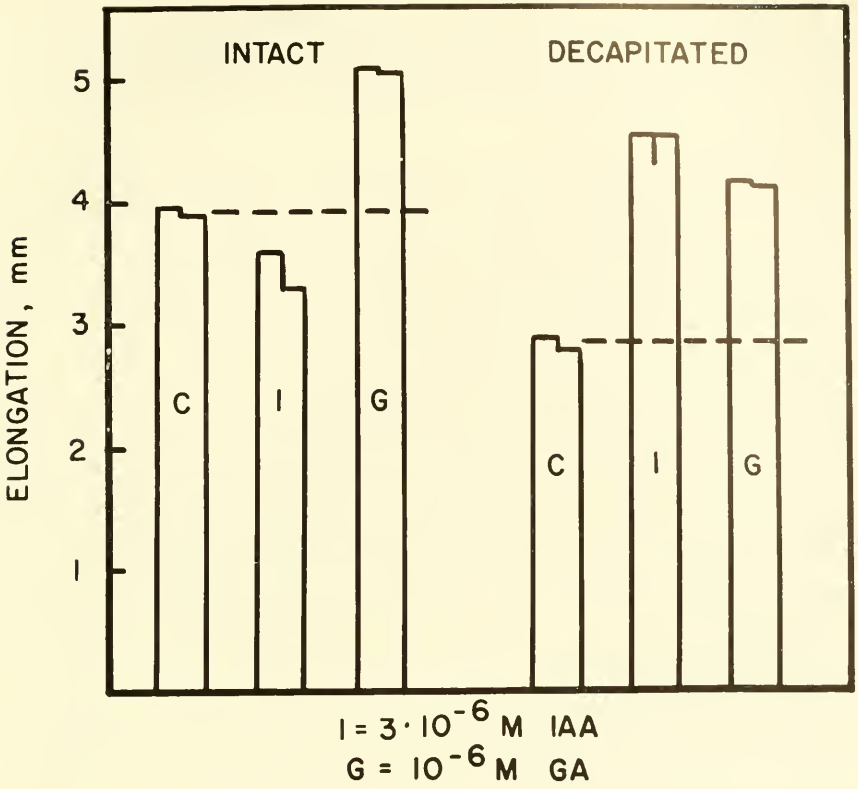

Fig. 4. Effects of decapitation of test plants on responses of S1 sections to GA and IAA. Decapitation 6 hrs. before taking of sections.

treatment are contradictory. If indeed they do, it still remains to be shown that such increased levels are causally related to the growth induced by gibberellin rather than merely correlated with it. GA promotion of elongation in the presence of inhibitory IAA levels (Figure 2) is again difficult to reconcile with a mechanism invoking increased auxin production, transport, or binding as the mechanism of GA action, although it can be argued that exogenously applied IAA is not physiologically equivalent to an increase in native pea auxin, whatever its nature. Proponents of such a view would then have to postulate at least partially different modes of action for native and exogenous auxins.

The two preceding hypotheses are relatively clear, and appear unsatisfactory on the available evidence. In contrast, possible actions of gibberellin on the availability of molecular sites for auxin action are more difficult to formulate clearly, and thus to evaluate. The data

of Figure 2, which injured the previous hypotheses, are harmless here: it would be reasonable to expect GA-induced elongation in the presence of inhibitory auxin levels if GA increased the other component of the auxin reaction system rather than increasing or protecting auxin itself.

A major source of support for this general view can be found in numerous reports of systems in which a response to GA is dependent upon, or greatly increased by, the presence of exogenous auxin. The most striking such system has been described by Kuse (6). GA-IAA synergisms have also been found for the elongation of sections from light-grown pea seedlings and in starved etiolated sections (2) although in our work with etiolated pea sections the effects of GA and IAA under all circumstances have been additive or subadditive. The existence of GA-IAA synergisms, however, does not demonstrate an auxin-mediated action of gibberellin; it may simply indicate that such systems are so strongly auxin-depleted that auxin is absolutely limiting to growth. It is notable that in some systems the action of kinetin (6-furfurylaminopurine) is similarly dependent upon the presence of auxin, yet it has not been concluded that kinetin acts directly on auxin metabolism (13). Returning to our own work, we note that there is an almost absolute dependence of GA-induced (but not of auxin-induced) elongation in S1 sections on the presence of sucrose. It would be unwise to conclude from this, however, that GA action is specifically mediated by carbohydrate metabolism. Although none of these conclusions can be rejected, they cannot be accepted on this sort of evidence.

It has been suggested (2) in support of auxin-mediated GA action that the lower response of subapical older tissue to GA is due to its lower auxin content. It is certainly evident that such tissue is auxin-limited, since added auxin can increase its elongation. However, the auxin occasions no increase in the GA response (Figure 1). Of course, since the apical and subapical sections differ considerably anatomically, it really is not to be expected that a single factor such as auxin would account for the difference in GA response.

If GA acts directly to increase a nonauxin component of the auxin reaction system, the relation between IAA- and GA-induced elongation in our experiments is surprisingly loose. Either previous decapitation of the plants or inclusion of PCIB in the test medium reduces the control growth and at the same time results in an increased response to IAA, thus creating a strong presumption of a change in the auxin relations of the tissues. In effect, either treatment appears to make auxin more limiting. Yet the elongation induced by GA re-

mains virtually unchanged by these treatments (Figures 3 and 4), a result which seems to render any direct relationship between GA and auxin action unlikely, although it cannot disprove it.

Alternative Views

If present evidence leads to a rejection of the concept that gibberellin action is auxin-mediated, it may still be useful to consider ways in which auxin and gibberellin might interact in growth phenomena. It has been proposed (2) that GA and auxin interact through some third factor. If this factor is conceived of as an auxin-destruction system, or a direct inhibitor of auxin action, such an hypothesis appears unlikely in view of the above evidence. If, however, it is regarded as some unspecified complex of unknown reactions, or, to expand it further, the plant tissue itself, the hypothesis is of course perfectly reasonable, if unspecific.

It was suggested in the experimental section that the increment of elongation induced by GA in a section is relatively independent of the presence of IAA or PCIB, although it is affected by the presence or absence of sucrose. Similarly, the increment (positive or negative) in elongation caused by IAA is about the same in the presence or absence of GA. Such results, in which the absolute effects of various treatments appear to be independent of each other, are frequently encountered in pea section growth tests, at least in this laboratory, and merit some consideration here. A digression into light physiology is necessary to provide further background.

In 1941 Schneider (11) reported that the absolute magnitude of the inhibition of elongation caused by red light in dark-grown *Avena* first internode sections was more or less constant, even when the total elongation of the sections was varied over a wide range by changing the auxin level. Hillman (5) extended these observations in studying the red light inhibition of the elongation of pea sections from dark-grown plants and the far-red promotion of sections taken from plants grown in red light. The absolute magnitudes of the red light inhibition or far-red promotion were unaffected by any but high levels of IAA, and were also independent of GA, although both growth substances affected total elongation. The conclusion was reached that a portion of "endogenous" growth was light-sensitive while GA- and IAA-induced growth was not. These results were obtained with long, subapical pea sections. Since then, Bertsch (1) has shown that in S1 sections, in which elongation is greatly promoted by sucrose, only that increment of elongation attributable to sucrose is labile to red light; the red light inhibition is the same in absolute units as the

sucrose promotion, while the increments obtained in buffer alone, or, again, with GA or IAA, are unaffected by light.

This somewhat extended account is presented solely to indicate that it may be unwise to regard the elongation of pea sections, or other organs, as homogeneous and limited by a single system through which all effects are to be explained. It behaves under certain conditions as if it consisted of separate "components" which add or subtract to give the total but frequently do not interact. This of course does not establish the objective reality of such components. In this connection it is worth recalling that a two-phase mechanism for root cell elongation, in which one phase is promoted while another is inhibited by auxin, has been proposed by Burström (3). Further investigation along analogous lines might uncover these and other phases in pea sections as well, and help provide data with which to judge the reality of the components mentioned here. It is well to remember that the only observations made in most work of this kind are on length or weight changes, with no close examination of histological or cytological changes. One cannot assume, for example, that two substances have the same action simply because each can cause a 2 mm. increase over the control. Although the additivity of various effects is often imperfect in the presence of optimal IAA, and always in the presence of superoptimal levels, as observed elsewhere (1, 5, 11) and as is evident in the few data presented here, it may still be useful to consider possible interpretations for independent elongation components on the assumption that the interactions are secondary.

Returning specifically to GA and IAA, this independence may arise from their action (at least when promoting growth) in different, spatially-separated systems. They may be limiting to the growth of different groups or types of cells within a single section. Alternatively, there may be, within a single cell, regions whose elongation, expansion, or differentiation is limited by these two different factors, and others as well.

Some evidence for this sort of view is given by the different responses, mentioned earlier, of apical and subapical tissues to GA and IAA. The two substances may affect different stages in cell development. Strong support for such a view can be found in Wareing's (15) report on the effects of GA and IAA on cambium in trees. Although the two synergize in promoting development, GA specifically promotes cell division, and IAA, differentiation; together they promote the formation of a large zone of normal wood, but their actions are nevertheless qualitatively distinct, and there is no reason to suppose that only a few biochemical steps intervene between them. Yet if growth had been measured only in some gross quantitative sense such

as bulk or fresh weight increases, one might conclude simply that GA and IAA both promote growth and that the two interact to produce a maximal effect on the same growth system. It should be noted in passing that perhaps one reason for attempts to show close linkage between gibberellin and auxin actions was the belief that the former, like the latter, promoted cell extension or expansion rather than cell division, although this generalization is not even completely valid for auxin. In any case, several papers, such as that cited above and that of Sachs *et al.* (10), have shown in elegant fashion that GA can act as a potent cell-division factor as well.

To associate a single growth factor with one component of growth, as if others were not involved at all, is almost certainly an error, and one which we do not wish to commit. Probably all the known growth factors, and more, are necessary for each plant cell; experimental techniques are such as to identify as participating only those which can be made limiting to a given process. It seems unlikely, however, that all or even several act in a single biochemical process limiting many stages of development, and the value of thinking in terms of some such master growth reaction is questionable. It might be just as well to discard the general term "growth" entirely in such discussions and look in more specific morphological, cytological, and biochemical detail at the phenomena in question before proposing simple mechanisms for their interactions.

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Physiological Action of Gibberellin With Special Reference to Auxin

Since Yabuta *et al.* (31) isolated gibberellins from the culture medium of *Gibberella fujikuroi* (Saw) Wollenweber, it has been observed that gibberellin has the effect of causing hyperelongation in many higher plants. I became interested in the mode of action of gibberellin and began a study comparing it with auxin.

At the start I had to use a crude preparation of gibberellin. However, it became evident that gibberellin is a growth substance quite different from auxin. As summarized in Table 1, gibberellin was negative in the *Avena* test and in the pea test at any concentration up to 1,000 mg/l. A 1 per cent lanolin paste of gibberellin did not inhibit, but accelerated, the growth of lateral buds of kidney bean and of etiolated pea seedlings. Gibberellin was not active in inducing callus formation on the cut surface of tomato and sunflower seedlings. Gibberellin A (GA_1) did not promote the root formation of etiolated pea stem, but was rather inhibiting (Table 1).

The root growth of cucumber seedlings was not affected by from 1 to 100 mg/l of GA_1. This agrees with the result of Brian *et al.* (5). Whaley and Kephart (28) have reported that the root growth is significantly stimulated by gibberellic acid (GA) in a certain genotype (strain 854) of maize but not in another (strain 857). These results led to the conclusion that gibberellin is a type of growth-regulating substance quite different from auxins as represented by indole-3-acetic acid (IAA) (11, 12).

In order to confirm this conclusion, the interaction between gibberellin and auxin was studied. Ten mg/l of GA_1 were added to a concentration series of IAA and 1-naphthaleneacetic acid (NAA).

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Table 1. Differences in activities of gibberellin and auxin (11, 12).

Test	IAA	GA_1
Avena standard.	+	−
Pea split stem	+	−
Tomato epinasty	+	−
Callus formation	+	−
Bud inhibition	+	−
Root formation	+	−

Elongation of etiolated pea stem sections showed that the effect of GA_1 was additive to the effect of 0.0001 mg/l through 10 mg/l of the auxins (12). Purves and Hillman (23) obtained similar results. Even when auxin inhibited the shoot growth of cucumber seedlings by being supraoptimal in concentration, GA_1 added to the auxin solutions manifested its own growth-promoting effect independent of the auxin effect (Table 2). As to the root growth of cucumber seedlings, GA_1 showed no effect; and GA_1 with auxin inhibited growth. Inhibition by NAA of the lateral bud of kidney bean was reversed completely by 5 per cent GA_1-lanolin paste. GA_1 is inhibitory to root formation and is antagonistic to the root-forming activity of IAA. All these results show that gibberellin works quite differently from auxin. The inhibition of shoot growth by coumarin (CM) and maleic hydrazide (MH) was reversed by GA_1 (Table 2) and the inhibition of root growth by CM and MH was not reversed, nor increased (Table 3). Hence gibberellin differs also from these substances in its physiological action.

In order further to confirm that the reaction sequence caused by gibberellin is different from that caused by auxin, the effect of GA was observed in combination with various types of anti-auxins. As shown in Figures 1, 2, 3, and 4, 4-chlorophenoxyisobutyric acid

Table 2. Effect of gibberellin A on IAA-induced growth inhibition of cucumber seedling (11).

Concn. of GA_1, Mg/L	Concentration of IAA, Mg/L							
	0.0		0.05		0.5		5.0	
	Root*	Shoot†	Root*	Shoot†	Root*	Shoot†	Root*	Shoot†
0	100	100	93	98	54	64	28	62
50	103	125	81	121	54	110	28	100
100	105	139	90	131	52	100	30	111

* In each column, differences among the values are not significant at the 5 per cent level.

† In each column, differences among the values are significant at the 5 per cent level.

Table 3. Effect of gibberellin A on coumarin-induced growth inhibition of cucumber seedling (11).

Concn. of GA_1, Mg/L	Concentration of Coumarin Mg/L							
	0.0		1		20		30	
	Root*	Shoot†	Root*	Shoot†	Root*	Shoot†	Root*	Shoot†
0	100	100	100	105	60	62	48	54
50	100	135	105	124	55	87	48	73
100	100	135	99	147	53	101	..	..

* In each column, differences among the values are not significant at the 5 per cent level.

† In each column, differences among the values are significant at the 5 per cent level.

(4-CIBA), 3-chlorophenoxyisobutyric acid (3-CIBA), 2,4,6-trichlorophenoxyacetic acid (2,4,6-T), and 2-methyl-1,4-dihydronaphthoquinone (K_3) reduce the effect of auxin when the auxin concentration is low, but not when it is high enough. Hence they seem to be competitive with auxin, as already pointed out by McRae and Bonner (16), Ingestad (10), and Fransson (7). In contrast, the anti-auxins used made GA completely ineffective even up to a high concentration, 300 mg/l. The inhibition of GA effect by anti-auxins shall be considered later. Here it is to be noted that anti-auxins are not competitive with GA, while they are competitive with auxin.

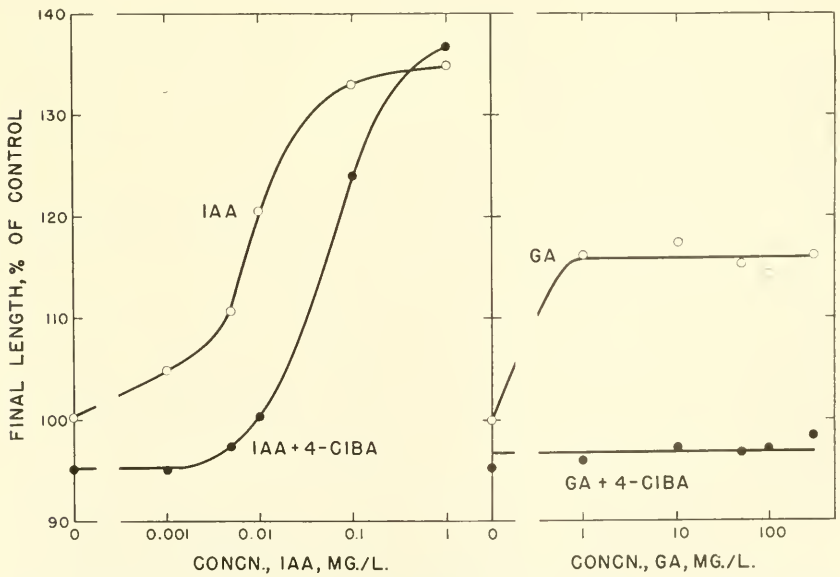

Fig. 1. Interaction of 15 mg/l of 4-chlorophenoxyisobutyric acid (4-CIBA) with IAA and gibberellic acid in the elongation of pea stem sections (13).

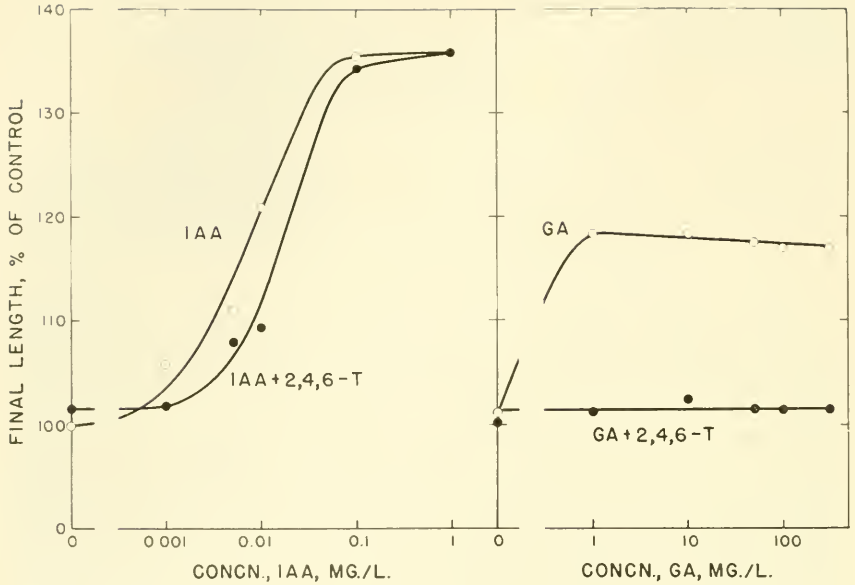

Fig. 2. Interaction of 30 mg/l of 3-chlorophenoxyisobutyric acid (3-ClBA) with IAA and gibberellic acid in the elongation of pea stem sections (13).

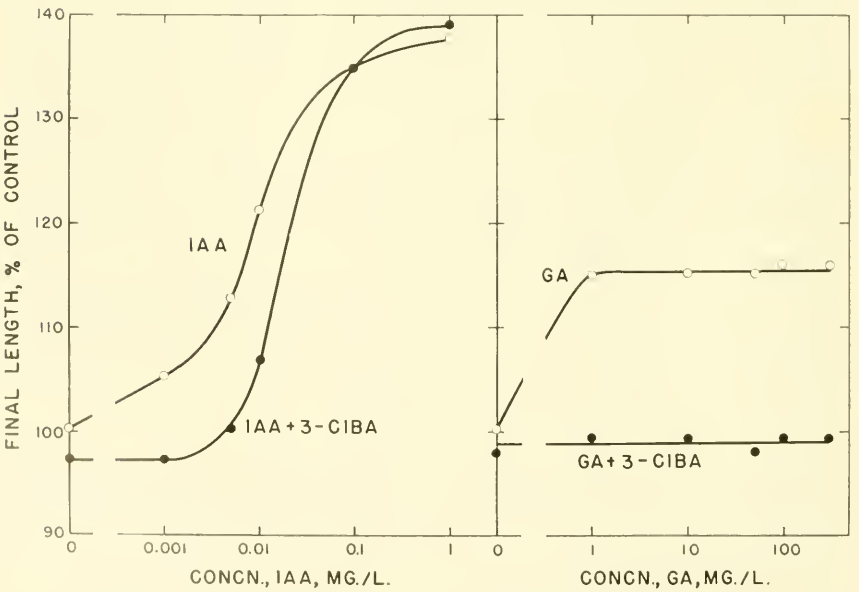

Fig. 3. Interaction of 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) with IAA and gibberellic acid in the elongation of pea stem sections (13).

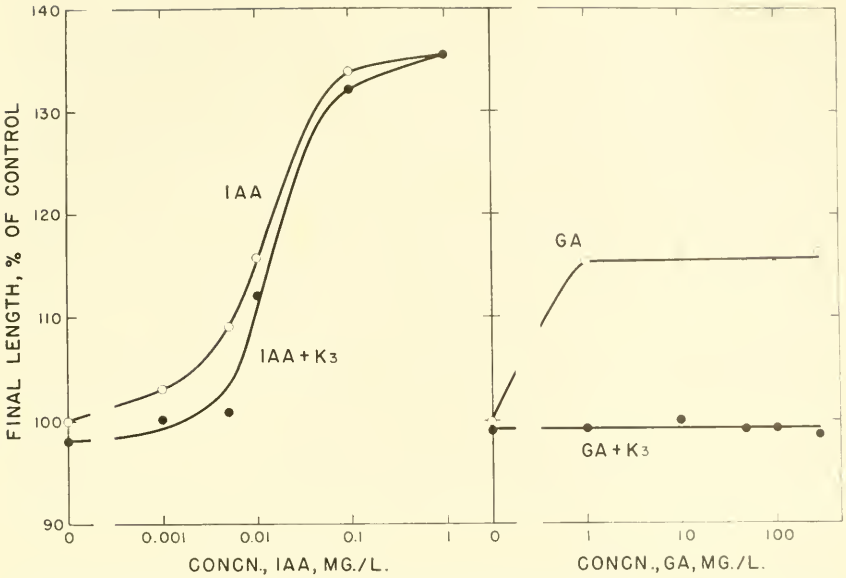

Fig. 4. Interaction of 2-methyl-1,4-dihydronaphthoquinone (K_3) with IAA and gibberellic acid in the elongation of pea stem sections (13).

The series of experiments so far presented may suggest that the reaction sequence caused in tissues by gibberellin differs from that caused by auxin, or more specifically, that probably the physiological receptor for gibberellin is different from that for auxin. Curtis (6) found that, using *Phaseolus vulgaris* 'Black Valentine,' the inhibitory effect of the filtrate from the culture medium of *Aspergillus niger* could be reversed by GA but not by IAA, and inferred that GA operates through a system different from that of IAA.

The complete inhibition of the GA-induced elongation by anti-auxins, as described above, was reversed by the concomitant addition of IAA or indole-3-acetamide. This reversing effect of IAA, however, could not be substituted by other growth factors such as amino acids (L-leucine, L-histidine, tyrosine, L-phenylalanine, L-methionine, DL-threonine, L-hydroxyproline, DL-iso-leucine, aspartic acid, DL-ornithine, L-arginine, L-alanine, L-proline, glycine, L-lysine, DL-valine, L-glutamic acid), vitamins (thiamine, riboflavin, ascorbic acid, vitamin E, vitamin K, pyridoxine, pantothenic acid, folic acid, nicotinamide, vitamin B_{12}), diphenylurea, biotin, casein hydrolysate, and yeast extract. Hence the effect of GA seems to be caused by some process involving auxin, as already suggested by Brian and Hemming (4), Kuse (15), and Galston and Warburg (8).

On the other hand, Applegate (2), using seedlings of *Zinnia ele-*

gans, reported no difference between the effect of GA and mixtures of GA and TIBA in various concentrations, and concluded that auxin was not definitely responsible for GA-induced cell elongation.

As to the mode of action of gibberellin, Pilet (21), Pilet and Wurgler (22), and Stutz and Watanabe (25) think that gibberellin operates through its effect on the IAA oxidase, namely through raising the auxin level in plant tissues. Nitsch (18) reported that GA treatment of some woody plants increased their auxin content. However, my experiments using combinations of gibberellin with auxins and anti-auxins have suggested that the effect of gibberellin involves a physiological sequence different from that of auxin (12, 13).

Brian and Hemming (4) have shown that GA had no stimulatory nor inhibitory effect on the IAA oxidase prepared from etiolated pea seedlings. Kato and Katsumi (14) also tested the effects of GA and GA_1 on the activity of IAA oxidase prepared from etiolated pea shoots. As is shown in Figure 5, neither was stimulating nor inhibiting. GA had no effect even at high concentrations and at various pH values.

According to my unpublished experiments, 0.1 to 1.0 mg/l solutions of crude gibberellin accelerated the multiplication of fronds of *Lemna paucicostata,* 0.5 mg/l being the optimum. Fronds grown in

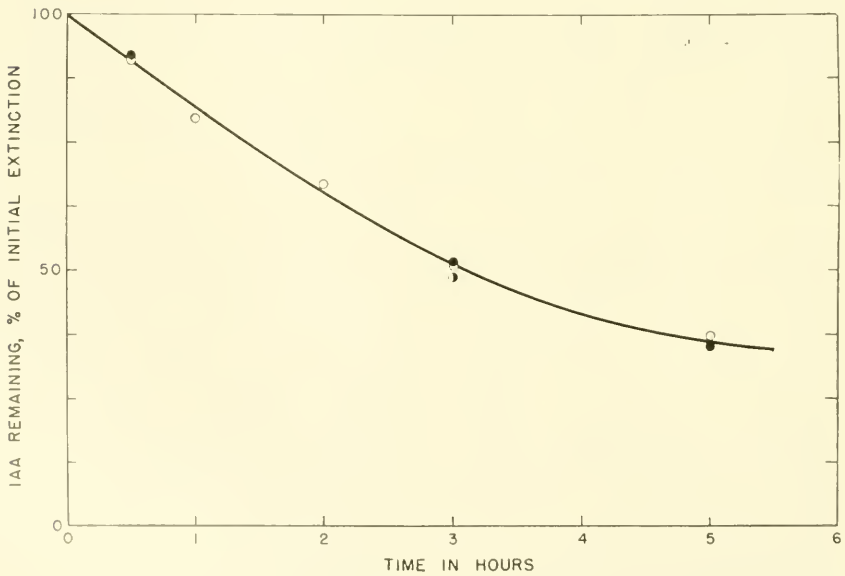

Fig. 5. Lack of effect of gibberellic acid and gibberellin A on the activity of indole-3-acetic acid oxidase (14).

the presence of gibberellin were smaller and lighter green in color than normal ones. With IAA, however, multiplication of the frond was inhibited by 1 mg/l and stimulated by 0.5 mg/l; fronds grown in the latter concentration were larger in area and somewhat deeper green in color than the controls. Therefore, the hypothesis that the gibberellin action is due to a change in the auxin content in plant tissues should be revised. There are many reports (1, 2, 3, 6, 9, 17, 20, 24, 26) which do not conform with the hypothesis of Pilet (21), Pilet and Wurgler (22), and Stutz and Watanabe (25).

There remains, then, the problem of the mechanism of action of gibberellin. Without entering into this difficult problem, I wish to mention that some hormone-like factors are needed for the growth effect of gibberellin. The necessity of auxin is already discussed. Vlitos and Meudt (27) demonstrated, by using etiolated pea cuttings, that some factor(s), regarded as existing in the shoot apex, is involved in GA action. Brian and Hemming (4) and Galston and Warburg (8) postulated that a third factor is required for gibberellin to be effective. Since Wittwer and Bukovac (29) and Wittwer *et al.* (30) showed that the photoperiod was an important factor controlling the plant response to gibberellin, it is presumed that some factor(s) produced under proper photoperiods is necessary for the gibberellin action.

The necessity of these factors should be kept in mind when the action mechanism of gibberellin is considered and also when the bioassay of gibberellin is attempted. It should also be noted, on the other hand, that such factor(s) may be contained naturally in certain kinds of strains of plants and not in others (19, 28).

SUMMARY

Gibberellin is a growth-promoting substance quite different in nature from auxin. Its growth effect is not due to a change in auxin level of the affected tissue. The reaction site of gibberellin in plant tissues is different from that of auxin.

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An Analysis of Gibberellin-Auxin Interaction and Its Possible Metabolic Basis

It has now become clear that in plant tissues which respond both to gibberellin and to auxin, several kinds of interactions are possible between these compounds. In such objects as the sub-apical etiolated pea epicotyl section, the growth increment produced by a joint application of the two substances is usually considerably less than, and never significantly more than, the expected sum of the growth increments produced by the separate administration of these compounds (7, 18). On the other hand, in such objects as the sub-apical green pea stem section (1, 7), the sweet potato petiole (14), fruit cells (15, 21), and in starved or otherwise pretreated etiolated tissues (7, 9, 24) a true supra-additivity or synergism has been reported to exist between the growth effects produced by these compounds. In view of the fact that several theories of auxin-dependent gibberellin action have been proposed (1, 7), it seemed desirable to analyze this situation more systematically in a single plant tissue, to attempt to determine the conditions under which synergistic interaction occurs. This has been a major aim of the work here described. In brief, we, and Purves and Hillman (19) of this laboratory, have found, in contrast with previously published reports (7, 9, 24), that etiolated pea tissue, no matter how pretreated, does not show a synergistic interaction between auxin and gibberellin. On the other hand, green pea stem tissue derived from plants grown under 8-hr. daily photoperiods almost invariably manifests a marked synergism. Prolongation of the daily duration of illumination to 16.5 or 24 hrs. of high light intensity results in a diminution or even a disappearance of this synergism, except under special conditions.

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In view of the fact that synergistic interactions between auxin and gibberellin do occur in certain tissues, a second aim of this work has been an attempt to elucidate some metabolic basis for the growth interaction. In this search we have concentrated on the peroxidases, which are known to inactivate auxins in vitro (5) and which have been implicated in certain growth and developmental phenomena (3). In corroboration of previous reports (10) we have found a marked effect of gibberellins on the peroxidase activity of sensitive cells, especially in dwarf plants. We have further studied this effect in detail by an electrophoretic separation of peroxidase into its various component fractions, and by delineation of the particular fractions which are affected by gibberellin.

GROWTH EXPERIMENTS WITH PEA STEM SECTIONS

Materials and Methods

'Alaska' peas, purchased from Associated Seed Growers, New Haven, Connecticut, were soaked in tap water for 4 hrs. and sown in polyethylene containers in water-saturated vermiculite. Etiolated plants were grown for 7 days in a dark cabinet in a dark room maintained at ca. 27° C., and were exposed only briefly at harvest to a dim green safelight, produced by wrapping a 15 watt Sylvania green fluorescent tube with three layers of green and three layers of amber du Pont cellophane. Subapical 5 mm. sections were cut with a guillotine, as previously described (6). Ten such sections were permitted to grow overnight in the dark in 5 ml. of growth medium in a 7.5 cm. petri dish. This medium consisted of a 1 per cent sucrose solution containing 0.02 *M* KH_2PO_4-Na_2HPO_4 buffer, pH 6.1, plus 10^{-4} *M* gibberellic acid (GA) and 10^{-6} or 10^{-5} *M* indole-3-acetic acid (IAA) where indicated. The GA was obtained from Dr. P. W. Brian of Imperial Chemicals Industries and the IAA from Nutritional Biochemicals Co. Both were made up as 10^{-3} *M* stock solutions and stored in the dark in the refrigerator for no longer than one month. Growth of the sections was measured to the nearest 0.1 mm. under a dissecting microscope after about 18 hrs. Group averages and standard error of the mean were computed. In all experiments here reported, the standard error was 5 per cent of the mean or below. Growth was also measured by obtaining fresh weights of the groups of ten sections after gentle blotting.

Green pea plants were grown in three controlled-condition rooms maintained at ca. 23° C., and at photoperiods of 8, 16.5, and 24 hrs. The light intensity at the growing tables was ca. 1,500 foot candles, coming from a bank of mixed fluorescent and incandescent lights. Plants on the growing tables were automatically subirrigated twice

daily with a nutrient solution composed of 120 g. Hyponex (Hydroponics Chemicals Co., Copley, Ohio) per 100 l. tap water. Subapical 5 mm. stem sections were obtained from 14- to 18-day-old plants with a double-bladed cutting tool. Green sections were grown under high intensity (ca. 1,200 foot candles) fluorescent light in a medium containing sucrose and buffer as above, and GA and IAA, where indicated, at 10^{-4} *M*. These conditions have recently been determined in our laboratories to be optimal for the growth of such sections, but space limitations preclude detailed description of such experiments here. Measurement of the growth of green sections was made by the same methods as detailed above.

Results

Typical results with etiolated peas are shown in Table 1, which gives the growth increments produced by IAA and GA alone, and the two together, when supplied to (a) sections from completely etiolated plants, (b) sections from etiolated plants exposed to weak red light 24 hrs. prior to harvest, (c) etiolated sections pretreated with GA or control media for 30 min. and then transferred to growth solution containing or lacking IAA, and (d) sections from 100 mm. long etiolated epicotyls treated basally for 30 min. with GA, then excised and placed in growth solutions containing IAA. In the latter two series the pretreatment with GA followed previously published methods (7) of immersion in 1 per cent sucrose and buffer + 10^{-4} *M* GA for the indicated period. The completely etiolated sections show a large response to GA, a larger response to IAA, and less than additive effects of the two compounds together. Red light pretreatment reduces the endogenous growth and response to auxin, as previously reported (4), as well as the response to GA. GA pretreatment, either to sections or

Table 1. Absence of GA-IAA synergism in etiolated pea epicotyl sections derived from plants treated in various ways. All sections initially 5 mm. long. All figures are averages of two closely checking means, each derived from ten sections growing in a single dish. Data in lower section of table represent a separate experiment.

Treatment	Endogenous Growth, Mm.	Δ L, IAA, Mm.	Δ L, GA, Mm.	Δ L, GA + IAA, Mm.		
				Calc.	Obs.	Obs.-Calc.
Etiolated	1.30	2.63	1.15	3.78	3.24	−0.54
Red pretreatment	1.11	2.32	0.72	3.04	2.75	−0.29
GA pretreatment (sections)	1.84	1.49	1.29	2.78	1.92	−0.86
GA pretreatment (epicotyls)	1.39	1.85	1.21	3.06	2.17	−0.89

to 100 mm. long epicotyls, yields below additive growth increments in the presence of IAA. Thus, it is clear that in no instance is there any GA-IAA synergism. This disagrees with some of our previous results (7) for reasons which we cannot at present explain. Drs. W. S. Hillman and W. K. Purves have also obtained data like those in Table 1, and pointed out the non-synergistic interaction to us. Their results will be published elsewhere.

Similar experiments were performed with stem sections derived from green peas grown under 8, 16.5, and 24 hr. daily light periods. The results are shown in Table 2, from which the following conclusions can be drawn: (a) Endogenous growth and response to exogenous IAA are lower in sections derived from the 8-hr. photoperiod plants than in those from 16 and 24 hr. plants. (b) GA response is independent of the photoperiod of the parent plant and is markedly lower in green tissue than in etiolated tissue. Although GA response in the

Table 2. GA-IAA synergism in green pea stem sections as affected by daily duration of light to which the parent plant was exposed. Details as in Table 1.

Daily Duration of Light, Hrs.	Endogenous Growth, Mm.	Δ L, IAA, Mm.	Δ L, GA, Mm.	Δ L, GA + IAA, Mm.		
				Calc.	Obs.	Obs.-Calc.
8	0.85	1.87	0.40	2.27	2.89	+0.62
16.5	1.11	2.72	0.52	3.24	3.44	+0.20
24	1.15	2.93	0.45	3.38	3.26	−0.12

absence of IAA is small, it is not completely lacking as in the experiments of Brian and Hemming (2). (c) Marked GA-IAA synergism occurs in the 8 hr. sections, none in the other sections.

An experiment was next performed to study the effect of passage of GA through various lengths of green stem tissue on its subsequent interaction with IAA administered to sections excised from the pretreated stems. Previous experiments had reported such effects to be large (8). For this purpose, green plants were harvested from the 8, 16.5, and 24 hr. photoperiod rooms, decapitated just below the terminal bud, cut to lengths of 20, 50, or 100 mm., and then freed of all leaves. These leafless stems were then immersed basally in GA (10^{-4} M + 1 per cent sucrose + buffer) or control solutions for 1 hr. After this, apical sections were excised, placed in IAA-containing (or control) solutions, and permitted to grow for 18 hrs. The results are shown in Table 3. It can be seen that in all three groups there was a synergism manifested in the 100 mm. lengths, while only the 8 hr. group showed synergism at the 20 mm. length (and in the 5

Table 3. Effect of length of stem through which GA passes on degree of subsequent synergism with IAA in section growth.

Photoperiod, Hrs.	Length of Stem, Mm.	Endogenous Growth, Mm.	Δ L, IAA, Mm.	Δ L, GA, Mm.	Δ L, GA + IAA, Mm.		
					Calc.	Obs.	Obs.-Calc.
8	20	0.86	2.63	0.19	2.82	3.23	*+0.41*
	50	0.88	2.77	0.33	3.10	3.14	+0.04
	100	1.12	2.12	0.29	2.41	3.20	*+0.79*
16.5	20	0.98	2.38	0.51	2.89	3.01	+0.12
	50	1.05	2.75	0.43	3.18	3.44	+0.26
	100	1.38	2.41	0.18	2.59	3.11	*+0.52*
24	20	1.07	2.35	0.20	2.55	2.81	+0.26
	50	0.80	3.09	0.68	3.77	3.24	−0.53
	100	0.91	2.73	0.28	2.91	3.56	*+0.55*

mm. sections themselves, as in Table 2). One puzzling fact is that no series showed synergism at the 50 mm. length of pretreated stem. We cannot at the moment explain this, but it seems not to obviate the conclusion that in stem tissue from the longer photoperiod plants, which did not show GA-IAA synergism in 5 mm. sections, synergism was obvious in the longest stems treated. These results thus corroborate and extend our previous findings (7).

The possibility remained that it was the 1 hr. gap in time between GA and IAA treatments, rather than the length of stem, which was responsible for the synergism induced in the previous experiment. This was tested by dipping excised 5 mm. sections from each of the different photoperiod groups into GA-sucrose-buffer or control solutions for 1 hr., then transferring them to growth solutions containing or lacking IAA for an additional 17 hrs. The results, shown in Table 4, indicate that the time lapse is not the important factor, in that synergism is clearly manifested once again only in the 8 hr. photoperiod peas. In a subsequent experiment (Table 5) it was found that, in the 8 hr. peas, the growth increments due to GA, as well as the GA-IAA synergism, were induced equally by exposure of the 5 mm. sections to 1, 10, or 60 min. of 10^{-4} *M* GA. These effects resemble similar ones for IAA already described (6), and indicate that timing of

Table 4. GA-IAA synergism in green pea stem sections pretreated with GA for 1 hr. and later exposed to IAA.

Photoperiod, Hrs.	Endogenous Growth, Mm.	Δ L, IAA, Mm.	Δ L, GA, Mm.	Δ L, GA + IAA, Mm.		
				Calc.	Obs.	Obs.-Calc.
8	0.93	1.96	0.32	2.28	3.05	*+0.77*
16.5	1.06	2.26	0.48	2.74	3.03	0.29
24	1.05	2.42	0.32	2.74	3.02	0.29

GA and IAA "pulses" are not important in determining either the growth or degree of synergistic interaction.

In summary, it appears that GA-IAA synergism is not demonstrable in etiolated tissue, but shows up in appropriately treated green tissue. The factors that enhance the synergism in green tissue seem to be short duration of daily illumination and possibly passage of GA through a considerable length of stem.

THE EFFECTS OF GA ON PEROXIDASES IN DWARF PEAS AND CORN

Several previous investigators have reported that dwarf plants in such widely divergent genera as *Phaseolus, Epilobium, Zea,* and *Pisum* have much greater peroxidase activity than their normal counterparts (8, 12, 20, 23). In view of the fact that GA is known to alter the phenotype of certain dwarfs to normal (1, 17), it seemed important to discover whether the peroxidase activity of such GA-treated plants was also altered to the normal pattern. The plants used in these investigations were dwarf peas, 'Progress No. 9' and tall peas, 'Alaska,' both obtained from Associated Seed Growers, and a segregating population of corn, yielding 75 per cent tall plants and 25 per cent dwarf-1

Table 5. Effect of duration of pretreatment with 10^{-4} *M* GA on degree of synergism with IAA in green pea sections derived from 8 hr. photoperiod plants.

Pretreatment Time, Min.	Endogenous Growth, Mm.	Δ L, IAA, Mm.	Δ L, GA, Mm.	Δ L, IAA + GA, Mm.		
				Calc.	Obs.	Obs.-Calc.
1	0.87	1.91	0.23	2.14	2.77	*+0.63*
10	0.91	2.04	0.34	2.38	2.81	*+0.43*
60	0.99	2.21	0.14	2.35	2.94	*+0.59*

mutants, obtained from Dr. B. O. Phinney, of the University of California at Los Angeles. The genetic differences between the dwarf and tall peas are partially unknown and probably complex, but the dwarf-1 *Zea* mutant is known to differ from the wild type by a single gene (17).

The peas were grown in the light as described above, except that the temperature was maintained at ca. 17° C., rather than 23° C. Fourteen days after planting, half of the plants were treated with 1 μg. GA in 0.003 ml. ethanol, applied to the stipules enclosing the fifth internode. At various intervals after treatment, the plants were harvested and the fifth internodes excised and homogenized with a prechilled mortar and pestle in 0.025 *M* pH 6.1 phosphate buffer (1 g. fresh wt/10 ml homogenate). The homogenate was then stored in a deep freezer in Lusteroid centrifuge tubes until further use. The peroxidase activity was unchanged by such storage.

Seven days after planting, the corn was treated with 1 μg. GA in 0.003 ml. ethanol applied to the tip of the first leaf as it emerged from the coleoptile. Two and four days later, the basal third of the first leaf sheath (the rapidly elongating region) of 20 to 30 plants was excised, combined, and homogenized and stored as above.

Prior to assay, the tissue homogenate was centrifuged at 2,000 $\times$ gravity for 10 min. and the clear supernatant made up to standard volume and used for peroxidase and protein nitrogen determinations, according to previously-published procedures (16, 22). The usual substrate for peroxidase determinations was pyrogallol, but IAA and guaiacol were also used extensively. Most of the data were obtained with a Klett-Summerson photoelectric colorimeter, but a Spectracord recording spectrophotometer was also employed in later studies, especially with IAA. The results with pyrogallol as substrate are presented in Table 6. It is clear that GA greatly promotes the growth of both dwarfs, while markedly lowering the peroxidase activity per unit protein N. In the dwarf corn, the peroxidase activity of both dwarf and normals rises with increasing age, and the depressive effect of GA appears to result from a prevention of this normal increase. The GA produces much smaller effects on tall corn, and is entirely without effect on tall peas. Thus, based on pyrogallol as a substrate, GA can be said to depress the abnormally high peroxidase activity of dwarf plants.

When the experiment of Table 6 was repeated with guaiacol as a substrate, then GA was found to increase, rather than decrease, the peroxidase activity of the dwarf tissue. This corroborates a recent report (10) on the effect of GA on the peroxidase of rice, in which guaiacol was used as a substrate. These opposite results forced us to

Table 6. The effect of GA application on the growth and peroxidase activities of dwarf and tall peas and corn. Peroxidase substrate was pyrogallol; data obtained on Klett-Summerson photoelectric colorimeter with a blue filter.

Plant	GA Treatment	Length of Corn Leaf Sheath or Pea Internode in Mm.		Peroxidase Activity in MμM Purpurogallin/ μg Protein N/Min	
		2 days	4 days	2 days	4 days
Dwarf corn	−	15 ± 0.6*	20 ± 0.5	52	73†
Dwarf corn	+	32 ± 0.9	44 ± 1.6	45	56†
Tall corn	−	42 ± 0.9	53 ± 1.5	45	55
Tall corn	+	53 ± 1.2	64 ± 25	40	52
Dwarf pea, 'Progress No. 9'	−	5.3 ± 0.13	....	38†	..
Dwarf pea, 'Progress No. 9'	+	9.1 ± 0.43	....	28†	..
Tall pea, 'Alaska'	−	21.3 ± 0.92	....	21	..
Tall pea, 'Alaska'	+	21.6 ± 0.83	....	19	..

* Standard error.
† Difference significant at the 1 per cent level by analysis of variance.

the conclusion that GA induces some alteration of the peroxidase complex of enzymes in the plant such that the relative activities toward different substrates are altered. This is not altogether surprising, since it is well known that the peroxidases of plants such as horseradish and sweet potato are resolvable by electrophoresis into about five components (11, 13). We therefore decided to subject the peroxidases of the dwarf and normal corn to electrophoretic separation, in an attempt to delineate further the nature of the changes in peroxidase activity produced by GA.

The procedure was as follows: 75 g. dry potato starch was washed repeatedly with distilled water, then dried by filtration on a Büchner funnel. To the dried starch were added 33 ml. of buffer 0.02 *M*, pH 6.1, KH_2PO_4-Na_2HPO_4. The resulting slurry was poured into a trough 40 × 2.5 × 1.0 cm. and ca. 9 ml. of exuded solution removed by filter paper blotters. A segment 1 cm. long was then removed from the middle of the block, and replaced by a slurry of 1.2 ml. of centrifuged plant homogenate (see paragraph below) in 1.8 g. dry washed starch. The block was then placed in an E-C Co. electrophoresis apparatus and exposed to 400 V (ca. 2.8 mA) for 12 hrs. in a cold room maintained at 2° C. After 12 hrs., the block was cut into 1 cm. segments, each of which was placed in a centrifuge tube containing buffer. The

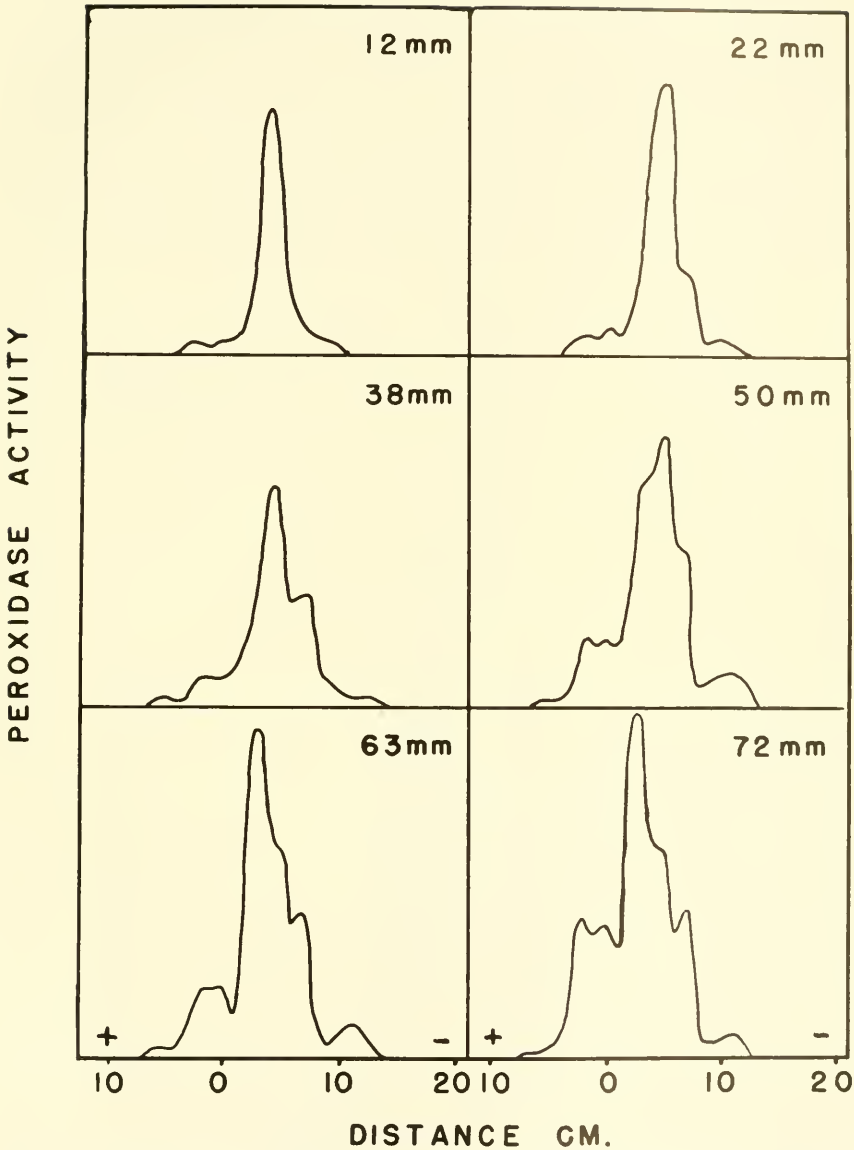

Fig. 1. Electrophoretic patterns of peroxidases in the normal leaf sheath of corn as a function of length of the sheath. Guaiacol substrate.

starch was stirred thoroughly and centrifuged down, the supernatant being drawn off with a pipette and used for the peroxidase assay.

For each electrophoretic analysis, the second leaf sheaths of ca. 30–40 plants were harvested. The final homogenate represented ca. 250 mg. fresh weight of tissue per ml. This homogenate was centrifuged at ca. 20,000 × gravity for 15 min. and the precipitate discarded. The supernatant liquid was saturated with $(NH_4)_2SO_4$, and the precipitate removed by centrifugation (20,000 × gravity, 15 min.) 3 hrs. later. The precipitate was dissolved in 2.5 ml. buffer, then dialyzed for a total of 20 hrs. against three successive volumes of 200 ml. buffer in the cold room. The final residue in the dialysis bag was again clarified

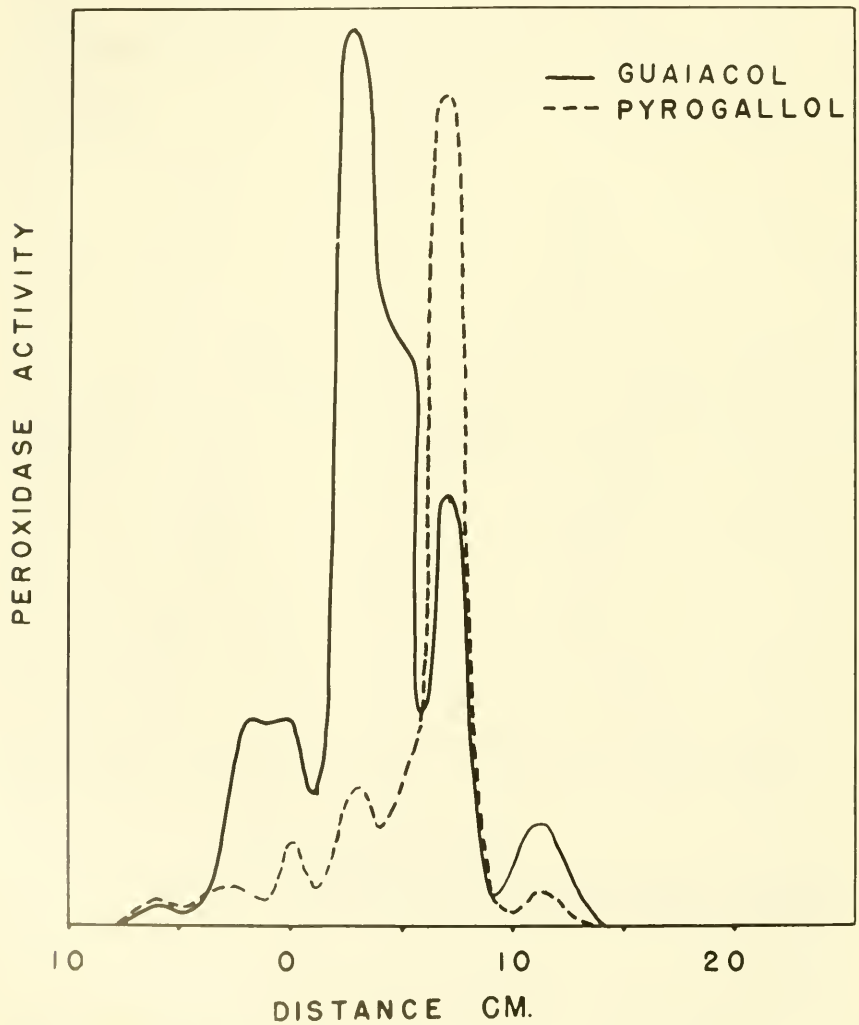

Fig. 2. Electrophoretic pattern of peroxidases from normal leaf sheath of corn. Guaiacol or pyrogallol substrate.

by centrifugation, and 1.2 ml. of the total of ca. 2.5 ml. then used in the electrophoretic separation.

The electrophoretic peroxidase patterns are shown in Figures 1, 2, and 3. Figure 1 shows the developmental pattern of peroxidases in the normal leaf sheath as it grows from a length of 12 mm. to a length of 70 mm. in the course of 6 days. Clearly, there is initially a single major peak, which gradually becomes more complex until there are ultimately at least five major components. Pyrogallol and guaiacol give the same general spectrum, differing only in the relative height of the various peaks (Figure 2).

Figure 3 shows that at the latest stage of development studied,

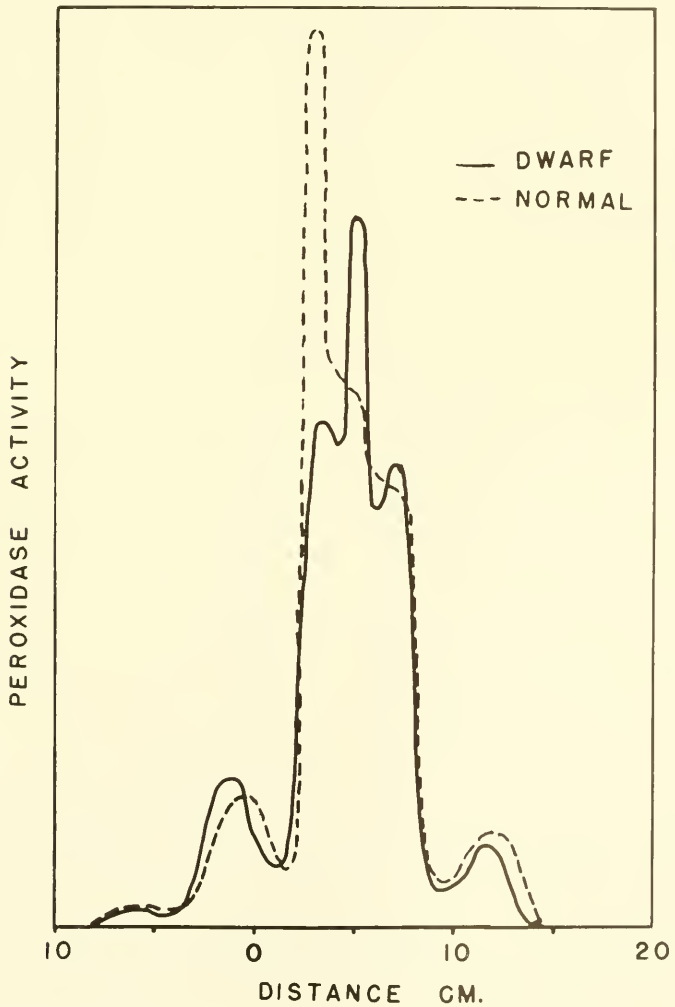

Fig. 3. Electrophoretic pattern of peroxidases from leaf sheath of normal and dwarf corn. Guaiacol substrate.

the dwarf differs from the normal mainly in elevation of one major peak and depression of another with guaiacol as a substrate. When the dwarf is treated with GA and the peroxidase assayed with guaiacol, these two peaks are reciprocally affected (Figure 4). It thus appears that GA treatment results in a qualitative change in the peroxidase components of the dwarf corn leaf sheath, the changes induced resembling those produced by the normal allele.

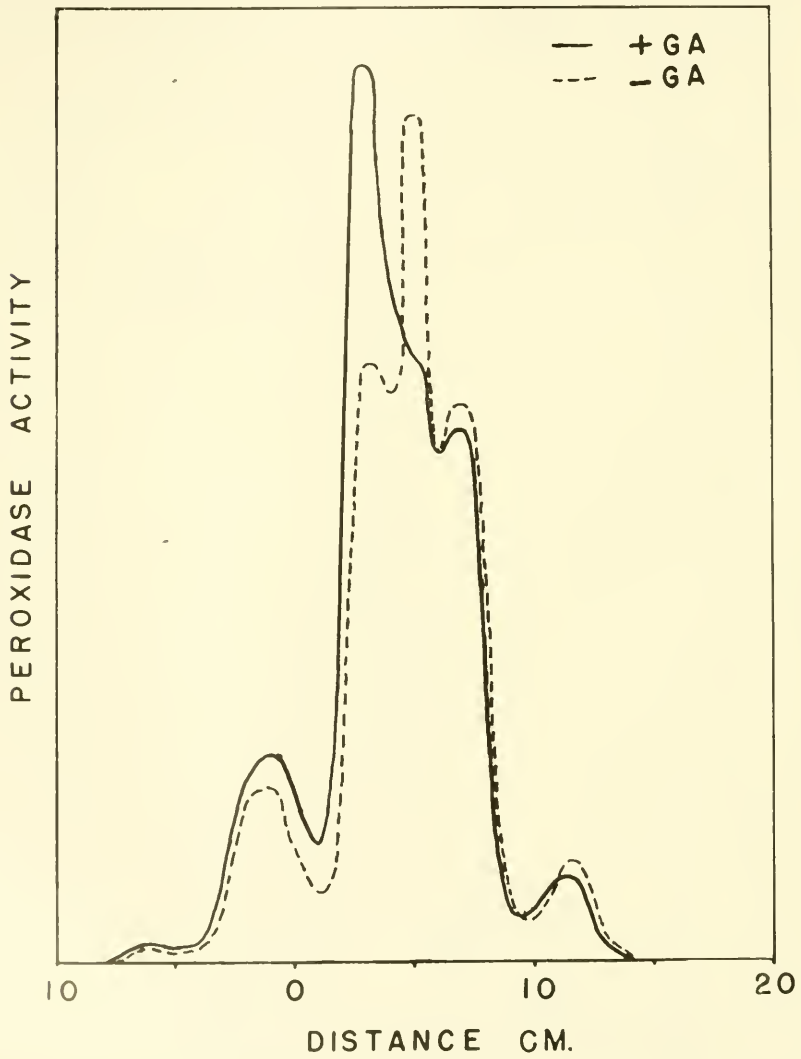

Fig. 4. Electrophoretic pattern of peroxidases from leaf sheath of dwarf corn treated with GA. Guaiacol substrate.

DISCUSSION

The significance of the GA-IAA synergism for the control of growth in certain green tissues, and of the absence of such synergism in etiolated tissues is at present impossible to ascertain. In another article in this volume, Hillman and Purves present considerable evidence that, in etiolated pea tissue at least, GA and IAA operate through largely independent pathways. A similar analysis has not yet been performed for green stem tissue.

What is the significance of these findings on peroxidase levels as affected by GA? In view of the uncertainty concerning the in vivo function of peroxidase, little of a definite nature can be stated. However, the following points seem suggestive of some significance, at least. (a) The changes induced by GA on peroxidase in dwarfs are detectable at least as early as the growth effects can be noted. They are therefore probably not merely distant and secondary consequences of the alteration of growth by GA. (b) The simple nature of the differences between the peroxidase pattern of the normal and dwarf plants seems consistent with the fact that they differ in a single gene. (c) The relatively simple and specific effect of GA on the peroxidase pattern seems consistent with the biochemical amelioration of a simple genetic abnormality.

Considerably more work is obviously needed to assess these interesting possibilities more adequately.

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DISCUSSION

Dr. Burström: Do you think, Dr. Galston, that the change in peroxidase activity, which amounted to something between 25 and 40 per cent, can account for an increase in growth by 100 per cent or more? It is difficult to compare the amounts or the enzyme activities,

since, when you have two pieces of plants growing at 100 per cent difference in elongation, you have a different morphological pattern. You have, of course, referred activity to amount of protein, which is the most natural way, but it does not necessarily follow that this is the right expression of the real activity of the enzyme when you have such a fundamental difference in the organization of the tissues, as you must have.

Dr. Galston: Your point is certainly well taken. Remember, however, that although we produced only a 40 per cent change in over-all peroxidase activity, we have produced very much greater changes in one or two of the electrophoretically separable peroxidase peaks. The changes can be of the order of several hundred per cent when calculated for the individual peroxidase components. As for the mechanism by means of which a peroxidase could inhibit growth, we do not know. It may destroy auxin, it may make lignin, or it may do other things. All we can say is that we have here a specific reversal by gibberellin of a biochemical abnormality in a single gene dwarf mutant. Whether this has significance for the control of growth is for the future to tell.

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The Influence of Gibberellic Acid on Indole-3-acetic Acid Disappearance From Solutions Containing Excised Pea Stem Tissues and Indole-3-acetic Acid Oxidase

Since 1957 it has been widely recognized that the gibberellins can markedly modify plant growth and development including, for example, changing the quantity of solid matter in various tissues (2, 7, 23), replacing the light requirement for germination of light-sensitive seeds (14), and influencing the time of flowering (17, 26). Their most characteristic property, however, is the ability to promote stem elongation in many plants, including tall and dwarf varieties of pea, the latter showing the greater increase in growth rate (3). Using seedlings of 'Alaska' pea grown in complete darkness, Lockhart (20) obtained little or no response to applied gibberellins. When seedlings the growth of which had been inhibited by red light were treated, their growth rate was restored to that in darkness. On the other hand, with the dwarf 'Progress No. 9,' growth rate in darkness was increased under the influence of gibberellins, but this enhanced growth was also equalled by gibberellin-treated dwarfs grown in light.

Rather different results have been obtained by Brian and Hemming (4) with segments excised from stems of dwarf 'Meteor' pea. No increased elongation with gibberellic acid (GA) in light was obtained with sections from light-grown plants, whereas in the dark, with sections from dark-grown plants, Brian, Hemming, and Radley (6) ob-

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tained small elongations. However, Brian and co-workers appear to have used different techniques in these studies, and it has been recently shown by Purves and Hillman (25) that, at least for dark-grown plants, the size of sections used and position on the stem from which they are excised are of importance in determining response.

The results of Brian and Hemming (4) are of interest in that they show a synergism between GA and indole-3-acetic acid (IAA). Such synergisms have not always been obtained (15, 25). The less recent literature has been summarized by Stowe and Yamaki (26). In Brian and Hemming's experiment, section growth in light was examined in a basal medium of 2 per cent sucrose + phosphate buffer, medium + IAA, medium + GA, and medium + GA and IAA. While GA alone had little or no effect, when in the presence of IAA it increased section elongation over that obtained in IAA alone. Brian and Hemming offered no hypothesis to account for these results, but it seemed to the present authors that the synergistic enhancement of IAA-induced growth by GA could result from an IAA-sparing action brought about, for example, by the blocking of an IAA-destroying system with GA. This possibility has been examined in the experiments reported in the present paper by observing the rate of disappearance of IAA from solutions containing excised pea stem tissues or pea breis of IAA-oxidase enzyme in the presence and absence of GA. Growth of stem tissues was not measured in this study.

MATERIALS AND METHODS

Pisum sativum, 'Progress No. 9,' (Sharpe Seed Co.) was used except when stated to the contrary. GA (Imperial Chemical Industries, Ltd.) and IAA (British Drug Houses, Ltd.) were used at a concentration of 10 mg/l in all experiments.

Estimation of IAA was made colorimetrically using the Salkowski reaction technique of Gordon and Weber (10) to develop the red Fe-IAA complex which was measured with a Hilger photoelectric absorptiometer (1 cm. cell and Ilford 604 filter). GA with Salkowski reagent gave no absorption (against reagent as a blank in the machine), and no interference occurred with IAA-color development when GA and IAA solutions were mixed and allowed to stand for 5 to 120 min. before addition of reagent. The relationship between absorption and concentration of IAA is linear, and absorptions of four IAA concentrations are given below:

IAA Concn. (mg/l)	2.5	5	10	20
Absorption Units	0.044	0.122	0.267	0.528

Enzyme breis for IAA-oxidase experiments were prepared from 7-day-old plants with third internodes extending. Growth was in the dark at 27° C. with occasional brief irradiation with weak green

light from a tungsten lamp for observation. Ten g. of frozen epicotyls were ground in a chilled mortar with $M/50$ KH_2PO_4-Na_2HPO_4 buffer (pH 6.4), filtered through washed cheesecloth, and made up to 110 ml. with buffer. Storage was at —15° C. in the dark. Enzyme activity of this preparation was low, so a preliminary experiment, based on a design used by Hillman and Galston (11), was carried out to determine the quantities of $MnCl_2$ and 2,4-dichlorophenol that had to be added to the reaction mixture to give a suitable rate of IAA destruction. A reaction mixture of $10^{-4}M$ 2,4-dichlorophenol, $10^{-5}M$ $MnCl_2$, $0.02M$ phosphate buffer, and enzyme at X 1/10 of the prepared concentration was chosen.

Experiments on rate of IAA disappearance from solutions containing stem sections were carried out with plants grown in the dark in the manner described above. Ten-mm. sections were cut, one per stem, from just below the first node. After randomizing, four lots of 42 sections were withdrawn, and each lot was placed in 14 ml. of test solution in a 250 ml. wide-necked conical flask. Two ml. was previously removed for an absorption reading at zero time: two flasks were controls which were prepared for reagent blanks in the absorptiometer. Preliminary experiments using $0.0166M$ phosphate buffer (pH 6.4) and no other addendum gave little IAA destruction; therefore $10^{-4}M$ 2,4-dichlorophenol and $10^{-5}M$ $MnCl_2$ were incorporated into the test solution. Flasks were rocked at approximately 90 oscillations per minute at 27° C. Whenever 2 ml. aliquots of solution were withdrawn for IAA determinations, six sections were removed from each flask to maintain uniform tissue/solution ratios. Manipulations for experiments in the dark were carried out in weak green light (Ilford G907 filter).

Experiments with apical tissues were carried out in a similar manner to those with stem sections except that only $0.0166M$ buffer was used and no addendum as a suitable rate of IAA destruction was obtained. To prepare the tissues, as many ensheathing leaves as possible were stripped with fine forceps from around the apex, and the remaining bud together with 10 mm. of attached stem was excised. Closer stripping could be carried out with experiments in the light than in the dark, while dark-grown plants were easier to prepare than light-grown plants.

Plants grown in light were raised in a greenhouse and were used after 21 days when five or six internodes were present. In experiments carried out in light, "warm white" fluorescent tubes were used which gave an intensity of 135 foot candles at the level of the plant material in the conical flasks. Under these conditions, this light did not destroy any IAA when an irradiated solution (with addenda) was repeatedly sampled over a period of 212 min.

RESULTS

Experiments With Enzyme Breis

In pea and other plants an enzyme system occurs (IAA-oxidase) which destroys IAA (18, 27). It was necessary, therefore, to examine whether GA has any sparing action upon rate of IAA destruction when incubated with this enzyme system. The result of one experiment is shown in Figure 1A. In all experiments, absorption is plotted against time of sampling aliquots from the reaction mixture. It is seen that as the concentration of IAA decreases, GA has no effect upon the rate of IAA destruction. Similar results were obtained when this experiment was repeated, and also when a third experiment was carried out with a brei prepared from 'Alaska' peas.

As the activity of crude preparations of IAA-oxidase of peas can be influenced by light (9), it was decided to examine the effect of GA on rate of IAA destruction when reaction mixtures with enzyme brei were light-irradiated throughout the experimental period. A brei from dark-grown plants was used, as light-grown plants contain an inhibitor of the IAA-oxidase system (9). The experiment was similar to that shown in Figure 1A except that it was carried out under fluorescent light tubes. The result was similar to those shown in Figure 1A in which GA had no influence on rate of IAA destruction, while another two experiments, one of which is shown in Figure 1B,

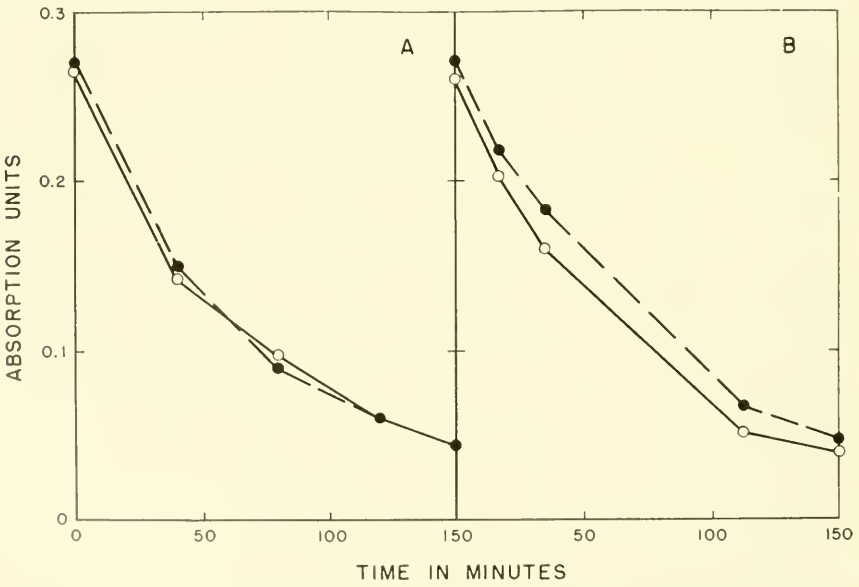

Fig. 1. Rate of destruction of indole-3-acetic acid by indole-3-acetic oxidase enzyme in the presence (solid lines) and absence (broken lines) of gibberellic acid in darkness (A) and in light (B).

showed an enhanced rate of IAA destruction with GA over part of the experimental period. In no experiment did GA exert a sparing action.

Experiments With Stem Sections

During the summer of 1957 extensive preliminary experiments were carried out with stem sections, following their use by Brian and Hemming (4), to investigate whether GA influences rates of IAA destruction when sections are incubated in solutions of hormone. Sections were cut serially from the entire length of stems of light-grown plants and were randomized before use. Rather variable sparing action results were obtained which, apart from variation caused by restricted sampling of sections, suggested that tissues of different physiological ages and states should be separated. This was done in all the following experiments carried out during the summer of 1958.

Results of experiments in the dark with sections from dark-grown plants are shown in Figure 2. Results in Figure 2C were obtained with 'Alaska' pea. A sparing of IAA destruction with GA was obtained in all experiments; however, in Figure 2B it was appreciable while in Figure 2A it was smaller, and in Figure 2C it was present over approximately only a 3 hr. period.

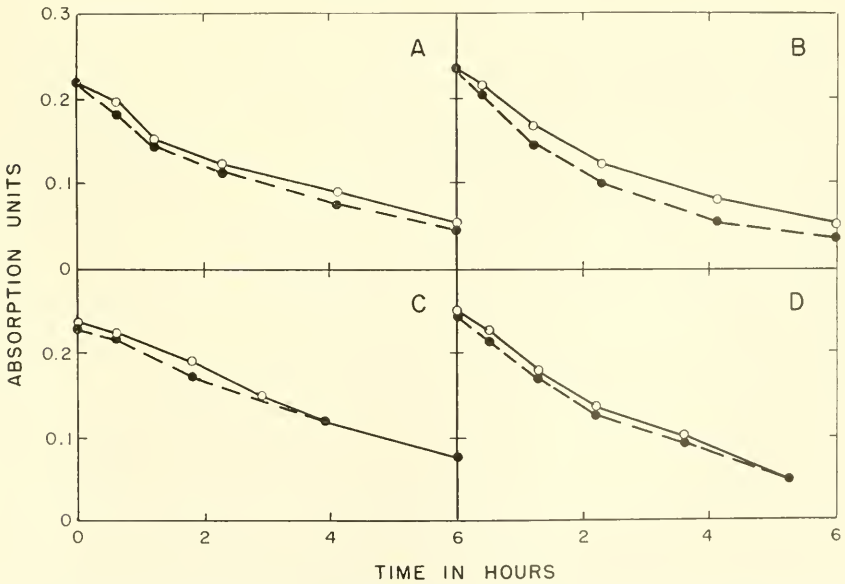

Fig. 2. Rate of destruction of indole-3-acetic acid by pea stem sections in the presence (solid lines) and absence (broken lines) of gibberellic acid in darkness (**A, B, C**) and in light (**D**). 'Alaska' peas were used for the experiment in **C** and 'Progress No. 9' for the remainder.

One experiment was carried out with dark-grown material in light under fluorescent tubes (Figure 2D). GA treatment resulted in a small but consistent sparing of IAA destruction.

Experiments With Stem Apices

Results of experiments with apical tissues are shown in Figures 3 and 4. Several curves differ from those of Figure 2 in being convex rather than concave upwards; the rate of disappearance of IAA tends to increase with time rather than decrease. Experiments carried out in the dark with tissues from dark-grown (Figure 3A, B, C) and light-grown plants (Figure 3D) show, with the exception of Figure 3C, no sparing of IAA destruction with GA; in Figure 3C there is a tendency to sparing. In Figure 3A and B it is thought that the separation of the curves after approximately 4 and 2.5 hrs., respectively, resulted from accidental removal of apical tissues from the hormone solutions during shaking in the dark (this occurred only with apical tissues, and when the volume of experimental solution became small).

In contrast with the above experiments in the dark, experiments in the light using apices from dark-grown (Figure 4A and B) and light-grown plants (Figure 4C to F) show a greater range of IAA sparing. Marked sparing is shown in Figure 4C, while in the remainder of the experiments there is a progressive decrease until, in Figure 4F and possibly in Figure 4D, no IAA sparing occurs.

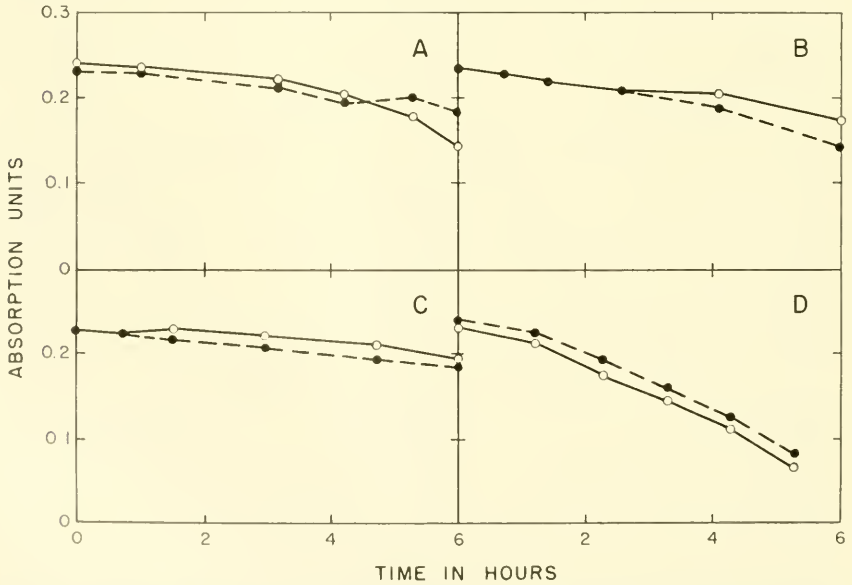

Fig. 3. Rate of destruction of indole-3-acetic acid by apical pea stem tissues with (solid lines) and without (broken lines) gibberellic acid in darkness. Plants grown in darkness (**A, B, C**) and in light (**D**).

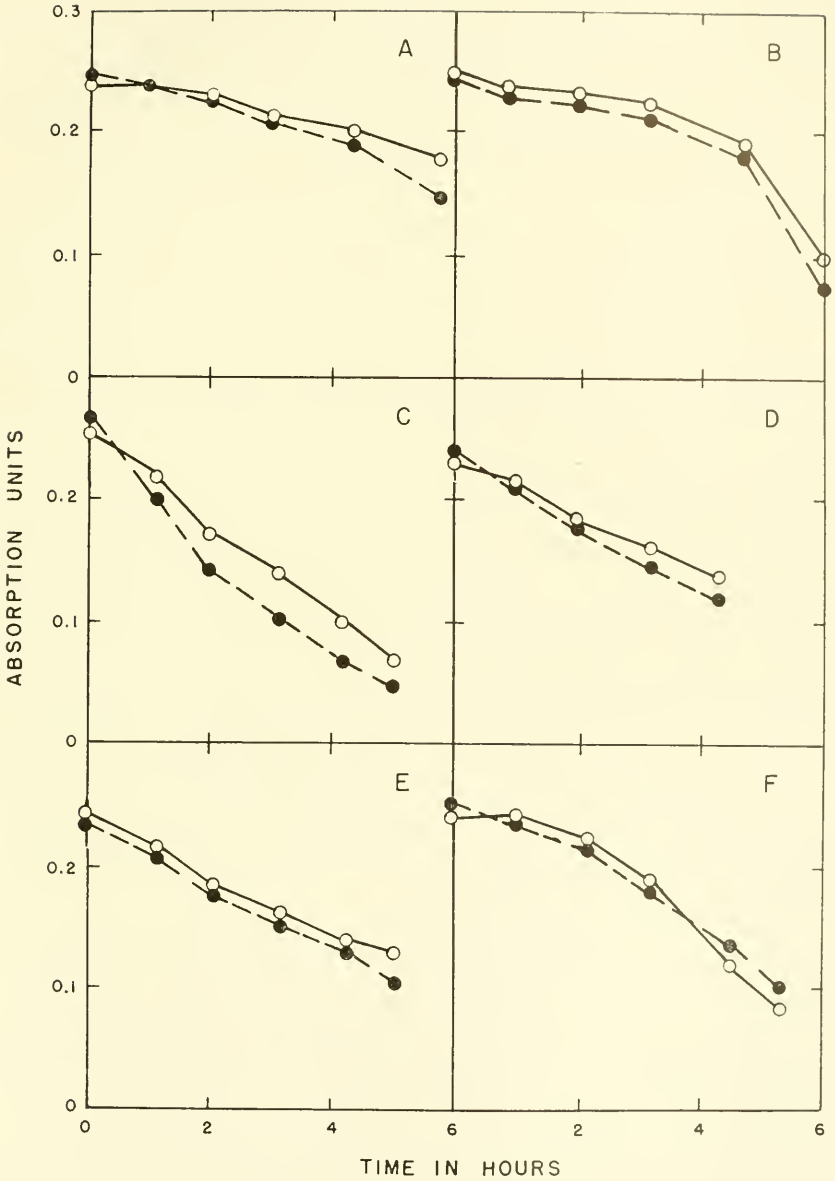

Fig. 4. Rates of destruction of indole-3-acetic acid by apical pea stem tissues in the presence (solid lines) and absence (broken lines) of gibberellic acid in light. Plants were grown in darkness (**A, B**) or in light (**C, D, E, F**).

DISCUSSION

The present study has shown that GA at a concentration of 10 mg/l has no influence on the IAA-oxidase system of pea in vitro in the dark, while in the light on some occasions it may result in an enhanced rate of IAA destruction. Results similar to the former have been obtained by Brian and Hemming (5) and Kato and Katsumi (16) using IAA-oxidase preparations from pea roots and shoots.

The present study has also shown that GA can influence rate of IAA destruction by excised pea tissues, and on some occasions the rate is reduced thus producing a sparing of IAA. The sparing effect is frequently not marked but varies considerably in magnitude from a mere trend (Figure 3C) to an appreciable effect (Figure 4C). This sparing accounts for the growth synergisms obtained by Brian and Hemming (4); also, our range of variation is consistent with the range of growth synergisms shown in Brian and Hemming's data, i.e., small synergisms ranging to marked ones.

Brian and Hemming (5) state that they have considered the possibility that GA might reduce rate of auxin destruction, but have been unable to detect any effect of GA on IAA destruction by pea internode sections; however, they do not mention the method and techniques used; therefore it is not possible to assess the reason for their failure. On the other hand, Nitsch (24) has shown that prior application of GA to an intact plant (*Rhus typhina*) causes the level of endogenous auxin in the apical tissues to be raised, an observation that may be accounted for by an auxin-sparing action of the type described in the present work. Nitsch's results (obtained by *Avena* straight-growth bioassay of ether-soluble materials after separation by paper chromatography) are important for they are a direct approach to the examination of effects of GA on the naturally-occurring hormone system of plants.

The possible mechanisms whereby an auxin-sparing phenomenon may be brought about by GA are next discussed. Before carrying out our early experiments, the results of Brian and Hemming (4) were considered in conjunction with those of Lockhart (20), and for a preliminary hypothesis it was suggested that GA acts by interfering in a light-mediated system of auxin destruction; the same hypothesis has been speculatively put forward by Vlitos and Meudt (28). As it is necessary to view hypotheses against the background of sparing actions shown in Figures 2, 3, 4, and 6, it is helpful first to summarize the latter more concisely (Table 1). In assessing the scoring in this table, the consistency of the sparing action through time was taken into account as well as the magnitude of the action. It may

Table 1. A summary of the sparing of indole-3-acetic acid destruction (+ . . . .) obtained in Figures 2, 3, and 4. (−) Indicates no sparing action occurred.

Stem Sections		Apical Tissues			
Dark-grown		Light-grown		Dark-grown	
Light exp.	Dark exp.	Light exp.	Dark exp.	Light exp.	Dark exp.
2D +	2A ++	4C ++++	3D −	4A ++++	3A −
	2B ++++	4D +++		4B +	3B −(?)
	2C ++	4E +(?)			3C +
		4F −			

be noted that shades of opinion in rating the score (e.g., Figure 2C to be +? or Figure 3C to be ++?) do not alter the final deductions drawn. Table 1 shows that with apical tissue experiments carried out in light (columns 3 and 5) there are sparing actions of almost all magnitudes, whereas in the dark (columns 4 and 6) sparing actions are either absent or not marked. There is no sharp division between experiments carried out in light and in darkness, but the largest sparing actions are shown in the light. In contrast with this distribution of sparing action, stem section experiments have an emphasis on sparing in experiments carried out in the dark, while in light a single experiment of low sparing permits no opinions to be formed. However, it may be noted that again no sharp division exists between experiments carried out in light and in darkness.

If one now views the light-mediated auxin destruction hypothesis against the results of Table 1, it is evident that at least two disturbing points prevent a satisfactory union between the two. The most obvious lies in the sparing actions shown in the dark (column 2), for with the above hypothesis sparing should not occur under these conditions. The second concerns the marked variation in magnitude of sparing actions referred to above. If GA were to intercede directly in a light-operated hormone-destroying mechanism, one might reasonably expect sparing actions to be present more consistently and to be less variable in magnitude.

Further hypotheses have been considered by Brian and Hemming (5) to account for the enhancement of growth by GA. The possibility that GA may combine with auxin(s) in the plant to form a more active compound is considered and dismissed. Our own data do not readily fit into any scheme based on this idea and it does not appear to be a profitable line to pursue. Similarly, the possibility that GA

increases auxin in the plant by increasing the amount formed does not readily lend itself to the interpretation of our data in terms of any known scheme. The possibility that GA retards auxin destruction is considered by Brian and Hemming (5), but since they were unable to obtain a sparing action they took the matter no further. To sum up our own views on one aspect of this hypothesis, it is felt that there is no close relationship between light treatment and GA sparing actions, and that any relationship between the two involves another factor(s).

An hypothesis favored by Brian and Hemming is that GA affects some metabolic process which normally limits growth even though auxin is present in non-limiting amounts. They discuss this idea at length in relation to their own and other data and are led to suppose that in the intact plant there is an inhibitory system which limits growth rate and that GA treatment can be envisaged as a neutralization of this inhibitory system. Thus, GA plays no direct or positive part in cell extension, but that, by neutralizing an inhibition of extension, it releases the full potentialities of the auxins present. This hypothesis is attractive as it is potentially versatile enough to account for our own data; it is compatible with our own views and has some supporting evidence cited by Brian and Hemming (5).

A naturally-occurring system which answers to the above requirements is the IAA-oxidase enzymes and their inhibitor(s). One may raise arguments objecting to this choice; however, it is profitable to discuss the system and to defer objections for later discussion. The facts required to construct a theory have been briefly summarized by Galston (8), but only limited data have been published in detail (12). In light-grown 'Alaska' pea considerable IAA-oxidase activity can be demonstrated in young stem and bud tissues while in the leaf a large amount of inhibitor is present. Some inhibitor is present also in the stem, the highest concentration occurring in apical tissues and a gradient existing down the plant (presumably the stem too). The level of inhibitor in the youngest leaves (i.e., part of the apical tissues) may be raised by treatment of the entire plant for 2 days with $10^{-5}M$ GA (administered via the roots), while inhibitor level in leaves may also be raised progressively by increasing the length of exposure of the plant to light. This light effect on inhibitor level has been further investigated by Hillman and Galston (12) who have shown that in vitro IAA-oxidase activity of dark-grown 'Alaska' plants is greatly inhibited by red light given to the intact plants before harvest. The inhibition is reversible by near infrared radiation, i.e., far-red (29, p. 384), given immediately after the red light, but not more than 1 hr. afterwards.

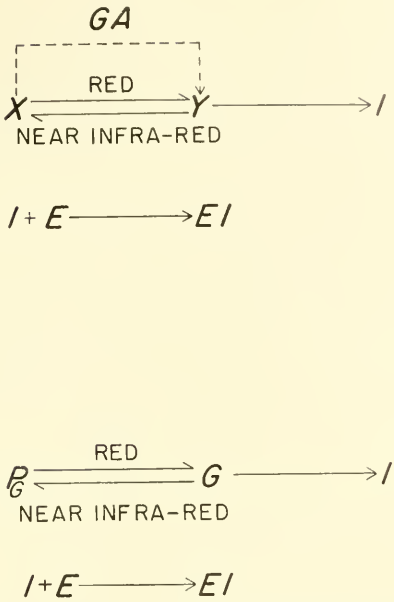

Fig. 5. Hypothetical schemes showing possible relationships between gibberellic acid (GA) above and gibberellins (G) below and the inhibitor (I) of the indoleacetic acid oxidase enzymes (E).

In dwarf 'Laurel' peas, less inhibitor is found when corresponding parts with tall types, e.g., 'Alaska,' are examined (8).

To construct a scheme as in Figure 5 (top) one may imagine that when IAA-oxidase inhibitor (*I*) associates with the IAA-oxidase enzyme (*E*), an inactive complex (*EI*) is formed which will not destroy IAA; the kinetics of the reaction, however, need not concern us. The level of *I* in a given plant part is influenced by a number of factors. Galston (8) states that GA raises the level of *I* in the youngest leaves, and we shall assume that this generally holds in other plant parts, in particular the stem. Red light is thought to raise the level of *I* also, and near infrared radiation to oppose the reaction. These points, shown diagramatically in Figure 5 (top), are linked as follows. *X* is a precursor of *I* and does not inhibit *E*. The reaction $X \rightarrow I$ does not take place in a single step but passes through at least one intermediate compound, *Y*. *Y* but not *I* can be changed back to *X* by near infrared radiation, while in the absence of this radiation *Y* is slowly changed to *I* by an unknown process. It is necessary to postulate the intermediate compound, *Y*, to account for the negative effect of near infrared radiation when applied 1 hr. after red light.

Before elaborating on the above scheme, it is desirable first to examine how well the whole inhibitor hypothesis accounts for the results of Figures 2, 3, and 4. During the course of experimentation an impression was obtained that large sparing actions were associated with rapid rates of IAA inactivation and conversely smaller sparing actions with less rapid rates. Data were analyzed and the results are shown in Figure 6. Average rates of IAA destruction (no GA present) over the first 3 hr. period are plotted against differences between average rates when GA is present and absent. This period was chosen as rates of IAA destruction in many experiments were fairly uniform over this interval. Rates are expressed in absorption units to permit data from the 1957 season to be included, while points are joined merely to show trends more clearly. Figure 6 shows that the sparing action with GA tends to increase as the rate of IAA destruction increases. Expressing this in terms of Figure 5, as the rate of IAA destruction increases (i.e., as E increases relative to I and EI) so the effectiveness of GA increases *showing the same effect as an increase in* I. Expressed alternatively, if EI is high and E is low, there will be a low rate of IAA destruction and only a small effect of GA provided the effect of GA can be equated to I. Thus, our own data are consistent with Galston's statement that GA increases the level of I in the plant. It may be noted that Lockhart (21) has observed a similar effect: intact dark-grown plants which do not respond to applied GA give the greatest GA-induced growth promotion when decapitated (very high I in intact plants results in no growth with applied GA; however, if I is markedly reduced by decapitation of these plants, the GA-induced growth relative to the intact plants will possibly be at a maximum).

It is now possible to consider the results of Table 1 against the background of the inhibitor scheme. It will be recalled that concentration of inhibitor is high at the apex and a gradient exists down the plant. The concentration in the sections of the second column will be lower than that in the apices of the sixth column, and therefore there will be a tendency for greater sparing actions to occur in the former; this expectation is reflected in the table. Comparison of light- and dark-grown materials (i.e., column 3 with 5, and column 4 with 6) cannot be made readily as inhibitor levels of light- and dark-grown stems do not appear to have been compared. Comparison of experiments carried out in light and in darkness (column 3 with 4, and column 5 with 6) suggests that inhibitor level in the former was less than the latter on some occasions. This variation may have been due to several factors, the most important possibly being insufficient control over the quality of light used during experimentation. A sec-

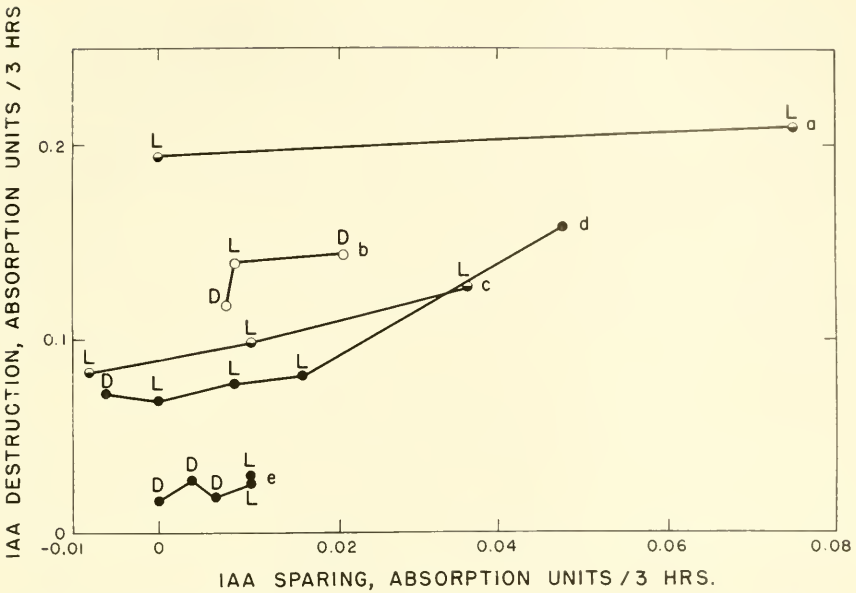

Fig. 6. Average rate of indole-3-acetic acid (IAA) destruction over the first 3 hr. period (vertical axis) plotted against the difference between the average rates of destruction over the same period in the presence or absence of gibberellic acid (horizontal axis). Curves (**a**) and (**c**) represent experiments (1957) with stem sections from light-grown material; curve (**b**) represents section experiments (1958) from dark-grown material; curves (**d**) and (**e**) represent experiments (1958) with apical tissues. Experiments shown in (**a**) were carried out with 'Lincoln' peas; experiments shown in curves (**b**) to (**e**) with 'Progress No. 9' peas. Experiments of curves (**d**) and (**e**) were carried out with light- and dark-grown material, respectively. The letter over each point indicates whether the experiment was conducted in light (L) or in darkness (D).

ond factor was the differing amounts of leaf tissue removed when apical tissues were being prepared for excision. Other factors may be associated with disturbances resulting from excision, a point discussed by Brian and Hemming (5).

As the present data are consistent with an inhibitor interpretation in the scheme of Figure 5 (top), it is profitable to consider this system further. The formation of *I* is influenced by a red/near infrared reaction, and it is constructive to consider the literature. Johnson and Liverman (13) state that near infrared-induced dormancy in summer-grown tomatoes can be reversed by GA producing a striking effect on stem elongation; the same dormancy can be broken also by red light, auxins, or cool weather (19). Lockhart (22) notes that the GA-induced elongation of dwarf bean seedlings may be markedly enhanced by a 2 to 5 min. exposure of the plants to red light. This effect may be reversed by subsequent exposure of the plants to near infrared

radiation. Other literature relating GA to these light effects has been discussed by Brian (1). Thus, it would appear the applied GA in some systems cannot only by-pass the effects of red/near infrared radiations but the two may mutually reinforce each other. To accommodate this, an attractive modification of the scheme in Figure 5 (top) is to suggest that red/near infrared radiations are influencing the naturally-occurring gibberellins (Figure 5, bottom). *X* now becomes P_G, a gibberellin precursor, while *Y* becomes *G*, its corresponding gibberellin. The mechanism of increase in *I* under the influence of *G* remains an unknown process. It may be noted that Brian (1) has been led to postulate a similar relationship between gibberellin(s), its precursor(s), and red/near infrared radiations as a result of theoretical considerations on mechanisms of flowering in plants.

Although it is possible to discuss further this scheme (Figure 5, bottom) in relation to published literature, discussion becomes diffuse due to lack of adequate biochemical data. Discussion also becomes diffuse when published data, which do not appear directly to support the scheme of Figure 5, are considered. For example, choice of the IAA-oxidase inhibitor for *I* may be objected to on the ground that growth interactions between hormone and GA are observed with 2,4-dichlorophenoxyacetic acid and 1-naphthaleneacetic acid and these compounds are degraded less readily than IAA (5) and may not go through the IAA-oxidase system. If, however, one accepts this criticism without considering further the data in the literature on which it rests, it does not alter the concept that GA operates through an inhibitor system, but merely removes the scheme in Figure 5 without replacing it with any known inhibitor system. In view of the statements of Galston (8) on the relationship between GA and *I*, it would seem preferable to use Figure 5 (bottom) to design biochemical experiments to test the validity of the scheme in this figure.

SUMMARY

An examination has been made of the rates of disappearance of indole-3-acetic acid (IAA) in the presence and absence of gibberellic acid (GA) from solutions containing either IAA-oxidase enzymes from pea or excised stem tissues of pea. IAA destruction with the enzyme system in vitro was not consistently influenced by GA in darkness or in light. With apical tissues (apex, young leaves + 10 mm. immature stem) from light- and dark-grown plants, rate of IAA destruction in light was decreased by GA, thus causing an IAA-sparing effect, while in darkness GA had little or no effect upon rate of destruction. However, there was no sharp division between the light and dark treatments, some experiments in light showing little or no sparing action and thus resembling the dark experiments.

With stem sections taken from just below the first node of dark-grown plants, rate of IAA destruction in darkness and in light was decreased by GA. The sparing actions obtained varied in size as for apical tissues. The IAA sparing action is discussed in terms of current theories on the mechanism of action of GA-induced growth. The data are consistent with the hypothesis that GA leads to the production of an inhibitor which retards an auxin-destroying system. Evidence from the literature that this inhibitor could be an inhibitor of the IAA-oxidase enzyme system is considered. The possibility that the effect of red and near infrared (far-red) radiations on growth could be brought about through an inhibitor/gibberellin-mediated system is pointed out.

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DISCUSSION

Dr. Waygood: I'd like to point out that there is no inhibitory effect of gibberellin on a purified IAA oxidase system. The effect of IAA and gibberellin shown in the slide by Brian and Hemming is not a synergistic effect.

Dr. Housley: Our results do not conflict with Dr. Waygood's first statement that there is no inhibitory effect of gibberellic acid on a purified IAA oxidase system. With respect to his second statement, perhaps the use of the term synergistic in the present context is best left for each individual worker to accept or substitute otherwise according to preference.

Dr. Andreae: I should like to ask what justification is there to equate loss of IAA from solution with destruction? We found with similar experiments that as little as one-fifth to as much as all of the IAA lost from solution accumulates in the tissues as indoleacetylaspartic acid.

Dr. Housley: One may certainly account for the disappearance of IAA from solution other than by its destruction; the example you mention is conjugation with some other entity. In our preliminary experiments we frequently obtained little or no disappearance from solution (i.e., no decrease in absorption) over a period of 6 or more hours; however, if suitable amounts of manganese ion and 2,4-dichlorophenol were incorporated in the solution, IAA began to disappear rapidly. This dependence upon addition of these addenda for IAA disappearance led us to suppose that the acid was being removed from solution primarily as a result of its destruction by the IAA oxidase enzymes.

Dr. Purves: I'd like to describe one experiment in connection with the theory put forth by Dr. Housley. In etiolated pea epicotyl sections under certain conditions you can get a very low IAA optimum, and can get a concentration curve showing an IAA optimum at $10^{-7}M$ with concentrations above $10^{-6}M$ being inhibitory to the growth rate. That is, the growth produced in the presence of high auxin concentration is actually lower than that of the controls. Now, if gibberellin is to act by virtue of an auxin-sparing mechanism, you would expect the treatment under these conditions with GA leading to an increased auxin content would lead to a further inhibition in the presence of inhibitory IAA concentrations. However, we find that the increment of growth produced by gibberellin is almost as great in the presence of inhibitory auxin concentrations as in the absence of auxin, suggesting that gibberellin cannot act by an auxin-sparing mechanism.

Dr. Housley: Dr. Purves' work might suggest that gibberellin is not acting by an IAA-sparing mechanism alone and perhaps that more than one mechanism in his experimental system is operating. Such a mechanism, for example, could involve chemical combination of gibberellin with endogenous auxin of pea sections forming a complex with novel growth properties as has been postulated by Phillips, Vlitos, and Cutler (Contr. Boyce Thompson Institute. 20, 111–120. 1959) to account for their gibberellic acid — endogenous auxin studies on pea.

With respect to Dr. Purves' suggestion that gibberellin cannot

act by an auxin-sparing mechanism, one is rejecting a biochemical mechanism by considering data derived from a complex growth phenomenon, namely auxin-induced inhibition of growth. Such consideration requires caution. Our own work consists of examination of a biochemical reaction; no observations on growth are made. We point out that our results are consistent with Brian and Hemming's growth synergisms obtained with IAA and gibberellic acids at optimal and sub-optimal concentrations.

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Interaction of Gibberellic Acid and Auxin in Extension Growth of Pea Stems

The garden pea (*Pisum sativum*) is an excellent plant for study of the role of gibberellins in internode extension. Of particular interest are comparisons of tall and dwarf varieties and comparisons of intact internodes with internode sections floating on substrate solutions. The following observations have been made in the course of our work on this plant (3, 4):

(a) The rate of internode extension in tall varieties of pea is far greater than that in dwarfs, yet sections from comparable internodes of tall and dwarf varieties extend at approximately the same rate, whether incubated in plain buffer or in solutions containing sucrose or indole-3-acetic acid (IAA). The rate of extension of sections on IAA/sucrose in short-term experiments is much greater than that of comparable tissues in intact dwarfs, but nearly the same as that of internode tissues in intact tall varieties.

(b) Though intact dwarf pea plants give a great response to exogenous gibberellic acid (GA), the response of sections is small, and only demonstrable in the presence of an auxin.

(c) Intact dwarfs give a much greater response to GA than intact tall peas, yet sections from both kinds respond to nearly the same extent.

These observations indicate that the difference in growth rate between the tall and dwarf varieties is not determined in the internode tissue but elsewhere; similarly, the differential response of tall and dwarf peas to exogenous GA is not due to an innate difference in competence of their internode tissues. Indeed, we have evidence that the apical bud is of great importance in this connection. However, we have taken the view that detailed knowledge of the factors governing

the response of sections to GA is necessary before full value can be derived from comparisons of internode sections with similar tissues in their normal anatomical context.

We have used sections from peas grown in light (green sections), studying their extension in light, because we feel that such a system is closer to conditions in the intact plant than is the more usual system of etiolated sections growing in darkness, and that more valid comparisons between internode sections and intact plants can therefore be made. The results now reported supplement those given in an earlier publication (4).

METHODS

Unless otherwise stated, sections have been cut from the dwarf pea, 'Meteor.' The procedure used for study of growth of these sections has been described elsewhere (4). One modification of that procedure has been made: 0.75 ml. medium is used for each section instead of 0.5 ml. We have found this to give slightly enhanced extension. Unless otherwise stated, the initial length of sections was 5.1 mm., and growth took place over 24 hrs. at 15° C. in a light intensity of about 800 foot candles.

RESULTS

Interaction Between Auxins and Gibberellic Acid

We have already shown that GA alone has little or no effect on extension of 'Meteor' pea stem sections, but that it will induce increased extension in the presence of IAA (4). We have since confirmed this in many experiments. Similar synergism is exhibited be-

Table 1. Interaction between auxins and gibberellic acid in promotion of extension of 'Meteor' pea stem sections. Mean final lengths (mm.) of sections. Basal medium: 1 per cent sucrose in phosphate buffer.

Auxin	Concn., μg./ml	Mean Final Length of Sections, Mm.		
		Gibberellic acid, μg./ml		Effect of gibberellic acid
		0	10	
NAA	4	9.3	10.6	+1.3
NAA	1	8.6	9.8	+1.2
2,4-D	1	10.4	11.6	+1.2
2,4-D	0.25	9.9	10.9	+1.0
IAA	10	9.6	10.9	+1.3
IAA	2.5	9.0	9.9	+0.9
None		6.9	7.1	+0.2
None		6.9	7.2	+0.3
None		7.1	7.0	−0.1

Significant difference between means: 0.36 (P=.05), 0.48 (P=.01), 0.62 (P=.001).

Table 2. Interaction between light and sucrose in promotion of extension of pea stem sections, using two pea varieties. Mean final lengths (mm.) of sections. Basal medium: 10 μg/ml indoleacetic acid in phosphate buffer.

Lighting Conditions	Sucrose, Per Cent 0	0.5	1.0	2.0	4.0
	'Meteor' (Dwarf)				
Light	8.6	9.1	9.4	9.0	8.3
Dark	6.5	7.1	7.4	7.2	6.6
Effect of light	+2.1	+2.0	+2.0	+1.8	+1.7
	'Improved Pilot' (Tall)				
Light	8.1	8.7	8.7	9.0	7.9
Dark	6.5	7.5	7.7	8.2	7.3
Effect of light	+1.6	+1.2	+1.0	+0.8	+0.6

Significant differences (P = .01) between means: 'Meteor,' 0.42; 'Improved Pilot,' 0.44.

tween GA and such auxin analogues as 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Table 1). This result is relevant to discussions of the mode of action of GA and is mentioned again later.

Effect of Light

Galston and Baker (7) showed that light enhanced extension of green sections; they showed that it could be largely replaced by sucrose, though their data also showed that sucrose could not completely substitute for light. In our experiments (Table 2) different varieties of pea differed in their response to light. With sections from the tall variety 'Improved Pilot' the effects of light and sucrose were less than additive (light × sucrose interaction significant at P = 0.001), indicating partial replacement of light by sucrose. Sections from the tall variety 'Alaska' behaved similarly; this was the variety used by Galston and Baker. But with sections from the dwarf pea 'Meteor' the effects of light and sucrose were approximately additive (light × sucrose interaction not significant at P = 0.05). Thus, particularly in 'Meteor,' light has some effect of importance which cannot be replaced by sucrose. In the presence of optimal sucrose light has the effect of enhancing the response to IAA (Table 3), 2,4-D and NAA (Table 4). In experiments not reported here in detail we have found that this is a high intensity light effect and that the response is proportional to the length of exposure to light.

The response to GA also is dependent on light (Table 5). Factorial experiments in which light, IAA, and GA are supplied separately and in combination (Table 6) suggest that the dependence of the GA response on light is a consequence of the known GA × IAA interaction

Table 3. Interaction between light and indoleacetic acid (IAA) in promotion of extension of 'Meteor' pea stem sections. Basal medium: 1 per cent sucrose in phosphate buffer.

IAA μg/ml	Mean Final Length, Mm.		Effect of Light
	Light	Dark	
0	6.5	5.7	+0.8
0.1	7.0	6.1	+0.9
1.0	7.8	6.5	+1.3
10.0	9.1	6.9	+2.2
100.0	9.3	6.9	+2.4

Significant difference between means: 0.40 (P = .05), 0.53 (P = .01), 0.68 (P = .001).

Table 4. Interaction between light and 1-naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) in promotion of extension of 'Meteor' pea stem sections. Basal medium: 1 per cent sucrose in phosphate buffer.

Auxin	Mean Final Length, Mm.		Effect of Light
	Light	Dark	
NAA, 5 μg/ml.	8.8	6.9	+1.9
NAA, 0.5 μg/ml.	7.8	6.6	+1.2
NAA, 0.05 μg/ml	7.0	6.3	+0.7
2,4-D, 1 μg/ml	9.3	6.9	+2.4
2,4-D, 0.1 μg/ml.	8.8	6.7	+2.1
2,4-D, 0.01 μg/ml.	7.5	6.2	+1.3
None	6.9	6.2	+0.7
None	7.0	6.1	+0.9

Significant difference between means: 0.28 (P = .05), 0.37 (P = 0.01), 0.48 (P = .001).

Table 5. Interaction between light and gibberellic acid in promotion of extension of 'Meteor' pea stem sections. Basal medium: 10 μg/ml IAA + 1 per cent sucrose in phosphate buffer.

Hrs. of Light	Mean Final Length, Mm.		Effect of Gibberellic Acid
	Gibberellic acid μg/ml		
	None	10	
0	7.4	7.7	+0.3
6	8.4	9.1	+0.7
12	9.2	10.2	+1.0
18	9.7	10.9	+1.2
24	9.9	11.1	+1.2

Significant difference between means: 0.38 (P = 0.05), 0.50 (P = .01), 0.65 (P = .001).

Table 6. Interaction between light, gibberellic acid, and indoleacetic acid in promotion of extension of 'Meteor' pea stem sections. Basal medium: 1 per cent sucrose in phosphate buffer.

IAA μg/ml	GA μg/ml	Mean Final Length, Mm.		Effect of Light
		Light	Dark	
10	0	7.5	6.5	+1.0
	0.01	8.5	6.7	+1.8
	0.1	8.7	6.8	+1.9
	1.0	8.8	6.8	+2.0
	10.0	8.6	6.7	+1.9
0	0	6.1	5.7	+0.4
	0.01	6.0	5.7	+0.3
	0.1	6.2	5.8	+0.4
	1.0	6.1	5.8	+0.3
	10.0	6.1	5.8	+0.3

Significant difference between means: 0.25 (P = 0.05), 0.33 (P = .01), 0.43 (P = .001).

and also the necessity for light if IAA is to have its full effect.

It is, of course, equally true to say that light has its greatest effect in the presence of an auxin and that its effect is still further enhanced if GA is supplied (Table 6). One possible interpretation of this light effect is that some product of photosynthesis, other than sucrose, is essential for the growth reaction in which auxin intervenes. A number of possible early products of CO_2-fixation have been tested, including glucose-6-phosphate, glucose-1-phosphate, fructose-1,6-diphosphoric acid, fructose-6-phosphoric acid, 2-phosphoglyceric acid, and 3-phosphoglyceric acid, but none of these had light-replacing properties greater than sucrose. Nevertheless, some indirect evidence that a product of photosynthesis may be involved has arisen from work with specific inhibitors.

Effect of L-Azaserine and 6-Diazo-5-oxo-L-norleucine

Two antibiotics, azaserine (AZS) and 6-diazo-5-oxo-L-norleucine (DON), which have marked tumor-inhibiting properties, have been shown by Calvin and his colleagues (1, 11) to have specific inhibitory effects on the CO_2-fixation pattern of photosynthesizing algae. Both substances are powerful inhibitors of IAA-induced extension of green sections (Table 7). Furthermore, doses which have little effect on extension of sections in darkness cause a marked inhibition of extension in light. Thus these substances appear to inhibit those growth processes initiated by exposure to light. Specificity, if it exists, is not complete because higher doses will inhibit extension in darkness. Their anti-tumor activity has been attributed (5, 9, 10) to interference with biosynthesis of purines or some amino acids, and their effects can to some extent be reversed by some purines, cyclic amino acids,

Table 7. Effect of azaserine (AZS) and 6-diazo-5-oxo-L-norleucine (DON) on response of 'Meteor' pea stem sections to light. Basal medium: 10 μg/ml indoleacetic acid + 1 per cent sucrose in phosphate buffer.

Inhibitor	Concn., μg/ml	Mean Final Length, Mm. Light 24 hrs.	Dark 24 hrs.	Effect of Light
None		9.5	7.5	+2.0
AZS	20	8.7	7.0	+1.7
AZS	100	7.5	6.8	+0.7
DON	1	8.1	7.4	+0.7
DON	5	7.4	7.2	+0.2

Significant difference between means: 0.36 (P = 0.05), 0.48 (P = 0.01), 0.61 (P = 0.001).

and L-glutamine. In their effect on photosynthesis they leave the carbon cycle untouched but block transamination reactions, and the effect of AZS has been partially reversed by L-glutamine (1). We have examined the possibility of reversal of DON and AZS inhibition of section extension and have tested a large number of purines, pyrimidines, nucleosides, and amino acids. The only substance which has shown any evidence of reversal is L-glutamine, but the effect is at most very slight. Thus we have little information concerning the mode

Table 8. Effect of starvation of 'Meteor' pea stem sections on response (**A**) to indoleacetic acid (10 μg/ml) and sucrose (2 per cent) and (**B**) to gibberellic acid. Data from three experiments in each case. [Mean final lengths of sections (initial length 5.1 mm. untreated, 5.4 mm. after starvation treatment). Basal media: (**A**) phosphate buffer, (**B**) 10 μg/ml IAA + 2 per cent sucrose in buffer.]

A

Medium	Untreated Sections, Mm. (i)	(ii)	(iii)	Mean	Starved Sections, Mm. (i)	(ii)	(iii)	Mean
—	6.8	6.6	6.3	6.6	6.2	6.1	6.8	6.0
IAA	8.6	8.0	8.1	8.2	7.6	7.2	7.1	7.3
S	7.6	6.6	6.2	6.8	6.6	6.5	6.2	6.4
IAA + S	9.1	8.1	8.7	8.6	10.2	8.3	9.2	9.2

B

GA, μg/ml	Untreated Sections, Mm. (i)	(ii)	(iii)	Mean	Starved Sections, Mm. (i)	(ii)	(iii)	Mean
0	9.1	8.1	8.7	8.6	10.2	8.3	9.2	9.2
10	9.9	10.0	9.2	9.7	10.2	8.8	9.0	9.3
Effect of GA	+0.8	+1.9	+0.5	+1.1	0	+0.5	−0.2	+0.1

Significant difference between means of all experiments: 0.28 (P = .05), 0.37 (P = .01), 0.48 (P = .001).

of action of these inhibitors of section growth, but we consider their effect relevant both because they appear to block the response of sections to light, and, in a number of preliminary experiments, to GA.

Effect of Starvation of Sections

We have shown (4) that rapid washing of sections in distilled water affects their subsequent growth. This led us to investigate in greater detail the effect of pretreatment of sections in aerated distilled water over longer periods. A 3 hr. treatment has two obvious effects on subsequent section extension. In the first place, it much reduces extension in buffer or buffer plus IAA, but normal extension is restored on addition of sucrose (Table 8A); this is our main justification for interpreting it as a starvation treatment. Secondly, it very strikingly reduces the response to GA even when optimal IAA and sucrose are present (Table 8B). We cannot decide, on the basis of the data at present available, whether this is due to removal of some cofactor necessary for growth, or to removal of an inhibitor normally reversed by GA.

CONCLUSIONS

Our knowledge of the mode of action of gibberellic acid has not been greatly advanced by these experiments, but they have shown that several factors are involved in the response of green sections to GA, and that the response of green sections to light merits further investigation.

The response to GA is conditional on the presence of an auxin. We previously reported that synergism could be demonstrated not only between GA and IAA but also between GA and synthetic auxin analogues, but we did not then (4) give experimental evidence for the statement. We have since repeated the relevant work several times and it is now quite certain that a response to GA from green pea sections can be induced by supplying either IAA, 2,4-D, or NAA (Table 1). A very tempting explanation of IAA $\times$ GA synergism is that GA directly or indirectly inhibits metabolic destruction of IAA, and suggestions have been made that the activity of IAA-oxidase systems may be altered (6, 12), with a good deal of supporting evidence. However, the fact that GA has a synergistic relationship with such substances as NAA and 2,4-D, which are much less susceptible to metabolic breakdown, throws considerable doubt on this theory, unless it be supposed that such substances as 2,4-D and NAA themselves induce section extension by displacing endogenous IAA from some physiologically inactive complex, as originally envisaged by Skoog (13). There is little evidence that this does occur.

The response of green sections to auxins, and therefore indirectly

the response to GA, is dependent on high-intensity light. This cannot be explained purely in terms of photosynthetic formation of sucrose. This effect of light appears to be blocked by the inhibitors AZS and DON, which are known to affect the pattern of photosynthetic CO_2-fixation in algae in pathways other than those concerned in sucrose formation. They also block the response of sections to GA. From what is known of their mode of action in other systems, it can be suggested that the effect of light on green section growth is perhaps concerned with biosynthesis of purines or amino acids, pathways blocked by AZS or DON by their effect on transamination reactions. In this connection it is interesting to note (2) that treatment of germinating barley grain with gibberellic acid results in considerable increases in transaminase content.

Starvation of sections before use has little subsequent effect on extension if sucrose is supplied. Such sections no longer respond to exogenous GA, and it seems probable that they have been depleted of some other substance necessary for the response to GA.

It has already been suggested (4, 8) that other factors besides auxin are involved in stem extension responses to GA. The results reported above offer further support for this view.

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DISCUSSION

Dr. Sachs: Dr. Brian, could you comment on what you meant by your evidence and that of Dr. Vlitos, concerning the role of the apical bud in the intact plant?

Dr. Brian: If you excise the apical bud from a pea plant and apply gibberellic acid, you don't get anything like the response that you get when it is there.

Dr. Sachs: I meant to ask about the size of the apical bud.

Dr. Brian: A pretty large size.

Dr. Sachs: In this case you perhaps removed the region where growth actually takes place by cell division. Evidence indicates that the whole region of shoot histogenesis exists within 2,000 to 3,000 microns, and perhaps up to 10,000 microns in some plants, below the apical meristem. The apical meristem itself is completely inoperative in shoot histogenesis.

Dr. Brian: I would agree, but certainly the parts corresponding to the section which increases in length by cell extension have not been removed.

Dr. Nitsch: I would like to make two comments. The first one bears on what has just been said. Using oat seedlings, we have been able to demonstrate that gibberellic acid acts mainly on the very young, meristematic tissues, whereas auxins act on somewhat older tissues, located further down the coleoptile or first internode (Nitsch, J. P., and Nitsch, C. — Bul. Soc. Bot., France, in press). The other comment concerns the changes caused by gibberellic acid in the metabolism of the endogenous growth substances. We have been studying this question ever since we discovered that the application of GA to a sumac or a bean plant produces a great surge in the amount of endogenous growth substances (Nitsch, J. P., and Nitsch, C. — *In*: Photoperiodism and Related Phenomena in Plants and Animals. R. B.

Withrow, ed., AAAS, Washington, D.C., pp. 225–242. 1957). Actually, not one, but possibly three to four compounds appear, or at least increase in the tips of bean seedlings after the application of gibberellic acid. (Nitsch, J. P. — XVth Interntl. Hort. Cong., Nice. 1958; Nitsch, J. P., and Nitsch, C.–Bul. Soc. Française Physiol. Vég. 5:20–23. (1959.)

Dr. Galston: I would like to say that, like Dr. Brian, we have recently been working almost exclusively with the green sections that we started with 10 years ago, and we feel much as Dr. Brian does that they have a great many advantages for this kind of work. We completely agree with him that sugar replaces part but not all of the enhancing effect of light upon growth. We agree also that the extra promotive effect of light is a high-intensity reaction. We also feel that the previous photoperiod to which the plant has been exposed has a very marked effect on the response of these excised green sections to gibberellin.

There is one respect in which our results differ from those of Dr. Brian. We do not ever find that the sections fail to respond to gibberellin in the absence of auxin. We always get a small but significant increase in growth with gibberellin alone, and this effect is greater, the greater the previous photoperiod to which the plants had been exposed prior to excision of the sections.

Dr. Brian and I have also used a somewhat similar approach in attempting to inhibit photosynthesis. I would like to mention one other compound that we have used which behaves rather like DON and azaserine. This is a compound called fenuron which belongs to the urea herbicide group. It is 3-(phenyl)-3,3-dimethylurea. The *p*-chlorophenyl analogue (CMV or monuron) has been widely used in the inhibition of the Hill reaction. We find that with this compound we can almost completely inhibit photosynthetic activity of green sections but not affect their ability to grow heterotrophically in the presence of sugar. We have found to our surprise that fenuron acts as an auxin or auxin synergist in the dark in the presence of sugar. This is a rather surprising effect for this type of compound. Of course, Drs. Steward and Shantz have reported the isolation of 1,3-diphenylurea from coconut milk and have shown that this has some growth-promoting ability in tissue culture. I would guess, then, that we are dealing here with a new class of auxins.

Mr. Barlow: In your starved sections, is it a question of actually leaching in water, or have you for instance kept them on damp filter paper? Do you also get the same effect of starvation by simply keeping the whole plant in darkness before cutting the section out? Apropos of that, how does the growth of the section compare with

that of the same region on the plant, since these increases that you obtained, of 10 or perhaps 20 per cent at the most, seem rather small?

Dr. Brian: I can't answer the first question with any certainty. Certainly by just putting them on filter paper you do get the same kind of effect, but it isn't so pronounced. I called it starvation. I'm not at all sure that it isn't starvation plus leaching. The second point is that certainly the response to gibberellin is never as great as the growth the same piece of tissue would make in the intact plant. The over-all extension is, if you are using a dwarf section, however, very much greater than the extension of the same tissue in the intact plant.

Dr. Tolbert: Dr. Haber and I have, some years ago, extensively studied the distribution of carbon-14 in the products from long and short term photosynthetic $C^{14}O_2$ fixation by gibberellin-treated plants. We found no difference, that could be detected by paper chromatography, in the rate or amount of C^{14} incorporated into the sugars, amino acids, or organic acids, of plants treated with gibberellin as compared to controls.

Dr. Lockhart: I'd like to make a comment on the importance of the tip in the response to gibberellic acid. I also agree with the other workers that in light-grown plants or plants that have been treated with light, the response to gibberellin is very much reduced if you remove the tip. However, if the plants are grown and maintained in continuous darkness, then the tip appears to be completely unnecessary for a response to gibberellin — the tip then can be completely replaced in the promotion of elongation by gibberellic acid. This is true with the 'Alaska' — a tall variety, but not when a dwarf variety is used.

Dr. Evenari: First of all, do you know anything about the action spectrum of this interesting high-intensity light effect? We know now, as far as germination is concerned, there is definitely a red-far-red low-energy, and a red-far-red high-energy process involved. And it would be very interesting to know what the action spectrum of your own high-energy effect is; that part which is not photosynthetic. Also is there any effect of a dark period? I mean, is there any difference between continuous light or interruption by dark periods.

Dr. Brian: I'll take your second point first. So far as we have investigated it, the dark period has no effect except that it isn't light. We've only just started work on the spectral effects and I really can't make any useful comment on this at all.

Dr. Gordon: Considering the irradiances employed, may I suggest an alternative explanation for the light effect you observed? Your own experiments have demonstrated the interaction between

auxin and gibberellin in increasing tissue extension. Now, similar irradiances will increase the ability of legume shoots to convert tryptophan to indoleacetic acid. Light will even more markedly enhance the ability of *Coleus* roots to form auxin or indoleacetate from tryptophan. Could we not infer, therefore, that light caused an enhanced auxin biosynthesis in your experimental material, and that you were simply measuring the interaction between the two growth substances?

Dr. Brian: I don't think that explanation is possible because the effect of light on sections not supplied with exogenous auxin is very small, and the effect of light is greatest when you have optimal exogenous auxin. But even when you've gone beyond the optimal of exogenous auxin so that growth is not quite as good as it would be with a somewhat lower concentration, light still has an increasing effect. I don't think, therefore, that the production of further auxin in the tissue itself could explain the results.

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Inhibition of Gibberellin Action by Auxin

In 1956 we started a search for a simple, yet specific, test for gibberellins; something comparable to the section test for auxins. We found it in the *Avena leaf base section test* and first reported on it at the Stanford AIBS meetings (3). In this test, a section of the basal portion of the coleoptile which includes the enclosed embryonic shoot is taken. It is this shoot which emerges from the coleoptile as a response specific to gibberellin. We have repeated this test more than 100 times, always with the same general results: gibberellin promotes growth, auxins inhibit the growth of this shoot, while kinetins promote it very slightly. In the beginning the variability of the growth of the individual sections was considerable, but gradually we have learned to reduce this to a minimum.

TECHNIQUE OF THE LEAF BASE SECTION TEST

Planting and Preparation

Wooden flats such as are used in nurseries are filled with approximately 2 inches of rather coarse Vermiculite, covered with a tin lid, and steam sterilized at 100° C. for at least 3 hrs. One-half pint of 'Kanota' oat seeds are placed in a one-quart jar, 5 to 10 mg. of Ceresan is added, and the jar is filled with water. The seeds are soaked for 1 hr. The oats are drained and spread on the sterile Vermiculite. The flat is then soaked with water, covered with the tin lid, drained, and put to germinate at 30° C. for 24 hrs. After germination, the seeds are covered with 0.5 inch sterile Vermiculite, saturated with water, the lid replaced, and the flat drained. With the lid still on, the flat of oats is taken to a physiological darkroom (27° C. and 80 to 90 per cent relative humidity). Here the lid is removed and the flat placed in a

dark chamber. The oats are grown in the dark, interrupted only by short periods of red light from a neon tube of ruby red glass.

The Test Solution

Solutions are prepared prior to cutting the sections. The stock solution contains 2 per cent sucrose and 0.02 *M* phosphate buffer (Na_2HPO_4 and KH_2PO_4). Double-strength regulator solutions are made so that equal parts of it can be added to the buffer to give a final concentration of 1 per cent sugar and 0.01 *M* buffer at a pH of 6.2. One ml. of buffer and 1 ml. of test solution thus give the final 2 ml. of solution in which the sections are placed.

Preparation of Beakers

Fifty-ml. beakers are heat sterilized in paper bags, as are the watch-glasses which later will serve as covers. The beakers and cover glasses are stored in the bags and remain sterile for some time. The test solutions are placed in the beakers and kept covered with the watch-glasses. No special effort is made to sterilize the solutions themselves.

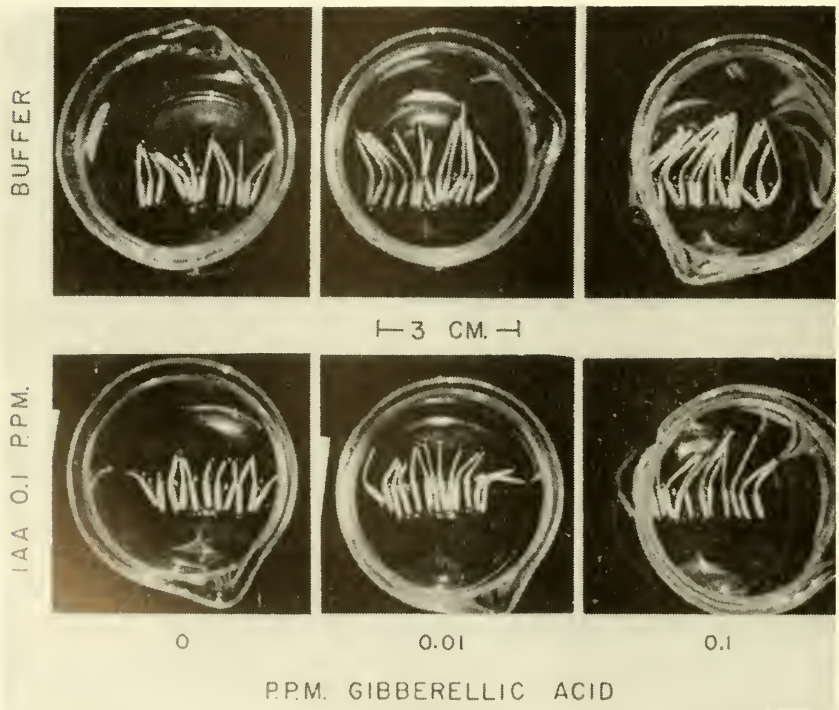

Fig. 1. *Avena* leaf section test. The leaf is shown as it grows out of the basal 5 mm. of the coleoptile. Duration of the test: 48 hrs.

The Test

On the fifth day the oats are ready for use. The length from the coleoptile tip to the node is approximately 20 to 30 mm. Twenty-four hrs. prior to use, the flat is jarred gently in order to pack the Vermiculite and prevent the oats from growing crooked. Seedlings with a coleoptile length between 20 and 30 mm. are selected in red light for the test. The shoots are cut at the base and floated in a dish of water. After all the shoots necessary for a test have been selected and placed in the water, a 5-mm. section is cut from the base of each coleoptile by means of two parallel razor blades mounted in a special holder. The section includes the coleoptilar node, and the lower cut is made just below it. The shoots are laid on a piece of glass under which is placed a paper with a line on it (Figure 1). The node is placed on the line and the lower razor blade is lined up with this line as a cutting guide. New razor blades are always used, as dull blades may injure the leaf, causing it to grow out crooked from the coleoptile. Immediately after cutting, the sections are floated in a dish of distilled water and held there until they are ready to be put into the final test solutions. When a sufficient number of sections has been cut, they are drained through cheesecloth and, using the cheesecloth as a sack, they are dipped 10 to 15 times in a 0.2 per cent Clorox solution (100 p.p.m. active chlorine). The Clorox is not rinsed off. Without this method of

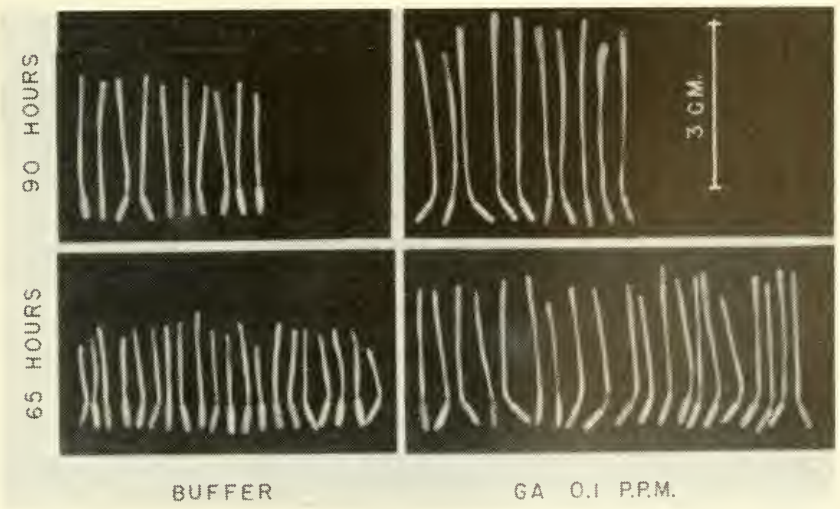

Fig. 2. Basal 5 mm. coleoptile sections of *Avena* with leaves growing out of them. Leaf growth is promoted by the addition of low concentrations of gibberellic acid to the basic medium of phosphate buffer and sucrose. The sections floated in 2 ml. of solution on a shaker in the physiological darkroom at 27° C. and were occasionally exposed to red light for observation.

disinfection, it was found that microorganisms will grow on the leaves, and when this happens, growth is abnormal and inhibited. The addition of a low concentration of isopropyl alcohol to the test solutions has also been used to keep them relatively free of interfering microorganisms. The Clorox-dipped sections are then placed in 50-ml. beakers containing 2 ml. of test solution. The beakers are covered with watchglasses and placed on a slow shaker for 48 hrs. All of these operations have taken place in the physiological darkroom (Figure 2).

The objective of this new test was not so much to assay extracts of plant material for their gibberellin content, but rather to provide a convenient means of studying gibberellin action. We want to report here on a striking inhibition of the effect of gibberellic acid (GA) by other regulators, specifically indole-3-acetic acid (IAA) (Figure 3). Because inhibition of kinetin by auxin has been reported in the literature (5), kinetin was included in some of the tests. As the results in Figure 4 show, kinetin does inhibit the effect of gibberellic acid at the same low concentrations at which indole-3-acetic acid is effective. While IAA by itself inhibits growth, kinetin alone appears to promote growth.

When low concentrations of indole-3-acetic acid are applied together with gibberellic acid, the effect of the latter is much suppressed (Figures 3 and 4). The entire GA curve is dropped. This in-

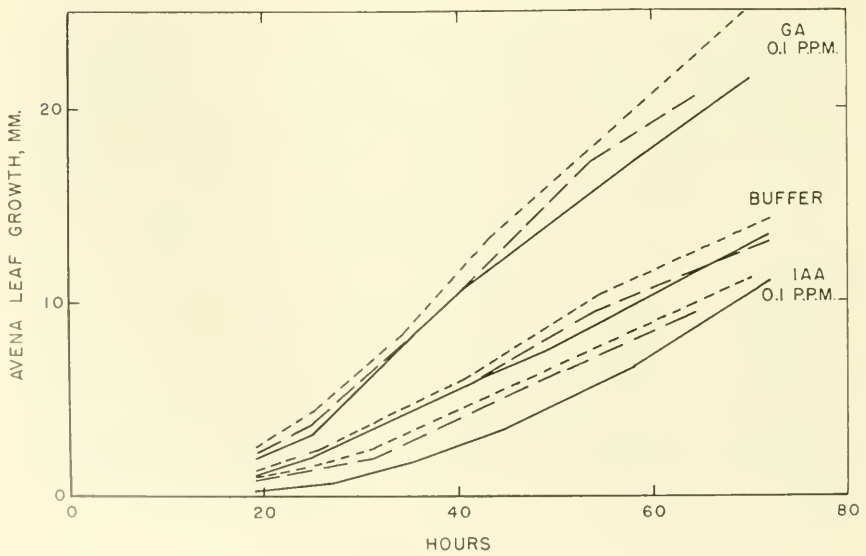

Fig. 3. *Avena* leaf growth from basal coleoptile sections as influenced by low concentrations of gibberellic acid (GA) and indoleacetic acid (IAA). Gibberellic acid promoted leaf growth, while indoleacetic acid inhibited it. Three tests were run on consecutive days; each line is the average of 24 sections.

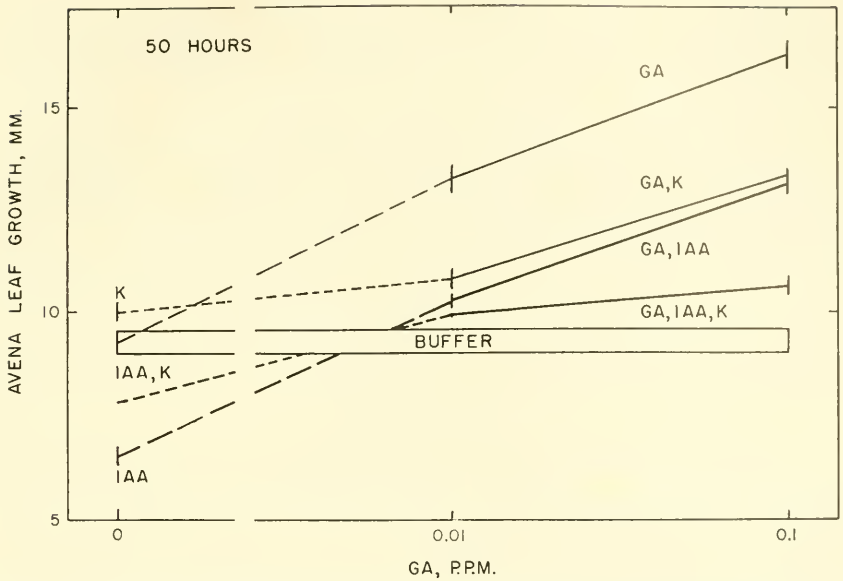

Fig. 4. Interaction of hormones demonstrated in the *Avena* leaf section test. Averages of 7 tests comprising 150 sections for each value plotted. Vertical lines: magnitude of error. Duration: 50 hrs. Gibberellic acid (GA, 0.01 and 0.1 p.p.m.) promoted leaf growth. The addition of kinetin (K, 0.1 p.p.m.) and of indoleacetic acid (IAA, 0.1 p.p.m.) suppressed the GA-induced growth. Addition of a mixture of IAA and K (both at 0.1 p.p.m.) suppressed the GA effect even more, so that growth in a solution with all three regulators was nearly identical to that of the buffer in sugar solution without regulators.

hibition of gibberellin by auxin has been found in a large number of tests, although the magnitude of the inhibition was variable. If this phenomenon of gibberellin action inhibited by auxin is a general one, it has some interesting consequences (2).

Bud Inhibition

The promoting agent here is gibberellin and the inhibiting agent, auxin. This was proven by the work of Kato (1).

Fruit Drop

If one assumes that certain stages of fruit growth are gibberellin dependent, it follows that auxins will inhibit this. This interrelation was demonstrated on cotton by Walhood (4). It also explains why auxin enhances the June drop of apples.

Root Growth

In the past it had been assumed that elongation of roots is auxin dependent (2). Suppose it were gibberellin dependent — and there is

nothing in the literature that would disagree with such a view — then the well-known inhibition of root elongation by auxins could easily be explained.

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DISCUSSION

Mr. Barlow: We have used a leaf base test similar to that described by Dr. van Overbeek. It was a slight modification of the test published by Margaret Radley (Ann. Bot., N.S. 22: 297. 1958) in which the wheat leaf base is used instead of the *Avena* leaf base. We didn't observe this depression of GA stimulation by IAA. I'm wondering if it is partly due to the age of the coleoptile which is used. Radley recommended rather old, long, coleoptiles and used a basal 1-cm. section. In that case, of course, the coleoptile section itself doesn't extend, but the leaf grows considerably. I also noticed in your photographs that the leaf only seemed to grow out of one end of the section. In our experience, the leaf expands on both sides, leaving the coleoptile section in the middle. Could you tell me if the growth of that leaf is due primarily to enlargement of the cells, or is it due to cell division? I have had a very brief look at this and, as far as I could see, most of the increase in length was due to cell extension. Right at the base of the leaf, however, it became extremely difficult to say whether, in fact, the cells were growing (in Prof. Thimann's sense of the word growth) or whether in fact the leaf base was adding cells.

Dr. van Overbeek: I can answer part of those questions. We cut the section below the node; when we cut it above the node, the leaf grows out on both sides. We have found variability at times, and we thought that part of the answer was the age of the seedling. We tried different ages and still found this inhibition. In oat seedlings, age apparently is not the principal factor in the inhibition of GA stimulation by IAA. Whether the elongation was cell division or cell enlargement, I do not know. We do have the complete bud here, and since there is a certain amount of lag in growth response to GA, it is possible, although unlikely, that cell division is involved. I think it is growth in the sense of Thimann's elongation.

Dr. Brian: I would like to add that the test that Miss Radley uses is basically similar to this, although the plants are cut *above* the node and wheat is used instead of oats. Certainly we have never encountered any inhibition by IAA as you have suggested. We do regard it as an almost specific test for gibberellins, but occasionally we find that there is a response in leaf extension due to auxin. Although we use this technique in our work on screening for natural gibberellins, we always confirm the results by using one of the tests involving a genetic dwarf.

Other Plant Growth Regulators

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Séparation des Substances de Croissance d'Extrait de Maïs Immature

Les recherches qui font l'objet de cette note ont porté sur différents extraits de Maïs immature conservés dans l'éthanol à 50 pour-cent à la glacière.

Dans plusieurs publications (1, 4, 5, 6, 7), nous avons déjà exposé comment nous étions arrivés à la conclusion que les extraits de Maïs immature contenaient d'autres substances que les auxines déjà connues. Après avoir essayé différentes méthodes de séparation par solvants, par chromatographie, ou par dialyse, qui se sont montrées insuffisantes, nous avons eu recours à des séparations fondées sur les fonctions chimiques des corps obtenus dans ces extraits. C'est ainsi que nous avons utilisé les échangeurs d'ions synthétiques.

SOMMAIRE DES EXPERIENCES

Séparation en Trois Groupes

(A) L'extrait de Maïs immature, en milieu éthylique à 50 pour-cent est versé sur une colonne de résine échangeuse d'ions, polystyrène sulfoné de formule générale $R - SO_3H$, préparée par un traitement à l'acide chlorhydrique à 10 pour-cent, et rincée jusqu'à neutralité par de l'eau désionisée. Cet échangeur d'ions, sous sa forme acide, échange ses ions H^+ contre les cations métalliques et organiques qu'il fixe. L'extrait de Maïs traité est en particulier débarrassé de tous les cations organiques tels que les acides aminés et les petits polypeptides.

La résine est ensuite rincée. Les eaux de lavage sont ajoutées à l'extrait déjà traité. Une solution d'ammoniaque $2N$ est alors versée sur l'échangeur pour en libérer tous les cations organiques. Certains, tel l'arginine, ne sont élués qu'avec difficulté. Après évaporation sous vide entre 30 et 35° C. de cet éluat ammoniacal, les corps sont remis en

solution dans l'eau, et le volume ramené à celui de l'extrait initial. Ceci constitue le *Groupe A* qui contient tous les cations organiques de l'extrait de Maïs.

(B) Après ce traitement par échangeur acide, l'extrait de Maïs est versé sur une colonne de résine synthétique, polystyrène ammonium quaternaire, de formule générale $(R')_4$ N-OH, se comportant comme une base forte, préparée par une solution de soude à 4 pour-cent, et rincée jusqu'à neutralité. En passant sur cette colonne, l'extrait de Maïs, échange tous les corps présentant une fonction acide, même extrêmement faible, contre les oxhydriles de l'échangeur.

(C) Après passage de l'extrait de Maïs, la colonne de résine est rincée et les eaux de rinçage ajoutées au filtrat qui ne contient alors ni acide, ni base, mais seulement des substances ne présentant que peu ou pas d'activité chimique, dont des sucres (glucose, lévulose, saccharose), des lipides, et une ou plusieurs petites protéines solubles dans l'alcool éthylique à 50 pour-cent. Ce filtrat, appelé par nous "filtrat total" constitue le *Groupe C.*

L'échangeur basique est traité par une solution de soude à 4 pour-cent, contenant un nombre d'équivalents plus grand que ne l'exigerait la capacité totale de la colonne. L'éluat sodique est ensuite traité par un échangeur acide pour ôter toute trace de soude, et le filtrat acide constitue le *Groupe B.*

Étude de l'Activité de ces Trois Groupes

L'activité de ces trois groupes de substances A, B et C, a été essayée à l'aide de culture de tissu.

Les tissus de Topinambour révèlèrent que les trois groupes étaient actifs. Aucun, pris seul, ne montrait cependant une activité semblable à celle de l'extrait total, mais en ajoutant au Groupe A de l'acide indole-3-acétique on obtenait des résultats équivalents ou dépassant ceux atteints avec l'extrait de Maïs total. Le Groupe B, seul ou avec adjonction d'auxine synthétique, montrait une activité analogue à celle de l'auxine synthétique ajoutée sur le milieu. Enfin, le Groupe C ne semblait agir qu'en présence d'auxine.

Etudiés de cette manière, voici les coefficients d'accroissement obtenus en Table 1.

Il fallait trouver un essai biologique permettant de distinguer entre substances d'élongation et substances de division cellulaire. Pour celà, nous avons utilisé le "Test" mis au point par Jablonski et Skoog (3).

Ce test utilise le parenchyme médullaire de Tabac. En présence d'auxine ce tissus présente peu ou pas de divisions cellulaires, mais un allongement considérable des cellules qui provoque souvent un éclatement des tissus (Figure 1).

Table 1. Coefficients d'accroissement de l'activité de trois groupes d'extrait de maïs immature.

Témoin sans auxine	0,2
Témoin avec IAA ($2{,}8 \times 10^{-7} M$)	2,5
Extrait de maïs total (10 pour-cent)	6,8
Fraction A seule (10 pour-cent)	6,6
Fraction A (10 pour-cent) + IAA	7,1
Fraction B seule (10 pour-cent)	4,6
Fraction B (10 pour-cent) + IAA	4,9
Fraction C seule (10 pour-cent)	1,3
Fraction C (10 pour-cent) + IAA	5,2

Le Groupe A, même seul, agit comme une substance de division cellulaire. Le parenchyme médullaire de Tabac cultivé en présence de ce groupe de substances répond par des divisions cellulaires intenses au niveau des deux ou trois premières assises de cellules. Les cellules néoformées sont beaucoup plus petites que les cellules préexistantes du parenchyme dont souvent elles remplissent la cavité. L'adjonction d'auxine synthétique augmente l'activité de cette fraction, mais ne semble pas augmenter beaucoup la grandeur des cellules formées qui donne un tissu très ferme.

Fig. 1. Cultures de Moelle de Tabac.
A — Témoin sans auxine. **B** — Témoin + IAA $1{,}5 \times 10^{-6}$. **C** — Fraction cathodique du Groupe A. **D** — Fraction du Groupe A, non fixée par un échangeur basique fort. **E** — Fraction du Groupe C dialysée non fixée par la Dowex 21 K. **F** —Fraction du Groupe C fixée par la Dowex 21 K. **G** — Fraction du Groupe A de pHi $\geqq 7$. **H** — (même fraction que J). **I** — Sac dialysé + IAA $1{,}5 \times 10^{-6}$. **J** — Sac dialysé sans auxine. **K** — Dialysat + IAA $1{,}5 \times 10^{-6}$. **L** — Dialysat sans auxine.

Le Groupe B montre, au contraire, une activité analogue à celle de l'auxine synthétique : le tissu de moelle de Tabac cultivé en présence de cette fraction réagit par des élongations cellulaires et peu ou pas de division cellulaire. Le tissu formé est gorgé d'eau et extrêmement fragile.

Le Groupe C qui se montrait peu actif, seul, avec les tissus de Topinambour, manifeste avec le test de Skoog une activité de division cellulaire très grande.

Les cellules sont petites, par rapport à celles du parenchyme initial, mais plus grandes que celles formées en présence du Groupe A. Les tissus prolifèrent abondamment avec ou sans adjonction d'auxine synthétique.

Après ces résultats, nous ne nous sommes plus occupés du Groupe B qui semble ne contenir que des auxines naturelles dont nous connaissions déjà la présence dans nos extraits. Il se peut du reste que le traitement par les échangeurs basiques forts de ces substances, entraine la destruction partielle des plus fragiles d'entre elles.

Essais de Fractionnement du Groupe A

Poursuivant nos recherches, nous avons voulu une technique qui pourrait séparer les différents cations du Groupe A, en se basant toujours sur leur activité chimique. La chromatographie bi-dimensionnelle nous avait, en effet, montré qu'il y avait dans ce groupe plus de 20 substances positives à la ninhydrine. L'électrophorèse nous a semblé convenir parfaitement pour séparer ces corps. Dans un champ électrique donné continu, à un pH donné, les corps organiques du Groupe A se séparent suivant leur point isoélectrique. Nous avons mis en route une électrophorèse continue sous une tension de 400 v. et une intensité qui s'est stabilisée à 18 milliampères. Le tampon de pH 3,95, était constitué par un mélange d'acide acétique glacial et de pyridine : (acide acétique 200 ml. + pyridine 60 ml. pour 20 l.). L'arrivée des substances, sur le rideau de papier Whatman No. 3 MM, se faisait non au milieu, mais environ au quart de la largeur vers l'anode. Pour séparer 2 l. du Groupe A, l'électrophorèse a duré un mois. Les fractions étaient recueillies régulièrement et analysées par électrophorèse simple. Nous avons obtenu 28 fractions, mais en rassemblant celles qui contenaient les mêmes substances, nous les avons réduites à 7.

Le test de Skoog nous montra que l'activité du Groupe A se portrait du côté des substances les plus électropositives. De ce fait, nous pouvions éliminer tous les corps qui s'étaient portés vers l'anode, en particulier les acides aspartique et glutamique en quantité très importante.

A la suite de cette constatation, connaissant la capacité qu'ont les échangeurs ammonium quaternaire, base forte, de fixer presque tous

les acides aminés, à l'exception des plus basiques, comme l'arginine, le Groupe A a été traité par un de ces échangeurs. Le filtrat qui ne contenait plus que trois ou quatre substances réagissant à la ninhydrine, a montré une activité très nette. Donc, l'activité du Groupe A ne restait pas fixée sur l'échangeur basique fort. Une résine acide faible, du type général R — COOH qui fixe tous les corps ayant un point isoélectrique plus grand que 5 et pouvant être tamponnée, nous offrait des possibilités de séparation plus précise. Avec cette résine préparée par une solution d'acide chlorhydrique, 0,1*N*, on a traité le Groupe A. L'élution faite par l'acide formique, 0,1*N*, a donné différentes fractions traitées ensuite par une résine basique faible pour éliminer l'acide formique. La fraction active contient encore plusieurs substances positives à la ninhydrine, et aussi, tout comme la fraction active de l'électrophorèse, une substance donnant les réactions des bases puriques, mais ne correspondant, semble-t-il, à aucun corps connu de cette famille, par même à la kinétine.

Nos recherches se poursuivent en ce moment, mais nous savons déjà : que l'activité du Groupe A n'est pas due à l'ensemble des aminoacides qui s'y trouvent, ce qu'on aurait pu supposer d'après certains travaux sur les hydrolisats de caséine (2) ; que cette activité serait limitée actuellement à un groupe de 5 ou 6 substances de point isoélectrique compris entre 6 et 9, et serait peut-être dû à celle de ces substances qui donne les réactions des bases qu'on trouve liées aux acides nucléiques.

Essais de Fractionnement du Groupe C

Le Groupe C ne présentant aucune activité chimique, — à part ses sucres réducteurs — nous a obligés à recourir à un moyen physique de séparation : la dialyse. Ce groupe contient, nous l'avons dit, une ou plusieurs petites protéines. L'électrophorèse de ce groupe n'ayant donné aucun résultat pour le moment, la dialyse semblait convenir pour la séparation des petites molécules, les sucres en particulier.

Nous avons fait des dialyses de deux sortes, contre de l'eau distillée et contre de l'alcool. Nous avons mené ces opérations en chambre froide entre 0° et —3° C., avec agitation par agitateur magnétique. Chaque fois nous avons mis 100 ml., en présence de 8 fois 2000 ml.

(a) *Dialyse contre* H_2O. Lorsqu'on dialyse la fraction C contre de l'eau, par suite d'un phénomène de pression osmotique, l'eau pénètre dans le sac dialysé au point de tripler le volume de liquide initialement mis à dialyser. Par ailleurs, ce liquide se trouble et à la longue un précipité se dépose.

Le dialysat étudié par chromatographie isopropanol-eau et essayé avec le test mésocotyle de Nitsch et Nitsch (8) montre une zône active au Rf 0,80. Aucune réaction colorée n'a pu être obtenue. Si on traite le

dialysat par un échangeur d'ions basique exceptionnellement fort (Dowex 21 K), cette substance n'est pas fixée, alors que certains sucres semblent être fixés par cet échangeur, et on retrouve dans le filtrat la même substance au même Rf. Il ne peut être question d'un *ester*- car on sait que les échangeurs forts sont capables de scinder en leurs éléments des molécules extrêmement unies. D'après les résultats obtenus jusqu'ici avec le test de Skoog, on ne peut dire si cette substance active sur le test mésocotyle, est également responsable de l'activité de division cellulaire obtenue avec cette nouvelle fraction sur la moelle de Tabac. Tandis que les éléments retenus sur l'échangeur n'auraient qu'une activité de division cellulaire qui semble, en apparence, moins importante que la fraction non fixée par 21 K. Au cours de la dialyse passent dans l'eau des substances qui se montrent très actives. Mais, malgré un lavage abondant, puisque la partie dialysée est traitée par 8×20 fois son volume (c'est-à-dire que s'il y avait 2 g. de substance active dans 100 ml. au départ, il n'en resterait plus à la fin que 0,0008 μg.), le sac dialysé dans H_2O reste très actif du point de vue du test de Skoog, mais aucunement du point de vue du test mésocotyle. Le sac dialysé contre de l'eau de la manière qu'on vient de dire, et soumis ensuite à une dialyse contre de l'alcool à 50 pour-cent (5 fois 2000 ml.) :

1) retrouve le volume initial : la pression osmotique qui se manifeste dans l'eau ne se manifeste plus dans l'alcool;
2) le trouble disparait, les substances qui avaient précipité se trouvent remises en solution.

Ce dialysat alcoolique, qui succède donc à une très importante dialyse contre de l'eau, chromatographié dans isopropanol-eau, manifeste une activité pour le test mésocotyle au Rf 0,80 environ, comme pour les deux cas cités plus haut.

L'activité du sac demeure, mais semble nécessiter la présence d'acide indole-acétique pour se manifester d'une manière importante.

(b) *Dialyse contre de l'alcool à 50 pour-cent.* Les dialyses faites entièrement contre de l'alcool à 50 pour-cent donnent des résultats analogues. Dans ce cas, pourtant, la dialyse entraîne des substances lipidiques solubles dans l'alcool qui ne dialysent que peu ou pas, lorsque la dialyse est faite contre de l'eau. Le dialysat et le sac dialysé sont actifs sur le test de Skoog. L'activité du sac est renforcée par la présence d'auxine. L'évaporation à sec du sac dialysé :

1) contre de l'eau et 2) contre de l'alcool

laisse une matière sèche d'un poids assez considérable de l'ordre de 920 mg/l, alors que le dialysat alcoolique contient 100 mg. de substances qui n'ont jamais pu arriver à être dessèchées parfaitement, car il y avait proportionnellement beaucoup de substances lipidiques.

La matière sèche du "sac" est très hétérogène. Elle contient en particulier les matières protéiques résiduelles de l'extrait de Maïs.

RÉSUMÉ

Les substances du Groupe A quoique très nombreuses, semblent pouvoir être assez facilement séparées en s'adressant à leur fonction chimique et la substance active isolée de cette manière, serait une substance qui provoquerait la division cellulaire dans le cas du test de Skoog. Les cultures faites sur les fractions les plus cationiques du Groupe A qui seraient inactives sans auxines, manifestent une activité considérable avec l'acide indole-3-acétique.

Les substances du Groupe B sont déjà presque toutes connues ; elles sont du type "auxine", peut-être devrait-on y joindre les acides gibbérelliques.

Les substances du Groupe C qui provoquent une division cellulaire très active du parenchyme médullaire de Tabac, échapperaient à toute séparation chimique. Soluble dans l'eau, davantage encore dans l'alcool à 50 pour-cent, elles semblent être complexes : la dialyse ne permettant pas de faire une séparation d'activité. Nous ne pouvons dire encore s'il s'agit de plusieurs substances ou d'un complexe protidique qui libérerait peu à peu une substance active dialysable, d'ou viennent cette activité qui demeure d'une part du côté de la fraction protidique du Groupe C dans le sac dialysé et cette activité qui dialyse avec les glucides d'autre part. Il ne peut, en tout état de cause, *être question* d'acide indole-3-acétique ni de ses esters. Le traitement par échangeur basique extrêmement fort les aurait ou détruits ou fixés. Par ailleurs, le test de Skoog qui réagit en leur présence manifeste une activité de division cellulaire et non d'élongation, comme en présence d'auxine.

Notons qu'après le fractionnement du Groupe A, les cultures faites avec les substances de pHi > 6 et < 9 donnent des tissus ayant des cellules plus grandes et un tissu moins ferme que ce qu'on obtient avec le Groupe A non fractionné, et le Groupe C.

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Growth Substances and Plant Tissue Cultures

Because of the dramatic effect obtained by applying gibberellins to intact plants and the natural occurrence of at least one of these compounds in higher plants (5), it seemed desirable to study the effect of gibberellin in plant tissue culture systems where many environmental and growth variables may be controlled, and where many of the complicated influences of morphogenetic development in higher plants are not present. Furthermore, the percentage of cells that are meristematic (or at least nondifferentiated) is quite large compared with that in intact plants.

The present paper includes data from experiments with 49 strains representing 25 different species. The effects of various levels of the gibberellins, variations in the media used, type of tissue, source of the tissue, as well as physiological and pathological state of the tissues involved, are considered.

The main purpose of the present paper is to survey a large number of cultures to determine if any correlations can be made between the response of tissue cultures to gibberellin and characteristics of the tissues or the conditions under which they are grown, as well as to compare the resultant data with those obtained from intact plants.

MATERIALS AND METHODS

Gibberellin

The gibberellin used was Pfizer lot #76088, which is a mixture of gibberellin A and gibberellic acid (GA). Parallel tests were run in many cases with potassium gibberellate, with similar results. Solutions were made up at 10 times the highest test level, the pH ad-

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justed, and the solutions sterile-filtered through sintered glass. The amount necessary to give the desired level was added to the autoclaved agar medium just before solidification. Recent results in our laboratory, however, indicate a surprising stability of this mixture to heat. Only about one-third of the activity was lost after autoclaving for 15 min. at 15 lbs. pressure. The activity was measured in the dwarf pea test and with avocado tissue.

Media

Three synthetic media (White's, LP, and 24) and modifications of each were used to grow the varied types of tissues included in these tests. The medium (20 ml.) was added to 1 × 6 in. Pyrex test tubes, plugged with nonabsorbent cotton, and sterilized by autoclaving for 15 min. at 15 lbs. pressure. The pH level of all media was adjusted to between 5.0 and 6.0 before autoclaving.

For many tissues the media were supplemented by 2,4-D (6 p.p.m.) and coconut milk (18 per cent by volume). The coconut milk added to culture media is prepared by collecting the liquid from 100 mature coconuts. This pooled coconut milk is filtered, dispensed into flasks, autoclaved, and stored at 5° C. Before use, it is filtered to remove precipitated protein. In this processed form it is then added to media at 18 ml. per 100 ml. of medium, and re-autoclaved.

Tissue Culture Methods and Experimental Conditions

The inoculum for each test level was weighed and divided into 5 tubes. The final wet weight in the 5 tubes was divided by the inoculum wet weight to give an index of growth, termed the "growth value." This is the only term used in the present investigation to express increments of growth. Growth was in an air-conditioned culture room at 21° C. in diffuse light.

EXPERIMENTAL RESULTS

Dosage-response experiments were set up with gibberellin levels varying from 0.1 p.p.m. to 100 p.p.m., using several different kinds of tissues. The responses of the 4 tissues selected for this study were either stimulatory or depressive (Table 1). The *Rumex* (sorrel) virus tumor tissue was stimulated at 10 p.p.m., while the broad bean cotyledon tissue was stimulated from 0.5 to 5 p.p.m. *Taxus* pollen tissue was slightly depressed at 10 p.p.m., strongly at 100 p.p.m. Avocado cotyledon tissue was extremely sensitive, being strongly inhibited at 5 p.p.m., and killed at 10 p.p.m. (Figure 1A).

Because of the large number of tissues to be evaluated in the present work, it was necessary to decide on one test level of gibberellin

Table 1. Types of growth response of plant tissue cultures to various concentrations of gibberellin.*

Tissue	Gibberellin Level in P.P.M.						
	0	0.1	0.5	1.0	5.0	10.0	100.0
Rumex	4.0 4.6	... ...	... ...	5.4 4.1	... ...	6.1 5.8	2.4 2.0
Persea	5.2	5.4	5.2	4.9	2.0	dead	...
Taxus	7.2	...	...	7.0	...	5.5	4.0
Vicia	2.9	3.1	3.7	3.8	3.7	3.1	...

*Ratio of fresh weight at end of test over initial fresh weight.

for comparative purposes. In view of the results shown in Table 1 as well as similar experiments in this and other laboratories (3, 6, 7, 10), the level chosen was 10 p.p.m. A stimulatory or inhibitory effect at 10 p.p.m. indicates the general response of a tissue. However, a lack of response is not conclusive. Broad bean cotyledon is an example of a tissue which is stimulated at a level below 10 p.p.m. At 10 p.p.m. no effect is apparent, yet there is significant stimulation at 0.5 to 5 p.p.m.

The 49 tissues selected for inclusion in this paper represent 17 families and 25 species. The cultures came from diverse origins within the plants: pollen, root, stem, leaf, tuber, prop root, cotyledon, and petiole. The time in culture of these tissues varies from

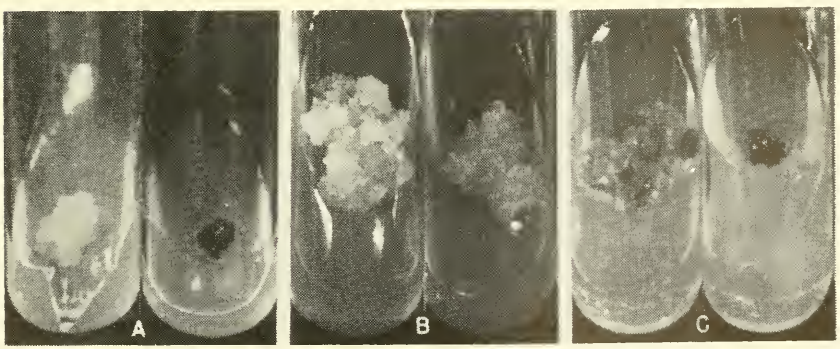

Fig. 1. **A.** The effect of gibberellin on the growth of a tissue culture from avocado cotyledon. **Left,** untreated; **right,** 10.0 p.p.m. gibberellin. **B.** Increased growth of avocado cotyledon tissue culture by removal of 2,4-D from the medium. **Left,** White's medium with coconut milk; **right,** the same medium with 6.0 p.p.m. 2,4-D. **C.** Necessity of 2,4-D for growth of 'Pontiac' potato tuber tissue in culture. **Left,** White's medium with coconut milk and 6.0 p.p.m. 2,4-D; **right,** the same medium without 2,4-D.

Table 2. Effect of gibberellin on growth of various strains of plant tissue cultures.

Classification	Plant Sources of Cultures: Latin Name	Common Name	Plant Part	Medium*
GYMNOSPERMS				
Ginkgoaceae	*Ginkgo biloba*	maidenhair tree	pollen	WMCM
	Ginkgo biloba	maidenhair tree	pollen	WMArg
Taxaceae	*Taxus* sp.	yew	pollen	W-2,4-D-CM
ANGIOSPERMS				
Monocotyledons				
Amaryllidaceae	*Agave toumeyana*	century plant	leaf	W-2,4-D-CM
	Agave toumeyana	century plant	seedling	W-2,4-D-CM
Dioscoreaceae	*Dioscorea composita*	Mexican yam	tuber	W-2,4-D-CM
Gramineae	*Zea saccharata*	sweet corn	prop root	LP-Ye-pCl
	Zea saccharata	sweet corn	root	W-2,4-D-CM
Dicotyledons				
Apocynaceae	*Vinca rosea*	periwinkle	stem	LP
Aquifoliaceae	*Ilex aquifolium*	holly	root	W-2,4-D-CM
Asclepiadaceae	*Stapelia* sp.	carrion flower	leaf	W-2,4-D-CM†
Cactaceae	*Opuntia monacantha*	cactus	stem	LP
	Opuntia monacantha	cactus	stem	LP
Euphorbiaceae	*Croton glabellus*	croton	root	W-2,4-D-CM
Lauraceae	*Persea americana*	avocado	cotyledon	W-2,4-D-CM
Leguminosae	*Melilotus officinalis*	yellow sweet clover	root	LP
	Melilotus officinalis	yellow sweet clover	root	LP
	Melilotus officinalis	yellow sweet clover	root	LP
	Melilotus officinalis	yellow sweet clover	root	LP
	Melilotus officinalis	yellow sweet clover	root	LP-Ye-pCl
	Melilotus officinalis	yellow sweet clover	root	W-2,4-D-CM
	Melilotus officinalis	yellow sweet clover	stem	LP-Ye-pCl
	Melilotus officinalis	yellow sweet clover	stem	LP-Ye-pCl
	Melilotus officinalis	yellow sweet clover	stem	24-Ye-pCl
	Melilotus officinalis	yellow sweet clover	root	24-Ye-pCl
	Phaseolus vulgaris	pole bean	hypocotyl	W-2,4-D-CM
	Phaseolus vulgaris	pod pole bean	stem	W-2,4-D-CM
	Phaseolus vulgaris	pinto bean	stem	W-2,4-D-CM
	Phaseolus vulgaris	pinto bean	root	W-2,4-D-CM
	Phaseolus vulgaris	pinto bean	cotyledon	W-2,4-D-CM
	Phaseolus vulgaris	bean	stem	W-2,4-D-CM
	Phaseolus vulgaris	bush bean	cotyledon	W-2,4-D-CM
	Pisum sativum	garden pea	cotyledon	W-2,4-D-CM
	Pisum sativum	garden pea	root	W-2,4-D-CM†
	Vicia faba	broad bean	cotyledon	W-2,4-D-CM
	Vigna sinensis	black cow pea	root	LP-Ye-pCl
Malvaceae	*Hibiscus syriacus*	Rose-of-Sharon	root	W-2,4-D-CM
Polygonaceae	*Rumex acetosa*	sorrel, dock	root	24
Portulacaceae	*Portulaca oleracea*	pigweed	stem	W-2,4-D-CM†
Rosaceae	*Rosa*	'Paul's Scarlet' rose	stem	W-2,4-D-CM
	Rosa	'Better Times' rose	stem	W-2,4-D-CM
	Rosa	'Better Times' rose	petiole	W-2,4-D-CM
Solanaceae	*Datura stramonium*	thorn-apple	stem	W-2,4-D-CM
	Nicotiana tabacum	tobacco	stem	LP
	Nicotiana langsdorfii X glauca	tobacco hybrid	stem	WMCM
	Solanum tuberosum	potato	tuber	W-2,4-D-CM
	Solanum tuberosum	potato	tuber	W-2,4-D-CM
Compositae	*Helianthus annuus*	sunflower	petiole	LP
	Helianthus annuus	sunflower	stem	LP

* W = White's basal medium (13); WM = White's basal medium with 10X KH_2PO_4 (11); CM = coconut milk (20% by volume); Arg = l-arginine HCl, 100 p.p.m. (11); 2,4-D = 2,4-dichlorophenoxyacetic acid, 6 p.p.m.; LP = modification of medium 24 with low phosphate, 1

Comments	Response to Gibberellin			
	No. of Expts.	Growth Value**		Per Cent Change from Control
		Control	Gibberellin (10 p.p.m.)	
Culture 81056	4	8.6	10.2	+20
Culture 5857	3	3.1	4.2	+35
Culture from C. D. LaRue	1	7.2	5.5	−25
Winkleman culture	3	3.1	2.7	−13
Superior culture	1	2.4	1.4	−42
	2	3.2	3.1	− 3
'Golden Cross Bantam' with corn stunt virus	1	3.6	3.6	0
'Golden Cross Bantam'	1	2.4	1.5	−35
crown gall	1	5.1	4.1	−20
	2	22.5	19.5	−14
	1	1.2	1.0	−17
Red pigment, crown gall	3	3.3	dead	dead
No pigment, crown gall	1	1.8	1.3	−13
	1	8.0	5.9	−26
	2	6.2	dead	dead
Chemically induced, habituated	6	3.4	3.7	+17
C_{10} crown gall (white)	1	3.0	3.7	+23
C_{10} crown gall (green)	2	1.6	1.4	−10
C_{10} crown gall (differentiated)	1	2.3	3.1	+34
	1	16.8	16.4	− 3
	2	4.2	4.9	+12
C_{10} crown gall	1	14.3	12.2	−15
C_{10} chemically induced	1	7.7	5.8	+ 3
C_{10} virus tumor	1	1.9	3.5	+84
C_{10} virus tumor	1	5.0	5.6	+12
	4	22.7	19.1	−16
	1	11.1	10.6	− 5
	2	15.5	16.7	+ 8
	2	19.0	15.0	−21
	2	20.0	20.0	0
'Golden Pod'	1	16.0	14.2	+12
'Golden Wax'	1	20.8	10.1	−56
	2	3.9	2.8	−28
'Dwarf Progress'	2	2.4	2.2	− 8
	4	2.8	3.6	+28
	1	2.6	2.6	0
	1	9.9	5.0	−50
virus tumor	4	4.7	5.7	+18
	1	6.2	5.0	−19
	2	15.4	9.2	−40
	1	12.8	5.6	−57
	1	7.2	6.0	−17
	3	9.1	7.1	−22
crown gall	6	4.2	4.9	+12
Hybrid	1	15.0	15.0	0
'Pontiac' (red)	5	8.4	8.9	+ 6
'Katahdin' (white)	3	3.7	4.2	+14
crown gall	5	14.6	7.3	−50
crown gall	1	13.8	8.2	−41

millimole (8); 24 = synthetic medium for virus tumors with high phosphate, 8 millimole (1); Ye = Mead-Johnson yeast extract, 5 mg/l; pCl = parachlorophenoxyacetic acid, 6 p.p.m.

** Ratio of fresh weight at end of test over initial fresh weight.

† 0.6 p.p.m., 2,4-D.

rather recent isolates to tissues which have been maintained in vitro for 15 years. The media used include both synthetic and supplemented types. The methods of induction that led to the establishment of these strains include hormonal, crown gall, virus, and genetic.

The results of a large number of experiments incorporating 10 p.p.m. of gibberellin in the nutrient media are shown in Table 2. These show that, while a few tissues are increased in their growth and others show no response, the majority are retarded. For example, all three strains of rose tissue were retarded; avocado cotyledon and the pigmented cactus stem crown gall were killed.

The five strains of tissue from monocotyledonous species were either reduced or showed no effect. These strains are from three different families and represent leaf, seedling, and root cultures.

Severe depression of tissue growth was noted for two crown-gall tissues of sunflower, one from the stem and the other from the petiole. Growth values at 10 p.p.m. gibberellin were approximately one-half the control values. The results of five experiments with petiolar crown-gall tissue of sunflower are presented in Table 3. Variations in growth among these experiments are due principally to differences in the physiological state of the inocula. Nevertheless, the response obtained, in this case depression, is consistent. Of the other crown-gall tissues tested, some were depressed (*Vinca* and *Melilotus* stem). Only two were stimulated to any appreciable extent (*Melilotus* root crown gall, white and differentiated), and these responses were not so striking as the depressions.

Growth of three strains of tissue from the Leguminosae was increased by gibberellin; *Vicia faba* (broad bean) cotyledon tissue was

Table 3. The variation in growth of *Helianthus* petiole crown-gall tissue between experiments. All experiments run for 4 weeks on LP medium.

Initial Weight, Mg.		Final Weight, Mg.		Growth Value*	
Control	Gibberellin	Control	Gibberellin	Control	Gibberellin
275	280	4,250	2,680	15.4	9.5
230	245	2,060	1,070	13.1	6.5
245	260	1,795	880	7.3	3.1
245	210	5,755	2,715	23.4	13.5
215	215	2,975	755	13.8	4.1
Average growth value of five experiments				14.6	7.3

* Ratio of fresh weight at end of test over initial fresh weight.

most responsive. Growth of eight strains from this family was reduced; bush bean cotyledon tissue by as much as 50 per cent of the control growth.

The four fastest-growing cultures were all inhibited. These are holly root, pole bean hypocotyl, pinto bean root, and bush bean cotyledon. Of the slowest-growing cultures, sweet clover virus stem tumor was stimulated, while sweet clover root crown gall, the nonpigmented cactus stem crown gall, and *Stapelia* leaf tissue were slightly inhibited.

Over the last several years, we have found a few cultures that change somewhat in appearance. These "variants" have been segregated and maintained as separate cultures. Since they are morphologically distinct from the parent culture, they were tested to determine their growth response to added gibberellin. *Melilotus* root crown gall (white and green) and *Opuntia* stem crown gall (red and white) show that the variants respond differently to gibberellin from the parent cultures. Whether these "variants" are true somatic mutations, merely segregants from mixed cell populations, or represent other phenomena is not known.

Because of the possibility that the auxin-gibberellin relationship in specific tissues might play a part in their response to gibberellin, two types of experiments were set up. In the first type, two tissues were selected that grow on a synthetic medium (LP) with no added auxin. One tissue (tobacco stem) is stimulated by added gibberellin, whereas the other (sunflower petiole) is inhibited. Both tissues are crown galls. In the second type, two tissues were used that were maintained on a medium supplemented with 2,4-D and coconut milk. The growth of one of these was promoted by added gibberellin (broad bean cotyledon), the other was killed (avocado cotyledon). These two tissues are both normal.

Before discussing the results of these experiments, it is well to point out the effect on growth of the removal of 2,4-D from the medium. The growth of avocado is increased by removal of 2,4-D (Figure 1B). 'Pontiac' potato tuber tissue, on the other hand, dies when 2,4-D is removed from the medium, even on the first subculture (Figure 1C). Similar results are obtained with holly and yam tissues.

The results with the two crown-gall tissues are shown in Table 4. Tobacco, which is stimulated by gibberellin at 10 p.p.m., is inhibited by 2,4-D at 1 p.p.m. The addition of both substances causes an intermediate response. Sunflower, which is inhibited by gibberellin at 10 p.p.m., is also inhibited by 2,4-D, but to a greater extent. Both substances together give the same effect as 2,4-D alone.

The responses of the two normal tissues to gibberellin and 2,4-D

Table 4. The effect of gibberellin and 2,4-D on the growth of tobacco and sunflower crown-gall tissues in LP medium.

Additive	Concn., P.P.M.	Growth Value*	
		Tobacco stem	Sunflower petiole
None	..	5.1	47.3
GA	10	6.2	14.5
GA and 2,4-D	10 1	3.7	2.2
2,4-D	1	2.0	2.2

* Ratio of fresh weight at end of test over initial fresh weight.

are presented in Table 5. Avocado is killed by gibberellin alone or in the presence of 2,4-D, and its effect is apparently independent of the level of 2,4-D used. The growth of broad bean is increased by gibberellin. This effect is found in the presence or absence of 2,4-D.

DISCUSSION

The growth response of intact plants to applied gibberellins is characterized by high sensitivity and considerable variability from one type of plant to another. Similar responses of plant tissue cultures are also observed in the presence of gibberellins. No strict correlation was found between the response to applied gibberellin and the inciting agent of tissue growth, the age of the culture, the type of medium, or the plant part from which cultures were obtained. Netien (6, 7) and Henderson (3) found the gibberellins inhibitory to the growth of plant tissue cultures of *Scorzonera, Daucus, Rubus, Helianthus tuberosus,* and *H. annuus.* Schroeder and Spector (10), on the other hand, obtained a significant growth increase at 5 to 25 p.p.m. gibberellin with fresh explants of the mesocarp of *Citrus medica.*

The results from the pollen tissues of *Taxus* and *Ginkgo* are in agreement with the work of Chandler (2), who found that the pollens of some plants were stimulated in germination and tube elongation, while other pollens were retarded. Growth of the *Ginkgo* pollen tissue was increased by the addition of 10 p.p.m. gibberellin to the basal medium; this was true for two strains of the tissue. *Taxus* pollen tissue, on the other hand, was retarded.

An interesting observation is the effect of gibberellin on the color of all bean cultures. As is shown in Table 2, there are bean cultures that fall into each category: some are stimulated, some depressed, and

some not affected in their growth. However, the color of all the bean cultures is darkened, from white to light yellow, from light yellow to dark yellow, from dark yellow to brown, depending on the color of the untreated tissue. Apparently there is an effect on one or more enzyme systems involved in pigment formation that is unrelated to the growth effects.

Radley and Dear (9) have shown that coconut milk contains gibberellin-like substances as determined by the dwarf pea test. This is mentioned because many of the tissues used in the present work were grown on media containing coconut milk. The levels reported by Radley and Dear are for concentrated coconut milk. When the gibberellin-like activity of unconcentrated coconut milk is calculated from their data, the level is below the amount necessary to obtain a growth response by a standard dwarf pea test. This level is also below that to which the tissues respond.

It is possible that the gibberellin response of plants is related to the auxin state of the particular plant, plant organ, or tissue to which it is applied. Vlitos and Meudt (12) have emphasized this by contrasting the responses obtained with intact plants and isolated plant parts. Intact plants respond more to applied gibberellins, whereas the auxin response is exaggerated in isolated plant parts. In addition to the effect of isolation from the parent plant, the tissue culture responses discussed here are obscured somewhat by the presence of plant hormones in many of the media. Moreover, the tissues on media that lack such hormones are, with one exception (*Melilotus* root,

Table 5. The effect of gibberellin and 2,4-D on the growth of avocado and broad bean tissue cultures in White's medium plus coconut milk.

Additive	Concn., P.P.M.	Growth Value*	
		Avocado	Broad bean
None	....	4.8	3.7
2,4-D	0.6	3.6	3.1
2,4-D	6.0	1.6	2.5
GA	10.0	0.9	4.5
GA and 2,4-D	10.0 0.6	0.8	3.5
GA and 2,4-D	10.0 0.6	0.9	3.3

* Ratio of fresh weight at end of test over initial fresh weight.

chemically induced, habituated), all crown-gall or virus tumor tissues, and the latter have been shown by Kulescha (4) to have endogenous levels of auxin much greater than homologous normal tissue.

The results with tissues cultured on media containing gibberellin and/or 2,4-D suggest that these two substances operate separately. This suggestion is best supported by the results with the crown-gall tissues, normally grown on a synthetic medium without added auxin. Added gibberellin causes a 20 per cent increase in the growth of tobacco crown-gall tissue and more than a 60 per cent decrease in the growth of sunflower crown-gall tissue, whereas the addition of low levels of 2,4-D reduces the growth of both tissues.

SUMMARY

Forty-nine strains of tissue from 25 species of plants have been tested for their growth response to gibberellin at 10 p.p.m. in tissue culture. Depression of growth is the most pronounced effect, but the growth of several tissues is promoted. Probably the most interesting effect is the lethal action of gibberellin on two of the cultures. As a group, monocotyledonous tissues were depressed in growth. There is no apparent correlation between response to gibberellin and characteristics of strains of tissue such as agents inducing proliferation, plant parts from which the tissues were obtained, age of the cultures, or media supporting tissue growth. Tests with 2,4-D and gibberellin added singly and together suggest that these two growth substances act separately.

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Growth Factors in the Tomato Fruit[1]

The time at which one of us (J.P.N.) first became interested in the juice of tomatoes as a source of growth factors goes back to January, 1949, when an effort was made to grow excised ovaries in sterile culture. At the suggestion of Mr. E. F. Vacin, who had been using tomato juice (TJ) to improve the growth of orchid seedlings, tomato juice was added to the media which were to receive excised tomato ovaries. This resulted in the successful development of tomato fruits in sterile culture (20, 21, 22). Tomato juice was tried, then, on standard strains of tissue cultures, namely crown-gall strains of *Helianthus annuus* (De Ropp's PIII), of *Nicotiana tabacum* (Morel's), and of *Opuntia monacantha* (Morel's). In all these cases, after an initial slow start, growth burst into vigorously proliferating cultures (23). The fact that none of the strains used required auxin to grow indicated at once that one was dealing here with a factor quite different from an auxin. A more extensive study of this factor was indicated. It is clear from the literature that other workers also observed biological effects resulting from the addition of tomato juice.

PHYSIOLOGICAL EFFECTS OF TOMATO JUICE

Stimulation of Growth in Microorganisms

Some 20 years ago, tomato juice was a popular source for "bios" factors for bacteria, yeasts, etc. (see, e.g., 4, 18, 19). Interest in the "bios" substances faded, however, as they were progressively identified

[1] A part of the work reported here was supported by the National Science Foundation (Grant G-4046) and the National Institutes of Health, Bethesda, Md. (Grant RG-4840).

as vitamins, amino acids, and other chemically-defined factors. In 1942 tomato juice was still reported to contain an unidentified growth factor for hemolytic streptococci (6), and the possibility remains that there are, indeed, unidentified growth factors for microorganisms in tomato fruits.

Inhibition of Seed Germination

In any event, one of the effects of tomato juice which became known first was its inhibitory effect upon seed germination. After the early reports of Oppenheimer (30) and Reinhard and Gorelik (36), Köckemann (11) extended this type of investigation to other fruits and gave a name, blastocholine, to the principle present in the juice of many fleshy fruits which inhibits the germination of seeds. Larsen (14) found that the extract of ripe tomatoes contained a complex of substances which were inhibitory in the *Avena* curvature test; he could divide the ether extract into three fractions, each of which had inhibitory properties. Ozorio de Almeida *et al.* (31), on the other hand, reported that the factor inhibiting the germination of tomato seeds was insoluble in ether or chloroform, but soluble in alcohol, whereas Sartory and Meyer (37) found that the ether or chloroform extracts of tomatoes inhibited the germination of *Lepidium* seeds. Konis (12) observed that the inhibitory effect of tomato juice disappeared at low concentrations, under which circumstances, on the contrary, a promoting effect became visible. He thought that the inhibitor was a volatile substance, which could be partially destroyed by boiling.

Stimulation of the Growth of Immature Embryos

Tomato juice has been reported to stimulate the growth of very young embryos of *Hordeum* (9). Its effect could be more or less duplicated by casein hydrolysate or sodium nucleate. Meyer (17) and Vacin and Went (42) added tomato juice to media prepared for the asymbiotic development of orchid seed in sterile culture, which resulted in a marked stimulation of growth. The stimulative effect of tomato juice upon immature embryos is reminiscent of that of coconut milk (43, 44).

Growth of Excised Ovaries

The autoclaved aqueous extract of both green and ripe tomatoes was found to stimulate the growth of excised tomato ovaries which had been treated with 2-naphthoxyacetic acid to stimulate parthenocarpy (24), as shown in Figure 1A. Confirmation of these results was reported with the use of a different, nonsterile technique (16).

Growth of Roots

Roots often form on the pedicels of tomato ovaries cultivated in vitro. The tomato juice which was added to the sterile medium was

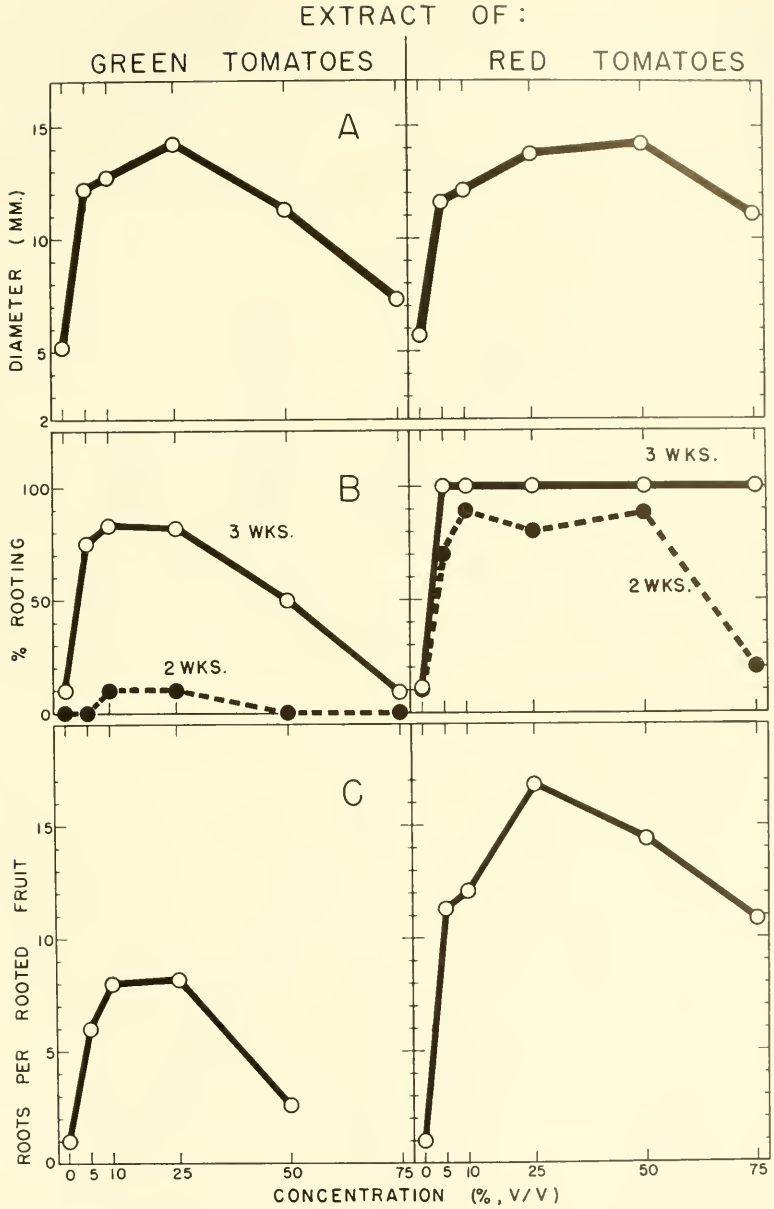

Fig. 1. Effects of juice of green *(left)* and red *(right)* tomatoes *(Lycopersicon esculentum)* on the following processes: **A**: growth of excised, unpollinated tomato ovaries planted on artificial media at bloom, after having received 1 drop of an aqueous solution containing 100 mg/l of 2-naphthoxyacetic acid; measurements after 9 weeks. **B**: percentage of peduncles of the above fruits which formed roots after 2 and 3 weeks. **C**: average number of roots produced per rooted fruit after 3 weeks. Each point is the average of 5 to 10 replicates. (From 24)

found to stimulate the development of these roots in an astonishing manner. Sucrose at a concentration of 5 per cent generally inhibits the elongation of adventitious roots produced on the floral peduncles, as contrasted with lower concentrations which allow perfect growth of the same primordia. The addition of tomato juice to the medium, however, allowed roots to develop profusely, even though the sucrose concentration was 5 per cent (24), as shown in Figure 1, B and C.

Growth of Tissue Cultures

Investigations over a period of years with various strains of tissue cultures showed that tomato juice stimulated the growth of normal tissues, tissues habituated to auxin, crown-gall tissues, and also tissues derived from pollen or endosperm.

Crown-gall tissues. Since crown-gall tissues are known to grow well without any added auxin, no synthetic auxin was added to the media. Under these conditions, De Ropp's PIII strain of crown-gall tissues of *Helianthus annuus* and Morel's crown-gall strains of *Nicotiana tabacum* and *Opuntia monacantha* (23) and *Parthenocissus tricuspidata* (3) all responded to the addition of tomato juice to the medium by an increase in fresh weight up to five times that of the controls, for the optimum concentration of 10 per cent (v/v) of TJ (Figure 2). Gau-

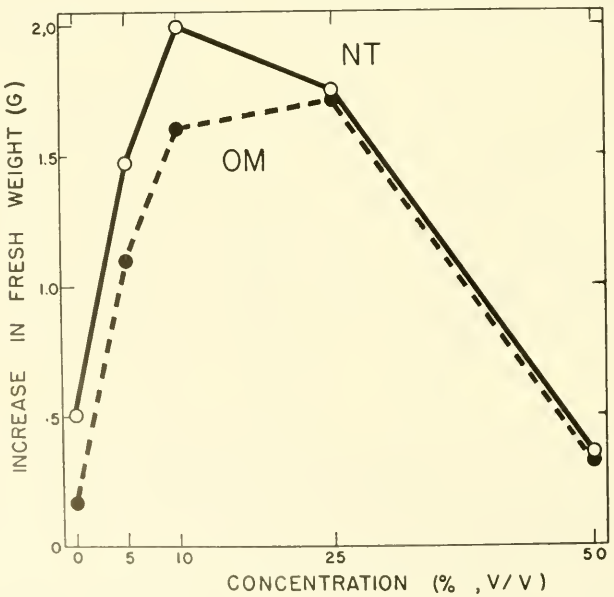

Fig. 2. Increase in fresh weight on addition of tomato juice to tissue cultures of Morel's crown-gall strain of *Nicotiana tobacum* (NT) and *Opuntia monacantha* (OM), after 5 weeks and 9 weeks, respectively. Each point represents the average of 6 (NT) or 10 (OM) replicates. (From 23)

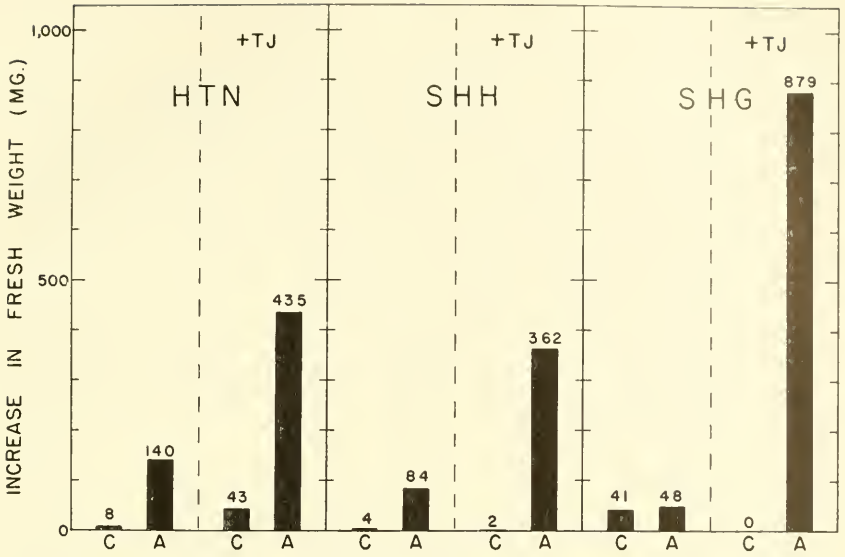

Fig. 3. Synergistic effect of a synthetic auxin and 10 per cent (v/v) of tomato juice (TJ) on the growth of the cultures of: normal *Helianthus tuberosus* tuber tissues (HTN), and Gautheret's strains of habituated (SHH) and crown-gall root tissues of *Scorzonera hispanica* (SHG). C: controls without auxin. A: = 2,4-dichlorophenoxyacetic acid (0.1 mg/l). The numbers on top of the black bars give the actual increases in mg. of fresh weight. (From 25)

theret's crown-gall strain of *Scorzonera hispanica,* however, was inhibited by TJ when small explants were used (3), but stimulated by TJ when larger explants were used (13). It was shown later by Nitsch and Nitsch (24) that this particular crown-gall strain also could be markedly stimulated by TJ even when small explants were used, providing an auxin was added to the medium (see Figure 3).

Habituated tissues. A preliminary experiment with Morel's habituated strain of *Rubus fruticosus* indicated a stimulatory effect of canned TJ. Although their fresh weight was much greater, the tissues grown on TJ were not as healthy looking as the controls; they were lightly colored, granular and friable, instead of being white and compact. New experiments, in which habituated strains of *Parthenocissus tricuspidata* of Morel and *Scorzonera hispanica* of Gautheret were used, showed only a marked growth inhibition by TJ (3). In these experiments no synthetic auxin had been added to the media, because it had been demonstrated that the habituated tissues proliferate very well in culture without added auxin (see 5). However, it was found later that the particular strain of habituated *Scorzonera hispanica* which had been used had, in fact, reverted to the auxin-requiring type. When a synthetic auxin was added to the media together with TJ, then the growth-stimulating effect of TJ became evident on the

strains of habituated *Scorzonera hispanica* (Figure 3) and of habituated *Parthenocissus tricuspidata* (25).

Normal tissues. Normal tissues, such as those of Morel's normal strain of *Parthenocissus tricuspidata* (3) and the tuber tissues of *Helianthus tuberosus* (24) responded to TJ by a clear-cut increase in growth, providing a synthetic auxin was added to the medium (Figure 4). Tissues from roots of *Scorzonera hispanica,* from tubers of *Solanum tuberosum* or from fruit parenchyma of apples did not grow on media containing TJ plus a synthetic auxin.

Special tissues. LaRue (15) mentions tomato juice among other addenda tried in order to stimulate the proliferation of endosperm tissue in sterile culture, and Tulecke (41) used tomato juice in some of his cultures of *Ginkgo* pollen, which led to the formation of undifferentiated masses of tissue.

Induction of Bud Formation

Cultures of Gautheret's habituated strain of *Scorzonera hispanica* occasionally formed small buds when cultured on 5 per cent TJ. These buds could be made to develop until 1 or 2 small leaf primordia became visible. Later on, however, the buds died.[2]

The effects which have been listed here are varied, and they may well be due to different constituents of the tomato juice. We will restrict ourselves, therefore, to that effect which more specifically stimulates growth by increase in fresh weight in undifferentiated tissue cultures.

GENERAL PHYSIOLOGICAL PROPERTIES OF TOMATO JUICE

Looking for an explanation for the stimulative effect observed in tissue cultures, we have come to the following conclusions:

Active Principle in TJ Not an Auxin

Added auxins have no stimulatory effects on crown-gall cultures (10), in contrast to TJ. Moreover, to obtain a stimulation of growth with TJ on normal and certain other strains of tissue cultures, an auxin must be added to the medium (25).

[2] Using Skoog's technique, it has since been found that a purified fraction of the tomato juice which, alone, had no growth-promoting properties on tobacco pith tissue, produced a voluminous and healthy callus when combined with 1 mg/l of IAA. This is exactly what kinetin did to the same tissue. After some 6 weeks, many buds developed on the growing cultures, just as they developed on cultures grown on kinetin plus IAA. It can be concluded, therefore, that TJ contains a natural kinin. [See Nitsch, J. P. Présence d'une substance du type "cinétine" dans le jus de tomates. Bul. Soc. Bot. France. 107. 1960. (In press.)]

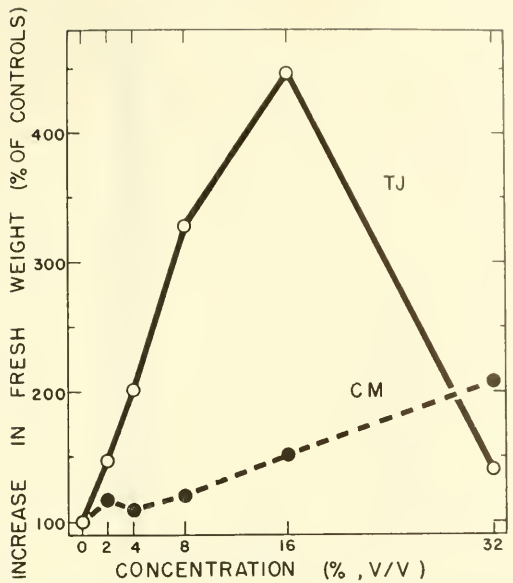

Fig. 4. Effect of various concentrations of tomato juice (TJ) and coconut milk (CM) on the growth of cultures of *Helianthus tuberosus* tuber tissues, 'Blanc commun' in the presence of IAA (1 mg/l). (Curve TJ from 24, curve CM, new data.)

Necessity of an Auxin

As a matter of fact, there seems to be a synergistic effect between auxins and TJ (Figure 3). This effect became very striking when various fractions were bioassayed (Figures 5 and 6).

Comparison With Coconut Milk

Tomato juice often produced a larger increase in fresh weight of various cultures than did a comparable concentration of coconut milk, as is shown in Table 1 and in Figure 4. With coconut milk, however, the cultures were generally more compact and healthier than with TJ.

Presence of an Inhibitory Principle

Often, however, the tomato juice became highly inhibitory, whereas coconut milk did not. In cultures of Morel's normal strain of *Parthenocissus tricuspidata,* for example, the toxic effects of TJ became evident as soon as the TJ concentration was increased above 5 per cent (v/v), whereas coconut milk promoted growth over that of the controls even at a 50 per cent concentration (3). Thus, it soon appeared that, in addition to growth-promoting factors, TJ also contained substances inhibitory to growth in tissue cultures and to a much greater degree than coconut milk.

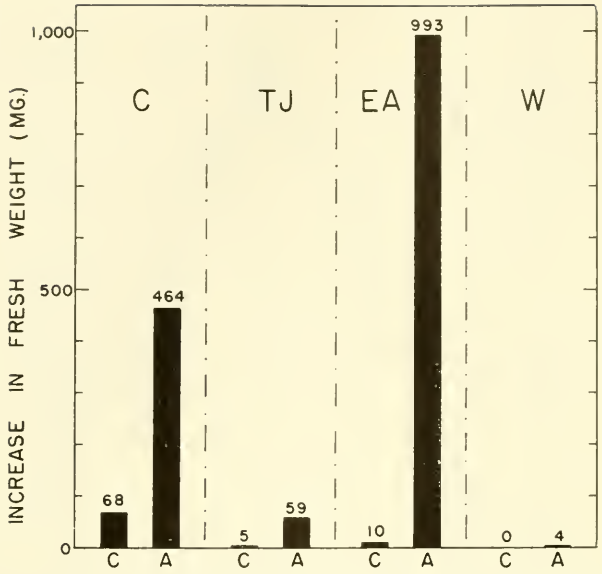

Fig. 5. Separation of an inhibitory principle from a growth-stimulating one by partitioning tomato juice between ethyl acetate and water. The fractions were tested on Gautheret's strain of habituated *Scorzonera hispanica* without (C) and with $5 \times 10^{-7} M$ indole-3-acetic acid (A). TJ = whole tomato juice (10 per cent, v/v). EA = ethyl acetate fraction. W = aqueous fraction. The numbers on top of the bars give the exact growth increments. (From 25)

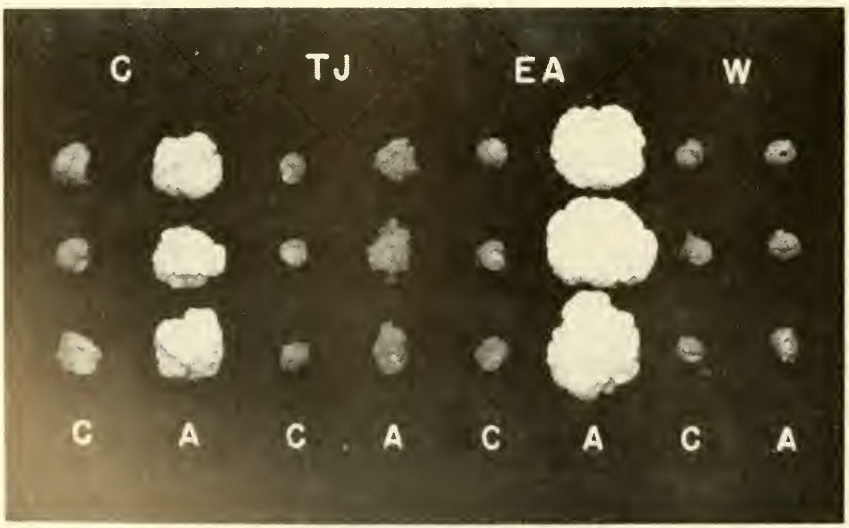

Fig. 6. Aspect of the cultures of *Scorzonera hispanica* (habituated strain) grown for 11 weeks on the media indicated in Figure 5.

Table 1. A comparison between the effects of tomato juice and coconut milk upon the increase in fresh weight of various tissue cultures.

Tissue	Concn. of Addendum (v/v), %	Increase in Fresh Weight, % of Control		Reference
		with TJ	with CM	
Nicotiana tabacum . . . . . (Morel's crown-gall strain)	10	202	107	Nitsch, unpublished
Helianthus tuberosus . . . . . 'Blanc commun' (normal tuber tissue)	8	328	120	Nitsch, unpublished
Parthenocissus tricuspidata. . . . . (Morel's normal strain)	5	297	138	3
Parthenocissus tricuspidata. . . . . (Morel's crown-gall strain)	10	238	186	3

CHEMICAL WORK

Bioassays

To measure the biological activity of the different fractions which have been isolated, the following two assays were used:

The Jerusalem artichoke test. The difficulty of securing large quantities of crown-gall tissue which would be homogeneous prompted us to use the xylem parenchyma of tubers of the P-17 strain of Jerusalem artichoke (*Helianthus tuberosus)* with which we had worked previously (27, 28). According to the procedure outlined (27), cylinders of tissue, generally 15 mg. in fresh weight, were removed aseptically from the tubers and planted on sterile media. The increase in fresh weight of the cultures was determined 21 days later.

The basal medium contained the following substances: the mineral salts of the N_1 solution (27) or those of an N_2 solution which had the same composition as the N_1 one, except that the sodium diphosphate had been replaced by an equivalent amount of commercial sodium hexametaphosphate (Calgon); sucrose (50 g/l); IAA (5×10^{-7} *M*); and Difco Bacto agar (10 g/l). The pH was always adjusted to 5.5 with HCl or NaOH before autoclaving. The media were autoclaved for 15 to 20 min. at 15 lbs. pressure.

The auxin was added to the medium routinely because (a) Jerusalem artichoke tuber tissues need an auxin in order to proliferate, (b) the aim was to pick up substances other than auxins, and (c) the factors present in TJ seemed to have a synergistic effect with auxins.

Auxin tests. To determine the auxin activity of various fractions, the first internode test and the sensitized coleoptile tests were performed as described previously (26).

Stability of the Tomato Juice Factor

The tomato juice factor (TJF) was found to be relatively stable when autoclaved with the medium at pH 5.5 for 20 min. at 15 lbs. pressure. On the other hand, an experiment showed that autoclaved TJ (pH 5.0) left at room temperature under sterile conditions but exposed to air through a cotton plug had lost its activity one month later.

Extraction Procedures

The TJF was extracted from both dried and fresh material. After many preliminary experiments, the two following methods were found to give good results:

From dried material. Fresh tomatoes were frozen at — 21° C., then broken into pieces while in the frozen state, and lyophilized. The resulting dry powder was extracted stepwise with the following solvents (Figure 7):

1. Petroleum ether (b.p. 30 to 60° C.): This produced a yellow liquid which fluoresced red in UV light and contained an inhibitory principle which was partially destroyed by autoclaving.
2. Benzene: The extract did not seem to contain any active substances.
3. Ethyl acetate: The dark green extract did not seem to contain active substances.
4. Cold methanol: The light green extract was partitioned between ether and water containing 0.5 per cent concentrated HCl. The ether fraction, which picked up all the chlorophyll, had little biological activity. The golden yellow aqueous fraction, on the contrary, contained both growth-promoting and growth-inhibiting substances.
5. Boiling methanol: This fraction had the greatest growth-promoting activity, together with some growth-inhibiting activity.
6. Boiling water: This fraction still had appreciable growth-promoting activity.

In short, the stepwise extraction procedure showed that: (a) petroleum ether extracts an inhibitor which is partially destroyed by boiling, and (b) the growth-promoting factor is preferentially water-soluble, together with a strong inhibitor which is also water-soluble.

From fresh material. Fresh tomatoes were stored frozen until needed. They were autoclaved for 10 to 15 min. at 15 lbs. pressure, in order to inactivate all the enzymes. The juice was then pressed out, filtered, and the remaining pulp washed with enough hot water to obtain a

total weight of juice equal to the initial weight of the tomatoes. The pH of this juice was generally around 4.0.

Separation of the Stimulating and Inhibiting Principles

A difficulty arose from the fact that both the growth-promoting principle and the strong inhibitor were generally extracted together. Among the various methods tried to separate them, two techniques gave satisfactory results, namely partitioning between water and ethyl acetate and adsorption on activated charcoal.

Extraction with ethyl acetate. When the crude juice or an aqueous solution containing the active principles was shaken with ethyl acetate, the growth-promoting principle moved, at least in part, to the ethyl acetate layer, the inhibitor remaining in the water layer (25). Experiments showed that more of the growth-promoting factor moved to the ethyl acetate layer at acid pH values (2.0) than at slightly alka-

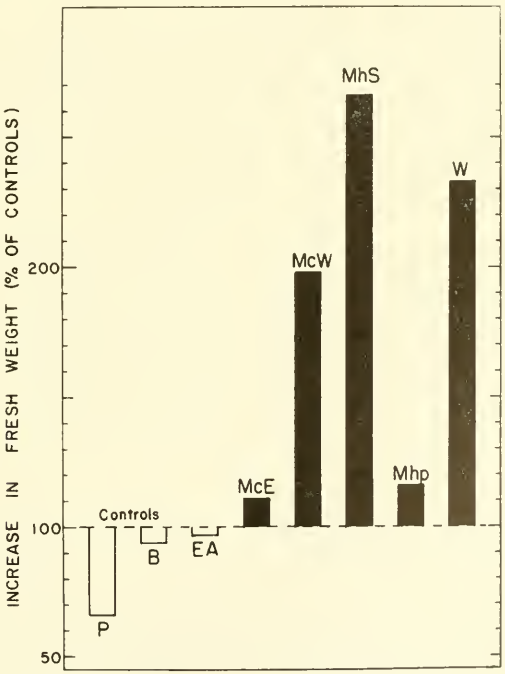

Fig. 7. Effects on *Helianthus tuberosus* cultures obtained from stepwise extraction of lyophilized tomato fruits with the following solvents: P: petroleum ether (b.p. 30 to 60° C.), B: benzene, EA: ethyl acetate, Mc: cold methanol, Mh: hot methanol, W: hot water. The Mc extract has been partitioned between ether (McE) and water (McW) under acid conditions. The Mh extract, upon concentration and standing in the cold, has separated into a white precipitate (Mhp) and the supernatant (MhS). All fractions were tested on *Helianthus tuberosus,* strain P-17, at concentrations equivalent to 20 per cent (v/v) of the original juice. Each value is the average of 12 replicate cultures. Differences less than 15 per cent are not statistically significant.

line ones (8.0). Thus a first method to separate the water-soluble inhibitor from the growth-promoting principle is to partition the aqueous extract with ethyl acetate at an acid pH (Figures 5 and 6).

Adsorption on charcoal. A second method, which gave even slightly better results in that it apparently isolated a greater quantity of the growth-promoting material, made use of activated charcoal. The crude juice, or an aqueous extract, was treated with activated charcoal and filtered. The inhibitory principle remained in the filtrate while most of the growth-promoting activity remained adsorbed on the charcoal. The growth-promoting principle was eluted with boiling methanol. Since this eluate contained also substances of the auxin type, it was further partitioned between ether and water at pH 3.0 (HCl), the auxins moving to the ether layer and the TJF remaining in the water layer.

Further Purification of the TJF

Further purification of the growth-promoting principle was achieved through the use of paper and column chromatography. With the mixture ethyl acetate (9) + glacial acetic acid (3) + H_2O (4) (v/v), three different active components could be separated on either paper strips or cellulose columns, the Rf values being: substance I (0.15), substance II (0.45), and substance III (0.95).

GROWTH FACTORS IN TOMATO FRUITS

The various techniques which had been briefly mentioned here have not yet led to the identification of any single compound which would be responsible alone for the biological properties of tomato juice. Instead, the TJF seemed to split into numerous constituents, many of which had a small activity on one or the other biological tests.

Inhibitors

Even the inhibitory effect of TJ was found to be due to at least three groups of substances, namely:

Petroleum ether-soluble inhibitor. This was extracted with petroleum ether from either dried or fresh material and was present in green and in ripe tomatoes. As was already reported by Konis (12), this inhibitor is partially eliminated by autoclaving. It is probably an essential oil.

Ether-soluble inhibitors. This fraction was reported by Larsen (14) and Hemberg (7) to contain substances which inhibit curvature in the *Avena* test. One of these inhibitors may be salicylic acid which was found to move at the same position as the inhibitory ether fraction on paper chromatograms and which was found to inhibit the

elongation of coleoptile sections. Another might be ferulic acid which was reported by Akkerman and Veldstra (1) as being one of the components of Köckemann's blastocholine. In our tests, ferulic acid had an inhibitory effect upon the growth of the first internodes of *Avena* seedlings in the presence of small amounts of IAA. Upon the growth of Jerusalem artichoke tissues, however, ferulic acid had no visible effect, at least at concentrations ranging from 10^{-6} to 10^{-4} *M*. Ferulic acid, on the other hand, has been reported by Hemberg (7) and by Reinders-Gouwentak and Smeets (35) to have a synergistic effect with auxins.

Water-soluble inhibitors. The aqueous fraction was the one which contained the most powerful inhibitors after autoclaving. Their chemical nature has not yet been determined.

Chlorogenic Acid and Its Derivatives

In addition to ferulic acid, Akkerman and Veldstra (1) extracted caffeic acid from tomatoes and claimed that it was a component of the blastocholine complex. Later on, however, it was reported (7, 35) that caffeic acid not only was not an inhibitor but was actually an auxin synergist. In our tests with Jerusalem artichoke tissues, caffeic acid (at least between 10^{-8} and 10^{-4} *M*) gave a small synergistic effect with IAA. Since caffeic acid is actually a component of chlorogenic acid, which has been reported in tomatoes (8), we have also tested chlorogenic acid[3], quinic acid, a mixture of caffeic and quinic acids, and cynarine[4] which contains two molecules of caffeic acid per molecule of quinic acid (32). None of these compounds gave evidence for a stimulation of growth at greater than 10 to 15 per cent concentrations ranging from 10^{-8} to 10^{-4} *M*. Toxic effects occurred at higher concentrations.

On the contrary, quite unexpected results were obtained when these compounds were tested on the *Avena* first internode and coleoptile bioassays for auxins. None of them had any auxin activity when used alone. In conjunction with low concentrations of IAA, however, they showed a marked synergistic effect, a rather pronounced response being obtained with chlorogenic acid (29). The effect of chlorogenic acid could be ascribed to its caffeic acid moiety, the quinic acid part having no growth-promoting effect (Figure 8). Similar results were obtained with coleoptile sections. Chlorogenic acid had an effect even when it was not given at the same time as IAA to the first internode or coleoptile sections. Thus, these sections could be first soaked in chlorogenic acid for 1 hour, then taken out of this solution

[3] Kindly supplied by Dr. A. C. Hulme.

[4] Kindly supplied by Dr. L. Panizzi.

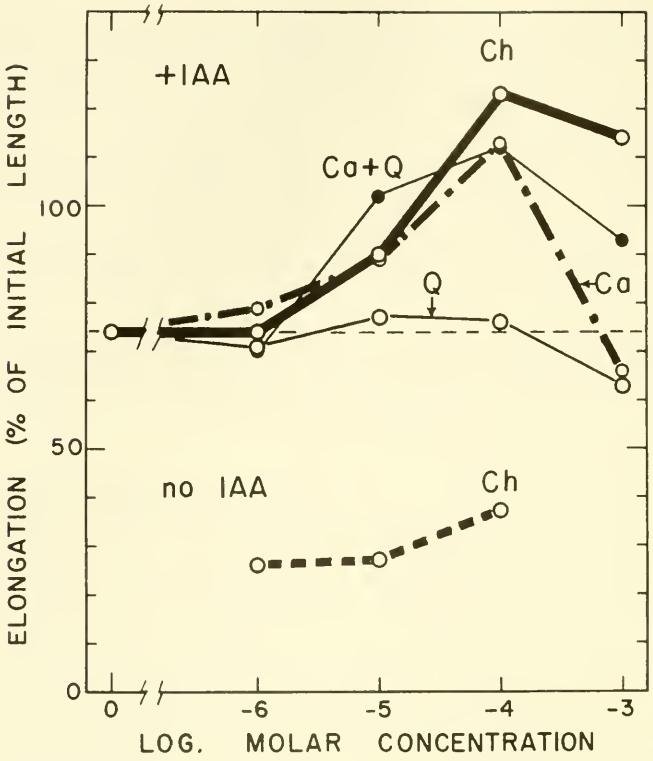

Fig. 8. Synergistic effects of chlorogenic acid (Ch), caffeic acid (Ca), quinic acid (Q), and caffeic + quinic acids at equimolar concentrations (Ca + Q) upon the elongation of *Avena* first internodes. The concentration of IAA was 10 μg/l; initial length 4 mm.

and transferred to tubes containing IAA only. An enhancement of the IAA effect could be observed under these conditions. Such a result may perhaps be ascribed to the reported inhibitory effect of chlorogenic acid on IAA oxidase (33).

Carboxylic Acids

Since the fraction containing the TJF was acid, the two most prominent acids of tomato juice, malic and citric acid, were tested on tissue cultures. The effect was slight (Table 2).

Amino Acids

Tomato juice contains, of course, many amino acids, such as glutamic acid, glutamine, arginine, tryptophane (2), γ-aminobutyric acid (38), etc. It has been shown (28) that glutamic acid and glutamine have a stimulatory effect upon the growth of Jerusalem artichoke tissue cultures in the presence of IAA. A mixture of amino acids, such

Table 2. Activity of various known components of tomato juice and related compounds on the increase in fresh weight of tissue cultures. (Differences smaller than 15 per cent are generally not statistically significant.)

Compound	Autoclaved or Filtered	Species and Strain	Molar Concn. $\times 10^{-5}$ or Ml/L‡	Fresh Weight, Per Cent of Control
Malic acid	autoclaved	*Helianthus tuberosus*, 'Blanc commun'	10	112
			1	100
			0.1	95
Malic acid	filtered	*H. tuberosus*, P-17	100	106
			10	97
Malic acid and citric acid	autoclaved	*Nicotiana tabacum**	220	
			190	100
Casein hydrolysate†	autoclaved	*N. tabacum**	1 ml.	103
			1 ml.	113
			1 ml.	114
Casein hydrolysate	filtered	*H. tuberosus* P-17	200 (N)	136
	autoclaved		200 (N)	141
Glutathione	filtered	*N. tabacum**	5.2	208
Glutathione and ascorbic acid	"		5.2	216
	"		14.0	
Glutathione	autoclaved		5.2	73
Glutathione and casein hydrolysate	"		5.2	
	"		1 ml.	119
Ascorbic acid	filtered	*N. tabacum**	14	188
			100	134
			10	99
			1	83
Epicatechin	filtered	*H. tuberosus* P-17	10	139
	"		1	100
	"		10	104
	"		1	92
	"		0.1	126
	autoclaved		1	107
Catechin	filtered	*H. tuberosus* P-17	10	116
	"		1	109
	"		10	100
	"		1	107
	"		0.1	92
	autoclaved		1	100
Quercetrin	filtered	*H. tuberosus* P-17	2.2	125
			0.2	112
			0.02	119

* Morel's crown-gall strain of *N. tabacum*.

† Enzymatic casein hydrolysate (vitamin free) 0.5 per cent solution obtained from the Nutritional Biochemicals Corp., Inc., Cleveland, Ohio.

‡ When the same concentrations appear twice in this column the experiments have been repeated at different times.

as those contained in casein hydrolysate, was found to promote the growth of the Jerusalem artichoke tissues also as well as that of crown-gall tissues (Table 2). Thus, it is possible that part of the TJ effect is due to its amino acid content.

Reducing Compounds

Tomato juice also contains reducing compounds, such as ascorbic acid, glutathione (40), phytoene (34), etc. Ascorbic acid and glutathione were found to enhance the growth of the strains of tissue cultures which we stimulated by TJ (Table 2). Epicatechin[5], at the concentration of 10^{-4} *M*, also gave some growth stimulation, more than catechin.[5]

Flavones

The yellow color of substances I, II, and III, and the fact that these colors deepened markedly under alkaline conditions suggested to us that these compounds could be flavones. Although the precise chemical nature of these three substances has not yet been determined, we suspect that they are glycosides, at least substances I and II. Quercetrin was found to stimulate slightly the growth of Jerusalem artichoke tissues (Table 2). The presence of biologically active glycosides of flavones in TJ would be of special interest in view of the report that a leucoanthocyanin is one of the active components of coconut milk (39).

Sugars

The fraction which produced the most intense growth effects upon the growth of Jerusalem artichoke tissues was, when concentrated, of the consistency of a sugar syrup. From this fraction a whitish compound could be precipitated out with ether or acetone. This substance, called substance S, was biologically active.

Auxins

Methanol extracts of tomatoes at various stages of development yielded several growth substances of the auxin type which were separated by paper chromatography. These substances, with their relative biological importance, R_f values, and color reactions are listed in Table 3. It should be noted that, in addition to biologically active substances, several other spots giving a color with the Ehrlich reagent for indoles were found to have no growth-promoting effect upon the elongation of *Avena* first internodes.

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[5] Kindly supplied by Dr. K. Freudenberg.

Table 3. Substances active on the *Avena* first internode test obtained from methanol extracts of lyophilized, immature tomatoes chromatographed on Whatman No. 3 MM paper in 80 per cent isopropanol redistilled over Zn and KOH.

Substance	R_f	Intensity of Biological Response	Color With Ehrlich's Reagent
A	0.05	+	.....
B	0.20	+++	purple*
C	0.35	++	.....
D	0.50	++	purple*
E	0.65	+++	.....
F	0.90	+	.....

* It is not yet known if the growth-promoting substances are actually Ehrlich-positive. One can say only that their position coincides with an area which reacts with Ehrlich's reagent.

SUMMARY

At the present time, the chemical identification of all the active components of tomato juice is not yet completed. The work presented here, therefore, is more a progress report than the account of a completed task. The wide range of biological effects exerted by tomato juice may well be due to different, unrelated constituents. In any case, we have been able to separate an inhibitory principle which can be split further into three categories: petroleum ether-soluble, ether-soluble, and water-soluble compounds. In the growth-promoting fraction of tomato juice, some compounds, such as chlorogenic acid and derivatives, were found to act as auxin synergists on the first internode and coleoptile sections of *Avena*. Other compounds promoted the growth of *Helianthus tuberosus* tuber tissues, but only when an auxin was added simultaneously. Such results gain interest when one considers that the tomato is one of the fruits which can be set easily with applied auxins. It is conceivable that the applied auxins work in conjunction with the synergists which are naturally present in the ovary of this species.

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DISCUSSION

Dr. Siegel: The reference made by Dr. Nitsch to the chemical reducing activity of growth factors prompts me to call attention to the generality of this relationship and to the manner in which it ties together many points discussed here. The main concept in our paper in this volume is that most organisms must, of necessity, live in an environment which can and does attack continuously their metabolites, catalyses, and architecture.

Oxygen is the only common electron acceptor which can support bioenergetic processes effectively, yet the hazards to life of its aerobic environment have been well documented. Our contribution to this

volume contains a number of recent references to oxygen toxicity, but a fuller appreciation of the phenomenon can be gained by examination of older studies (Clements, Carnegie Inst., Washington, 1921). The toxic effects of oxygen on plants were known to Scheele (1777), Huber and Senebier (1801), etc. The benefits of reduced (sub-atmospheric) oxygen levels were appreciated by Sedebeck (1881) and Jaccard (1893). We have, on the whole, relegated such nonchemical effects and observations to the physiology of our earlier day, and pursued the study of chemical growth factors without regard to nonmetabolic oxidation. Now we are confronted with observations which suggest that the functions of growth factors be re-examined, keeping in mind the duality of oxygen as part of the biological milieu.

Dr. Stowe was concerned about the discrepancies in growth rate between sections and intact organs. He sought to narrow the gap, finally by application of unsaturated lipids. Drs. Crosby and Vlitos discovered that fatty substances could supplant conventional auxins as growth factors. Dr. Crosby recognized the antioxidant character of some indolic auxins. Dr. Marrè discussed growth inhibitions which he associated with *oxidized* ascorbic acid, and speculated that ascorbic acid might also be a growth promoter (but only, we suggest, in reduced form). Dr. van Overbeek alluded to the importance of acid and the reducing environment of archeozoic times but did not distinguish between oxygen uptake, respiration, and energetic processes, thus leaving the issue unsettled. Drs. Muir and Hansch, and Dr. Thimann considered the electronic features of organic compounds as directly related to their auxin or auxin-like properties. From their comments we would assume that electron delocalization, polarization, and local electron densities are important in relation to attachment of the hormone to its acceptor. Now, the significance of electrical forces in the localization of hormones is obvious, but it was somewhat surprising that those who treated these matters with such facility did not carry their consideration of electron mobility beyond the primary events of auxin-acceptor interaction.

The true mechanism of action of auxin or other growth factors is hardly to be found in a consideration of attachment, nor in deliberations on the biophysics of the cell wall. Thus, a knowledge of the factors which enable auxin to be delivered to the right place in the cell still leaves unanswered the question: "What does the auxin do when it gets there?" No laboratory has yet answered this question, but we contend that important elements of the answer reside in those electronic properties which are particularly meaningful when considered together with oxygen toxicity. "Antioxidant" and "reducing" are operational terms. They apply to molecules, or ions, which are characterized more fundamentally by their high electron availability.

The property we seek resides in pi-electron donors and atoms with nonbonding electrons, singly or in combination, hence, in the indoles, aryloxy compounds and other aromatic substances which have occupied so much of our interest.

It does not follow that all antioxidants (or electron donors) will be growth promoters, or even regulators. The growth-promoting potentialities of these compounds can only be realized if they are presented to the cell in suitable form. Antioxidants of different structure which promote growth may operate through different limiting pathways, some acting as auxins, some as auxin-sparing agents, and others as more uniquely localized protectants or cofactors.

ULRICH NÄF
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On the Physiology of Antheridium Formation in Ferns[1]

CONTROL OF ANTHERIDIUM FORMATION IN DIFFERENT GROUPS OF FERNS

Döpp (5) demonstrated that an extract from mature prothalli of *Pteridium aquilinum* hastened the onset of antheridium formation in young prothalli of this fern species by a few days and in the prothalli of *Athyrium filix-mas* by a few weeks. Döpp envisaged the possibility that the promotion of antheridium formation was the result of nonspecific growth inhibition. Subsequent investigations (9), though, led to the conclusion that the activity of the extract must be attributed to a specific factor which controls antheridium formation during normal development.

An assay was devised which took advantage of the observation that the prothalli of *Onoclea sensibilis* failed to form any antheridia spontaneously under the prevailing conditions of culture but responded readily if extract from mature prothalli of *Pteridium aquilinum* was added (see Figure 1A, B). Conditions were further defined under which the extract from 7-week-old prothalli of *Pteridium aquilinum* was active to a dilution of 1:30,000. This increased the activity obtained by Döpp by a factor of about 300. Under these same conditions of culture the active substance accumulated to almost as high an activity in the medium (9). The available data show that this substance must be active at a dilution of 1.6×10^{-9} or less (9).

Studies on the activity spectrum of the factor disclosed that it was active toward the tested representatives of seven out of the nine subgroups of the family Polypodiaceae listed by Eames (7): The As-

[1] This investigation was supported in part by research grants (NSF G-3225 and NSF G-6144) from the National Science Foundation.

plenioids, Pteroids (5), Onocleoids, Blechnoids, Dryopteroids (9), Gymnogrammoids (6), and the Woodsioids (11). Among nonpolypodiaceous species, only *Dennstaedtia punctilobula* (Dicksoniaceae) was found to be responsive. The substance failed to promote antheridium formation even at the highest available concentration (½ strength *Pteridium* medium that was active toward the prothalli of *Onoclea sensibilis* to a dilution of 1:30,000) in the following fern species: *Polypodium aureum* (Polypodiaceae), *Lygodium japonicum, Anemia phyllitidis* (Schizaeaceae), *Osmunda claytoniana,* and *O. cinnamomea* (Osmundaceae).

Studies on fern species that were unresponsive toward the *Pteridium* factor led to the demonstration of a substance that controls

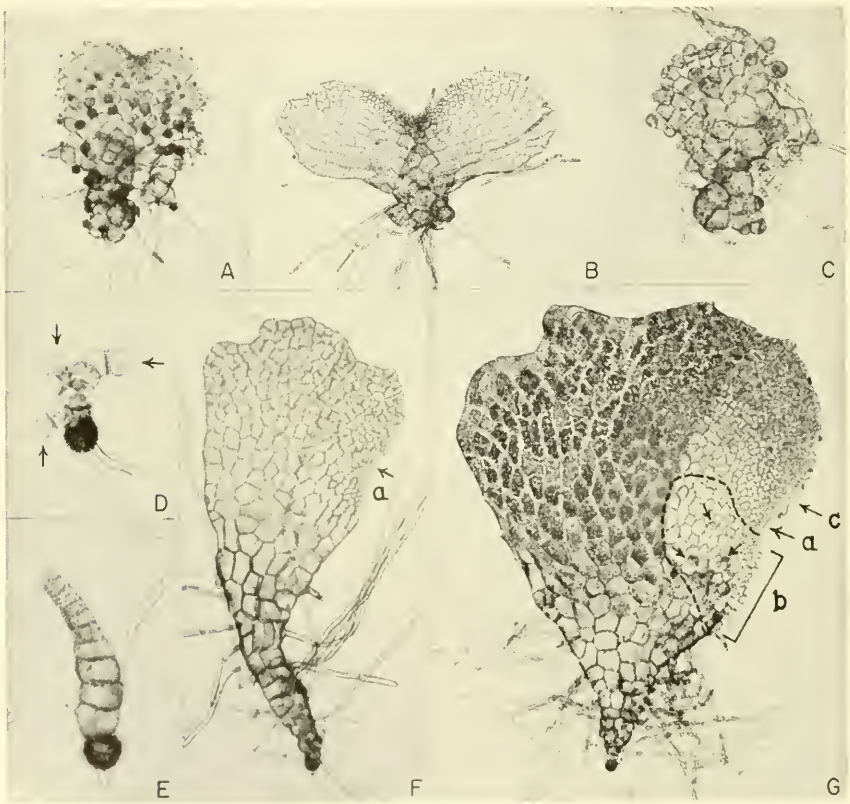

Fig. 1. **A.** Gametophyte of *Onoclea sensibilis,* 19 days old, 33 X, grown in the presence of *Pteridium* medium 1:250. **B.** Same as **A**, except grown in the absence of *Pteridium* medium. **C.** Incipient ameristic prothallus of *Pteridium aquilinum,* 20 days old, 55 X. **D.** Gametophyte of *Anemia phyllitidis,* 12 days old, 94 X, grown in the presence of *Anemia* medium 1:30. **E.** Same as **D**, except grown in the absence of *Anemia* medium. **F.** Gametophyte of *Anemia phyllitidis,* 25 days old, 41 X. **G.** Gametophyte of *Anemia phyllitidis,* 35 days old, 30 X.

antheridium formation in *Anemia phyllitidis* (11) but is inactive toward the species used to assay for the *Pteridium* factor. In *Lygodium japonicum* antheridium formation seems to be controlled by still another substance (12) even though this species belongs to the same family as *A. phyllitidis* (Schizaeaceae).

The application of the *Anemia* factor to young cultures of *Anemia phyllitidis* led to the onset of the antheridial phase while the prothalli were still at a juvenile (filamentous) phase (Figure 1D, arrows indicate antheridia). Control prothalli (Figure 1E) were invariably free of antheridia even at the much more advanced stage of development pictured in Figure 1F.

It is apparent from these studies that antheridium formation is controlled by different substances in different groups of ferns. It should also be stressed that within the wide range of species responsive to the *Pteridium* factor, the minimally effective concentrations vary widely. Thus the prothalli of *Dennstaedtia punctilobula* failed to respond unless they were supplied with the *Pteridium* factor at a concentration about 125 times higher than was necessary in the prothalli of *Onoclea sensibilis* and of *Pteridium aquilinum* itself. In the prothalli of *Woodsia obtusa* the minimally effective concentration of the *Pteridium* factor exceeded that required for antheridium formation in *Onoclea sensibilis* by a factor of about 25 (11).

The possibility must therefore be considered that the factors controlling antheridium formation in these species are actually different but structurally so closely related that the factor produced by *Pteridium aquilinum* is capable of bringing about antheridium formation also in *Dennstaedtia punctilobula* and in *Woodsia obtusa* if it is supplied at a high enough concentration. Raper (14) also considers the possibility that hormonal specificities account for the failure to obtain oospores in some of the attempted interspecies and intergeneric crosses of water molds.

The above results raise a question of biological specificity. The studies of Kluyver and Van Niel have drawn attention to the similarity, even identity, of basic biochemical patterns in taxonomically widely separated organisms. It is tempting to postulate that the metabolism associated with antheridium formation, i.e., an event that we conceive of mainly in morphological terms, is also similar in different fern species. The above results may be reconciled with such a postulate if we consider that the induction of an antheridium is likely to involve many reactions and compounds. Antheridium formation in *Pteridium aquilinum, Anemia phyllitidis,* and *Lygodium japonicum* might thus be controlled by different factors because different reactions became rate-limiting during evolution. Alternatively, we might be witness to evolution on a molecular level. On this as-

sumption the inducing molecule has undergone a gradual structural modification probably concomitantly with changes in a receptor molecule. The isolation and characterization of the three factors should yield pertinent information. In the meantime, an attempt is being made to assay for similarity between the various factors based on the postulates that one factor may be a precursor of the other or that one factor may behave as a chemical analogue of the other and thus interfere with its synthesis or with the function it performs in the initiation of antheridia.

LOSS OF SENSITIVITY TO THE ANTHERIDIUM-INDUCING FACTOR

Many individuals in a gametophyte population of *Pteridium aquilinum,* and of many other fern species, have an early antheridial phase which is, however, terminated as the prothalli attain the archegonial phase. Why do these prothalli discontinue the formation of antheridia? The hypothesis may be proposed that they discontinue forming the antheridium-inducing factor.

It was shown, however, that the active substance becomes available at progressively higher concentrations as more and more prothalli stop forming antheridia and begin to produce archegonia instead (10). This suggests that the gametophytes continue to elaborate the antheridium-inducing factor while they form archegonia. It must be pointed out, however, that even mature cultures contain a small percentage of gametophytes which form antheridia only throughout the life of the culture (the so-called ameristic prothalli; see section beginning on page 716). Accordingly, it may be these ameristic prothalli rather than the archegonium-bearing prothalli which account for the continued elaboration of the active substance by maturing cultures of *Pteridium aquilinum.* Döpp (5) effected a separation, necessarily incomplete, of the two types of prothalli and found the active factor to be present in the extract of either type if at somewhat greater concentration in the archegonium-bearing prothalli. This evidence does not unequivocally support Döpp's conclusion that archegonium-bearing prothalli produce antheridial factor. Rather, the active substance found in the archegonium-bearing prothalli might have been produced at an earlier, antheridial stage. Moreover, the active substance found in either type of prothallus might have been taken up from the medium into which it was secreted by the other type of prothallus. These objections were met by isolating archegonium-bearing prothalli, one per flask, and assaying the media for antheridium-inducing activity at intervals over a period of time. Such studies showed (see Table 1) that archegonium-bearing prothalli actually produced large amounts of the antheridal factor (10).

Table 1. Number of media (out of 20 at each interval) that have antheridium-inducing activity to the indicated maximal dilutions.

Dilution of Medium	Time in Days		
	3	13	23
1/2	17	. .	. .
1/10	3	2	. .
1/50	.	16	2
1/250	.	2	12
1/1,250	.	.	6

Discontinuance of antheridium formation could not, therefore, be ascribed to a discontinuance of antheridial factor production. The alternative hypothesis suggested itself, that the maturing prothalli become insensitive to antheridial factor, i.e., that they lose the ability to respond with antheridium formation to the presence of that factor. This hypothesis was tested first on the prothalli of *Onoclea sensibilis* which were particularly suited to such an investigation because they failed to form antheridia spontaneously under the prevailing conditions of culture but formed them readily in response to added antheridial factor. Prothalli of this species were transferred at intervals of 2 days, starting 8 days after inoculation, to new medium containing the active factor at a concentration series ranging from 1/2 to 1/31,250 full strength *Pteridium* medium. Table 2 shows that, whereas nearly all 10-day-old prothalli were sensitive to the antheridial factor, nearly all 14-day-old prothalli were insensitive to it. Subsequent investigations on isolated prothalli showed that the individual prothallus became insensitive within a period of about 2 days or less. Following this interval, a gametophyte fails to form antheridia even at the highest concentration of the antheridial factor, i.e., a concentration

Table 2. Number of prothalli (out of 20) that formed antheridia when transferred from basic medium to medium containing antheridial factor at different intervals following inoculation of the spores.

Dilution of *Pteridium* Medium	Interval in Days				
	8	10	12	14	16
1/2	20	20	7	2	0
1/10	20	19	6	0	0
1/50	20	20	5	1	0
1/250	20	20	6	2	0
1/1,250	20	20	5	2	0
1/6,250	20	19	5	1	0
1/31,250	7	6	1	0	0
Control	0	0	0	0	0

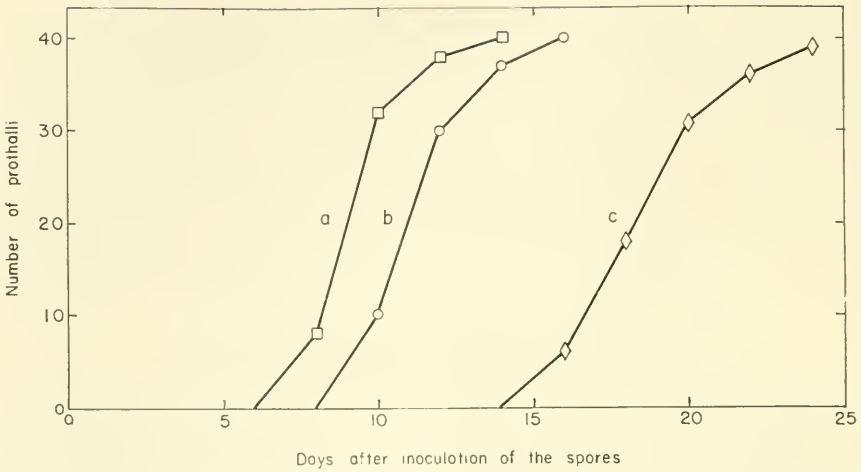

Fig. 2. Sequence of developmental stages in prothalli of *Onoclea sensibilis*. (a) Numbers of prothalli (out of 40) that have attained heart shape. (b) Numbers of prothalli (out of 40) that have become insensitive to the antheridial factor. (c) Numbers of prothalli (out of 40) that have initiated one or more archegonia.

15,000 times higher than that sufficient to induce antheridia in prothalli just 2 days younger.

Figure 2 relates the onset of this loss of sensitivity to two other major events in the development of *Onoclea* gametophytes: attainment of heart shape and of the archegonial phase. The results recorded in this figure show that the prothalli became insensitive to the antheridial factor about 2 days after attaining heart shape and 6 to 7 days prior to the attainment of the archegonial phase.

In cultures of *Pteridium aquilinum* the first insensitive prothalli were observed in 7-day-old cultures. The studies on this fern species further showed that the organization of the meristem, the activity of which results in the attainment of heart shape, again precedes the loss of sensitivity to the antheridial factor (10).

Similar studies were carried out on *Anemia phyllitidis*. Figures 1F and 1G show that the prothalli of this fern species have a lateral meristem, a phenomenon encountered in only few fern species. The first antheridium is invariably initiated by a marginal cell a short distance back of the meristematic initial. Marginal cells cut off subsequently by the meristematic initial also give rise to antheridia so that ultimately a whole row of them can be seen (marked *b* in Figure 1G). In contrast, the marginal cells given off by the initial toward the tip of the prothallus fail to form antheridia even in the presence of added *Anemia* factor. Instead, some of them give rise to hook-shaped hairs (arrow *c*). Once the first antheridium has been initiated

marginally, others will arise also on cells inside the prothallus (three of ten such antheridia are indicated by arrows).

The area of spontaneous antheridium formation always remains restricted, however. Thus, antheridia never arise in the part of the prothallus anterior to the lateral meristem. Again the zone of antheridium formation in the posterior region does not stretch across the whole prothallus; instead, it extends about half way or less inside the prothallus from the margin of the cell plate which bears the lateral meristem (zone is marked by broken line on Figure 1G). The other half of the posterior region remains free of antheridia except for the occasional occurrence of one to three antheridia in marginal cells.

Observations following the application of the active substance to cultures of various ages showed that the very young prothallus responded with antheridium formation throughout most of its body. As the age of the prothalli increased, though, the responding area gradually contracted to that described for spontaneous antheridium formation. The loss of sensitivity in the anterior part of the prothallus preceded the contraction in the responsive area of its posterior region (11).

Döpp (6) showed that the excision of the meristem from archegonium-bearing, i.e., insensitive, prothalli of *Pteridium aquilinum* led to the formation of antheridia in the regenerating fragments. The same phenomenon was encountered in *Onoclea sensibilis* (author's results). The studies which were carried out on wings, cut from insensitive, archegonium-bearing gametophytes, further showed that antheridium initials in these meristemless fragments did not appear, even at the highest available concentration of antheridial factor, until 7 to 14 days after the wings were removed. In contrast, 9-day-old, i.e., still sensitive, *whole* prothalli will give rise to antheridium initials within a period of between 2½ and 3 days after the antheridial factor has been applied. Thus the removal of the meristem leads to the restoration of sensitivity with a considerable time lag. Further observation showed that antheridia were formed only in those regions of the meristemless prothalli which had undergone a considerable amount of cell division. This correlation between antheridium formation and cell division indicates that something is diluted out before the cells become sensitive again.

As already mentioned, the application of the antheridium-inducing factor to 9-day-old whole prothalli led to the appearance of antheridium initials within a period of between 2½ and 3 days at all tested concentrations. If the antheridial factor was withdrawn from the medium 1½ days after it was applied, then no antheridia were formed.

THREE TYPES OF GAMETOPHYTES IN CULTURES OF *PTERIDIUM AQUILINUM*

Mature gametophyte cultures of *Pteridium aquilinum,* and of many other homosporous, leptosporangiate fern species, contain two types of prothalli. Some bear antheridia only and are commonly designated the male prothalli. They lack a meristem (hence their diffuse growth habit and random shape) and are for this reason occasionally also referred to as the ameristic prothalli. This latter designation is used in this report for reasons that will become apparent below. Others bear only archegonia and are commonly called the female prothalli. Such observations on mature cultures led to the concept that the female prothalli failed to form antheridia under normal conditions of culture or formed them only rarely, especially under atypical conditions of culture (2, 4, 5, 8, 16). In contrast, Czaja (3) came to the conclusion that the female gametophytes formed antheridia regularly at an early stage of development but discontinued their formation as they attained the archegonial phase.

It could be demonstrated that the archegonial phase is, in many prothalli of *Pteridium aquilinum,* actually preceded by an antheridial phase. At the same time it was shown that some individuals of the gametophyte population, to wit, the most rapidly growing and developing ones, attained the archegonial phase without a prior antheridial phase (10).

The occurrence of the two types of archegonium-forming prothalli led to the hypothesis that the prothalli of *Pteridium aquilinum* become insensitive to the antheridial factor before they begin to produce it at effective concentrations. This hypothesis was considered established when it was demonstrated that all individuals of the gametophyte population *failed* to form antheridia if they were grown one per flask, and that all prothalli formed antheridia if they all were exposed to antheridial factor while they were still sensitive to it (10).

The antheridia a gametophyte forms thus arise in response to antheridial factor that is secreted into the medium by more rapidly developing individuals of the gametophyte population (which themselves have already become insensitive to it). Accordingly, the most rapidly developing gametophytes in a culture of *Pteridium aquilinum* attain the archegonial phase without a prior antheridial phase because they are without a supply of antheridial factor while they are still sensitive to it.

A gametophyte population of *Pteridium aquilinum* thus contains three types of individuals: Archegonium-forming prothalli *with* a

prior antheridial phase, archegonium-forming prothalli *without* a prior antheridial phase, and the ameristic prothalli which produce only antheridia even in mature cultures.

The mechanism underlying the formation of ameristic prothalli remains to be determined. Why do they fail to form archegonia even in mature cultures? The search for the factors concerned with the formation of these ameristic prothalli must also take into account the striking differences between them and the archegonium-forming prothalli with regard to both size and shape. Thus, the surface area of ameristic prothalli amounts to less than a hundredth that of the archegonium-bearing prothalli in mature cultures; again their shape is highly irregular due to their diffuse growth habit while the archegonium-bearing prothalli have the well-known heart shape that results from the activity of a characteristically organized meristem.

Prantl (13) observed that the formation of ameristic prothalli is favored by conditions that interfere with growth, e.g., poor mineral supply, crowding, and poor lighting. He accordingly advanced the hypothesis that male prothalli are gametophytes retained at a juvenile stage due to adverse conditions of nutrition. This hypothesis accounts at first sight for most of the distinguishing characteristics of ameristic prothalli. Closer observation, though, reveals a number of discrepancies. Thus, young prothalli pass through a fairly regular sequence of cell divisions and quickly organize a growing region long before they attain the size of the average ameristic prothallus. The hypothesis, therefore, does not account for the diffuse growth habit and random shape of ameristic prothalli. Again, Prantl's hypothesis calls for a continuum in sizes among the individuals of the gametophyte population. While such a continuum can be readily observed in younger cultures, it gives way in maturing cultures to a steadily widening hiatus between the size range of ameristic prothalli, on one hand, and that of archegonium-bearing prothalli on the other hand.

A clue to the mechanism that underlies the formation of ameristic prothalli derived from an attempt to detect such prothalli at an incipient stage of development. Observations on young cultures of *Pteridium aquilinum* convinced the author that prospective ameristic prothalli did not begin to differ until they attained the antheridial phase. At this stage of development they gave rise to antheridia not only in the maturing region of the gametophyte but in the meristematic region itself (frontal row of antheridium-bearing cells, Figure 1C). In contrast, the meristematic region of antheridium-bearing prothalli that subsequently proceeded to form archegonia remained free of antheridia at all stages of development. As indicated, such incipient ameristic prothalli still had a clearly recognizable meristem

and their shape conformed to that of other individuals at a comparable stage of development. The diffuse growth habit and random shape of ameristic prothalli is thus acquired subsequently.

The questions arose: What are the conditions that favor the formation of antheridia by meristematic cells, and can such antheridium formation account for the distinctive characteristics of ameristic prothalli?

The described incipient ameristic prothalli were observed to be among the smallest individuals of the gametophyte population. The hypothesis may thus be proposed that antheridium formation by meristematic cells is favored by conditions of slow growth.

It was demonstrated that all individuals of the gametophyte population will give rise to antheridia in their meristematic cells and subsequently acquire the characteristics of ameristic prothalli if, firstly, they are cultivated under conditions of slow growth and if, secondly, they are all supplied with antheridial factor while they are still sensitive to it. The requirement for added antheridial factor is understood if it is recalled, firstly, that the formation of ameristic prothalli is related to antheridium formation (in the meristematic region) and, secondly, that the most rapidly developing individuals (archegonium-forming prothalli without a prior antheridial phase) are not otherwise exposed to antheridial factor during their sensitive period.

The question as to how slow growth favors the formation of antheridia by meristematic cells has not as yet been answered. It may be pointed out, however, that the meristematic cells of rapidly growing prothalli divide at a rate of at least one per day. On the other hand, the appearance of antheridium initials follows the application of antheridial factor with a delay of between 2½ and 3 days. It is possible, therefore, that the meristematic cells of rapidly growing prothalli remain free of antheridia because they divide before the induction of an antheridium can take effect.

It was emphasized above that incipient ameristic prothalli are of no more irregular shape than archegonium-forming prothalli at a comparable stage of development. The formation of antheridia in the meristematic region is, however, soon followed by a breakdown in meristematic growth. The meristem ceases to operate as a functional unit and cell division resumes in the basal region of the gametophyte. The formation of antheridia in the meristematic region thus releases the potentiality of maturing cells for cell division which is suppressed in the presence of an actively functioning (antheridium-free) meristem. Antheridium formation by meristematic cells thus brings forth the same response as the excision of the meristem itself (1).

The assumption of heart shape is preceded by, and dependent on,

a change in the organization of the meristem. In turn, the attainment of heart shape invariably precedes the loss of sensitivity to the antheridial factor and the attainment of the archegonial phase (10). The failure of ameristic prothalli to assume heart shape (and their resulting diffuse growth habit), their failure to become insensitive to the antheridial factor and to form archegonia may thus be traced to the breakdown in meristematic growth at an early stage of development. The wide hiatus in sizes between the ameristic and the archegonium-bearing prothalli of mature cultures remains to be explained. Clearly, the observation that ameristic prothalli arise from the most slowly growing individuals cannot account for this hiatus.

Antheridium formation entails a diversion of growth potential from the formation of vegetative cells to that of antheridial cells (9). Also, it has long been known that a vegetative cell bears not infrequently two or even three antheridia, mostly at different stages of development. A vegetative cell might thus give rise to new antheridia repeatedly after the previously formed, ephemeral structures have fallen apart. The resulting diversion of growth potential from the formation of vegetative cells to that of antheridial cells could fully account for the ever widening hiatus between the sizes of ameristic prothalli which form antheridia even in mature cultures and the archegonium-forming prothalli which either lack a prior antheridial phase or discontinue it at an early stage of development.

It was stressed above that the antheridia a prothallus forms are initiated in response to antheridial factor produced by more rapidly developing individuals of the gametophyte population which themselves have already become insensitive to it. The ameristic prothalli, like the archegonium-forming prothalli with a prior antheridial phase, thus are the result of interaction between gametophytes. The above results are further in agreement with the postulate that the several distinctive characteristics of ameristic prothalli are all the consequence of antheridium formation (in the meristematic region). These distinctive characteristics should therefore disappear if the prothalli are removed from the interaction with other individuals of the gametophyte population.

Ameristic prothalli taken from mature cultures actually gave rise to heart-shaped lobes, became insensitive to the antheridial factor, and attained the archegonial phase after they were washed and transferred, one to a flask, to new medium.

ON THE PHYSIOLOGY OF REPRODUCTION IN *PTERIDIUM AQUILINUM*

It should be of interest to consider the question as to how these three types of prothalli relate to sexual reproduction. If in all prothalli an antheridial phase were followed by an archegonial phase,

then there would be little overlap between prothalli at an antheridial stage and prothalli at an archegonial stage of development. Accordingly, sexual reproduction would be hindered.

Ameristic prothalli which form antheridia indefinitely thus appear to be functional by supplying male gametes when the other two types of prothalli have already attained the archegonial phase. With regard to archegonium-forming prothalli without a prior antheridial phase, the following argument seems pertinent. Antheridium formation diverts growth potential from the formation of vegetative cells to that of antheridial cells, thus delaying the onset of the archegonial phase (see section beginning on p. 712). The occurrence of archegonium-forming prothalli without a prior antheridial phase thus minimizes the time lag between the first appearance in the culture of antheridium-bearing and of archegonium-bearing prothalli and thereby hastens the onset of sexual reproduction.

Several characteristics of prothallial development combine to interfere with the simultaneous occurrence of male and female sex organs on the same prothallus, especially the failure of ameristic prothalli to form archegonia and the lack of a prior antheridial phase in one of the two types of archegonium-forming prothalli. The more rapid attainment of the archegonial phase by prothalli *without* a prior antheridial phase results, during an initial period of time, in the presence of prothalli that bear either antheridia only or archegonia only. As the prothalli *with* a prior antheridial phase attain the archegonial stage, the last-initiated antheridia do not fall apart until one to three archegonia have been initiated (10). It is unlikely, though, that the first-initiated archegonium matures before the last-initiated antheridia have fallen apart. The simultaneous occurrence of male and female gametes on the same prothallus is, however, occasionally observed later because antheridia may arise on the basal outgrowths which some of the archegonium-bearing gametophytes form at late stages of development.

These characteristics of prothallial development which interfere with the simultaneous occurrence of male and female sex organs on the same prothallus would seem to be functional by interfering with self-fertilization. This interpretation, though, appears invalidated by Wilkie's report that the prothalli of *Pteridium aquilinum* are self-sterile (15). If this finding can be confirmed, then the mentioned characteristics of development could still be considered functional in terms of preventing wasteful contact between incompatible gametes. In self-fertile species, however, these characteristics of development would clearly serve to minimize the chance of self-fertilization.

Preliminary investigations actually indicate that *Polypodium aureum* (personal communication from M. Ward, 1959) and *Onoclea sensibilis* (author's result) are self-fertile.

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DISCUSSION

Mr. Barlow: What do you think happens in natural conditions? You say that this factor is required from elsewhere, rather than the prothallus itself.

Dr. Näf: It has been demonstrated that a prothallus does not begin to produce the antheridial factor at effective concentrations until after it has become insensitive to it. The prothallus will therefore fail to form antheridia unless it is supplied with it from "elsewhere," i. e., with antheridial factor secreted into the substrate by the more rapidly growing and developing individuals of the gametophyte population. I would think that this is the case also in nature.

Mr. Barlow: This is rather curious from the biological point of view, because many mechanisms are available for distributing plants so they get away from each other. This would seem to be one in which it is essential that two or three individuals developing from the spores of the same fern should be present together before they can complete their life cycle, which is rather unusual.

Dr. Näf: It is perhaps not too rare that the completion of a life cycle depends on the proximity of two or more individuals. Raper's investigations on *Achlya* may be recalled in this connection. He showed that the formation of both male and female sex organs is a function of hormones produced not by the individual that forms the sex organ but by individuals of the opposite sex. Raper's name also recalls the myxomycetes, a quite different type of organism. The work of various investigators has led to the conclusion that the aggregation of the amoebae depends on a substance, termed acrasin, which is secreted predominantly by one type of individual and is effective on another. It would not be too difficult to add to these examples in which the completion of a life cycle depends on interaction between individuals by a chemical messenger, and therefore on proximity between them.

Returning to the bracken fern, it must also be emphasized that there are a number of developmental mechanisms which tend to prevent the simultaneous occurrence of male and female gametes on the same prothallus. In addition, Wilkie has recently demonstrated that this fern species is self-sterile. Thus, both the formation of the zygote and of the antheridium are consequent upon the occurrence of the prothalli in clusters.

Dr. Galston: I'd like to ask two questions. Is the formation of archegonia under the control of any such factors as you have described or only antheridial formation? Can you tell us anything at this time about the stability of the antheridium-promoting factor in vitro?

Dr. Näf: Preliminary investigations are in agreement with the hypothesis that archegonium formation is also under the control of a demonstrable factor, although it is probably not secreted into the medium. The experimental results as well as certain theoretical considerations further indicate that this factor can be demonstrated only if it is assayed against the prothalli of species which have special characteristics with regard to archegonium formation. I should like to emphasize that many more experiments will be required before this can be considered established. The *Pteridium* factor is quite stable. Specifically it is stable to autoclaving at the pH of the medium and to boiling for 10 min. at pH 2, but labile to boiling for the same length of time at pH 12.

Dr. Morel: When an adult gametophyte is cut into small pieces and put into a medium, it regenerates new gametophytes. What is the situation with this new gametophyte; is it the same as when you start with a spore?

Dr. Näf: I cannot give a definitive answer to this question because this problem has not been thoroughly investigated. Some incidental observations suggest, however, that the situation in regenerating gametophytes is the same as in gametophytes grown from spores.

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Ascorbic Acid As a Growth Hormone

According to the generally accepted definition, a growth regulating hormone is a substance which is produced within an organism, can be translocated far from its site of synthesis, and is active, at low concentration, in influencing the rate or the modalities of the process of growth. There is no doubt that ascorbic acid (AA) satisfies in the higher plants the two conditions of endogenous synthesis and of translocation. As to the third condition, considerable evidence has accumulated in recent years, showing that the concentration of AA and of its derivatives is an important factor in the control of the rate of growth.

In this survey of the contributions of our laboratory in this field, the following points will be considered: (1) The effects of treatment with AA on the growth of whole plants and of isolated plant parts; (2) the effects of treatment with AA on some physiological processes different from growth; (3) some metabolic changes accompanying the effects of AA on growth and related processes; and (4) the effects of auxin on the AA system.

THE EFFECTS OF TREATMENT WITH ASCORBIC ACID

Intact Plants

In 1950 Tonzig and Trezzi (20) showed that the growth of the shoot organs of oats, peas, beans, lupine, and castor beans is markedly inhibited by treatment with AA, applied either as lanolin paste to the base of the shoot or added as a solution to the nutrient medium (20). This effect was mainly due to the inhibition of cell elongation, although also the rate of cell division was decreased. Determinations of AA contents in the treated tissues showed that the experimentally

induced increase of this compound was, in most cases, within what can be considered a physiological range (20 to 100 per cent increase). It is well known, in fact, that large changes of the AA level may occur under physiological conditions, as a function of light intensity or of temperature (3). The inhibiting effect of AA treatment on cell elongation was confirmed by experiments in which geotropic and phototropic bending of stems and coleoptiles, as well as bending caused by unilateral application of auxin (indole-3-acetic acid, IAA), was inhibited by the application of a lanolin-AA paste to the shoot bases (21).

In further experiments Tonzig and Bracci have shown that in some cases cell division can also be markedly inhibited by treatment with AA. In fact, no root nodules developed on the roots of pea plants infected with *Rhizobium leguminosarum* when AA at concentrations ranging from 0.1 to 0.05 per cent was added to the medium. Similarly, treatment with AA prevented the effect of the bacteria on the induction of calluses in wounded pea stems (19).

On the whole, these experiments on intact plants have shown that an increase of the internal level of AA effectively inhibits growth in a number of species and under various experimental conditions. Moreover, experiments in which AA changes in the different plant parts were determined confirm that AA is easily translocated in the plant, translocation upward being much more rapid than translocation downward.

Isolated Plant Parts

Growth by elongation of etiolated pea internode segments and *Avena* coleoptile sections appeared strongly inhibited by AA at concentrations higher than 10^{-3} *M* (12, 20). Very low concentrations of AA occasionally induced a slight stimulation, an effect which has been recently investigated by Chinoy *et al.* (1).

The fact that the stimulating effect of AA at very low cencentration appears relatively weak and scarcely reproducible, when compared with the much more consistent inhibitory effect of AA concentrations high enough to induce a significant increase of the internal level of this compound, seems to indicate that the endogenous contents of AA in the materials investigated are very close to or even higher than the concentration required for maximal growth. This condition obviously makes the study of the growth inhibiting component more susceptible to experimental investigation. On the other hand, there seems to be no ground to assume that the inhibitory component of the AA effect has a less important physiological role, in the intact plant, than the eventual stimulating component.

THE EFFECTS OF ASCORBIC ACID ON PROCESSES DIFFERENT FROM GROWTH

Plasma Viscosity

Plasma viscosity, measured as change of time of plasmolysis in hypertonic solutions, was shown by Tonzig and Trezzi (23) to be decreased by AA treatment under a number of experimental conditions and in quite different plant materials. Also the water-holding capacity markedly decreased in the AA treated tissues (22). This suggested that changes of the physico-chemical state of the cytoplasm could play an important role in mediating the effects of AA on water uptake and on growth (18, 22).

Respiration

The oxygen uptake of pea internode segments was markedly inhibited by the presence in the medium of AA at concentrations higher than 3×10^{-3} *M*. At concentrations which inhibit growth by 40 to 50 per cent, an apparent 20 per cent decrease in respiration rate was observed; this value rose to above 35 per cent inhibition when the data were corrected for the O_2 uptake due to the enzymatic oxidation of AA in the medium (Figure 1). A very strong AA oxidase activity appeared to develop at the cut surfaces of the segments (16, 25) (Table 1).

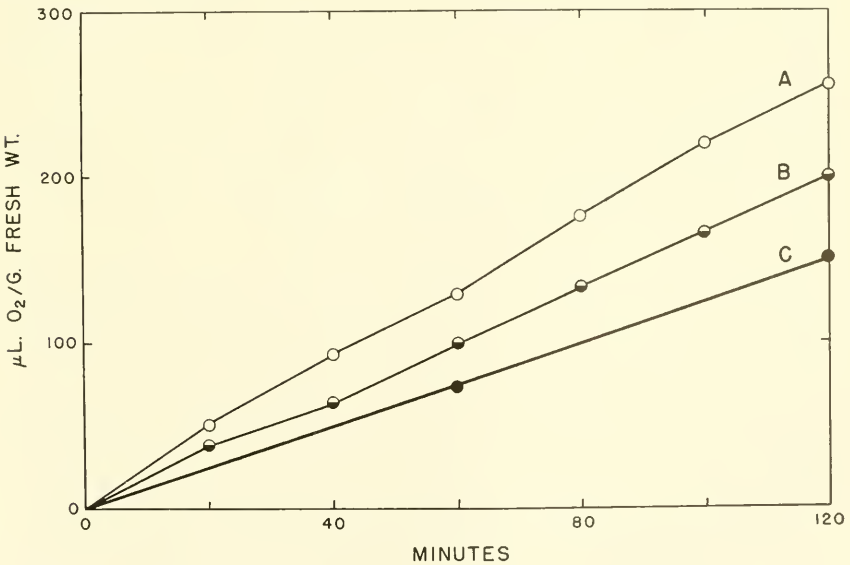

Fig. 1. The effects of ascorbic acid treatment on O_2 uptake of pea internode segments. **A**, control; **B**, ascorbic acid (2×10^{-3} *M*) corrected for autooxidation only; **C**, ascorbic acid (2×10^{-3} *M*) corrected for autooxidation and enzymatic oxidation.

Table 1. The effects of ascorbic acid (AA) on growth and internal contents of AA and dehydroascorbic acid (DHA) in pea internode segments. (E. Marrè and G. Laudi, unpublished.)

Molar Concn. of AA in the Medium*	Growth As Per Cent Increase of Fresh Wt.	AA, μ Moles/g Initial Fresh Wt.	DHA, μ Moles/g Initial Fresh Wt.
0	25	4.5	0.72
5×10^{-4}	21	5.1	0.88
2×10^{-3}	18	5.3	0.95
6×10^{-3}	17	5.5	1.15
10^{-2}	14	5.3	1.35
5×10^{-2}	13	4.7	1.65

* Medium buffered with 0.05 *M* phosphate, pH 6. For experimental conditions see (5).

Glutathione Oxidation/Reduction Equilibrium

In the pea internode segments, AA at growth inhibiting concentrations consistently increased the ratio of oxidized to reduced glutathione (7). This effect seems quite interesting, as it has been shown that the oxidation-reduction state of glutathione is an important factor in growth regulation (6, 7).

It has to be emphasized that all of these physiological parameters (plasma viscosity, water holding capacity, respiration, reduced to oxidized glutathione ratio, as well as growth by cell extension and cell division), are also influenced — under identical experimental conditions — by auxins. However, the auxin induced effects are always opposite in direction to those induced by treatment with AA.

METABOLIC CHANGES ACCOMPANYING THE EFFECTS OF ASCORBIC ACID ON GROWTH AND RELATED PROCESSES

The fact that AA seems to behave, at least in most cases, as an antagonist of auxins, suggested the possibility of some kind of biochemical competition between the two types of substances or between their active derivatives. This hypothesis appears consistent with the results of experiments showing that the amount of free (easily extractable) auxin increases, while bound auxin decreases in pea stem segments treated with AA. Moreover, diffusion of auxin from isolated oat coleoptile sections was accelerated by treatment with AA (24).

A different approach to the problem of the mechanism of action of AA was suggested by some investigations on its fate when externally supplied. Moreover, the effect of AA and its derivatives on metabolic systems in vitro was investigated.

A first series of experiments showed that AA, when supplied as

such, accumulated in the treated tissues largely in the oxidized form, dehydroascorbic acid (DHA) (Table 2). It was also observed that the inhibition of respiration and growth is much better correlated with the internal concentration of DHA than with that of AA (12) (Figure 2). Moreover, pretreatment of the pea internode segments with 0.5 per cent diethyl dithiocarbamate (an inhibitor of AA oxidase), which markedly inhibited the accumulation of DHA within the treated tissues, consistently reduced and in some experiments completely suppressed the inhibiting effect of AA treatment on growth (17) (Table 3). These results were interpreted as evidence that DHA, rather than AA, is the real inhibitor in the case of the treatment with AA. In support of this view, a series of experiments on the effects of AA, DHA, and 2,3-diketogulonic acid on metabolic systems in vitro, show that DHA at concentrations between 10^{-4} and 10^{-3} *M* markedly inhibits several dehydrogenase systems (14), as well as the oxidative and phosphorylative activity of mitochondrial preparations (2, 10) (Figure 2). This seemed to provide a satisfactory explanation for the inhibitory effect of AA on respiration in vivo, and also to suggest that at least part of the growth effect could depend on the inhibition of some step of respiratory metabolism.

Table 2. The effects of diethyldithiocarbamate (DIECA) pretreatment on growth inhibition by ascorbic acid (AA) (11).

AA in the Medium, Mg/Ml	Growth As Per Cent Increase of Fresh Wt. in 3 Hrs.		Per Cent Inhibition Due to AA	
	No pretreatment	Pretreatment with DIECA	No pretreatment	Pretreatment with DIECA
0.0	14.6	13.3		
2.0	10.7	13.4	26	0
0.0	9.5	8.7		
2.0	3.0	6.2	68	29
0.0	6.3	6.7		
2.0	4.6	6.7	37	0
0.0	12.9	11.3		
2.0	6.4	7.4	46	35
0.0	9.3	8.4		
2.0	5.8	7.8	38	7
0.0	6.0	6.5		
2.0	4.7	5.6	22	14
Average				
0.0	9.6	9.1		
2.0	5.8	7.8	40	14

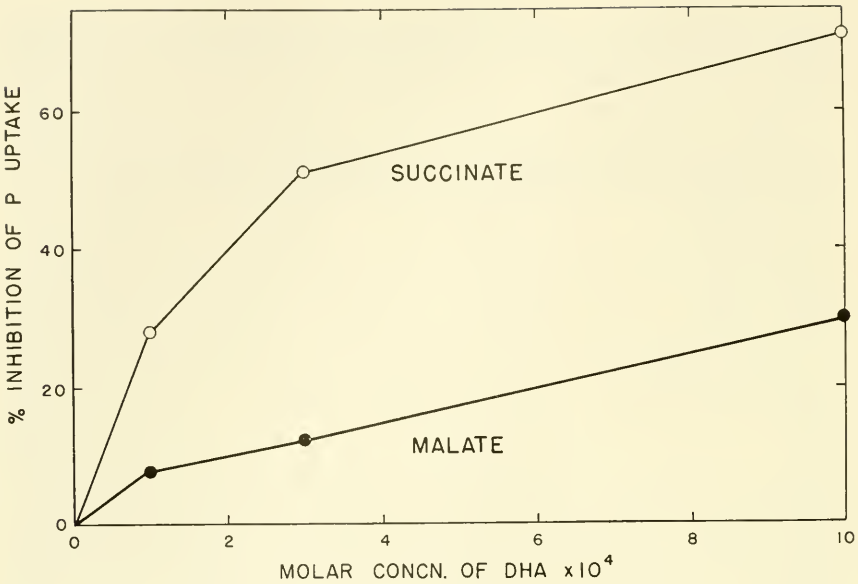

Fig. 2. The effects of dehydroascorbic acid (DHA) on phosphorylation coupled to malate and succinate oxidation of mitochondria from pea internode tissues (Forti, 2).

In addition, the decrease of the reduced to oxidized glutathione ratio in the AA treated tissues appeared fully consistent with the hypothesis that the real inhibiting agent is DHA, as the enzyme glutathione-dehydroascorbic acid reductase was found present in the *Avena* coleoptile and pea internode tissues (11, 27).

THE EFFECTS OF AUXIN ON ASCORBIC ACID METABOLISM

According to the working hypothesis suggested by the above-mentioned results, AA induced inhibition of growth and respiration might be conveniently interpreted as due to the rise of the DHA:AA ratio, followed by a corresponding decrease of the reduced to oxidized glutathione ratio, and by secondary effects such as changes of the physico-chemical state of cytoplasm and the activity of enzyme sys-

Table 3. The effects of increasing auxin concentrations on AA and DHA contents in pea internode segments (23).

	IAA Concentration in the Medium P.P.M.				
Effect Observed	0	10	100	1,000	3,000
Growth as per cent elongation .	4	16	12	4	1
AA, μg/gm initial fresh wt. . . .	403	429	415	391	381
DHA, μg/gm initial fresh wt. .	78	56	63	89	99

tems. The observation that — besides growth — plasma viscosity, respiration, and glutathione are also influenced by auxin, although in an opposite direction than by AA, indicated a close connection between the mechanisms of action of these substances. This hypothesis found further support in experiments showing that IAA markedly influences in vivo the oxidation-reduction state of AA (4, 9, 25). When added to the medium at concentrations up to the optimal for growth stimulation (10 p.p.m. for the pea internode segments), IAA increased the AA content and decreased the DHA content in the tissues. At higher, relatively growth inhibiting concentrations, IAA induced an opposite effect, that is, a decrease of the AA:DHA ratio (Table 4). It was also found that in etiolated pea seedlings the removal of the apex, a center of production for native auxin, causes a rapid increase of DHA and a decrease of AA. In all of these cases, the decrease of DHA paralleled growth stimulation, and its increase paralleled growth inhibition (13).

The possibility that auxin could affect growth and metabolism at least in part through a direct effect on enzymes controlling the oxidation-reduction state of the AA system prompted a series of in vitro investigations on this aspect of the problem. A relatively modest but significant inhibition of AA-oxidase activity of cell-free extracts or of partially purified preparation from pea internodes was observed in these experiments (5), which is in agreement with the previous results of Wagenknecht *et al.* (26) on different materials. Some inhibiting action of auxin on AA oxidase from lettuce seeds has also been recently reported by Mayer (15). However, in other experiments this in vitro effect of auxins on AA oxidation appeared poorly reproducible. It appears that further investigations on the mechanism of oxidation of AA in the intact tissues are needed before a fruitful study of a possible direct effect of auxins on AA oxidase can be demonstrated.

On the other hand, some investigations on the enzyme systems involved in the reductive metabolism of the AA system led to interesting

Table 4. The internal concentration of ascorbic acid (AA) and of dehydroascorbic acid (DHA) in segments of pea internodes treated with ascorbic acid.

	μM/g fresh wt.	
Time of Treatment	AA	DHA
0	1.7	0.37
120 min.	2.0	0.61

and reproducible results. Monodehydroascorbic acid reductase, an enzyme catalyzing the transfer of electrons from the reduced pyridine nucleotide coenzymes to a partially oxidized form of AA, was markedly inhibited by concentrations of indole-3-acetic and 2,4-dichlorophenoxyacetic acids higher than 10^{-4} *M*. This provides a good basis for the interpretation of the effect of superoptimal concentrations of auxin on the level of DHA in the intact cells. It also gives a first indication that a pyridine coenzymes-monodehydroascorbic acid reductase-AA system could play a significant role in electron transfer from respiratory substrates to oxygen (8).

On the whole, these attempts to demonstrate in vitro an action of auxin on AA metabolism, though not yet leading to definitive conclusions, seem to support the hypothesis that a direct control by auxin of the oxidation-reduction state of the AA system could be of importance in mediating the final physiological effects of the hormone.

SUMMARY

AA is considered to be a hormone involved in growth regulation. Almost all of the available evidence indicates that a rise in the AA supply of a tissue (or of its endogenous synthesis) induces a depression of the growth rate. This does not exclude that low amounts of AA could be promotive, or perhaps necessary, for growth.

The increase of AA in a tissue seems to inhibit growth essentially through the concomitant larger increase of DHA, which appears to be the real inhibiting substance. The inhibiting effect of DHA on respiratory enzyme systems in vitro provides a basis for the interpretation of the in vivo effects on respiration and glutathione oxidation-reduction state, as well as those on plasma viscosity and growth.

On the other hand, the mechanism of action of AA appears strictly connected with that of auxins. In fact, treatment in vivo with AA influences the distribution of auxin in the tissues; and, correspondingly, treatment with auxin affects the oxidation-reduction state of the AA system.

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DISCUSSION

Dr. Vlitos: I wonder if Dr. Marrè has any recent information on the possible role of ascorbigen?

Dr. Marrè: I don't have any direct information. The recent findings of Prochazka could be of considerable importance in this regard, especially if it were shown that ascorbigen is generally present in higher plants and not only in Cruciferae. If a derivative of auxin could bind with a precursor or a derivative of ascorbic acid, the compound thus formed could possibly interact with some enzyme system involved in the oxidation or reduction of ascorbic acid. This could help in understanding the effect of auxin on the reduced to oxidized ascorbic acid ratio in vivo and also the inhibiting action of auxin on ascorbic acid oxidase in vitro which has been reported in different laboratories. This in vitro effect is poorly reproducible, so that it seems more probable that it is due to some derivative of IAA formed in the cell-free preparations than to pure auxin. But, of course, at the present time this is just something between a working hypothesis and pure speculation.

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Coumarins and Their Role in Growth and Germination

Coumarin derivatives are of comparatively wide occurrence in plants. These compounds occur largely in the form of their glycosides; as a result, even when present in high concentrations they are frequently physiologically inactive [Dean (13), Wawzonek (80)]. Coumarin itself has been shown to occur as the lactone; although being very active physiologically, it is very difficult to detect, because of lack of a sensitive method. In most plants it probably occurs only in very small amounts. The glycoside of the opened lactone ring, melilotoside, has also been found in plants.

Coumarin does not give any very simple reactions and is not fluorescent. Only after alkaline hydrolysis does it give a diazo reaction and show fluorescence on exposure to ultraviolet light. This is due to the opening of the lactone ring which proceeds readily under alkaline conditions.

The coumarin molecule is stable but undergoes substitution reactions fairly easily. Samuel (71) has measured the charge density of coumarin, which resembles that of naphthalene. Thus, coumarin is attacked by nucleophilic reagents at position 4 and is susceptible to electrophilic substitution at positions 3, 6, and 8.

Evidence for metabolism of coumarin is available only in a few cases. Kosuge and Conn (25) studied the synthesis of coumarin in sweet clover and concluded that trans-cinnamic acid is the most effective precursor. Synthesis was much more efficient in shoots than in roots. Most of the other papers have been concerned with the breakdown of coumarin and its derivatives. James *et al.* (22) have shown that aesculetin, a hydroxycoumarin derivative, is oxidized by phenolase. Mead *et al.* (46) have shown that coumarin is hydroxylated in rabbits and that as a result 3, 7, and 8 hydroxycoumarins are formed but neither

4 nor 6 hydroxycoumarin. Mason (37) reviews a number of cases in which unsubstituted benzene or naphthalene rings are hydroxylated in animal tissues and Fernley and Evans (14) suggested a mechanism by which bacteria metabolize such compounds. Thus, it seems very possible that in plant tissues also, coumarin can be broken down either by ring opening and subsequent hydroxylation or it can be detoxicated by hydroxylation of the coumarin ring. The latter could be due to the action of some hydroxylating enzyme or to some mixed function oxidase [Mason (37)]. Phenolase itself has been shown to hydroxylate only monophenols but not unsubstituted benzene rings.

The only evidence available for coumarin breakdown in plants is that provided by Mayer (38) who showed that extracts of lettuce seeds (variety 'Progress') can destroy coumarin but that extracts of seeds pretreated with coumarin were unable to do so.

During the last eight or nine years numerous references to the physiological action of coumarin have appeared in the literature.

In animals coumarin has been shown to have a rather general hypnotic and narcotic effect (13, 80), while coumarins and especially furanocoumarins are toxic to fish (74). Little work on specific effects of coumarin on animal metabolism appears to have been reported.

At the cellular level it has been shown (2) that coumarin causes chromosome breakage in resting and in dividing nuclei of *Allium*. Quercioli (64, 65) and Steinegger and Leupi (75) have also shown that coumarin derivatives affect chromosomal behavior during mitosis. This effect results mainly in disorganized spindle formation (c-mitosis). Cornman (11) showed that very high coumarin concentrations inhibit mitosis. Winter (81), on the other hand, claims that low coumarin concentrations stimulated cambial division in seedlings of *Beta vulgaris*.

As regards plant material, most of the references are concerned with the inhibitory action of coumarin in growth and germination. Inhibition of germination by coumarin has been shown by many authors [Nutile (54), Sigmund (73), McLane and Murneek (35), Rousseau (70)] as well as in our laboratory. The latter work will be dealt with in detail later on. Inhibition of potato sprouting has been reported by Moewus and Schader (49). Growth inhibition has also been frequently reported; Audus and Quastel (5) commented on the phytostatic action of coumarin, and Audus (4) showed this inhibition to be reversible on the removal of coumarin. Root growth inhibition has also been often described, for example, by Goodwin and Taves (16), Pollock, Goodwin, and Greene (63), and Moewus (50). Burström (9), while studying the inhibitory action of coumarin on the growth of epidermis cells of roots, suggested that it affects cell wall tensibility. Other inhibitory effects which have been shown are abolition of

apical dominance in sugar cane by Burr (8), reduction of permeability by Guttenberg and Beythien (19), Guttenberg and Meinl (20), and Reiff and Guttenberg (66), and growth inhibition in chicory by Graillot (18). The inhibitory action of coumarin on barley growth and its possible use in brewing has been discussed by Cook (10) and by Kirsop and Pollock (24).

A specific effect of coumarin on dehydrogenases has been claimed. Marrè (36) states that coumarin, at concentrations of 10^{-3} *M* inhibits glucose-6-phosphate dehydrogenase activity in vitro. In contrast, Kuhn, Pfleiderer, and Schulz (26) claim that 10^{-2} *M* coumarin enhances by two- to threefold the citric acid dehydrogenase activity of maize seeds. No in vitro effect of coumarin (0.7×10^{-3} *M*) on dehydrogenase activity of lettuce seeds could be shown by Mayer, Poljakoff-Mayber, and Appleman (45) although when the seeds were germinated in the same concentration of coumarin, the development of dehydrogenase activity was inhibited in vivo.

A relationship between the effects of coumarins and auxins in growth and metabolism has been suggested by Libbert (32) and Reinders-Gouwentak and Smeets (67). But Libbert and Lübke (33, 34) failed to prove that scopoletin is identical with the natural inhibitor. Thimann and Bonner (79), using the straight growth test, found that coumarin inhibited the IAA induced growth of *Avena* coleoptiles, and showed that this inhibition could be reversed by BAL. This, however, is contradicted by San Antonio (72), who reported that coumarin does not inhibit *Avena* elongation in the presence of IAA. He, however, used the curvature test. Gortner, Kent, and Sutherland (17) assign to the coumarin derivatives, *p*-coumaric and ferulic acids, an effect on IAA oxidase. An inhibition of this enzyme system by coumarin itself has been reported by Blumenthal-Goldschmidt (6).

Reports of stimulatory effects of coumarin are of special interest. It seems very probable that the action of coumarin in stimulating or inhibiting growth and germination is closely related to its concentration. Germination stimulation similar to that caused by IAA was shown by Lavollay and Laborey (29) for barley, using 10^{-7} to 10^{-10} *M* coumarin, while Evenari observed stimulation by low concentration in lettuce. Van Sumere *et al.* (76) showed that coumarin enhanced the germination of uredospores of wheat rust. Miller and Meyer (47) found that coumarin in concentrations of 1 to 200 p.p.m. stimulated the leaf expansion of *Chenopodium* in the presence of KNO_3 and glucose. Winter (81), as already stated, observed stimulation of cambial activity in *Helianthus* and *Phaseolus* plants, caused by the application of 0.5 mg. coumarin per plant. Tarragan (77) demonstrated stimulation of the growth of tomato roots in tissue culture by very low cou-

marin concentrations (10^{-12} *M*). Norman and Weintraub (53) included coumarin in the list of chemicals causing root initiation. Gigantism in initial cells of *Marchantia,* caused by coumarin treatment, was reported by Rousseau (70). Buis (7) showed that 10 to 100 p.p.m. of coumarin affected both the size and number of sprouts of maize plants, an effect which may be closely related to the abolition of apical dominance (8).

Neumann (51, 52, and unpublished[1]) in our laboratory has shown that coumarin can stimulate the growth of a number of plant tissues. He showed that the elongation of sunflower hypocotyls is stimulated by coumarin concentrations of 250 p.p.m. and that inhibitory effects were only noted at 1,000 p.p.m. Similar effects have been shown by Neumann for pea stems, bean hypocotyls, and *Avena* coleoptiles, although in this latter material the effect was less pronounced and limited to a very narrow range of concentrations. This stimulation was similar to that obtained with 1-naphthaleneacetic acid. Thus the stimulatory action of coumarin on the growth of plant tissue is not confined to any one plant tissue but is a more general response. It seems as though coumarin can act as a genuine auxin since, first, in various ranges of concentrations it stimulates elongation of stem tissue; secondly, though it generally inhibits growth of roots, in very low concentrations it stimulates the latter; thirdly, it stimulates the expansion of leaf tissue, cambial activity, and root initiation. The recent findings of Misra and Patnaik (48) also lend support to such a view.

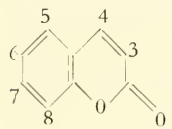
The relation between structure and activity of coumarin and its derivatives has been studied in detail only in a few cases. Goodwin and Taves (16) have shown that coumarin is the most powerful root growth inhibitor but some of its derivatives were almost as active as coumarin itself, namely, 7,8-dihydroxycoumarin, 7,8-dihydroxy-4-methylcoumarin, 8-methylcoumarin, and coumarin-3-carboxylic acid. 3-Methyl substitution greatly diminished the inhibitory action on root growth (Table 1). San Antonio (72), on not very convincing evidence, claimed that the lactone structure is not essential for inhibitory action of coumarin on growth and germination. He tested only few derivatives, and these under special conditions. Mayer and Evenari (43) have shown that germination inhibition by coumarin is definitely a function of the unsaturated lactone structure. All derivatives of coumarin were less active than coumarin itself. However, it is of special significance that very appreciable differences were observed by Mayer and Evenari between the response of wheat and lettuce seed to coumarin,

[1] *Added in proof:* Neumann, J. The nature of the growth-promoting action of coumarin. Physiol. Plant. 13: 328–341. 1960.

indicating specific differences between species in their response. Goodwin and Taves, and Mayer and Evenari stress the importance of the unsaturated lactone linked to an aromatic nucleus.

The only data available on stimulatory action as related to structure are those of Neumann (51). He found that both 3- and 4-hydroxycoumarins are growth inhibitors at concentrations at which coumarin itself stimulates (Table 1). 3-Methyl and 3-chloro substitution reduced stimulatory activity of coumarin considerably, but the substances still had some activity at concentrations at which the hydroxy derivatives were inhibiting. Trans-*o*-coumaric acid and melilotic acid (dihydro-*o*-

Table 1. The effect of various coumarin derivatives on germination and root growth.*

Derivative of 	Effect on Germination of Lettuce Seed	Effect on Root Growth of *Avena*	Effect on Stem Growth of Sunflower
Parent compound. .	Strong inhibitor	Strong inhibitor	Stimulator
3-Methyl-	Weak inhibitor	Weak inhibitor	Weak stimulator
4-Methyl-	. . . . .	Weak inhibitor	
5-Methyl-	. . . . .	Weak inhibitor	
6-Methyl-	. . . . .	Weak inhibitor	
7-Methyl-	. . . . .	Weak inhibitor	
8-Methyl-	. . . . .	Strong inhibitor	
3-Hydroxy-	Very weak inhibitor	Very weak inhibitor	Inhibitor
4-Hydroxy-	Very weak inhibitor	. . . . .	Inhibitor
6-Hydroxy-	Very weak inhibitor	. . . . .	Stimulator
7-Hydroxy-	Very weak inhibitor	. . . . .	
3-Chloro-	Weak inhibitor	. . . . .	Weak stimulator
6-Chloro-	Weak inhibitor	. . . . .	
Coumarin-3-carboxylic acid.	Very weak inhibitor	Strong inhibitor	
7,8-Dihydroxy-	Almost not an inhibitor	Strong inhibitor	
7,8-Dihydroxy-4-methyl-	. . . . .	Strong inhibitor	
7-Hydroxy-4-methyl-	Almost not an inhibitor	Very weak inhibitor	
5,7-Dihydroxy-	Almost not an inhibitor	Very weak inhibitor	
6-Methoxy-7-hydroxy-	Almost not an inhibitor	Very weak inhibitor	

* The results on germination were taken from Mayer and Evenari (44): Inhibition index of 1.0 in Mayer and Evenari = strong inhibition. Inhibition index 0.9 to 0.25, weak inhibitor. Inhibition index <0.25, very weak inhibition. The results of root growth were taken from Goodwin and Taves (16): First group—strong inhibition; second group—weak inhibition; third group—very weak inhibition. The results on stem growth were taken from Neumann (52).

coumaric acid) were less active in promoting elongation than coumarin. Here again the need for the lactone structure seems clearly established. The fact that both 3- and 4-hydroxy substitution converts coumarin from stimulator to inhibitor is somewhat surprising. On theoretical grounds it would be expected that 3-substitution affects the ring quite differently from 4-substitution. From the work with coumarin derivatives it seems that the double bond is of special significance in the stimulation of growth, as well as in its inhibition. Norman and Weintraub (53) also stressed the importance of this double bond, and pointed out that all the naturally occurring active derivatives have the 3 and 4 positions free.

The 3- and 4-hydroxycoumarins are not only themselves inhibitors but also counteract the stimulation caused by coumarin. Hydroxy substitution may markedly affect the activity of other sites in the coumarin molecule and change the ease of ring opening. The 4-hydroxycoumarin can enolize readily with the carbonyl group but it is less easy to visualize such an effect for the 3-hydroxycoumarin. 6-Hydroxycoumarin was almost as active as the unsubstituted coumarin in promoting extension growth. This work of Neumann is the first attempt to elucidate the connection between the structure and growth stimulatory action of the coumarin derivatives and clearly requires extension.

The fact that germination inhibition and growth stimulation require somewhat different structural configuration is of special importance (Table 1). Germination and growth are differently affected by various agents and they are apparently two different processes, germination being a process of its own and not simply a result of embryo growth. Both processes involve numerous steps, one or more of which may be rate limiting. Altering the rate of such a step will materially affect the whole process and will consequently be of great importance in the development of the organism. Coumarin may be such an agent which acts on different rate-limiting steps in growth and germination.

In our laboratory an attempt was made to elucidate the mode of action of coumarin by studying the effect of coumarin on various metabolic changes in germinating lettuce seeds, as well as its effect on the activity of various enzymes both in vitro and in vivo. These results are summarized in Table 2.

Generally speaking, coumarin, when applied to whole seeds, i.e., in vivo, inhibits germination and therefore various enzymes are not formed or do not increase in activity. However, proteinase, and a neutral lipase are very markedly inhibited by coumarin in vitro as well (Table 2). It is known that even dry seeds already contain a considerable number of enzymes and their presence can be demonstrated

Table 2. The effect of coumarin in vivo and in vitro on metabolism and enzymatic activity in germinating lettuce seeds and seedlings, as related to the activity in water-germinated lettuce.

Metabolite or Enzyme	Effect in vivo (When Germinated in Coumarin)	Effect in vitro (When Coumarin Added to Reaction Mixture)	Reference
Sucrose utilization	Not affected during first 24 hrs. but later almost completely blocked		55
Glucose accumulation	Completely blocked		55
Fat utilization	Fat breakdown blocked		55, 61
Free fatty acid liberation	Inhibition during 48 hrs. and stimulation during next 24 hrs.		61
Volatile fatty acids	Increase in amount in first 48 hrs. then decrease		61
Phytin breakdown	Prevents phytin breakdown		40
Proteinase	Activity remains more or less on same level as in dry seeds	Inhibits activity of enzyme extracted from imbibed seeds and young seedlings	57
Lipase (neutral)	Inhibits increase in activity. Activity remains as in dry seeds	Inhibits strongly	68
Lipase (acid)	Inhibits increase in activity. Activity remains as in dry seeds	No effect	68
Phytase	Strong inhibition	Slight inhibition	40
Ascorbic acid oxidase	No effect	No effect	41
Phenolase	Inhibition of activity	Slight stimulation of activity	42, 44
Catalase	No effect	No effect	56
Peroxidase	No direct effect. If no germination, no increase in peroxidase activity. Enzyme activity correlated to per cent germination	No effect	58
Dehydrogenases	Prevents increase of activity that usually follows germination	No effect	45
DPNH oxidase	No effect	No effect	42
Oxygen uptake of whole seeds	In beginning slight stimulation, then inhibition		31
Oxygen uptake of lettuce mitochondria	Inhibition of particulate activity parallel to inhibition of germination	20 per cent increase in oxygen uptake	59

in water extracts prepared from the seeds. But the amount of each of these enzymes, or their activity, is rather limited and increases during germination. The liberation, or the activation, of many enzymes is known to be closely linked with proteolytic cleavage of enzymogens or protein complexes of some kind. If such liberation of enzymes is a prerequisite for germination and growth, then some of the inhibitory action of coumarin might be the direct result of its inhibition of proteinase activity.

Ron and Mayer (69) have shown that coumarin at a concentration of 1.4×10^{-3} *M* completely prevents the growth of cultures of *Chlorella vulgaris*. A somewhat lower concentration (6.85×10^{-4} *M*) does not prevent cell multiplication but causes an increase in cell diameter. The diameter of the controls was 5 μ while that of cells grown in coumarin was 6.8 μ. This represents an increase by a factor of approximately 2½ in cell volume. The increase of cell size was paralleled by an increase in oxygen uptake per cell. However, when calculated per unit of dry weight, coumarin caused a decrease in oxygen uptake as the dry weight of cells grown in coumarin increased. Substances that induce an increase in oxygen uptake are usually considered as potential uncouplers of oxidative phosphorylation. There is a tendency to consider the coumarin derivatives as uncouplers; this is mainly based on the uncoupling action of dicoumarol (30), although dicoumarol differs from the naturally occurring coumarins, especially in the steric configuration near the double bond of the lactone. Ron and Mayer do not ascribe such an uncoupling action to the coumarin effect in *Chlorella,* but an uncoupling action of coumarin may be hinted at by the work of Levari (31) who showed that coumarin stimulates the respiration of lettuce seeds (variety 'Progress') during the first six hours of germination, and by the fact that coumarin slightly increased (20 per cent) the oxygen uptake of mitochondria (59). But in all these cases the results do not permit any conclusions, as they were not always calculated on a protein or a nitrogen unit, and no direct measurements of phosphorylation were made.

Aloni (1) studied the ability of lettuce mitochondria to liberate inorganic phosphorus from ATP (adenosine triphosphate). Addition of coumarin to such reaction mixtures did not cause any significant increase in phosphorus liberation. Apparently coumarin did not affect the ATPase activity of lettuce mitochondria.

The effect of coumarin on phosphorus metabolism of the seeds is especially interesting. Half of the phosphorus in dry lettuce seeds is present in the form of phytin. The breakdown of this phytin begins immediately with the onset of germination and phytin has disappeared completely in lettuce seedlings germinated for three days

(40). The phosphorus compounds in lettuce seeds were fractionated into trichloracetic acid (TCA) soluble, HCl soluble, and hydrolyzable during 7 min. at 100° C., and into residual phosphorus (Poljakoff-Mayber, unpublished). The TCA soluble fraction decreased with germination while the others increased. The TCA fraction contains phytin, phosphorylated sugars, as well as adenosine phosphates and small amounts of inorganic phosphate. As germination proceeded, the amount of ATP apparently decreased but increased again with subsequent growth. When the seeds were germinated in coumarin the ATP spot on the chromatograms did not decrease in size and the fructose 1,6-phosphate spot was absent (15). These observations may be related to the fact that phytin decomposition was prevented and phytase activity was almost absent in seeds germinated in coumarin. Since, as has been pointed out above, half of the phosphorus in dry seeds is present as phytin, coumarin may in this way block the inorganic phosphorus supply. On the other hand, the changes in the phosphorylated intermediates noted above may indicate a more direct interference in phosphate metabolism. In this connection it is of interest that mitochondria-free extracts of peas germinated in coumarin show glycolytic phosphorylation, i.e., ATP formation, to the same extent as do the controls. Thus, here neither the glycolysis nor the enzymes transferring phosphate to ATP were affected by the coumarin (Mayer, unpublished). It is possible that in lettuce glycolysis begins at a somewhat later stage of development than in peas and that the energy releasing processes are different during germination proper and during the subsequent growth. This is suggested by the changes in activity of the tricarboxylic acid cycle enzymes during germination (60).

Some interesting information can be obtained from a study of interaction between coumarin and thiourea and their effect on germination and growth. Thiourea stimulates the germination in the dark of light-sensitive lettuce seeds but strongly inhibits the growth of the lettuce seedlings. Treatment of seeds with coumarin makes them more sensitive to thiourea, i.e., the maximum stimulatory effect on germination is evident at lower concentrations of thiourea (2.5×10^{-2} *M*). The higher concentration (5×10^{-2} *M*), which in the absence of coumarin causes the maximal effect, is much less effective in its presence. As a result an optimum response curve to thiourea appears in coumarin-treated seeds which is absent in non-treated ones in the range of thiourea concentrations used (62). Thiourea and coumarin also interact in their effect on the growth of lettuce seedlings. An antagonistic effect of the two substances in their effect on germination is also reported by Lavollay and Laborey (27, 28). The interaction between the two substances in their effect on germination and growth may be

Table 3. The combined effect of gibberellic acid and coumarin on germination of 'Grand Rapids' lettuce seeds, at 25° C. in the dark.

Molar Concentration of Gibberellic Acid	Percentage Germination at Various Molar Concentrations of Coumarin			
	0	10^{-4}	3.3×10^{-4}	6.6×10^{-4}
0	74.0	13.5	2.0	0.0
0.95×10^{-4} (33 p.p.m.)	97.5	32.0	1.5	0.0
1.9×10^{-4}	100.0	45.0	8.5	0.7
3.8×10^{-4}	100.0	95.0	21.0	10.0

[2] *Added in proof:* Mayer, A. M. Joint action of gibberellic acid and coumarin in germination. Nature. 184: 826, 827. 1959.

through oxidative systems. Thiourea inhibits certain oxidative enzymes in lettuce (40, 42) and causes others to function at an earlier stage (60). Coumarin may be linked to this process via its interference with the phosphorus metabolism. Another possible link for this interaction is the coumarin-destroying enzymes. As already mentioned, lettuce seeds can metabolize coumarin and it is possible that this ability is modified by thiourea, as thiourea affects phenolase activity and inhibits its catecholase activity more than its cresolase, i.e., hydroxylating, activity (39, 42, 44). Thus, other hydroxylating enzymes containing metal as an active group may be similarly affected.

It is interesting to note that gibberellic acid reverses the inhibition of germination caused by coumarin (Mayer, unpublished[2]). This reversal is a function of both coumarin and gibberellic acid concentrations (Table 3). Such a reversal seems to be absent in growth inhibition of lettuce seedlings caused by coumarin. But Kato (23) reports also partial reversal of growth inhibition on cucumber shoots caused by coumarin. Both coumarin and gibberellin can be considered as naturally occurring growth substances. It is tempting to suggest that exogenous coumarin affects in some way the growth substance metabolism or turnover. IAA itself need not necessarily be involved, although the observation that coumarin inhibits IAA oxidase of pea epicotyls (6) suggests that it may be. The observation by Housley and Taylor (21) that the β-inhibitor from potatoes contains a coumarin derivative — scopoletin — may be relevant in this connection.

SUMMARY

Coumarin appears to be a widely distributed compound occurring in many plants and seeds. It can stimulate or inhibit growth and germination, depending on the plant species, the concentration, and the presence or absence of compounds modifying its action.

To elucidate the mode of its action, its effect on the metabolism and enzymatic activity in germinating lettuce seeds has been investigated. Various effects were observed, three of which seem to be promising in explaining the effect of coumarin: (a) The inhibition of proteinase activity, which may disturb the normal enzymatic equilibrium in the germinating seed; (b) the interference with phosphorus metabolism which is liable to disturb the normal oxidative and energy transferring processes; and (c) there is much evidence to suggest that coumarin has auxin-like activity and is closely concerned with the metabolism of other growth regulating substances in the plant.

It is likely that coumarin does not always act through all of these mechanisms. In fact it seems probable that the difference in response of different plants and plant tissues to coumarin is due to its affecting different pathways, or affecting different rate-controlling steps in the same pathway. In addition, the relative importance of the various metabolic pathways probably differs between species, and, therefore, a change in rate of the same step may be of varying significance in different species or even organs. Moreover, the mode and extent of coumarin metabolism probably differs in different plants. Much further research is still needed to clarify the problem.

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DISCUSSION

Dr. Wain: In the segment test experiments where coumarin was acting as an auxin, so to speak, is it possible that the coumarin ring was opened and ortho-hydroxy cinnamic acid formed? Since cinnamic acid itself is a growth substance, was the ortho-derivative tested for these effects?

Dr. Mayer: No, I don't think ring opening occurs. If you study all the derivatives, coumarin is far the most active. An additional point is that the 3-hydroxy and the 4-hydroxy are both very strong antagonists of coumarin stimulation in growth. It is a very interesting point that both the 3- and the 4-hydroxy coumarins, which are rather different in their chemical behavior, are antagonizing growth stimulation.

Dr. Burström: Your conclusion that coumarin may act as a growth promoter is very interesting, and I recall a paper by Dr. Thimann showing growth promotion by coumarin. I don't remember all of the material, but perhaps Dr. Thimann does. We experimented with several coumarin derivatives years ago, and found a most considerable root growth promotion by daphnetin. It increased root elongation by about 50 per cent, but we didn't follow this up. It may be of interest in connection with your results.

Dr. Thimann: Yes, we did publish some work on coumarin. It was rather different from what Dr. Mayer has described in that our work was done with sections in the presence of auxin. What happened here was that in low concentrations, and over a fairly wide range, coumarin promoted growth very strongly. At levels of about 10^{-3} *M* it inhibited equally strongly (78).

The inhibiting part of the curve was log-linear and below that, from 10^{-6} to 10^{-4} *M* was the wide range of growth promotion. At the time we reported this we also studied a smaller, but less stable molecule, proto-anemonine. It gave exactly the same results but much more powerfully. I do not think this is an auxin effect since it was observed in the presence of auxin.

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Studies With C^{14}-labeled Ethylene

Ethylene is a potent physiologically-active gas affecting plants in many diverse ways. Certain species and responses are affected by concentrations as low as one part per billion in the atmosphere (8). The earlier work on the effects of ethylene upon plants and its specific roles in plant metabolism has been reviewed by Crocker (8). The more recent research, particularly that centering around the possible role of ethylene in abscission and the physiology of ripening fruits has been reviewed by others (1, 5, 13, 20). Several of the responses (epinasty of leaves, proliferation of tissues, stimulation of rooting, coloring and ripening of fruits, and acceleration of flowering in pineapple and other species) and the levels of ethylene necessary to induce these are similar to those caused by the auxins. The effect on other responses (abscission of organs, apical dominance and inhibition of lateral buds, and breaking of dormancy) appears to be the opposite of those produced by the auxins.

Quantitative proof has been obtained for the biological production of ethylene by aerobically respiring fruits (5) and from excised and intact leaves (14, 19), and indirect evidence exists that other tissues produce the gas (8).

Although many data have been accumulated to suggest that ethylene plays an important role in plant metabolism, the exact mechanism of its mode of action or production is still unknown. Because of its natural production by plants, often in sufficient concentrations to affect physiological processes and to modify development, Crocker (8) considered ethylene to be a phytohormone. Other workers, however, have questioned the validity of this classification. Biale *et al.* (5) and Addicott and Lynch (1) doubt whether ethylene has regulatory func-

tion with particular reference to fruit ripening and abscission. Conclusive proof, however, that ethylene lacks biological significance in plants remains to be established.

A limited number of experiments have been conducted with radioactive ethylene (7, 15). Buhler *et al.* (7) noted that the uptake of carbon-labeled ethylene from the atmosphere surrounding fruits was small and they believed that ethylene is probably a terminal product in fruit and cannot be further metabolized. On the contrary, field observations (13) and the rapid uptake of ethylene-C^{14} (15) suggest absorption and metabolism in young, rapidly growing plants.

The results given in this paper report some of the findings obtained to date in a long-term project designed to elucidate the biological significance of ethylene. It is believed that the radiochemical approach coupled with other techniques should clarify the importance of ethylene in plant metabolism and the labeling of suspected substrates should aid in establishing its biological mode of production.

ABSORPTION AND TRANSLOCATION EXPERIMENTS

A number of experiments were conducted on the absorption and translocation of ethylene-C^{14}, some in conjunction with studies to obtain tissue for metabolite investigations. To date *Coleus* and cotton have been the test plants. The plants were grown in the greenhouse in Vermiculite or quartz sand supplied daily with nutrient solution.

In an initial experiment ten healthy cotton plants, 'Deltapine 15' at the six true leaf stage and five plants of the green-leaf variety of *Coleus blumei* (five leaf pair stage), were treated inside a Plexiglass chamber with 1,000 p.p.m. of radioactive ethylene of 1 mc/m*M* specific activity. In addition, ethylene-C^{14} recovered from a previous experiment was liberated from a mercuric-perchlorate complex inside the chamber according to the method of Young *et al.* (21). It is estimated that a total of 0.8 millicurie of radioactive ethylene was available in the chamber for the plants to absorb. The experiment was conducted in subdued light in a fume hood for 15 hrs. The unused ethylene was evacuated from the chamber into mercuric-perchlorate solution. Simple monitoring of the treated plants with a Geiger tube indicated appreciable activity in the leaves. Intact and detached leaves were lyophilized, mounted, and pressed against No-screen X-ray film for 30 days. The radioautograms showed that ethylene was absorbed readily and uniformly over the blades, petioles, and stems. Representative detached leaves are shown in Figure 1. It can be noted that the stipules absorb the gas rather heavily. The difference between the young and old cotton blades in the density of the main veins is probably due to greater self-absorption by the thicker veins of the older leaves.

Fig. 1. Radioautograms of cotton and *Coleus* leaves after absorption of ethylene-C^{14}. Film exposed for 30 days, negative print (light areas indicate radioactivity).

Entire cotton plants at the six true leaf stage were enclosed for a period of 5 hrs. in an atmosphere of about 625 p.p.m. of ethylene-C^{14}. During the treatment period the plants were subjected to 500 foot candles of light. The leaves were harvested and oven-dried at 50° C. for 8 hrs. Following grinding the dry leaf powder was pressed at 15,000 pounds per square inch for 3 min. into smooth 6.6 $cm.^2$ briquettes of uniform texture. The briquettes were counted in a Nuclear Measurement Model PC-3 proportional-gas-flow counter. The leaves contained a total of 2,600 c.p.m. with a specific activity of 2.0 cpm/mg of dry tissue.

Translocation Experiments

A series of experiments with cotton and *Coleus* indicated that when a single attached leaf was exposed to radio-ethylene it was absorbed rapidly and transported to other parts of the plant. The concentration of ethylene in the treatment chamber varied from 600 to 660 p.p.m. and the plants were irradiated with light of 1,000 foot candles intensity during the experiments. The treated and nontreated leaves were harvested separately, dried, ground, and the tissue counted as briquettes as described above. After 5 hrs. there was seven times more activity in the nontreated leaves of cotton than in the treated leaves; after 20 hrs. the nontreated leaves contained 30 times more activity than the treated leaves. From 5 to 20 hrs. the treated leaves increased in activity about eightfold. Essentially the same relative results were obtained with *Coleus* except the actual differences in activity between the treated leaves and the untreated leaves were not as great.

It was noted in a number of experiments that the amount of ethylene absorbed and transported is greatly influenced by whether "fresh" or "aged" ethylene is used. This will be discussed in a subsequent section. In the case of cotton it was found that after 5 hrs. the treated leaf had absorbed and transported to the nontreated leaves four times more "aged" ethylene than "fresh" ethylene.

Comparison of plants maintained at less than 1 foot candle of light to plants maintained at 1,000 foot candles during the treatment period showed that the fixation of ethylene was greater in the dark, but the translocation of the metabolites was governed by the same factors, particularly photosynthesis and transpiration, which influence normal translocation. Metabolite studies will be reported in a subsequent section. At the present time the transport form of ethylene is unknown, but it appears that several metabolites may be involved.

Cotton seedlings with two true leaves were fitted into 250 ml. suction flasks with only the roots sealed tightly inside the flasks. Each flask was filled with 200 ml. of distilled water and the roots immersed to different depths. The shoots of the plants outside the flask were illuminated with 1,000 foot candles of fluorescent light but the root systems were kept in darkness. Ethylene-C^{14} (4.8 ml.) was injected into the atmosphere above the roots to give 2,400 p.p.m. inside the flasks. After 17 hrs. the plants were harvested and the leaves and roots prepared for counting. The results (Figure 2A) show that ethylene may be absorbed by roots and lower stems and transported to the leaves. Up to 8 cpm/mg of dry weight of leaves were found after 17 hrs. in some experiments. It can be seen that the depth of immersion of the roots had little effect on the amount of metabolites present in the leaves.

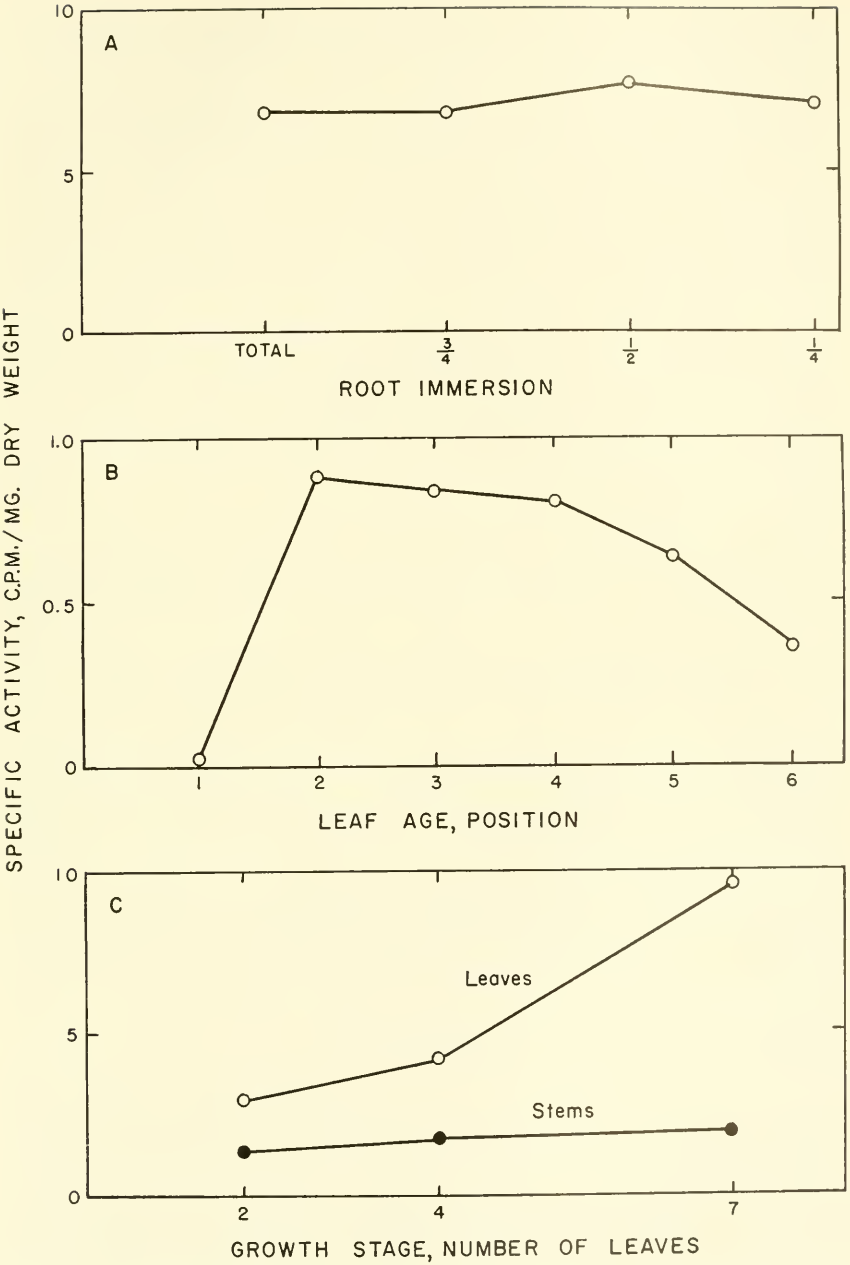

Fig. 2. **A.** Radioactivity in cotton leaves after absorption by roots immersed to different depths. **B.** Influence of leaf age on ethylene-C^{14} fixation in cotton. **C.** Absorption of ethylene-C^{14} by leaves and stems of cotton plants at three growth stages from young vegetative (2-leaf) to young fruiting (7-leaf).

A second series of cotton seedlings with two true leaves were treated in the same manner (2,000 p.p.m. of ethylene-C^{14} in the root medium). Plants were harvested for radioautographing 1, 2, 4, and 8 hrs. after introducing the ethylene. The intact plants were pressed between blotters and frozen. They were lyophilized while pressed, mounted, and radioautographed against No-screen X-ray film for one month. The radioautograms indicated that ethylene was absorbed rapidly by the roots and translocation of its metabolites occurred almost uniformly throughout the plant within the first hr. Maximum activity in the leaves was reached in 2 hrs.; however, after 4 hrs. activity decreased in the leaves indicating that the metabolites were being converted to a gaseous form and emitted. The nature of the released radioactive gas is being studied currently by means of the vapor fractometer and vibrating reed electrometer. Tentatively, the gas does not appear to be carbon dioxide.

Single Leaf Treatments

Single, detached, fully-expanded cotton leaves were treated in the light (2,000 foot candles) and in the dark ($<$ 1.0 foot candle) in a "lollipop" flask. The trials were conducted to determine the feasibility of short-term treatments in a manner similar to those used to study photosynthetic intermediates of carbon dioxide fixation. Considerably less fixation occurred in 3.5 to 5 hrs. in the detached leaf than when the single leaf was attached to the plant during treatment. There was three to five times more ethylene fixed in the dark than in the light, suggesting that photosynthesis is probably not involved directly in ethylene fixation by leaves.

When the treated leaves were oven-dried overnight at 60° to 70° C. about 79 per cent of the fixed radioactivity was lost. This suggests that a major portion of the fixed ethylene is in loose combination in the leaf. Therefore, lyophilization was used in most of the subsequent studies.

Influence of Leaf Age on Ethylene Fixation

The tops of six cotton plants in the early-fruiting stage were treated with 6.9 ml. of ethylene-C^{14} in an eleven-liter chamber. The atmosphere inside the chamber contained about 600 p.p.m. of ethylene. Light intensity was reduced to less than one foot candle during the 16 hr. exposure period. Leaves were harvested separately, numbered from 1 to 6 from the apex to the base, dried, ground, and pressed into uniform briquettes for counting. The results given in Figure 2B show that, in general, the younger the leaf the more ethylene it fixed. The number one leaves which were about 2 cm. in

width were the exception. The number two leaves contained approximately three times more activity than the oldest leaves (No. 6).

Coleus plants with four leaf pairs were treated with the ethylene recovered from the previous experiment with cotton. The experimental conditions were the same as used for cotton except 25 g. of sodium hydroxide pellets were placed in the chamber to absorb respiratory carbon dioxide and moisture. The youngest (No. 1 leaf pairs) and the oldest (No. 4 leaf pairs) fixed the most activity, while the intermediate aged leaves (No. 2 and 3) fixed the least, indicating a species difference from cotton.

Influence of Plant Age on Ethylene Fixation

Two cotton plants of three different age groups (early-fruiting the oldest) were treated with 6.20 ml. of ethylene-C^{14} to give about 350 p.p.m. of ethylene in the treatment chamber. The plants were maintained in the dark for 17 hrs. After treatment the leaves and stems of each plant were harvested separately and assayed for radioactivity.

The results (Figure 2C) indicate that the older plants absorbed more ethylene per unit dry weight and that the foliage contained higher levels than the stems.

METABOLISM OF EXOGENOUS ETHYLENE-C^{14}

"Fresh" Versus "Aged" Ethylene

In the absorption and translocation experiments as well as in other metabolite experiments it was noted that greater amounts of C^{14} were incorporated when "aged" ethylene was used compared to "fresh," pure ethylene, or the gas used immediately after releasing it from a light-proof, sealed vial. "Aged" ethylene denotes ethylene that had been recovered after experiments by mercuric-perchlorate absorption and regenerated from the complex for use (21), or ethylene that had been stored for a considerable period and then exposed to light, high humidity and temperature. The regenerated ethylene, which appears to be the most active, has been studied in some detail. The difference in absorption and fixation is illustrated by the following experiments.

Cotton plants were exposed to 10 ml. of "fresh" ethylene containing 0.4 mc. of ethylene-C^{14} (1,000 p.p.m.) for 15 hrs. in July, 1958. The leaves of the treated plants contained about 4 cpm/mg of C^{14}. Immediately following the experiment the unused ethylene was evacuated into mercuric-perchlorate and the complex stored in the dark at 0° C. for one month. The radio-ethylene was liberated from the complex (21) into a chamber containing cotton plants of the same age used in the July experiment. The plants were allowed to absorb the

ethylene for 15 hrs. and upon radioassay were found to have incorporated approximately 100 cpm/mg of C^{14}, or a twenty-five fold increase. The unused ethylene was again complexed by mercuric-perchlorate and was stored in the light in a glass vial at ambient temperatures for one year in the laboratory. Cotton seedlings were treated in the dark in an enclosed chamber for 15 hrs. with the regenerated ethylene. The treated plants possessed a specific activity of about 4,000 cpm/mg, or about a thousand-fold increase in the amount of C^{14} incorporated over that from the original "fresh" pure ethylene. Gas chromatography of nonradioactive ethylene and of the original and regenerated samples complexed for various periods of time indicate that ethylene is the only main constituent. Only minor amounts of carbon dioxide, which increases with the age of the regenerated ethylene samples, have been detected. The rate of incorporation into cell wall constituents and fixation by plants atypical of carbon dioxide indicate tentatively that the C^{14} from the "aged" samples is not contributed by $C^{14}O_2$. There is no doubt that C^{14} fixation from the "aged" ethylene is increased, but the significance of this must await the results of further tests.

Water-soluble Metabolites

Two experiments, one using "fresh" ethylene and the other using "aged" or recovered ethylene, were conducted with *Coleus* and cotton. After 15 hrs. exposure to ethylene-C^{14} the tissues were lyophilized, extracted, fractionated, purified, and the identification of some of the radioactive metabolites attempted by various procedures including column and paper chromatography, radioautography, and conventional chemical techniques. In general the radioactive metabolites were the same whether "fresh" or "aged" ethylene was used. Only the "aged" ethylene, however, gave sufficient activity in the metabolites for detailed studies. Therefore, all of the results with metabolites presented in this paper are those obtained using "aged" ethylene.

During extraction of cotton tissue it was found that one of the major radioactivity-containing fractions was water soluble. The bulk of the total radioactivity, however, remained in the residue and was not extractable by water, ether, alcohol, or other common solvents.

Characterization of the water soluble radioactive fraction was attempted first. Ten grams of lyophilized cotton or *Coleus* material were extracted by refluxing for two and one-half hrs. with 100 ml. of boiling water. This was followed by four 30-min. successive extractions using 25 ml. of water each time. The total extract was combined to give a final volume of 200 ml. and was used for subsequent fractionations. Aliquots of the stock solution were purified by fractional elu-

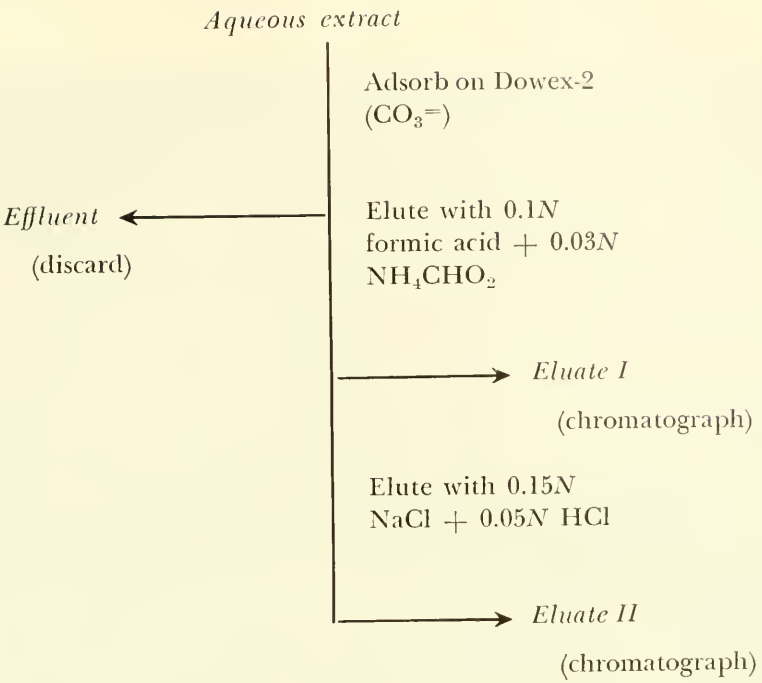

Fig. 3. Fractional elution scheme of water-soluble cotton metabolites.

tion from an anion exchange column as shown in Figure 3. The radioactive fractions were neutralized, reduced in volume, and chromatographed in two solvent systems.

Inspection of the histograms presented in Figure 4 shows at least two radioactive fractions were obtained by elution from the anion exchanger. The activity in the area of the second peak is almost 14 times that of the first peak. Strong adsorption on the column denoted a fairly acidic compound.

Chromatograms of the fractions, sprayed with Hanes and Isherwood's reagent (6), gave a positive test for phosphates and the duplicate radioautograms (Figure 5A, Sh 39 and 40) showed that the main radioactive spots corresponded with the molybdenum blue areas, but with two additional spots above them. The main metabolite from the anion exchanger was chromatographed in two solvent systems and the respective radioautograms are shown in Figure 5B, Sh 41 and 42. The same fraction after purification was chromatographed in Bandurski and Axelrod solvents (3) and sprayed for phosphate esters (Figure 5B, Sh 43 and 44). The presence of phosphate-containing compounds was demonstrated in all cases and they corresponded with

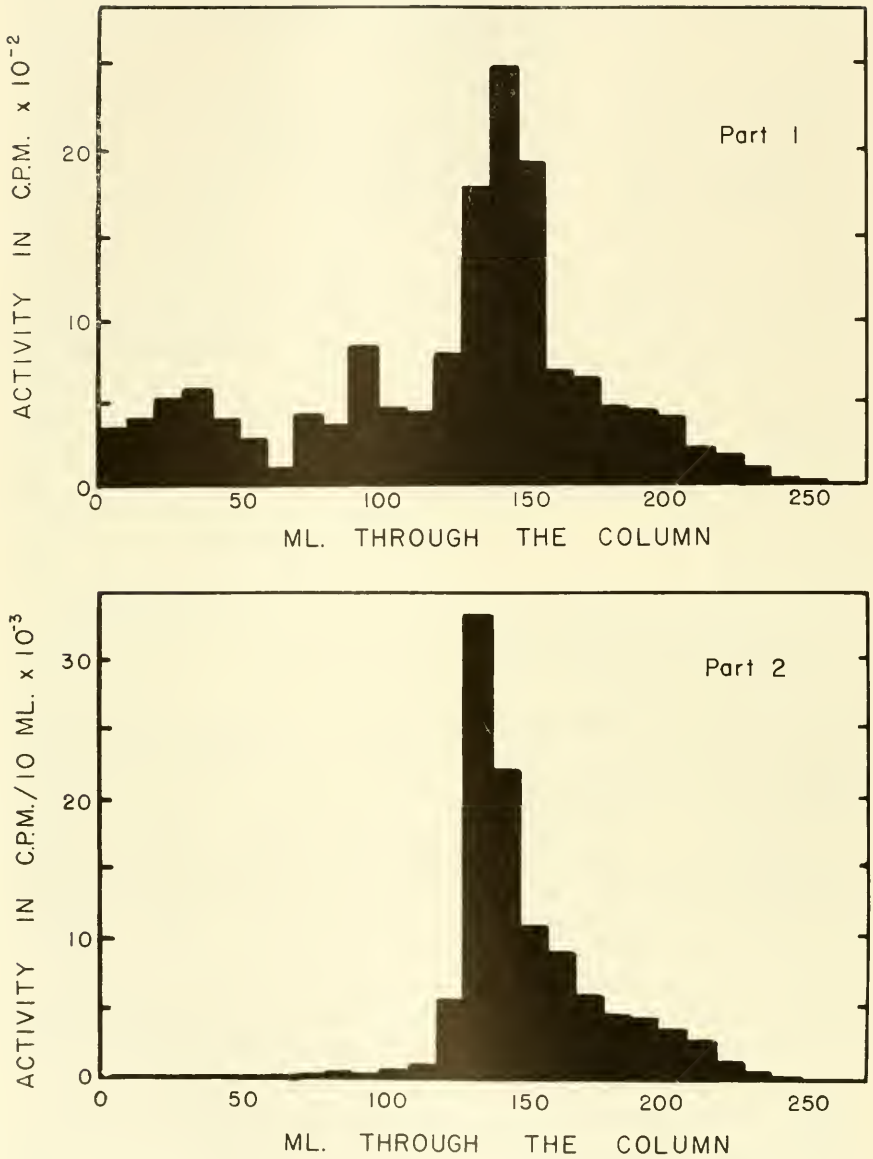

Fig. 4. Anion exchange chromatogram of water-soluble fractions from cotton. Parts 1 and 2 are a continued elution spectrum.

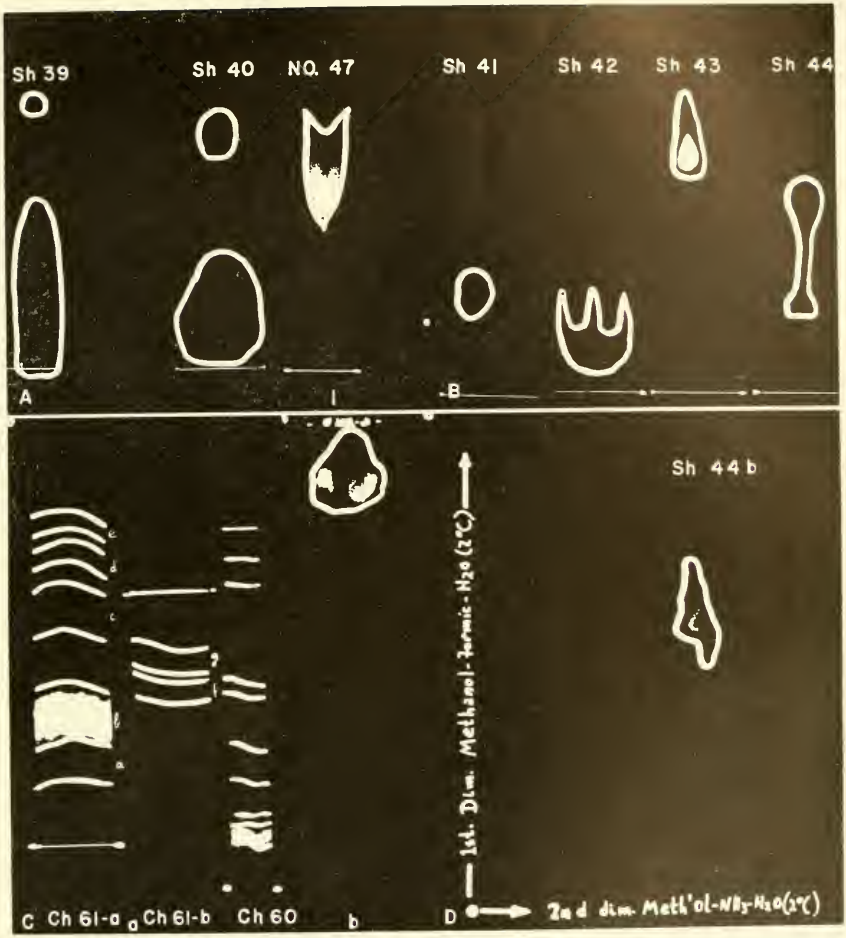

Fig. 5. **A.** Radiochromatograms of water-soluble fractions 1 and 2 from anion exchange column; Sh 39 and 40 are fraction 1 in two different solvents; No. 47 is fraction 2 in one solvent. **B.** Main metabolite chromatographed in two solvents before purification (Sh 41 and 42) and after purification (Sh 43 and 44). **C.** Ch 61-a and Ch 61-b — radiochromatograms of crude water extract from cotton showing seven radioactive bands. Ch 60 — same extract chromatographed in different solvent and b band eluted and re-chromatographed. **D.** Sh 44b — two dimensional radiochromatogram of purified cotton metabolite developed in Bandurski and Axelrod solvents.

the radioactive spots on the chromatograms. Another aliquot of the purified unknown was chromatographed in two dimensions with the same solvents (3) and gave a single, molybdenum blue spot; its autoradiogram showed only one spot (Figure 5D, Sh 44b) overlapping the molybdenum blue area of the chromatogram. The R_f values of the phosphorylated metabolite in six solvents are given in Table 1. Comparison of the R_f values of the two phosphorylated, radioactive spots with those of known phosphorylated compounds gave inconclusive results.

Table 1. R_f values of phosphorylated cotton metabolite.

No.	Solvent System	Hanes-Isherwood Reaction	R_f Values
39	95 per cent Ethanol:NH_3:H_2O (8:1:1)	+	0.35
40	Amyl alcohol:5 *M* Formic acid:H_2O (1:1)	+	0.27
41	Pyridine:Formamide:Ethyl acetate (1:2:1)	+	0.22
42	Phenol:H_2O (72:28)	+	0.10
43*	Methanol:88 per cent formic acid: H_2O (80:15:5)	+	0.59
44*	Methanol:NH_3:H_2O (6:1:3)	+	0.63

* Developed at 2° C.

Radiochromatograms made with the crude water extract from cotton showed at least seven radioactive compounds, but only two important ones coinciding with two fluorescent bands in the paper (Figure 5C, Ch 61-a and Ch 61-b). The principal metabolite exhibited a brilliant blue fluorescence and the less active one a dark blue fluorescence.

Two water soluble, phosphorylated radioactive metabolites were also demonstrated in *Coleus* tissue processed in the same manner. The second active fraction, after purification on the anion exchange column, contained more radioactivity than found in cotton. The radiochromatograms in six solvent systems (Figure 6) showed two radioactive spots on each chromatogram. R_f values of the two metabolites,

Table 2. R_f values of *Coleus* metabolites.

Chromatogram Number	Solvent System	Compound X	Compound Y
89	Ethanol:NH_3:H_2O (8:1:1)	0.08	0.19
91	*Tert*butanol:NH_3:H_2O (60:5:35)	0.29	0.42
92	Amyl alc.:5*M* formic acid (1:1) (org. phase)	0.13	0.04
93	Isopropanol:Formic acid: H_2O (35:5:10)	0.73	0.86

designated X and Y, in four of the solvents giving good separation are summarized in Table 2. When the aqueous extract was radiochromatographed in two dimensions following Bassham and Calvin's technique (4, pp. 17–24), at least 9 radioactive spots, with a major metabolite in the general area of the phosphorylated compounds, were demonstrated (Figure 6, 100). Two minor spots in the region where organic acids migrate and three others where amino acids usually appear were found (4).

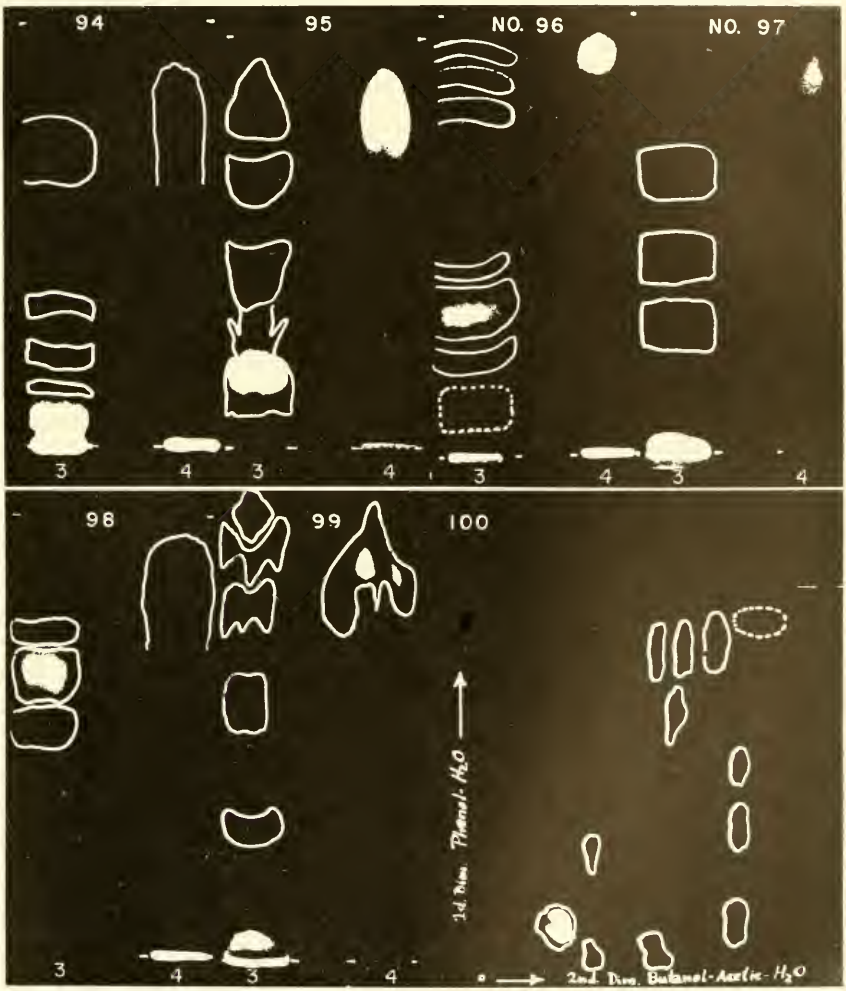

Fig. 6. Radiochromatograms of the radioactive fractions from *Coleus* in six solvent systems (Nos. 94, 95, 96, 97, 98, 99). In each case No. 3 is the water-soluble extract and No. 4 is the fraction soluble in petroleum ether (pigments). No. 100 — two dimensional radiochromatogram of aqueous extract from *Coleus,* according to Bassham and Calvin's technique, showing multiple radioactive metabolites.

Table 3. Distribution of radioactivity in *Coleus* material.

Fraction	Volume, Ml.	Counts Min Ml	Total Activity in Fraction, c.p.m.	Per Cent
80 per cent ethanol extract	200	7,030	1,406,000	99.2
Pet. ether extract	75	10,210	766,000	54.5
Aqueous residue	14 5	28,820	418,000	29.7
Second aqueous residue	60	370	22,200	1.5
Solid residue	wafer		10,700	0.8
Not accounted for			189,100	13.4
Total			1,416,700	100.0

Petroleum Ether-soluble Fraction

When lyophilized *Coleus* material was extracted with 80 per cent boiling ethanol, the alcoholic extract reduced in volume and extracted with petroleum ether, 54.5 per cent of the total activity remained in the petroleum ether-soluble fraction (Table 3). Radioassay of the chloroplast pigment fraction (Table 4) showed that carotene retained most of the activity. Single dimensional radiochromatograms in six solvent systems showed only one radioactive spot throughout the series, tentatively identified as carotenoids (Figure 6). Radiochromatography of the pigments in 1 per cent petroleum ether in *n*-propanol exhibited one radioactive spot superimposed on the carotene spot in the paper with an R_f value of 0.87.

A number of detailed experiments have been conducted with cotton and the results confirm those obtained with *Coleus*. Ether extraction of lyophilized cotton leaves, reduction in volume and extraction with petroleum ether (Figure 7), and radiochromatographing in five solvent systems have demonstrated that most of the radioactivity is associated with the carotene fraction. Strip counting of the chromatogram illustrated in Figure 8 showed that the radioactivity coincides with the carotene spot on the chromatogram.

Table 4. Distribution of radioactivity in *Coleus* pigments.

Fraction	Volume, Ml.	Counts/ Min/Ml	Total Activity	Per Cent
Pet. ether extract	60	4,045	242,700	100.0
Chlorophyll a	30	446	13,400	5.5
Chlorophyll b	30	205	6,150	2.5
Carotene	50	3,138	156,900	64.6
Xanthophylls	50	767	38,350	15.8
Not accounted for			27,920	11.5

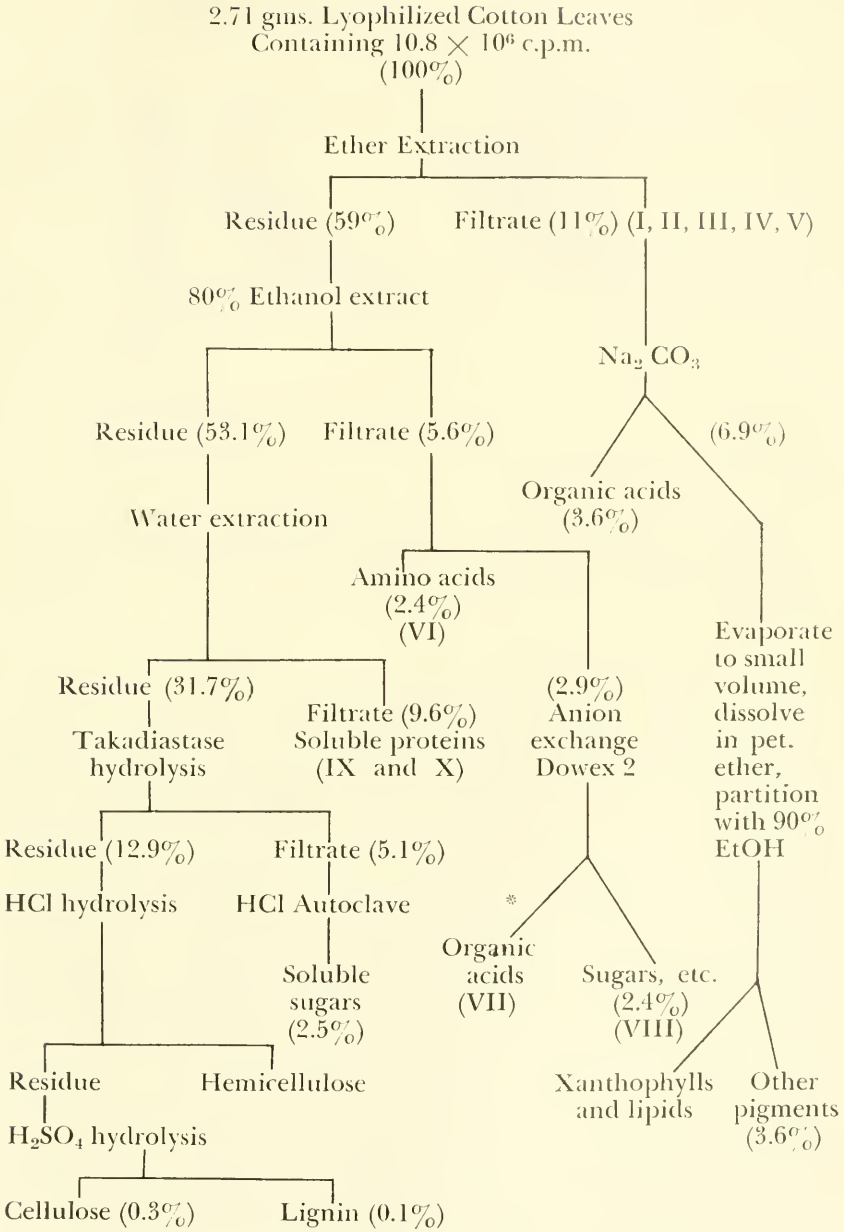

Fig. 7. Fractional extraction survey scheme of radioactivity in cotton leaves after 15 hrs. exposure to ethylene-C^{14}.

* The materials which adsorb to the anion exchange column cannot be eluted with 1*N* NH_4OH. 2*N* HCl causes a loss of the radioactive carbon.

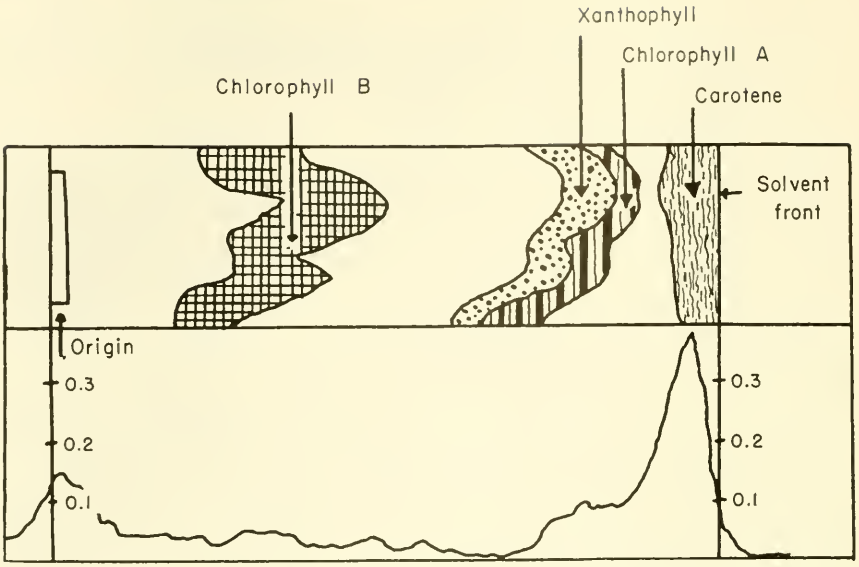

Fig. 8. Chromatogram of the pigment fraction of cotton (1 per cent petroleum ether in *n*-propanol) and strip count showing major metabolite coincides with carotene.

Survey of Activity in Cotton Leaves

Cotton leaves from plants exposed to ethylene-C^{14} for 15 hrs. in different experiments were extracted as shown in Figure 7 and the extracts chromatographed in five solvents. At least 18 radioactive metabolites were found in the different fractions. It can be noted that considerable activity remains in the residue after ether extraction and that approximately two-thirds of the activity is lost through the scheme by heating and hydrolysis. Three radioactive metabolites were found in the ether-soluble fraction, the major one being associated with carotene. Eight metabolites were present in the alcohol-soluble fraction, one of which has an amino acid-like structure which will be discussed in detail below. An alcohol-soluble metabolite was found in the sugar fraction but does not appear to be any of the known sugars; the other metabolites soluble in alcohol are probably organic acids. From five to nine radioactive, water-soluble metabolites have been isolated in different experiments. Two of the compounds containing high activity possess a phosphorus moiety and a third compound appears to be a protein. Acid or enzyme hydrolysis of the residual tissue shows a number of metabolites associated with the cell wall constituents.

Comparison of the amount of C^{14} from ethylene fixed in the various components of cotton to the amount of C^{14} fixed from $C^{14}O_2$ in

Table 5. Comparison between fixation of $C^{14}O_2$ by barley in the light (2) and ethylene-C^{14} fixation by cotton in the dark.

	Barley, 2.223 g. with 2×10^6 c.p.m.	Cotton, 2.71 g. with 10.8×10^6 c.p.m.
	(per cent)	(per cent)
Dried whole plant	100	100*
Ether soluble (I)	[12.5]	11
Chlorins (II)	6.1	6.9
Phytol (III)		
Carotenoids (IV)		
Other lipids (V)		
Amino acids (VI)	7.2	[< 2.4]
Acids (VII)	11.3	1
Sugars (VIII)	25.8	2.4
Soluble protein (IX)	4.0	9.6
Nonprotein aqueous (X)		
Cellulose (XI)	2.8	31.7
Lignins, etc. (XII)	[8.3]	

* Loss of radioactivity was primarily due to oven-drying of residues for counting purposes.

the light by barley (2) shows a relatively higher fixation in the cell wall constituents in the case of ethylene (Table 5).

Amino Acid Fraction

Chromatography of the amino acid fraction extracted by ethanol (Figure 7) revealed that the radioactivity appeared primarily in one amino acid-like compound. Only a trace amount of radioactivity was found in two or three other amino acids as indicated by two dimensional chromatography.

Based on R_f values in several solvents and other tests, the major radioactive compound was originally believed to be tryptophan. Considerable attention was given this compound because of its possible significance to indole-3-acetic acid biosynthesis. However, as shown in Table 6, the conclusion that the compound was tryptophan is not fully substantiated as shown by the R_f values in other solvents and the color reactions performed.

DISCUSSION

The present experiments were designed primarily to develop basic background information for more detailed experiments on the biogenesis, metabolism, and physiological functions of ethylene in plants. It was also hoped that the results would serve as a guide to means of increasing the amount of ethylene fixed, and by shortening the fixation period, to facilitate determining the sequence of metabolite formation.

Table 6. Chromatographic characteristics of the radioactive tryptophan-like metabolite from cotton leaves.

Solvent System	R_f Values	
	Unknown	Tryptophan
Butanol:acetic acid:water (100:22:50)	0.46	0.51
Ethanol:butanol:water (4:1:1)	0.31	0.35
Methanol:ammonia:water (80:5:15)	0.64	0.65
77 per cent Ethanol	0.37	0.37
70 per cent Isopropanol with NH_3 atmos.	0.76	0.82
Butanol:pyridine:water (1:1:1)	0.18	0.52
Butanol:acetic acid:water (60:15:25)	0.51	0.55
Isopropanol:NH_3:H_2O (8:1:1)	0.26	0.50
Isopropanol:NH_3:H_2O (20:1:2)	0.10	0.24
Isopropanol:NH_3:H_2O (80:5:15)	0.24	0.44

Spray Reagent	Color Reactions		U.V. Fluorescence	
	Unknown	Tryptophan	Unknown	Tryptophan
None			lt. blue	lt. blue
Ninhydrin	purple	purple	dk. purple	dk. purple
Salkowski	no color	yellow	none	none
p-Dimethyl aminobenzaldehyde	no color	purple	none	none
Ninhydrin in acetic acid	purple	purple	dk. purple	dk. purple
Isatin	gray	blue-gray	none	none

The results obtained shed additional light on the hormonal nature of ethylene. Quantitative evidence that ethylene is produced naturally by plants (5, 13, 19), that it is translocated and metabolized, and that it affects the basic reactions of plants (16, 17, 20) supports Crocker's contention (8) that ethylene should be classed as a phytohormone.

It is apparent from the data that ethylene is absorbed readily by the vegetative organs of the plant. The accumulation of radioactive carbon from ethylene apparently reached a maximum about two hrs. after administration. The amount absorbed increases with the dosage administered and is greatly influenced by the physiological activity of the plant or tissue and whether "fresh" or "aged" ethylene is used. Comparatively, only a small amount of "fresh" ethylene was fixed by *Coleus* or cotton plants. The changes occurring during the "aging" of ethylene are presently unknown. Based on field observations (13) apparently similar changes occur in ethylene that increase its reactivity for plants when it is exposed to atmospheric conditions.

Ethylene was absorbed in higher amounts in the dark than in the light under aerobic conditions and with lowered carbon dioxide content of the surrounding atmosphere. A large amount of the ethylene absorbed is unreacted or in loose combination in the plant since two-

thirds or more can be removed by oven-drying or other techniques and when introduced through the roots it is partially lost via the foliage.

The fixed ethylene apparently does not enter readily into the normal metabolic pathways that result in respiration to carbon dioxide. More ethylene is fixed by actively growing older plants but not necessarily by older leaves.

Translocation of ethylene-C^{14} or its metabolites was demonstrated by its movement from treated roots or leaves to other parts of the plant. When applied to roots, the highest concentration of radioactivity was found in the terminal meristem. The cotyledons of cotton plants in the two true leaf stage contained more radioactive metabolites than the true leaves. When applied to a single leaf, movement of radioactivity occurred to nontreatd leaves where it accumulated in higher amounts than in the treated leaf. Intact leaves absorbed and fixed larger amounts of ethylene-C^{14} than detached leaves. Although more fixation occurred in the dark, more metabolites were translocated under conditions permitting photosynthesis and transpiration.

The aqueous extracts from both cotton and *Coleus* plants contained at least two major radioactive metabolites with acidic properties since they were absorbed by and could not be eluted from anion exchangers with electronegative reagents. The major radioactive fraction from both sources eluted from the exchange column as the second peak in the elution sequence. After exposure to radioactive ethylene, as many as seven and nine metabolites were detected by radiochromatographing the water-soluble fraction of cotton and *Coleus,* respectively. This suggests either the reactivity of ethylene towards certain native plant materials or the entrance of the ethylene molecule into some major metabolic pathway. At present the former possibility seems more logical. Although the amount of radioactivity incorporated from "aged" ethylene-C^{14} compares favorably to the quantities of C^{14} from carbon dioxide fixed in the dark by tobacco (18) and by barley in the light (2), the distribution and proportion of the metabolites contained in the various soluble fractions of cotton suggest that the C^{14} was not incorporated as carbon dioxide. Fixation atypical of carbon dioxide is also indicated by higher radioactivity in the cell wall constituents but lower amounts in the soluble compounds than would be expected in 15 hrs. It is highly probable that ethylene is translocated both as the gas and in the form of water-soluble metabolites.

The major water-soluble metabolites in addition to being strongly electronegative possessed a phosphate ester moiety as indicated by the

positive Hanes and Isherwood reaction. It has been reported that certain phosphorylated organic compounds, mainly insecticides, are able to elicit ethylene-like symptoms in cotton, tomato, carnation, and other plants (11, 12, 13). All of these compounds have free or potential ethyl phosphate groups as parts of more complex molecules. It is possible that these ethyl phosphate-containing compounds are metabolized by plants to ethylene, or, more logically, ethylene enters into direct combination with one or more of the many fundamental phosphorylated compounds known to exist in plants. This suggests a relationship between ethylene and phosphorylation reactions and a feasible explanation of some of the striking effects of ethylene treatment on plants. For example, it would be attractive to propose as suggested in the literature (9) that ethylene functions by uncoupling phosphorylation from oxidation. This, however, is not the case since significant effects of ethylene upon phosphate uptake or on the transfer of phosphate from ATP to glucose could not be demonstrated with cytoplasmic homogenates or with mitochondria (17). Neither was ethylene-C^{14} incorporated by mitochondria (17). Other theoretical implications of ethylene upon phosphorylation could be postulated, but any hypothesis based on the available evidence would be largely speculation.

The observation by Buhler *et al.* (7) concerning the incorporation of ethylene specifically into succinic and fumaric acids has not been confirmed from the present material under study. Labeled organic acids do exist in the alcohol-soluble fraction.

In both *Coleus* and cotton the major portion of the labeled carbon in the ether-soluble fraction was found in association with the carotenes. Radioactivity in the xanthophylls suggests turnover from the carotenes by oxidation. Genevois in 1954 (10) expressed the opinion that carotenoids may arise from ethylene. Since some 65 per cent of the ether-soluble radioactivity was present in the carotenoid fraction, it may be assumed that ethylene has reacted with β-carotene or served as a precursor in its formation. The function of ethylene in inducing chlorosis, coloring, and ripening changes in green organs attaches physiological significance to these observations. A corollary observation is that catalase is inhibited by ethylene (15). The effects of ethylene upon the pigments and catalase, all found in close structural proximity on the chloroplasts, may have more significance than circumstantial evidence and speculation now permits.

Rapid fixation and a relatively high retention of the radioactivity from ethylene-C^{14} by cell wall constituents may prove to be important in explaining cell wall modifications induced by ethylene, including cellular growth, ripening, and abscission. The preliminary results obtained to date do not permit any positive conclusions in this regard.

In the same manner, the association of ethylene-C¹⁴ with a tryptophan-like compound suggests an explanation for ethylene in auxin-controlled responses, especially apical dominance and abscission. By altering or blocking indole-3-acetic acid synthesis, the cause for loss of apical dominance and the stimulation of abscission in plants exposed to ethylene becomes apparent. It has also been found that ethylene inhibits catalase but stimulates peroxidase activity (16). This may be important in explaining the effects of ethylene upon auxin-controlled responses. True assessment of the significance of possible ethylene-auxin interactions, however, awaits the results of experiments now in progress.

SUMMARY

Experiments with *Coleus* and cotton plants exposed to various levels of ethylene-C¹⁴ have provided quantitative evidence supporting the hormonal nature of ethylene. Both radioautography and direct counting of the radioactivity have confirmed that exogenous ethylene is absorbed and transported readily by the vegetative organs of the two species. The amount of ethylene absorbed in general increases with the dosage administered and is greatly influenced by the physiological activity of the plant or tissue and according to whether "fresh" or "aged" ethylene is used. The causes for the changes occurring during the "aging" of ethylene, as described in this paper, and the reasons for the increase in its biological reactivity, are presently unknown.

Considerable of the ethylene-C¹⁴ absorbed is unreacted or in loose combination in the plant since two-thirds or more of the absorbed radioactivity can be removed by oven-drying or other techniques and when introduced through the roots it is partially lost via the foliage. The transport form of ethylene is unknown, but the evidence suggests that it is translocated both as a gas and as water-soluble metabolites.

The "fixed" ethylene apparently does not enter readily into normal metabolic pathways that result through respiration in carbon dioxide. More ethylene is fixed by rapidly growing flowering plants than by younger plants, but not necessarily by the older, mature leaves. Intact leaves absorbed and fixed higher amounts of ethylene-C¹⁴ than detached leaves. Although more fixation occurred in the dark, more metabolites were translocated under conditions permitting photosynthesis and transpiration.

At least 18 radioactive metabolites were formed in leaves of plants after exposure to ethylene-C¹⁴ for 15 hrs. From 5 to 9 radioactive, water-soluble metabolites have been isolated in different experiments; two of these having acidic properties and giving a positive phosphate reaction contained the majority of the water-soluble radioactivity.

Three metabolites were found in the ether-soluble fraction, the major one being associated with carotene. Eight metabolites were found in the alcohol-soluble fraction; a peptide, because of its similarity to tryptophan, was studied in some detail. After solvent extraction of the treated tissue with various reagents, the bulk of the fixed radioactivity remains associated with the cell wall constituents. Evidence showing that fixation of the activity atypical of carbon dioxide was presented.

The physiological significance of the findings and the possible relationship of ethylene to phosphorylation reactions, chlorosis, coloring, and ripening changes, interaction with auxin and cell wall modifications are briefly presented and discussed.

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DISCUSSION

Dr. Thimann: As a footnote to Dr. Hall's studies of what happens to ethylene in the plant, I would like to mention some work we have been doing for the last three years on the conditions for the formation of ethylene. This work was carried out in collaboration with Dr. Stanley Burg, with the valuable assistance for instrumentation of Dr. Jan Stolwijk who joined us from Holland. I would like also to acknowledge the continued advice and criticism of Dr. Bruce Stowe. The first part of the work was largely devoted to improving the methods for determining ethylene, which are very insensitive. Experiments which require having three or four whole apples in a container for several hours are very unsatisfactory for the study of biogenesis, but fortunately Burg and Stolwijk were able to overcome this and, by skillful adaptation of gas chromatographic methods, were able to determine amounts of ethylene only about 1/1,000th of that required with the existing procedure of absorbing first in mercury perchlorate and then releasing and measuring the gas volume. I will mention only three of the points which have become clear from this study. The first is the very close relationship between the production of ethylene in plugs or slices of apple, and their respiration. It is possible with these small pieces of tissue to follow in parallel their oxygen consumption and their ethylene production, as a function of oxygen tension. The production of ethylene and oxygen consumption both have pO_2^{50} at between 1.5 and 2 per cent oxygen. Further, numerous respiration inhibitors all inhibit production of ethylene. Dinitrophenol, iodoacetate, fluoride, fluoroacetate, or, a little less satisfactorily, arsenite, all show the same thing: namely, that the influence on oxygen consumption is paralleled extremely closely by an influence on ethylene production and the level of the inhibitor which just causes a threshold effect is exactly the same for the two processes. These data taken together con-

vinced us that ethylene is produced by an oxidative reaction which is very closely related to the total consumption of oxygen. The second point is that there evidently is a sequence of reactions in the formation of ethylene of which oxidation is only a part. In pure nitrogen there is a very small production of ethylene, limited to the first 20 minutes or so, and after that it ceases. If tissues which have been anaerobic for 4 or 5 hrs. are exposed to oxygen for 5 min. only and again returned to nitrogen, those few minutes of oxygen allow a very considerable production of ethylene afterwards in nitrogen. There is evidence for the accumulation of an intermediate in nitrogen which afterwards is converted in oxygen to a precursor of ethylene.[1] Some experiments with tritiated water strongly suggest that one of the terminal reactions is a reversible dehydration because the tritium is incorporated into ethylene just about as rapidly as into the tissue water. Lastly, there are very peculiar osmotic phenomena concerned with ethylene production. We early found that slices of apple tissue are very different from slices of potato tissue or indeed from slices of any other tissue with which I am familiar in that when placed in water they do not become turgid. On the contrary, they leak and lose a considerable quantity of their constituents. This has some advantages in that they can take up complex organic substances. But their turgor diminishes and simultaneously they lose to a great extent their ability to produce ethylene. On the other hand, in low concentrations of a solute they become turgid, and this is particularly well shown in glycerol. In higher concentrations of solute they become flaccid again. A plot of the increase or decrease in fresh weight of the tissue, as a function of the glycerol concentration, shows that in water, they actually lose water. They gain water in moderate concentrations of solute, and in high concentrations they are plasmolyzed. The effect of these changes on the ethylene production is unexpected; in water, ethylene production goes down just as does the water-holding capacity, in the intermediate concentrations it goes up, and finally in the high concentrations it remains up. That is to say, the tissue can be perfectly plasmolyzed and flaccid and yet it produces ethylene at the full rate. On the other hand, if it is flaccid due to soaking in water it produces ethylene at less than half the control rate. Thus although the system is osmotically very sensitive, it is not sensitive to the water content of the cells themselves, but rather to the osmotic state of something inside the cells. KCl reacts exactly like glycerol, and $CaCl_2$ is a little less effective. This phenomenon and the very close relationship to oxygen consumption strongly suggest that the osmotic

[1] See S. P. Burg and K. V. Thimann, Proc. Nat. Acad. Sci. 45: 335–344, 1959; Plant Physiol. (In preparation.)

system which produces ethylene is probably the mitochondria and that when the solute concentration of the cell declines, as during water soaking, these particles become swollen and the swelling damages this particular enzyme system. High solute concentrations, such as would occur after plasmolysis, do not adversely affect the enzyme system.

Dr. Hitchcock: First, I would like to comment on the ethylene molecule and then on some of the responses induced by ethylene. Many growth regulator structures have been drawn on the blackboard, but I think this is one of the simplest for a growth regulator (CH_2:CH_2 drawn). Carbon monoxide with the simple structure C:O also has growth regulating properties. Indirect evidence for the translocation of ethylene, also carbon monoxide, throughout plants was obtained a number of years ago. When one leaf on a tomato plant was sealed in a flask containing ethylene or carbon monoxide, all leaves showed an epinastic response. Hall's work with radioactive ethylene now gives direct proof of the translocation of ethylene throughout the plant. Hall's finding that the stem contains only trace amounts of ethylene as compared to large amounts in the leaves appears to explain why there is no stem bending — only epinasty of leaves on plants exposed to ethylene. Ethylene induces many responses characteristic of growth regulators — epinasty of leaves involving cell elongation in the petiole or in the midrib, differences in flowering and the ripening, development and coloration of fruit, inhibition of nutational movements, and interference with the correlative movements of leaves and leaflets of plants such as *Mimosa pudica*. The last two are anaesthetic effects. Thus ethylene is unquestionably a growth regulator, but there is disagreement as to whether ethylene is a plant hormone.

Dr. Nitsch: I'd like to ask Dr. Hitchcock if the response to CO is observed in the light or only in darkness?

Dr. Hitchcock: I don't recall whether we had ever run tests in darkness alone as against light, so I can't answer that question.

Dr. Galston: I would like to ask several questions of Dr. Hall. First, do you know whether the entrance of ethylene into the leaf is through the stomata? For instance, if you permit a leaf to wilt completely, will the ethylene still enter?

Dr. Hall: We have not conducted experiments as you describe but ethylene is absorbed by the petiole. That is, if you remove the blade ethylene is still absorbed by the debladed petiole. Ethylene also enters through the stem or by the roots. So I would judge that it enters in other ways in addition to entering through the stomata.

Dr. Galston: I was struck by the fact that the ethylene appeared not to enter the very youngest leaf very effectively. I wonder whether

this might not be a reflection of the different ratio of surface area to mass of the leaf at this particular stage. If the youngest leaf were not spread out and the stomata exposed, the ethylene would not be able to enter. This could possibly explain the difference that you have noted in penetration of ethylene into leaves of different ages.

Dr. Hall: The youngest leaf you refer to, our No. 1 leaf, was partially expanded. In other words, it had emerged from the apical bud. Based on our work I believe that entrance of ethylene does not depend entirely on the stomata, but it may also penetrate the epidermis directly.

Dr. Forti: I would like to ask Dr. Thimann if he has any explanation about the inhibition of oxygen uptake and ethylene production by dinitrophenol at the level of 10^{-6} molar.

Dr. Thimann: The oxidation rate in many tissues is inhibited by dinitrophenol at somewhere near that. Actually apple is a little more sensitive than coleoptile or pea tissue or potato tubers by about a power of 10.

Dr. Shantz: Dr. Hall, as I understood your remarks with the "aged" ethylene, this was taken up by the plant at many times the rate of fresh ethylene. Have you also found by any method for the quantitative estimation of growth effects of ethylene, that the "aged" ethylene induced a correspondingly higher growth response than fresh ethylene?

Dr. Hall: The "aged" ethylene acts biologically similar to fresh ethylene. One response that would indicate that it has essentially the same effect as the fresh ethylene is that it induces rapid abscission of leaves, the same as the fresh ethylene. The "aged" ethylene also produces epinasty, chlorosis, and coloring. I might comment briefly about the possible relation of ethylene to smog. We have an air pollution problem in Texas where ethylene is being liberated from an industrial plant near the Gulf Coast causing rather serious damage to cotton and other crops in the area. Apparently "aging" of ethylene from this source also takes place in the atmosphere because the extent of the damage to plants depends upon certain atmospheric conditions.

I might add, Dr. Thimann, that we have already conducted experiments with castor bean mitochondria. It was thought that our finding that ethylene reacts with a phosphorylated compound and its well known stimulating effect upon respiration might be more than coincidentally related. In other words, it would be attractive to think that ethylene might be uncoupling oxidation from phosphorylation. However, we have not been able to demonstrate this with castor bean mitochondria. In fact, when using radioactive ethylene, we have not been able to demonstrate any measurable radioactivity in the mito-

chondria, yet they do undergo swelling in the presence of ethylene. Although we were not able to demonstrate any significant effect of ethylene upon oxidative phosphorylation, ethylene does affect mitochondria in a physical manner.

Dr. Bitancourt: I want to refer briefly to some experiments carried out several years ago with Miss Rossetti. We worked with the fungus, *Mucor spinosus,* which produces a gas which stimulates the growth of *Phytophthora.* When the air above cultures of *Mucor spinosus* was passed through water, sulfuric acid, or sodium hydroxide before passing over cultures of *Phytophthora,* growth stimulation still occurred. The gas produced by *Mucor spinosus* did not induce epinasty in tomato plants.

Dr. Ray: I, too, am a bit disturbed by the "aging" effect. I wonder whether even the original samples of ethylene contain trace amounts of this material derived by aging. It causes one to doubt whether any of the "metabolites" (so-called) of ethylene which are observed are in fact derived from ethylene and not from this other material since it apparently is fixed so much more readily by the plant. I also wondered whether you had thought of trying to wash the ethylene through towers of alkali, acid, or adsorbents of various kinds to see whether this couldn't be gotten rid of. I think this is a rather basic problem. Even the initial samples could be contaminated with trace amounts, could they not?

Dr. Hall: Yes, even a fresh sample of radioactive ethylene may have trace amounts of ethylene oxide present, but the content of ethylene oxide does not increase with age nor is ethylene oxide responsible for the increased reactivity of the "aged" sample. Scrubbing of the "aged" ethylene as you propose does not decrease its enhanced reactivity over that of fresh ethylene. Also in experiments where we have used both fresh and "aged" ethylene, we found essentially the same metabolites being formed after absorption, but much higher activity in the metabolites from the "aged" sample. To date we have not been able to show by gas chromatography and the vibrating reed electrometer that any other radioactive material is present in the "aged" sample except ethylene.

Dr. Teubner: First, I'd like to comment on some work at Michigan State on ethylene production in stored apples. I realize Dr. Thimann has some objections to working with intact pieces of tissue, but at any rate as horticulturists we must do this. Reviews by Biale (Ann. Rev. Plant Phys. 1: 183. 1950) and by Smock and Neubert (Apples and Apple Products. 486 pp. Interscience, New York. 1950) discuss the delay of the climacteric with high levels of carbon dioxide (5 per cent) and a low level of oxygen. Respiration of the fruit under these

conditions is quite drastically repressed. On removal and exposure to atmospheric conditions there is an increase in respiratory activity and concomitant with this is an increase in ethylene production. Using this response of apples to these conditions of storage, C^{14} labeled carbon dioxide was incorporated into the storage atmosphere for several days. Upon exposure to a normal atmosphere and collection of the evolved ethylene in mercuric perchlorate, it was found that a considerable portion of the fraction was active. I would also like to direct a question to Dr. Hall which is, How do you distinguish whether or not ethylene is metabolized before or after transport? Since only radioactivity is measured the ethylene could be metabolized within the leaf and the labeled material transported as some other metabolite.

Dr. Hall: At the present time we have not distinguished exactly when the ethylene is metabolized. We do not know whether it's before or after transport. However, it appears that most of the metabolism takes place in the leaf following absorption, and before translocation out of the leaf occurs. Apparently the leaves are the principal organs of metabolism, since much less metabolism occurs after absorption by roots or stems.

Dr. Thimann: I assure Dr. Teubner I have no objection whatever to intact tissue or anything else that works. It is only that if one needs to feed organic substances to the tissue, the slices are much better. But I would like to ask him if he can give any further details since his experiment suggests that carbon dioxide is converted to ethylene in some way. And so far our efforts to get evidence for this have signally failed. Not only carbon dioxide but many compounds that could be metabolized to give carbon dioxide seem not to transfer any label to ethylene, at least in short-term experiments. It is true that in long-term experiments, labeled sugar gives rise to labeled ethylene but the significance of that is hard to assess.

Dr. Teubner: I'm not sure how much further these studies have been carried. Complications arose in attempting to examine potential precursors before the apples released their ethylene. We feel sure there is an anaerobic fixation of carbon dioxide and incorporation into organic acid fractions which are then metabolized. The accelerated carbon dioxide production upon restoration to a normal atmosphere is reminiscent of an uncoupling effect. We attempted to examine the organic acids, but the quantity of malic acid in apples presented difficulties in specific activity determinations.

N. E. TOLBERT
Michigan State University

(2-Chloroethyl)trimethylammonium Chloride and Related Compounds As Plant Growth Substances[1]

(2-Chloroethyl)trimethylammonium chloride and certain other structurally related compounds act as plant growth substances (4). These compounds are analogues of choline, and trivial names have been formed from choline. Thus, (2-bromoethyl)trimethylammonium bromide has been called bromocholine bromide. The most characteristic growth change after treatment with these compounds is the development of stockier plants with shorter and thicker stems (5, 6). In most respects the appearance of plants after treatment with derivatives related to (2-chloroethyl)trimethylammonium chloride is the opposite from that obtained with gibberellin, and the effects can be reversed by gibberellin.

CHEMICAL SYNTHESIS AND BIOASSAY

The synthesis and analysis of the compounds have been reported (4). In the procedure an amine was reacted with a halogenated hydrocarbon under controlled conditions to produce a specific product, the structure of which was confirmed by C, H, N, and halide analyses, melting point, and the picrate salt melting point. Thus, trimethylamine and 1,2-dibromoethane at 40° C. formed (2-bromoethyl)trimethylammonium bromide, and (2,3-*n*-propylene)trimethylammonium bromide was prepared from allyl bromide and trimethylamine. Since these salts were infinitely soluble in water, aqueous solutions were simple to prepare and to use for treatment of the plants.

The activity of the compounds was assayed in a procedure with 'Thatcher' wheat seedlings (4). Excess aqueous solution of the chemicals was poured onto the soil once, 11 days after planting the seeds.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 2480.

Two weeks later the distance in millimeters between the base of the first leaf blade to the base of the second leaf blade was measured, and 4 weeks later the total stem height and the weight of the plant were recorded. After some chemical treatments, negative values for the measurement of height were recorded to indicate the distance in millimeters that the base of the second leaf blade lay below the base of the first leaf blade. In these cases the base of the second leaf blade had forced its way out through the sheath of the first leaf blade.

RESULTS

Active Structure

A general structure for the active derivatives is $(CH_3)_3N^+CH_2$-CH_2X where X is Cl, Br, or the $=CH_2$ group. The chemical names for the three most active compounds are (2-chloroethyl)trimethylammonium chloride, (2-bromoethyl)trimethylammonium bromide, and (2,3-*n*-propylene)trimethylammonium bromide. For the growth of wheat and tomatoes these compounds were effective from 10^{-6} to 10^{-2} *M* when applied to the soil; from 10^{-5} to $10^{-2}M$ when applied as a spray; from 10^{-7} to $10^{-3}M$ when applied in nutrient solution to tomatoes; and from less than 10^{-3} to $10^{-1}M$ when applied by seed treatment. For (2-chloroethyl)trimethylammonium chloride a $10^{-6}M$ solution is the same as 0.13 p.p.m.

A large number of chemicals with related structure were either synthesized or purchased and tested by the wheat bioassay. All compounds which were effective on reducing the height of 'Thatcher' wheat plants were also effective in the same proportion when used to treat a variety of other plants.

From the screening program certain correlations between structure and activity were developed: (a) A trimethylamine at one end of the molecule was essential for activity. (b) The carbon chain which contains the constituent X should be 2 carbons in length for maximum activity. When this chain was 1 carbon long, as in (bromomethyl)trimethylammonium bromide, the compound was less than 1/10 as active. When this chain was 3 carbons long, as in (3-bromopropyl)trimethylammonium bromide, the compound was 1/100 as active, and if this chain were 4 carbons long or branched, the derivative was inactive. (c) The derivatives which were found to be active have had a Cl, Br, or $=CH_2$ group substituent for X of the general structure. Other derivatives are still being tested. It is interesting to note that the natural-occurring derivatives, choline, betaine, and phosphorylcholine, were completely inactive. An iodo derivative was toxic. When X was an H, as in (ethyl)trimethylammonium bromide, there was only a trace of activity.

Effect on Growth of Wheat

When wheat seedlings were treated with one application of either of the three most active derivatives, they grew with much shorter and thicker stems than untreated plants (Table 1) (5). An example of this type of growth is shown in Figure 1. The treated plants were darker green in appearance; this greening phenomenon was particularly noticeable after treating tomato and tobacco plants (6). Leaves of the treated wheat plants were shorter in length and broader. There was no decrease in fresh, wet weight of wheat plants after soil treatments of $10^{-3}M$ or less, but higher concentrations of the chemical reduced total plant growth. Tillering of treated plants occurred shortly after treatment rather than later during maturation of untreated plants. The tillers developed and headed at about the same rate as the main stalk and produced the appearance of a more bushy plant. At maturity there was more uniformity in height of the treated plants, and heading in the treated plants occurred several days later than in the controls. Thus, wheat plants treated with (2-chloroethyl)trimethylammonium chloride developed into sturdier, shorter, and bushier plants.

Effect on Growth of Tomatoes

Growth and fruiting of tomatoes have been extensively tested with the three most active derivatives (6). Best growth results have been obtained with (2,3-*n*-propylene)trimethylammonium bromide, though the halogenated compounds were also very active. Plants grew short and sturdy with intensely green leaves and stems that were thicker

Table 1. Length of 'Thatcher' wheat plants after soil treatment with (2-chloroethyl)trimethylammonium chloride.*

Molarity	Weeks after Treatment	
	2	4
	(*Mm.*)	(*Mm.*)
0	34	260
10^{-6}	23	200
10^{-5}	15	153
10^{-4}	7	139
10^{-3}	1	91
10^{-2}	−3	78

* Twenty 'Thatcher' wheat seedlings in 8-inch pots of sand and loam soil were treated by pouring 500 ml. of solution of the chemical on the soil at 11 days after planting. The second leaf was visible at time of treatment. See text for description of the measurement of the plant height.

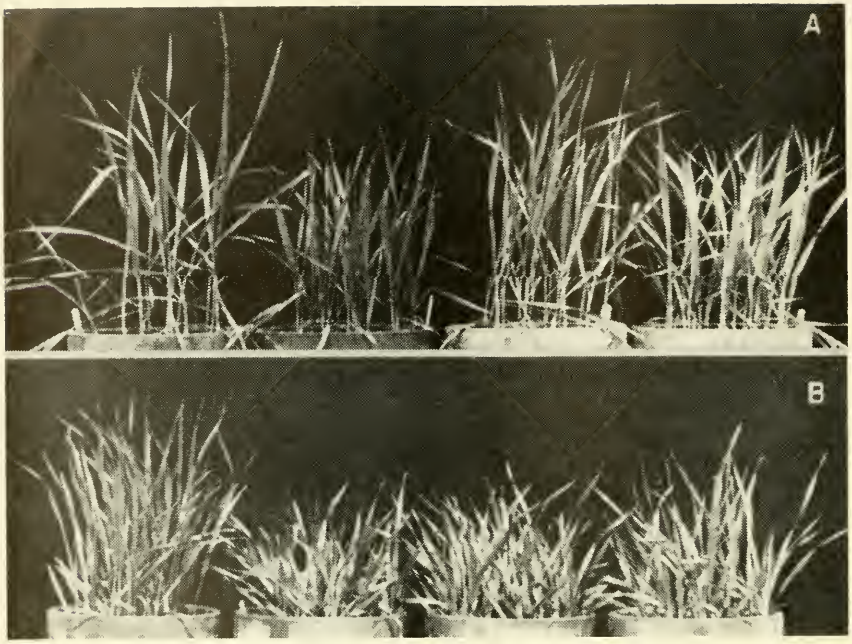

Fig. 1. **A.** 'Thatcher' wheat seedlings 2 weeks after one soil application of 10^{-3} *M* solutions: (left to right) none, (2-bromoethyl)trimethylammonium bromide, (3-bromo-*n*-propyl)trimethylammonium bromide, and (2,3-*n*-propylene)trimethylammonium bromide. **B.** Wheat plants from seed treated (left to right, 0, 10^{-1}, 10^{-2}, 10^{-3} *M*) with (2-chloroethyl)trimethylammonium chloride before planting.

and with more chlorophyll. Tap-root and leaf-stem ratios were reduced. Flowering was 3 to 10 days earlier and height of the first flower clusters was reduced. Thus, earlier and more prolific flowering and fruiting of market tomatoes, both under greenhouse conditions and in the field, were promoted.

Effect on Other Plants

The three active chemicals are being tested on a variety of other plants, and preliminary results only are available at the time of this symposium. A similar response has been obtained with other vegetable crops such as pepper, eggplant, cucumber, beets, and lettuce (6). Solutions of 10^{-5} to $10^{-3}M$, when applied to the soil, induced darker green growth of young sugar beet plants.[2] When cucumbers were treated by $10^{-5}M$ solutions, the internodes were very much shorter and tendril formation was abolished.[3] High concentrations, $10^{-2}M$

[2] Snyder, F. W., Tolbert, N. E., and Wittwer, S. H., unpublished.

[3] Mitchell, W. D., and Wittwer, S. H., unpublished.

(2-bromoethyl)trimethylammonium bromide, were necessary to shorten the stem and peduncle of chrysanthemums during the summer[4]; however, it did not damage the plants or delay flowering at concentrations less than $10^{-1}M$. In this respect (2-bromoethyl)trimethylammonium bromide was not as active as AMO 1618 (3).

Combined Action of (2-Chloroethyl)trimethylammonium Chloride and Gibberellins

The action of the (2-chloroethyl)trimethylammonium chloride derivatives in altering plant growth was the opposite from the action of gibberellin (GA). GA promoted stem elongation, spindly growth, and lighter green coloration of the leaves. (2-Chloroethyl)trimethylammonium chloride, on the other hand, induced growth with shorter stems, stockier plants, and darker green leaves than the untreated plants. When excess gibberellin and (2-chloroethyl)trimethylammonium chloride were applied together to wheat seedlings, the action of the gibberellin at first predominated, but later the (2-chloroethyl)trimethylammonium chloride became effective. When limited amounts of gibberellin and large amounts of the choline analogue were used, a complete variation in height from an elongated, to a normal, to a short and bushy plant could be obtained. The type of growth obtained was dependent upon the amount of each chemical applied. Plants may also be treated with either chemical separately for a period of time and then the first growth pattern reversed by treatment with the other chemical. These mutually antagonistic effects between (2-chloroethyl)trimethylammonium chloride and GA have been demonstrated with wheat and tomatoes (5, 6). The chemical structures of the two types of compounds are so entirely different that it is difficult to see how the two chemicals could be affecting the same growth-controlling processes. The normal appearance of plants after treatment with amounts of both chemicals which would produce their individual effects, however, must be explained.

Light Intensity

Plants grow tall or elongated in low light intensities or in restricted portions of the visible spectrum. The growth of plants after treatment with (2-chloroethyl)trimethylammonium chloride derivatives is similar in appearance to growth in full sunlight, and the growth after gibberellin treatment is similar to that obtained in low light. These qualitative comparisons provide thought for future experiments with (2-chloroethyl)trimethylammonium chloride as a substitute for high light intensity in the control of plant development.

[4] Lindstrom, R. S., and Tolbert, N. E., unpublished.

This phenomenon, however, becomes a problem in reproducing the results. All of our first experiments were carried out in the greenhouse in the winter when the available daylight intensity was low. Later, when the experiments were redone in April and May, the percentage decrease in stem length from the chemical treatments was less. The effects of the chemical had not changed markedly, but the length of the stems of the control plants was less since the plants were then growing in high light intensity. However, the general pattern of shorter and bushier growth after treatment with (2-chloroethyl)trimethylammonium chloride was still obtained during the summer months.

Effect of Temperature

The (2-chloroethyl)trimethylammonium chloride derivatives were most effective at lower temperatures. This was true for the growth of wheat which was tested at temperatures of 56° F. night and 70° F. day, 65° F. night and 75° F. day, and 80° F. night and 85° F. day. Also, with tomatoes in the greenhouse the effects were more pronounced under relatively cool (55 to 60° F.) temperatures and during the short days of fall and winter. Favorable responses have, however, resulted for both plants at higher temperatures as well as for the other plants so far tested. Thus, although the compounds are most effective near the optimum growth temperature, they may be valuable when applied to plants at temperatures where satisfactory growth is not readily obtained.

(2,3-*n*-Propylene)trimethylammonium Bromide

Only recently has it been realized that the propylene derivative was active. At this time it appears to be the most promising compound, since growth of sturdy plants with broad and green leaves without as great a reduction in over-all height of the plant was obtained when it was applied over a range of $10^{-2}M$ to less than $10^{-5}M$. Further, this compound on tomato plants appears to be very effective at the elevated temperatures and light intensities obtained in the summer (6).

Similarity of Action to Other Chemicals

Several other chemicals have been reported to cause plants to grow with shorter stems when applied to plants in low concentrations, and it was of interest to compare their structure and activity with the (2-chloroethyl)trimethylammonium chloride derivatives. AMO 1618, 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine carboxylate methylchloride has been reported to promote shorter stem growth of chrysanthemums and other plants (1, 3). AMO 1618

is not structurally related to (2-chloroethyl)trimethylammonium chloride except that both are quaternary ammonium salts. Both compounds promote short stem growth, and in both cases these changes are reversed with gibberellin. AMO 1618 was not active on wheat, but on the other hand (2-chloroethyl)trimethylammonium chloride was not very active on chrysanthemums. Thymohydroquinone and thymoquinone have been reported to induce wheat to grow with a shorter stem (2). The structure of thymohydroquinone 2-isopropyl-5-methylammonium chloride is also not related to chlorocholine chloride. Thymohydroquinone in our tests on wheat was toxic at $10^{-2}M$, caused dwarfing at $10^{-3}M$, and induced little effect at $10^{-4}M$.

Mode of Action

The effect of the (2-chloroethyl)trimethylammonium chloride derivatives as chemicals to induce growth of plants with shorter and thicker stems seems characteristic of a new type of plant growth substance. There is so far no evidence that these chemicals are naturally occurring nor any evidence as to their mode of action. They are not particularly toxic to either plant or animal. Since a minute amount of the chemicals, when applied once to young plants, affected the further growth and development of the plant during an entire growing season, it is likely that the compounds were not rapidly metabolized or bound into a part of the cell structure. The high degree in specificity of structure for biological activity suggests that they may affect an equally specific enzyme site. This can be postulated as a protein with one binding site for the trimethylamine end of the molecule and one site for the chloro, bromo, or $=CH_2$ group. The two active sites must be about the length of the ethyl, carbon chain apart.

(2-Chloroethyl)trimethylammonium chloride is structurally related to choline and betaine in that the number 2 carbon of the ethyl chain is halogenated instead of being an alcohol or acid group. From this similarity it may be postulated that these derivatives may be influencing lipid metabolism or transmethylation reactions. Alteration of either of these processes might cause altered cell development which would result in shorter plant growth as has been observed.

SUMMARY

Compounds of the structure $(CH_3)_3N^+CH_2\text{-}CH_2X$ are active as plant growth substances when X is Cl, Br, or $=CH_2$ group. Solutions of (2-chloroethyl)trimethylammonium chloride, (2-bromoethyl)trimethylammonium bromide, and (2,3-*n*-propylene)trimethylammonium bromide were active at $10^{-6}M$ when poured on the soil. Treated wheat plants grew with shorter and thicker stems and greener leaves

than control plants. These growth changes were similar to those produced by high light intensity and the opposite from those caused by gibberellin. The opposite types of growth induced by gibberellin or (2-chloroethyl)trimethylammonium bromide counteracted each other on the same plant.

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Improvement of Growth Regulator Formulation

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Improvement of Growth Regulator Formulation

In the use of growth regulators we are constantly attempting to obtain maximum effectiveness from minimum dosage. This high efficiency is desirable from two standpoints: first, economy, and second, distribution. Economy may or may not be important. With chemicals such as the indole derivatives cost is an appreciable factor; with 2,4-D and other phenoxy compounds used as regulators, the price is so low that cost is only nominal.

Distribution within the plant is essential to the function of most growth regulators which, by definition, act at a distance from the point of application. And with regulators used as herbicides distribution is imperative, as in the control of perennials from foliar application. In such use we are faced with the paradox of requiring living cells of the phloem to conduct a toxic chemical through leaves and stems at concentrations that must, possibly after some local accumulation, prove lethal in roots.

In the foliar application of regulators there are two mechanisms of uptake into the living mesophyll, cuticular and stomatal. However, the differences between these are not basic but rather relative. That is, cuticular absorption involves movement across a relatively thick fatty layer from an environment that may have a low relative humidity whereas stomatal absorption involves uptake across cell walls having a thin cuticle from an environment approaching water saturation. Both include diffusion of the chemical across cells walls, partition to the cytoplasm, and migration via the symplast to the vascular tissues.

Earlier work with dinitro compounds and pentachlorophenol (2) proved that buffering the spray solution on the acid side greatly increased penetration of the chemicals. This has been taken to indi-

Table 1. Absorption and translocation of 2,4-D as shown by bean bending.

pH	Degree of Curvature After Various Hours Following Application of 2,4-D							
	1	2	3	4	5	6	7	8
2	0	13	78	90	97	96	88	86
3	0	5	50	71	77	80	74	83
3.3	0	0	18	52	72	89	84	75
4	0	0	21	50	71	86	87	85
5	0	0	20	54	78	86	86	84
6	0	0	9	40	62	82	85	92
7	0	0	4	13	21	28	35	37
8	0	0	0	2	10	16	29	38
9	0	0	0	8	15	18	28	37
10	0	0	0	0	0	6	18	30

cate that repression of dissociation enhances uptake via a lipoidal route by making the applied chemical more lipoid soluble. More recent work (3) has shown this same relation to hold for 2,4-D, Table 1. Endothal is another compound that benefits from acidification of the formulation.

Work with maleic hydrazide (MH) by Zukel and associates (13) soon proved that acidification of this compound was of no avail, Table 2. Trials with the parent compound and with ester formulations likewise failed to improve uptake (7), Table 3. The fact that at present the only way to increase uptake of MH is to place the plant in a saturated atmosphere would seem to indicate that there are two distinct routes by which chemicals applied to leaves may move into the vascular channels, a lipoid route and an aqueous route. MH moves via the latter.

Considering first the lipoid route, it seems evident that the spray solution must wet the cuticle. From this solution the regulator mole-

Table 2. Weight in grams of growth of 'Bermuda' grass shoots sprayed January 11, 1951, with 0.5 per cent MH (DEA) adjusted to a series of pH values. (Values are averages of duplicate cultures.)

pH	Weights At Various Intervals After Application of MH						
	7/3/51	9/25/51	12/3/51	3/7/52	7/23/52	9/2/52	Average
Control	51.5	81.9	12.5	14.0	24.2	10.0	32.3
2	16.7	59.0	15.2	18.2	22.2	9.5	23.9
3	17.1	58.0	15.4	18.5	19.2	8.0	22.7
4	18.5	40.2	16.6	25.3	22.0	8.6	21.9
5	19.5	18.7	11.7	21.3	28.3	8.6	18.0
6	17.1	7.7	7.9	9.3	10.9	3.9	9.5
7	13.9	7.0	5.9	0.0	0.0	0.0	4.5
8	15.8	0.6	7.9	14.0	15.5	4.1	9.7
9	20.2	25.2	19.3	24.7	27.9	11.6	21.5
10	18.5	37.1	11.6	13.7	31.8	10.6	20.6

cules must adsorb to the cuticle (10), dissolve in the cuticle, diffuse through the cuticle, penetrate the ectoplast (plasmalemma), and pass into the mesoplasm. Here the molecules may migrate by symplastic movement (diffusion and protoplasmic streaming) to the vascular tissues where, experience indicates, they move rapidly in the phloem. Auxin (12), phenoxy compounds (4), dalapon (8), and some other regulators apparently traverse this route. There is evidence that they may also be actively absorbed from the phloem during the process of rapid transport (5, 6).

If 2,4-D is applied in solution as the acid, the pH is around 3.3. As the molecules cross the ectoplast they probably dissociate partially at the prevailing pH of 5 to 6. Entering the sieve tubes of the phloem they are present in the assimilate stream at a pH of around 7.0; thus dissociation must be almost complete. This dissociation process undoubtedly steepens the diffusion gradient and accelerates uptake.

Table 3. MH formulations rated according to the amount of regrowth shown by 'Bermuda' grass cultures.

Formulation*	Necrosis, † Per Cent	Regrowth ‡	Flowering ‡
MH, diethanoleamine salt, in 95 per cent ethanol	85	None	0
MH, diethanoleamine salt, in 5 per cent glycerine	70	None	0
MH, diethanoleamine salt, in 5 per cent Triton B-1956	95	Trace	0
MH, diethanoleamine salt, in 0.16 per cent benzene	75	Very slight	0
MH, diethanoleamine salt	70	Slight	0
N-acetyl MH	65	Slight	0
MH, diethanoleamine salt, in 1 per cent KCNS	60	Slight	0
MH technical	60	Moderate	0
MH, K salt	60	Moderate	0
MH, dodecylamine salt	60	Moderate	+
MH, Na salt	60	Moderate	0
MH, Mn salt	60	Dense	+
N-caproyl MH	40	Dense	+
MH, Mg salt	40	Dense	+
MH, Zn salt	40	Dense	+
N-benzoyl MH	30	Dense	+
MH, diethanoleamine salt, in 50 per cent glycerine	25	Mod.-dense	+
MH, Al salt	25	Dense	+
MH, Ca salt	20	Mod.-dense	+
MH, Cu salt	10	Dense	+
MH technical, no Vatsol	10	Dense	+
2-Ethyl hexoyl MH	0	Dense	+
N-benzoyl MH, no Vatsol	0	Dense	+
Control	0	Dense	+

* Spray applied March 22, 1950. MH was applied at 0.5 per cent active basis in all instances. All solutions except those noted contained Vatsol OT at 0.1 per cent.

† Data taken May 24, 1950.

‡ Data taken July 11, 1950. 0 = no flowers, + = some flowers present.

Because in the use of regulators as herbicides one is dealing with toxic compounds, formulation should be aimed at bringing about wetting of the cuticle, ordered uptake into the mesophyll, controlled concentration to avoid injury to sieve tubes, and accumulation to toxic levels in the roots. Balanced solubility in lipoid and aqueous phases is essential, and proper partitioning is required.

If regulators are absorbed via an aqueous route, there must be pores from the interior of the leaf to the outer surface which under saturated conditions are water filled. Electron microscope views of the cuticle and of cellulose indicate that such pores exist (9, 11). The aqueous medium in the leaf is undoubtedly a continuum with the saturation of the surface dependent upon the water balance of the

Fig. 1. Apoplastic movement of amitrol in bean leaf as shown by the dark wedge-shaped pattern of an autoradiogram. Symplastic movement also is shown by labeling of stem, roots, and trifoliate leaf. Dosage, 125 μg. as a drop at base of primary leaf. Treatment period 8 hrs.

Fig. 2. Symplastic movement of 2,4-D as shown by labeling of stem, roots, and bud. Dosage 50 μg. Treatment period 8 hrs.

leaf. Under saturated conditions the micro-capillaries must be filled to the surface; under stress, menisci must recede in the capillaries to depths proportional to the extent of the deficit.

Applied under conditions of saturation regulator molecules in the solution are able to diffuse directly into the living symplast via the water continuum. Applied under conditions of stress the micro-capillaries are blocked by entrapped bubbles and entry of the regulator is prevented. Inclusion of a liquid surfactant in the formulation enables the applied solution to creep around the bubbles and make contact with the water continuum.

Strugger showed some time ago (14) that dye molecules could move rapidly along the cell walls of mesophyll cells. Some molecules, such as amitrol and MH, may move in this way from the point of application to the periphery of a leaf. Figure 1 shows such movement

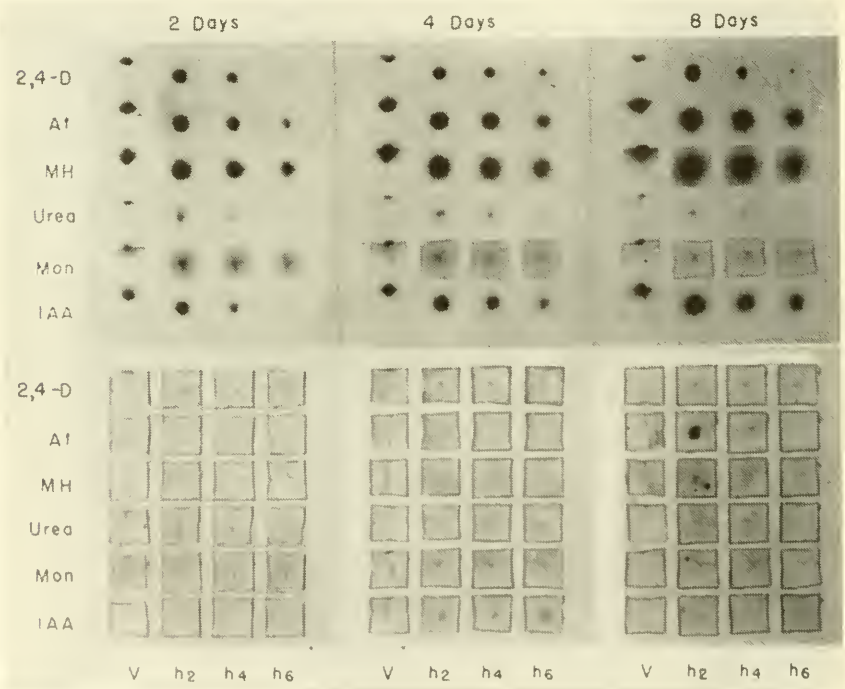

Fig. 3. Symplastic and apoplastic movements of compounds in potato tuber tissue as shown by autoradiograms after 2, 4, and 8 days. Top, autoradiograms; bottom, mounted tissue. Compounds tested were 2,4-D, amitrol (At); maleic hydrazide (MH), urea, monuron, and IAA. Columns represent median vertical slices through one set of tuber blocks (v); horizontal slices 2 mm. in thickness cut 2 mm. (h2), 4 mm. (h4), and 6 mm. (h6) from the top of another treated block. 2,4-D, IAA, amitrol, and MH show symplastic movement of compounds of increasing mobility. Monuron shows apoplastic movement. Urea was evidently hydrolyzed and the $C^{14}O_2$ lost by diffusion.

as contrasted with rapid uptake and movement in the symplast (Figure 2). Calcium also moves in this way when applied to the leaf surface. Monuron and simazin applied to leaves move in this way. Figure 3 shows contrasting symplastic and apoplastic movement in potato tuber tissue.

Recent studies by Clor (1) have shown that not only MH, but amitrol, urea, and 2,4-D are all subject to accelerated uptake and

Fig. 4. Urea translocation in cotton; autoradiograms, top and mounted plants, bottom. The plant on the left of each pair was unringed, the one on the right was steamringed below the cotyledons. The left pair of plants were covered with a polyethylene bag and developed a saturated atmosphere soon after treating. The right pair of plants were left in the greenhouse in a fairly dry atmosphere. Phloem movement to the roots was prevented by the ring in each environment. In the saturated atmosphere the urea moved via the xylem to the opposite cotyledon and into the top leaves.

Fig. 5. Comparative mobility of 2,4-D (2,500 p.p.m.), amitrol (5,000 p.p.m.), and maleic hydrazide (5,000 p.p.m.) in *Zebrina pendula*. Mature plants in depleted soil. Treatment 4 days with 0.5 μc.; **top:** radioautogram; **bottom:** mounted plants.

movement when the treated plant is placed in a saturated atmosphere. Not only is phloem movement increased; there is apparently a temporary reversal of the transpiration stream so that these compounds are taken into the xylem and moved down into the stem and then upward into untreated stem and leaves above the treated leaf. Also the untreated opposite cotyledon of cotton becomes labeled when C^{14}-labeled compounds are used. Figure 4 shows the results of such treatments.

One additional phenomenon needs consideration at this point; this is the possible transfer of molecules from phloem to xylem during their movement in the vascular channels. Figure 5 shows three *Zebrina* plants treated with 2,4-D, amitrol, and MH, respectively. The 2,4-D has penetrated the cuticle, entered the symplast, moved to the phloem, and on into adjacent stem tissue. However, because of binding in all living cells along the route it has moved only a few centimeters in the stem.

Amitrol after entering the phloem has moved to root and shoot tips; its movement has been limited to phloem. Under like conditions MH has moved throughout the phloem; it also has transferred to xylem and entered every transpiring leaf. This compound, like phosphorus, is evidently able to circulate in the plant. Dalapon seems to resemble MH in this respect.

Keeping these various responses in mind it is interesting to examine the constituents of a spray formulation and seek out their various functions. The surfactant plays several roles: it promotes wetting of the cuticle and hence may bring about stomatal uptake when stomata are open. The surfactant may serve as a filming agent and cosolvent holding the regulator molecules in a liquid layer in intimate contact with the cuticle when the water in the spray has evaporated. And if the surfactant dries to a liquid film of low surface tension and highly lipophilic properties it may creep along the surface of the cuticle, pass the entrapped bubbles in the micro-capillaries, and make contact with the water continuum. These various func-

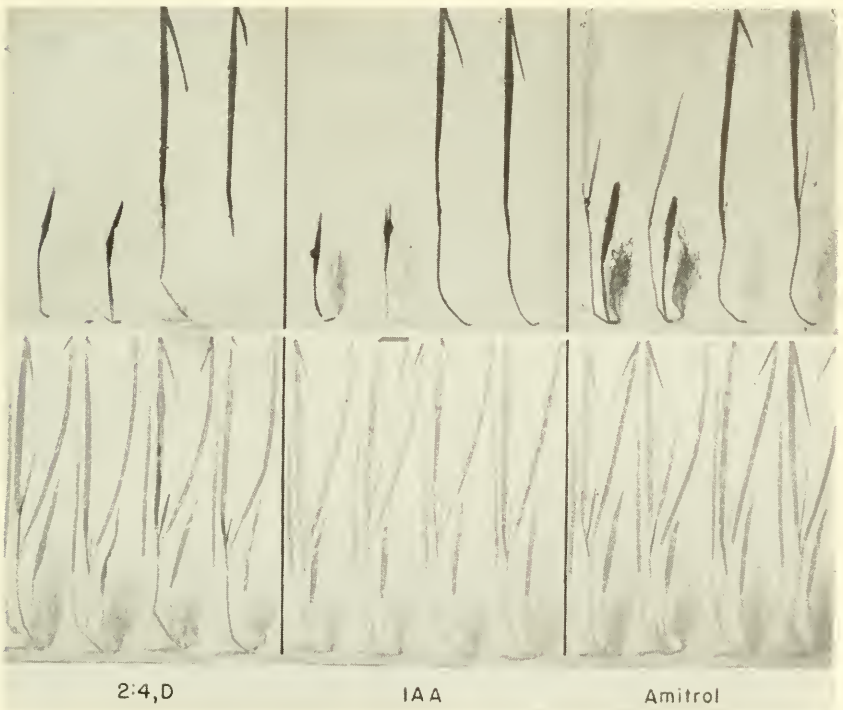

Fig. 6. Comparative mobility of 2,4-D, IAA, and amitrol in barley. Pair of plants on left in each group treated on leaf no. 1; right-hand pair treated on leaf no. 4. Dosage, 0.05 μc. Treatment time, 24 hrs.

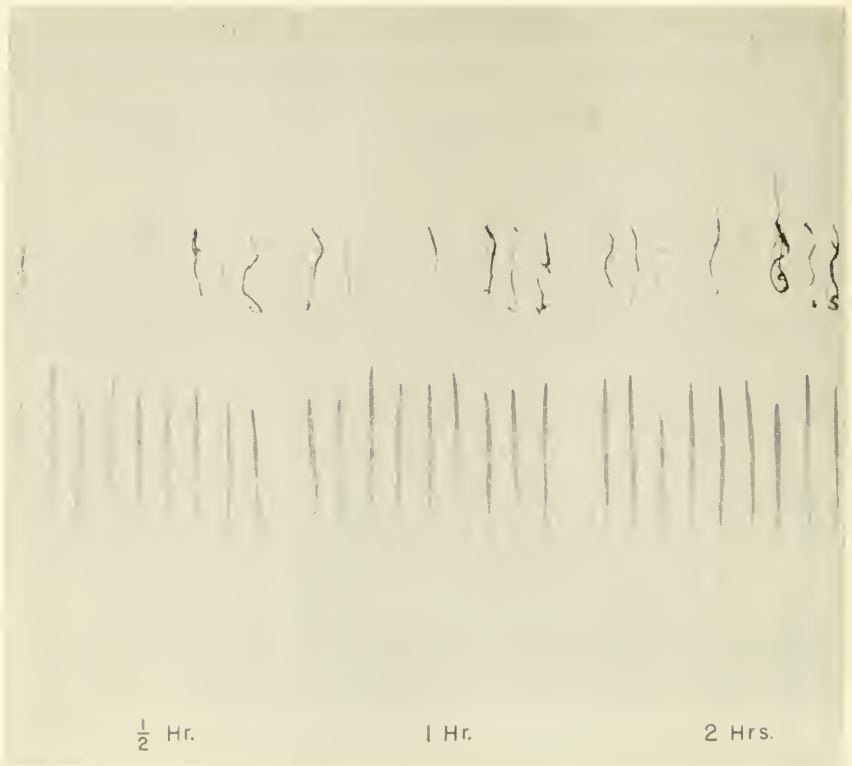

Fig. 7. Comparative uptake of nine labeled compounds by roots of barley seedlings. Autoradiograms above, mounted plants below. Dosage 0.01 μc. per 4 ml. per plant. Treatment times as indicated. Compounds from left to right: 2,4-D, amitrol, MH, urea, monuron, dalapon, simazin, $P^{32}O_4$, IAA.

tions explain the virtues of a surfactant for use in sprays on plants operating under water stress.

Humectants such as glycerin (Table 3), glycols, and $CaCl_2$ aid in the attainment of liquid-liquid contact between the spray solution and the water continuum in the cells. They may do more harm than good under saturated conditions as they bring about run-off of the spray solution.

Oils are used as filming agents where 2,4-D is used in controlling woody plants. It holds the toxicant in intimate contact with the cuticle or bark and serves to regulate penetration to a slow ordered movement.

Recent work by Leonard (personal communication) has shown that ammonium thiocyanate included in an amitrol formulation (Amitrol-T) protects the treated foliage from rapid contact action

and hence makes for a slow but continued uptake of the toxicant. The treated foliage remains green for several days after control plants have been completely killed, yet regrowth is almost completely inhibited.

Relative mobility is a property inherent in the regulator molecules. Figure 5 shows one series, Figure 6 a similar series in barley. If we could find a molecule that translocates as freely as MH and is as toxic as 2,4-D, many of our problems of perennial weed control would be solved.

Finally, turning to soil application, there are inherent differences in molecules with respect to uptake and distribution. Here we have very little basic information. The following observations have been made. When C^{14}-labeled 2,4-D, IAA, amitrol, MH, urea, dalapon, monuron, or simazin, or P^{32} as phosphate are applied to roots of barley seedlings, 2,4-D, monuron, simazin, and IAA are absorbed sufficiently to autograph strongly in 30 min. Monuron and simazin move into the tops in 2 hrs. and continue to accumulate to the 8th day.

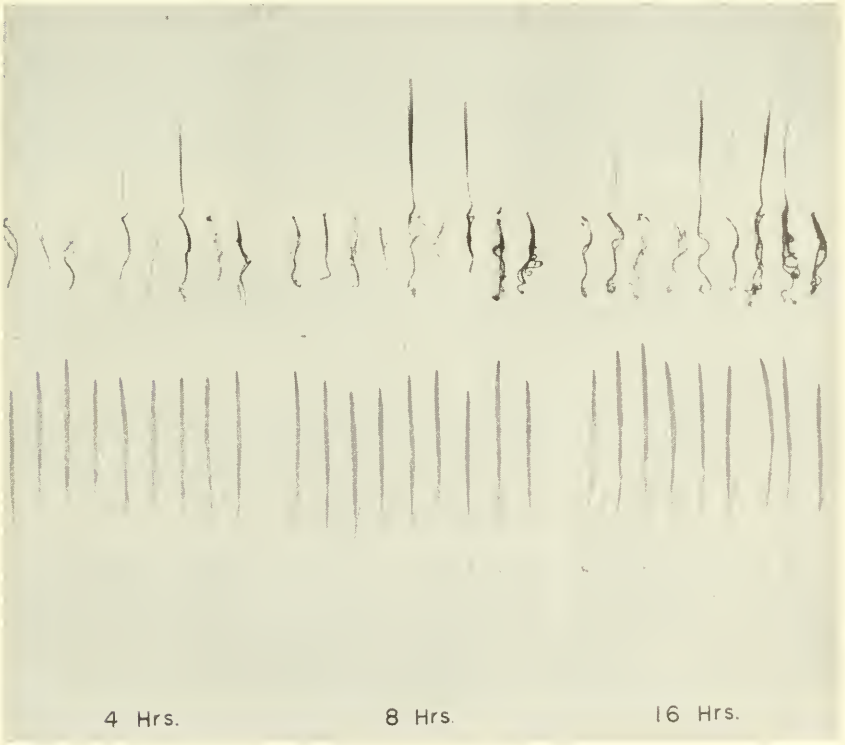

Fig. 8. Same as Figure 7, treatment times 4, 8, and 16 hrs.

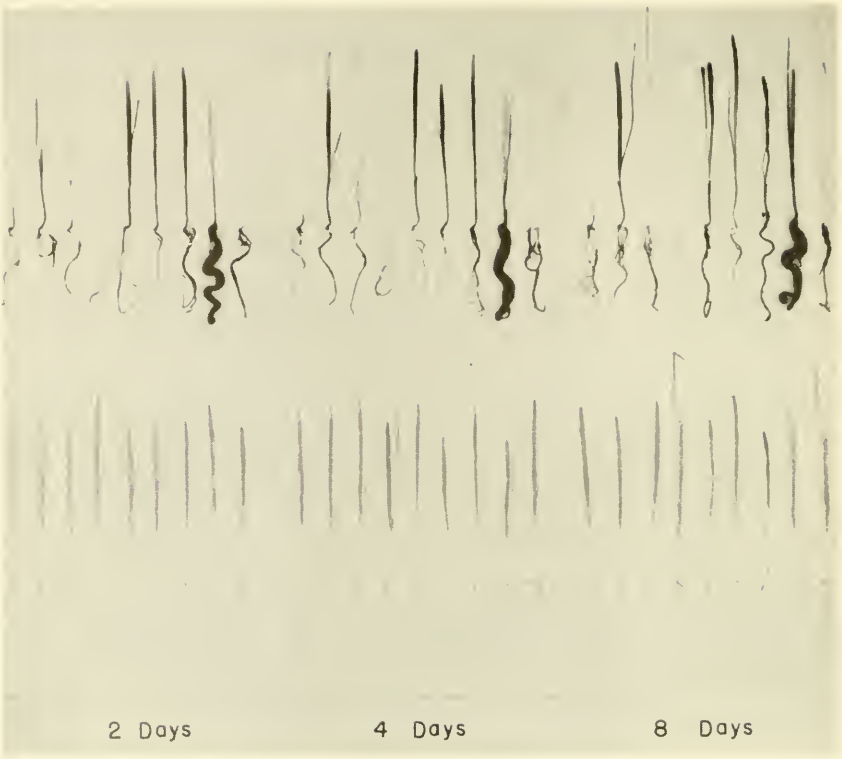

Fig. 9. Same as Figure 7, treatment times 2, 4, and 8 days.

Next to move to the tops, in order of appearance are: amitrol and PO_4 in 4 hrs.; by 8 hrs. dalapon, IAA, and MH; after 2 days 2,4-D at very low concentration; after 4 days urea, or some compound carrying its C^{14}.

After 8 days, concentrations in roots from high to low are in the following order: PO_4, IAA, simazin, monuron, amitrol, 2,4-D, MH, dalapon, urea. In tops the order is simazin, monuron, amitrol, PO_4, dalapon, MH, IAA, 2,4-D, and urea. Figures 7, 8, and 9 show the results of this experiment.

Formulation of soil-borne chemicals usually aims at solubility as related to penetration and leaching, at particle size as related to rate of solution, at pelleting as a means of distribution and of selectivity, and at pellet composition as related to partitioning.

From these various observations it seems that formulation holds much promise for obtaining maximum usefulness of regulator molecules. Synthesis and screening are furnishing new compounds and

should continue to do so. The conscious design of more effective plant growth regulators for agricultural use is an ever present challenge to pesticide workers and biochemists.

ACKNOWLEDGMENT

Much of the work reported here was supported by A.E.C. Contract AT(11-1)-34 Project No. 9 and Project No. 38.

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DISCUSSION

Dr. Henderson: What form of 2,4-D was used and where was the label? Do you believe that 2,4-D does not enter as the whole molecule?

Dr. Crafts: We used 2,4-D acid, labeled in the carboxyl position. We're convinced that with this compound the bulk of the labeling in our autograms is 2,4-D. With maleic hydrazide, and some of the substituted benzoic acids we're fairly certain that these molecules are stable enough that our autograms show the distribution of the orig-

inal compound. Now with IAA I must admit that I have no idea what it is that's moving, but we hope eventually that we can find out. I suspect it may be a conjugate.

Dr. Thimann: Could you say why you are convinced that radioactivity in the 2,4-D autograms represents unchanged 2,4-D?

Dr. Crafts: My conclusions are based on several observations and on Dr. Weintraub's work showing that the decarboxylation of 2,4-D goes on at a relatively slow pace. We can also extract these compounds from the roots and get bud suppression and leaf deformation typical of 2,4-D. We are now engaged in chromatographing the extracts of these and hope to follow this much further.

Professor Blackman: We have heard a good deal about the effects of surfactants *per se*. Perhaps I might point out that if you take a series of different types of wetting agents and increase the concentration past the point where there is any further and appreciable lowering of the surface tension of water then, though the surface tension is not changed, there will still be effects of concentration on the level of retention following a spray application. Moreover there will be significant interactions between the species and the type of surfactant. One of the many queries that arise is whether measurements of surface tension by accepted methods employing static conditions are applicable to droplets impinging on the leaf surface.

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Some Comments on Growth Regulators With a Potential in Agriculture[1]

The topic for discussion is the design of more effective plant growth regulators for agricultural use. This paper will propose that a number of effective growth regulators for agriculture are already designed and that they simply await the recognition that will follow their wider testing. Although a full range of variously substituted aryloxyalkyl carboxylic acids has been studied in the laboratory in biological tests of relative potency, only a few have thus far found horticultural use. If one names the 2-methyl-4-chloro-, the 4-chloro-, the 2,4-dichloro-, and the 2,4,5-trichlorophenoxyacetic, alpha-propionic, and gamma-butyric acids, he has very nearly covered the field.

It is interesting that although the phenoxyacetic series has been in use for some time, the phenoxypropionic and butyric analogues are much more recent arrivals on the commercial scene, usually for a higher degree of selective weeding, and the propionic derivatives also for crop control in circumstances where the acetic derivatives would be quite injurious. Wain (6) and co-workers have adequately pointed out that the relative activities of the butyric and acetic analogues derive from merely quantitative considerations in some plants, but qualitative differences in others. It is the qualitative aspect that I wish to stress, for in this may lie the key to greater usefulness of other phenoxyalkyl carboxylic acids in crops too sensitive to those mentioned.

For instance, the pineapple plant may be forced into unseasonal flowering by 2,4-dichlorophenoxyacetic acid (2,4-D) at rates from 5 to 10 g/acre (rates such as would be encountered in spray drift from herbicidal applications of 2,4-D at 1 to 2 lb/acre). This can result

[1] Published with the approval of the Director as Technical Paper No. 268 of the Pineapple Research Institute of Hawaii, Honolulu, Hawaii.

Table 1. Some substituted phenyl- and phenoxyalkyl carboxylic acids with herbicidal properties on young broad-leaf weeds.

Acid*	Herbicidal Rate, Lbs/Acre†	Pea-Stem Test, Per Cent of Activity of NAA‡	Induces Flowering in Pineapple
2,4-Dichlorophenoxyacetic	0.25	96	yes
2-Methyl-5-bromophenoxyacetic	2	137	no
2-Methyl-5-chlorophenoxyacetic	2	115	no
2,5-Dichlorophenoxyacetic	2	57	no
α-2,4-Dichlorophenoxyvaleric	2	68	no
3-Chloro-5-methylphenoxyacetic	2	7	no
3,5-Dimethylphenoxyacetic	2	0	no
3-Ethylphenoxyacetic	8	110	no
2-Methylphenylacetic	2	287	no
2,5-Dimethylphenylacetic	8	98	no
2-Chlorophenylacetic	2	111	no
2,4-Dichlorophenylacetic	4	150	no
3,4-Dichlorophenylacetic	8	75	no
4-Chlorophenylmercaptoacetic	2	29	no
3-Chlorosalicylic	4	0	no

* All except 2,4-D and 3-chlorosalicylic acid (Dow Chemical Co.) and 4-chlorophenylmercaptoacetic acid (Evans Chemetics, Inc.) synthesized by R. W. Leeper, Pineapple Research Institute, Chemistry Department.

† Sprays at 100 gallons per acre; chemical dissolved in dimethylformamide and applied with triethanolamine and a wetting agent.

‡ Data of M. J. Kent, Pineapple Research Institute, Chemistry Department: 1-naphthaleneacetic acid used at 200 p.p.m. as a standard for comparison.

in loss of the crop, and for this reason such compounds are not used for weed control in pineapple fields. The pineapple plant is forced to flower by the α-propionic and γ-butyric analogues of 2,4-D also. However, within the halogen- or alkyl-substituted phenoxyacetic acids, several of the 2,5-substituted and the 3,5-substituted compounds have some herbicidal activity, but all such compounds have thus far failed to force pineapples to flower. Results with some of these compounds and others are shown in Table 1. Pybus *et al.* (5) have recently reported on the effectiveness of certain phenylacetic acids.

Of these compounds, all have had more or less activity in the split pea-stem test for growth regulator activity with the exception of 3-chlorosalicylic acid and the 3,5-substituted compounds. The latter are herbicidal apparently owing to some contact, or less general systemic, activity. Such materials, then, although much less effective than 2,4-D as herbicides, would merit further investigation in crops exceptionally sensitive to 2,4-D since they might be tolerated in drift amounts. Although some of them have the advantages of systemic herbicidal activity, they would be no more injurious to the crop than some nongrowth-regulator chemicals in a misdirected contact spray.

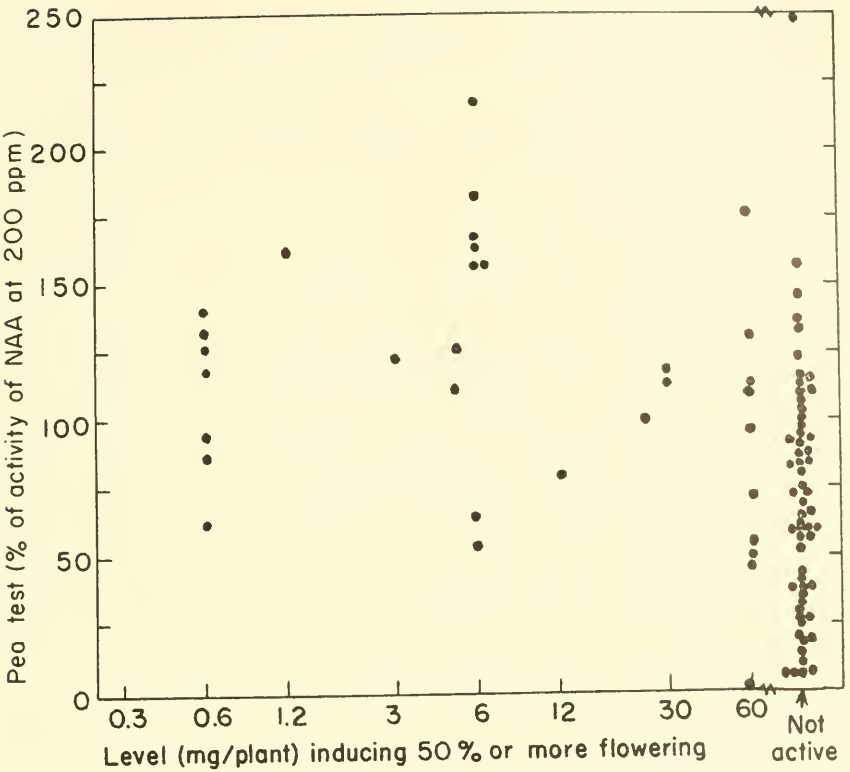

Fig. 1. Lack of correlation between activity in the split pea-stem curvature test and induction of flowering in the pineapple. Data for 92 substituted phenoxyalkyl carboxylic acids, omitting data for 53 others inactive in the pea test which were also inactive in flower induction.

The emphasis in the previous paragraphs was on toxicity, but the point should be recognized that there are qualitative differences in morphogenic responses between compounds which assay as "auxin" in common in *growth* responses.

From Figure 1 it can be readily seen that there is no particular correlation between activity in the split pea-stem curvature test and ability to induce flowering in the pineapple plant. Of the 92 variously substituted phenoxyalkyl carboxylic acids shown active in the pea test, only 33 were active in flower induction. The compounds inactive in flower induction ranged from 6 to 249 per cent of the activity of NAA in the pea test, and were tested in many cases on pineapple at rates up to a toxic level.

Another example was reported some time ago by Leopold (4, p. 302) in connection with pineapple. He pointed out that the use of

1-naphthaleneacetic acid (NAA) for flower induction in this plant resulted in a suppression of slips.[2] However, *p*-chlorophenoxyacetic acid (PCA) induces slip production in plants forced with NAA. These two materials, although both auxins in growth responses and although both will induce flowering in the pineapple, are actually antagonistic in this other morphogenic response. When the two are applied at various rates in combination, the NAA offsets the slip-producing activity of PCA. Other instances of antagonism in morphogenic effects have been reported by the writer, e.g., that of indole-3-acetic acid for NAA, and of indole-3-acetic acid for indole-3-butyric acid in the flowering-induction response in the pineapple (2, 3). The horticulturist seeking a chemical to give a morphogenic response cannot afford to assume that if one growth regulator is active, the activity of all others will be in the same direction, and only to a greater or lesser extent.

A further point which has been but little investigated is the range of morphogenic responses assignable to various levels of application, short of outright toxicity. As an example of the importance of the proper level of growth regulators to obtain a particular response, the inhibition of flowering in pineapple by NAA may be mentioned. A number of years ago, Clark and Kerns (1) reported that NAA at a concentration of 10 p.p.m. induces flowering, but suppresses flowering at 1,000 p.p.m.

The induction of flowering by NAA has been a regular practice for a number of years, but the chemical inhibition of flowering on

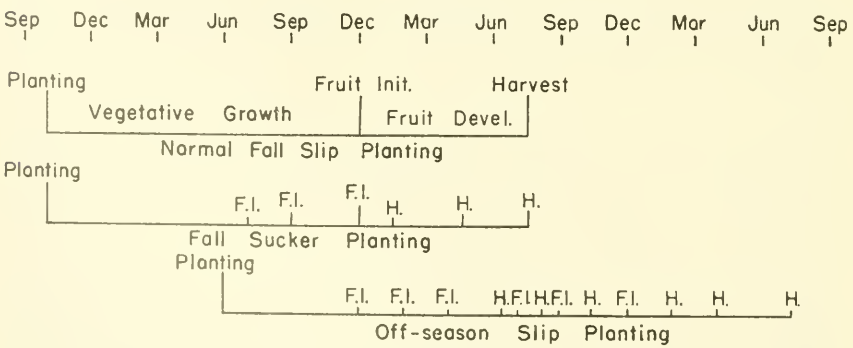

Fig. 2. A sketch of the circumstances of pineapple crop production under Hawaiian conditions. Year-around harvest (H.) is desirable for cannery operations. However, several periods of fruit initiation (F. I.) in a given field are costly. (See text.)

[2] The slip is morphologically a fruit, but is overwhelmingly vegetative rather than fruitlike in its development and is the preferred planting material for succeeding crops. It is borne on the peduncle just as is the fruit, in distinction to the crown, a vegetative organ borne on the top of the fruit, and the sucker, which is a branch off the main stem. Slips and suckers normally develop at the time of natural fruit initiation, and the crown is initiated after the florets of the inflorescence have been laid down.

pineapple plantations is not yet on a commercial scale. A moment's digression to Figure 2 will illustrate the problem. Although the crop from slips planted in the fall initiates inflorescences quite uniformly in December of the succeeding year and is harvested as a unit the following summer, to handle all the crop in this way would exceed the fruit-packing capacity of the canneries. Consequently, plantations are compelled to harvest the year around, and it is in trying to make the population in a field of fall-planted suckers, or in a field planted in an off-season, behave as a unit that the problem arises. Commonly, in such fields, a percentage of the plants will differentiate in one month, more in another couple of months, and so forth as shown in Figure 2. Fruits and plants of different physiological ages in the same field increase costs by increasing the number of harvest periods and of harvest rounds within these periods, and they make maintenance of optimum fertilization and insecticide schedules quite impossible. Moreover, precocious fruits from small plants are often small. The development of an acceptable inhibitor of precocious flowering is therefore quite important.

Figures 3 and 4 will show why the use of NAA at 1,000 p.p.m. to inhibit flowering has not become a general practice, despite the seriousness of the problem. Figure 3 represents an untreated 15-month-old nonfruiting pineapple plant stripped down and the leaves arranged from left to right according to their position on the stem (oldest leaves at the bottom). It will be seen that as the pineapple plant grows the length of the longest leaf produced increases with age of the plants to a maximum. It will continue to hold this maximum until flowering

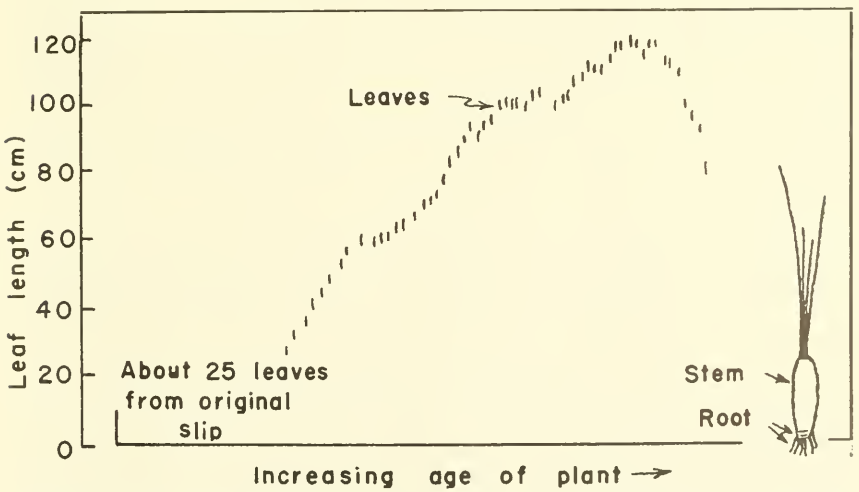

Fig. 3. Lengths of leaves of nonfruiting pineapple plant 15 months after planting. Oldest leaves at left; each mark one leaf tip. (Leaves with broken tips omitted.)

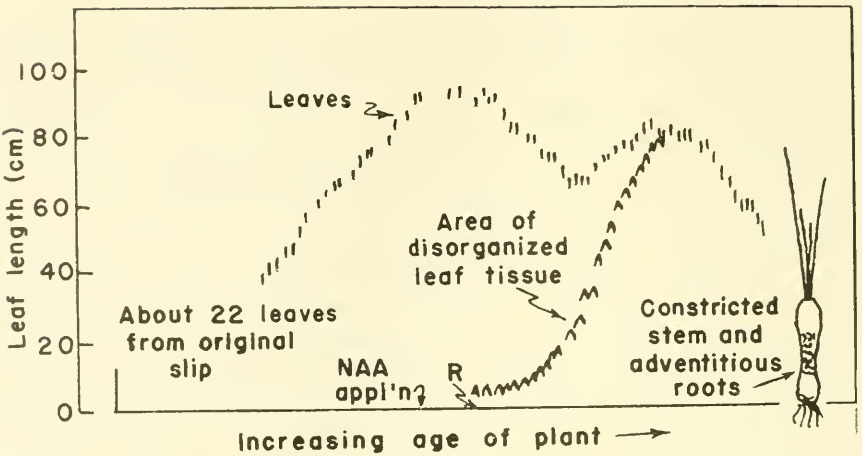

Fig. 4. Representation of 15-month-old pineapple plant 5 months after treatment with NAA at 1,000 p.p.m. Some rotting of leaf bases and proliferation of adventitious roots on basal leaf tissue at R.

is induced either naturally or artificially. (The shorter leaves on the right are the immature leaves of the growing point at the stem tip). Figure 4 shows a similar plant stripped down about 5 months after application of NAA at 1,000 p.p.m. Note that from the time of application the growth of the developing leaves was severely curtailed, there was some damage to the leaf tissue, some rotting and root formation on the basal white leaf tissue, a marked constriction and elongation of the stem, and an unusual proliferation of roots for some distance up the stem. The constriction of the stem and the evidence of disturbed growth in the leaves is eventually outgrown, and succeeding leaves attain again the previous maximum length, provided the plant is not induced to flower naturally or artificially. However, the total mass of green tissue which must contribute to the fruit has been reduced much below that illustrated in Figure 3. Reduced plant size generally means reduced fruit size, and when a standard can size for sliced pineapple must be met, this is not acceptable.

Quite apart from the commercial aspects, is the shortened leaf an indication of an induced juvenility? There is some circumstantial evidence in favor of this view. The leaves on 'Smooth Cayenne' pineapple are normally spineless except at the tip. (This is, of course, the first tissue laid down in the growth of these monocotyledonous leaves.) When excessive levels of NAA have been used (and incidentally also when the plant resumes growth after a period of drought), some spines may be produced on the leaves just below the area of constricted growth. Parenthetically, it may be suggested that this is further evi-

dence that ontogeny repeats phylogeny, because it is suspected that the 'Smooth Cayenne' plant was derived from a spiny ancestor.

Two points can be made here in respect to Figure 4. One is that the quantitative differences in rate of application of NAA have qualitatively different effects in differentiation responses. And the second point, which is suggested by this, is that other growth regulators may provide the same desirable over-all effect of inhibition of flowering without some of the undesirable (or at least nonessential) side effects of proliferation of roots, constricted stems, and spininess. We have some evidence that this is so.

From these illustrations, I should like to derive the principle that a good deal of work remains yet to be done with the substituted aryloxyalkyl carboxylic acids and other recognized growth regulators, common or uncommon. Much *will* be done if horticultural workers will refuse to take for granted that all these materials behave the same and vary only in the degree of activity. Admittedly, it is scientifically less satisfying to move into a program of testing without a well-developed hypothesis. We do have some well-developed hypotheses in the testing program for crop control in pineapple production, but these were formulated only after empirical testing of several hundred compounds. Some of the earlier hypotheses, as was inevitable, required modification as the body of test results increased and new evidence became available. However, the net result has been the development of information useful in enlarging our conceptual framework along with its practical value.

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DISCUSSION

Professor Blackman: Since we are discussing tropical applications, perhaps another example might be given where existing knowledge and existing compounds can be used as a basis for a research program. I am referring to the program on the stimulation of latex flow

in old rubber trees which is being conducted at the Rubber Research Institute in Malaya. The new clones of rubber trees in Malaya are many times as productive as the unselected trees planted 30 to 40 years ago. There is a great need to replace the old trees as rapidly as possible, but they cannot all be replaced at once because then there would be no rubber on the estate. It has been found that if esters of substituted phenoxyacetic acids are applied below the tapping panel in an oil vehicle, the rubber flow is appreciably increased for as much as six months. It is relevant to the discussion of the *Avena* test that 2,4,5-T causes the greatest stimulation, followed by 2,4-D. Substitution in the 6 position, dimethyl substitution, or monochloro substitution, all greatly decrease the activity. The effects of other growth regulators are now being explored.

Dr. Fawcett: How do 3,4-dichloro- and 2-methyl-4-chlorophenoxyacetic acids compare with 2,4-D for the promotion of flowering?

Dr. Gowing: In connection with Prof. Blackman's comments, 4-chlorophenoxyacetic acid, 2,4-D, and 2,4,5-T show about the same order of effectiveness in induction of flowering in pineapple as in stimulating the flow of latex. The 2,4,5-T is the most active of the group.

Dr. Fawcett asked about the 2-methyl-4-chloro- and the 3-methyl-4-chlorophenoxyacetic acids. The 3-chloro-4-methyl compound has 249 per cent of the activity of 1-naphthaleneacetic acid in the split pea-stem curvature test but doesn't induce flowering in pineapple. However, the 3-methyl-4-chloro compound does induce flowering. Similarly, the 2-chloro-4-methyl compound has no activity in flower induction but has considerable activity in the split pea-stem curvature test, and the 2-methyl-4-chloro compound (MCP) is active in both tests. MCP is active in flower induction at about the same level as 2,4-D (0.6 mg. per plant for 50 per cent or more induction). The 3,4-dichloro compound induces flowering in the pineapple at about 3 mg. per plant for 50 per cent or more effectiveness. Consequently, it is excluded from commercial consideration as an herbicide in pineapple plantings.

Dr. Sachs: In view of the multiplicity of the responses observed by Dr. Gowing, such as effects on flower initiation, herbicidal effects, etc., I am wondering about the mechanism and site of action of these substances. Is there any universal point of view that can be developed? Will it be one Kingdom of Heaven or seven? I think there has been a general impression that the primary site of action of most growth regulators is in the cell walls, probably by causing them to become soft and thereby letting them extend. I don't hold to this view, or at least I don't see how one can jump from softening a cell wall to getting a nucleus to divide to getting flowers to be initiated.

Dr. Gowing: "In my Father's house there are many mansions."

Dr. Thimann: There are some rather striking differences between the effectiveness of growth substances in simple growth tests and their effectiveness in the production of parthenocarpic fruits. An important point to bear in mind is that in different types of tests like these, there is a very wide difference in timing. After the substance is applied to the pineapple, how many weeks or months later, Dr. Gowing, is it that the flower initials appear?

Dr. Gowing: It varies from 50 to 120 days.

Dr. Thimann: In contrast, a growth test is usually done in 24 hrs. So one must consider that the determining factors for activity in a long time test are going to include not only activity *per se,* but the ability of the substance to be adsorbed or combined on other constituents of the plant and its ability to be metabolized or destroyed, or at least made inactive. In considering those different phenomena that can intervene between application and finally getting the effect, we will probably get the explanation of these differences.

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Physical-Chemical Factors of Surfactants in Relation to Their Effects on the Biological Activity of Chemicals

It is not possible to state the exact manner in which wetting agents or other types of surfactants may be employed to increase the effectiveness of biologically active materials. In the first place, the term surfactant, which is a contraction of surface active agent, denotes any organic substance which possesses surface active properties. A great number of surfactants have appeared over the past 30 years. Primarily, the big boom started with the expansion of the soap industry immediately after World War I. Development of synthetic detergents was accentuated greatly during World War II. During the past ten years there has been a tremendous boost in the number of surface active agents of all types. The variety of commercial applications of surfactants is almost phenomenal. From the usage standpoint, surfactants may be classified as soaps, detergents, wetters, emulsifiers, dispersants, spreaders, thickeners, solubilizers, etc. In this paper are presented some of the physical-chemical properties of surfactants which I believe warrant consideration in future experimentation. It also calls attention to a great bulk of information which has been brought together and summarized by Schwartz and Perry (1). This book brings up to date most of the surfactant information as it existed up to 1947. Developments which occurred from 1947 to 1957 are summarized in a second volume by Schwartz, Perry, and Berch (2).

We have previously examined some of the important biological applications of surfactants. Dr. Phinney has demonstrated greatly enhanced uptake of gibberellic acid from surfactant solutions. Crosby and Vlitos have found certain naturally-occurring long-chain alcohols and acids, which undoubtedly exhibit some surfactant properties, to have growth stimulatory activity. In the same manner, Stowe has ob-

tained growth promotion from Tween 20, a polyoxyethylene sorbitan monolaurate. He has also employed surfactants in application procedures to emulsify and stabilize fatty materials. Classic uses of surface active agents in growth-regulator and herbicide research have presumably been associated with improved wetting, sticking, or spreading qualities. They have also been used to emulsify or disperse poorly soluble or nonsoluble compounds in aqueous and/or oil systems.

The majority of surfactants have a characteristic type of surface activity with respect to the energy relationships of the interfaces formed. The definite free energy relationships of these interfaces are measured in two different ways. They are referred to as surface tension for the liquid-gas interface and as interfacial tension for the liquid-liquid and liquid-solid interfaces. In Figure 1A are illustrated the generalized ranges of values for these free energies found for solutions of most surfactants. From a value of 72 dynes/cm for pure water, surface tension falls rapidly as surfactant concentration is increased to approximately 0.1 per cent. At higher surfactant concentrations the surface tension remains more or less constant. Interfacial tension relative to surfactant concentration behaves similarly. The latter is usually measured between an aqueous and a Nujol or mineral oil phase. Since the effective biological concentrations of surfactants in general are greater than 0.1 per cent, one must assume that biological effectiveness is better correlated with other properties than surface energies.

One point which may be significant in explaining biological effectiveness is the fact that actual measurements of surface and interfacial tensions do not always coincide with theoretical values. A great many surfactants show the initial rapid drop in tension but at higher concentrations exhibit a slight increase in tension. The lowest point on the tension curve appears to correlate with the formation of colloidal micelles and is referred to as the critical micellar concentration. Since the end of the steep drop in tension is usually found in the concentration range from 0.01 to 0.1 per cent, it seems probable that at biologically effective levels surfactants are behaving as strong colloidal systems.

Several other properties of surfactants undergo marked changes in regions corresponding to the critical micellar concentration. One of these is the relative conductivity of the solution. In Figure 1B the conductivities of an homologous series of alcohol sulfonates are illustrated. As concentration is increased, very little change in electrical conductivity is found for the C-2 to C-7 homologues. Higher members of the series, however, exhibit a rapid drop in relative conductivity

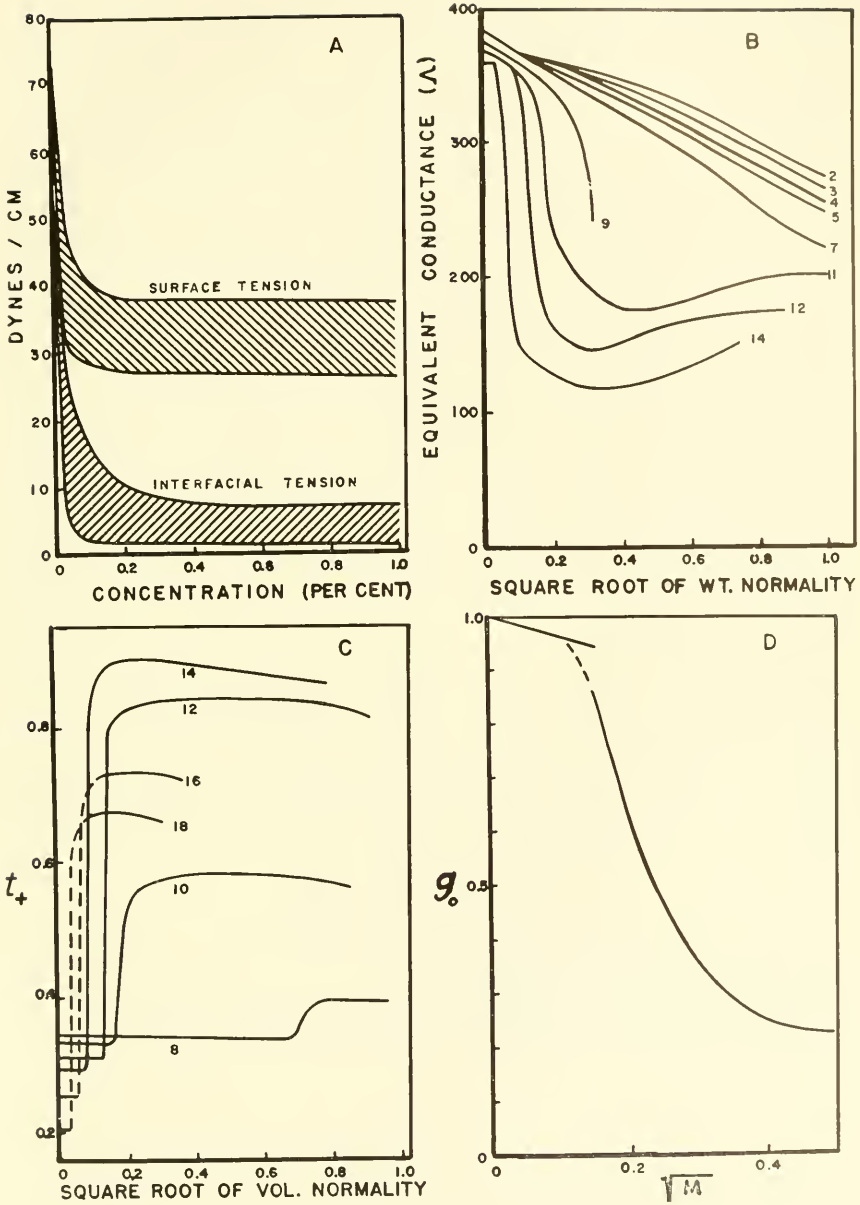

Fig. 1. Effects of surfactant concentration on several properties of surfactants. **A.** Generalized ranges of values for surface tension and interfacial tension for the majority of surfactants. **B.** Equivalent conductances for a series of alkyl sulfonic acids (numbers indicate length of the carbon chains). **C.** Cation transference numbers for a series of alkyl amine hydrochlorides. **D.** Osmotic coefficient of sodium tetradecylsulfate. (Modified after Schwartz and Perry (1) by permission of Interscience Publishers, Inc., New York.)

in the region corresponding to the critical micellar concentration. One can conceive what tremendous effects could result from placement of such a solution on the charged surface of a cell wall and how these effects could be transmitted through the aqueous continuum in the cuticle and cell wall to the charged cytoplasmic skeleton.

Electrophoretic and osmotic properties of surfactant solutions are also modified anomolously as concentration of the surfactant is increased. In Figure 1C we see the cation transference numbers for an homologous series of alkyl amine hydrochlorides plotted against concentration of surfactant. Abrupt changes occur in cation transfer after we pass the C-8 moiety. Among higher homologues the magnitude of the change is increased as chain-length increases to C-14, but thereafter the magnitude is lessened. The effect of concentration of a representative surfactant on the osmotic coefficient, as determined by freezing point depression, is plotted in Figure 1D. The osmotic coefficient of surfactant systems is considerably less than predicted values at concentrations commonly employed in industrial and biological work. In the case of the representative surfactant used in the illustration, the breakpoint occurs at a concentration of approximately 0.8 per cent.

One cannot comment at the present time on the importance of all of these phenomena to the behaviors of biologically active chemicals. They are factors, however, which conceivably could materially influence penetration, translocation, and chemical activity. The purpose of this presentation has been to draw attention to those factors other than surface tensions which may be influential in the biological activity of systems containing surfactants.

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The Next Steps

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The Probable Future of Auxinology

The only way in which we can forecast the future of a human activity is by projecting past trends into the future. This does not allow us to predict with certainty; it only allows us to predict a probability, just as the weather forecaster predicts the weather. He says, "Well, I can measure some things today, and I can look around and see how things were yesterday, and I can see how things probably will be tomorrow if the situation develops as similar situations have developed in the past." He forecasts a probability, and as you know about weather forecasters, they are often wrong but never in doubt. And in this same spirit, tempered by a certain amount of humility, I make my own forecast of the probable future of auxinology.

Let us start with the matter of auxin conferences. We've had quite a number now. The first one was held, I understand, in Paris in 1937, and it was attended by about 20 people. In 1949 a conference was held in Wisconsin and the official participants numbered about 45. At the third auxin conference at Wye in 1955, the participants numbered about 65. Today, in 1959, we have over 100 people in attendance. Twenty in 1937, 45 in 1949, over 100 in 1959. It is clear that the doubling time for the number of people who attend an auxin conference is about 10 years. And this enables us to predict, then, that 100 years from now, at the conference to be held in 2059, there will be approximately 100,000 people in attendance. It's a heartwarming prospect.

There is another trend we can also foresee. Quite evidently the number of people who attend auxin conferences is increasing more rapidly than is the total number of people in the world. The time must therefore inevitably come when everyone, each single individual on the earth's surface, will be an auxinologist. And if we bear in

mind the relevant rates of increase of people and of auxinologists, we can determine by simple calculation that the crossover point, the time at which all people will be auxinologists, will be in the year 2229. (Don't trust the last figure.) At that time there will be approximately ten billion people on the earth's surface, and they will all be auxinologists. Isn't that a wonderful thought?

We know further that an attendant at an auxinology conference submits a paper which appears in a book, and that on the average, the individual paper contributes five pages to this book. The book that results from our conference in the year 2229 will therefore consist of fifty billion pages. If we bind the proceedings of the conference up in volumes, each of 1,000 pages (which is a handy size), they will constitute approximately 50 million volumes. And if we put these volumes on a library shelf, they will occupy a shelf approximately 120 miles in length. My trusted agent in the Library of Congress has studied the reading habits of scientists, and he assures me that the biologist on the average reads down the shelf of biological literature at the rate of about 12 inches a year. This means, therefore, that if an individual wishes to read the Proceedings of the Auxinology Conference of 2229, he must realize that the task is going to take him about one million years.

This isn't such a good prospect, and in fact our forecast begins to appear rather ridiculous. It has evidently been made on too unsophisticated a basis. Let us therefore start afresh and base our forecast on an entirely different model. We know that all human activities undergo a grand period of growth. They rise and attain a maximum only to decline again. This is true of human activities, of human innovations. Innovations are innovated; they appear; they increase in number or importance; and then they disappear as they are supplanted by some new innovation. Take, for example, bows and arrows. They were invented and they increased until some large number of them were present on the earth's surface, and now they're almost gone again. Or take horses on the North American continent. They were introduced in the year 1515. They increased, attaining substantial numbers, and reached a maximum about the time of the first World War. Since the end of the first World War they have catastrophically declined in numbers until they have now almost disappeared.

This same behavior is characteristic of the exploitation of fields of human activity. Take an oil field. We find an oil field and some people put down a well, and they start extracting the good stuff out of the ground. They get it, and other people start putting down more and more oil wells and they pump out more and more oil each year. But finally the oil gets harder to find, and you have to pump it from

deeper and deeper. And so the rate of production of oil goes down and down and ultimately declines to zero. This has actually happened to oil fields in our country. It is an interesting fact that oil production during the exploitation of an oil field first increases with time along an S-shaped curve. It then reaches a maximum and this is followed by decline also along an S-shaped curve. The curve which describes production as a function of time is symmetrical about its maximum. Rate of production rises and falls along a symmetrical bell-shaped curve, a curve which resembles appropriately enough that which describes how growth rate of the *Avena* coleoptile section rises and falls as we increase auxin concentration. Perhaps, then, the model that we should use for forecasting the future of auxinology is not just an unsophisticated doubling every so often but rather that of the exploitation of a field.

Perhaps we should think about the exploitation of a field of knowledge. And so I have investigated auxinology from this standpoint too. With what kinetics is auxin lore being extracted from nature?

I have approached this question by studying how many papers are written each year on auxinological subjects, and I have found out how this number has changed with time. Now we can't just count the number of papers that are produced each year because some of them aren't very good, will never be read again, and don't really constitute an extraction of knowledge from nature. As measure I have used, therefore, the yearly number of auxinological papers which someone else has thought worthwhile to cite. This is a better measure. These are essentially contributions to the theory of auxinology — contributions which have influenced other workers.

Now to kinetics. The first paper of modern auxinology was, of course, Professor Went's contribution published in the year 1928, so in that year there was one paper. During the next year two or three papers appeared, and in the next year four or five. The number of papers per year on auxinological subjects thereafter increased steadily and exponentially, following a nice S-shaped curve, attaining a maximum in the years 1948 to 1954. And, I have found to my horror and dismay, that we are already on the declining limb of the curve; we have seen the rise and are now witnessing the decay of auxinology. We may predict that the yearly number of auxinological papers will be down to half-maximal in about the year 1965, and that the last paper on auxinology will be written about 1985.

This would appear to be a rather dismal conclusion. However, it isn't actually quite so gloomy as it seems, or rather it places gloom where no gloom is really intended. I think that you will agree

that this sort of rise and fall is what we must expect in the exploitation of any and all fields of knowledge. Let me make this a point more concretely by reference to a field of knowledge that has already been thoroughly exploited and explored. About 40 years ago, biologists suddenly became aware of the importance of hydrogen ion concentration. We learned that there is such a thing as pH and that we are supposed to measure it. There ensued a great gold rush of biologists to measure the pH of everything — insides and outsides of cells, different parts of cells, the soil — everything one might imagine was pH-measured. And papers concerning the pH of objects of biological origin appeared with rapidly increasing frequency. Numbers of papers per year in this field attained a maximum and then subsequently shrank. The rise and fall of works on pH has followed the kinetics of our exploitation model. What does this mean? It doesn't mean that we're less interested in pH than we used to be. It just means that everybody knows that there's such a thing as pH and that when you do an experiment you should measure the pH, that you've got to be careful about it and put buffers in solutions, and do all the things that biologists do to take cognizance of the fact that the pH of the solution is an important variable. We have essentially incorporated pH lore into biological wisdom and we use it as an everyday part of our equipment for being a biologist.

It is therefore my prediction that by the year 1985, although the last paper specifically dedicated to auxinology will have already appeared in print, auxinology won't really disappear, it will just be incorporated into the total body of classical biology in the same way that pH lore has been assimilated. When people study growth matters, they'll know that there's auxin, that auxin is made in certain places, that it goes around the plant and that it makes leaves not fall off or makes cells increase in size, or makes fruits be parthenocarpic — they'll know the things that auxin does, whatever it does, and this wisdom will be used in assessing the possible existence of any new growth factor, in discussing the interaction of other growth factors, in discussing the ways in which climatic factors influence plants, etc. Auxin lore will just be a part of the background information which we have at our disposal to help us evaluate new facets of the behavior of plants. It will be a part of general plant biological wisdom. And I think that this is not too dismal a prospect. We can look forward perhaps to a decreasing interest in auxinology as a specific subject in its own right, but we can look forward to its becoming increasingly a part of the biological body. I forecast that this change will be attended by an alteration in the educational level at which we offer instruction about auxin matters. There was a time in which people

learned about auxin only during their postdoctoral careers. I learned about auxinology in graduate school. There are many people in attendance at this conference who first learned about auxinology as undergraduates. I predict that by 1985 students will learn about auxin in high school or possibly even in junior high school. It will just be everyday stuff.

We have outlined one trend in auxinology: it is going to become a part of classical biology.

There is another trend that I think we can foresee. We must recognize the debt which plant physiology owes to the discovery of auxin and to the development of the whole field of experimental auxinology. In certain respects the development of auxinology has, I think, made plant physiologists much more into cell biologists. It has interested plant physiologists in things on a cellular level, in finding out how the auxin works and finding out what this has to do with respiration and so on. The study of auxin-controlled plant growth has resulted in the accelerated development of plant physiology along the lines of the study of plant behavior on a cellular level. This is one thing that auxinology has done for plant physiology. A second thing auxinology has done is that it has been responsible, perhaps more than any other single force, for making botanists aware of the existence of chemistry. Auxinology has really brought chemistry to plant physiology and it has taken plant physiologists to chemistry. It has had a very great effect in making plant physiology a more chemical and, I will say, a more sophisticated subject.

We all know that in 1935 it was first demonstrated that it is possible to make compounds synthetically which possess auxin activity and which are therefore synthetic substitutes for naturally-occurring auxin. This discovery resulted in a major gold rush in auxinology. Chemists mysteriously appeared from everywhere — I don't know where chemists appear from — there must be lots of chemists who are technologically unemployed, and who, when some new field opens up, rush in to fill the vacuum that has been created. Anyway, chemists appeared from everywhere to synthesize tens of thousands of compounds, which were then tested for possession or nonpossession of auxin activity. As everyone knows, some of these compounds turned out to have useful auxin activity, to be able to do things such as be herbicides or be abscission inhibitors, and so on. And so to the development of auxinology in this chemical way we owe, to a very considerable extent, the development of agricultural chemical plant physiology. And I think that we can foresee clearly that this trend will continue far into the future, to a future far more distant than I can foresee for you now. People will continue to make more and

more plant regulators. They will find compounds that tell plants to grow, or tell plants not to grow, or tell plants to make sugar, or tell them to please make amino acids. The day will inevitably come when every aspect of the activity of every kind of plant will be supervised by the giving to that plant of an appropriate chemical. The poor little plant just won't have any private life of its own at all.

However, I don't really refer to this as a long-range prospect for auxinology. I think that what auxinology has done is to enunciate and develop the concept of the supervision of plant activities by the application of chemical substances and that the agricultural chemical industry which is based upon this concept is now already very much wider than the concept of auxinology itself. It started a whole new field, and a field which has grown far beyond the bounds of auxinology. Here, then, is another trend which we can foresee for the long-range future.

When we look into the shorter-range future, I think we can get some glimpses of trends in auxinology, trends which are apparent to everyone who has attended the present conference. In the first place, it is quite apparent that some day, any decade now, we shall finally succeed in finding out how the auxin does its work. This is an immediate task of plant physiologists, and some day it will be found out. It is really rather a disgrace to the profession of plant physiology that the nature of auxin action has not yet been revealed. This sad state of affairs is due to the fact that we embarked on the study of how auxin does its work insufficiently prepared with basic knowledge. We have known too little of the constitution of plant cells and of the constitution of the cell wall, and we have been insufficiently prepared with knowledge of the basic cell biology of the plant. We have had, in fact, insufficient knowledge of the basic cell biology of any kind of creature. But now as we get this new knowledge, we may hope that we will be able to solve the problem of how the auxin does its many kinds of work which are manifested in cell elongation, bud and root inhibition, prevention of abscission, etc. And in its effort to attain a solution to the problem of how auxin works we can, I think, forecast that auxinology and indeed plant physiology generally will become what I will call, pardon the expression, more sophisticated, will become a more rigorous science. It will become more physical, more biophysical if you will, more biochemical. Plant physiologists to come will become more enzymological because enzymology is a craft which can obviously contribute to the solution of our problems. Plant physiologists will indulge more in model making, in stochastic research, that is, in the making of conceptual models which can then be tested for correspondence with real life. Plant physiology will, in

short, progress until it resembles in its mode of operation the sciences of biochemistry and of biophysics today. So this is another trend.

We know, too, that because there is such a thing as auxin, one might well look for other kinds of growth factors. That there are other kinds of growth factors is now amply attested by the fact that we know about some of them. We know about gibberellin, for example, and therefore all of the kinds of questions that have been asked about auxin can now be asked about gibberellin, too. When all of the problems of auxinology are solved, it will not mean that we have nothing to do. We can always convert ourselves to working on gibberellin, or working on the isolation of flowering hormone, or some other lesser known growth factor. There is an old saying, or if there isn't such an old saying, there ought to be one, that "Old auxinologists never die, they turn into gibberellinologists." So there are many things to do in the study of plant growth factors other than auxin itself. I predict, therefore, that within another 25 years certainly, the chemical nature and mode of action of a vast array of further plant hormones will be understood.

What, now, is going to happen after plant physiologists have gotten busy and found out about the nature of all of these other growth factors and know how all of them do their work? What will people do then? I have a suggestion. It seems to me that really the most basic biological problem that confronts us, now that the problem of the nature of genetic information and its replication has been wound up, is the problem of differentiation. We have mentioned differentiation many times during the course of this conference. No one has explained anything about differentiation; we just say to each other, "Oh, that's differentiation," or "This substance influences differentiation," or something of similar ilk. We fail to understand differentiation because we haven't yet really had a tool which would enable us to approach the subject in a productive experimental way. How does differentiation take place? It is my prediction that another generation from now this may well be one of the principal studies to which people who would have been auxinologists in the olden days will turn their talents. And I have a little suggestion, too, about how the approach to the study of differentiation might possibly be made. I want to make this suggestion now because perhaps some of us can be thinking about it and perhaps we can jump the gun a little and get on with the study of differentiation.

It is implicit in the thinking of every biologist and has been implicit, too, to a small extent in our discussion at these meetings, that when cells of common cell ancestry differentiate along different pathways and turn into different kinds of cells, these different kinds of

cells are different because they possess different kinds of enzymes. They have different enzymatic complements. They have, as it were, different enzymatic spectra. One type of cell will have lots of enzyme A and little of enzyme B, while of a second type, the converse will be true. And as a matter of fact, I believe that most students of biology now begin to be pretty well convinced that differentiation with respect to enzymatic composition is not only an accompaniment of the differentiation process but is very probably the cause of differentiation itself.

How do cells of common descent and of common genetic constitution get different enzymatic complements? It is a tenet of modern biology, of course, that each enzyme in the cell is produced under the control of a specific gene, a gene which sits in the nucleus and says to the cell, "Please make me some of this kind of enzyme." For each enzyme there is a responsible gene. This is the central dogma of our time. We now know a little bit about how enzyme molecules are made. This is information which has accrued to cell biologists in very recent years. We know that enzyme molecules are made of amino acids and that the specificity of an enzyme molecule, the fact that it is this enzyme instead of some other enzyme, depends in a great measure upon the sequence of amino acids in the peptide chain of which the molecule is made. We might say that an enzyme is a message written in a 20-letter alphabet — the 20-letter alphabet of the 20 amino acids which occur in protein molecules. And we know, too, that enzyme molecules are synthesized in the cell, or even outside the cell, by special little objects which sit in the cytoplasm and do this task — objects which we in the West call microsomes and which, in the East, people call microsomal nucleoprotein particles. And the evidence is pretty good that each one of these kinds of particles carries the information required to make one — and only one — kind of enzyme molecule.

The newer cell biology tells us, too, that microsomal particles contain their information in the form of nucleic acid — information written in language of ribonucleic acid, RNA. This information is somehow transmitted to and used in the assemblage of the amino acids in the specific sequence of a specific enzyme. The microsomal particles in turn are made within the nucleus, and we don't know how this happens. We know only that microsomal particles are made in the nucleus, that they are made in the nucleus only in the presence of deoxyribonucleic acid, the DNA of the genetic material. The implication is that the message contained in the DNA of the nucleus is somehow printed off in the language of RNA, and then extruded into the cytoplasm in the form of microsomes which are responsible for the assemblage of enzyme molecules.

Now let us get back to differentiation. We know that every cell of the multicellular organism, so far as we can tell today (and this is backed by extensive embryological information) possesses all of the genes which are needed for the assemblage of the whole plant or the whole animal. It is quite obvious, though, that all cells do not use all of the genetic information, do not make all of the enzymes which are needed to make the whole creature. Take a pea plant for example. It has genes in it which tell the cotyledons how to make the reserve protein of pea cotyledons. But this protein does not occur in all parts of the pea plant. The genes for making reserve protein of pea cotyledons only do their work when they sit in the cells of a pea cotyledon and do not do their work when they sit in a cell of a root or stem or leaf or flower. Here, then, we return to differentiation. It is quite evident that there is some entity within the nucleus which says to the gene either, "Please be active and make appropriate microsomes which can go out and make the enzyme for which I contain the message," or this entity, whatever it is, says to the gene, "Please be inert and do not send out your message." Differentiation consists of the sequential and properly programmed turning on and off of the genetic information of the nucleus. Perhaps part of this programming is information contained in the DNA. As one of my students once said, "Perhaps part of the information in the nucleus is information on when to use the information." But I don't know what it is that programs the nucleus. Perhaps one of the things that turns the genes off and on in the nucleus is auxin itself. This would indeed be jolly, but I don't believe it. But let's find out what it is that turns genes off and on during the course of development, even if it does not turn out to be auxin. In any case the study of differentiation is clearly enough one of the important tasks of the plant physiology of the future.

But let us now return to auxinology, the treasury of auxin lore. What does the future hold for our chosen discipline? My principal forecast is that auxinology, as a specific science apart, will ultimately come to an end. Everything will be found out. But auxinology as a part of basic biological lore will be immortal.

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