CHEMICAL CONSTITUTION AS RELATED TO GROWTH REGULATOR ACTION[1]

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Introduction

Recent volumes of this series have included excellent reviews of the progress and new interpretations in the field of growth regulators, particularly the auxins [Bonner & Bandurski (11), Gordon (22), van Overbeek (43) Veldstra (60)]. A survey and appraisal of the publications which have appeared since these reviews were prepared would have little value. Although Veldstra's review of the literature concerning the significance of chemical constitution in the action of growth regulators is as detailed and analytical as might be desired, it is essentially an interpretation of the available data in terms of a physicochemical mode of action of regulators with the reservation that proof of the supposition must await the identification of the reactive entity within the cell. No such identification has been accomplished and this review will not attempt to resolve the question of physicochemical activity versus chemical reactivity. Rather, it will consider certain aspects of the general problem of analysis of chemical constitution and activity together with the experimental facts which an hypothesis relating constitution and activity must explain.

Analysis of Activity of Regulators

Plant tissue and response.—The principal problem of an appraisal of all the literature pertaining to the chemical constitution of plant growth regulators lies in the correlating and reconciling of data derived from many different biological responses such as the formation of roots on stem cuttings, induction of parthenocarpic fruit development, epinasty of leaves, inhibition of root growth, elongation of coleoptile and epicotyl tissue, and reversal of curvature resulting from tissue tension in slit epicotyls (pea test) and coleoptiles. An attempt to correlate and reconcile all such data must assume that these growth responses in all plants are controlled by the same hormone, indoleacetic acid, having the same mode of action in each response.

Such an assumption was unwarranted when auxin *a* and auxin *b* were considered to be growth hormones (70), and is subject to question now because of the isolation of indoleacetonitrile from plant tissues (26) as well as the evidence of unknown growth hormones obtained by paper chromatog-

[1] The survey of the literature pertaining to this review was concluded in November, 1954.

raphy (7, 54). Yet the assumption is fundamental in the suggestion that the activity of a compound, as measured in different responses, may reflect the relative penetration of the compound to reactive sites or other unknown properties of the compound (65), and that the relative activities of different compounds in a single response are unreliable measures of effect in the common growth reaction. The same assumption is responsible for the interpretation of the phenomenon wherein a compound which induces a response in the tissue of one species but not in that of another, must have been converted in the former to a structure similar to those which cause the same or similar response in both types of tissue (43, 57). The assumption appears to be involved also in the explanation of effects of compounds which do not follow the pattern of relationship of activity and concentration shown by other regulators and are therefore regarded as having an unknown interaction (synergistic action) with the hormone rather than a direct effect similar to that of the hormone (5).

There are numerous instances in which we are unable to reconcile data from different plant responses for the same molecular structures and, in some cases, the most logical interpretation questions the above assumption. In fact, Audus (6) has suggested that the effects of various regulators on the growth of roots can be interpreted more logically in terms of control by a native inhibitor rather than by indoleacetic acid.

Recent studies of molecular structure in the action of regulators for the most part have employed either the straight growth of segments of *Avena* coleoptiles, or curvature of slit epicotyls of pea (pea test), or both tests for growth activity. For many compounds the data of these tests cannot be reconciled (39, 56, 60, 65). The complex nature of the pea test and the participation of large numbers of wounded cells as well as other features make it less certainly a strict test of effects of growth regulators on the elongation process, yet we may only conjecture reasons for the differences between the two test methods. In this connection and also in the general consideration of growth in the pea, the recent discovery of another nitrile compound in plant tissue may have considerable significance.

The first nitrile to be isolated from plant tissue and found to have growth effects was indoleacetonitrile (26) which is more effective as an auxin for *Avena* coleoptile tissue than indoleacetic acid (9). It was isolated from cabbage and since has been reported from other tissues (7). The discovery of the second nitrile with growth effects came about through the investigation of the substance in the seeds of *Lathyrus odoratus* which causes the skeletal abnormalities characteristic of lathyrism in man and domestic animals. Schilling & Strong (47) and Dasler (15) have isolated and identified a crystalline substance which causes the skeletal changes in rats and found it to be β-(γ-L-glutamyl)-aminopropionitrile. Although a diet of seeds of *Pisum sativum* does not give symptoms of lathyrism in the rat, the addition of a water-soluble extract of the seeds does produce the symptoms in the monkey (53). Preliminary investigations of the activity of this

substance in the straight growth of coleoptile sections indicate that it is an antagonist toward indoleacetic acid at low concentrations and that the antagonism is readily overcome with higher concentrations of indoleacetic acid (37). Similar results were obtained with aqueous extracts of seeds of sweet pea and garden pea. Thus, it appears that the investigation of nitrile metabolism in plants may have an important bearing on the control of growth by hormones.

Stowe & Thimann (54) have reported that indoleacetic acid can be detected by paper chromatography in solutions of indoleacetonitrile on which *Avena* coleoptile sections grow for 24 hr. as well as in extracts of the coleoptile tissue, thus indicating that the tissue converts the nitrile to the acid. Thimann (57) has also reported that although the nitrile has no activity in the pea test, both the pressed sap and the ether extract of sections of coleoptiles grown for 48 hr. on a solution of the nitrile cause sufficient response in the pea test to indicate a conversion of more than 50 per cent of the nitrile by the coleoptile tissue.

Another type of metabolic transformation of growth regulators which has been the subject of recent study is β-oxidation of long side chains. Synerholm & Zimmerman (55) suggested such a transformation leading to the active acetic acid homologue to explain their observation that in a series of ω-2,4-dichlorophenoxyalkylcarboxylic acids only those with an even number of carbon atoms in the side chain caused epinasty in tomato plants. Fawcett, Ingram & Wain (16) have studied the oxidative degradation of phenoxy acids with varying lengths of side chain within the tissues of the flax plant and have obtained evidence that β-oxidation does, in fact, occur. Such acids with an odd number of carbon atoms in the side chain gave rise to appreciable quantities of phenol while those with an even number yielded only traces. In this investigation the degradation product of acids with an even number of carbon atoms in the side chain would be phenoxyacetic acid and no activity was displayed in epinasty or elongation of *Avena* coleoptile sections, yet in the pea test, acids with an odd number were active and those with an even number were inactive. Fawcett, Ingram & Wain suggest that in the tissue of the pea a specific intermediate product in the degradation of the side chain is responsible for the difference in response.

Further study of the growth-regulating activity of 4-chloro-, 2,4-dichloro-, and 2,4,5-trichloro-phenoxy acids in relation to their β-oxidation has been made by Wain & Wightman (68). In the 4-chloro- and 2,4-dichloro series the acids with even numbers of carbon atoms in the side chain which would yield active acetic derivatives caused elongation of sections of wheat coleoptiles, epinasty of tomato leaves, and curvature in the pea test, whereas the acids with odd numbers of carbon atoms were inactive. In the 2,4,5-trichloro series, a similar relationship of chemical constitution and effect was noted in the elongation of the coleoptile tissue. However, only the acetic acid caused an effect in the epinasty and pea tests, thus indicating a lack of conversion in the pea and tomato which Wain & Wightman suggest may be

due to the effect of nuclear substituents in the β-oxidation mechanism or an effect on the penetration and movement within the tissue. When the pea test was made with solutions on which coleoptile tissue had grown for 72 hr. the response was the same as for coleoptile tissue, indicating the conversion of the higher homologues to the acetic derivative. The conversion of the butyric acid derivative was demonstrated by paper chromotography.

Kinetic analysis.—The most important contribution to the analysis of activity of growth regulators in recent years has been the discovery of McRae, Bonner & Foster (19, 32, 33, 34) that the elongation of *Avena* coleoptile sections induced by exogenous regulators may be described and analyzed by the methods of study of enzyme kinetics proposed by Michaelis and Menten. This discovery provides a means of determining the nature and properties of the reactive entity within the plant cell. The kinetic analysis of elongation has already given precise explanations of the inhibition of growth by high concentrations of auxin (19), the antiauxin effect of molecular structures similar to auxins (32, 33), and the interaction of chemically different auxins (34). The possibility of determining the binding affinities between regulators and the reactive entity within the cell as reported by Foster (18) affords the first critical approach to the question of chemical activity as opposed to physicochemical activity.

These investigators employ a kinetic treatment according to the basic equation

$$\mathrm{E} + \mathrm{S} \underset{}{\overset{K_S}{\rightleftarrows}} \mathrm{ES} \xrightarrow{k} \mathrm{E} + \text{Products} \qquad 1.$$

widely used in enzyme kinetics (20). In this equation **E** represents an enzyme system in the coleoptile section, S the growth regulator, ES the active complex which is the growth limiting factor under the experimental conditions, K_S the dissociation constant of the complex ES, and k the velocity constant relating concentration of ES to product formation which is growth in this application. This equation may be expressed as

$$v = \frac{V_{\max}[\mathrm{S}]}{K_S + [\mathrm{S}]} \qquad 2.$$

where $V_{\max}$ is the maximum growth rate obtained when sufficient regulator, S, is present to saturate all enzyme molecules, E. In the above equation v is the reaction velocity (growth rate of the coleoptile section) and K_S and $V_{\max}$ are constants. Writing the equation as its reciprocal we obtain

$$\frac{1}{v} = \frac{K_S}{V_{\max}[\mathrm{S}]} + \frac{1}{V_{\max}} \qquad 3.$$

Plotting $1/v$ against $1/[\mathrm{S}]$ would thus give a straight line and the data for growth rate as related to concentration of auxin obtained by McRae, Foster & Bonner (34), when so plotted, do give a straight line (Fig. 1) thus confirming the assumption that the auxin reacts with a plant substrate according to equation 1.

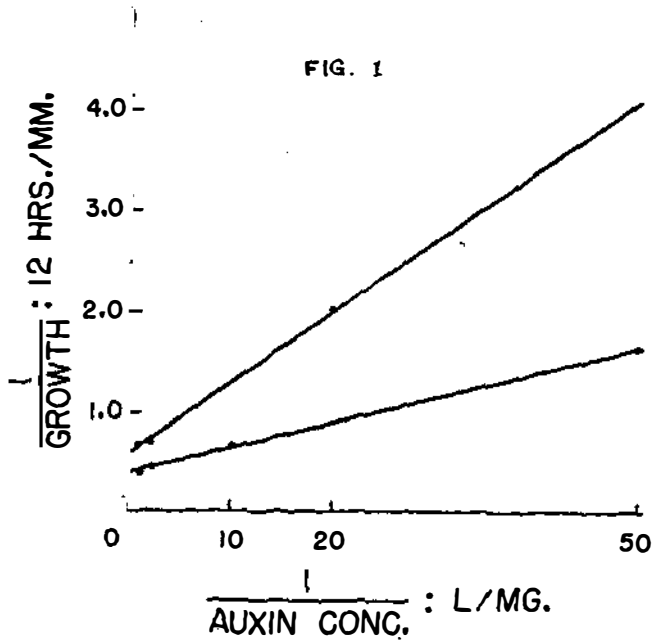


FIG. 1. The reciprocal of growth rate of *Avena* coleoptile sections plotted as a reciprocal function of the concentration of 2,4-dichlorophenoxyacetic acid (upper line) and indoleacetic acid (lower line) [after McRae, Foster & Bonner (34)].

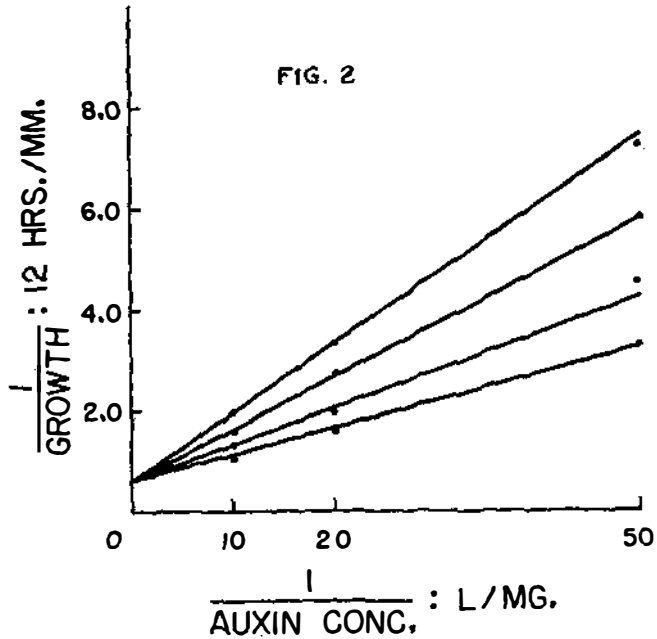


FIG. 2. The reciprocal of growth rate of *Avena* coleoptile sections plotted as a reciprocal function of the concentration of 2,4-dichlorophenoxyacetic acid alone (bottom line) and in the presence of 0.1 mg./l., 0.5 mg./l., and 1.0 mg./l. of 2,6-dichlorophenoxyacetic acid (upper lines in order of ascent) [after McRae & Bonner (32)].

Assuming that an inhibitor reacts with the same substrate or enzyme as does the auxin, equation 1 becomes

$$E + I \overset{K_I}{\rightleftarrows} EI \qquad 4.$$

where I represents the inhibitor, EI the inactive inhibitor-enzyme complex, and K_I the dissociation constant for the complex EI. When an inhibitor is present equation 3 becomes

$$\frac{1}{v} = \frac{1}{V_{max}}\left[K_S + \frac{K_S[I]}{K_I}\right]\frac{1}{[S]} + \frac{1}{V_{max}} \qquad 5.$$

Comparing equation 3 with equation 5 it is seen that when $1/v$ is plotted against 1/[S] the competitive inhibitor increases the slope of the resultant line by the factor $K_S[I]/K_I$. The intercept, $1/V_{max}$, does not change. The experimental data of McRae & Bonner (*32*, *33*) pertaining to the effects of phenoxyisobutyric and diortho-substituted phenoxyacetic acids on elongation of *Avena* coleoptile sections conform with this kinetic formulation (Fig. 2) and establish the competitive action of these regulators and auxins. For the auxin 2,4-dichlorophenoxyacetic acid, the plotting of the reciprocal of growth rate against reciprocal of concentration in the absence and presence of 2,6-dichlorophenoxyacetic acid shows that with increasing concentration of the latter the slope of the line increases but all lines have a common intercept.

Foster, McRae & Bonner (19) have shown that inhibition of growth by auxins at higher concentrations would be expected if the auxin reacts by a two-point attachment mechanism. The active complex would have an auxin molecule attached at two points on the substrate; as the concentration of auxin increases, the probability of two auxin molecules attaching at the two substrate sites increases and the number of two-point attachments of one auxin molecule forming the active complex progressively diminishes. If the kinetic formulation of equation 2 is altered to include the possibility of formation of an inactive complex between the enzyme and substrate, Foster, McRae & Bonner obtain the expression

$$v = \frac{V_{ex}[S]}{K_S' + [S] + [S]^2/C} \qquad 6.$$

where V_{ex} (corresponding to V_{max}), K_S' and C are experimentally determinable constants and $[S]_2/C$ is a measure of the probability that a second molecule of auxin, S, will become attached to the substrate before the first has consummated its two-point attachment. The experimental data are described accurately by this equation (Fig. 3).

Recently the validity of this kinetic analysis of elongation has been challenged by Bennet-Clark & Kefford (8). They interpret their data to indicate that a linear relationship does not exist between elongation and time at most concentrations of auxin, and thus the measurement of elongation

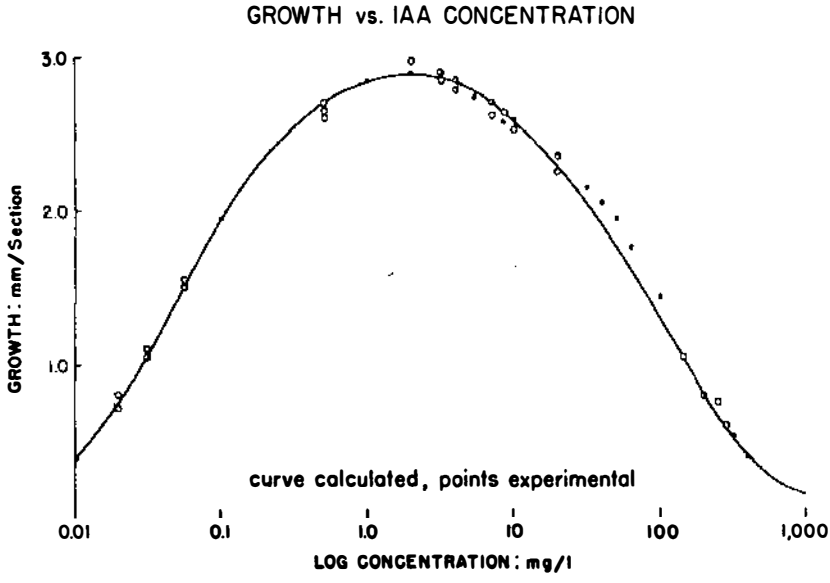


FIG. 3. Growth rate of *Avena* coleoptile sections plotted as a function of indoleacetic acid concentration. The solid line is the curve calculated from equation 6 and the individual points are data from five different experiments [after Foster, McRae & Bonner (32)].

after 12 hr. is not a measurement of the initial velocity of reaction as required for the kinetic analysis. A slightly sigmoid curve was claimed for low concentrations of indoleacetic acid suggesting a "lag phase." However, these data were obtained with coleoptile sections growing in 3 per cent sucrose without potassium maleate buffer at pH 4.5. Bonner & Foster (12) have shown that the data of Bennet-Clark & Kefford do, in fact, conform to a linear relationship within experimental error and that in the presence of the potassium maleate buffer the growth rate of coleoptile sections is constant over a wide range of concentrations of indoleacetic acid at time intervals of 10 min. to 20 hr.

The existence of a "lag phase" as envisioned by Bennet-Clark & Kefford has been demonstrated much more conclusively for 2,6-dichlorobenzoic acid, *ortho* chlorobenzoic acid, and 2,3,5-triiodobenzoic acid by Carroll (14). In these instances the "lag phase" appears logically explainable in terms of a slower reaction involving displacement of electron-attracting substituents by the nucleophilic substrate through an intermediate complex (23). It is not surprising that the precisely quantitative data required for the kinetic analysis of elongation are greatly dependent upon the experimental conditions. There is no more reason to believe that the exogenous sucrose supply constitutes a "normal" condition than that a balancing supply of potassium

does, and the interaction of these factors may be of fundamental importance in the growth reaction (14, 73). The pH of the medium in which growth of the sections occurs is also a very important factor. It appears that the pH of 4.5 selected by McRae & Bonner is the optimal condition for maximum growth (73).

In expressing the kinetic parameter, V_{max}, or maximum velocity of the growth reaction, as a constant, the ratio of the increment of growth to the original length gives a value independent of the initial length of the section which may be preferable to its expression in millimeters of increment (73). Ordinarily, the velocity values are obtained by the subtraction of growth occurring in the absence of the regulator from that in the presence of the regulator. Under certain experimental conditions and in the presence of certain regulators, the growth may be less than in the control medium as found by Ingestad (25). In this situation Ingestad proposes that the endogenous auxin concentration can be calculated and a value obtained for total auxin concentration. With the corrected concentration values, straight line reciprocal plots are obtained, and Ingestad suggests this result reflects the endogenous action of the regulators.

Use of buffer systems.—The pH of the medium may be of importance in the assay of weak acids for activity in the growth reaction of plant cells. The initial assay of the activity of 2,4,6-trimethylphenoxyacetic acid indicated a small but significant elongation effect of this compound (41). Subsequently, it was found that the elongation occurred as a result of the pH of some of the more concentrated solutions of the acid, for when the acid was adjusted to pH 5.6 no elongation effect was obtained, and low pH levels provided by hydrochloric acid alone were found to result in greater elongation than that which occurs in more alkaline media.

In testing such acids over a wide concentration range, the secondary effect of pH may be prevented either by adjusting the pH of the solution or by using a buffer system. The latter procedure, though seemingly the obvious choice, introduces additional problems for most buffers contain ions which may directly affect the growth of the tissue and the selection of the pH level for the system is in itself arbitrary. Simon & Beevers (49) have shown that as the pH of a solution of a weak acid is lowered to the pK of the acid the concentration of the acid required for a standard response becomes minimal. Since the pK values of the synthetic regulators show some variation, the use of a buffer system at uniform pH would not assay the maximum activity of all of the acids. Further, the pK values for many of the compounds would be at such a low pH as to be toxic to the tissue.

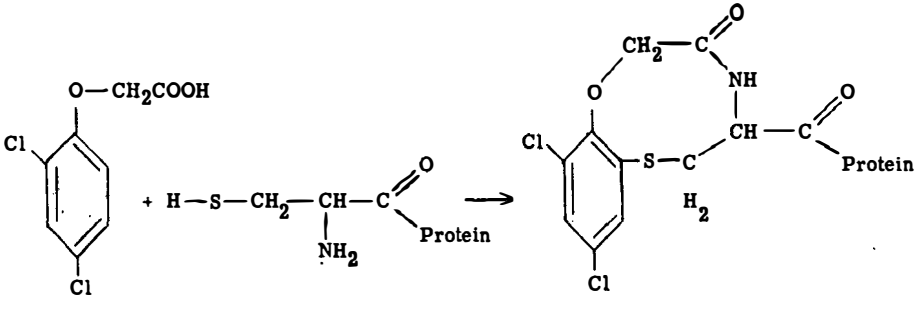
The most important difficulty in the use of buffer systems occurs in the testing of molecular structures possessing very slight activity in elongation. Low concentrations of phenoxyacetic acid have no effect on the growth of *Avena* coleoptile sections in water solutions. At concentrations of 2 or $3\times10^{-4}M$ adjusted to pH 5.6 the acid causes a small but significant growth

effect (39). Ingestad (25) found, however, that the acid at 1 and $3\times10^{-5}M$ in $10^{-3}M$ sodium citrate buffer of pH 4.5 inhibited the growth of coleoptile sections. A similar effect of the buffer system has been noted by Åberg & Khalil (5) on the antagonism of the (−) isomer of α-2,4,5-trichlorophenoxypropionic acid in which 10^{-6} and $10^{-6}M$ concentrations in Na_2HPO_4-KH_2PO_4 buffer at pH 5.9 caused inhibition of the growth of coleoptile sections. Smith, Wain & Wightman (52), however, found no inhibition of growth at concentrations of 0.01 to 20 p.p.m. in water solutions. There is no evidence to substantiate any interpretation other than an effect of the buffer system on the coleoptile tissue.

Regulators of the Aryloxy Acid Series

With the discovery of the growth-regulating properties of the chlorinated phenoxyacetic acids by Zimmerman & Hitchcock in 1942 (74), this group of analogues acquired fundamental significance in the analysis of chemical constitution as related to physiological activity. The study of the effects of substitution of halogens and methyl groups in the 2, 4, and 6 positions of the benzene ring revealed that substitution of both *ortho* positions caused complete loss of stimulatory effect on elongation (41). This observation was the basis for an hypothesis that the position on the benzene ring adjacent to the point of attachment of the side chain is directly involved in the growth reaction by a nucleophilic substitution together with the attachment of the carboxyl group of the side chain to a basic group (24).

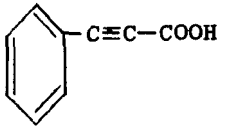
Such a two-point reaction is suggested to take place between the regulator and a cysteinyl unit of a protein as follows in Reaction I:



Reaction I

This hypothesis affords a very satisfactory explanation of the activity of *cis*-cinnamic acid in elongation and the inactivity of the *trans* form as well as other *cis-trans* acids of this type (24). If a growth regulator reacts at two points with two points of a plant substrate then a ring structure will result. It is a well-known fact (21) that cyclic structures containing a *trans* double

bond form with difficulty except in very large rings. Phenylpropiolic acid (see cut)



Phenylpropiolic acid

has been found to be inactive in the elongation of *Avena* coleoptile sections (40) and this is to be expected since rings containing triple bonds are highly strained unless they are very large (21).

Wain has reported (66) that 2,4-dichloro-6-fluoro- and 2,4-dibromo-6-fluoro-phenoxyacetic acids have high activity in the elongation of *Avena* coleoptile sections. In these compounds both *ortho* positions are substituted and the *ortho* reaction hypothesis does not explain their activity. Although more study of fluorine-substituted compounds is required to evaluate the significance of these results, it is possible that a reversible reaction at the *ortho* position, i.e., an adsorption reaction, would proceed with little more hindrance by a small fluorine atom than that by a hydrogen atom. If, however, the reaction is in the nature of the formation of a new covalent bond, then the F^- could be removed by a displacement mechanism. It has been shown (72) that fluorine is much more easily removed from an electron-rich benzene ring than are the other halogens. Wain has suggested that the reason for the activity of 2,4-dichloro-6-fluorophenoxyacetic acid compared with the inactivity of 2,4,6-trichlorophenoxyacetic acid is that in the first molecule the side chain may be free to rotate because of the small size of the fluorine atom, while the two large chlorine atoms in the second structure prevent rotation. However, this hypothesis fails to explain the activity of other regulators with both *ortho* positions substituted.

In 1951 Leaper & Bishop prepared all mono-, di-, and trichlorophenoxyacetic acids and tested them for activity in the inhibition of root growth, leaf epinasty, and initiation of roots on stems (27). Because of the inactivity of the 3,5-, 2,3,5-, and 3,4,5-acids in these tests they proposed that an open position *para* to the *ortho* position is required for activity and the active molecule is involved in the formation of quinoid compounds in plant cells. Wain & Wightman have examined these same compounds for activity in the straight growth of *Avena* coleoptiles and in the pea test (67). They find that in both tests the 2,3-, 2,3,4-, 2,3,5-, and 3,4,5-acids are active. Since these compounds do not have an open position *para* to the *ortho* position, the hypothesis of Leaper and Bishop is invalid. Further evidence against the hypothesis is the fact that 2,5-dihydroxyphenylacetic acid which should form a quinone readily is less active than phenylacetic acid; 4-chloroindoleacetic

acid, which is more active than indoleacetic acid, would require displacement of the chlorine for *para* quinone formation or destruction of the five-membered ring; and 2-methyl,5,7-dichloro-3-indoleacetic acid is active although all positions are blocked against quinone formation (39).

Thimann (56) has found that 2,6-dichlorophenoxyacetic acid has some activity in the pea test and proposes a novel electronic interpretation in which occupation of one or more of the *ortho* or *para* positions of the benzene ring results in the intensification of the nucleophilic influence at the remaining positions and occupation of all three positions prevents all reaction. The 3,5- acid would not have activation of the *ortho* or *para* positions and its inactivity would be explained. However, in this view the 2,6- compound should have activity approaching that of the 2,4-substituted ring which it does not, and alkyl substituents such as methyl groups should be very effective activators, which they are not. That 2,6-dichlorophenoxyacetic acid does not cause elongation of *Avena* coleoptiles has been reported by several workers (32, 39, 42, 65). The activity reported for the compound by Thimann has been suggested to be the result of the presence of active impurities (33). Wain, however, (65) states that activity of the compound in the pea test is dependent upon exposure of the pea seedlings to red light during growth.

This effect of red light may involve the formation of a reactive entity (receptor) within the cells as described by Liverman & Bonner (29), it may be due to the opening of the stomata allowing for high internal concentrations of the compound in the cortical tissue, or it may reflect some fundamental difference in the growth mechanism such as the participation of nitriles. In the pea test, it appears quite unlikely that activity dependent upon exposure to red light has any direct significance in the relationship of structure and activity. The fact that the greater part of our knowledge concerning chemical constitution and activity in plant growth can be applied to varied plant responses has caused undue emphasis to be placed upon conflicting data from different tests in the selection of a plausible interpretation. With more complete knowledge of the growth reaction in *Avena* coleoptile tissue, the differences in growth responses of other tissues may become valuable clues to the variation in the reaction among different tissues.

Recently an effect of the length of side chain on the activity of di-*ortho*-substituted phenoxy acids has been reported by Osborne *et al.* (42). Although the di-*ortho*-substituted phenoxyacetic acids were without effect in causing elongation of coleoptile sections, some activity was found for phenoxypropionic and phenoxybutyric acids with either chlorine atoms or methyl groups in both *ortho* positions. The composition of the medium in which the growth effects were observed was not described. The stimulatory effect on elongation was not obtained with similar compounds in which the 4-position was substituted (2,4,6-trichlorophenoxypropionic acid and 2-methyl-4,6,-dichlorophenoxypropionic acid) which suggests the possibility

of the di-*ortho*-substituted phenoxypropionic acids entering into the reaction at the *para* position when the *ortho* position is blocked.

In the study of the optical isomers of α-2-naphthoxypropionic acid, Smith & Wain (50) found the L-isomer to be inactive in the pea test and the elongation of *Avena* coleoptile sections while the D-isomer was active. Furthermore, 2-naphthoxyisobutyric acid was inactive in both tests. Although recognizing that the optical isomers may combine with an optically active cell constituent and thus form two compounds with different properties of which only one might have activity, they prefer an explanation involving three specific groupings of the molecule, the carboxyl group, the unsaturated ring, and an *alpha* hydrogen atom arranged around the asymmetric carbon atom, which must be stuitably placed for contact with the receptor groups. Thus, in the D-isomer these three groups are in the proper spatial arrangement and in the L-isomer they are not. The inactivity of the α-isobutyric acid is thus the result of its lack of the essential α-hydrogen atom.

Recently, an elaborate study by Wain and co-workers, employing six growth responses including the pea test and straight growth of coleoptile sections, has compared the activity of the acetic, α-propionic, α-butyric, and α-isobutyric side chains in substituted phenoxy acids and naphthoxy acids (17). In tests dependent strictly upon cell elongation the isobutyric acids were inactive, but in the pea test slight activity was shown by several isobutyric acids and high activity was shown by 2,4,5-trichlorophenoxyisobutyric acid. This observation may be regarded as additional evidence of the unique character of the response in the pea test. Wain and his coworkers have also shown that the inactive optical isomer is antagonistic toward the effect of the active isomer and that di-*alpha*-substituted phenoxyacetic acids have an antagonistic effect toward active acids (51, 52).

An extensive study of the effects of 19 pairs of optical isomers on the growth of flax roots has been made by Åberg (2). He interprets his data as indicating a similar characteristic of D-forms having auxin activity and L-forms being antiauxins except in the phenoxy compounds where the L-forms have neither auxin nor antiauxin properties. However, antiauxin properties are difficult to establish by this test since they are determined by the stimulation of root growth and such stimulation is invariably small for flax roots (1). Since Matell has been able to demonstrate that (+)-α-phenoxypropionic acid has the same configuration as D-(−)-α-alanine and ethyl D-(+)-lactate (30) and that the (+)forms of α-2-methyl-4-chlorophenoxypropionic acid and α-(2,4-dichlorophenoxy)-*n*-butyric acid have the D-configuration (31), it seems likely that all of the most active members of enantiomorphic pairs belong to the D-series.

Attractive as Wain's hypothesis may be for the explanation of the activity of one of two optical isomers and the requirement of an α-hydrogen atom, these characteristics of molecular structure may find explanation also in chemical reactivity. The difference in activity of optical isomers resulting

from an asymmetric carbon atom may be due to differences in reaction rates resulting from interference in combining with another asymmetrical center. Such interference has been shown in the slower reaction of the L form of mandelic acid with L-meththol in esterification compared with the rate of reaction of the D form of the acid (21). The effect of *alpha* substitutions in the side chain can be explained by the steroic hindrance of the carboxyl group (71); two alkyl groups on the α-carbon atom would so reduce the reactivity of the carboxyl group that the molecule would be without biological effect.

Certain observations are not in accord with the hypothesis of a three-point reaction. Phenoxyacetic acid with two α-hydrogen atoms should be more active than α-methylphenoxyacetic acid but the latter is much more active than the former. In this instance, a subsidiary effect of structure on penetration into cells may explain the difference in activity, for Mitchell and his co-workers have found remarkable movement of α-methoxyphenylactic acid and other *alpha*-substituted regulators within the plant (36, 46), and between plant roots. Molecular structures such as the substituted benzoic acids which have no α-hydrogen atoms should not induce elongation, yet, some do. Other compounds with activity such as *cis*-cinnamic acid do not have the three specific groupings in the same spatial relation as in the regulators with saturated side chains, and their precise fit to the receptor sites would not be possible. Finally, Wain's hypothesis does not explain the lack of growth-promoting activity for the di-*ortho*-substituted regulators.

Inherent in the concept of chemical constitution determining physiological activity is the interference in such action resulting from the presence of similar but not identical molecular structures. The study of such interference should yield equally significant data concerning the mechanism of the activity but interference, in contrast to the stimulation of growth, may be a nonspecific effect. McRae & Bonner (33) have pointed out that the only rigorous method for distinguishing between interference of a competitive nature and that arising from such effects as the destruction of reactive groups is the kinetic analysis formulated by Lineweaver and Burk.

In 1949, Bonner (10) described the antagonistic action of 2,4-dichloroanisole in the auxin effect. This observation was questioned by Audus & Shipton (6) but has been reaffirmed by McRae & Bonner (33). However, Thimann also regards 2,4-dichloroanisole as only a growth inhibitor and not an antiauxin (66). Competitive inhibition has been demonstrated for the di-*ortho*-substituted phenoxyacetic acids (32), 4-chloro- and 2,4-dichlorophenoxyisobutyric acids (33), phenoxyacetic acid, and α-methylphenoxyacetic acid (25) in the elongation of *Avena* coleoptile sections. Furthermore, McRae & Bonner (33) have demonstrated that 2,4,6-trichloroanisole which lacks a carboxyl group and, in addition, has both *ortho* positions blocked for reaction, functions neither as an auxin nor as an antiauxin. They have therefore suggested that those compounds reported as antiauxins may be classified

according to structure: (*a*) aryloxy compounds with a free *ortho* position but lacking a carboxyl group; (*b*) compounds with a carboxyl group but lacking a free (reactive) *ortho* position; (*c*) compounds with steric hindrance preventing reaction of the carboxyl group; (*d*) compounds with auxin activity but of low V_{max}; and (*e*) substituted benzoic acids lacking auxin activity. It appears that the most logical explanation of molecular structure in relation to competitive interference is furnished by the hypothesis of a two-point reaction between the growth-promoting regulator and the plant substrate involving the carboxyl group and an *ortho* position.

Similar difficulties are encountered in attempting to relate chemical constitution to antiauxin activity in different plant responses as are found in relating constitution to auxin activity. Åberg (1) found that 2,4-dichloroanisole and other α-naphthyl and 2,4-dichlorophenoxy derivatives were without conspicuous antagonism against the inhibition of growth of flax roots by 2,4-dichlorophenoxyacetic acid. He concluded that a carboxyl group was essential for attachment of the auxin antagonist. However, in a study of the effect of monoalkyl substituents other than methyl groups on the α-carbon atom of aryl- and aryloxyacetic acids it was found that the steric hindrance preventing reaction of the carboxyl group gave rise to antagonistic action (3). The antagonism of the latter compounds can best be explained by incomplete reaction of the carboxyl group (salt formation without subsequent amidization).

Åberg (4) has also found that molecules, in which the *ortho* position is blocked for reaction such as the 2,6-dichloro- and 2,4,6-trichlorophenoxyacetic acids, have an antagonistic action against the inhibition of root growth resulting from 2,4-D and that substitution of large alkyl groups in the *ortho* or *para* positions of phenoxyacetic and phenoxypropionic acids increases the antagonism of such molecules. The presence of chlorine in 2-isopropyl-4-chloro-5-methylphenoxyacetic acid significantly increased the antagonism as was found for 2,4,6-trichlorophenoxyacetic acid in comparison with the 2,6-dichloro acid.

Growth Regulators of the Substituted Benzoic Acid Series

Since the discovery by Bentley of the activity of 2,3,6-trichlorobenzoic acid in the elongation of *Avena* coleoptile sections, the substituted benzoic acids have been of considerable interest with respect to the relationship of chemical constitution and growth-regulator activity. In a study of a selected series of *ortho*-substituted benzoic acids, Muir & Hansch (38) found that those with any stimulatory effect upon elongation contained an electron-attracting group (halogen or nitro group) in the *ortho* position. On the basis of this common structural feature and the well-established electronic theory of organic reactions, it was suggested that the reaction of these compounds involved the displacement of the electron-attracting group whereas in the phenoxyacetic acid derivatives the reaction occurs by hydrogen displacement.

Hansch *et al.* (24) were able to demonstrate the release of chloride from 2,6-dichlorobenzoic acids during the growth of *Avena* coleoptile sections, thus substantiating the hypothesis.

Minarik *et al.* (35) have reported the results of tests of more than 200 substituted benzoic acids on the growth of cucumber roots and the responses of the bean plants. All but three of the 30 compounds listed by them as active in causing responses of bean plants have a halogen atom in the *ortho* position. Furthermore, of 35 compounds found to stimulate elongation of cucumber roots none contained a halogen atom in the *ortho* position and the most active were 3-nitro-4-halogenbenzoic acids of which 3-nitro-4-fluorobenzoic acid was shown to antagonize the inhibition of elongation induced by 2,4-D.

The hypothesis of a reaction involving the displacement of the *ortho* halogen atom in the substituted benzoic acids does not account for the slight activity of 2,6-dimethylbenzoic acid or the lack of activity of 2,4-dichlorobenzoic acid in the elongation of *Avena* coleoptile sections (38, 39), nor does it explain the activity of the 2,6-dimethyl-3-nitro- and 2,6-dimethyl-3-halogen-substituted benzoic acids in the pea test reported by Veldstra & van de Westeringh (63) or their activity in the elongation of *Avena* coleoptile sections (40).

Veldstra (59) has found that the ultraviolet absorption spectra of the di-*ortho*-substituted compounds indicates steric inhibition of resonance with the carboxyl group forced into a position at right angles to the benzene ring. This finding would substantiate his hypothesis of activity dependent upon a nonpolar portion of the molecule (ring system) associated with an acidic polar group (carboxyl group) situated out of the plane of the ring as far as possible or as frequently as possible (62, 64). However, many di-*ortho*-substituted benzoic acids are inactive in the pea test and the coleoptile test (38, 59). Most of these compounds have a substituent in the *para* position and it appears that substitution at this position eliminates stimulatory activity in elongation irrespective of the spatial position of the carboxyl group or the nature of the *ortho* substituents. However, the complete lack of activity of 2,6-dimethoxybenzoic acid (38) is directly opposed to the hypothesis that di-*ortho* substitution brings about activity simply by holding the carboxyl group out of the plane of the ring. It is possible that di-*ortho* substitution of the benzoic acids forces reaction at the *meta* position when a halogen atom or nitro group is present, as suggested above for reaction at the *para* position in di-*ortho*-substituted phenoxypropionic acid.

The lack of activity of phenoxyacetic acids, phenylacetic acids and indoleacetic acids substituted in both *ortho* positions is certainly opposed to any general interpretation of effect on elongation caused by the holding of the carboxyl group out of the plane of the ring. Whereas, 2,4,6-trichlorophenoxyacetic acid is completely inactive in stimulating elongation of coleoptile tissue, 2,4,5-trichlorophenoxyacetic acid is very active.

Although the hydrophilic/lipophilic balance of the molecule may be of real import in penetration, etc., an explanation of the difference in activity of these molecules in terms of hydrophilic/lipophilic ratios and consequent differences in adsorption characteristics is quite unlikely. When such characteristics were investigated with the dropping mercury electrode of the polarograph as suggested by Veldstra (58, 62), Paleg & Muir (45) found that the suppression effects on the oxygen maximum were in no way correlated with the effects of the molecules on elongation. Small suppression effects were found for substituted benzoic acids causing elongation and great suppression effects were found for substituted phenoxyacetic acids not inducing elongation. Furthermore, within a series of related compounds an active molecule may have a suppression effect that is greater, less or the same as the suppression effect of inactive molecules.

Muir & Hansch (38) have pointed out that the benzoic acid ring is electron deficient in contrast to the electron rich rings of phenylacetic, phenoxyacetic, and indoleacetic acids. Such consideration indicates that the electron rich and the electron poor rings would vary greatly in their tendency to be held at a biological interface. The specific type of ring structure required to confer regulator activity indicates that simple physical adsorption is an inadequate explanation of the mode of action. Although cyclohexene acetic acid has been reported to have some activity in the pea test (69), no other regulator has been found which does not have an aromatic ring. The fact that compounds such as cyclohexane and decahydronaphthalene with saturated, nonaromatic rings are not active (62) is quite surprising if one assumes that the function of the ring is merely physical adsorption. A systematic attempt by Veldstra & Booij (62) to find acids with nonaromatic rings which possessed activity was unsuccessful.

If one regards the ring structure as a site for substitution at an *ortho* position, then the nonactivity of the nonaromatic compounds is readily explained since aromatic rings are unique in undergoing a substitution reaction with the displacement of a hydrogen atom. This mechanism has been discussed in detail elsewhere (38, 39) where it was shown that H^+ would be easily removed from phenoxyacetic acid by an oxidative displacement while this same mechanism would not operate in the benzoic acids since the H^+ is extremely difficult to remove from the electron-deficient ring. Thus, the activity of the benzoic acids depends upon the presence of a group of the type X^- (halogen or nitro) at the *ortho* position which can be displaced easily.

The activity noted for benzoic acids with methyl groups in both *ortho* positions does not necessarily invalidate the two-point reaction hypothesis as has been claimed by Veldstra (60). Methyl groups have been shown to be displaced under mild conditions from compounds such as 2-methyl-4-methoxy-1,4-benzoquinone (13). Also it is conceivable that the methyl groups could be oxidized to carboxyl groups and this electronegative group would undergo the displacement reaction.

Nature of the Growth Reaction

An hypothesis relating chemical constitution to growth regulator activity necessarily describes reactivity characteristics of the plant substrate. If the growth regulator induces elongation by consummation of a two-point attachment through the carboxyl group and an *ortho* position, it is very likely that the substrate reacts at a basic group and a nucleophilic group. If the substituted benzoic acids can induce elongation, the minimum distance between reactive groups on the substrate must be sufficient to allow for combination of such acids through the carboxyl group and an *ortho* position. The cysteinyl structure fits these requirements and the sulfhydryl group as the nucleophilic reactant provides an explanation for the selective inhibition of elongation by iodoacetate and other reagents reacting with sulfhydryls as well as the inhibitory effects of unsaturated lactones on elongation of plant cells (24). The selection of the sulfhydryl group as the nucleophilic reactant is indicated also by the reports of reaction between coenzyme A and indoleacetic acid as well as other auxins presumably through formation of thioethers (28, 48).

McRae & Bonner (33) have noted the fact that the inactivity of 2,4-dichlorophenoxyisobutyric acid cannot be explained on the basis of steric hindrance by the α-methyl groups preventing approximation of the carboxyl group and *ortho* position. They have thus proposed another characteristic of the substrate in the form of a barrier between the two positions. The lack of activity of the compound may, however, be due to failure of formation of the requisite amide linkage (24) as the α-methyl groups may permit salt formation between the carboxyl group and basic amino group but prevent subsequent amide formation.

The nucleophilic condensation at the *ortho* position with a sulfhydryl group of the substrate would not be expected to be reversible. Thus, compounds with auxin activity but of low V_{max}, such as 2,3,5-triiodobenzoic acid in which nucleophilic condensation occurs, should display nonreversible antagonism as has been reported (11). Similarly, the interference of aryloxy compounds with free *ortho* positions but lacking a carboxyl group would not be expected to be overcome by added auxin although McRae & Bonner (33) have reported that the inhibition of 2,4-dichloroanisole is reversible.

Inhibition of elongation resulting from the reaction of unsaturated lactones such as coumarin with sulfhydryl groups of the cysteinyl units should not be reversible and Bonner (11) has found that it is not. McRae & Bonner (33) have pointed out that the inhibition induced by maleic hydrazide is not competitive and it would not be if the molecule reacts with sulfhydryl groups. Van Overbeek (44), however, has reported that the inhibitory effects of N-2,4-dichlorophenylmaleimide and related maleimides are reversible. He found that if sections of maize coleoptiles elongating in the presence of indoleacetic acid and either *trans*-cinnamic acid or the maleimide were trans-

ferred after five hours to similar solutions in which the concentration of the indoleacetic acid were 100-fold greater, their subsequent elongation took place at the same rate as did that of sections transferred from low to high concentrations of indoleacetic acid without inhibitor. Such results would also be obtained if the elongation of the sections was determined, not by the actual number of reactive sulfhydryl sites at any one time but by the continuous production of such sites. Reversibility of sulfhydryl reaction would not be involved. Similar considerations may apply also to those instances of limited reversibility reported for such compounds as 2,4-dichloroanisole, in which the competition is for newly formed sulfhydryls and not for those having undergone nucleophilic condensation.

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