STUDIES ON THE KINETICS OF AUXIN-INDUCED GROWTH

Thesis by

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ABSTRACT

The auxin-induced growth reaction of the Avena coleoptile has been treated by methods of classical enzyme kinetics. The kinetic treatment makes it possible to characterize the growth promoting activity of an auxin by two parameters, K_s and V_{max}. These express respectively the affinity of the auxin for the auxin-receptive site within the plant and the ability of the complex thus established to promote growth.

Treatment of Avena section growth by the methods of enzyme kinetics has made it possible to determine rigorously whether or not inhibitors of auxin-induced growth are true antiauxins and act by competing with auxin for the auxin-receptive site within the plant. Certain auxin-inactive compounds have been shown to possess antiauxin activity. Among such substances are 2,4-dichloroanisole, 4-chloro- and 2,4-dichlophenoxyisobutyric acids, and 2,6-dichloro- and 2,4,6-trichlorophenoxyacetic acids. Each of these substances can be considered as derived from an active auxin (2,4-dichlorophenoxyacetic acid) by elimination of one of the structural features essential to auxin activity and thereby capable of combining at one point of the auxin-receptive site but incapable of consummating the two-point attachment requisite for auxin activity.

It is shown that chemically different auxins compete with one another for the same receptive sites within the plant. Auxins of low V_{max} are capable of inhibiting or augmenting the activity of auxins of greater V_{max}. Whether inhibition or promotion result depend on the concentrations of the two substances and the differential

in V_{max} exhibits apparent antiauxin activity. This activity is shown to be different from competitive inhibition of true antiauxins.

The relationship between Avena section growth rate and auxin concentration has been demonstrated to be predictable on the basis of a requirement for two-point attachment of the auxin molecule to some receptive entity within the plant.

The growth inhibition resulting from high auxin concentrations is not alleviated by antiauxins but rather the auxin inhibition is augmented by the presence of sufficiently high antiauxin concentrations. A necessary corollary to the single point attachment antiauxin concept and the bimolecular complex formation concept for inhibitory auxin concentrations is therefore confirmed.

A preliminary investigation concerning herbicidal activity of mixtures of an antiauxin and an auxin on bean plants is presented. The data obtained do not unequivocally establish that inactive bimolecular auxin-receptor complex formation at high auxin concentrations is a factor contributing to herbicidal action of 2,4-D, but the possibility that this is in fact so is considered.

A cultural technique for obtaining isolated flax root clones is described and data for some experiments with an isolated flax root clone are presented. The inhibitory action of certain antimetabolites on the growth of young tomato plants is described.

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GLOSSARY OF ABBREVIATIONS

IAA	indole-3-acetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
NAA	naphthalene-1-acetic acid
TIBA	2,3,5-triiodobenzoic acid
PBA	γ-phenylbutyric acid
4-CPIB	4-chlorophenoxyisobutyric acid
2,4-DCPIB	2,4-dichlorophenoxyisobutyric acid
2,6-DCPA	2,6-dichlorophenoxyacetic acid
2,4,6-TCPA	2,4,6-trichlorophenoxyacetic acid
2,4-DCA	2,4-dichloroanisole
2,4,6-TCA	2,4,6-trichloroanisole
2,4,6-TCPP	2,4,6-trichlorophenoxypropanone

GENERAL INTRODUCTION

Growth, a fundamental attribute of living organisms, is a manifestation of an integrated chain of biochemical reactions. Higher plants, as well as other living organisms, have in common the biochemical characteristic that growth is governed by organic compounds which exert their effects at exceedingly low concentrations. It is characteristic of such plant growth substances that they are also hormones, synthesized in one tissue or organ and transported to distant sites where they exert their biochemical, physiological or morphological effects on growth. Among the plant growth substances, the auxins are the best known and have been most intensively studied. Historically, the term auxin was first applied to certain naturally occurring compounds possessed of the power of regulating cell elongation. It was subsequently found that related synthetic (non-native) compounds are able to duplicate the effects of native auxins in their influence upon the cell elongation phase of growth. We now know that auxins influence many aspects of plant growth in addition to cell elongation. This investigation is however concerned with basic principles of auxin physiology, principles which will be studied in relation to cell elongation.

There are certain plant tissues which do not grow (essentially) unless auxin is supplied. It is characteristic of such systems that there is a quantitative relation between auxin supplied and growth. In a sense, then, growth of such tissue might be viewed as an enzymatic reaction with the plant the enzyme and the hormone the substrate.

In the present investigation, we will take this thought and see how well it applies to auxin-induced growth. Growth of excised Avena coleoptile sections will be treated as an enzymatic reaction and by classical enzyme kinetics. To do this, such extensions as are needed and appropriate will be made. It will be seen that this way of viewing growth is a very useful one. It gives us new information on:

1. the basis of structural specificity of auxins,
2. the basis of structural specificity of antiauxins,
3. the reasons for the seemingly strange interactions of auxins,
4. the basis of herbicidal activity.

STRUCTURAL REQUIREMENTS FOR AUXIN ACTIVITY. During the past two decades much effort has been directed towards the determination of the structural features which a compound must possess in order to be active as an auxin (1-23). It is now apparent that there is a definite structural pattern which is prerequisite to auxin activity even though the many types of active molecules might at first seem quite unrelated. The features which auxin-active molecules possess in common, a matter first systematically studied by Koepfli et al. (1), may now be rather extensively codified and understood in terms of the two-point attachment concept of auxin action (20, 21, 22, 23).

On the basis of the compounds known to promote cell elongation, Koepfli et al. (1) set forth the following qualifications as those which a molecule must possess in order to elicit the cell elongation response.

1. It must possess a ring system as nucleus.
2. It must possess a double bond in this ring. (Subsequently Went (8) made this and the following qualifications more precise by stating that the side chain must be adjacent to the ring double bond.)
3. It must possess a side chain.
4. It must possess a carboxyl group (or a structure readily converted to a carboxyl, such as an ester or nitrile) in the side chain at least one carbon atom removed from the ring.
5. It must be capable of assuming a particular spatial relationship with respect to the ring and the carboxyl group.

The structural qualifications for auxin activity, as presented above, have not remained unchallenged. Veldstra (2, 3), who holds that the function of an auxin is a physical one and upon some boundary, reduced to two the structural requirements for auxin activity. Veldstra's proposed requirements are that the compound:

1. must possess a basal ring system (non-polar part) with high interface activity,
2. must possess a carboxyl group (polar part)--in general a group of acidic character--in such a spatial position with respect to the ring system that on adsorption of the active molecule to a boundary, this functional group will be situated perpendicularly to the plane of the ring system.

As indicated in Veldstra's second requirement, the carboxyl group qualification has been broadened to include groups of acidic character such as naphthalene-1-nitromethane (3). That qualification 3 of Koepfli et al. must also be revised has become evident in recent years. The substituted benzoic acid, 2-bromo-3-nitrobenzoic acid, was shown to be active in causing curvatures of tomato petioles (4) and to be weakly active in the split pea test (9, 10). Whether or not substituted benzoic acids were in fact active in causing cell elongation remained a controversial issue until Bentley (24) found that 2,3,6-trichlorobenzoic acid and its aldehyde have high activity in the Avena coleoptile section test. Thimann (26), has shown that 2,3,6-trichlorobenzoic acid is also highly active in the split pea test (see also 10, 26). That other substituted benzoic acids are active in straight growth of isolated Avena coleoptile sections has been reported by Muir and Hansch (23). Because of the fact that some of these compounds are inactive in the split pea test and inactive in promoting cell elongation in corn coleoptile sections (27), it has been suggested (16) that the substituted benzoic acids do not act like typical auxins. Veldstra (10,17) has also shown that some α-naphthoic acids are active in the split pea test.

In spite of the fact that the qualifications of Koepfli et al. have undergone some modification, it is nevertheless evident that in order for a molecule to be active as an auxin it must have a specific molecular architecture. The ultimate significance of this architecture becomes evident through the two-point attachment

concept of Muir and coworkers (20, 21, 22, 23). This concept, in addition to recognizing the qualifications of Koepfli et al. and their modifications, stresses the importance of a free or potentially substitutable position on the unsaturated ring ortho to the side chain or carboxyl group. The significance of the ortho positions of phenylacetic, phenoxyacetic, phenylbutyric and indoleacetic acids became apparent when it was realized that blocking both ortho positions by substitution converted the active auxin molecules into molecules incapable of promoting elongation of Avena coleoptile sections (20, 21). For example, 2,4-dichloro- and 2,4-dibromophenoxyacetic acids have high activity in the Avena section test but 2,4,6-trichloro- and 2,4,6-tribromophenoxyacetic acids are completely inactive. Veldstra and Booij (9) account for such inactivity by suggesting that trihalogenation increases the lipophilic character so that a required lipophilic-hydrophilic balance is upset. Paleg and Muir (29) have however pointed out that moving the halogen from the 4- position of 2,4-dichloro or 2,4-dibromophenoxyacetic acids has little effect on the lipophilic character of the benzene nucleus but nevertheless these diortho halogenated phenoxyacetic acids are inactive in the Avena section test.

The preceding observations, and others, relative to the importance of the ortho position suggested to Muir et al. (20) that in order for a compound to be active as an auxin it must be capable of undergoing a two-point attachment through the carboxyl group and the ortho group to some substrate within the cell. Furthermore, the fact that electronegative substituents in the aromatic ring

enhance the activity of phenoxyacetic and phenylacetic acids suggested (21) that the portion of the substrate within the cell which reacts at an ortho position is nucleophilic in nature. To complete the two-point attachment concept, Hansch et al. (23) proposed that the ortho position may react with a protein-bound cysteine sulfhydryl group and that the essential carboxyl group may become involved in a salt or amide type linkage with an amino group.

In contrast to the situation in phenylacetic, phenylbutyric, phenoxyacetic and indoleacetic acids, diortho substitution in the benzoic acids does not necessarily give rise to an auxin-inactive molecule (22). In fact substitution by an electron-rich reactant is enhanced when electronegative atoms or groups are present at one or both ortho positions of benzoic acids. The additional requirement which molecules must possess to be active as auxins may now be summarized by stating that a position ortho to the side chain or carboxyl group must be capable of displacement by an electron-rich plant substrate (23). Further considerations on the ortho effect have been presented by Thimann (25) who proposes two new rules for the relation between structure and activity of auxins. These proposed rules are that a compound

1. must possess two free positions para to one another, and
2. must possess one free position ortho to the carboxyl group containing side chain.

It is immediately apparent that rule 2 is that already proposed by Muir et al. (20). The first rule is based primarily on observations made by Leaper and Bishop (29) who pointed out that chlorophenoxyacetic

acids which have high physiological activity are substituted in positions in the benzene ring para to each other. Finally, it has been suggested by Wain (30) and by Smith et al. (31) that, in addition to carboxyl group and free ortho position requirements, the α-hydrogen atom of the side chain in aryloxy-acids may be requisite for auxin activity.

It has become evident during the course of the present work that available data concerning molecular architecture of compounds active as auxins may be conveniently summarized, with few if any exceptions, in the requirements for a carboxyl group and for a critical electron density in the aromatic nucleus at positions ortho to the carboxyl group or side chain and for an appropriate spatial configuration between these functional groups.

PRIMARY AUXIN ACTION. We have seen that although many chemically different organic compounds are active as auxins there is nevertheless a certain molecular configuration essential for activity. That there is a design common to physiologically active molecules would seem to demand that they participate in a unique biochemical reaction. The many different effects of applied auxins on whole plants, plant organs, tissues and enzyme systems (7, 13, 14, 15, 16, 32-38 and many others), might at first glance appear to exclude the possibility that auxins do in fact exert their effects upon a single biochemical reaction. It has become increasingly evident however that the different manifestations of auxin action are in fact secondary and are the result of a single common or

primary basic reaction which can be discovered when the experiment is made sufficiently brief. This subject has been adequately treated in recent reviews by Bonner and Bandurski (15) and others (9, 11, 16, 38, 39, 40) and it need only be pointed out that the study of auxins in relation to cell elongation is thus simultaneously the study of auxin-induced growth process in general.

Many investigations have been directed toward determining the primary auxin action through studies on the effects of auxins upon: (1) properties of the cell wall, (2) changes in chemical composition of the plant, (3) enzymes in vivo and in vitro, (4) relation between growth and respiration, and (5) water uptake. Data available at present are insufficient to exclude the possibility that the effect of auxin on increasing the plasticity of the cell wall is a direct rather than a secondary one. The available evidence does not however support the thought that there is a direct relation between the second and third of these effects and the primary action of an auxin (15, 40). Evidence does, on the other hand, suggest that respiration and water uptake are intimately associated with the action of an auxin. Since the work of Reinders (41, 42) and of others who followed her (43-49 and others) demonstrated that auxin-induced water uptake in plant tissues is an aerobic process, it has become increasingly clear that promotion of active water uptake is the primary function of auxin. Furthermore, the increase in respiratory rate resulting from the presence of auxin in the cells appears to be a response to the energy-consuming process of water accumulation (49).

It is evident then that although auxins evoke profound biochemical, physiological and morphological changes in plants, the primary auxin action may be the promotion of active water uptake.

THE ANTIAUXIN CONCEPT. The fact that a specific molecular architecture is a property of auxins and that auxins are implicated in the basic metabolic process of water accumulation suggests that further information may be obtained concerning the site of auxin action and the nature of the biochemical reaction through studies of compounds which interfere with the function of auxins. Compounds which interfere with the function of a metabolite and induce deficiency symptoms of the metabolite are called antimetabolites (metabolite antagonists or metabolite competitive inhibitors). The term metabolite is used in the broad sense in that it includes all substances utilized by an organism for maintenance of life.

It is now well recognized that many antimetabolites bear a close structural resemblance to the metabolite itself. Quastel (49a) clearly recognized that the metabolism of a substrate could be inhibited by molecules, structurally similar to the substrate, which are themselves incapable of undergoing enzymatic transformations. Approximately 12 years ago, Woods (50) reported that the bacteriostatic action of sulfanilamide and related sulfonamide drugs was completely prevented by p-aminobenzoic acid. This discovery led to a rapidly developing field in the study of antimetabolites. Antimetabolite investigations have been directed primarily toward the discovery of new chemotherapeutic agents and secondarily toward an exploration into the biochemical reactions

in which metabolites are involved. Considerably less attention has been directed toward a determination of the relationship which exists between the antimetabolite and the enzymatically active centers at which the antimetabolite exerts its inhibitory role. In recent years, however, specific inhibitors have been found which have aided in defining something of the nature of the enzymatically active centers of certain di- and polypeptidase enzymes as well as affording insight into the manner by which a substrate combines with its enzyme through multiple attachment points (51-55). From such studies, it is becoming increasingly evident that certain functional groups of the substrate interact with oriented and exposed functional groups on the enzyme. A consequence of this intimate relationship is that certain specific structural changes result in the conversion of a metabolite into an antimetabolite.

Demonstration that a compound acts as an antimetabolite (competitive inhibitor) can be achieved by either of two general methods. The first method, that of Lineweaver and Burk (56) or some variation thereof (57), is generally employed when quantitative data are desired. This method has been used in this investigation and is described in detail later. The second method, commonly called inhibition analysis (58, 59) is that most often used in antimetabolite investigations. The inhibition analysis method provides satisfactory qualitative data for most antimetabolite studies. Although this method is qualitative in nature, certain conditions must be fulfilled in order that antimetabolite activity may be ascribed to a substance. The basic condition is that the

effect of the antagonist be proportional to the amount of metabolite over a wide concentration range of the metabolite (59). In general, to analyze for this proportionality, the molar inhibition ratio, antagonist/metabolite, required to produce 50 per cent inhibition (or half maximal effect) is used. The ratio, antagonist/metabolite, is called the molar inhibition ratio and the numerical value for this ratio is termed the inhibition index. In antimetabolite studies, then, experiments are designed in such a manner that sufficient antimetabolite is added to increasing concentrations of metabolite so that 50 per cent inhibition may be determined at a number of concentration levels. If at a number of these concentration levels (four or more) the molar inhibition ratio remains constant, then the antagonism is competitive and the inhibitor may be described as a competitive inhibitor. On the other hand, if the ratio does not remain constant, then the antagonism is not competitive and use of the molar inhibition ratio or inhibition index is of little significance (59).

It is evident from the foregoing and the kinetic considerations to be discussed later, that an antimetabolite, or competitive inhibitor, is a substance which combines with the same catalytically active center of an enzyme as does the metabolite itself. Furthermore, a fundamental difference between the antimetabolite and the metabolite is that the antimetabolite is incapable of undergoing the enzymatic transformation which is characterisitic of the metabolite.

Let us now turn our attention from the general considerations of antimetabolites and consider only those substances which interfere

with the activity of auxins. Compounds which antagonize the action of auxins and induce symptoms of a lowered effective auxin concentration have been called antiauxins. It has been suggested (15) that the term antiauxin be restricted to those substances which are themselves inactive, or essentially so, as auxins but which satisfy the requirements of competitive inhibitors. It is in this sense that the term antiauxin is employed in this investigation.

It is important that the term antiauxin be restricted to those substances which compete at the auxin-active site within plants for there are other ways in which compounds may bring about lowered auxin concentrations in plants. For example, a substance may enhance the destruction of endogenous auxin through activation of indoleacetic acid oxidase (60, 61), and thereby produce auxin deficiency symptoms. Again, auxin deficiency symptoms may be induced by substances which inhibit synthesis of the native plant auxin. It is clear that in either of these instances auxin applied to the plant in the presence of such substances may alleviate the auxin deficiency symptoms but not quantitatively as is characteristic for the applied auxin alleviation of inhibitions induced by true antiauxins. The behavior of such compounds then is not that of competitive inhibitors.

Thus it may be seen that in order to ascribe true antiauxin activity to a substance it must be demonstrated that the relationship which exists between antiauxin inhibition and auxin alleviation of such inhibition is the appropriate quantitative relationship.

It is the purpose of the present investigation to show that the auxin-induced growth reaction of the isolated Avena coleoptile section can be treated by the methods of classical enzyme kinetics. Thereafter, using the Avena coleoptile section as experimental material, the investigation deals consecutively with:

(a) application of the Lineweaver and Burk kinetic analysis of competitive inhibition to the auxin-induced growth reaction and presentation of experimental evidence upon which is based a general formulation of the structural requirements for antiauxin activity,

(b) kinetics of auxin interactions,

(c) kinetics of auxin-induced growth inhibition,

(d) integration of the antiauxin concept and auxin-induced growth inhibition.

And finally, the investigation considers preliminary experiments on the application of kinetics to the study of auxins as herbicides.

MATERIALS AND METHODS

The Avena seeds (var. Siegeshafer) used throughout the investigation were first sterilized for ten minutes in a 1% NaOCl solution containing a pinch of detergent and were then soaked for one hour in distilled water. They were next sown in stainless steel trays containing vermiculite previously moistened with distilled water and allowed to germinate. Plantings for the majority of the experiments were made at the same hour of the day. Germination and seedling growth occurred in a standard Avena room, under low intensity red light (with occasional orange light) at 25-26°C and a relative humidity of 90%, for 82-84 hours. At this time a selection was made for those plants which possessed coleoptiles 2.75 to 3.25 cm. in length. Since the residual growth of excised coleoptile sections (that due to endogenous auxin) depends on the length of the coleoptile and since it is important for this work to obtain reproducible growth in the absence of added auxin, the selection of uniform coleoptiles contributes an important element. To this selection is due, in a considerable measure, the fact that the present results are both more precise and more reproducible than those heretofore recorded for growth of excised Avena sections. Additional water sprinkled over the seedlings approximately 24 and 12 hours prior to harvesting facilitated the handling of the coleoptiles and increased the sensitivity of the isolated sections to added auxins, particularly to indoleacetic acid.

From each selected coleoptile one 5.0 mm. section was cut, 2-3 mm. from the tip of each hollow coleoptile cylinder (leaves removed), with a double bladed cutting tool. After cutting, all sections were randomized and lots of 20 sections distributed to petri dishes containing 20 ml. of solution. A basal medium of Pyrex redistilled water, 3% sucrose and 0.0025M potassium maleate buffer (62) (pH 4.50) was employed. To this medium the auxins and compounds undergoing test as antiauxins, adjusted to pH 4.5 were added. The sections were incubated in the dark at 25-26°C and length measurements made under a microscope after incubation for 12 hours.

The variability to be expected within a single experiment as well as that to be expected between experiments can be judged from tables 1 and 2, respectively. In these tables the confidence limits have been determined according to the method described by Brownlee (63), and are calculated at the 1% probability level. The occasional experiments which for unknown reasons yielded a degree of precision less than that of table 2 were forthwith discarded. Most of the growth data presented in the paper are averages of two or more experiments (40 or more coleoptile sections per treatment) carried out on different days.

It is customary in auxin investigations to express concentrations in mg./l. or p.p.m. Therefore, this practice has been followed and data are given in mg./l. Since it is, however, usual to express kinetic constants in terms of molarity the appropriate conversions from mg./l. concentrations to molarities have been made in the summary tables.

TABLE 1

MEAN GROWTHS OF LOTS OF 20 AVENA COLEOPTILE SECTIONS AND THE CONFIDENCE LIMITS FOR REPLICATIONS WITHIN ONE EXPERIMENT.

Concentration of IAA	Growth: mm./section/12 hours					
	Replication 1		Replication 2		Replication 3	
mg./l.	Mean	Confidence Limits	Mean	Confidence Limits	Mean	Confidence Limits
0.0	0.41	0.39-0.43	0.41	0.38-0.44	0.43	0.40-0.46
0.01	0.90	0.87-0.93	0.89	0.86-0.93	0.91	0.87-0.95
0.05	1.61	1.56-1.66	1.57	1.52-1.62	1.62	1.58-1.66
0.1	1.96	1.88-2.04	1.96	1.90-2.02	2.02	1.97-2.07
0.5	2.63	2.56-2.70	2.65	2.58-2.72	2.61	2.56-2.66
1.0	2.91	2.85-2.96	2.95	2.86-3.04	2.92	2.85-2.99

TABLE 2

MEAN GROWTH OF LOTS OF 20 AVENA COLEOPTILES SECTIONS AND THE CONFIDENCE LIMITS FOR REPLICATIONS BETWEEN EXPERIMENTS.

Concentration of IAA		Growth: mm./section/12 hours				
		Day 1		Day 2		Day 3
mg./l.	Mean	Confidence Limits	Mean	Confidence Limits	Mean	Confidence Limits
0.0	0.41	0.38-0.44	0.41	0.38-0.44	0.44	0.41-0.47
0.01	0.89	0.85-0.93	0.90	0.86-0.94	0.87	0.83-0.91
0.05	1.55	1.50-1.60	1.60	1.56-1.64	1.58	1.53-1.63
0.1	1.95	1.87-2.01	1.96	1.91-2.01	2.01	1.94-2.08
0.5	2.60	2.54-2.66	2.66	2.60-2.72	2.61	2.54-2.68
1.0	2.88	2.80-2.96	2.88	2.80-2.96	2.95	2.88-3.03

KINETICS OF AUXIN-INDUCED AVENA COLEOPTILE SECTION GROWTH

OPTIMUM SUCROSE CONCENTRATION. In Avena coleoptile section growth experiments, as first used by Bonner (64), a carbohydrate source was not added to the basal medium. Subsequently Bonner (65) used sucrose, and Schneider (66) investigated the effect of varying sucrose concentration on IAA induced Avena section growth and found 1% to be optimal. Later Bonner (67) reinvestigated optimum sucrose concentration for section growth and found the optimum at 2-3% sucrose. Bentley (68) also investigated the effect of sucrose on section growth and obtained quite variable results after 48 hours incubation with 1% sucrose. Since the variability found by previous workers may be due to differences in experimental technique, it was decided to determine optimal sucrose concentration for the present system.

The effect of sucrose concentration on Avena coleoptile section growth at various IAA concentrations is shown in fig. 1. It is apparent that there is a definite optimum at 3% sucrose in the presence of 1.0 mg./l. IAA. In the absence of IAA or in the presence of low or inhibitory concentrations of IAA, growth in 2 or 4% sucrose is less markedly different or not significantly different from growth in the presence of 3% sucrose. Although 3% sucrose is essentially optimal for IAA induced Avena section growth, it was considered possible that this concentration would not necessarily be optimal for growth promoted by other auxins. However, fig. 2 indicates that 3% sucrose is optimal also for a considerable range

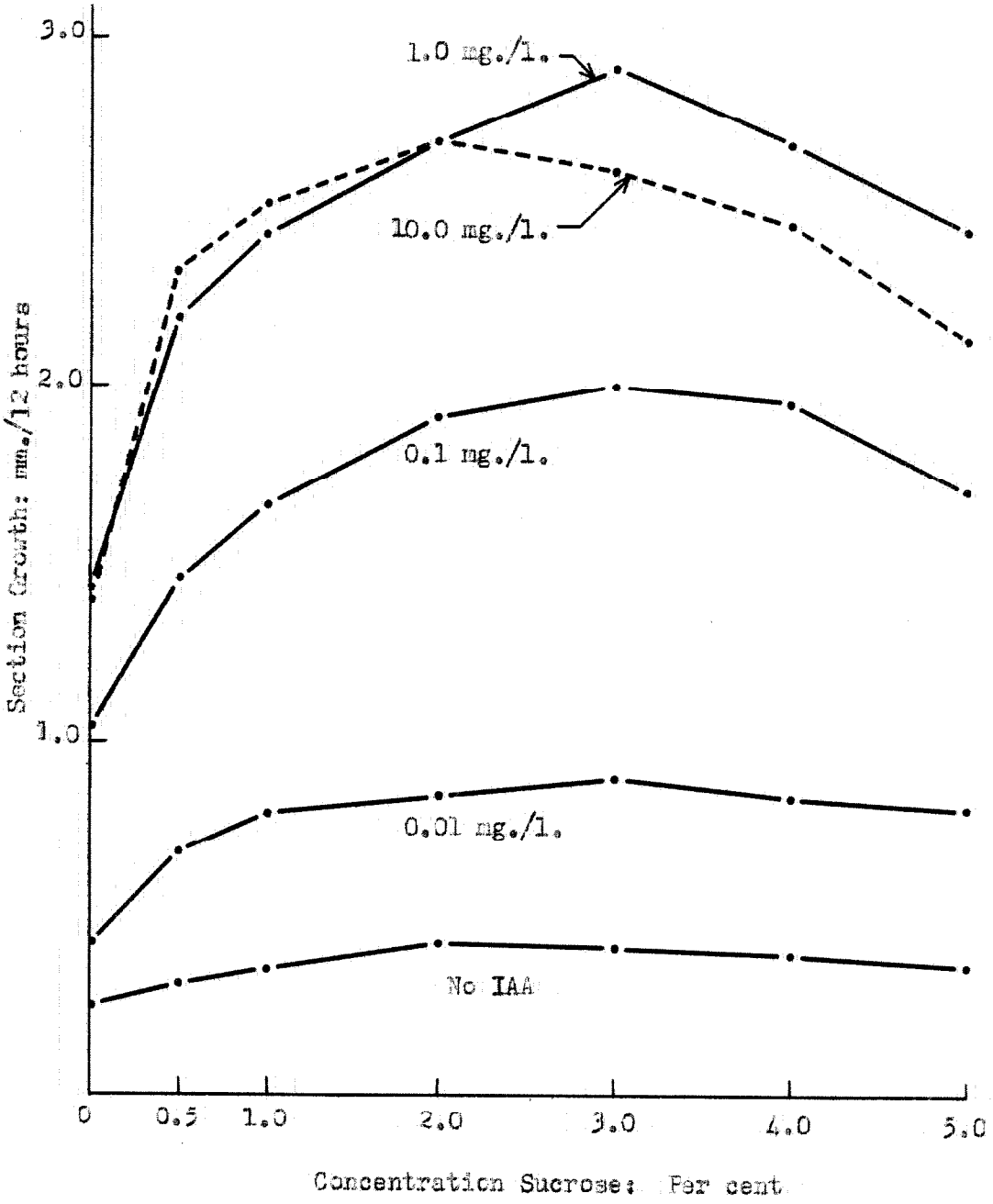


Figure 1. EFFECT OF SUCROSE ON IAA INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

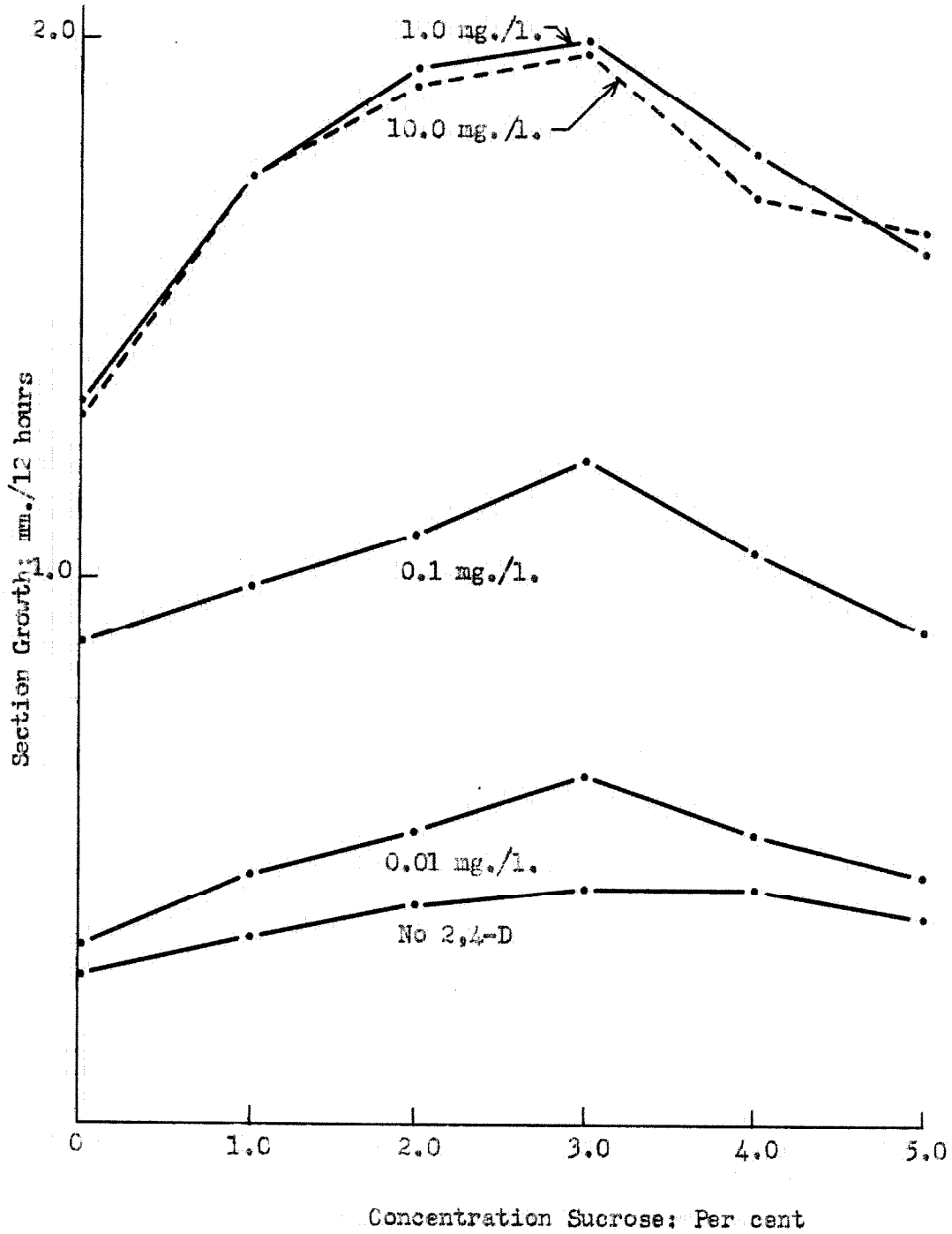


Figure 2. EFFECT OF SUCROSE ON 2,4-D INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

of 2,4-D concentrations. Three per cent sucrose was therefore chosen as addendum to the basal medium in all auxin-induced section growth experiments.

EFFECT OF POTASSIUM CHLORIDE. For the type of study carried out in this investigation, it was evident from the beginning that a large number of treatments would be used in each experiment. Furthermore, the nature of the treatments was to be such that growth differences obtained had to be the result of auxin treatments or effects of other compounds undergoing investigation and not due to extraneous materials such as inorganic ions. Since many of the compounds to be investigated are acids, it is necessary to use a base such as KOH to adjust the pH to 4.5. It was considered necessary then to determine the effect of K^+ ion and/or Cl^- ions as KCl on Avena coleoptile section growth.

Potassium chloride has been reported to influence Avena section growth. Thimann and Schneider (69) found that addition of KCl in the absence of auxin had no effect on section growth. In the presence of auxin, KCl was inhibitory above 0.01M although under some conditions it promoted growth at lower concentrations. It was reported by Bentley (68) that 0.01M KCl seemed inhibitory to sections incubated in auxin for 48 hours. Cooil (70) found that KCl had little effect on Avena section growth in the range between 0.001M and 0.01M. For sections incubated 20 hours, 0.01M KCl was slightly inhibitory but this concentration was not inhibitory to sections incubated for 40 hours.

The effect of added KCl on IAA and on 2,4-D induced section growth is shown in fig. 3 and 4, respectively. In these experiments, as in all other experiments, the basal medium contained 0.0025M potassium maleate buffer. It is clear from the figures that the influence of added KCl on growth is essentially identical for both auxins. In the absence or presence of auxin there is no significant effect of KCl on growth up to approximately 0.003M KCl. At a KCl concentration of 0.01M there is slight growth inhibition at IAA and 2,4-D concentrations of 1.0 mg./l. Growth is slightly inhibited by 0.01M KCl in the presence of 10.0 mg./l. 2,4-D and at other concentrations of both IAA and 2,4-D section growth is definitely inhibited by KCl concentrations higher than 0.01M. These results indicate that the addition of KOH and HCl, at concentrations sufficient to affect solution of and to adjust pH of compounds under investigation, will have no effect on auxin-induced Avena section growth.

PROGRESS CURVES FOR AUXIN-INDUCED GROWTH. The considerations of classical Michaelis-Menten (71) enzyme kinetics can be applied only to systems for which initial reaction rates are measured. The time course of auxin-induced growth of the Avena coleoptile section was determined by measuring section growth over periods up to 48 hours. The results obtained for the auxins used in the investigation as well as the time course of growth in the absence of auxin are presented in fig. 5. It is apparent that the growth velocity of the sections is essentially constant up to 18 hours in the absence of auxin and in the presence of 1.0 mg./l. IAA. The growth velocity

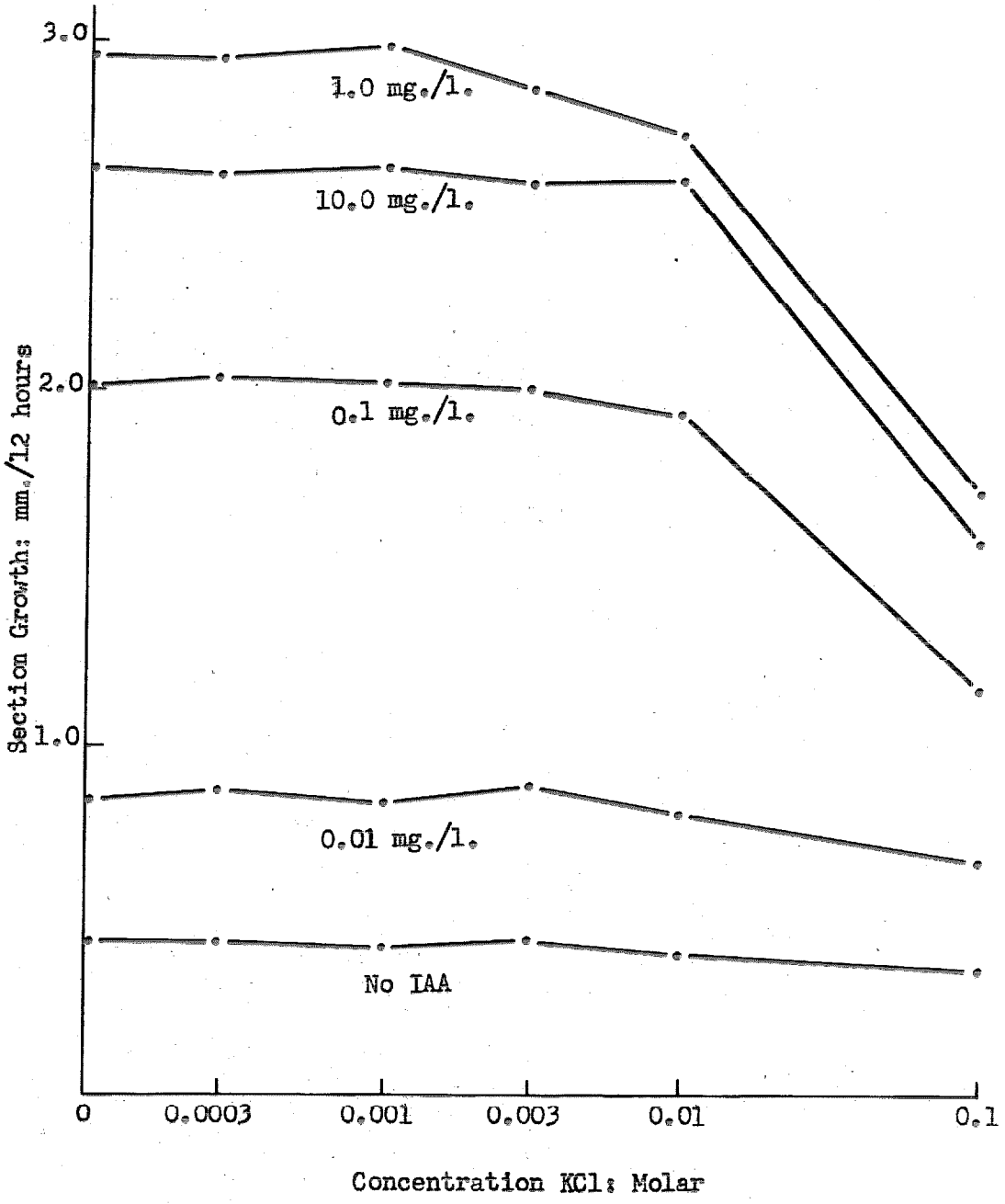


Figure 3. INFLUENCE OF KCl UPON IAA INDUCED AVENA COLEOPTILE SECTION GROWTH.

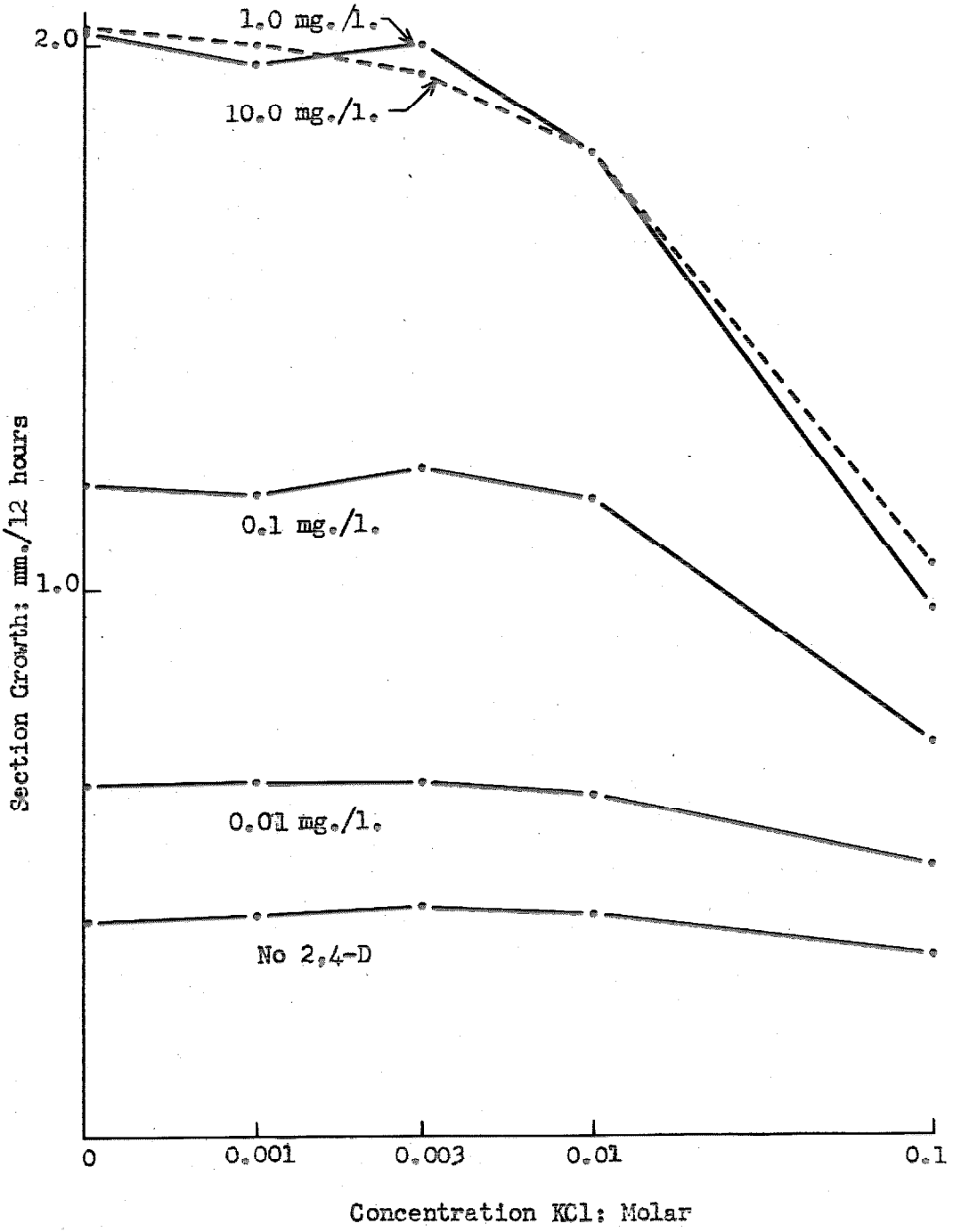


Figure 4. INFLUENCE OF KCl UPON 2,4-D INDUCED AVENA COLEOPTILE SECTION GROWTH.

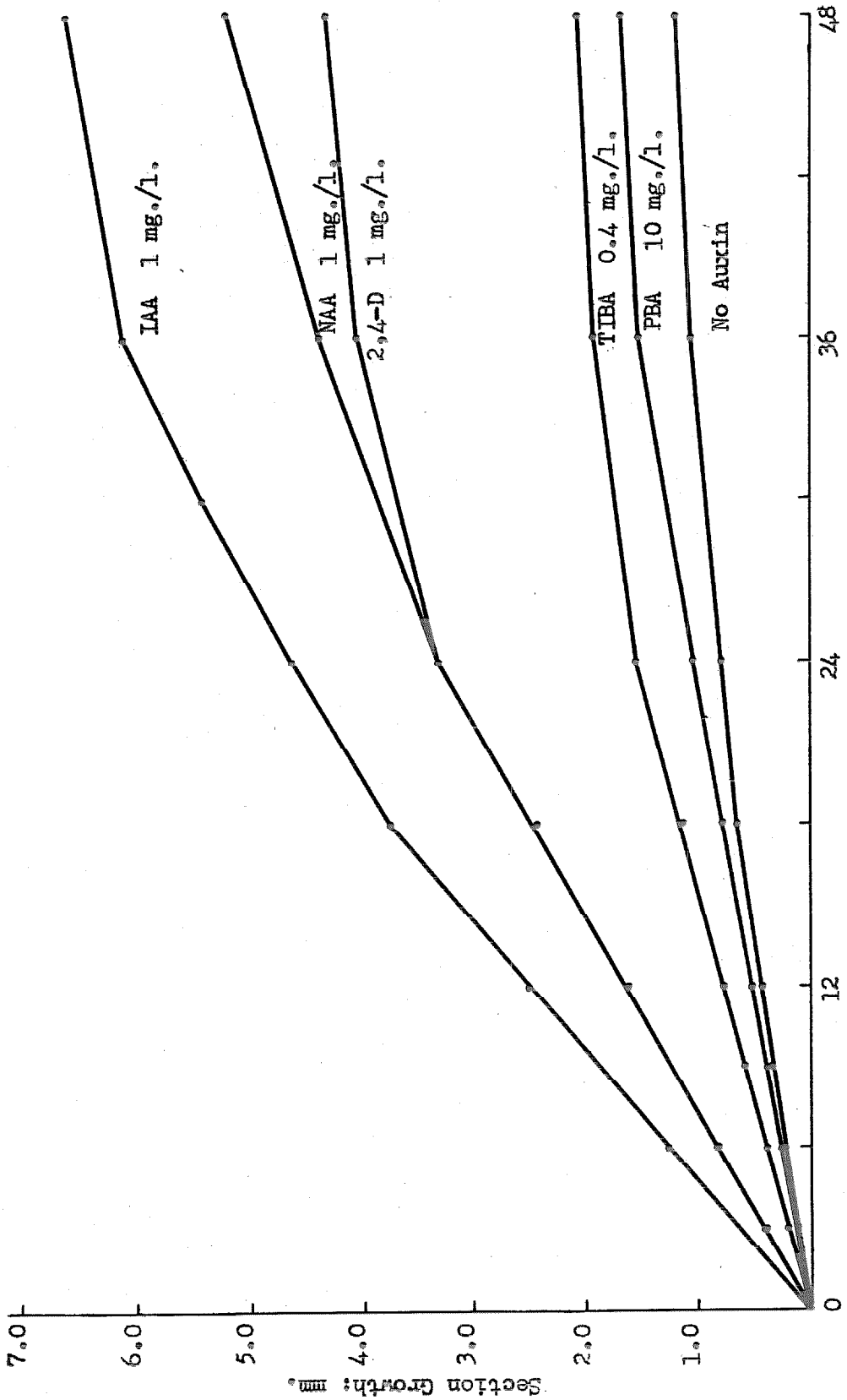


Figure 5. GROWTH OF AVENA COLEOPTILE SECTIONS AS A FUNCTION OF AUXIN CONCENTRATION. GROWTH IN PRESENCE OF AUXIN EQUALS TOTAL GROWTH MINUS GROWTH IN ABSENCE OF AUXIN.

of the sections is similarly constant up to 24 hours in the presence of 10.0 mg./l. PBA, 0.4 mg./l. TIBA, 1.0 mg./l. 2,4-D and 1.0 mg./l. NAA. It may be assumed that the initial growth velocity remains constant over a similar interval with other concentrations of the same auxins. In any case, an incubation time of 12 hours has been used in all subsequent experiments. This interval which includes only the first portion of linear time-growth curves of fig. 5 gives then an estimate of the initial growth velocity.

KINETIC TREATMENT OF AVENA COLEOPTILE SECTION GROWTH. It will now be shown that the growth response of the Avena coleoptile to added auxin can be formally treated by the enzyme kinetics of Michaelis and Menten (71). These considerations have as their basis the assumption that an enzyme catalyzes a reaction by first combining with substrate to form an intermediate enzyme-substrate complex which then decays to form the reaction products and to regenerate free enzyme. This situation is expressed in equation 1,

$$(1)\quad E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k} E + \text{Products}$$

where the equilibria involve the following quantities:

E: - Enzyme (in the present case, the coleoptile section or the auxin receptive entity within it)

S: - Substrate (in the present case, auxin)

ES: - Enzyme-substrate complex (in the present case, coleoptile-auxin complex)

K_s: - Dissociation constant of the complex ES

k: - Velocity constant relating concentration of active complex to rate of product formation (in the present case, growth)

Let us now express equation 1 in terms of reaction velocity (v). The maximum velocity (V_{max}) of the growth reaction is attained when the substrate concentration is sufficiently high to ensure that all available enzyme is saturated with S to form ES. Under these conditions, $E_{TOTAL} = ES$ and $V_{max} = k.E._{TOTAL}$, the kinetic consequences of equation 1 may now be expressed as in equation 2, where v is the reaction velocity.

$$(2)\quad v = \frac{V_{max}\,[S]}{K_S + [S]}$$

It is apparent that v (which in our case is section growth rate) is dependent upon $[S]$ and upon the constants K_S and V_{max}. Since K_S and V_{max} are constants, equation 2 is that of a rectangular hyperbola for which V_{max} is the value of v which is asymptotically approached as $[S]$ is increased, and for which K_S is the value of $[S]$ at which one half V_{max} is attained. That the data obtained for IAA and for 2,4-D induced growth of Avena coleoptile sections at concentrations below 4.4×10^{-6}M fit such hyperbolae are shown by the data of fig. 6A.

A more critical test of the applicability of equation 2 may be obtained by plotting the reciprocal of reaction velocity (1/v) as a function of the reciprocal of substrate concentration ($1/[S]$). This relation is expected to be a straight line for systems which follow Michaelis-Menten kinetics as is evident from equation 3 which is the reciprocal of equation 2. That the hyperbolae of

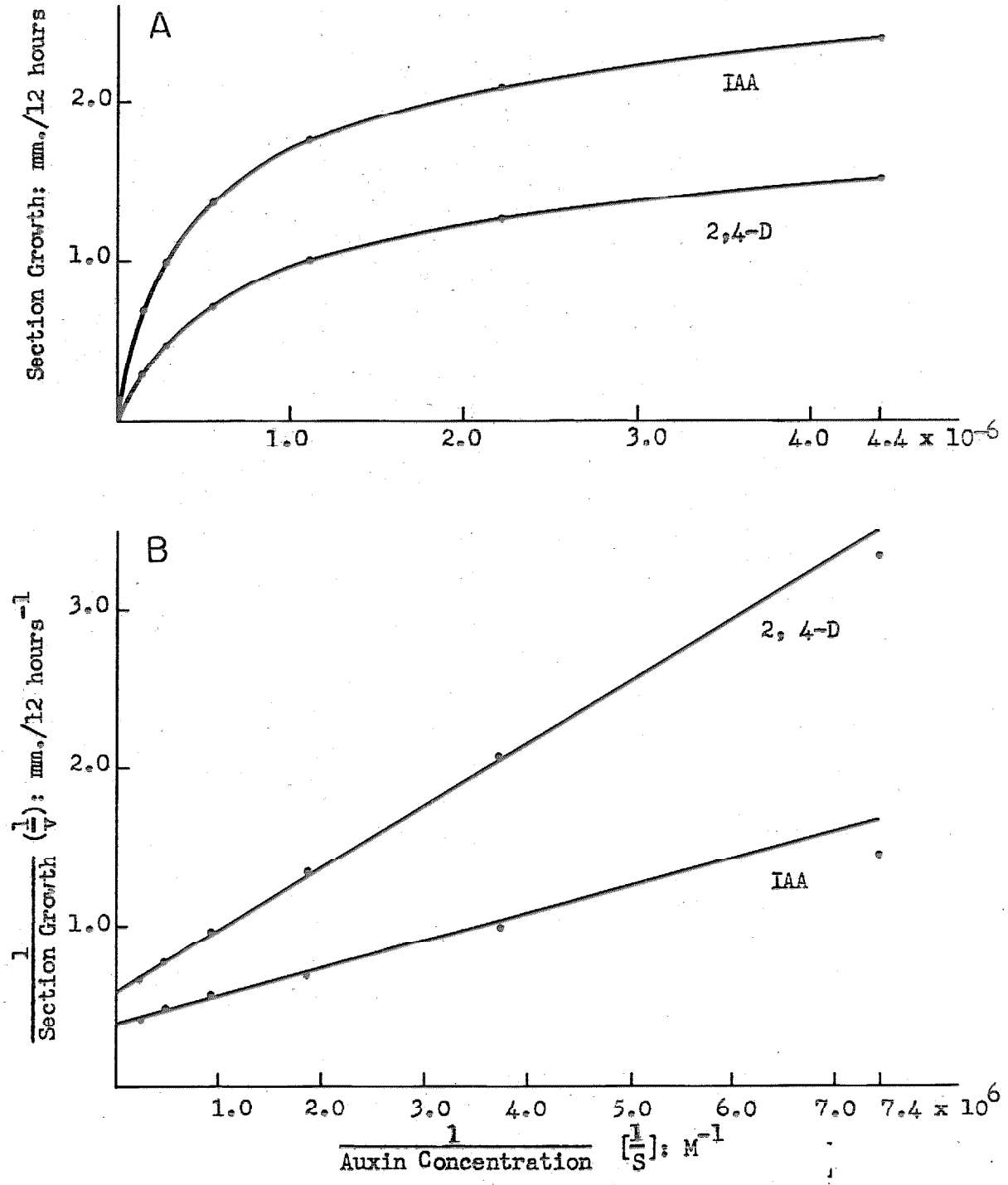


Figure 6. GROWTH OF AVENA COLEOPTILE SECTIONS AS A FUNCTION OF AUXIN CONCENTRATION. A. GROWTH PLOTTED AS A LINEAR FUNCTION OF AUXIN CONCENTRATION. B. RECIPROCAL OF GROWTH PLOTTED AS A RECIPROCAL FUNCTION OF AUXIN CONCENTRATION.

fig. 6A for IAA and for 2,4-D induced coleoptile

$$(3) \quad \frac{1}{v} = \frac{K_s}{V_{max}[S]} + \frac{1}{V_{max}}$$

section growth reaction do in fact yield thelinear relationship expected from equation 3 is shown in fig. 6B. The reciprocal plots of fig. 6B provide also a convenient method for the determination of the two parameters, V_{max} and K_s, which characterize the two different aspects of the activity of an auxin toward plant tissue. Thus the ordinate intercept, $1/V_{max}$, may be used to determine V_{max}, which is a measure of the effectiveness of an auxin if it is given in a concentration sufficiently high to elicit the maximum growth rate of which it is capable. The slope K_s/V_{max}, on the other hand, may be used to determine K_s which is a measure of the concentration of the auxin needed to elicit a growth response, and specifically, the concentration needed to elicit half maximum growth rate.*

KINETIC TREATMENT OF COMPETITIVE INHIBITION. - Competitive inhibitors are compounds which compete with the substrate for the substrate-receptive sites of the enzyme. This complex, unlike enzyme-substrate complex, does not, however, undergo further reaction. This may be formulated following Lineweaver and Burk (56) as in equation 4.

$$(4) \quad E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI$$

---

*In the application of the above considerations to Avena coleoptile section growth, it is assumed, as in classical enzyme kinetics, that the concentration of sites is small with respect to the concentration of auxin. That the total concentration of receptive sites remains constant with time is indicated by the fact that growth rate is constant over the period of measurement.

I: - Inhibitor

EI: - Inactive enzyme inhibitor complex (in the present case, coleoptile-inhibitor complex)

K_I: - Dissociation constant for the complex EI

We may now express the combined consequences of equation 1 and 4 (equation 5) in terms of reaction velocity (v) where this quantity is now the velocity in the presence of inhibitor. Thus the degree of inhibition exerted by a competitive inhibitor depends upon the relative substrate and inhibitor concentrations as well as upon the constants K_S and K_I. The maximum velocity (V_{max}) is attained when the substrate is present in such excess

$$(5) \qquad v = \frac{V_{max}[S]K_I}{K_S \cdot K_I + K_S[I] + K_I[S]}$$

over inhibitor that all of the enzyme is in the form ES and essentially none in the form of EI. Again, it is more convenient to use the reciprocal form of equation 5, which then becomes

$$(6) \qquad \frac{1}{v} = \frac{1}{V_{max}} \left[K_S + \frac{K_S[I]}{K_I} \right] \frac{1}{[S]} + \frac{1}{V_{max}} .$$

Comparing equation 6 with equation 3 it is apparent that in plots of $1/v$ against $1/[S]$, the presence of a competitive inhibitor increases the slope of the resultant line by the quantity $\frac{K_S[I]}{K_I}$. The intercept, $1/V_{max}$, on the other hand, remains constant and is not influenced by the presence of inhibitor. Thus the inhibition caused by a competitive inhibitor (antiauxin) in the presence of low auxin concentration is alleviated by increasing auxin concentrations. This does not imply that sufficiently high auxin concentrations

need completely overcome the antiauxin-induced inhibition. Since high auxin concentrations are of themselves inhibitory, it is essential for the present purposes to work within the range of auxin concentrations where this secondary inhibition is not appreciable.

CHEMICAL STRUCTURE AND ANTIAUXIN ACTIVITY

Introduction

This part of the investigation is concerned with the chemical characteristics and physiological properties of molecules which possess antiauxin activity. Relatively little attention has been directed towards the discovery of compounds which are able to antagonize the action of auxins. In the fields of chemotherapy, pharmacology, and bacterial nutrition, however, the study of metabolite antagonists (antimetabolites) has contributed extensively to our understanding of the sequence and nature of reactions involving such materials as the vitamins, hormones, and amino acids. It is, therefore, of interest to attempt to discover what structural modifications are required to convert an auxin molecule into an antiauxin. These compounds possess the practical importance that they may be used to lower the effective auxin concentration in plants and may thus be employed for desirable experimental (e.g. control of flower induction) and agricultural (e.g. leaf defoliation) ends.

For the present purposes, an antiauxin will be taken to be a compound which is itself devoid of auxin activity but which is capable of antagonizing competitively the action of an active molecule. Several substances have already been reported to be active as antiauxins and a comprehensive survey of such compounds has been made by Bonner and Bandurski (15). The 2,4-D analog, 2,4-dichloroanisole, for example, possesses the ability to antagonize auxin-induced growth (72). Although cis-cinnamic acid is

active as an auxin, its inactive isomer, trans-cinnamic acid, has been found by van Overbeek et al. (73) to have antiauxin properties. Maleic hydrazide is reported by Currier et al. (74) and Leopold and Klein (75, 76) to possess antiauxin activity. Stereoisomerism may also contribute antiauxin properties as has been shown for the two forms of certain α-arylpropionic acids by Smith et al. (31). Thus the (+) isomers of the (+)-α-(2-naphthoxy)-, (+)-α- (2,4-dichlorophenoxy)-, and (+)-α-(2,4,5-trichlorophenoxy)-propionic acids are active in the Avena coleoptile section test but the growth activity of the (+)-isomers can be partially or completely antagonized by the corresponding inactive (-)-isomers.

Compounds which possess weak auxin activity may also act antagonistically towards auxins of higher activity. Skoog et al. (77), in the first study of this matter, found that γ-phenylbutyric acid interacts competitively with indoleacetic acid in the Avena curvature test. An ability to antagonize more active auxins has also been reported for 2,3,5-triiodobenzoic acid by Galston (78), de Waard and Florschütz (79), Thimann and Bonner (80), and Weintraub et al. (81).

That auxins (except at very low concentrations) inhibit root growth (82) is among the better known facts of auxin lore. On this basis compounds other than auxins which are capable of increasing root growth or which can overcome auxin-induced root inhibition, have been described as antiauxins or auxin antagonists. No rigorous proof that this is so has however been presented. Among the materials first found to considerably promote root growth were 2,4-dichloro-

anisole (6), p-chlorophenoxyisobutyric acid (83, 84) and indole-3-isobutyric acid (84). Burström (85) has also found that phenoxyisobutyric acid as well as its 2- and 3- monochloro-derivatives are active in increasing root growth. It has subsequently become clear that a variety of substituted phenoxyisobutyric acids possess this property, including 2,4-dichloro-, 2,4,5- and 2,4,6-trichloro- and 2,3,4,5,6-pentachlorophenoxyisobutyric acids (85). The sulfur containing compounds 2,4-dichlorophenyl-sulfoneacetic and 2,4-dichlorophenylsulfoxideacetic acids have been shown by Wilske and Burström (86) to behave similarly. Minarik et al. (87) have found 3-nitro-4-fluorobenzoic acid to be the most active of thirty-five substituted benzoic acids which increase rate of root elongation. Åberg (88, 89, 90) obtained increases of root growth with 1-(naphthyl-methyl-sulfide)-propionic acid and auxin-induced root inhibitions were alleviated by the compound. Åberg (89) has also shown that a number of α-(naphthyl-methyl-sulfide or selenide)-alkylcarboxylic acids, in addition to increasing root elongation, act to alleviate 2,4-D induced root inhibition. Increases of root growth have been obtained with (-)-α-(2-naphthoxy)-propionic acid and furthermore the inhibition induced by the auxin-active (+)-isomer and by 2,4-dichlorophenoxyacetic acid can be alleviated by the (-)-isomer (88). Åberg (90) and Audus and Shipton (91) have demonstrated that the inhibition produced by 2,4-dichlorophenoxyacetic acid may be counteracted by the weak auxin phenoxyacetic acid.

Since rigorous proof of antiauxin activity has not previously been provided for any growth test, it was considered desirable to

first investigate compounds to which antiauxin activity has been attributed on qualitative grounds. The compounds chosen for the initial phase of this work were those which do not have auxin activity but which have been shown to increase rate of root growth. It will now be shown that certain of these compounds as well as other compounds not only inhibit auxin-induced Avena section growth but also that they do so in a strictly competitive manner.

Results

The growth effects of varying concentrations of IAA and of 4-CPIB alone and in combination, on Avena coleoptile sections, are summarized in table 3. It is apparent the 4-CPIB inhibits IAA induced section growth and that the inhibition increases with increasing concentrations of 4-CPIB. Further, for any given concentration of 4-CPIB the inhibition decreases with increasing concentrations of IAA up to a concentration of 1.0 mg./l. The growth values obtained for an inhibitory IAA concentration in the presence of 4-CPIB are also included in table 3. These data are not suitable for Lineweaver-Burk competitive inhibition treatment. It may be noted that the IAA inhibition at a concentration of 10 mg./l. is not alleviated by 4-CPIB. The significance of this fact will be discussed in a later section. That 4-CPIB competitively inhibits the action of IAA is shown in the double reciprocal plot ($1/v$ against $1/[S]$) of fig. 7. The lower line is that obtained from data on growth in the presence of IAA alone while the upper lines are those obtained in the presence of IAA but with increasing concentrations

TABLE 3

EFFECT OF 4-CPIB ON IAA INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration	Concentration of IAA mg./l						
	0.00	0.02	0.05	0.1	0.5	1.0	10.0
	Growth in absence	Growth in presence of IAA minus growth in absence of IAA					
mg./l	mm./section/12 hrs.	mm./section/12 hrs.					
0.0	0.44	0.59	1.14	1.56	2.12	2.45	2.10
0.1	0.43	0.48	0.97	1.39	2.08	2.45	2.12
0.5	0.42	0.41	0.84	1.25	2.02	2.41	2.11
1.0	0.43	0.33	0.68	1.08	1.92	2.39	2.12

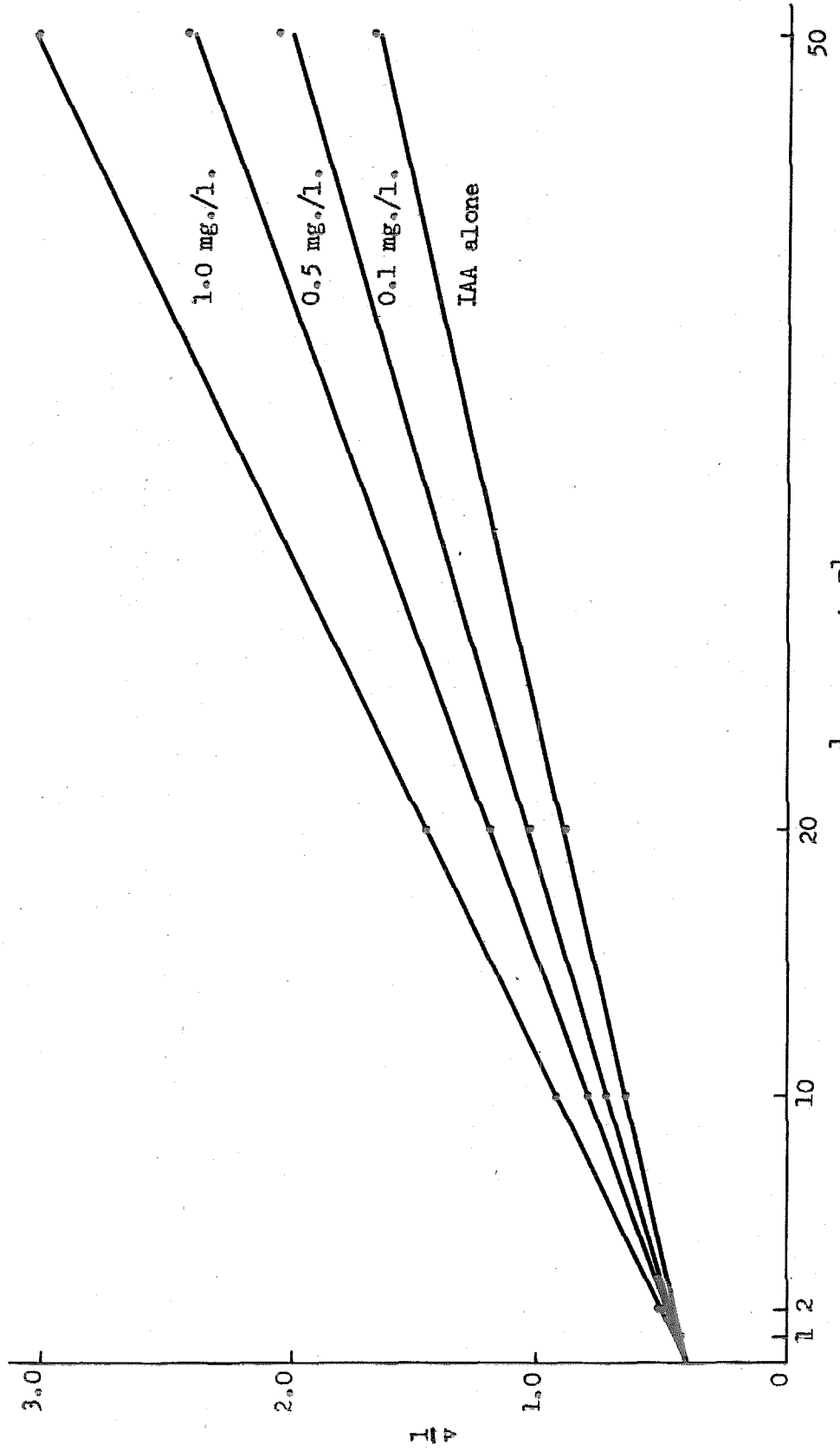


Figure 7. INHIBITION OF IAA INDUCED GROWTH BY 4-CPIB. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION. CONCENTRATIONS SHOWN ARE THOSE FOR 4-CPIB.

of 4-CPIB. The resultant family of straight lines possesses increasing slopes but a common ordinate intercept. The requisites for competitive inhibition are then fulfilled by 4-CPIB which is therefore a true antiauxin.

The data of table 4 show that similar results are obtained for the inhibition of 2,4-D induced section growth by 4-CPIB. The appropriate graphical treatment of the data of table 4, given in fig. 8, shows again that the criteria for competitive inhibition are satisfied. 4-CPIB is therefore an antiauxin toward 2,4-D as well as toward IAA.

A compound closely related to 4-CPIB, 2,4-DCPIB, also exhibits antiauxin activity toward IAA and 2,4-D in the Avena section test as is shown in tables 1 and 2 of Appendix I. It is evident that 2,4-DCPIB possesses no auxin activity and it is clear also that the compound inhibits both IAA and 2,4-D induced coleoptile section growth. In addition, increasing concentrations of IAA or of 2,4-D alleviates the inhibitions brought about by 2,4-DCPIB. The competitive nature of 2,4-DCPIB inhibition in the presence of IAA or of 2,4-D is evident from the double reciprocal plots of fig. 9 and 10 respectively.

We will now compare in quantitative terms the effectiveness of the auxins IAA and 2,4-D with the two antiauxins discussed above. The parameters, K_S and V_{max}, which describe the growth activity of IAA and of 2,4-D in the experiments of fig. 9 and 10 are recorded in table 5. The characteristic parameters, K_I, of each of the competitive inhibitors 4-CPIB and 2,4-DCPIB are also included. It

TABLE 4

EFFECT OF 4-CPIB ON 2,4-D INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 4-CPIB	Concentration of 2,4-D mg./l. 0.00	0.02	0.05	0.1	0.5	1.0	10.0
	Growth in absence of 2,4-D	Growth in presence of 2,4-D minus growth in absence of 2,4-D					
mg./l	mm./section/12 hrs.	mm./section/12 hrs.					
0.0	0.44	0.23	0.51	0.71	1.39	1.56	1.57
0.1	0.42	0.18	0.41	0.64	1.34	1.53	1.54
0.5	0.44	0.13	0.29	0.57	1.29	1.54	1.53
1.0	0.43	0.09	0.21	0.39	1.18	1.52	1.52

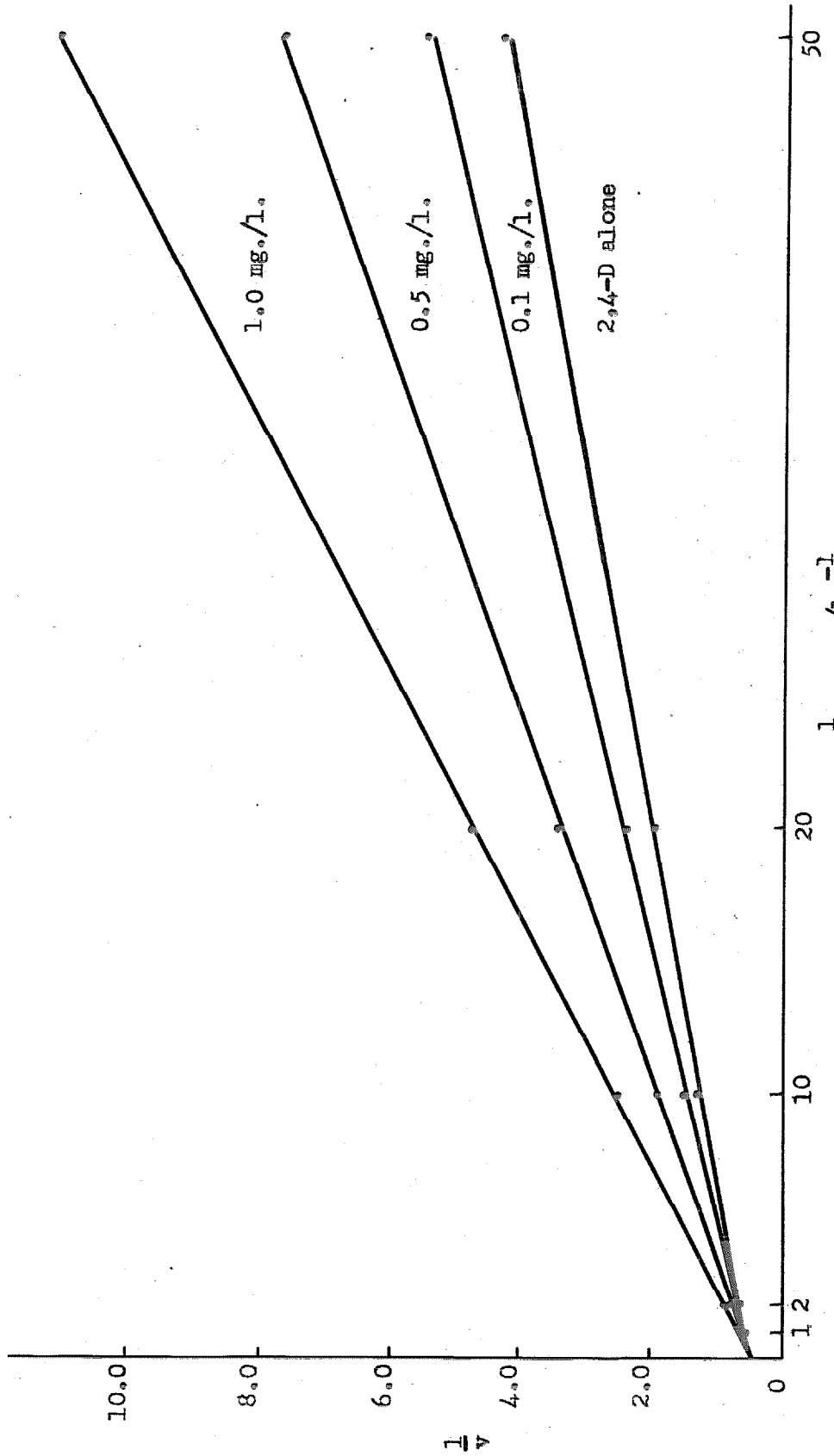


Figure 8. INHIBITION OF 2,4-D INDUCED GROWTH BY 4-CPIB. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION. CONCENTRATIONS SHOWN ARE THOSE FOR 4-CPIB.

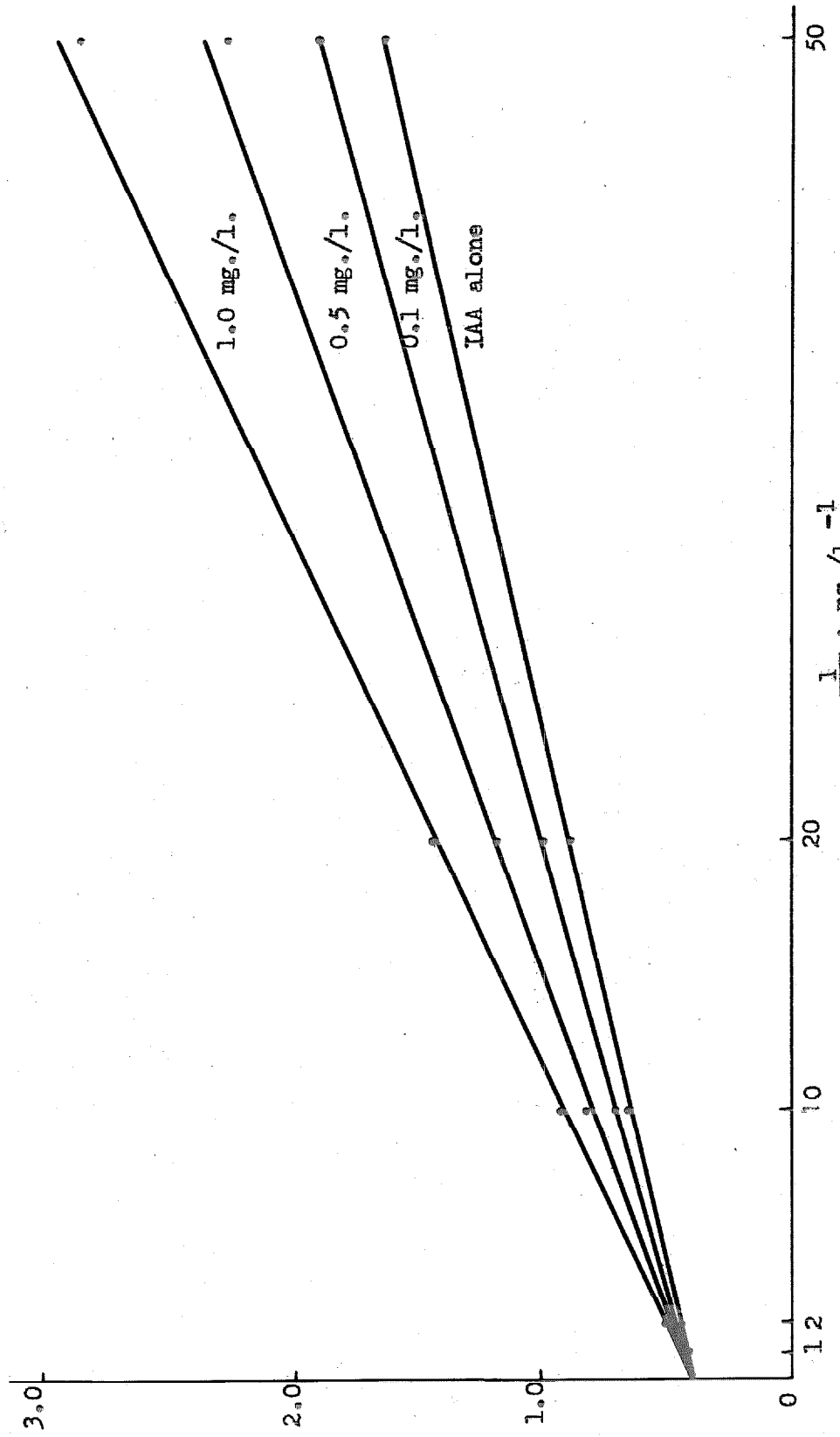


Figure 9. INHIBITION OF IAA INDUCED GROWTH BY 2,4-DCPIB. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION. CONCENTRATIONS SHOWN ARE THOSE FOR 2,4-DCPIB.

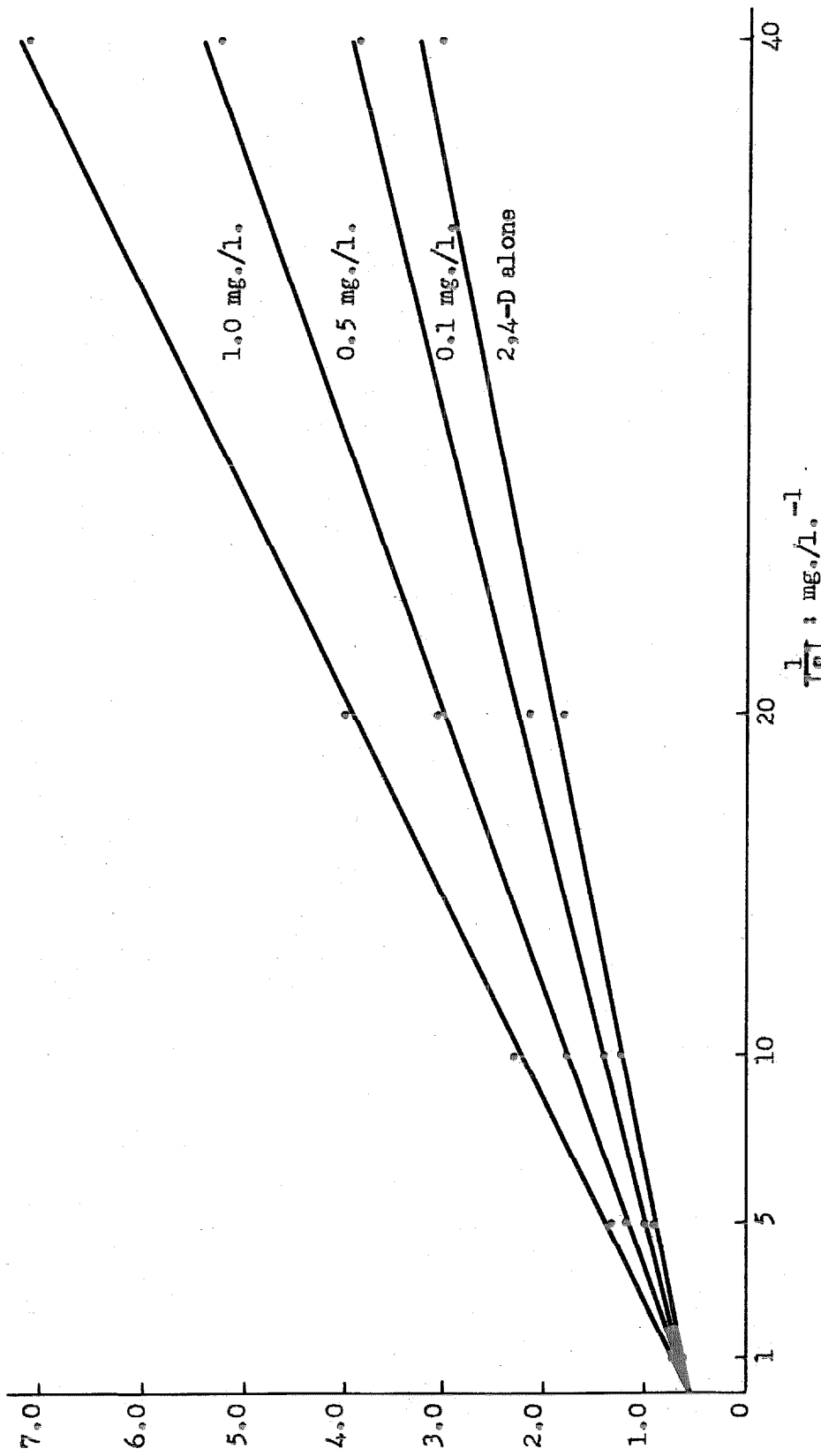


Figure 10. INHIBITION OF 2,4-D INDUCED GROWTH BY 2,4-DCPIB. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION. CONCENTRATIONS SHOWN ARE THOSE FOR 2,4-DCPIB.

TABLE 5

CONSTANTS WHICH CHARACTERIZE THE ACTION OF CERTAIN AUXINS (IAA AND 2,4-D) AND ANTIAUXINS(4-CPIB AND 2,4-DCPIB) ON THE GROWTH OF AVENA COLEOPTILE SECTIONS.

Substance	K_s (molar)	K_I (molar)	V_{max} mm./section/12 hrs.
IAA	3.6×10^{-7}		2.56
2,4-D	5.6×10^{-7}		1.71
4-CPIB			
in presence of IAA		3.1×10^{-6}	0.00
in presence of 2,4-D		2.6×10^{-6}	0.00
2,4-DCPIB			
in presence of IAA		3.8×10^{-6}	0.00
in presence of 2,4-D		3.2×10^{-6}	0.00

can be seen that the K_s of IAA is slightly lower than that of 2,4-D. IAA is, therefore, effective in this system in lower concentrations than is 2,4-D. The V_{max} which characterizes the action of IAA is also considerably higher than that for 2,4-D. IAA is, therefore, intrinsically more effective in promoting Avena section growth than is 2,4-D. The value of K_I for 4-CPIB is substantially identical with that for 2,4-DCPIB and both are roughly ten fold greater than the K_s values for IAA and 2,4-D. These antiauxins possess, therefore, an affinity for the plant growth system of the coleoptile which is some ten times less than that of the auxins. Since the affinity of an antiauxin for the receptive site within the plant depends only on the nature of the antiauxin and of the site, it follows that the K_I values should be independent of the nature of the auxin against which the antiauxin is tested. That this is so is shown by the fact that the K_I values for 4-CPIB in the presence of IAA and in the presence of 2,4-D are essentially identical. The same is true with 2,4-DCPIB for which K_I is the same in the presence of either of the two auxins.

The influence of varying concentrations of IAA and of 2,6-DCPA alone and in combination, on the growth of Avena coleoptile sections is shown in table 3 of Appendix I. It is clear that 2,6-DCPA is itself inactive over the concentration range tested. It is also apparent that 2,6-DCPA inhibits the section growth induced by IAA and that this inhibition increases with increasing concentrations of 2,6-DCPA. The inhibition by any given concentration of 2,6-DCPA does, however, decrease as the concentration of IAA is increased.

That 2,6-DCPA competitively inhibits the action of IAA is shown in fig. 11 by application of the Lineweaver-Burk formulation for competitive systems. The lower line is that obtained from the data on growth with IAA alone while the upper lines are those obtained from the data on growth in the presence of IAA but with increasing concentrations of 2,6-DCPA. The common intercept and different slopes of the lines fulfill the requirements of competitive inhibitors. Therefore 2,6-DCPA is a true antiauxin.

Entirely similar results for the inhibition of 2,6-DCPA of section growth induced by 2,4-D are shown in table 4 of Appendix I. The Lineweaver-Burk treatment of the data of this table is given in fig. 12 and shows that this inhibition is also a competitive one. Therefore, 2,6-DCPA is an antiauxin toward 2,4-D as well as toward IAA.

2,4,6-TCPA, like its analog 2,6-DCPA, is an inhibitor of section growth induced by auxin. The Lineweaver-Burk treatment for the data on the interaction of 2,4,6-TCPA with IAA and 2,4-D are presented in fig. 13 and 14, respectively. The growth values from which these graphs are obtained are contained in tables 5 and 6 of Appendix I. It is evident that, again, the criteria for competitive inhibition are fulfilled and 2,4,6-TCPA is therefore an antiauxin toward IAA and 2,4-D induced coleoptile section growth.

The constants which describe the activities of IAA, 2,4-D and 2,6-DCPA and 2,4,6-TCPA are recorded in table 6. This table gives, first, the experimental values for K_s and V_{max} of IAA and of 2,4-D obtained from the data of fig. 9 and 10. Again it can be seen that

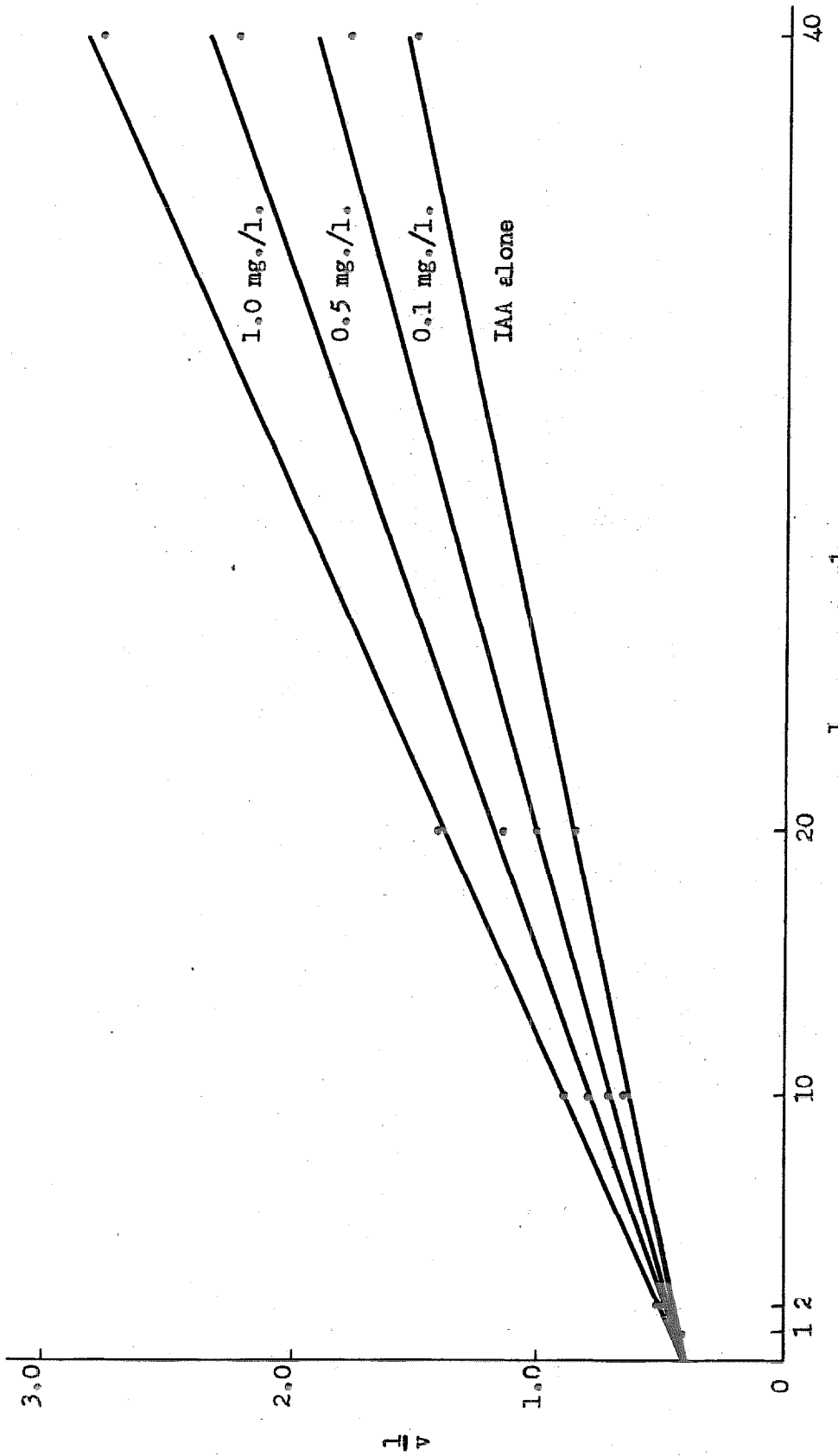


Figure 11. INHIBITION OF IAA INDUCED GROWTH BY 2,6-DCPA. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION. CONCENTRATIONS SHOWN ARE THOSE FOR 2,6-DCPA.

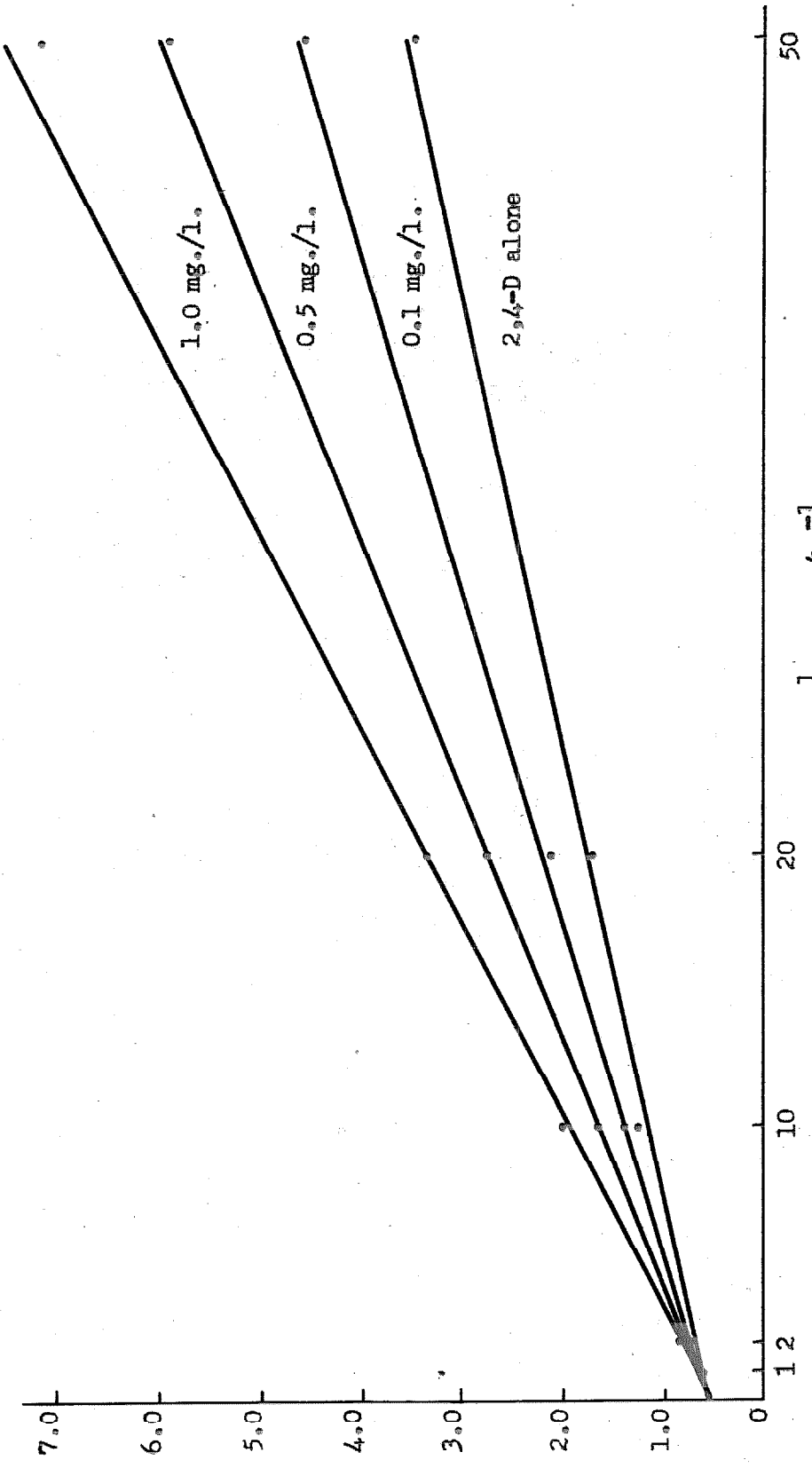


Figure 12. INHIBITION OF 2,4-D INDUCED GROWTH BY 2,6-DCPA. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION. CONCENTRATIONS SHOWN ARE THOSE FOR 2,6-DCPA.

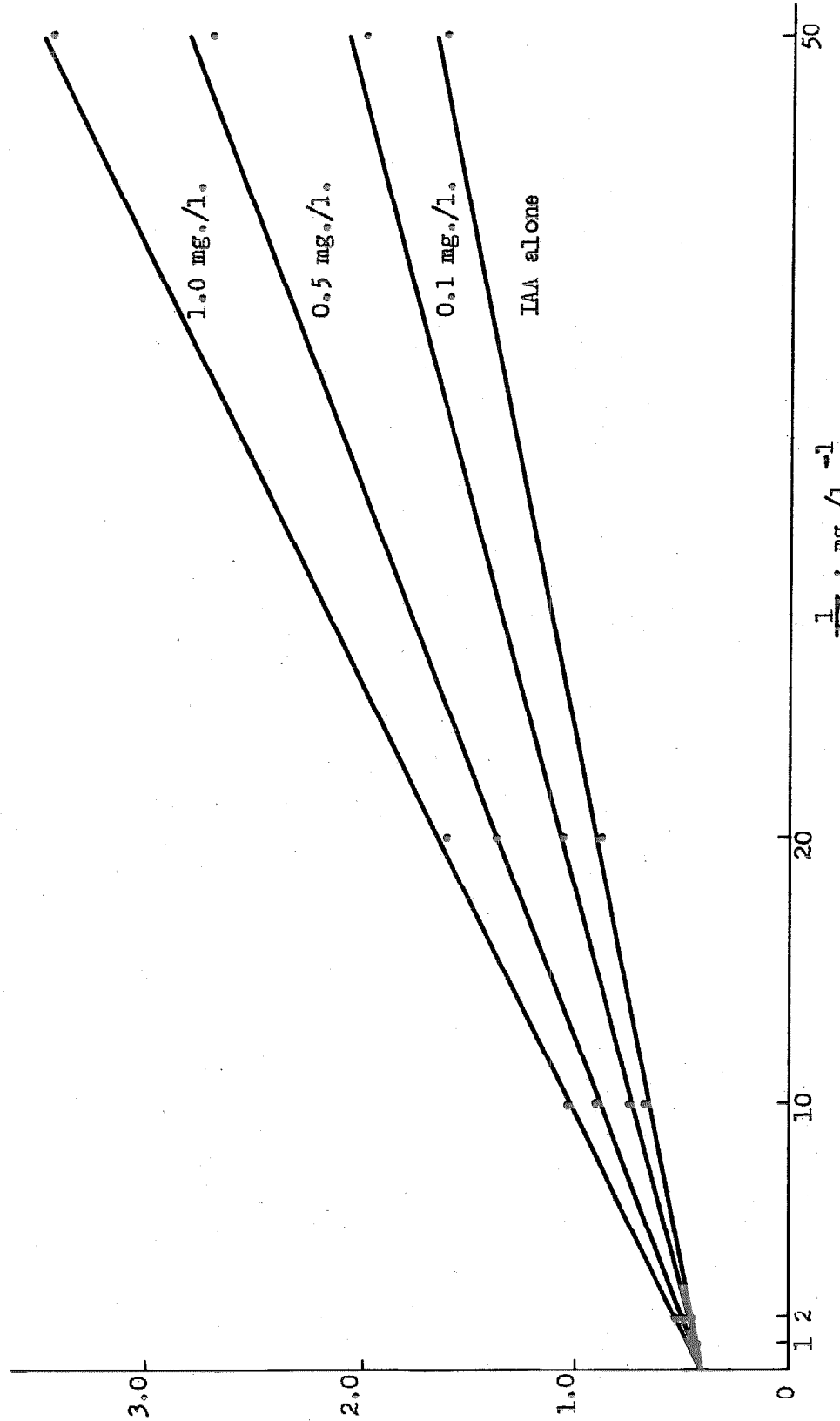


Figure 13. INHIBITION OF IAA INDUCED GROWTH BY 2,4,6-TCPA. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION. CONCENTRATIONS SHOWN ARE THOSE FOR 2,4,6-TCPA.

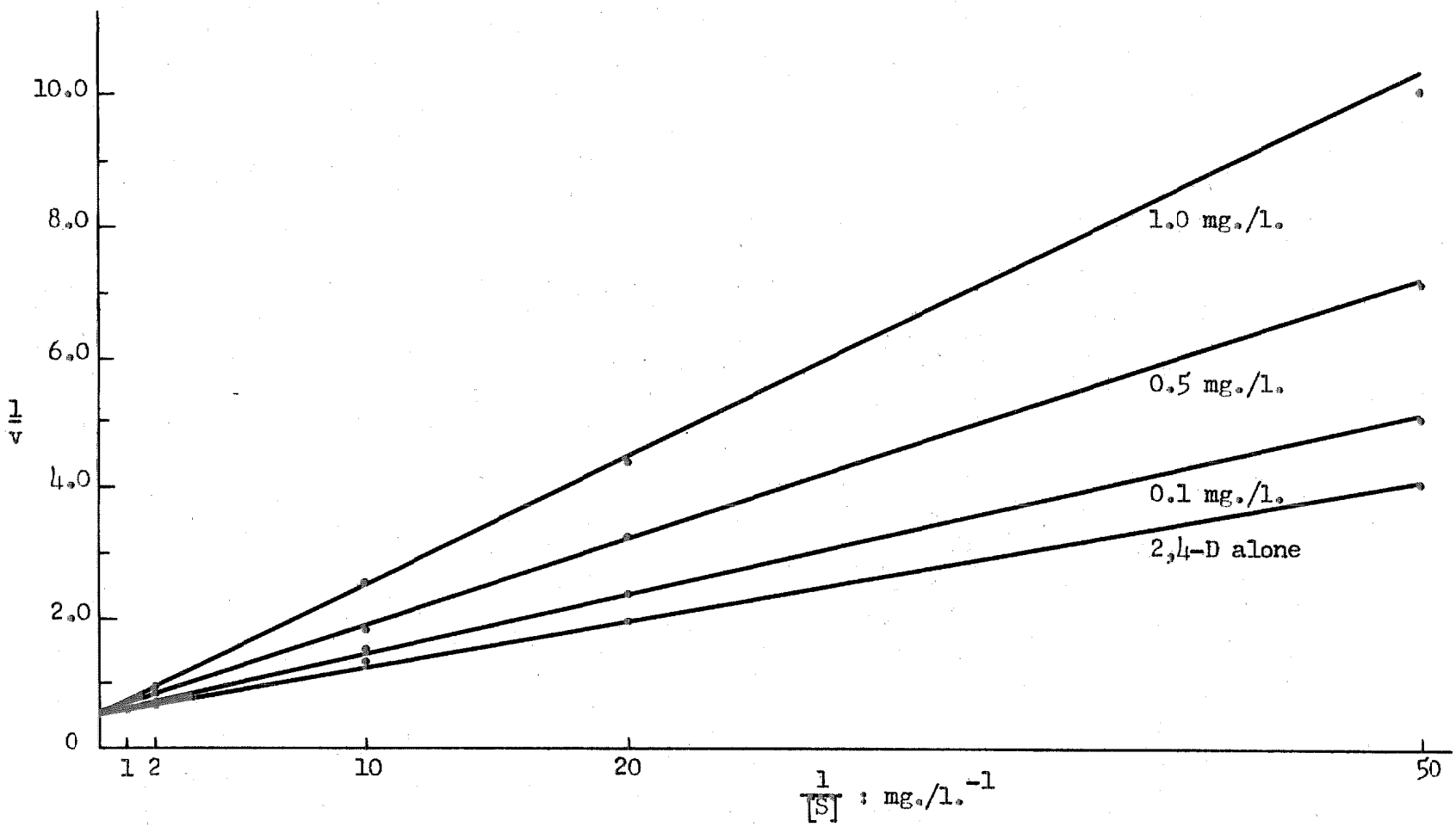


Figure 14. INHIBITION OF 2,4-D INDUCED GROWTH BY 2,4,6-TCPA. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION. CONCENTRATIONS SHOWN ARE THOSE FOR 2,4,6-TCPA.

TABLE 6

CONSTANTS WHICH DESCRIBE IN ENZYME KINETIC TERMS THE ACTIVITIES OF CERTAIN AUXINS AND ANTIAUXINS

	K_s (molar)	K_I (molar)	V_{max}
			(mm./section/12 hrs.)
IAA	3.55×10^{-7}	-	2.60
2,4-D	5.15×10^{-7}	-	1.76
2,6-DCPA			
in presence of IAA		4.0×10^{-6}	0.00
in presence of 2,4-D	-	3.7×10^{-6}	0.00
2,4,6-TCPA			
in presence of IAA	-	2.0×10^{-6}	0.00
in presence of 2,4-D	-	2.1×10^{-6}	0.00

the K_s for IAA is lower than that for 2,4-D. The V_{max} for IAA is also considerably greater than that of 2,4-D. The second portion of the table shows that the K_I for 2,6-DCPA in the presence of IAA is substantially identical with that which obtains in the presence of 2,4-D. This is true also for 2,4,6-TCPA. The K_I of 2,4,6-TCPA is approximately one-half that of 2,6-DCPA and 2,4,6-TCPA, is, therefore, a slightly more effective antiauxin than is 2,6-DCPA.

That IAA and 2,4-D promoted growth of Avena coleoptile sections may also be inhibited competitively by the compound 2,4-dichloroanisole (2,4-DCA) is evident from the double reciprocal plots of fig. 15 and 16 obtained from the data in tables 7 and 8 of Appendix I, respectively. 2,4-DCA is, therefore, a true antiauxin. The constants which describe the activities of IAA, 2,4-D, and 2,4-DCA are recorded in table 7. Again it is apparent that the inhibitor constant K_I for the antiauxin is essentially identical whether it is determined in the presence of IAA or of 2,4-D. It is evident, in addition, that the K_I for this antiauxin is approximately ten fold greater than are the K_I's for 4-CPIB or 2,4-DCPIB. 2,4-DCA is therefore less effective as an antiauxin than are the other two materials.

Discussion

The data presented above have shown that the growth response of Avena coleoptile sections to auxins conforms with some precision to the expectations of classical enzyme kinetics. The fact that this auxin-induced reaction can be so formulated and treated provides us not only with a rigorous method for testing whether or not growth

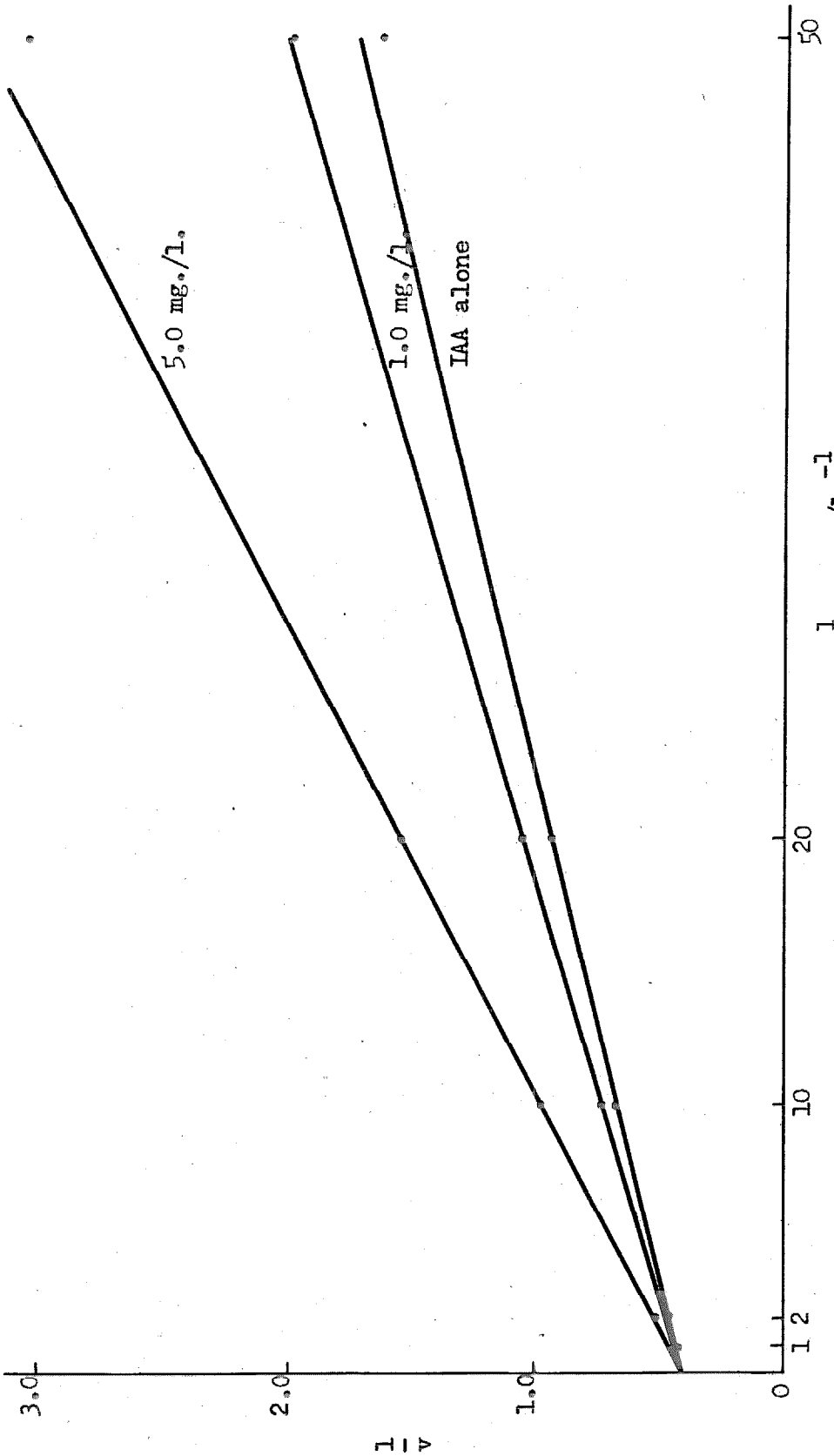


Figure 15. INHIBITION OF IAA INDUCED GROWTH BY 2,4-DCA. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION. CONCENTRATIONS SHOWN ARE THOSE FOR 2,4-DCA.

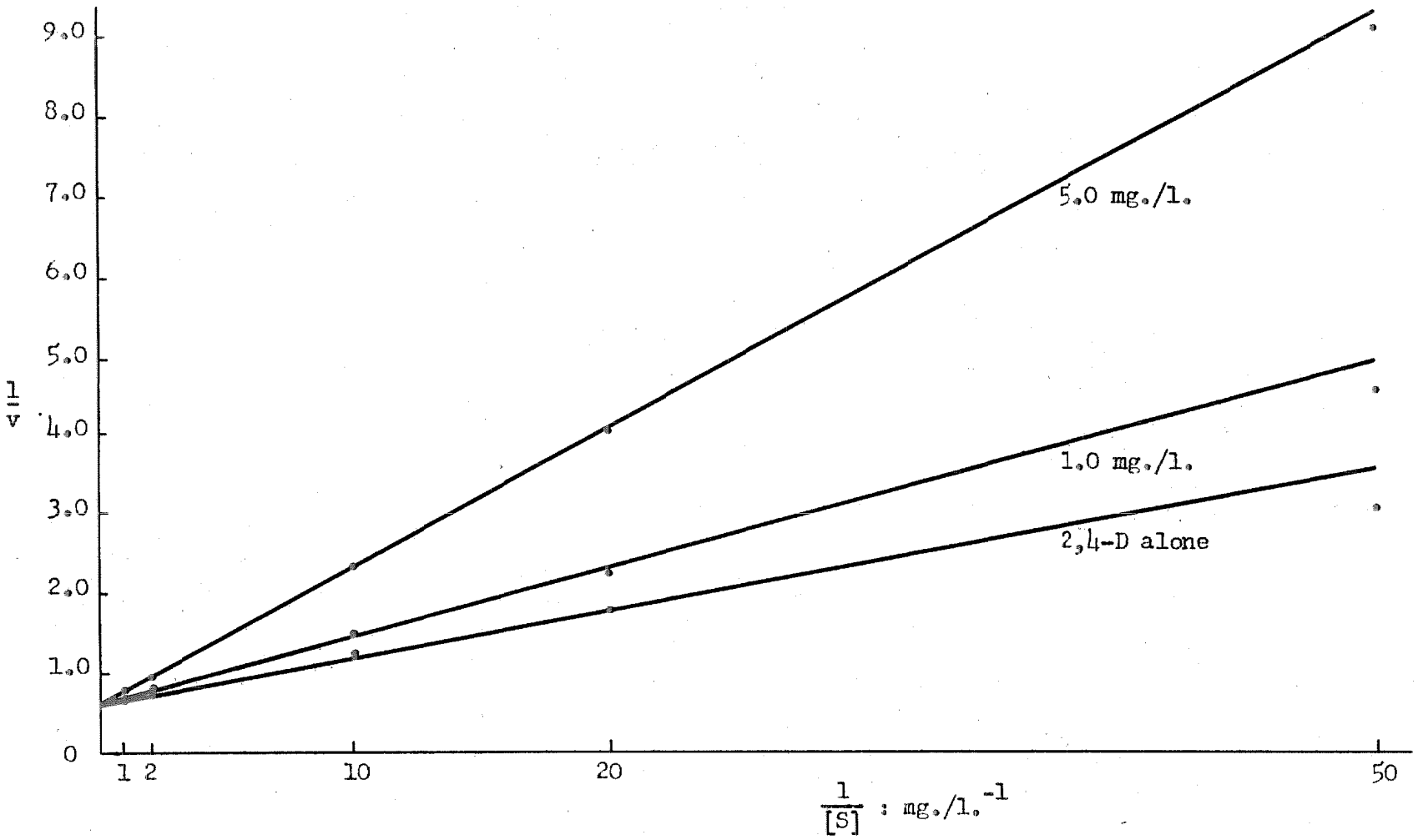


Figure 16. INHIBITION OF 2,4-D INDUCED GROWTH BY 2,4-DCA. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION. CONCENTRATIONS SHOWN ARE THOSE FOR 2,4-DCA.

TABLE 7

CONSTANTS WHICH CHARACTERIZE THE ACTION OF CERTAIN AUXINS (IAA AND 2,4-D) AND THE ANTIAUXIN 2,4-DCA ON THE GROWTH OF AVENA COLEOPTILE SECTIONS.

Substance	K_s (molar)	K_I (molar)	V_{max}
			(mm./section/12 hrs.)
IAA	3.6×10^{-7}		2.56
2,4-D	5.0×10^{-7}		1.73
2,4-DCA			
in presence of IAA		1.8×10^{-5}	0.00
in presence of 2,4-D		1.7×10^{-5}	0.00

inhibitors act as auxin competitors but provides us also with a convenient and quantitative method for the comparison of the activity of various auxins and antiauxins.

It has been customary in the past to describe the activities of diverse auxins in one of two general ways. Thus, on the one hand, activities have been described in terms of the concentration range over which the substance is effective or alternatively of the concentration range required to bring about some standard response. Or, on the other hand, compounds have been compared, usually qualitatively, in terms of the magnitude of the response which they can elicit. These two separate facets of auxin activity are exactly those described respectively by the parameters K_s and V_{max} of the present treatment. Thus K_s may be regarded as that concentration of an auxin which causes a half maximal effect, regardless of the magnitude of this maximal effect. V_{max} on the contrary is a measure of the maximal response elicited by an auxin when the latter is supplied in non-limiting concentrations. The parameter, K_s, possesses of course the dimensions of concentration and the K_s values for individual auxins may therefore be compared directly not only as between varied auxins but also as between varied plants and type of response. The parameter V_{max}, on the contrary, possesses the dimensions of response per unit time and will, therefore, be unique to each type of response to which kinetic treatment is applied. It is, of course, none the less possible to compare the relative values of V_{max} for each of a series of auxins as between varied kinds of responses.

The general methods for estimation of K_s and V_{max} for auxin-induced growth responses are evident from fig. 6A and 6B. It may be noted that the dissociation constant K_s for 2,4-D has been found to be ca. 1.6 times as great as that for IAA. This signifies then that the affinity of IAA for the auxin-receptive sites within the plant is ca. 1.6 times as great as is the case for 2,4-D. That these sites when combined with IAA are intrinsically more active in promoting Avena coleoptile growth than when combined with 2,4-D is shown by the fact that the V_{max} for IAA is ca. 1.5 that of 2,4-D.

Just as the constants K_s are a measure of the affinity of an auxin for auxin-receptive sites within the coleoptile, so the constants K_I are a measure of the affinity of growth inhibitors for sites within the tissue. Since the inhibitors which have been here considered have all been shown to act competitively toward auxins we may conclude that the constants K_I are a measure of the affinity of the inhibitors for the same sites for which the constants K_s measure auxin affinities.

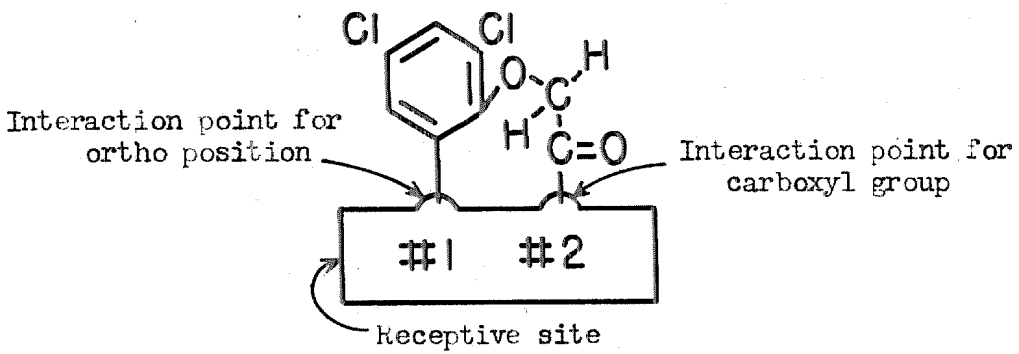
It is now apparent that molecules possessing structures which bear a close resemblance to an active auxin may possess antiauxin activity. Since, however, all auxin analogues are not antiauxins it appears that specific structural modifications of an auxin are required in order to produce an antiauxin. The nature of the structural modifications essential to antiauxin activity appear to be understandable in terms of the two-point attachment concept of auxin activity proposed by Muir et al. (20). According to this concept

which is illustrated in fig. 17A, an auxin molecule undergoes attachment at an appropriate site within the plant through the carboxyl group as well as through interaction at the reactive ortho position. A molecule to be active as an auxin must be able to consummate such two-point attachment.

It is apparent that certain of the antiauxins described above are simply 2,4-D molecules in which one or the other of the two essential reactive groups has been eliminated. This is true of 2,4-DCA in which the essential carboxyl group of 2,4-D is eliminated. We may surmise that the resulting molecule is still able to make a single point attachment through the free ortho position to position #1 on the receptive site (see fig. 17B). Even though 2,4-DCA cannot consummate the two-point attachment requisite for activity, it is nonetheless able to prevent 2,4-D or other auxin molecules from making such attachment. Antiauxins also result when the essential ortho positions of 2,4-D are blocked. This modification is illustrated by the antiauxin 2,6-DCPA in fig. 17C. Again the antiauxin may be surmised to be able to make a single point attachment, this time through the carboxyl group to position #2 of the receptive site. It is, however, unable to interact through either of the two halogen substituted ortho positions. The compound 2,4,6-TCPA is similarly an antiauxin of this type.

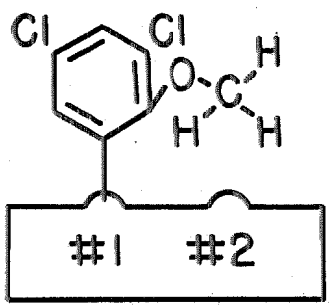
Still another change may be made in the 2,4-D molecule to convert it to an antiauxin. It is apparent from the early data on the inactivity of trans isomers of compounds active in the cis form, that, as summarized in fig. 17A, a proper spatial relationship

A



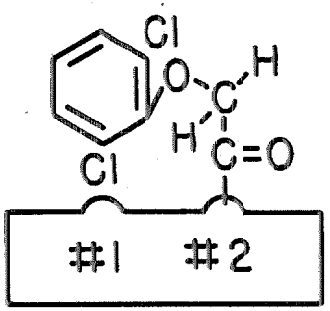
2,4-DICHLOROPHENOXYACETIC ACID
(2 point attachment)

B



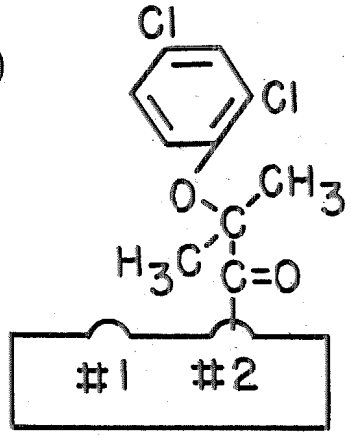
2,4-DICHLOROANISOLE
(ortho attachment only)

C



2,6-DICHLOROPHENOXYACETIC ACID
(carboxyl attachment only)

D



2,4-DICHLOROPHENOXYISOBUTYRIC ACID
(2 point attachment blocked)

Figure 17.

must exist between the essential side chain carboxyl group and the essential ortho position. It is possible then to interfere with this required spatial arrangement by introducing appropriate bulky groups or other forms of steric hindrance into the side chain. Such a modification is illustrated by 2,4-DCPIB in fig. 17D and a similar argument applies to 4-CPIB. Thus steric hindrance in the side chain permits of single point attachment but prevents the consummation of the two-point attachment which is essential to auxin activity.

Let us now consider what quantitative conclusions can be drawn from our knowledge of the values of K_I for these varied types of inhibitors. According to the formulation above, the inhibitor constants should provide us with the opportunity to determine separately the affinities of each interaction point of the auxin molecule for its respective group on the receptive entity. The data of table 8 summarize the values of K_I for each of the inhibitors studied in the present investigation. The ΔF values for the inhibitors (as calculated from K_I) are also included in this table as are values for K_s and ΔF for the auxins IAA and 2,4-D. These values are averages of all experiments. The K_I values for the diortho substituted phenoxyacetic acids, 2,6-DCPA and 2,4,6-TCPA, are closely similar and are about ten fold less than that for 2,4-DCA. Thus 2,4-DCA, a compound lacking a carboxyl group, does not possess as great an affinity for its reactive site within the coleoptile as do those antiauxins which possess a free carboxyl group but no reactive ortho position. 2,4-DCA is, therefore, less effective as an antiauxin. These differences in binding affinities are reflected

TABLE 8

FREE ENERGY OF FORMATION AND EQUILIBRIUM CONSTANTS FOR ESTABLISHMENT OF SOME AUXIN AND ANTIAUXIN COMPLEXES WITH AVENA COLEOPTILE SECTIONS.

Substance	K_s (molar)	K_I (molar)	ΔF (cal./mole)
IAA	3.6×10^{-7}		-8800
2,4-D	5.3×10^{-7}		-8500
2,6-DCPA		3.9×10^{-6}	-7400
2,4,6-TCPA		2.1×10^{-6}	-7900
4-CPIB		2.9×10^{-6}	-7500
2,4-DCPIB		3.5×10^{-6}	-7400
2,4-DCA		1.8×10^{-5}	-6500

in the ΔF of binding which is approximately 1000 cal./mole less for 2,4-DCA than for those antiauxins possessing a free carboxyl group. The fact that the values of K_I for 4-CPIB and 2,4-DCPIB are essentially identical to those for the diortho substituted antiauxins, which have free carboxyl groups, but are quite different from the K_S value for 2,4-DCA or the K_S value for 2,4-D indicates that the halogen substituted phenoxyisobutyric acids interact through the carboxyl group in preference to interacting through the free ortho position or through both positions. This, of course, is to be expected since the carboxyl group possesses an approximately ten fold greater affinity for its site than the ortho position does for its site.

Although a spatial configuration has been assigned to 2,4-DCPIB in fig. 17D, it is obvious that this planar representation does not properly present the three dimensional state of affairs. It might properly be asked whether the presence of the two α-methyl groups does actually prevent proper approximation of carboxyl and ortho groups. The Hirschfelder model of 2,4-DCPIB reveals that this molecule is capable of assuming a configuration in which the spatial arrangement of the carboxyl group relative to the free ortho position is not greatly different from that in 2,4-D. The inactivity of 2,4-DCPIB cannot therefore be attributed merely to prevention by the bulky methyl groups of proper approximation of carboxyl and reactive ortho groups.

That the phenoxyisobutyric acids are completely without auxin activity in the Avena coleoptile section test suggests then that

still another factor is involved in the inability of these molecules to consummate two-point attachment. Such a factor might well be the presence of an additional barrier or protuberance in the receptor site between positions #1 and #2. That such a hindrance or barrier does in fact exist is indicated by the ΔF values of table 8. If there were no barrier between positions #1 and #2 it would be expected that the ΔF for the two-point attachment of 2,4-D would approximately equal the sum of the ΔF's for attachment at the two separate points. This is not so since the ΔF for 2,4-D is considerably less than the sum of the ΔF's for the two types of antiauxins. Thus once a single point attachment has been made the second attachment is more difficult than is the case if the molecule is starting from scratch. It may be suggested, therefore, that the inactivity of the phenoxyisobutyric acids as auxins and their activity as antiauxins is to be accounted for by the presence of a barrier between positions #1 and #2 of the auxin-receptive entity. The bulky methyl groups on the α-carbon atom of the side chain prevent the side chain carboxyl group and the free ortho position of the phenoxyisobutyric acids from assuming the intimate spatial orientation essential to two-point attachment although single point attachment is nonetheless possible. A similar explanation suggests itself for the lack of auxin activity of one stereoisomeric form of α-aryloxy propionic acids and the auxin activity of the enantiomorphs.

Smith et al. (31) have suggested that the α-hydrogen atom of the side chain in active aryloxy-acids is somehow essential to auxin

activity. While the evidence available relative to the activity or the inactivity of stereoisomeric forms of α-aryloxy propionic acids and the phenoxyisobutyric acids might at first seem to lend support to this hypothesis, the available kinetic evidence does not favor a three-point attachment concept. It will be shown in a later section of this investigation that the kinetics of the auxin growth reaction at high auxin concentrations is compatible with a requirement for a two-point attachment of the auxin molecule to the receptive entity within the plant. Furthermore, the two-point attachment concept is equally applicable to the auxin active substituted benzoic acids which do not possess an α-hydrogen atom, to the aryloxy-acids and to other types of active auxin molecules.

The evidence presented above indicates then that an antiauxin results when an active auxin molecule capable of consummating two-point attachment, is so modified that it is only able to undergo attachment at a single point to the auxin receptive entity within the plant. There are at least three such changes which can be made in an auxin of the phenoxyacetic acid series and these modifications may be summarized as follows:

(1) Elimination of the carboxyl group and retention of an unsubstituted ortho position (e.g. 2,4-DCA).

(2) Blocking of both ortho positions by substitution, with retention of the carboxyl group (e.g. 2,6-DCPA).

(3) Prevention of proper spatial relationships between carboxyl and reactive ortho groups, for example, by

bulky methyl substituents on the α-carbon (e.g. 4-CPIB). An unsubstituted ortho position is not essential.

As a critical test of the foregoing concept the compound 2,4,6-TCA was examined for antiauxin activity. 2,4,6-TCA, a compound related to 2,4-DCA, lacks a carboxyl group and has both ortho positions blocked. It should not, therefore, be active as an antiauxin. Table 9 shows the effect on Avena section growth of 2,4,6-TCA alone and in the presence of IAA. It is evident that 2,4,6-TCA, at concentrations up to 5.0 mg./l., is inactive as an auxin and does not inhibit IAA induced section growth. Entirely similar results have been obtained with 2,4,6-TCA when 2,4-D is used as the auxin. This compound in which both reactive groups are absent is, therefore, neither an auxin nor an antiauxin.

As mentioned earlier, Smith et al. (31) have suggested that a compound of the aryloxy-acid series to be active must have a hydrogen atom on the α-carbon of the side chain. In an attempt to examine more critically the two-point attachment concept as contrasted with what is essentially a three-point attachment concept of Smith et al., the compound 2,4,6-TCPP was tested for antiauxin activity. 2,4,6-TCPP, a compound in which the hydroxyl of the carboxyl group of 2,4,6-TCPA is replaced by a methyl group, which has both ortho positions blocked, and in which the carboxyl group is eliminated but the α-hydrogen atoms of the side chain are present. If the α-hydrogen atoms of aryloxy-acids are involved

TABLE 9

EFFECT OF 2,4,6-TCA ON IAA INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 2,4,6-TCA	Concentration of IAA mg./l. 0.00	0.02	0.05	0.1	0.5	1.0
	Growth in absence of IAA	Growth in presence of IAA minus growth in absence of IAA				
	mm./section/12 hrs.	mm./section/12 hrs.				
0.0	0.42	0.61	1.18	1.57	2.22	2.54
0.1	0.42	0.64	1.16	1.59	2.17	2.47
0.5	0.43	0.63	1.14	1.54	2.18	2.46
1.0	0.41	0.61	1.19	1.54	2.20	2.50
5.0	0.40	0.59	1.17	1.56	2.16	2.52

in a point of attachment to the site within the plant, then 2,4,6-TCPP should be an antiauxin. There is, however, the possibility that the carbonyl group of the side chain may interact with the auxin active site and thereby mask competitive inhibitive properties of the compound. Complete inactivity of the compound would therefore be required in order to critically rule out participation of the side chain α-carbon hydrogen atoms in a three-point attachment to the active site within the plant. Alternatively, if the compound possesses the characteristics of a competitive inhibitor, the inhibition may not be assigned unequivocally to the hydrogens of the side chain α-carbon.

The data of table 9 (Appendix I) show that 2,4,6-TCPP, at concentrations of 10.0 and 50.0 mg./l., inhibits growth of Avena sections both in the absence and presence of IAA, whereas 2,4,6-TCPP concentrations of 0.1 and 1.0 mg./l. do not significantly inhibit growth. It may also be noted that there is much less tendency for increasing IAA concentrations to alleviate 2,4,6-TCPP inhibition than is true in the auxin-antiauxin systems. This fact becomes more apparent when the data are presented, as in fig. 18, in the usual double reciprocal plots. That the compound is not a competitive inhibitor is evident from the distinctly different ordinate intercepts. Furthermore, with increasing concentrations of 2,4,6-TCPP, the slope and the intercept increase by the factor $1 + \frac{[I]}{K_I}$ and the ratio, slope/intercept, is constant. These conditions fulfill the requirements for non-competitive inhibition (56). 2,4,6-TCPP appears therefore to be a non-competitive inhibitor.

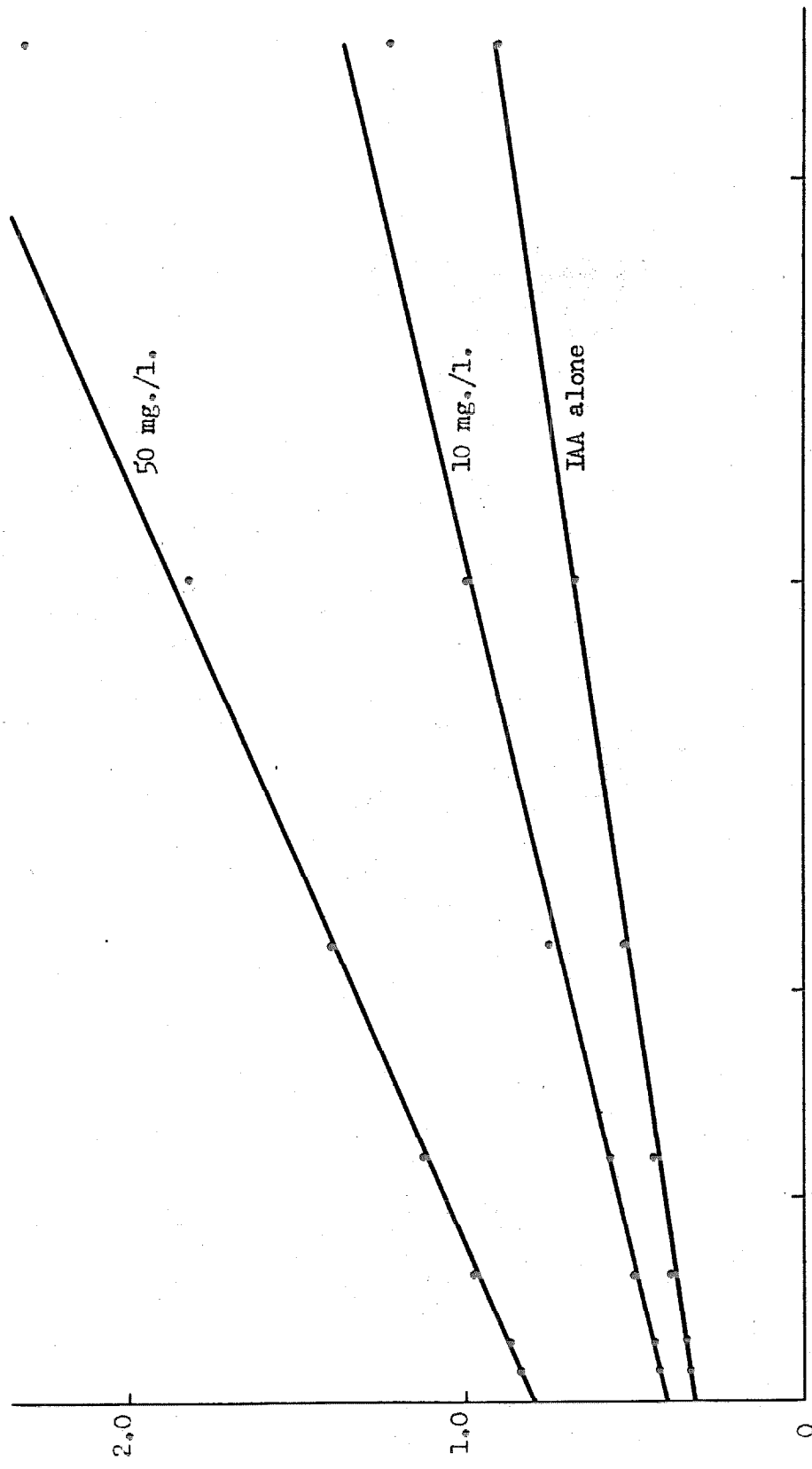


Figure 18. INHIBITION OF IAA INDUCED GROWTH BY 2,4,6-TCPP. DATA PLOTTED ACCORDING TO NON-COMPETITIVE INHIBITION TREATMENT. CONCENTRATIONS SHOWN ARE THOSE FOR 2,4,6-TCPP.

That the compound also inhibits 2,4-D induced section growth is apparent from the data of table 10 (Appendix I) and the double reciprocal plot of fig. 19. Again, the slopes and the intercepts of the lines are such that the criteria for non-competitive inhibition are satisfied. It is not immediately apparent why, at low 2,4-D concentrations in the presence of 2,4,6-TCPP, the inhibition is less than that expected on the basis of inhibition obtained in the presence of higher 2,4-D concentrations. The slope and the intercept have been determined, however, from the data on the linear portion of the curves.

The question arises as to whether or not the data in these experiments are sufficiently precise to eliminate the presence of weak competitive type inhibition in the system. The data of table 10 summarize the important features of the non-competitive action of 2,4,6-TCPP in the presence of IAA and in the presence of 2,4-D. It is apparent that the K_I of the inhibitor is independent of the inhibitor concentration and independent of the auxin. Furthermore, it is clear that the factor $1 + \frac{[I]}{K_I}$ increases by approximately the same amount and that the ratios slope/intercept are essentially constant. The precision of the data therefore supports the contention that competitive inhibition is not masked by non-competitive action of 2,4,6-TCPP.

Let us now assume that the above statement is not valid but rather let us make the assumption that competitive inhibition is masked by non-competitive action of 2,4,6-TCPP. An estimate of the minimum K_I which 2,4,6-TCPP can have as a competitive inhibitor may

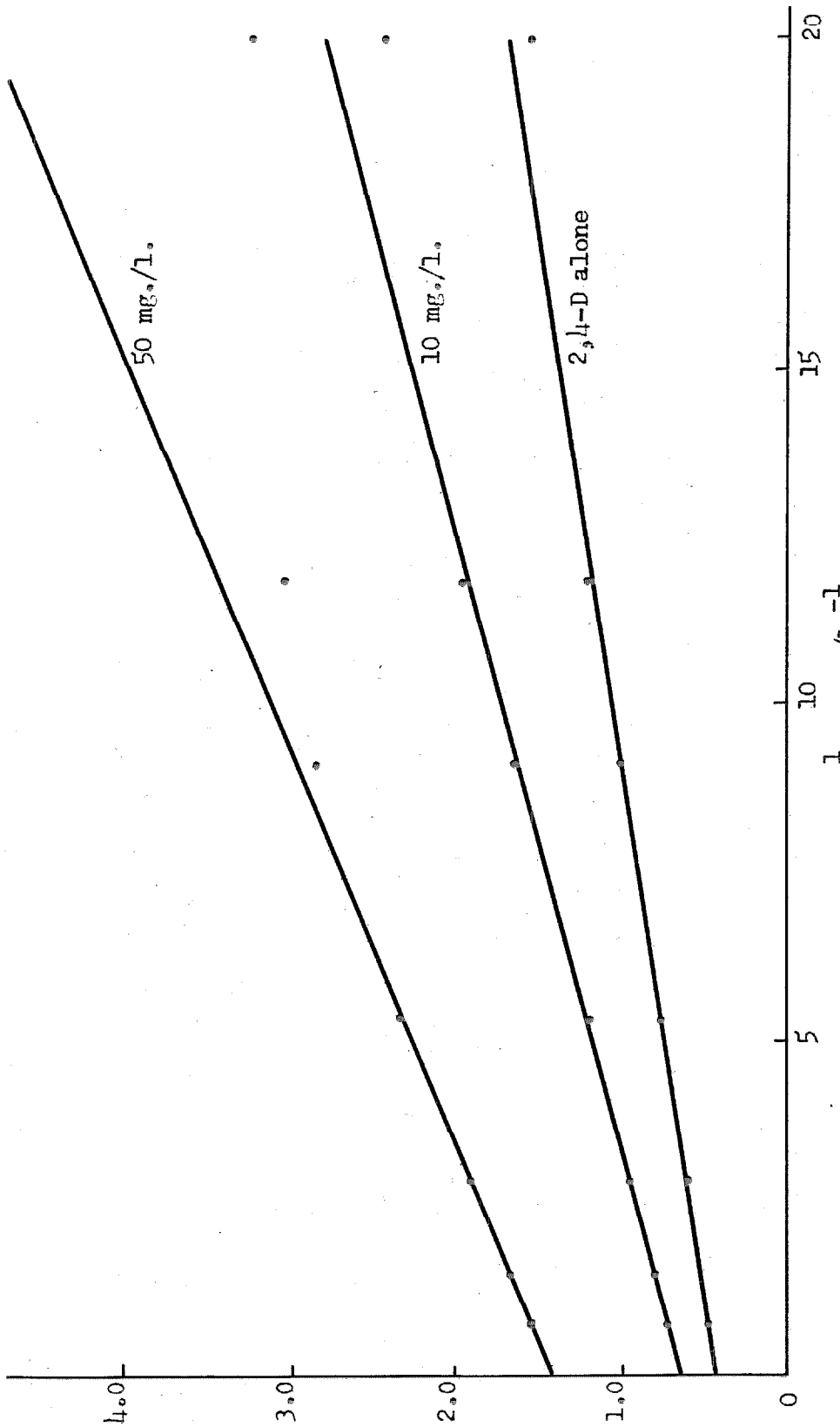


Figure 19. INHIBITION OF 2,4-D INDUCED GROWTH BY 2,4,6-TCPP, DATA PLOTTED ACCORDING TO NON-COMPETITIVE INHIBITION TREATMENT. CONCENTRATIONS SHOWN ARE THOSE FOR 2,4,6-TCPP.

TABLE 10

SUMMARY OF DATA PERTAINING TO 2,4,6-TCPP AND ITS ROLE AS A NON-COMPETITIVE INHIBITOR.

Substance	K_I	$1+\frac{[I]}{K_I}$	slope/intercept
IAA alone	-	0	-
in presence of 10.0 mg./l. 2,4,6-TCPP	1.1×10^{-4} M	1.37	0.076
in presence of 50.0 mg./l. 2,4,6-TCPP	1.2×10^{-4} M	2.67	0.077
2,4-D alone	-	0	-
in presence of 10.0 mg./l. 2,4,6-TCPP	1.2×10^{-4} M	1.33	0.119
in presence of 50.0 mg./l. 2,4,6-TCPP	1.1×10^{-4} M	2.81	0.109

be determined from the following expression:

$$\frac{\text{Slope without inhibitor}}{\text{Slope with inhibitor}} = 1 + \frac{[I]}{K_I} .$$

By substituting various K_I values and I values of 10 or 50 mg./l. into the above expression, a family of straight lines which have the same V_{max} as that for IAA in the presence of 10 or 50 mg./l. 2,4,6-TCPP and which have a slope as calculated from the above expression may be obtained. Suppose a K_I value of 1×10^{-3} M and an I value of 10 or 50 mg./l. are selected and substituted into the expression, straight lines may be obtained which lie above the lines for IAA in the presence of 10 or 50 mg./l. TCPP (see fig. 18). Now if the growth rate at any point along the theoretical line is determined, the rate is found to differ from the experimental IAA induced growth rate in the presence of inhibitor by approximately 10-15 per cent. This means that if the experimental variability is greater than about 10%, then 2,4,6-TCPP as a competitive inhibitor may have a K_I less than 1×10^{-3} M. If, however, the experimental variability is less than 10% then the K_I value may not be less than 1×10^{-3} M and may be greater than this value. It is evident from table 10 that the experimentally variability is not greater than 10%. 2,4,6-TCPP as a competitive inhibitor, therefore, may not have a K_I less than 1×10^{-3} M.

Let us now compare this value, 1×10^{-3} M, with the K_I for 2,4,-DCA (see table 7). It is evident that if 2,4,6-TCPP were a competitive inhibitor, its affinity for the auxin-receptor site may not be more than fifty times less than the affinity of 2,4-DCA for

the same site. Thus it may be concluded that if 2,4,6-TCPP has antiauxin activity by virtue of its ability to make a single point attachment through the α-carbon atom of the side chain to the auxin-receptor site, the affinity for the site is very low. It may therefore be further concluded that the binding through a third attachment point in aryloxy-acids, if such an attachment point does in fact exist, is of little significance as compared to the ortho position and the carboxyl group bindings of the auxin to the receptor site.

It is of interest to inquire in how far compounds which have earlier been described in the literature as antiauxins fit the requirements for single point attachment outlined above. A list of such compounds is presented in table 11. It is at present convenient to divide the list into the following five classes:

Class I: - Aryloxy compounds with free ortho position but lacking a carboxyl group.

Class II: - Compounds with carboxyl group but having ortho positions blocked.

Class III: - Compounds with steric hindrance in side chain.

Class IV: - Compounds with auxin activity but of low V_{max}.

Class V: - Substituted benzoic acids lacking auxin activity.

The first three classes of antiauxins include the types of compounds which have been discussed in this investigation. Compounds of low V_{max} (class IV) are not true antiauxins although the present kinetic treatment may be satisfactorily applied to their interaction in the plant with auxins of higher V_{max}, as will be shown in a later section. Since it has been shown by Muir and Hansch (22)

TABLE 11

A LITERATURE SUMMARY OF COMPOUNDS REPORTED TO POSSESS ANTIAUXIN ACTIVITY (AUXIN ANTAGONISTS)

Class	Substance	Nature of Antagonism	Author
I	2,4-Dichloroanisole	Competitively inhibits IAA and 2,4-D induced growth of Avena coleoptiles.	Bonner (72) This investigation
		Increases rate of cucumber root elongation.	Thompson et al. (6)
		Antagonizes phenoxyacetic acid induced inhibition of cress root growth.	Audus and Shipton (91)
		Antagonizes 2,4-D induced inhibition of flax root growth	o Aberg (92)
II	2,6-Dichlorophenoxyacetic acid	Competitively inhibits IAA and 2,4-D induced growth of Avena coleoptiles.	This investigation
		Increases rate of isolated flax root elongation.	See Appendix II
	2,4,6-Trichlorophenoxyacetic acid	Competitively inhibits IAA and 2,4-D induced growth of Avena coleoptiles.	This investigation
		Increases rate of isolated flax root elongation.	See Appendix II

TABLE 11 (cont.)

Class	Substance	Nature of Antagonism	Author
III	4-Chlorophenoxyisobutyric acid	Increases rate of wheat root elongation. Antagonizes IAA induced inhibition of wheat root growth.	Burström (83, 84)
		Competitively inhibits IAA and 2,4-D induced growth of Avena coleoptiles.	This investigation
	2,4-Dichlorophenylsulfoxideacetic acid	Increases rate of wheat root elongation.	Wilkse and Burström (86)
	2,4-Dichlorophenylsulfoneacetic acid	Increases rate of wheat root elongation.	Wilkse and Burström (86)
	3-Indoleisobutyric acid	Increases rate of wheat root elongation.	Burström (84)
	α-(1-Naphthylmethylsulfide)-propionic acid	Increases rate of flax root elongation and antagonizes IAA, NAA and 2,4-D induced inhibition of flax root growth.	Åberg (88, 89)
		Antagonizes (+)-α-(2-naphthoxy)-propionic acid induced inhibition of flax root growth.	Åberg, (88)
		Antagonizes phenylacetic, γ-phenylbutyric, cyclohexaneacetic and (+)-α-phenoxypropionic acids induced inhibition of flax root growth	Åberg (90)

TABLE 11 (cont.)

Class	Substance	Nature of Antagonism	Author
III	Phenoxyisobutyric acid	Increases rate of wheat root elongation.	Burström (85)
		Antagonizes α-phenoxypropionic acid induced inhibition of wheat root growth.	Burström (85)
		Antagonizes 2,4-D induced inhibition of flax root growth.	Åberg (90)
	2-Chlorophenoxyisobutyric acid	Increases rate of wheat root elongation.	Burström (85)
	3-Chlorophenoxyisobutyric acid	Increases rate of wheat root elongation.	Burström (85)
	2,4-Dichlorophenoxyisobutyric acid	Increases rate of wheat root elongation.	Burström (85)
		Competitively inhibits IAA and 2,4-D induced growth of Avena coleoptiles.	This investigation
	2,4,5-Trichlorophenoxyisobutryic acid	Increases rate of wheat root elongation.	Burström(85)
	2,4,6-Trichlorophenoxyisobutyric acid	Increases rate of wheat root elongation.	Burström (85)
	2,3,4,5,6-Pentachlorophenoxyisobutyric	Increases rate of wheat root elongation.	Burström (85)

TABLE 11 (cont.)

Class	Substance	Nature of Antagonism	Author
III	(-)-α-(2-Naphthoxy)-propionic acid	Increases rate of flax root elongation and antagonizes 2,4-D and (+)-α-(2-naphthoxy)-propionic acid induced inhibition of flax root growth.	Åberg (88)
	(+)-α-(1Napththoxy)-propionic acid	Increases rate of flax root elongation.	Åberg (88)
	Trans-cinnamic acid	Antagonizes IAA, 2,4-D, NAA and cis-cinnamic acid induced growth of pea sections.	van Oberbeek et al. (73)
	α-(2-Naphthylmethyl-sulfide)-propionic acid	Increase rate of flax root elongation and antagonizes 2,4-D induced inhibition of flax root growth.	Åberg (89)
	1-(Naphthylmethyl-sulfide)-propionic acid	Increases rate of flax root elongation and antagonizes 2,4-D induced inhibition of flax root growth.	Åberg (89)
	2-(Naphthylmethl-sulfide)-propionic acid	Increases rate of flax root elongation and antagonizes 2,4-D induced inhibition of flax root growth.	Åberg (89)

TABLE 11 (cont.)

Class	Substance	Nature of Antagonism	Author
III	α-(1-Naphthylmethyl-sulfide)-isobutyric acid	Increases rate of flax root elongation and antagonizes IAA, NAA and 2,4-D induced inhibition of flax root growth.	Åberg (89.95)
	α-(2-Naphthylmethyl-sulfide)-isobutyric acid	Increases rate of flax root elongation and antagonizes 2,4-D induced inhibition of flax root growth.	Åberg (89)
	1-Naphthylmethyl-selenideacetic acid	Increases rate of flax root elongation and antagonizes 2,4-D induced inhibition of flax root growth.	Åberg (89)
	2-Naphthylmethyl-selenideacetic acid	Increases rate of flax root elongation and antagonizes 2,4-D induced inhibition of flax root growth.	Åberg (89)
	δ-(Naphthylmethyl-selenide)-n-valeric acid	Increases rate of flax root elongation and antagonizes 2,4-D induced inhibition of flax root growth.	Åberg (89)

TABLE 11 (cont.)

Class	Substance	Nature of Antagonism	Author
III	(-)-α-(2-Naphthoxy)-propionic acid	Antagonizes (+)-isomer induced growth of Avena coleoptiles.	Smith et al. (31)
	(-)-α-(2,4-Dichlorophenoxy)-propionic acid	Antagonizes (+)-isomer induced growth of Avena coleoptiles.	Smith et al. (31)
	(-)-α-(2,4,5-Trichlorophenoxy)-propionic acid	Antagonizes (+)-isomer induced growth of Avena coleoptiles.	Smith et al. (31)
IV	γ-Phenylbutyric acid	Antagonizes IAA induced Avena curvatures.	Skoog et al. (77)
	2,3,5-Triiodobenzoic acid	Antagonizes IAA induced Avena curvatures.	Galston (78) de Waard and Florschütz (79) Thimann and Bonner (80)
		Antagonizes IAA, NAA, β-3-indolepropionic acid and γ-3-indolebutyric acids inhibition of abscission of first internode of Black Valentine bean seedlings.	Weintraub et al. (81)

TABLE 11 (cont.)

Class			
IV	Phenoxyacetic acid	Antagonizes 2,4-D induced inhibition of flax root growth.	Åberg (9)
		Antagonizes 2,4-D induced inhibition of cress root growth.	Audus and Shipton (91)
V	3-Nitro-4-fluorobenzoic acid	Increases rate of cucumber root elongation and antagonizes 2,4-D induced inhibition of cucumber root growth.	Minarik et al. (87)

that the rules which govern activity in the substituted benzoic acids are superficially different from those which govern activity in the phenylacetic and phenoxyacetic acid series, it is necessary for the present to consider benzoic acids separately from classes I and II. Class V, therefore, consists of auxin inactive substituted benzoic acids which possess antiauxin properties. It is evident at once from table 11 that the great bulk of substances to which antiauxin activity has been attributed fall into catagories I, II and particularly III of this paper. The present formulation of the basis of antiauxin activity is therefore broadly applicable.

There have been conflicting reports concerning the auxin or antiauxin activity of certain of the compounds listed in table 11. Thus Audus and Shipton (91) in a study of root growth failed to find any general interaction of 2,4-DCA with auxins, and have concluded that 2,4-DCA may not be an antiauxin. The methods used by Audus and Shipton are, however, quite unsuitable to the quantitative study of this matter since the concentrations of 2,4-DCA which they used were in general high enough to be themselves inhibitory to root growth. In the few experiments in which Audus and Shipton used non-inhibitory concentrations of 2,4-DCA they actually showed that the substance alleviates auxin-induced root growth inhibition. It is evident from the investigations of Åberg (88, 89, 90, 92), who has demonstrated clear cut reversal of auxin-induced root growth inhibition by antiauxins, that this result is obtained only with concentrations of antagonist which are not of themselves inhibitory or only slightly so. If the two-point attachment concept for auxin activity applies to root

growth, it is to be expected that inhibitions brought about by inhibitory concentrations of an antiauxin will be additive to auxin-induced inhibitions. It is true that 2,4-DCA is a less effective antiauxin than either the substituted isobutyric acids or the diortho substituted phenoxyacetic acids. The present experiments do however unequivocally establish this compound as a true competitive auxin inhibitor in the Avena section.

It has been shown that 2,6-DCPA is without growth promoting activity for the Avena coleoptile and the same is true of 2,6-DCPA in the pea test (93). 2,6-DCPA has also been found to be without auxin activity by Synerholm and Zimmerman (94), Leaper and Bishop (29), and by Hansch and Muir (21). The pea test activity attributed to the compound by Thimann (25) is no doubt owing to active impurities in the samples used, particularly since there is a marked negative correlation between activity and melting point of his several preparations.

It is of interest to note that Osborne and Wain (30) have found that certain aryloxyisobutyric acids (several of those listed in table 11 as antiauxins) induce positive curvatures in the pea test but are inactive in other tests involving cell elongation. Although it is evident that there are differences in chemical specificity between the pea curvature test and the Avena coleoptile section test, no satisfactory explanation has been presented to account for the difference.

The lone substituted benzoic acid listed in class V is representative of a large number of benzoic acids which appear to belong

to this category. Minarik et al. (87) have shown that 3-nitro-4-fluorobenzoic acid is the most active of thirty-five benzoic acid derivatives capable of increasing rate of elongation of cucumber roots although it has not been established that the action is a competitive one. It has not as yet been shown whether the substituted benzoic acids other than 3-nitro-4-fluorobenzoic acid are capable of antagonizing auxin-induced root inhibition or auxin promoted growth as in Avena sections. Neither do we know at the present time whether these compounds function as antiauxins by single point attachment at the ortho interaction point, at the carboxyl interaction point of the receptive site within the plant or otherwise. As this information becomes available it will doubtless be possible to assign the substituted benzoic acids to their appropriate places in class I or II.

There are at least two further general groups of compounds which have the ability to inhibit auxin-induced growth responses. These groups, certain phenols and certain unsaturated lactones respectively, have not been included in table 11 because it is quite clear for the unsaturated lactones, at least, that their action is not competitive (15, 80). With respect to the phenols, however, the matter is not quite so clear. Auxin-induced root inhibitions have been alleviated slightly by 1-naphthol (95) and by 2,4-dichlorophenol (91). Since the information available concerning these compounds does not demonstrate that they interact competitively with auxins, the two phenols have not been included in table 11.

The compound maleic hydrazide, which undoubtedly exerts its apparent antiauxin action in the enolic form, has likewise not been placed in table 11. Kinetic treatment of the results obtained by Leopold and Klein (76) indicates that maleic hydrazide interacts with auxins in a manner which is qualitatively but not quantitatively that expected of an antiauxin. Thus, the data of Leopold and Klein show that maleic hydrazide inhibition is disproportionately greater than expected at low auxin concentrations. It has been found during the present work with Avena coleoptile sections that maleic hydrazide in concentrations of 1, 10 and 50 mg./l. is disproportionately inhibitory in the presence of low auxin concentrations. That this is so is evident for the interaction between maleic hydrazide and 2,4-D as shown by the double reciprocal plots of fig. 20 (see data in table 11 of Appendix I). Maleic hydrazide is not, therefore, a strictly competitive inhibitor as are the antiauxins considered in the present study.

The extensive work of the past two decades on the relation of chemical structure to auxin activity has shown that three features of the molecule are of critical importance to the biological activity of the substance. We may summarize the considerations of the present paper by a formulation of the principles which govern antiauxin activity. An antiauxin appears in general to be merely an auxin-like molecule in which one but not all of the features essential to activity is lacking.

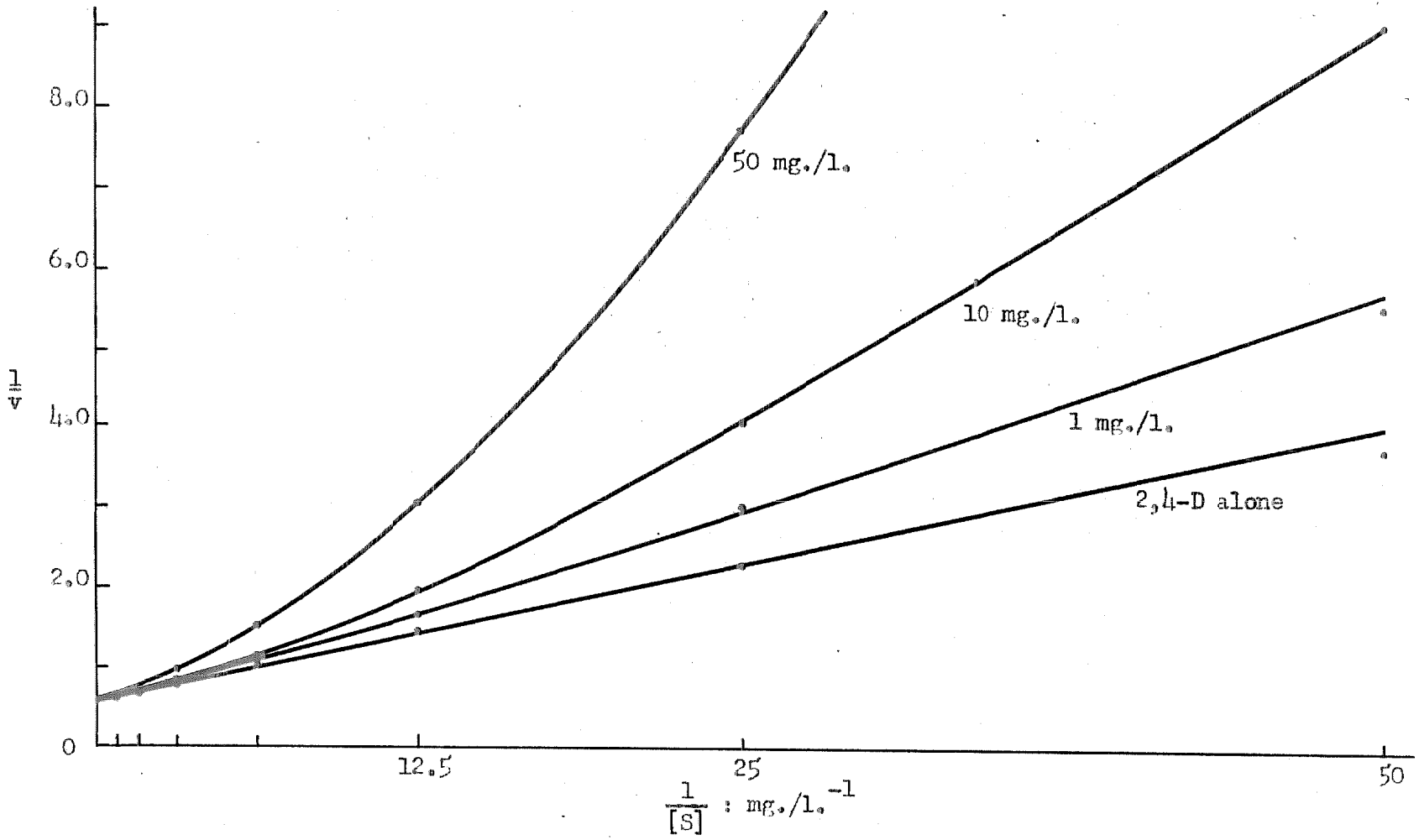


Figure 20. INHIBITION OF 2,4-D INDUCED GROWTH BY MALEIC HYDRAZIDE. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION. CONCENTRATIONS SHOWN ARE THOSE FOR MALEIC HYDRAZIDE.

Summary

1. The auxin-induced growth of the Avena coleoptile section may be formally treated by the methods of classical enzyme kinetics. The kinetic treatment, which is based on the supposition that auxin exerts its effect through and becomes attached to an auxin-receptive site within the plant, makes it possible to characterize the growth promoting activity of an auxin by two parameters, K_s and V_{max}. These express respectively the affinity of the auxin for the auxin-receptive site within the plant and the ability of the complex thus established to promote growth.

2. Treatment of Avena section growth data by the methods of enzyme kinetics makes it possible to determine rigorously whether or not inhibitors of auxin-induced growth are true antiauxins and act by competing with auxin for the auxin-receptive site within the plant.

3. Certain auxin-inactive derivatives of 2,4-dichlorophenoxyacetic acid (2,4-D) act as strict antiauxins in the Avena section. Among such substances are 2,4-dichloroanisole, 2,4-dichlorophenoxyisobutyric acid, 4-chlorophenoxyisobutyric acid, and the diortho substituted phenoxyacetic acids, 2,6-dichloro- and 2,4,6-trichlorophenoxyacetic acids.

4. The affinity of the auxin-receptive sites within the plant for indoleacetic acid is slightly greater than for 2,4-D. The affinities of these same sites for the substituted phenoxyisobutyric acids are approximately ten fold less and are approximately equal to the affinities for the auxin-inactive diortho substituted phenoxyacetic acids.

The affinity of the auxin-receptive sites for 2,4-dichloroanisole is still another ten fold less. 2,4-dichloroanisole is therefore a less effective antiauxin than either of the other two groups of materials.

5. Each of the substances here investigated and shown to be an antiauxin can be considered as derived from an active auxin by elimination of one of the structural features essential to auxin activity. This may apparently be accomplished in any of the following three ways:

a. elimination of the essential carboxyl group

b. elimination of the essential reactive ortho group

c. elimination of proper spatial relationships between groups a and b as by introduction of bulky groups in the side chain.

6. A survey of the literature has shown that most if not all presently known auxin antagonists which are themselves without auxin activity may be characterized as belonging to categories a, b, or c above.

7. Earlier investigations of various authors have indicated that a compound to be active as an auxin must be capable of consummating a two-point attachment to an auxin-receptive site within the plant. The results of the present investigation suggest that a compound to be active as an antiauxin must be capable of combining at one point but incapable of simultaneously combining at both points of this same receptive site.

KINETICS OF AUXIN INTERACTION

Introduction

It is proposed in this section to study experimentally the conditions under which one auxin may influence the action of a second chemically different auxin and to determine the principles according to which this interaction occurs. Again, the experimental work on which this discussion is based has all been done using elongation of excised oat coleoptile sections as the measure of physiological activity. Perhaps the first detailed study of this kind was that of Skoog et al. (77) who investigated the effect of PBA upon IAA induced growth of the Avena coleoptile. PBA is itself an auxin although its activity in the Avena curvature test is only 1×10^{-5} that of IAA. Although the growth promoting activity of PBA in the Avena coleoptile section test is so small as to have escaped Skoog et al. (77), such activity has been found by Muir et al. (20) and has been confirmed in the present study. PBA is not only less active than is IAA in the Avena section test but is in addition active only over a smaller concentration range.

The inhibition of the action of IAA by PBA reported by Skoog et al. may be shown by appropriate treatment to be a competitive one (15). It appears then that an active auxin may be competitively inhibited in its effect within the plant by other auxins of lesser activity. Observations generally similar to those made concerning the action of PBA have since been made by Thimann and Bonner (80)

on TIBA. Phenoxyacetic acid which possesses but little activity as an auxin has similarly been shown to antagonize the action of more active auxins. Thus Åberg (90) found this compound effective in counteracting 2,4-D induced inhibition of the growth of flax roots while Audus and Shipton (91) have shown that it relieves 2,4-D induced inhibition of cress root growth.

It would seem reasonable to suppose in a qualitative way that the action of a more active auxin might be inhibited by a less active auxin through some sort of competition for the site within the plant to which auxins become attached in order to exert their effect. This supposition would imply not only that auxins become attached in the plant but also that diverse auxins exert their effects through attachment at a common site. That these suppositions are correct has been strongly indicated by the fact that any given antiauxin inhibits the effects of many, perhaps of all, different auxins (73, 95).

As was shown earlier, the action of auxins in promoting the growth of the Avena coleoptile sections can be formally treated by the methods of classical enzyme kinetics. It was further shown that the activity of an auxin can be characterized by two parameters which correspond to the K_s and V_{max}, respectively, of Michaelis-Menten (71) kinetics. Thus, the parameter K_s, is a measure of the affinity of a given auxin for the auxin-receptive site or point of attachment within the plant. The ability of the complex, once formed to promote growth is measured by the parameter V_{max}. The way in which two auxins interact within the plant to determine growth rate

will be shown in the present section to be dependent upon the value of the constants, K_S and V_{max}, for each of the interacting materials.

Results

EQUILIBRIUM CONDITIONS OF AUXIN-INDUCED AVENA SECTION GROWTH.

Treatment of auxin-induced Avena coleoptile section growth by the classical methods of enzyme kinetics presupposes that equilibrium conditions prevail between free auxin and auxin bound to the receptive site within the coleoptile. The validity of this assumption may be readily demonstrated by the results of experiments in which Avena sections are first grown for a short period in a solution containing IAA as the growth substance and then transferred to a solution containing 2,4-D or vice versa. Transfer from one solution to the other occurred in each case after a six hour interval and measurements were made at three hour intervals for a total of 12 hours. The data of table 12 and fig. 21 show that sections growing at a rate determined by the concentration of IAA in the medium changed this growth rate to that appropriate to the concentration of 2,4-D when transferred to a fresh medium containing this substance as the active component. Transfer in the reverse direction (2,4-D to IAA) yielded similar results. Thus, after transfer of Avena sections from a medium containing IAA to one containing 2,4-D, the receptor sites become equilibrated with the new growth substance. The time needed for consummation of the new equilibrium condition is evidently short as compared to three hours since sections

TABLE 12

EFFECT ON GROWTH OF AVENA COLEOPTILES OF TRANSFERRING SECTIONS FROM IAA* TO 2,4-D* AND VICE VERSA.

Medium	Time of Measurement (hours)			
	3	6	9	12
	Growth in mm./section/3 hours			
IAA for 12 hrs.	0.68	0.65	0.69	0.65
2,4-D for 12 hrs.	0.45	0.43	0.44	0.44
IAA for 6 hrs. then	0.68	0.65	-	-
2,4-D for 6 hrs.	-	-	0.47	0.43
2,4-D for 6 hrs. then	0.45	0.43	-	-
IAA for 6 hrs.	-	-	0.68	0.65

* Concentration of IAA or of 2,4-D used was 2.89×10^{-6} M.

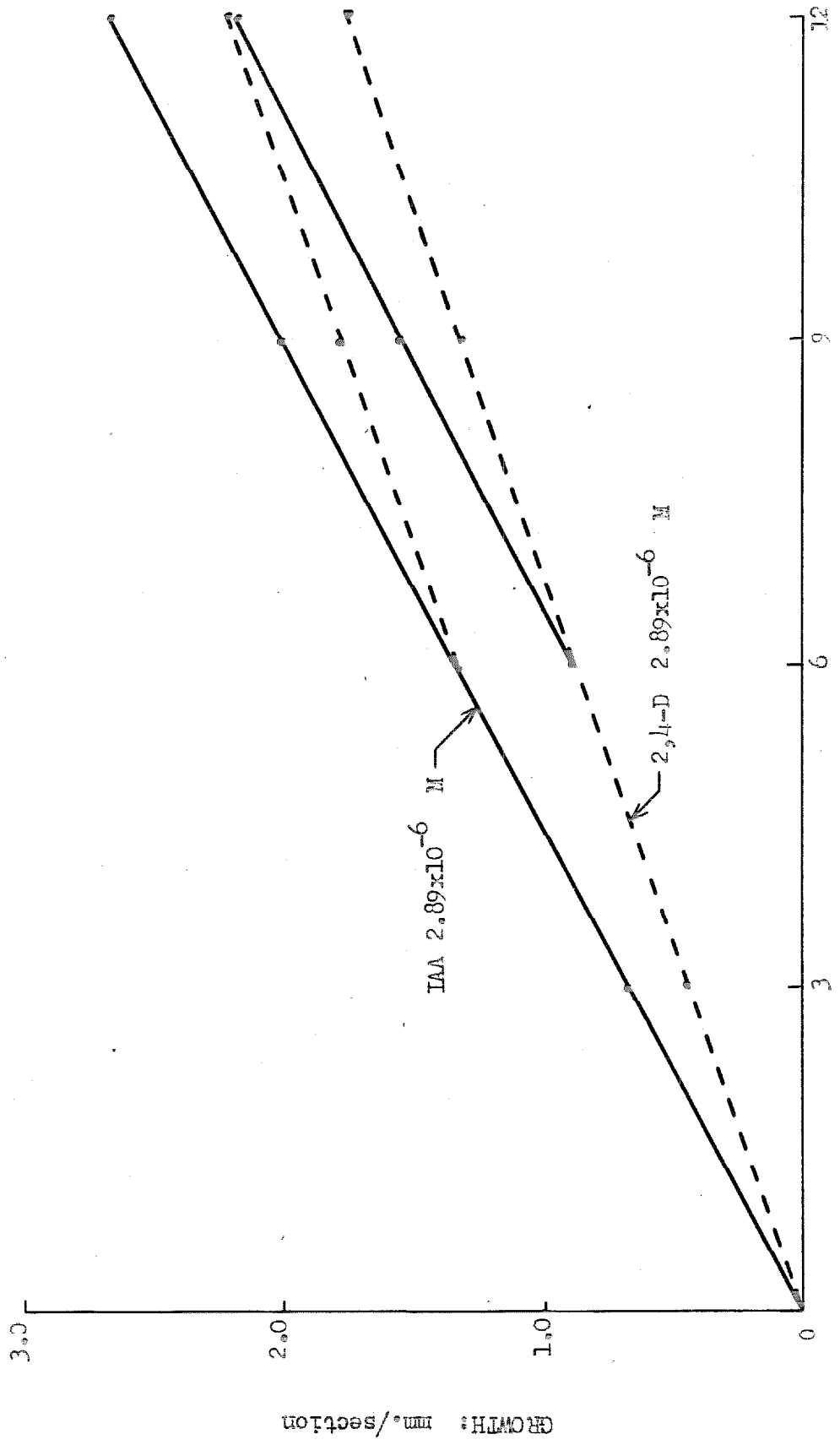


Figure 21. AVENA COLEOPTILE SECTION GROWTH IN THE PRESENCE OF IAA (SOLID LINE) AND OF 2,4-D (DASHED LINE), AFTER 6 HOURS INCUBATION, SECTIONS IN IAA WERE TRANSFERRED TO 2,4-D AND VICE VERSA FOR A FURTHER 6 HOUR PERIOD.

put in, for example, 2,4-D after having been in IAA grow at the same three hour rate as sections which are continuously in 2,4-D.

AVENA SECTION GROWTH IN PRESENCE OF AUXIN MIXTURES. Let us now consider what may be expected to happen if two chemically different auxins were to be simultaneously supplied to such sections. Suppose, for example, that auxin 1 (concentration S_1) and auxin 2 (concentration S_2), both act at the same site within the plant and that the two free auxins are in equilibrium with the bound forms ES_1, and ES_2, respectively. This situation is equivalent to that described by Foster and Niemann (96) for the competitive interaction of two substrates at a common enzymatic site. For this case the overall reaction velocity (growth rate) may be shown (96) to be given by the expression in equation 7.

$$(7) \qquad v = \frac{V_{max_1} \frac{[S_1]}{K_{s_1}}}{1 + \frac{[S_1]}{K_{s_1}} + \frac{[S_2]}{K_{s_2}}} + \frac{V_{max_2} \frac{[S_2]}{K_{s_2}}}{1 + \frac{[S_2]}{K_{s_2}} + \frac{[S_1]}{K_{s_1}}}$$

The quantities V_{max_1}, and V_{max_2} of equation 7 represent respectively the individual quantities, V_{max}, for the two substrates S_1 and S_2 while K_{s_1} and K_{s_2} represent the respective affinity constants. The first term of equation 7 gives the growth elicited by auxin 1 while the second term gives the growth due to auxin 2. Thus for a single auxin ($[S_2]=0$) equation 7 assumes the form of original equation 2. The use of equation 7 is facilitated by its transformation to the reciprocal form. Systems which follow equation 7, must, in reciprocal

form, and for any given constant ratio of S_1/S_2 be expected to yield a linear relationship between $1/v$ and $1/[S_1] + [S_2]$. From this linear relationship it is then possible to compute the apparent parameters $V_{max(combined)}$ and $K_{s(combined)}$ for the complex system as a whole. The apparent constants for the two-auxin system are related to the constants derived earlier for the individual systems by equations 8 and 9 below:

$$(8)\quad V_{max(combined)} = \frac{V_{max_1}\frac{[S_1]}{K_{s_1}} + V_{max_2}\frac{[S_2]}{K_{s_2}}}{\frac{[S_1]}{K_{s_1}} + \frac{[S_2]}{K_{s_2}}}$$

$$(9)\quad K_{s(combined)} = \frac{[S_1] + [S_2]}{\frac{[S_1]}{K_{s_1}} + \frac{[S_2]}{K_{s_2}}}$$

Thus, from a knowledge of the constants V_{max} and K_s which describe the effects of individual auxins, we should be able to predict the overall effects of combinations of auxins. Conversely by measuring the parameters $V_{max(combined)}$ and $K_{s(combined)}$ obtained for growth in the presence of an auxin mixture and by comparing these values with those obtained by calculation from the appropriate parameters of single auxin systems we can determine whether or not the present formulation adequately describes the growth response of Avena sections to auxin mixtures.

That mixtures of auxins do indeed influence growth of Avena sections according to principles here outlined is shown by the data

of fig. 22. These present the data from experiments on the interaction in Avena section growth of IAA and 2,4-D given alone and in a variety of concentrations and molar concentration ratios. The reciprocal of initial growth rate (1/v) is plotted as a function of the reciprocal of total auxin concentration ($1/[S_{total}]$) for each of these combinations. The data for each individual auxin yield a straight line with characteristic slope and intercept as expected from the similar experiments of fig. 6B. The data for the auxin mixtures yield in each case a family of straight lines lying between the lines for the individual auxins as predicted by equation 7. The slope and intercept of each line is determined by the molar concentration ratio between the two auxins. Table 13 gives a compilation of the values for the constants $K_{s(combined)}$ and $V_{max(combined)}$ as measured for the several pairs of auxins at various molar ratios and compares these values with those calculated from equations 8 and 9 using the previously determined values of K_s and V_{max} for the individual auxins. It is evident that there are no considerable differences between the measured and calculated values of the two combination parameters although there is a systematic deviation of the K_s values in favor of IAA of approximately 5 per cent.

Since the K_s and V_{max} values for NAA are approximately the same as those for 2,4-D, it is to be expected that results obtained for the interaction of IAA and NAA should be similar to those found for the interaction of IAA and 2,4-D. That this is so is evident from

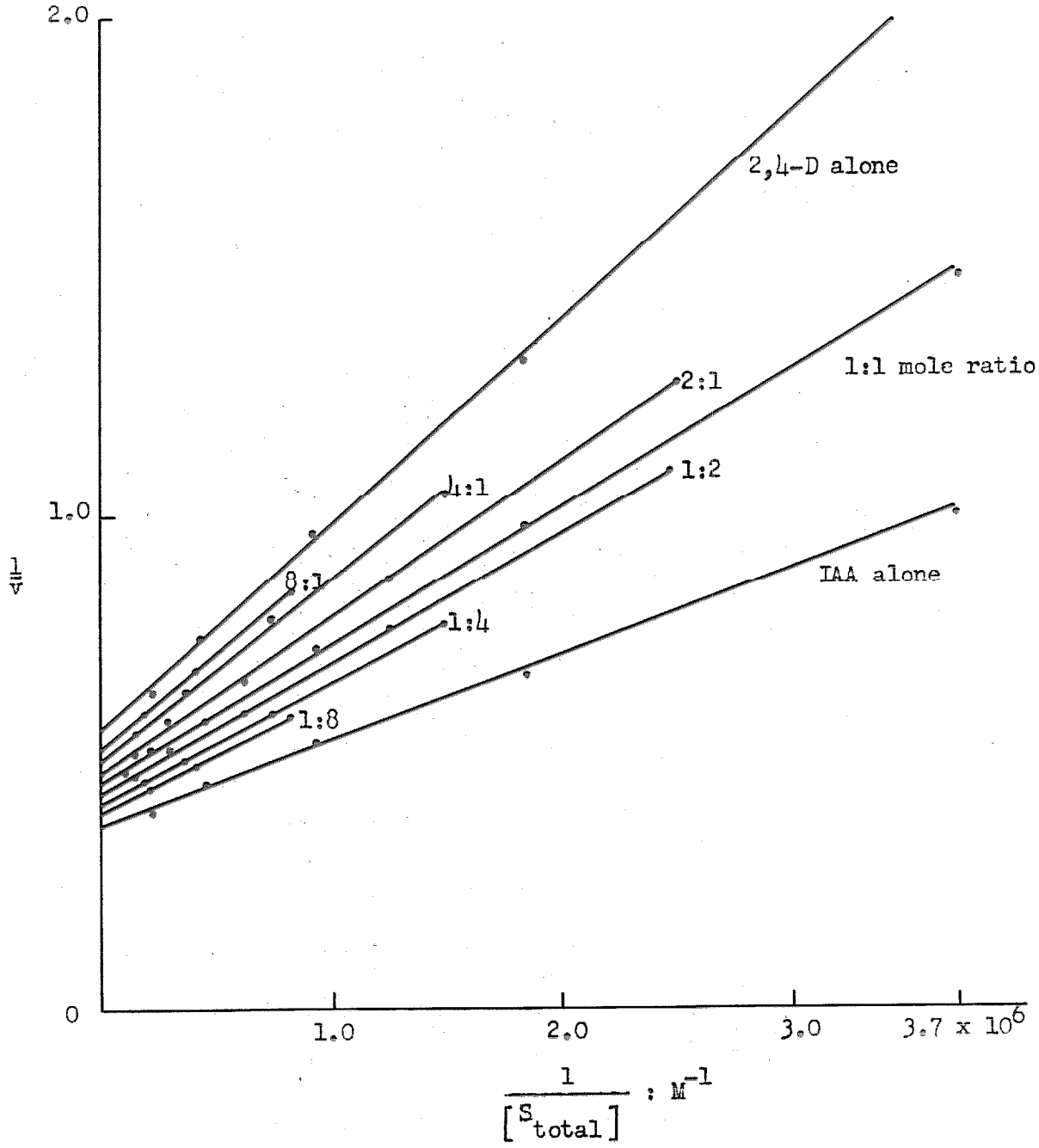


Figure 22. INTERACTION IN AVENA SECTION GROWTH OF IAA AND 2,4-D GIVEN INDIVIDUALLY OR IN A VARIETY OF CONCENTRATIONS AND MOLAR CONCENTRATION RATIOS. DATA PLOTTED ACCORDING TO THE METHOD OF FOSTER AND NIEMANN(96) AS A TEST FOR COMPETITION AT THE AUXIN-RECEPTIVE SITE.

TABLE 13

SUMMARY OF CONSTANTS K_s AND V_{max} FOR IAA AND 2,4-D ALONE AND IN COMBINATION AT VARYING MOLAR RATIOS.

Per cent composition in interaction mixture		K_s (molar)		V_{max} (mm./section/12 hrs.)	
		Observed	Calculated	Observed	Calculated
IAA	2,4-D				
100	0	4.6×10^{-7}	-	2.6	-
80	20	5.0×10^{-7}	5.2×10^{-7}	2.4	2.4
67	33	5.3×10^{-7}	5.5×10^{-7}	2.3	2.3
50	50	5.8×10^{-7}	5.9×10^{-7}	2.2	2.2
33	67	6.4×10^{-7}	6.7×10^{-7}	2.0	2.1
20	80	6.8×10^{-7}	7.0×10^{-7}	1.9	1.9
0	100	7.6×10^{-7}	-	1.7	-

fig. 23 where data obtained for the interaction of IAA and NAA are presented in the appropriate double reciprocal plots. (See table 12, Appendix I, for growth data.) Again, the data for the auxin mixtures yield a family of straight lines which lie between the lines for the individual auxins. Furthermore, it is apparent from table 14 that there is close agreement between the observed and calculated parameters. NAA, therefore, interacts with IAA in Avena section growth in a competitive manner as do IAA and 2,4-D.

Although the interactions between 2,4-D, NAA, and IAA are, as shown above, in general accord with the expectations of classical kinetics, there is nonetheless at least one aspect of this interaction which cannot be interpreted on the present basis. This is the inhibition of the action of low concentrations of IAA which is elicited by low concentrations of 2,4-D or NAA. That such inhibition occurs is evident from the curves of fig. 24 which have been obtained from the data of table 15. In this figure, growth rate of the Avena coleoptile section is plotted as a linear function of 2,4-D concentration. Each individual curve is that for a system of constant IAA concentration. The shaded area of each curve represents the concentration range over which 2,4-D inhibits growth at a constant IAA concentration. The sharp initial dip in each curve which represents inhibition by low 2,4-D concentrations is scarcely detectable in the double reciprocal plots of fig. 22 and 23 which there appear as straight lines. This inhibition is roughly competitive since it is most marked at low IAA concentrations.

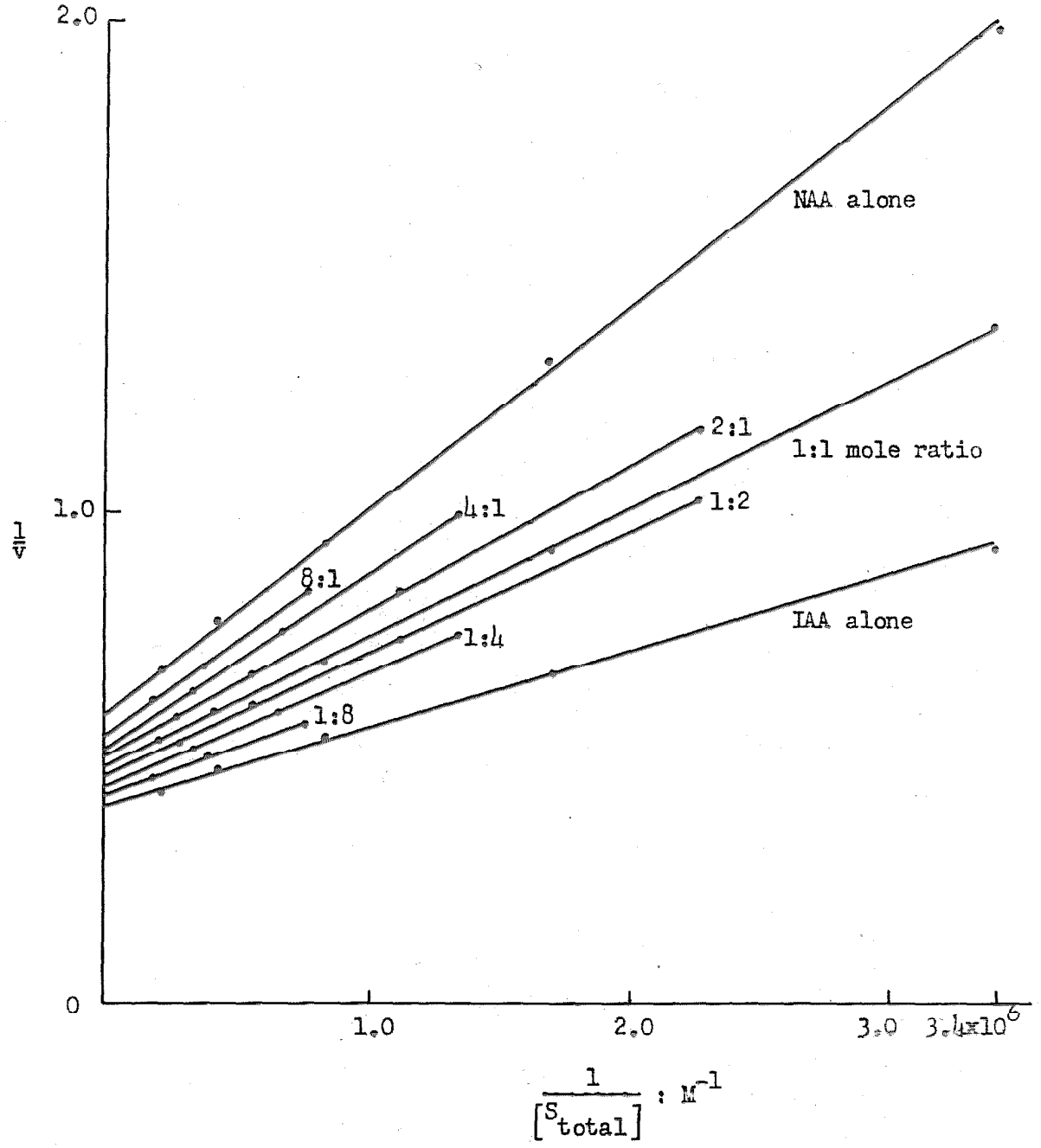


Figure 23. INTERACTION IN AVENA SECTION GROWTH OF IAA AND NAA GIVEN INDIVIDUALLY OR IN A VARIETY OF CONCENTRATIONS AND MOLAR CONCENTRATION RATIOS. DATA PLOTTED ACCORDING TO THE METHOD OF FOSTER AND NIEMANN (96) AS A TEST FOR COMPETITION AT THE AUXIN-RECEPTIVE SITE.

TABLE 14

SUMMARY OF CONSTANTS K_s AND V_{max} FOR IAA AND NAA ALONE AND IN COMBINATION AT VARYING MOLAR RATIOS.

Per cent composition in interaction mixture		K_s (molar)		V_{max} (mm./section/12 hrs.)	
		Observed	Calculated	Observed	Calculated
IAA	NAA				
100	0	4.4×10^{-7}	-	2.6	-
80	20	4.8×10^{-7}	4.8×10^{-7}	2.3	2.4
67	33	5.0×10^{-7}	5.1×10^{-7}	2.2	2.3
50	50	5.3×10^{-7}	5.5×10^{-7}	2.1	2.1
33	67	5.8×10^{-7}	5.9×10^{-7}	1.9	2.0
20	80	6.5×10^{-7}	6.4×10^{-7}	1.8	1.8
0	100	7.2×10^{-7}	-	1.6	-

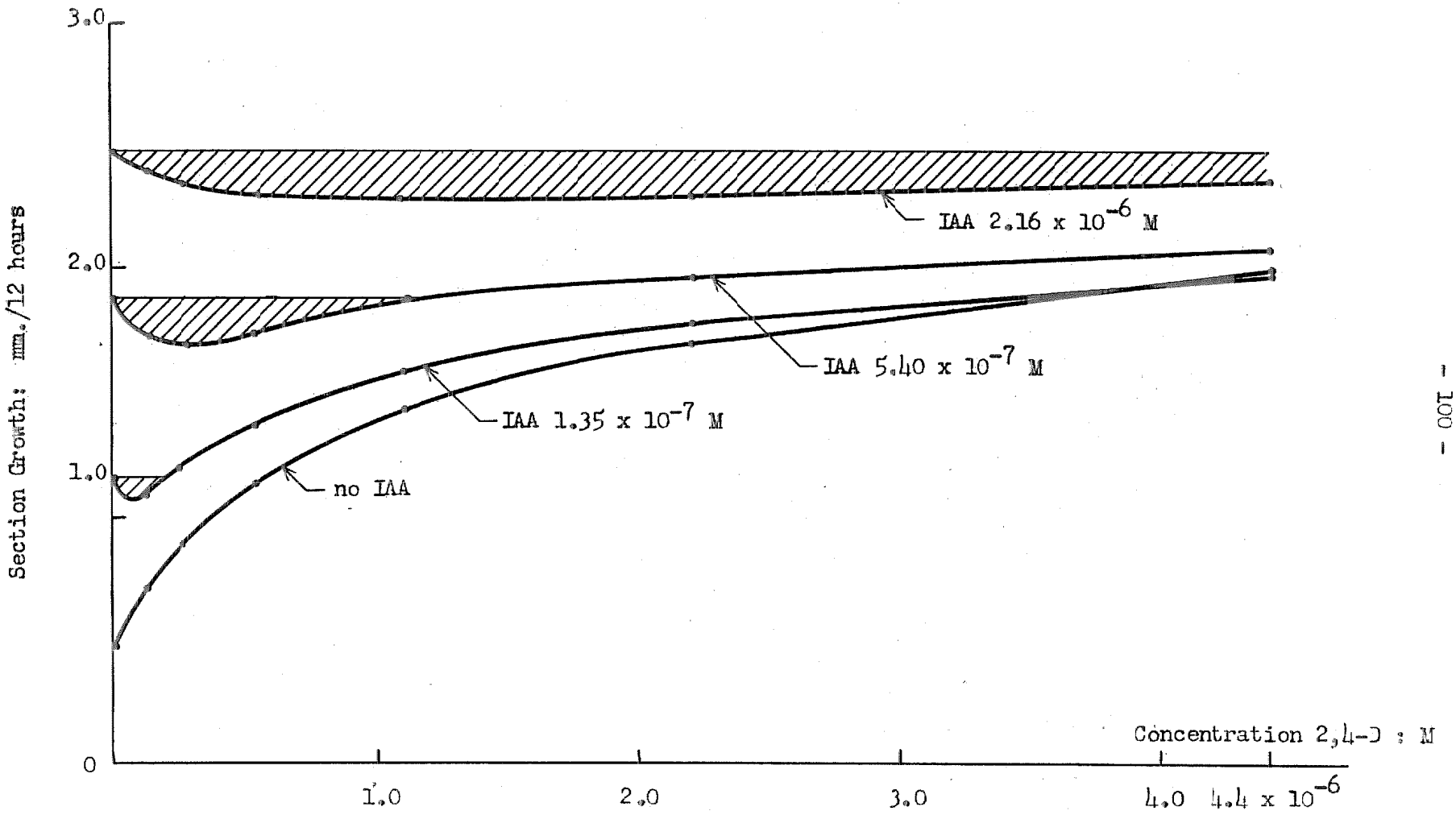


Figure 24. AVENA COLEOPTILE SECTION GROWTH AS A LINEAR FUNCTION OF 2,4-D CONCENTRATION IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF IAA. THE SHADED AREA OF EACH CURVE REPRESENTS THE CONCENTRATION RANGE OVER WHICH 2,4-D INHIBITS GROWTH BELOW THE LEVEL ATTAINED IN IAA ALONE.

TABLE 15

THE INTERACTION EFFECT OF IAA AND 2,4-D IN THE GROWTH OF AVENA COLEOPTILE SECTIONS.

Relative Concentration of 2,4-D*	Relative Concentration of IAA*						
	0	1	2	4	8	16	32
	Growth (mm./section/12 hrs.)						
0	0.42	1.12	1.41	1.87	2.19	2.49	2.81
1	0.72	1.09	1.33	1.70	2.06	2.36	2.84
2	0.90	1.20	1.42	1.70	2.09	2.34	2.70
4	1.17	1.37	1.56	1.75	2.06	2.33	2.66
8	1.44	1.67	1.89	2.09	2.26	2.26	2.36
16	1.70	1.79	1.85	1.96	2.11	2.27	2.52
32	1.97	1.96	2.01	2.05	2.17	2.33	2.46

* The relative unit of concentration, $1 = 1.348 \times 10^{-7}$ M.

Let us now consider what takes place when two auxins such as 2,4-D and NAA interact in growth promotion of Avena sections. Since the K_s and V_{max} values for these two auxins are closely similar, it is to be expected that section growth rate in the presence of 2,4-D and NAA mixtures would approximate the growth rate in either auxin alone. That this is so is clear from the data of fig. 25 where 1/v is again plotted as a function of $1/[S_{total}]$. (See table 13, Appendix I, for growth data.) It is apparent from fig. 25 that growth rates in the presence of 2,4-D and NAA at a 1:1 molar concentration ratio fall nicely between growth rates for 2,4-D alone and NAA alone. That mixtures of these two auxins behave in a manner consistent with the predicted interaction is apparent from table 16 which shows complete agreement between observed and calculated values for the constants K_s and V_{max}. Thus not only do 2,4-D and NAA compete with IAA according to the principles of auxin interaction but also 2,4-D and NAA compete with each other in accordance with the same principles.

It is of interest next to consider whether or not the preceding principles are applicable to interactions between an auxin which possesses a high V_{max} and one which possesses a V_{max} lower than those for either 2,4-D or NAA. An auxin which possesses such a low V_{max} is TIBA. The fact that this compound has auxin activity and has also been reported to possess antiauxin properties (78 - 81) makes it of particular interest. TIBA possesses the additional feature that it has been reported (25,80) to act synergistically toward

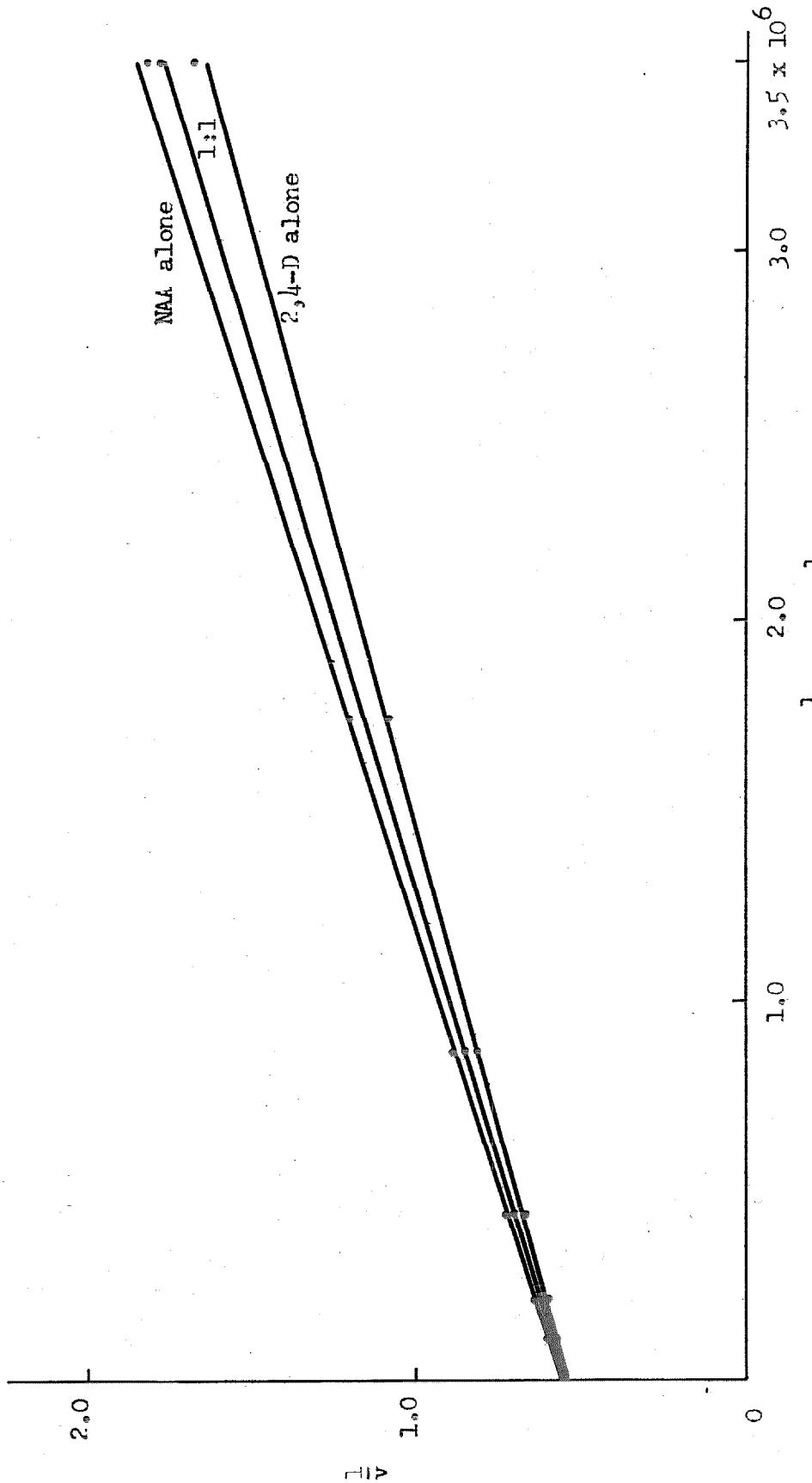


Figure 25. INTERACTION IN AVENA SECTION GROWTH OF 2,4-D AND NAA GIVEN INDIVIDUALLY OR IN A VARIETY OF CONCENTRATIONS AND 1:1 MOLAR CONCENTRATION RATIO. DATA PLOTTED ACCORDING TO THE METHOD OF FOSTER AND NIEMANN (96) AS A TEST FOR COMPETITION AT THE AUXIN-RECEPTOR SITE.

TABLE 16

SUMMARY OF CONSTANTS K_s AND V_{max} FOR 2,4-D AND NAA ALONE AND IN COMBINATION AT A 1:1 RATIO

Per cent composition in interaction mixture		K_s (molar)		V_{max} (mm./section/12 hrs.)	
		Observed	Calculated	Observed	Calculated
2,4-D	NAA				
100	0	5.87×10^{-7}	-	1.85	-
50	50	6.39×10^{-7}	6.39×10^{-7}	1.82	1.82
0	100	6.53×10^{-7}	-	1.79	-

and to increase growth response to low concentrations of IAA in the split pea test.

The data of experiments on the interaction of IAA and TIBA in Avena section growth are summarized in table 17 (see table 14, Appendix I, for growth data). It is evident that the K_s of TIBA is essentially the same as that for IAA while the V_{max} for TIBA is about one third that for IAA. TIBA then is an auxin which has approximately the same affinity for the active site within the plant as IAA but for which the intrinsic activity is considerably lower.

The data of table 17 reveal that growth rates obtained in mixtures of IAA and TIBA are not in agreement with growth rates predicted from the constants K_s and V_{max} of the individual auxins. It is clear that there is no consistent agreement between the observed and calculated values of K_s. Furthermore, the observed values for V_{max} are considerably higher than the calculated values. Thus, growth rates obtained at constant molar ratios are greater than those predicted and the straight lines obtained in the usual double reciprocal plots lie quite close to the straight line obtained for IAA alone. Fig. 26 presents a portion of the experimental data graphically. The growth rates for TIBA alone and for IAA alone as well as those for TIBA : IAA 4:1 molar ratio mixture are presented together with the straight line (dashed line) for the 4:1 molar ratio mixture calculated according to the usual method. It is clear that the calculated line lies considerably above the experimental line for this ratio. A final observation concerning the TIBA-IAA

TABLE 17

SUMMARY OF CONSTANTS K_s AND V_{max} FOR IAA AND TIBA ALONE AND INCOMBINATION AT VARYING MOLAR RATIOS.

Per cent composition in interaction mixture		K_s (molar)		V_{max} (mm./section/12 hrs.)	
		Observed	Calculated	Observed	Calculated
IAA	TIBA				
100	0	3.7×10^{-7}	-	2.6	-
67	33	3.7×10^{-7}	3.8×10^{-7}	2.5	2.0
50	50	3.7×10^{-7}	3.8×10^{-7}	2.5	1.7
33	67	3.9×10^{-7}	3.9×10^{-7}	2.2	1.5
20	80	4.8×10^{-7}	3.8×10^{-7}	2.1	1.2
13	87	4.5×10^{-7}	3.9×10^{-7}	2.0	1.1
0	100	3.8×10^{-7}	-	0.9	-

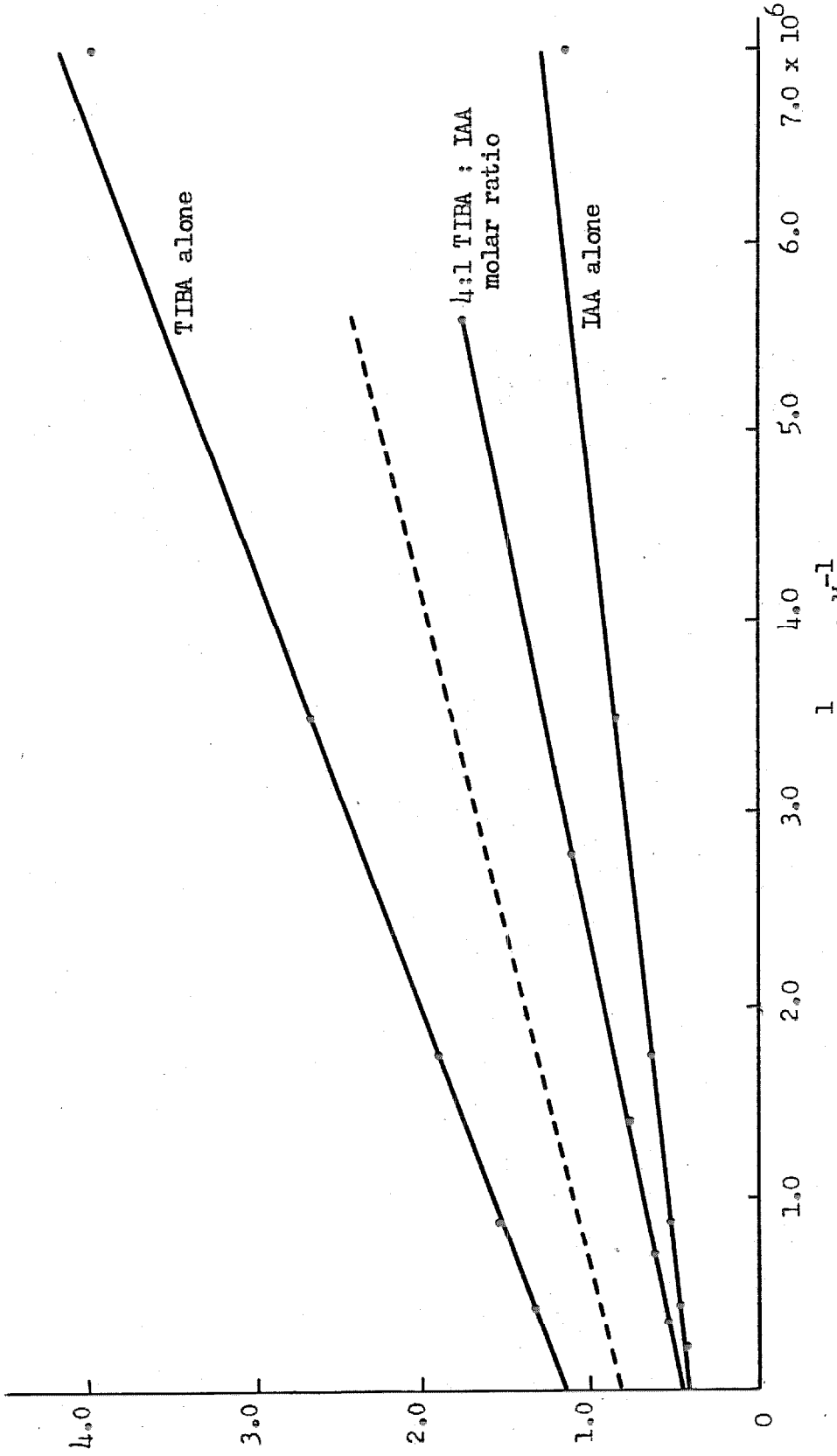


Figure 26. INTERACTION IN AVENA SECTION GROWTH OF 2,4-D AND NAA GIVEN INDIVIDUALLY OR IN A VARIETY OF CONCENTRATIONS AND 4:1 MOLAR CONCENTRATION RATIO. THE DASHED LINE IS THAT FOR CALCULATED TIBA : IAA 4:1 MOLAR CONCENTRATION RATIO. DATA PLOTTED ACCORDING TO THE METHOD OF FOSTER AND NIEMANN (96) AS A TEST FOR COMPETITION AT THE AUXIN-RECEPTOR SITE.

interaction data is that, although the growth rates obtained were considerably greater than those expected, TIBA did not act synergistically with IAA.

The TIBA-IAA interaction data may also be presented by plotting growth rate as a linear function of TIBA concentration. A portion of the data of table 14, Appendix I, is presented in fig. 27. It is apparent that growth promoted by TIBA is additive to IAA induced growth at IAA concentrations of 5.72×10^{-7} M and lower whereas at an IAA concentration of 9.14×10^{-6} M TIBA causes significant inhibition of IAA promoted growth. This decreased growth rate is not due to the type of inhibition under consideration in the present section. At an IAA concentration of 9.14×10^{-6} M, Avena section growth rate is essentially optimal; so the addition of more auxin makes the total auxin concentration supraoptimal and hence a lowered section growth rate results. The reason for this decreased growth rate will become apparent in a later section of this investigation.

As mentioned earlier, the auxin PBA has weak activity in the Avena section growth test and has been reported to possess anti-auxin activity. It is of interest then to determine whether this compound follows the principles developed above for interaction kinetics for auxins. Experiments similar to the preceding were carried out on the interaction of IAA and PBA in the growth of Avena sections and the data (see table 15, Appendix I, for growth values) are summarized in table 18. It is evident from the data in this table that PBA is considerably less active in promoting coleoptile section growth than is IAA. The V_{max} for PBA is however

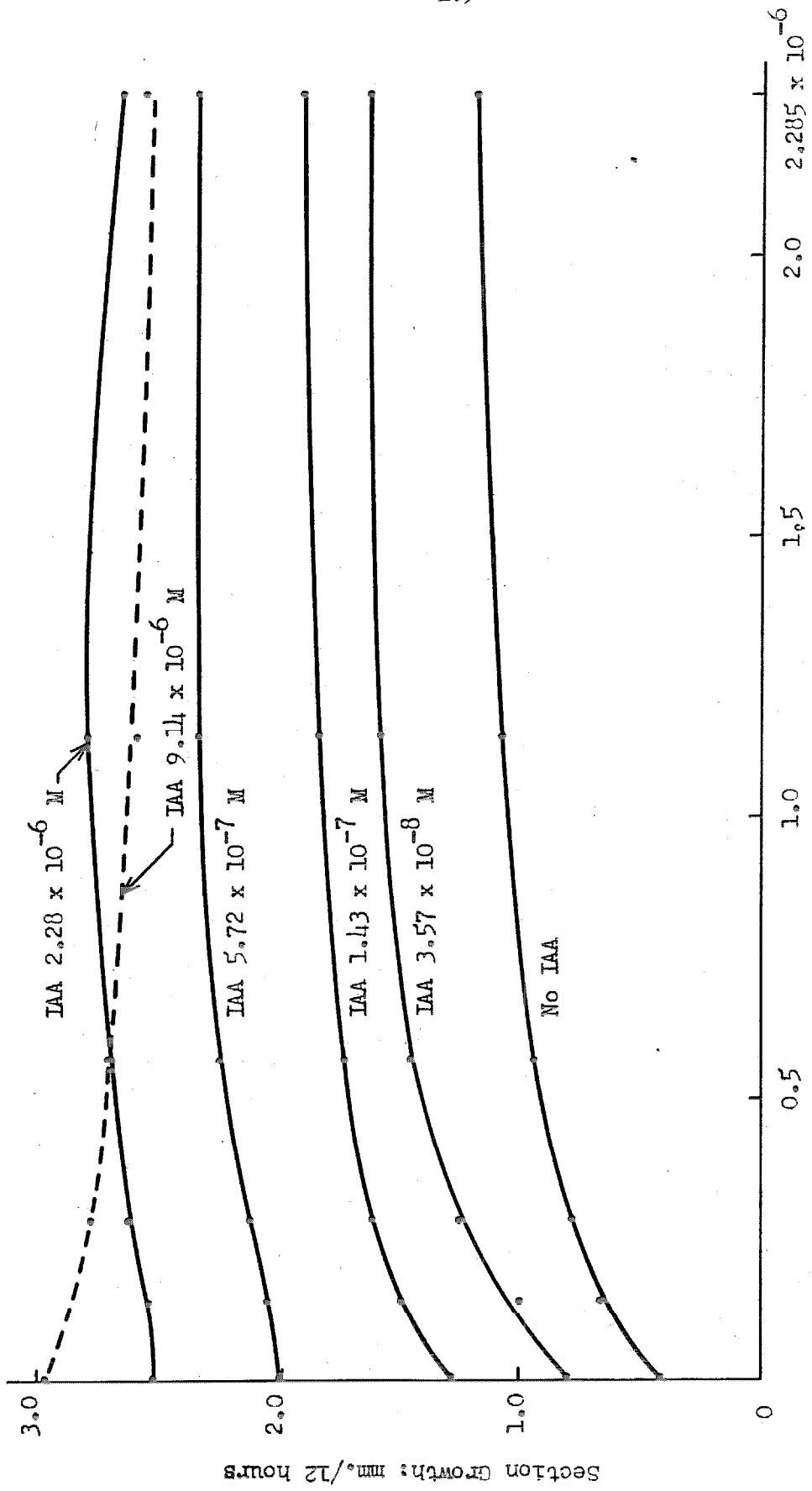


Figure 27. AVENA COLEOPTILE SECTION GROWTH AS A LINEAR FUNCTION OF TIBA CONCENTRATION IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF IAA.

TABLE 18

SUMMARY OF CONSTANTS K_s AND V_{max} FOR IAA AND PBA ALONE AND IN COMBINATION AT VARYING MOLAR RATIOS.

Molar Ratio IAA:PBA	K_s (molar) Observed	K_s (molar) Calculated	V_{max} (mm./section/12 hrs.) Observed	V_{max} (mm./section/12 hrs.) Calculated
100:0	4.0×10^{-7}	-	2.6	-
1:1	1.6×10^{-6}	0.8×10^{-6}	2.6	2.6
1:2	2.1×10^{-6}	1.2×10^{-6}	2.5	2.6
1:4	2.4×10^{-6}	1.9×10^{-6}	2.4	2.5
1:8	4.4×10^{-6}	3.4×10^{-6}	2.3	2.5
1:16	5.7×10^{-6}	5.6×10^{-6}	2.3	2.3
1:32	7.3×10^{-6}	9.1×10^{-6}	2.1	2.1
1:64	1.1×10^{-5}	1.3×10^{-5}	1.9	1.8
0:100	2.7×10^{-5}	-	0.9	-

approximately the same as that for TIBA. Interesting enough however is the fact that, whereas the K_s of TIBA and IAA are essentially the same, the K_s of PBA is about 100 fold greater. PBA therefore has less affinity for the active site within the coleoptile than has TIBA for the same site; but once the active complex is formed, PBA and TIBA induce approximately the same amount of growth. The remaining data of table 18 show that there is rather close agreement between the observed and calculated values for $V_{max(combined)}$. This situation does not hold quite so well with respect to the calculated and observed $K_{s(combined)}$ values. At high PBA : IAA molar ratios, the K_s values are in good agreement. At molar ratios of 8:1 and lower, experimental K_s values are somewhat higher than those predicted from the constants for the individual auxins. PBA therefore interacts competitively with IAA, quantitatively, only at high PBA : IAA molar ratios.

Another feature of PBA induced coleoptile section growth is the fact, as is evident from its high K_s, that the compound has auxin activity only at relatively high concentrations as compared to IAA. This together with its low activity, make it impractical to plot the interaction data for PBA and IAA in its entirety. A portion of the data is however presented in fig. 27 where the double reciprocal plots for PBA alone and IAA alone are shown together with interaction reciprocal plots for PBA : IAA molar ratios of 32:1 and 16:1. Since the calculated and experimental straight lines for these ratios very nearly coincide, the former have not been added to fig. 28.

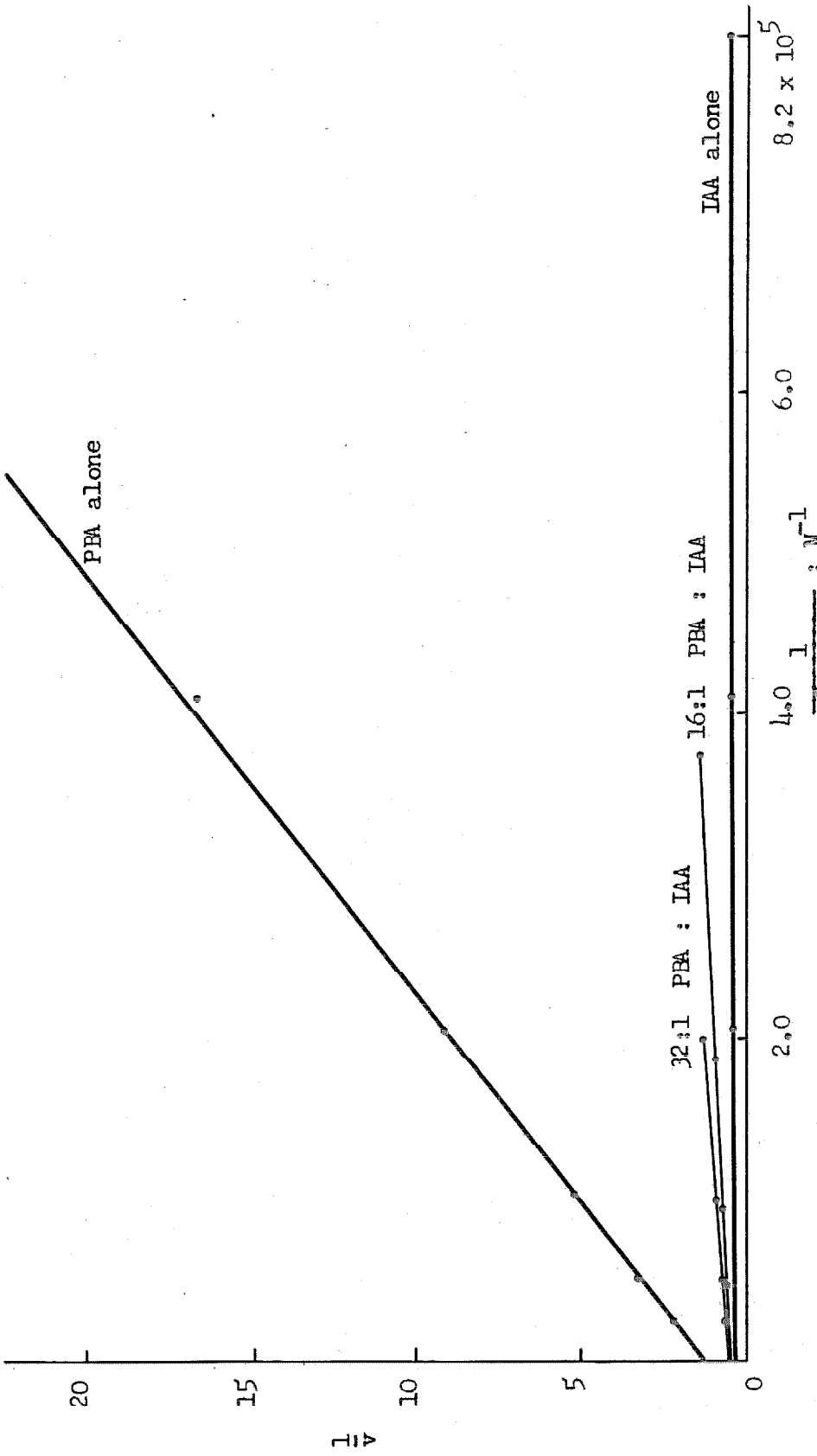


Figure 28. INTERACTION IN AVENA SECTION GROWTH OF IAA AND PBA GIVEN INDIVIDUALLY OR IN A VARIETY OF CONCENTRATIONS AND MOLAR CONCENTRATION RATIOS. DATA PLOTTED ACCORDING TO THE METHOD OF FOSTER AND NIEMANN (96) AS A TEST FOR COMPETITION AT THE AUXIN-RECEPTOR SITE.

Growth rates for the PBA-IAA interaction experiment may also be conveniently presented as a linear function of PBA concentration. When this is done as in fig. 29, curves similar to those for 2,4-D-IAA interaction data of fig. 24 are obtained. Again, each curve of fig. 29 is that for a system at constant IAA concentration and the shaded areas of the curves indicate the concentration range over which PBA effectively lowers the over-all auxin response. It is clear from the figure, as well as from the data of table 15, Appendix I, that concentrations of PBA which are of themselves incapable of promoting Avena section growth are nevertheless able to interact competitively with IAA and to decrease the IAA induced growth response. PBA in low concentrations acts qualitatively as an anti-auxin.

Let us now consider weak auxins as PBA in their role as anti-auxins. To do this we may again make use of the data of table 15, Appendix I. It is immediately apparent from this table that growth promoted by IAA is decreased in the presence of low PBA concentrations in a manner similar to antiauxin inhibition. That this is so is more clearly evident when a portion of the data is presented in the typical double reciprocal plots as in fig. 30. It is clear from this figure that PBA at the two lowest concentrations seemingly satisfies the requirements of a competitive inhibitor. Actually similar straight lines may be obtained for the four higher PBA concentrations. These however have been omitted because they fall quite close to the lines of fig. 30. There is also a tendency,

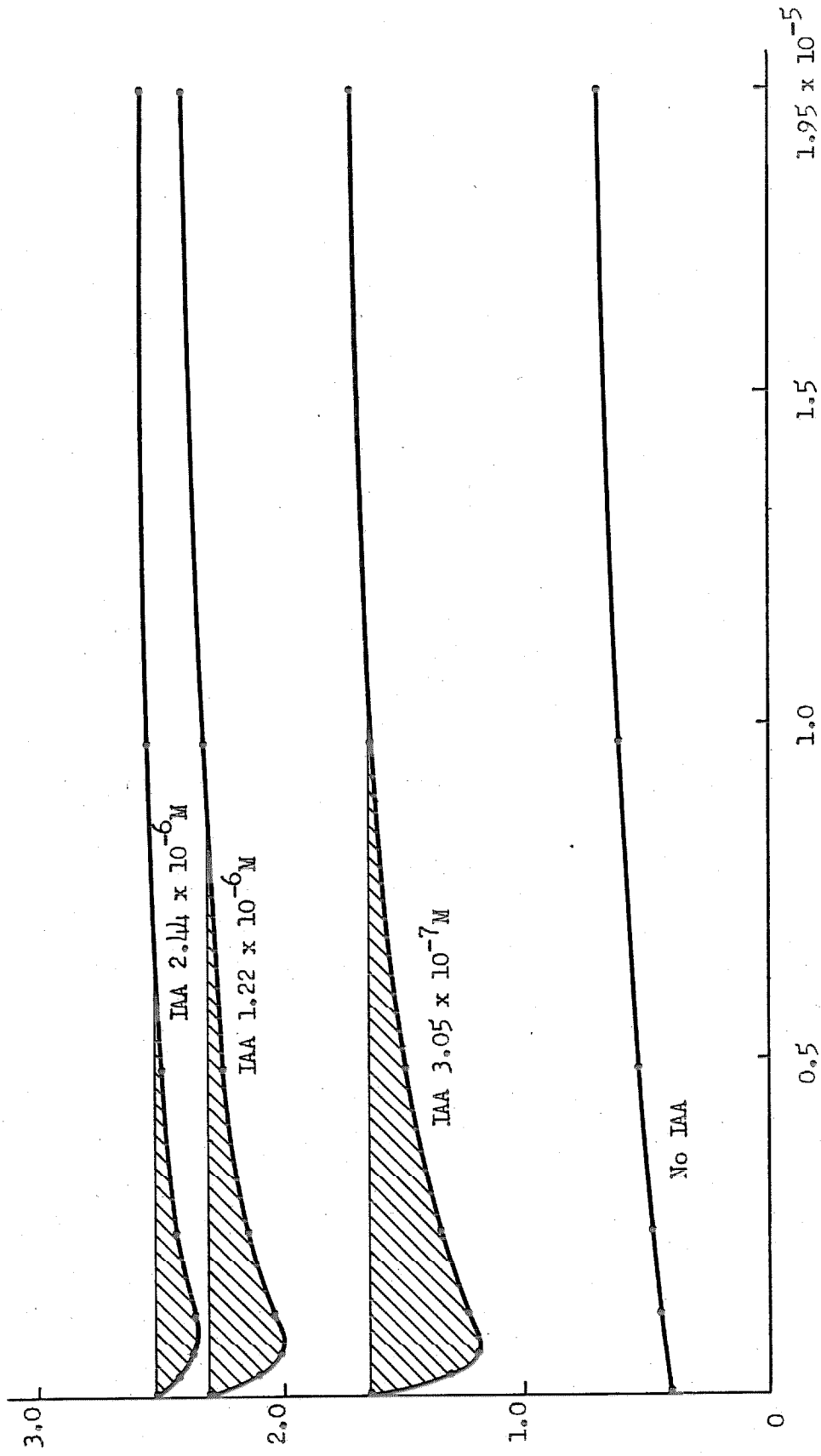


Figure 29. AVENA COLEOPTILE SECTION GROWTH AS A LINEAR FUNCTION OF PBA CONCENTRATION IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF IAA. THE SHADED AREA OF EACH CURVE REPRESENTS THE CONCENTRATION RANGE OVER WHICH PBA INHIBITS GROWTH BELOW THE LEVEL ATTAINED IN IAA ALONE.

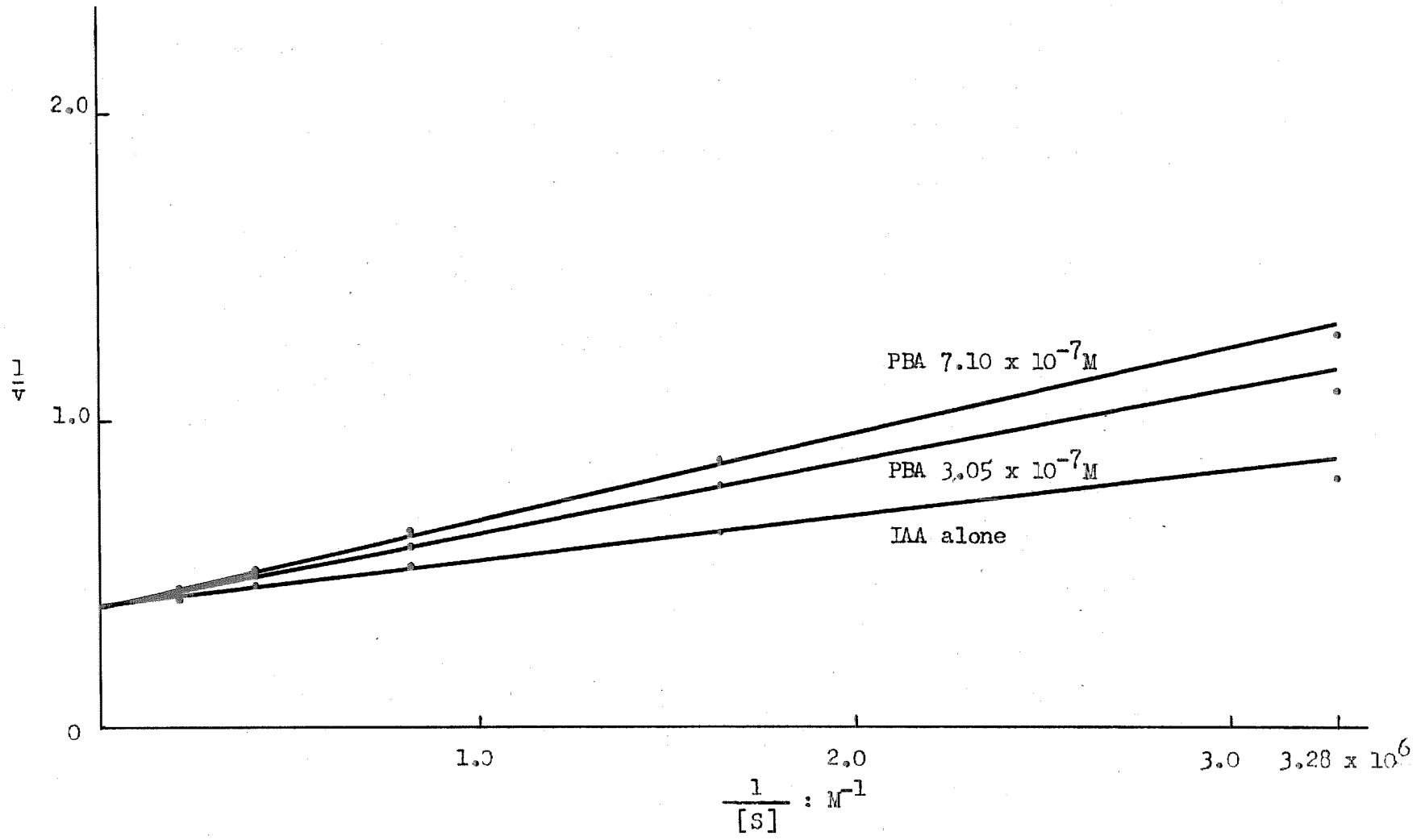


Figure 30. INHIBITION OF IAA INDUCED AVENA SECTION GROWTH BY PBA. DATA PLOTTED ACCORDING TO THE LINEWEAVER-BURK TREATMENT AS A TEST OF COMPETITIVE INHIBITION.

with increasing PBA concentrations, toward downward curvature of the lines at low IAA concentrations. Furthermore, instead of the slope of the lines increasing with increasing PBA concentrations, the slopes decrease and approach the slope for IAA alone. Nevertheless reasonably accurate straight lines may be drawn and the apparent K_I for PBA may be estimated for all six concentrations.

Since lines have not been included in fig. 30 to illustrate the above point, the slopes are presented in table 19. It is clear from this table that the slope of the lines decreases from a PBA concentration of 1.42×10^{-6} M to a concentration of 1.14×10^{-5} M. Included in table 19 also is the apparent K_I constant for each inhibitory PBA concentration. It is clearly evident that the apparent K_I is not constant for different PBA concentrations. (The fact that the K_I is not constant is the reason for calling it an apparent K_I). With a 64-fold increase in PBA concentration there is an approximately 800-fold increase in apparent K_I. The fact that this is so, in contrast to the constant K_I of a true antiauxin, is sufficient reason for not considering auxins of low V_{max} among the true antiauxins.

Discussion

The experiments described above demonstrate that different auxins interact in the Avena coleoptile section in a manner compatible with the hypothesis that they react at a common site. They have shown furthermore that the nature of the interaction is dependent upon at least four parameters which are respectively: (a) the K_s for each auxin, (b) the V_{max} for each auxin, and (c) and (d) the concentrations

TABLE 19

SUMMARY OF THE SLOPE AND APPARENT K_I CONSTANT WHICH CHARACTERIZE THE INHIBITING PROPERTIES OF PBA ON IAA INDUCED AVENA SECTION GROWTH.

Concentration of PBA (molar)	Slope	Apparent K_I (molar)
0.0	1.57×10^{-7}	-
3.05×10^{-7}	2.35×10^{-7}	6.3×10^{-7}
7.10×10^{-7}	2.74×10^{-7}	8.3×10^{-7}
1.42×10^{-6}	2.56×10^{-7}	2.0×10^{-6}
2.84×10^{-6}	2.13×10^{-7}	4.4×10^{-6}
5.68×10^{-6}	1.77×10^{-7}	4.2×10^{-5}
1.14×10^{-5}	1.62×10^{-7}	5.0×10^{-4}

of each auxin. The result of such interaction is that one auxin such as 2,4-D may augment or diminish the effects of a second auxin such as IAA depending on the absolute concentration of each. Thus the data of table 15 show that 2,4-D acts to diminish the growth response elicited by moderate or high concentrations of IAA. 2,4-D acts, in general, to augment the growth response elicited by low concentrations of IAA. The major interaction effects between 2,4-D and IAA are due to the fact that even though 2,4-D has slightly less affinity (higher K_s) for the active site within the plant than does IAA, it also possesses considerably lower intrinsic activity (V_{max}) than does IAA. When 2,4-D is added to a system which contains a concentration of IAA greater than some certain critical value, which is a function of the characteristic parameters of the system $\left(S_{critical}=K_{s_1}\frac{V_{max_2}}{V_{max_1}-V_{max_2}}\right)$, the 2,4-D replaces IAA at a portion of the available receptor sites. The sites occupied by 2,4-D contribute less to over-all growth rate than do sites occupied by IAA. Even though some previously occupied sites may also be occupied by 2,4-D, the over-all effect will nonetheless be to diminish growth rate. At concentrations of 2,4-D below the critical value, the addition of 2,4-D should result in an increased growth rate because, in this region, there are a relatively large number of unoccupied sites available for combination with 2,4-D. Superimposed upon this effect is however an inhibition which is particularly marked in the case of very low 2,4-D concentrations and which is not predicted by the present treatment. This qualitative description of the

kinetic consequences which result from the presence of 2,4-D in a system which contains IAA applies similarly to the effects of NAA in the presence of IAA and of NAA in the presence of 2,4-D.

The competitive interaction of auxins of low V_{max} with auxins of high V_{max} do not follow with precision the general principles outlined above. At high PBA : IAA molar ratios PBA behaves as expected, while at lower molar ratios there is a significant deviation. That the same or similar situation does not pertain to TIBA-IAA interaction is probably due to the fact that sufficiently low concentrations of the auxins were not employed. Thus it is not possible to obtain, from the available data, high TIBA : IAA molar ratios.

It is not surprising however that experimentally there is a significant departure from the theoretical with the two auxins of low V_{max}. It is evident from the data presented that approximately 50 per cent of the maximum section growth rate induced by these auxins at the end of a 12 hour period is due to endogenous auxin. When a situation such as this prevails, an endogenous growth concentration factor should be added to the exogenous auxin concentration. This however is impossible to do in the present experiments because the endogenous growth concentration factor for PBA and for TIBA are considerably greater than that for IAA. If the requisite factor is added, then the experimental design is inappropriate for obtaining data at constant molar ratios. The question may be asked then as to why in the PBA : IAA interaction experiment the observed and calculated constants, K_s and V_{max}, were in close agreement at high PBA : IAA

molar ratios. The probable answer to this is that at high molar ratios the endogenous growth concentration factor correction is relatively unimportant as compared to the high exogenous PBA concentrations while at lower exogenous PBA concentrations, and therefore low molar ratios, the correction factor is of considerable significance.

Let us now consider the possible steps which may be taken to overcome the experimental difficulties encountered when considering the interaction between auxins of low and high V_{max}. First, the endogenous growth concentration correction factor may be determined for each auxin. This correction factor is then added to the appropriate exogenous auxin concentration so that constant molar ratios, which include the correction factor, may be obtained. This approach has not however been taken. The second and more desirable method to meet the problem is to have a growth system which is devoid of endogenous auxin. With this in mind a simple experiment was carried out in an attempt to deplete the Avena sections of endogenous auxin.

Sections approximately 10mm. in length were cut from the tip of each coleoptile cylinder. A 2-3mm. tip was removed from one group of sections and the tips were retained on another group of sections. Both groups of sections were placed in distilled water on a shaker for 12 or 24 hours. After 12 or 24 hours, 5.0mm. sections were cut from each coleoptile and incubated for 12 hours with IAA at 1.0 mg./l., TIBA at 0.4 mg./l., a combination of both auxins and in the absence of auxin. The results of this experiment show that the endogenous growth rate is reduced by approximately 30 per

cent and the exogenous growth rate is reduced by over 50 per cent, the absolute reduction being dependent upon the pretreatment. This method of pretreatment did not eliminate the endogenous growth of the sections and it was felt that any simple method which might be employed to reduce endogenous growth would result in considerable reduction of exogenous auxin-induced growth rate. No further work was carried out along this approach.

Competitive interactions of the present type may serve to make understandable such bewildering responses of intact plants to auxin mixtures as those described by Hitchcock (97) who applied 2,4-D and/or IAA to tomato plants. The repression of response to 2,4-D by low concentrations of IAA and augmentation of responses to 2,4-D by higher concentrations of IAA recorded by Hitchcock are responses which are consistent with the interactions presented above.

The principles which govern the interaction of two different applied auxins may also be involved in the interaction of an applied exogenous auxin with the native endogenous auxin of the plant. In the pineapple, for example, an interesting anomaly is that synthetic auxins such as 2,4-D or NAA supplied to the plants at low concentrations induce flowering in spite of the fact that flowering under other conditions appears to be associated with a decrease in the amount of endogenous auxin. Bonner and Bandurski (15) have suggested that synthetic auxins may act as antagonists to the native auxin of the pineapple plant. According to this view, the synthetic auxins would at appropriate concentrations compete with the native auxin in such a manner that the effective auxin concentration within the plant would be lowered.

Auxin interactions of this type are to be anticipated on the basis of the present findings. That this is so is apparent from the curves of fig. 24. In terms of the effects of an exogenous auxin on a plant or plant organ which already contains native IAA, the shaded areas of fig. 24 would represent the concentration range over which applied 2,4-D will effectively lower the over-all auxin response. It is evident that this type of interaction may account for the induction of flowering in the pineapple plant by applied 2,4-D or NAA provided only that the native auxin of this plant be present in concentrations which do not saturate all of the receptive sites and that flowering is in fact associated with low effective auxin level. Responses similar to the above should also be obtained with applied PBA, as is evident from fig. 29.

It would appear to be of importance for the understanding of all such auxin interactions that the activity of each auxin be understood and characterized in terms of the parameters K_s and V_{max}.

Summary

Chemically different auxins such as IAA, 2,4-D and NAA compete with one another for the same receptive sites within the plant. That this is so has been shown by the following steps:

(a) formulation of the quantitative relations between growth rate and auxin concentration which would be expected on the basis of competition of auxins for the same sites.

(b) derivation of the parameters $K_{s(combined)}$ and $V_{max(combined)}$ for systems containing two different auxins in terms of the constants

K_s and V_{max} for each auxin alone.

(c) demonstration that the values $K_{s(combined)}$ and $V_{max(combined)}$ measured experimentally for growth rate in the presence of auxin mixtures are identical with those calculated on the basis of (b) above.

Auxins of low K_s and low V_{max} are capable of inhibiting or augmenting the activity of auxins of similar K_s and greater V_{max}. Whether inhibition or promotion result depends on the concentrations of the two substances and upon the differential in V_{max} for the two substances. Inhibition may be such that the auxin of low V_{max} exhibits apparent antiauxin activity. This activity is shown to be different from competitive inhibition of true antiauxins.

The principles according to which chemically different auxins interact in promotion of growth of the Avena coleoptile section appear to be largely understandable in terms of classical enzyme kinetics. Although the correctness of this formulation has been verified only for the Avena coleoptile, these same principles may perhaps be applicable to further plant systems.

KINETICS OF AUXIN-INDUCED GROWTH INHIBITION

Introduction

It is characteristic of a great number of biologically active substances that the responses which they elicit are twofold, low concentrations of the material promoting a particular activity, and higher concentrations inhibiting it. This is the case with the auxin-induced growth responses of plants. Excised Avena sections grow in length when they are floated in solutions containing low concentrations of auxin and this growth is a simple function of auxin concentration as was shown in fig. 6A. With increasing auxin concentration, however, growth rises to a maximum only to decline as auxin concentration is still further increased. This behavior is not restricted to Avena section growth but is found to apply also to other classical auxin responses such as Avena curvature test, split pea test, and pea epicotyl section test (64, 67, 69, 76, 77, 98-105). Little attention has however been focused on the significance of growth inhibition at supra-optimal auxin concentrations. The few suggestions which have been made concerning this phenomenon, those of Skoog et al. (77), Veldstra and Booij (9) and Thimann (14), will be returned to below.

It has been shown above that a molecule in order to be active as an auxin must satisfy a number of structural requirements among which is the requirement for two suitably positioned reactive groups. These two groups, a carboxyl group located in a side chain attached

to an unsaturated cyclic nucleus (1) and a substitutable group of critical reactivity in the nucleus ortho to the side chain (20) appear to be the functional groups through which the auxin molecule makes attachment to some receptor entity within the plant. The combined auxin, bound through its two reactive positions to two functional sites of the receptor entity, appears to be the form which is active in eliciting growth responses. Although the exact chemical nature and biochemical function of the auxin-receptor complex is not known, the existence and character of the complex can be deduced from kinetic considerations of the data presented in earlier sections of this investigation and from the study of the relationships of chemical structure to physiological activity of synthetic auxin analogs. It is the purpose of this part of the investigation to show that the inhibition of growth induced by supra-optimal concentrations of auxin is a natural and indeed an inescapable consequence of the two-point attachment by which auxin is bound within the plant to form the functional entity.

Results

KINETIC TREATMENT OF TWO-POINT ATTACHMENT. The kinetic treatment used in this investigation thus far has dealt implicitly with systems in which attachment to enzyme is consummated through a single point. Let us now consider the manner in which two-point attachment may be expected to alter this picture (106, 107). This may be done by employing an extension of the Michaelis-Menten treatment (71). A molecule of auxin (S) might be expected to become first

associated with one of the two receptive sites on the enzymatic entity and then, by becoming associated with the second receptive site, to consummate the two-point attachment essential to enzymatic (growth) activity. This sequence of reactions will predominate at low auxin concentrations. At higher auxin concentrations, however, there is an appreciable probability that two auxin molecules may simultaneously approach the receptor entity and simultaneously react with it, each combining through a single reactive group. Such interaction will yield an inactive auxin-receptor complex in which each auxin molecule prevents the other from consummating a two-point attachment.

The several equilibria which relate the monomolecular and bimolecular substrate-receptor complexes are illustrated in fig. 31. These equilibria involve the following quantities:

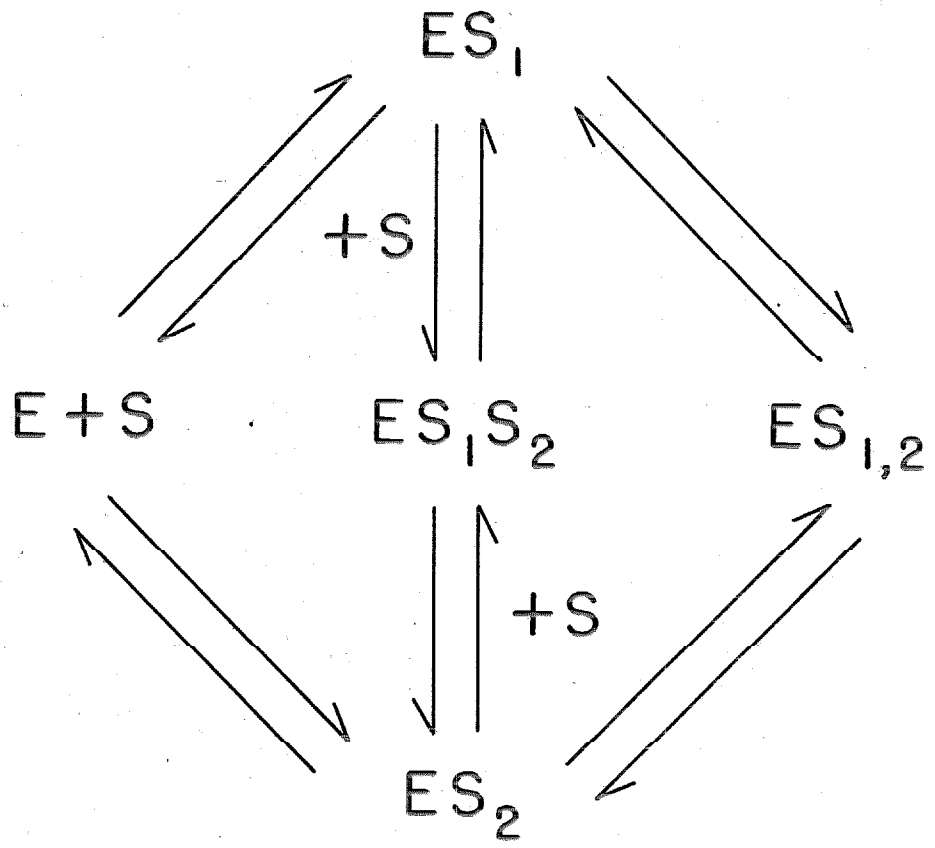
E: - Receptive entity of the plant.

S: - Auxin.

ES_1: - Auxin-receptor entity complex combined through point 1.

$ES_{1,2}$: - Auxin-receptor entity complex combined through points 1 and 2. This complex, the enzymatically active one, may be presumed to be formed from either ES_1 or ES_2.

ES_1S_2: - Bimolecular auxin-receptor entity complex in which two auxin molecules are involved, one attached through point 1, the other through point 2. This complex is presumed to be enzymatically inactive.



$$\text{GROWTH} = k[ES_{1,2}]$$

Figure 31.

EQUILIBRIA RELATING THE FORMATION OF ACTIVE RECEPTOR-SUBSTRATE COMPLEX ($ES_{1,2}$) TO THE FORMATION OF INACTIVE RECEPTOR-SUBSTRATE COMPLEX (ES_{1,S_2}).

In the classical enzyme kinetics only the equilibria which result in the formation of the active complex, $ES_{1,2}$ are considered. If we include in the consideration the formation of the complex ES_1S_2, the classical expression relating reaction velocity to substrate concentration may be shown to be altered to the form given in equation 10.

$$(10) \qquad v = \frac{V_{ex}[S]}{K_s' + [S] + [S]^2/C}$$

This expression differs from that of classical kinetics in that it includes a term $[S]^2/C$ which is a measure of the probability that a second molecule of substrate will become attached to the receptor entity before the first molecule has consummated its two-point attachment. The reaction velocity v, which is proportional to $ES_{1,2}$, is expressed in terms of S and the experimentally determinable constants V_{ex}, K_s' and C. Equation 10 predicts a maximum reaction velocity (growth rate) at a substrate concentration of $\sqrt{K_s' \cdot C}$. This maximal rate is decreased below the rate which would be achieved in a classical system by the amount to which the inactive complex ES_1S_2 is formed in the system at the substrate concentration $\sqrt{K_s' \cdot C}$.

Equation 10 will now be applied to data derived from experimental determination of the relation of auxin concentration to rate of Avena coleoptile section growth. We shall first evaluate the constants V_{ex} and K_s' which correspond to the constants V_{max} and K_s of classical enzyme kinetics. These two constants may be determined

in the low range of auxin concentration in which the formation of the bimolecular complex ES_1S_2 is negligible. This is most readily done on the reciprocal plot of fig. 32. V_{ex} is the extrapolated intercept on the ordinate while K_s' is the slope divided by the intercept. The remaining constant C, which is a measure of the probability that two molecules will react with the two receptor sites of the reactive entity before either has had a chance to consummate its two-point attachment, must be evaluated in the region of high concentration in which the formation of this complex predominates. This is done as shown in fig. 33 which presents growth velocity as a function of the reciprocal of the substrate concentration. It can be seen that as the auxin concentration is increased, the growth velocity becomes inversely proportional to it and from the limiting slope which is $V_{ex}C$, the value of C may be determined.

It appears then that at the extremes of the auxin concentration range auxin affects growth in the manner predicted by equation 10. This equation describes accurately the effects of IAA on the growth response of Avena coleoptile sections over the entire range of auxin concentrations to which this organ is sensitive, a range of one hundred thousand fold. That this is so is shown in fig. 34. Since the range of concentration is so large, the data are presented in the form of initial growth velocity as a function of the log of auxin concentration. The curve of fig. 34 is that for equation 10 using the values for the constants determined as outlined above.

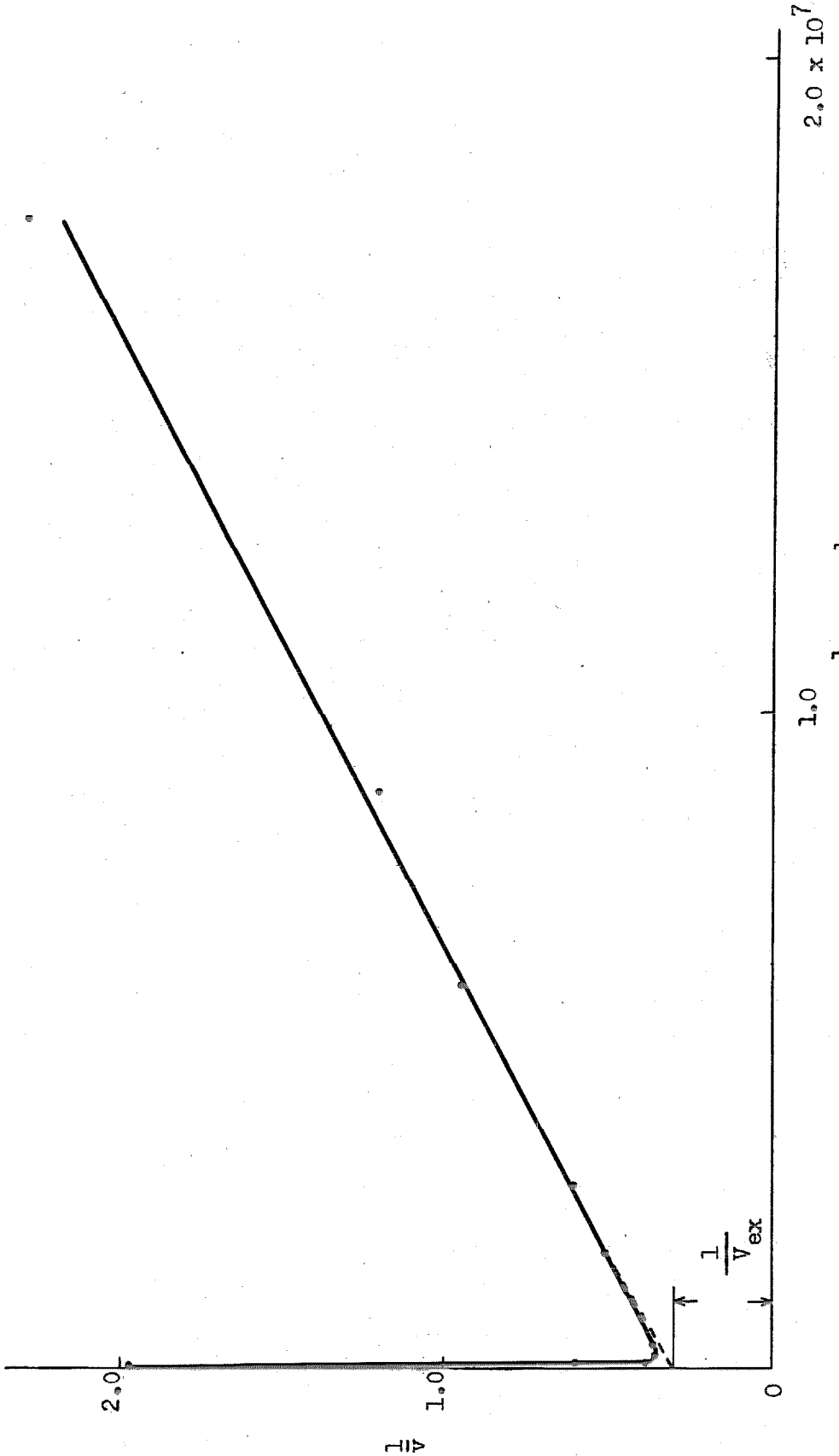


Figure 32. RECIPROCAL OF AVENA COLEOPTILE SECTION GROWTH RATE PLOTTED AS FUNCTION OF RECIPROCAL OF IAA CONCENTRATION.

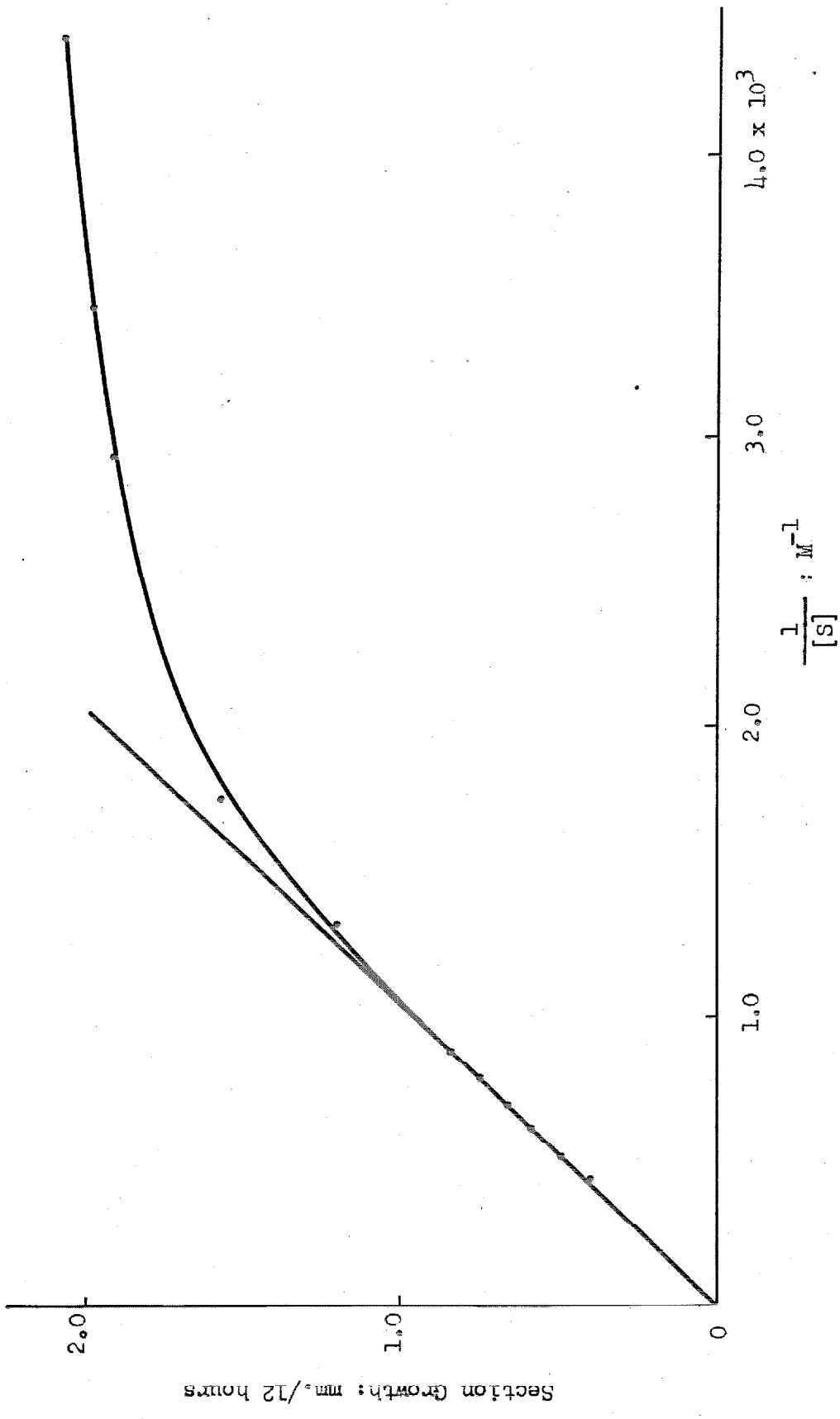


Figure 33. AT HIGH AUXIN (IAA) CONCENTRATION, GROWTH OF AVENA COLEOPTILE SECTIONS IS INVERSELY PROPORTIONAL TO THE SUBSTRATE CONCENTRATION. THE LIMITING SLOPE OF THE LOWER PORTION OF THIS CURVE = $V_{ex}C$.

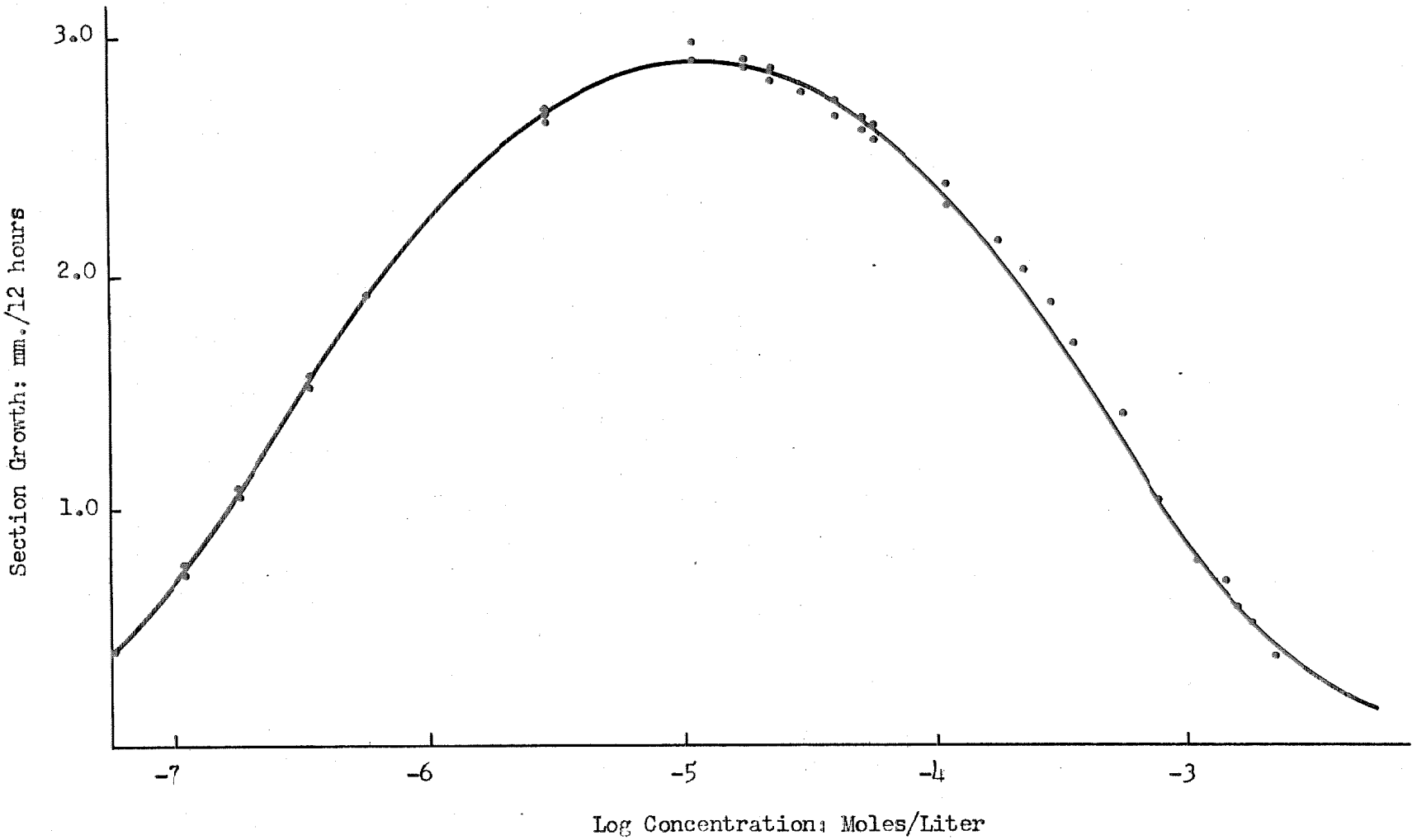


Figure 34. AVENA SECTION GROWTH AS A FUNCTION OF IAA CONCENTRATION. THE SOLID LINE IS THAT FOR EQUATION (10): THE POINTS REPRESENT EXPERIMENTAL DATA.

The individual points of fig. 34 represent data from five separate and complete experiments. These fit the curve within experimental error over the entire 100,000 fold range of concentrations. That the maximum growth rate occurs at a substrate concentration of $\sqrt{K_s' \cdot C}$ is also verified by the data.

The synthetic growth substances such as 2,4-D and NAA are, like the naturally occurring IAA, active in promoting growth of Avena coleoptile sections at low concentrations and active in inhibiting growth at higher concentrations. That the kinetic relations of 2,4-D and of NAA in Avena section growth are also in accord with the two-point attachment concept is evident from the data of fig. 35 and 36. This is true even though the constants V_{ex}, K_s' and C for these substances differ appreciably among themselves and differ also from those determined for IAA, a fact summarized in table 20.

It is next of interest to consider the behavior of auxins of low V_{max} at supra-optimal concentrations. That TIBA and PBA inhibit growth at high concentrations is apparent from the data of fig. 37 and 38, respectively. Furthermore it is evident that the kinetics of TIBA promoted growth follow the predications of equation 10 rather closely over most of the concentration range. At concentrations at or near the optimal however, the observed growth rate is somewhat higher than that predicted.

The behavior of PBA is less well in accord with the expectations of equation 10. In addition to a deviation in observed growth rates at or near optimal concentrations, the inhibition at high concentrations is greater than that expected.

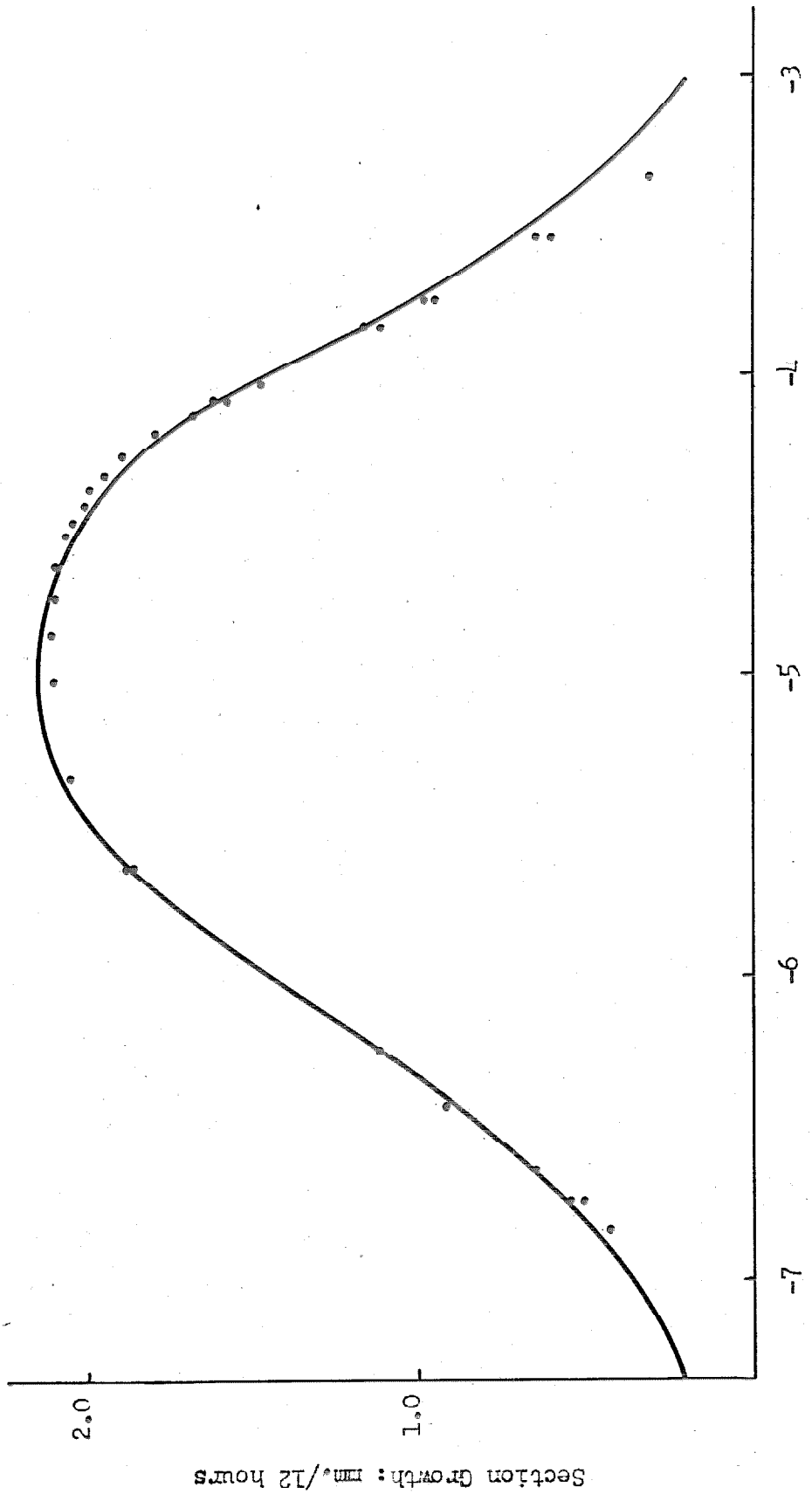


Figure 35. AVENA SECTION GROWTH AS A FUNCTION OF 2,4-D CONCENTRATION. THE SOLID LINE IS THAT FOR EQUATION (10): THE POINTS REPRESENT EXPERIMENTAL DATA.

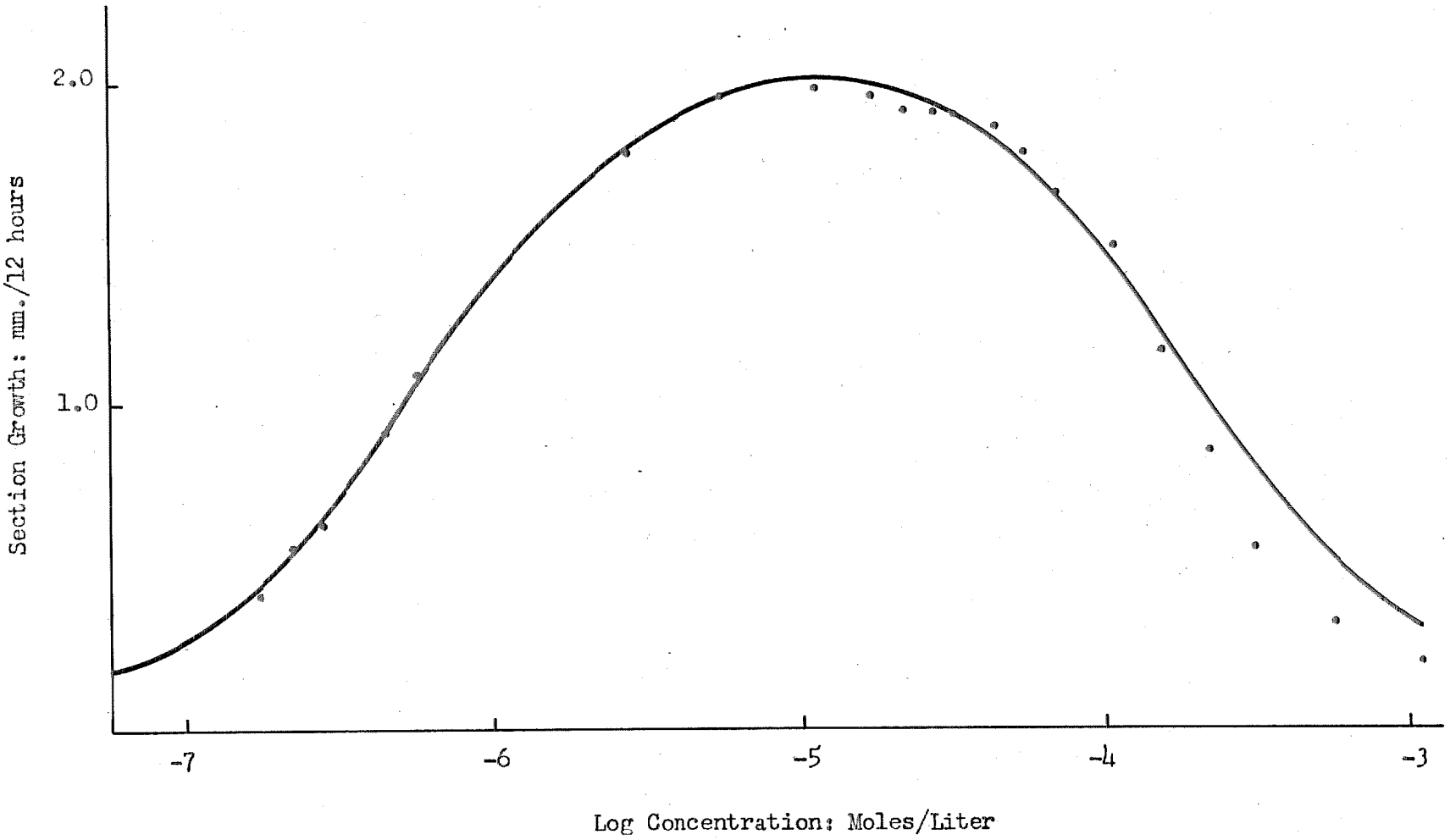


Figure 36. AVENA COLEOPTILE SECTION GROWTH AS A FUNCTION OF NAA CONCENTRATION. THE SOLID LINE IS THAT FOR EQUATION (10): THE POINTS REPRESENT EXPERIMENTAL DATA.

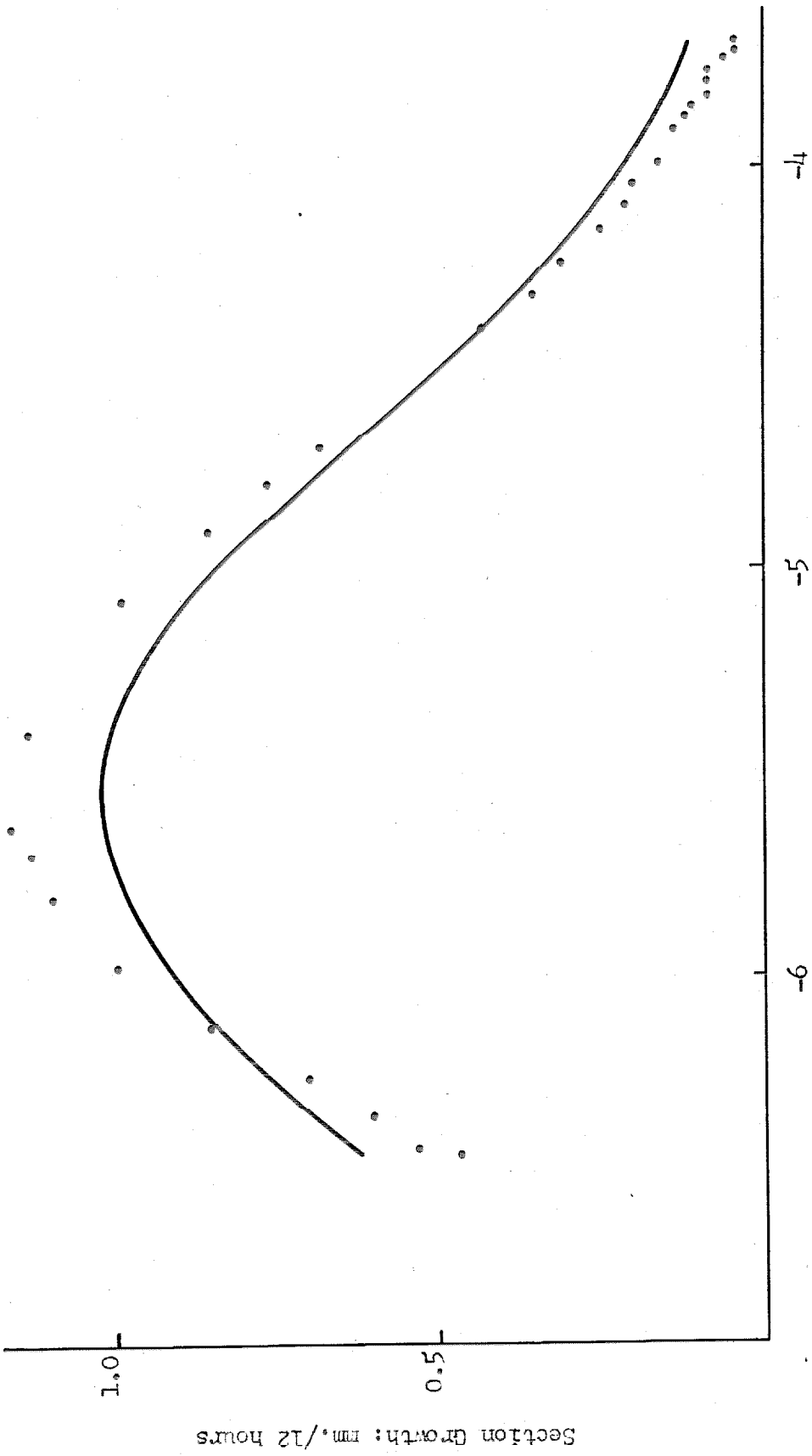


Figure 37. AVENA SECTION GROWTH AS A FUNCTION OF TIBA CONCENTRATION. THE SOLID LINE IS THAT FOR EQUATION (10): THE POINTS REPRESENT EXPERIMENTAL DATA.

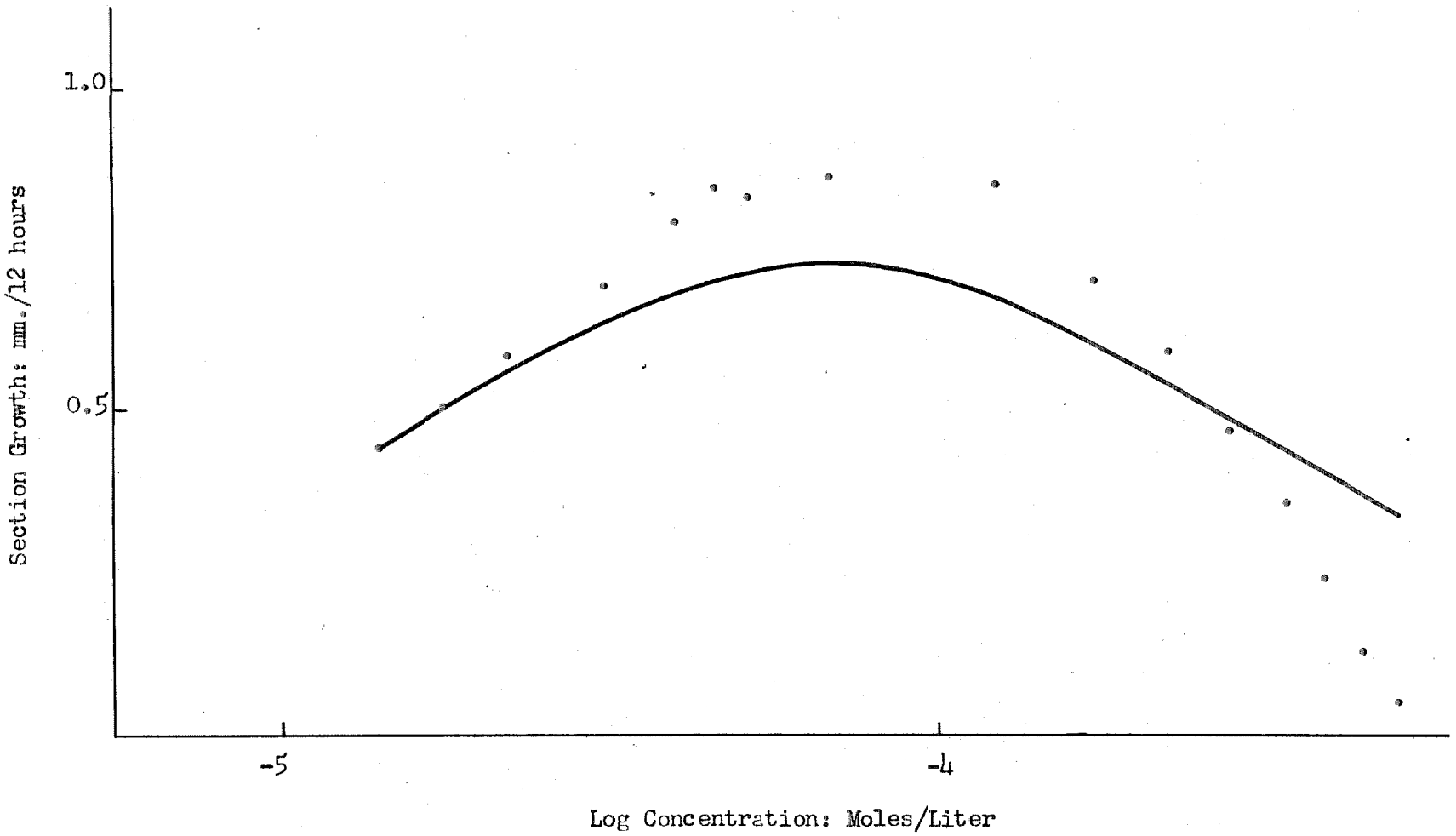


Figure 38. AVENA SECTION GROWTH AS A FUNCTION OF PBA CONCENTRATION. THE SOLID LINE IS THAT FOR EQUATION (10): THE POINTS REPRESENT EXPERIMENTAL DATA.

It is not immediately clear why growth rates for auxins of low V_{max} depart significantly from two-point attachment kinetics. It is suggested however that one of the following reasons or a combination of them may account for such deviations. First, in order to experimentally verify equation 10 accurately, it is necessary to apply the endogenous growth concentration correction factor. The concentration, in terms of exogenous concentration, which gives the observed endogenous growth rate, is determined by extrapolation to zero growth rate. This concentration is then added to all exogenous concentrations to give the S of equation 10. The precision with which this factor may be determined depends on the V_{max} of the auxin. The factor for high V_{max} auxins may be obtained more precisely than for low V_{max} auxins. Furthermore, if this factor is low, as it is with high V_{max} auxins, it is of significance only at low exogenous concentrations. If, on the other hand, the factor is high, as with low V_{max} auxins, it is of importance over a greater exogenous concentration range. It is evident then that failure of low V_{max} auxins to follow two-point attachment kinetics with precision may be due to an inadequate estimate of the endogenous growth concentration correction factor. Secondly, since the same correction factor is used to determine the constants K_s' and V_{ex}, it is clear that these constants may be more inaccurately estimated for low V_{max} auxins than for auxins of high V_{max}. Finally, the constant C is also more difficult to determine accurately for auxins of low V_{max} than for high V_{max} auxins. In view of these considerations, the relatively poor fit to two-point

kinetics found for TIBA and PBA as compared with auxins of higher V_{max} are not unexpected. While there is a possibility that other features of the system, as yet unrecognized, may complicate the kinetics for auxins of low V_{max}, any conclusion that there are such factors is not yet an obligatory one.

The constants which relate growth of Avena coleoptile sections to TIBA and PBA are included in table 20. The constant C for TIBA is approximately ten fold less than for the other auxins studied. The optimal concentration for TIBA induced growth is similarly lower than for the other auxins. PBA induced growth, on the contrary, is optimal at a higher concentration. Growth promoted by PBA differs from the other auxins in one other respect which is evident from the data of fig. 38. The growth activity of this auxin is restricted to a much smaller concentration range. The probable reason for this is to be found in the large differential between V_{ex} and the observed maximal growth rate. The V_{ex} for PBA is 1.3mm./section/12 hours as compared to the experimental maximal growth rate of approximately 0.9mm./section/12 hours. Since nearly half this 0.9mm./section/12 hours is endogenous growth, it is clear that the observed maximal growth rate is approximately 50 per cent of V_{ex}. This fact implies that at optimal PBA concentration bimolecular complex concentration is relatively high and therefore PBA not only inhibits IAA induced section growth at appropriate concentrations but is also strongly inhibitory to itself. In the case of TIBA, the differential between observed maximal growth rate and V_{ex} is about

TABLE 20

SUMMARY OF THE PARAMETERS WHICH RELATE GROWTH OF AVENA COLEOPTILE SECTIONS TO AUXIN CONCENTRATION

Auxin	V_{ex}, mm./12 hrs.	Ks', [a] moles/liter	C, [b] moles/liter	$\sqrt{Ks' \times C}$, [c] moles/liter
IAA	3.1	3.4×10^{-7}	4.0×10^{-4}	1.2×10^{-5}
2,4-D	2.3	5.5×10^{-7}	1.4×10^{-4}	0.9×10^{-5}
NAA	2.1	6.4×10^{-7}	1.7×10^{-4}	1.1×10^{-5}
TIBA	1.3	3.7×10^{-7}	2.0×10^{-5}	0.4×10^{-5}
PBA	1.3	2.7×10^{-5}	2.1×10^{-4}	7.7×10^{-5}

a Concentration of auxin at which growth rate is 0.5 V_{ex} and $ES_{1,2} = E_{free}$.

b Concentration of auxin at which growth rate is 0.5 maximal due to formation of the complex which contains two auxin molecules. $ES_{1,2} = ES_1S_2$.

c Concentration of auxin at which growth rate is maximal.

15 per cent whereas for the other high V_{max} auxins the differential is approximately five per cent.

Discussion

The concept that auxins are bound within the plant to an appropriate receptor entity through which auxin-induced growth responses are mediated was originally based not only on the known binding of auxins to plant proteins (108) but also and more specifically on the fact that the kinetics of auxin action are in close accord with the expectations of classical enzyme kinetics as has been shown earlier in this investigation. The extension of this concept to include the specification that the binding must be consummated through a two-point attachment of the auxin molecule to the receptor entity was first suggested by the observation of Muir et al. (20), that a free ortho group of critical reactivity as well as a free carboxyl group are essential to auxin activity. The hypothesis that both of these groups are in fact essential to auxin activity and by implication essential to auxin binding has been strengthened by the finding that auxin-like molecules in which either of the two essential groupings is blocked or abolished are not only inactive as auxins but also are in fact competitive inhibitors of auxin action. The matters considered in this paper constitute a critical experimental test of the two-point attachment concept as applied to auxin action. If two-point attachment of auxin to its receptor is essential for the formation of the complex which

induces growth it follows that inhibition of growth by the formation of bimolecular complexes may be expected at higher auxin concentrations. The fact that auxin-induced growth inhibition not only does occur but also that it follows in elegant detail the kinetics of bimolecular complex formation provides strong support of the whole two-point attachment concept.

Of what practical use may it be to express and to think about auxin-induced growth inhibition in terms of the kinetics of two-point attachment? One evident application may be in the study of the herbicidal action of synthetic growth substances such as 2,4-D. It is a peculiarity of these materials that although they are exceedingly deleterious to particular plants at higher concentrations, they act at lower concentrations in a manner indistinguishable from that of IAA itself. We might therefore inquire in how far the herbicidal action of 2,4-D and related materials may be owing to the formation of bimolecular complexes of the kind here described and we might attempt to answer this question by determination of the extent to which herbicidal activity is a function of the second power rather than of the first power of the applied growth substance concentration.

The twofold action of auxin on plant growth appears then to have its basis in a requirement for two-point attachment of the auxin to its receptor, a requirement which makes possible the formation of an inactive bimolecular complex at higher auxin concentrations. It will be of evident interest to discover whether the twofold action of other biologically active substances may in some instances similarly reside in a requirement for multiple attachment.

Summary

The relationship between Avena coleoptile section growth rate and auxin concentration over a wide range has been demonstrated to be predictable on the basis of a requirement for two-point attachment of the auxin molecule to some receptive entity in the plant. In particular, the inhibition of growth at higher auxin concentrations, which may be the cause of herbicidal activity of certain chemicals, has been shown to be a natural and predictable consequence of the two-point attachment of the auxin molecule to the receptor entity within the plant.

INTEGRATION OF THE ANTIAUXIN CONCEPT WITH THAT OF AUXIN-INDUCED GROWTH INHIBITION

Introduction

In a previous section of this investigation we have seen that at auxin concentrations below the optimum, auxin-induced Avena coleoptile section growth may be competitively antagonized by an antiauxin. It was also pointed out that this does not necessarily imply that sufficiently high auxin concentrations will completely overcome the inhibitory effects of an antiauxin because relatively high auxin concentrations are of themselves inhibitory. The question arises then as to what effect an antiauxin will have on growth in the presence of inhibitory auxin concentrations.

It is evident from data on growth in the presence of antiauxins, as recorded in tables of this text and of Appendix I, that in no instance did the antiauxin at concentrations tested have any significant effect on auxin-induced growth inhibition. Bonner (67) found that 2,4-DCA did not alleviate growth inhibition at inhibitory auxin concentrations but rather it increased the inhibition. Similarly, van Overbeek et al. (73) have shown that the antiauxin trans-cinnamic acid, at a concentration of 30 mg./l. displays toxicity symptoms in the presence of relatively high auxin concentrations but not in the absence of auxin. Leopold and Klein (76), anticipating that an antiauxin should overcome inhibitory effects of high auxin concentrations, reported that growth inhibition resulting

from high IAA and NAA concentrations was alleviated by maleic hydrazide. The intimation that an antiauxin should overcome inhibition brought about by inhibitory auxin concentrations has also been made by Audus and Shipton (91).

The hypotheses that an antiauxin is an auxin-like molecule so modified that it is capable only of a single point attachment to the auxin-receptor site within the plant and that bimolecular complex formation predominates at high auxin concentrations require that growth inhibition brought about by high auxin concentrations will be enhanced by the presence of an antiauxin. Both represent mechanisms for the sequestering of auxin-receptor in the form of inactive complexes. The present section of this investigation deals then with the effect of antiauxins on Avena section growth in the presence of inhibitory auxin concentrations.

Results

The data presented on a logarithmic scale in fig. 39 show the effect of 4-CPIB on Avena section growth in the presence of a ten thousand fold concentration range of IAA. The upper curve of fig. 39 is that for growth in the presence of IAA alone and the lower curves are those for growth in the presence of IAA together with increasing concentrations of 4-CPIB. As is to be expected, from previous data, 4-CPIB inhibits coleoptile section growth at sub-optimal IAA concentrations. It is apparent also that the growth inhibition at supra-optimal IAA concentrations is increased by the presence of 4-CPIB (10 and 50 mg./l.). It is also evident that growth rate in the

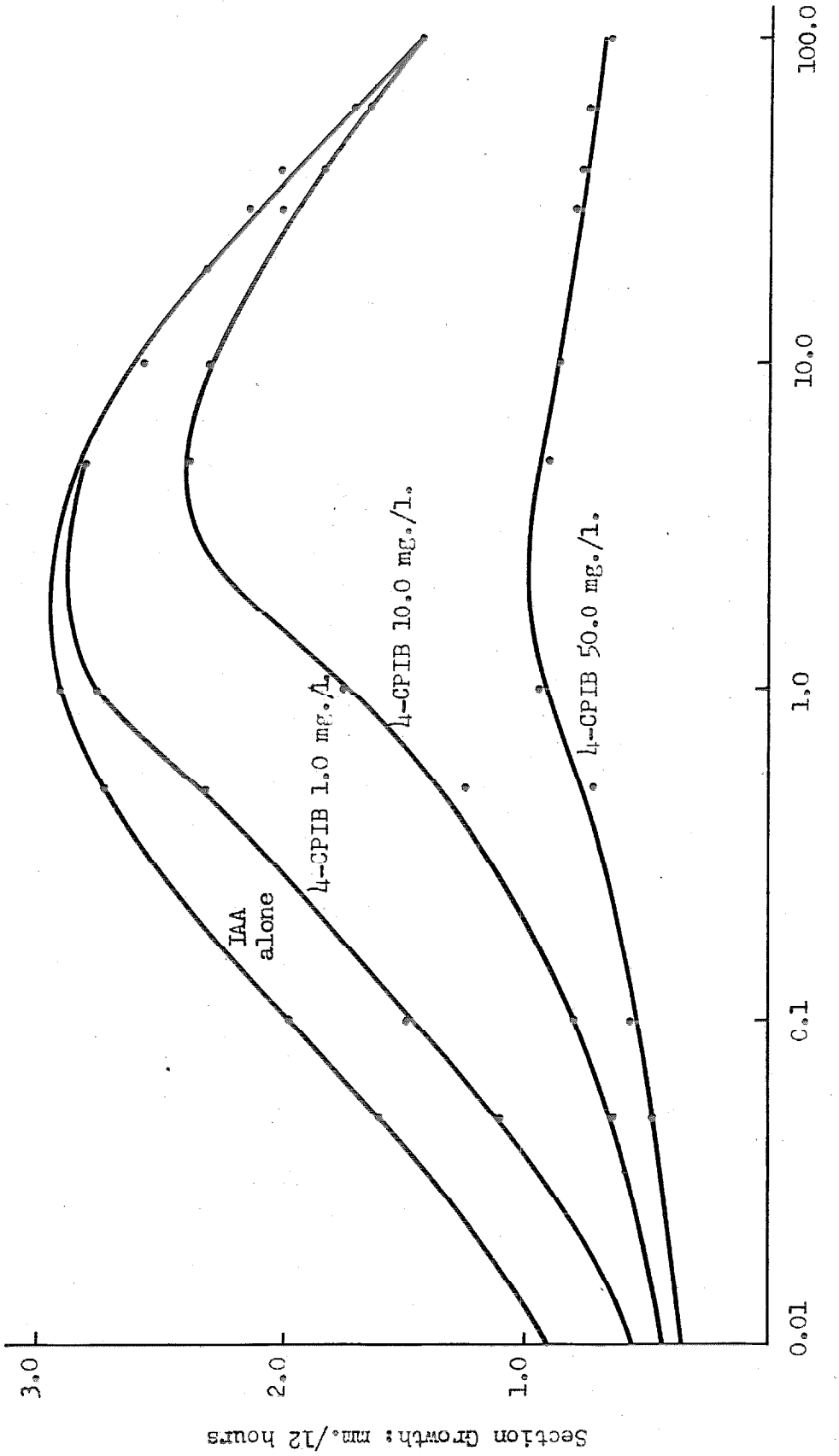


Figure 39. INHIBITION OF IAA INDUCED AVENA SECTION GROWTH BY 4-CPIB. THE UPPER CURVE IS THAT FOR IAA ALONE AND THE LOWER CURVES ARE THOSE FOR INCREASING CONCENTRATIONS OF 4-CPIB IN THE PRESENCE OF IAA.

presence of 10 and 50 mg./l. 4-CPIB approaches that for IAA alone at the high IAA concentrations. It may also be noted that in the presence of 10 mg./l. 4-CPIB, the growth optimum is shifted toward higher IAA concentrations. In the presence of 50 mg./l. 4-CPIB however the growth optimum shift is not so apparent. Finally, growth rates in the presence of 1 mg./l. 4-CPIB and IAA concentrations above 5 mg./l. (data not included in fig. 39) are not significantly different from growth rates in the presence of IAA alone. It is evident from the data presented above that the antiauxin, at sufficiently high concentrations, augments the inhibition induced by inhibitory concentrations of IAA.

The data of fig. 40 show that maleic hydrazide (50 mg./l.), like the antiauxin 4-CPIB, inhibits growth induced by low auxin concentrations and enhances the inhibition brought about by supra-optimal auxin concentrations. Maleic hydrazide, at concentrations of 1 and 10 mg./l., was also tested in this experiment. These concentrations however had no significant effect on section growth rate with 2,4-D concentrations between 1 and 100 mg./l.

Discussion

The data presented above, showing that the antiauxins 4-CPIB and maleic hydrazide enhance the growth inhibition caused by supra-optimal auxin concentrations, integrate and substantiate the single point attachment antiauxin concept and the bimolecular complex formation concept for inhibitory auxin concentrations. The fact that 4-CPIB causes a greater enhancement of inhibition in the presence of

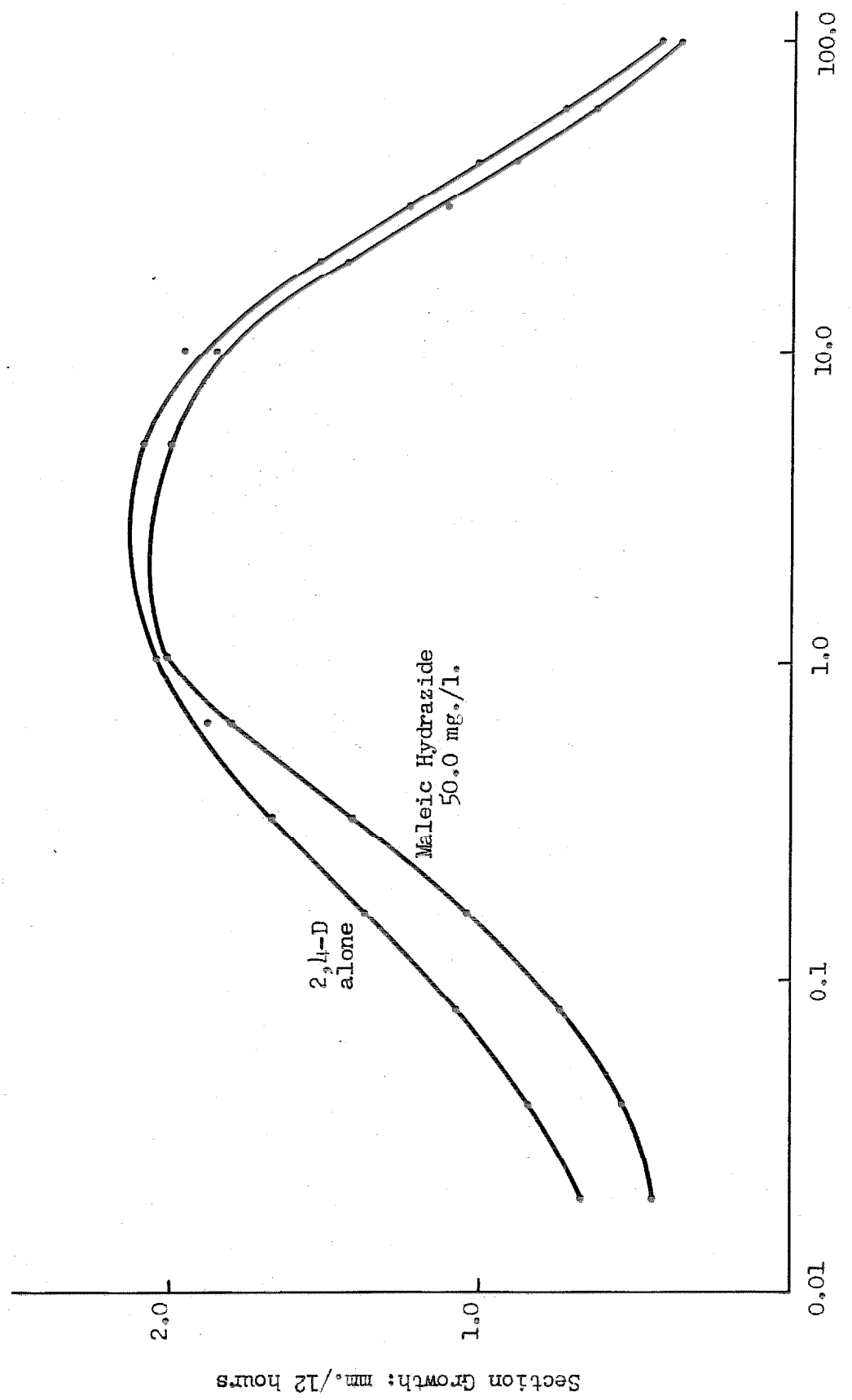


Figure 40. INHIBITION OF 2,4-D INDUCED AVENA SECTION GROWTH BY MALEIC HYDRAZIDE. THE UPPER CURVE IS THAT FOR 2,4-D ALONE AND THE LOWER CURVE IS THAT FOR MALEIC HYDRAZIDE IN THE PRESENCE OF 2,4-D.

supra-optimal auxin concentrations than does maleic hydrazide is compatible with the differences in the constants K_I for these two compounds. The K_I for 4-CPIB is approximately 3×10^{-6} M whereas the K_I for maleic hydrazide is between 3×10^{-5} and 3×10^{-4} M. Because of the anomalous behavior of maleic hydrazide, as is evident from the data of fig. 20, only a rough approximation of K_I for this compound may be obtained. Since the affinity of 4-CPIB for the auxin-receptive sites is from 10 to 100 fold greater than the affinity of maleic hydrazide for the same sites, it is to be expected that the additive inhibitory effects of 4-CPIB to supra-optimal auxin inhibition should be greater than those for maleic hydrazide.

A quantitative treatment for the behavior of a competitive inhibitor in the presence of a two-point attachment substrate is available (107). This treatment has not been applied to the present experiments because the available data are insufficient to justify it. It may be stated however that the quantitative treatment of Foster and Niemann (107) predicts that rates in the presence of the competitive inhibitor will approach the rate for substrate alone at inhibitory substrate concentrations. In addition, the treatment predicts that the optimum rate in the presence of inhibitor will shift toward higher substrate concentrations as the inhibitor concentration is increased. That the present data are at least in qualitative agreement with the above predictions, is evident from fig. 39.

In view of the results herein obtained with maleic hydrazide, it is of interest to inquire into the reason for the discrepancy

between the maleic hydrazide results obtained in this investigation and the results reported by Leopold and Klein (76). As mentioned earlier, Leopold and Klein, using the split pea test, found that maleic hydrazide alleviated growth inhibition resulting from high IAA and NAA concentrations. Leopold and Klein did not however use pH control in their split pea test experiments, since the strongly buffered solutions required resulted in flaccid plant material and unreliable results. As a substitute for pH control in the pea test, Leopold and Klein carried out a pH controlled experiment using pea root sections. They found that maleic hydrazide was able to overcome IAA induced inhibition of pea root section growth and concluded that alleviation of the auxin inhibition of growth by maleic hydrazide was independent of pH. It is quite clear however that the pea root section experiment has no bearing whatsoever on the split pea test. The conclusion which must therefore be reached is that the apparent maleic hydrazide alleviation of the inhibition resulting from high auxin concentrations, as observed by Leopold and Klein, might well have been due to a pH effect.

Summary

The growth inhibition resulting from high auxin concentrations is not alleviated by antiauxins but rather the auxin inhibition is augmented by the presence of sufficiently high antiauxin concentrations. A necessary corollary to the single point attachment antiauxin concept and the bimolecular complex formation concept for inhibitory auxin concentrations is therefore confirmed.

APPLICATION OF KINETICS TO THE STUDY OF AUXINS AS HERBICIDES

Introduction

Although the large variety of symptoms which result from application of many chemically different auxins to whole plants or plant organs and tissues have been intensively investigated during the past decade, an explanation in biochemical terms has not been found which satisfactorily accounts for these symptoms. That this is so is evident from the number of recent reviews which have brought together and integrated the tremendous body of facts pertinent to the multiplicity of auxin actions. Of utmost interest in recent years is that aspect of auxin activity, the herbicidal or weed-killing action, which brings about profound morphological, physiological and biochemical changes which ultimately cause death of the plant. In spite of intensive investigation in the herbicide field, it is generally conceded (109) that in no instance is there sufficient evidence available, relative to auxin-like herbicides, which permits a detailed description of the biochemical sequence of events which lead to the death of a plant. Nevertheless some suggestions have been made to account for the herbicidal action of auxins.

The most prominently held views concerning herbicidal action of auxins are those which pertain to changes in the level of metabolites such as carbohydrates, amino acids and proteins and to changes in respiratory levels (11, 15, 38, 40, 109-114). According to this school of thought, the auxin molecule interferes with one to many

enzymatic processes, an aberrant metabolism ensues, and brings about the eventual death of the plant. It has also been suggested (115, 116, 117) that auxins impede normal phloem function and transport of food and thereby cause death. Interference with the native auxin of the plant has also been proposed (7, 118, 119) as an explanation for herbicidal action. The fact that the naturally occurring coumarin derivative, scopoletin, increases in plants after 2,4-D treatment, has led to the suggestion (112) that toxicity brought about by increased scopoletin concentrations is the direct cause of the herbicidal action of 2,4-D. It has also been stated (8) that growth inhibitions of the herbicide type are not associated with an auxin-like structure but rather are more closely allied to the preparatory activity of hemiauxins. Ideas which are associated with the significance of growth inhibition at supra-optimal auxin concentrations (3, 7, 9, 14, 77) will be discussed below.

It was suggested in a preceding section of this investigation that formation of an inactive bimolecular auxin-receptor complex at high auxin concentrations might well account for the herbicidal action of 2,4-D and related synthetic auxins. It was further intimated that an appropriate approach to this problem might be to determine the extent to which herbicidal activity is a function of the second power rather than the first power of auxin concentration. Since it is difficult to obtain data expressing degree of killing in quantitative terms, it was decided to try and obtain indirect evidence which might support the bimolecular complex

formation concept of herbicidal action. This section of the investigation presents some preliminary experiments in this direction.

In the preceding section of this investigation it was shown that growth inhibition of the Avena coleoptile section resulting from high auxin concentrations was augmented by the presence of an antiauxin. It is to be expected then that if herbicidal action is a consequence of bimolecular complex formation, the killing of plants by an auxin such as 2,4-D should be enhanced by simultaneous application of an antiauxin.

Experimental and Results

A preliminary experiment, using 25 day old Red Kidney bean plants, was carried out in Dolk greenhouse in the fall of 1952. The trifoliate leaves of each plant were dipped into 2,4-D solutions alone, 4-CPIB solutions alone, or combinations of both compounds at concentrations indicated in fig. 41. The solutions contained a concentration of 4 drops/l. of Dow polyglycol 31 as wetting agent. In this experiment, in which the plants were not too uniform, seven plants were used per treatment. The photographs of fig. 41 were taken eight days after treatment. Up to a concentration of 100 mg./l. (treatments 6, 11, and 16), the antiauxin had no visible effects on the plants. 2,4-D treatment at the two lowest concentrations (treatments 2 and 3) caused little permanent injury to the plants. At 30 mg./l. 2,4-D (treatment 4)

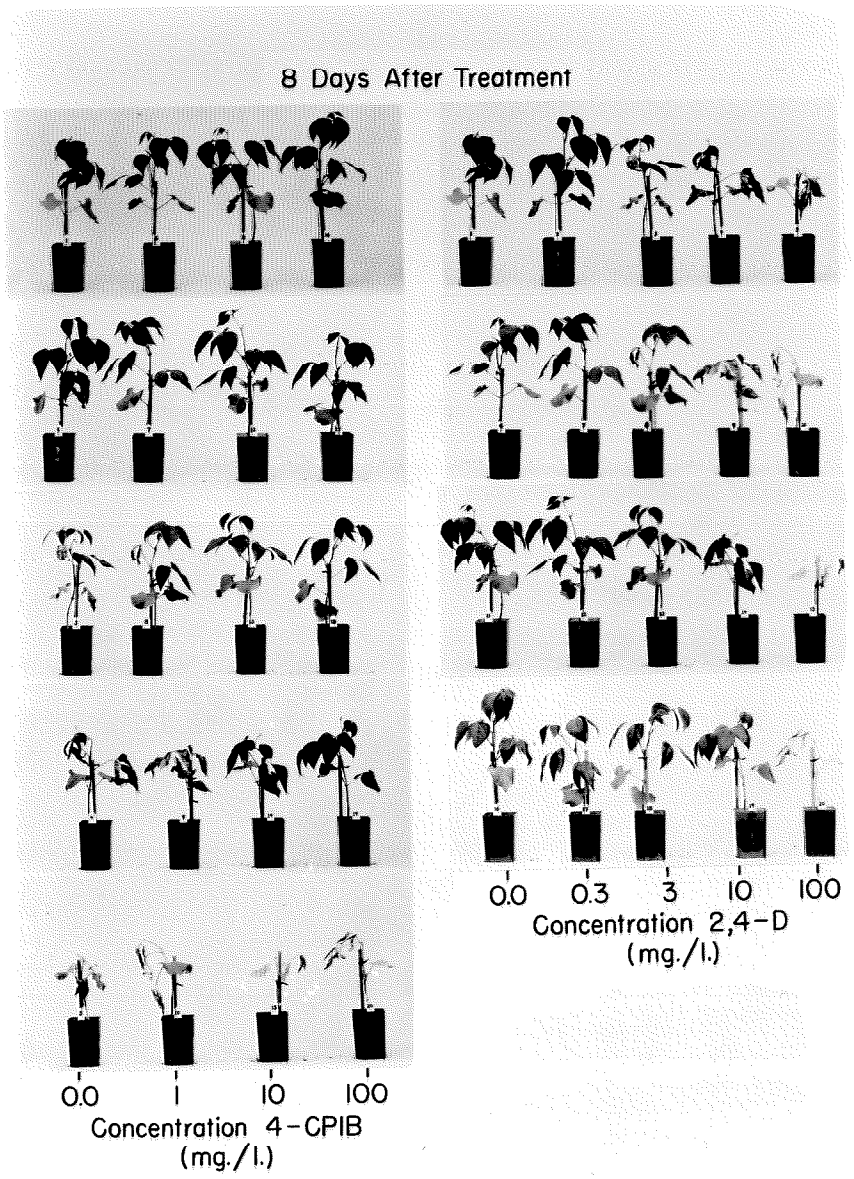

8 Days After Treatment
0.0
1
10
100
Concentration 4-CPIB
(mg./l.)
0.0
0.3
3
10
100
Concentration 2,4-D
(mg./l.)

Figure 41.

the plants were severely damaged but by the end of ten days meristems had recovered and new growth was starting to take place. The plants treated with 100 mg./l. 2,4-D (treatment 5) however showed little sign of recovery. It is further evident from fig. 41 that treatment with 4-CPIB augmented the injury symptoms of the lethal dose of 2,4-D (treatments 10, 15, 20).

Following this preliminary experiment, a second and more extensive experiment was carried out at Orlando greenhouse. Seventeen day old Red Kidney bean plants were treated with 2,4-D or 4-CPIB alone or in combination at concentrations shown in fig. 42 and 43. Dow polyglycol 31 was added to each solution and all were adjusted to pH 4.5. Primary leaves as well as trifoliate leaves of eight uniform plants were dipped in the appropriate solutions for each of the 35 different treatments. An additional treatment in which the trifoliate leaves only were dipped in 4-CPIB at a concentration of 1000 mg./l., was also included in the experiment.

The data presented in fig. 42 and 43 show that the results of this experiment, up to concentrations of 100 mg./l. 2,4-D and 100 mg./l. 4-CPIB, are similar to those of the first experiment. 4-CPIB alleviated the damage symptoms caused by 2,4-D concentrations below 30 mg./l. (treatments 11, 12, 18 and 19) and augmented the killing effects of 30 and 100 mg./l. 2,4-D (treatments 13, 14, 20 and 21). At 4-CPIB concentrations of 300 and 1000 mg./l. (treatments 23 and 30), the antiauxin induced localized injury. The trifoliate leaves were wrinkled and chlorotic, the primary leaves showed necrotic

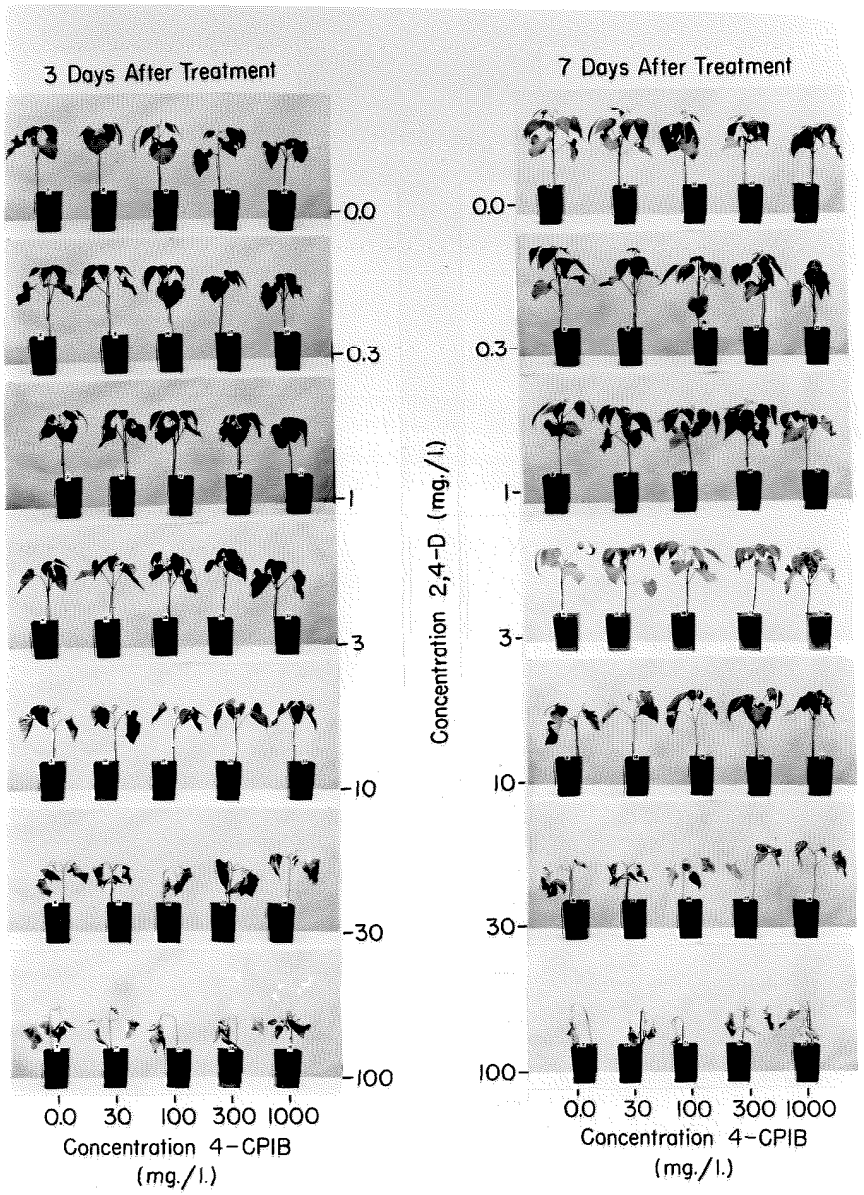

3 Days After Treatment
7 Days After Treatment
0.0
0.3
1
3
10
30
100
Concentration 2,4-D (mg./l.)
0.0 30 100 300 1000
Concentration 4-CPIB
(mg./l.)

Figure 42.

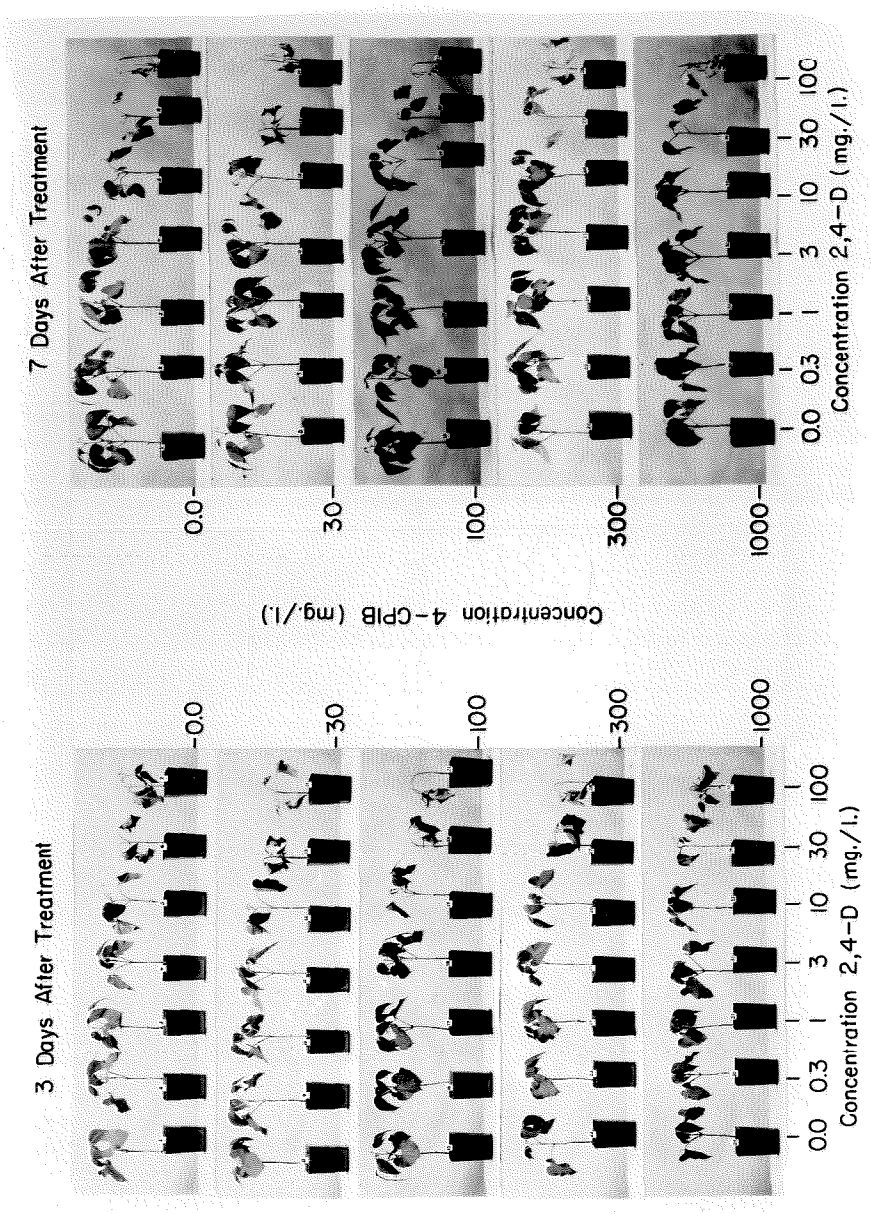

3 Days After Treatment
7 Days After Treatment
Concentration 4-CPIB (mg./l.)
0.0
30
100
300
1000
0.0 0.3 1 3 10 30 100
Concentration 2,4-D (mg./l.)

Figure 43.

areas, and the plants were stunted. There was no evidence of systemic injury. Absence of systemic injury was also apparent in those plants which received only trifoliate leaf treatment with the 4-CPIB concentration of 1000 mg./l. The trifoliate leaf symptoms were the same as those described above but primary leaves showed no visible injury. It is also evident from fig. 42 and 43 that 4-CPIB at concentrations of 300 and 1000 mg./l. alleviated sub-lethal doses of 2,4-D (treatments 25, 26, 27, 32, 33 and 34). The antiauxin, furthermore, alleviated the lethal effects of 100 mg./l. 2,4-D (treatments 28 and 35).

Preliminary work has been carried out in another type of experiment in an attempt to obtain further insight into the biochemical significance of herbicidal action. As mentioned previously, since it is difficult to express degree of killing of a whole plant in quantitative terms, it was considered possible that satisfactory data might be obtained from portions of leaves rather than the whole plant. Experiments have therefore been carried out using leaf discs from tobacco, cotton, bean and cocklebur plants. The data obtained thus far have been too variable to warrant their presentation. It may be stated, however, that leaf discs, floating on solutions containing 2,4-D at low concentrations, exhibit an increase in fresh weight whereas leaf discs, floating on solutions containing high concentrations of 2,4-D, show a decrease in fresh weight.

Discussion

It is apparent from the results presented above that the anti-auxin 4-CPIB alleviates the symptoms induced by low concentrations of 2,4-D but (under certain circumstances) enhances the killing effect of sub-lethal doses of 2,4-D. This fact is therefore consistent with the notion that inactive bimolecular auxin-receptor complex formation at high auxin concentrations is a factor in the herbicidal action of 2,4-D. Furthermore, the results obtained are in qualitative agreement with results reported by Hitchcock and Zimmerman (120). Hitchcock and Zimmerman obtained additive and antagonistic effects with combined treatments of 2,4-D and maleic hydrazide applied to tomato, sunflower, zinnia and snap bean plants.

The question which immediately arises is whether or not the amount of 2,4-D used for weed control is sufficiently high to lead to a concentration within the plant such that inactive bimolecular auxin-receptor complex formation predominates over active auxin-receptor complex formation. That inactive bimolecular complex formation within the plant resulting from herbicidal application of 2,4-D is possible is evident from the following considerations. An application of one kilogram 2,4-D (approximately five moles) per acre is sufficient to kill susceptible plants. There are of the order of one to ten tons of plant, wet weight, per acre and this is equivalent to a maximum of 10^3 to 10^4 liters of plant. The concentration of 2,4-D within the plant may therefore be of the order of 5×10^{-3} to 5×10^{-4} M. That bimolecular complex formation

predominates at such concentrations is evident from data presented previously. Since these considerations are compared to the Avena coleoptile section experiments, it would appear that monocotyledonous plants should also be killed by 2,4-D. It has been suggested (121) however that selective action toward dicotyledonous plants by 2,4-D like herbicides may be due to the fact that these compounds are not translocated readily in monocotyledonous plants. Thus concentrations required for bimolecular complex formation may not be attained in non-susceptible monocotyledonous plants.

In contrast to the concentrations of 2,4-D used for weed-killing, we find that considerably lower concentrations of auxin (10-50 grams per acre) are used for such physiological responses as promotion of flowering and prevention of fruit drop (10). Concentrations of 10-50 grams per acre, then, are of the order of 2.5×10^{-5} to 5.0×10^{-5} M within the plant. It is apparent from data presented previously that this concentration range is near the peak of the auxin activity curves. It is not unreasonable then to expect that while bimolecular complex formation may be a factor in the herbicidal action of 2,4-D, the positive effects such as inhibition of abscission may be due to the doubly attached monomolecular complex.

It is not immediately clear why an antiauxin may either enhance or alleviate the herbicidal effects of 2,4-D. It is suggested, however, that not only is the antiauxin translocated slowly within the plant but that it also interferes with the translocation of 2,4-D. Thus if the 2,4-D concentration is high, relatively low antiauxin

concentrations will not greatly interfere with translocation of 2,4-D and additive effects may be obtained. On the other hand, if the antiauxin concentration is high, translocation of 2,4-D is interfered with to such an extent that systemic herbicidal symptoms are alleviated.

Summary

A preliminary investigation concerning herbicidal activity of mixtures of an antiauxin and an auxin on Red Kidney bean plants is presented. Although the data obtained do not unequivocally establish that inactive bimolecular auxin-receptor complex formation at high auxin concentrations is a factor contributing to herbicidal action of 2,4-D, the possibility that this is in fact so is considered.

DISCUSSION

We have seen from the present investigation that in order for a substance to be active as an auxin it must be capable of consummating a two-point attachment to some receptive entity within the plant. The two functional groups through which this attachment is made appear to be the carboxyl group and a position in the cyclic nucleus ortho to the side chain carboxyl group. It is not immediately apparent that auxins have in common any other functional group which may be involved in the auxin-induced growth reaction. Furthermore, there is no evidence which is not consistent with the notion that the mere act of becoming two-point attached is sufficient to confer activity upon auxin molecules.

If it is the act of consummating two-point attachment which determines auxin activity, the question then arises as to why it is that various auxins possess markedly different V_{max}'s. An answer to this question may be found from the following considerations. The data presented in this investigation suggest that of every ten 2,4-D molecules present in the equilibrium mixture, within the coleoptile section, seven molecules are two-point attached while the remainder are single point attached. With an active auxin, such as 2,4-D, the energy required to complete the second point of attachment, once the first point of attachment is made, is relatively low. For the weak auxin PBA, on the other hand, only three molecules are two-point attached for every ten molecules present in the equilibrium

mixture and the energy required to consummate two-point attachment is considerably higher than that for 2,4-D. The evidence which supports this viewpoint is to be found in the fact that PBA, as an inhibitor, may have an apparent K_I which is considerably lower than its K_S, as an auxin. It may be tentatively concluded, therefore, that all auxins, once they are two-point attached may be equally active in promoting growth, and that the observed differences in V_{max} for various auxins are an expression of the proportions of two-point attached and of single point attached molecules in the equilibrium mixture.

The concept that auxin is involved in a chemical reaction which leads to growth is not a new one. Early investigations (see 39) have shown that there is a stoichiometric relationship between auxin and some substrate within the plant. Evidence for such a relationship has been deduced from the facts that there is a direct relationship between growth and the amount of auxin which disappears during cell elongation (122) and that an essentially direct proportionality exists between auxin concentration (linear or logarithmic) and growth. Further evidence that a stoichiometric relationship exists between auxin and some substrate within the cell has been based on the fact that, in some instances, when suitable corrections are made (123) different auxins possess the same molar activity in the growth reaction. Veldstra (3) has found, however, that acids derived from naphthalene possess different molar activities in the pea test even though corrections are made for the dissociation of

the acids. Similarly, van Overbeek et al. (73) have found that there is no correlation between auxin activity, in pea curvature or pea section tests, and molar concentrations which are corrected for the dissociation of the acids. That corrections for the degree of ionization of auxin active molecules of the phenoxyacetic acid series do not account for their relative growth activities in the Avena section test has been shown by Muir et al. (20). Audus (124) also failed to find a correlation between dissociation of auxins and their root elongation inhibitory activity. It is evident from the present investigation also that a correction for the degree of ionization of the auxins studied is not sufficient to account for the observed different activities.

Skoog et al. (77) have presented theoretical considerations which attempt to account not only for the varying physiological activities of different auxins but also to account for both the behavior of interacting auxins and inhibitions caused by high auxin concentrations. Skoog et al. based their considerations on the assumption that auxin acts as a coenzyme and the auxin active complex in turn reacts with a substrate. The considerations of Skoog et al. require that substances possess two properties which contribute to the physiological activity of auxins. First, the structural configuration must be such that the auxin molecule is able to occupy specific positions in a large molecular aggregate. Secondly, the auxin must have a specific chemically reactive group which interacts with the substrate. The relative activities of

different auxins, then, is visualized as being determined by their degree of affinity for the substrate and their degree of association with the apoenzyme-substrate complex. Skoog et al. (77) showed that the weak auxin PBA at low concentrations inhibits IAA induced growth of Avena coleoptile sections and that at higher PBA concentrations (but below inhibitory concentrations) growth was not inhibited. It is evident that the results which they obtained are similar to those obtained in this investigation. Skoog et al. account for the above behavior by assuming that PBA has the structural configuration essential to auxins but that it has low chemical reactivity and is therefore able to compete with the more active IAA for available positions in the enzyme-substrate system and thereby block the action of the more active auxin. It is apparent that while this hypothesis may account for PBA inhibition of IAA induced growth at low PBA concentrations, it is inadequate to account for the additive effects of IAA and higher concentrations of PBA.

Skoog et al. (77) also used the above hypothesis as a basis of an explanation for auxin-induced growth inhibition. They proposed that the auxin molecule may act as a sort of bridge between the appropriate enzymatic entity and a second molecule which forms the substrate of the enzyme. At higher auxin concentrations inhibition would be brought about according to this hypothesis by simultaneous saturation of both enzyme and substrate with consequent prevention of the formation of the active trimolecular complex. It is evident, however, from the present work that auxin-induced

growth inhibition is fundamentally a function of the second power of the auxin concentration. Corollaries to the Skoog et al. hypothesis which are required to make it fit the present experimental data are: (a) that the concentrations of enzyme and of the substrate must be approximately equal and both must be low, relative to auxin concentration, and (b) that the affinities of auxin for substrate and enzyme be approximately equal. An additional objection to the hypothesis of Skoog et al. is that it is difficult to visualize how auxins of low V_{max} can completely inhibit coleoptile section growth at lower concentrations than can high V_{max} auxins.

Skoog (7) subsequently extended the competition hypothesis and incorporated the idea that not only would the affinity of the auxin for the apoenzyme and substrate be of importance but also of significance would be the affinity of auxin for protein complexes containing bound native auxin. According to this view, auxins having high affinity for the protein complexes would displace the native auxin. As a consequence, release of native auxin would cause, at first, a stimulation of growth, and ultimately, an excessive loss of auxin might lead to loss of native auxin and in extreme cases would cause a complete destruction of the native auxin supplying mechanism. Skoog (7) suggested, as have others (118, 119), that this type of action may account for weed killing. According to the above hypothesis then, a weak auxin is incapable of actively displacing the native auxin and therefore growth promotion induced by such low affinity compounds is small. It is evident again, however,

that this concept cannot account for the fact that auxins of low V_{max} induce complete inhibition of section growth at lower concentrations than the active auxins which supposedly can readily displace the native auxin.

Let us now consider other mechanisms of auxin action which have as their basis a reaction involving three components or a two step reaction and attempt to determine if the data, upon which such mechanisms have been proposed, do in fact demand a trimolecular or two step reaction. Thimann (14) has suggested that auxins are substances which protect an enzyme, such as succinic dehydrogenase, from a naturally occurring inhibitor. Thus, it is proposed that in the absence of auxin the inhibitor occupies the active site on the enzyme and thereby prevents the substrate, essential for growth, from occupying the site. In the presence of auxin, however, the inhibitor is excluded from the active site because the auxin molecules have greater affinity than the inhibitor molecules for the active sites. On the basis of this interpretation it is immediately apparent that auxin, even at low concentration, should inhibit growth and not promote growth. For, if the auxin molecule prevents the inhibitor from occupying the active site on the enzyme, then it must also block the substrate from the active site; and since the reaction which the substrate undergoes is essential for growth, then growth must be inhibited and not promoted by auxin. Since according to the above view auxin should inhibit growth at all concentrations, Thimann's suggestions concerning auxin-induced growth

inhibition at high concentrations and its relationship to herbicidal activity are not consequences of his hypothesis and therefore need not be considered further.

It is of interest next to consider the views on physiological activity of auxins and mechanism of auxin action which have been presented by Veldstra and his collaborators (2, 3, 9, 10, 17, 18, 19). Since Veldstra presented his first hypothesis on mechanism of auxin action and physiological activity of auxins, it has undergone frequent revision and it is therefore difficult to present his views in a unified theory. For this reason most of the details of the investigations will be omitted and only the major features of Veldstra's ideas will be considered. Veldstra's first hypothesis stressed the view that the function of auxin is a physico-chemical influencing of a boundary which results in an altered permeability of cell membranes. Auxin was visualized as being adsorbed to the boundary with the ring system influencing permeability while the carboxyl group contributed to the physiological action proper with the possibility of a chemical reaction, in the usual sense, being ruled out. This hypothesis was based, in part, on polarographic studies which showed that there was a correlation between physiological activity and adsorbability of some auxins. Veldstra postulated that auxins at low concentrations exert an opening effect on the membrane and at high concentrations they have a condensing effect on the membrane. Veldstra considered that the condensing effect (decreasing permeability) would account for growth inhibition at high auxin concentrations and might also

account for herbicidal activity of compounds such as 2,4-D. Upon examining the hypothesis, using oleate coacervates as a model membrane and beet cells as a physiological membrane, Veldstra found that auxins exerted a turgescent effect only regardless of the auxin concentration. Furthermore, he found that there was an inverse correlation between degree of physiological activity and turgescent effect. These observations invalidate the original deduction but nonetheless Veldstra stayed with the idea that auxins exert their effects on the membrane but conceded to the view that auxin may also have some influence within the cell. This latter extension to his views permitted Veldstra to account for the fact that many compounds, such as 2,4,6-trichlorophenoxyacetic acid, were inactive as auxins even though they were more lipophilic than 2,4-D and similar active compounds. Subsequently Veldstra proposed that there must be a fairly precise balance between the hydrophilic and lipophilic parts of the auxin molecule. Thus, as the lipophilic character is increased to a point, activity is also increased; as the lipophilic character increased still further, however, auxin activity decreased. As a consequence, the compound strongly adsorbed to the cell membrane could not get inside the cell in concentrations sufficiently high for the primary reaction to take place. Furthermore, Veldstra stated that too strong an adsorption might induce an opening effect which in turn could disturb the normal equilibrium between the protoplasm and the outside of the cell. Veldstra further makes the assumption in his two-step reaction of auxins

that most of the auxin is adsorbed to the membrane and only a small amount of the total auxin is required for the primary reaction within the cell.

The question with which we are now confronted is whether or not Veldstra's adsorption theory is to be accepted in preference to a two-point attachment chemical reaction concept. As was mentioned above, the primary precept upon which Veldstra's theory is based is the fact that he found a correlation between physiological activity and surface activity with a few auxins. Paleg and Muir (28), however, have shown that there is no correlation of surface activity with physiological activity of 25 compounds tested in the Avena section test. Furthermore, according to the Veldstra hypothesis, a compound such as 2,4,6-TCPA which has greater affinity for the membrane than has 2,4-D should displace 2,4-D and make 2,4-D more available for the primary reaction inside the cell. 2,4,6-TCPA should therefore act synergistically with 2,4-D. It is evident from the present investigation that this is not so, in fact 2,4,6-TCPA inhibits 2,4-D induced growth. It would appear then, that the Veldstra theory on mechanism of auxin action, if at all valid, is not valid insofar as Avena section growth is concerned. In fact, a rather careful survey of the compounds which Veldstra has tested in his investigations reveals that his results provide more support for a two-point attachment concept than for his own theory.

Thus far we have seen that the two-point attachment concept, involving a chemical reaction between the auxin and some site within

the plant, satisfactorily accounts for the mechanism of auxin action without the requirement of either a third hypothetical substrate or a second step in the reaction. There is however another two separate step reaction theory for mechanism of auxin action which is apparently contradictory to the results of the present investigation. Went (see 125) has advanced the hypothesis that in the growing Avena coleoptile the decreased growth rate of the basal regions is due to an auxin deficiency whereas a factor, subsequently called "food factor," which comes from the seed or roots limits the growth rate of regions nearest the tip. According to this theory, auxin, in addition to exerting its primary action within the plant, also influences the transport of the food factor (a facilitation or preparatory reaction). Thus auxin applied to the tip of an Avena coleoptile attracts to it the food factor with which auxin must cooperate to cause growth. Adverse criticism of this hypothesis led Went to examine it experimentally. In the first series of experiments, Went (125) stated that only one type of experiment (par. 7) provided evidence for the hypothesis that growth of the tip zone was limited by the food factor. It is of interest therefore to consider this experiment and to determine whether or not an alternative explanation may be found to account for the apparent food factor requirement. Went (125) measured the growth distribution in an Avena coleoptile over consecutive 2, 4 and 10 hour periods. There were three 2 hour periods, two 4 hour periods, and one 10 hour period making a total observation period of 24 hours. It was found

that there was a gradual drop in the growth rate over all zones of the coleoptile (about 40mm. final length). Next, lanolin paste containing IAA was applied to the tip of a coleoptile, at the end of the second two-hour period, and the growth distribution was measured as previously. The concentration of auxin used was described as "excess auxin" (2.5 mg./gr. lanolin). Following the auxin application (i. e. after the third 2 hour period) it was found that there was a marked increase in the growth of the highest zone (upper 20mm.) while the lower zones grew at the control rate. In the successive periods, however, the growth rate of the upper zone dropped off with time and the growth rate of lower zones remained constant. The interpretation which was given to account for the above behavior was that the food factor was used up in the lower zones during its upward transport and therefore became limiting with time in the upper zone. It is apparent from the above description of the experiment that excess auxin applied to the tip of the coleoptile might well result in the following sequence of events:

1. auxin diffuses slowly into the upper zone of the coleoptile and induces the typical auxin growth promotion response,
2. with increasing time, however, an inhibitory concentration of auxin accumulates in the upper zone of the coleoptile and bimolecular complex formation brings about a decreased growth rate,
3. transport of auxin to the basal region is insufficient for bimolecular complex formation.

It may be seen that the above explanation may satisfactorily account for the results obtained by Went.

Later, Went (126) carried out another series of experiments with the Avena coleoptile and obtained data which suggested that the auxin-induced growth reaction proper was preceded by a preparatory reaction. Again the preparatory reaction consisted in an attraction of food factor toward the point of application of auxin. Went (126) found that if intact Avena coleoptiles were treated with weak auxins such as PBA or phenylacetic acid by application in lanoline to the tip, inhibition of growth occurred. Furthermore, if intact coleoptiles were pretreated for two or three hours with the above mentioned auxins and then cut into three or four sections and placed in IAA solution, it was shown that there was an increase (over controls pretreated with pure lanoline) in growth of the upper sections and a decrease (usually) in the growth of the basal sections. As a consequence of the above behavior, it was concluded that auxins, in general, have a dual effect; first, they redistribute other growth factors, and secondly, they react with these factors and cause cell elongation. This general statement was made in spite of the fact that pretreatment with IAA caused a marked inhibition of the growth of apical sections when subsequently transferred to IAA solution. Although the conclusion which Went has reached as a result of the above experiments is a reasonable one (with the exception of IAA pretreatment behavior), it is not necessarily a unique conclusion. A detailed examination of Went's

data reveals that they may be interpreted on the basis of the interaction experiments, between an auxin of low V_{max} with a high V_{max} auxin, presented in this investigation and similar experiments by Skoog et al. (77). That is to say, an auxin of low V_{max} may inhibit or augment the growth induced by a high V_{max} auxin (native or synthetic), the nature of the response being dependent upon the relative concentration of each auxin. The foregoing considerations, therefore, provide further evidence that insofar as auxin-induced cell elongation of the Avena coleoptile is concerned, it is not necessary to postulate a dual effect of auxin. Went (127) has shown, however, using the pea test, that the sensitivity to auxins may be increased by pretreating split pea stems with compounds closely related to, but not active as, auxins. This increased sensitivity was considered to lend support to the contention that auxins exert a dual effect in the growth reaction. It has been pointed out (128), however, that although Went has demonstrated the existence of two separate reactions in the pea curvature test, it does not necessarily follow that the reactions are part of a chain reaction. Furthermore, Skoog (77) has suggested that the preparatory reaction, which sensitizes split pea stems to auxins, may be due to an auxin-sparing action on the part of the preparatory substances (hemiauxins) and, there does not appear to be any evidence which might eliminate such a possibility. The existence of a dual effect of auxin as a chain reaction, as deduced from the behavior of hemiauxins in the split pea curvature test, has not necessarily been proven.

It would appear from the above considerations that the reaction between two functional groups of an auxin molecule and the receptive entity within the plant satisfactorily accounts for and describes the kinetic and structural specificity aspects of the phenomenon of auxin-induced cell elongation. We have seen, further, that the presence of a finite minimum number of two-point attached auxin molecules appears to be essential not only to normal plant growth but also even to the continued life of plant cells. If two-point attachment of auxin molecules is reduced below this lower limit by, for example, high auxin or antiauxin concentrations, the inevitable consequence is death of plant tissues.

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APPENDIX I

TABLES OF DATA

TABLE 1

EFFECT OF 2,4-DCPIB ON IAA INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 2,4-DCPIB	Concentration of IAA mg./l. 0.00	0.02	0.05	0.1	0.5	1.0	10.0
	Growth in absence of IAA	Growth in presence of IAA minus growth in absence of IAA					
mg.l.	mm./section/12 hrs.	mm./section/12 hrs.					
0.0	0.44	0.61	1.14	1.53	2.18	2.46	2.12
0.1	0.41	0.53	1.01	1.41	2.10	2.43	2.14
0.5	0.41	0.44	0.85	1.22	2.00	2.40	2.12
1.0	0.43	0.35	0.69	1.08	1.92	2.34	2.05

TABLE 2

EFFECT OF 2,4-DCPIB ON 2,4-D INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 2,4-DCPIB	Concentration of 2,4-D mg./l. 0.00	0.025	0.05	0.1	0.5	1.0	10.0
	Growth in absence of IAA	Growth in presence of 2,4-D minus growth in absence of 2,4-D					
mg./l.	mm./section/12 hrs.	mm./section/12 hrs.					
0.0	0.40	0.33	0.55	0.80	1.06	1.65	1.64
0.1	0.44	0.26	0.47	0.74	0.98	1.53	1.56
0.5	0.43	0.19	0.33	0.56	0.83	1.48	1.55
1.0	0.44	0.14	0.25	0.44	0.76	1.40	1.55

TABLE 3

EFFECT OF 2,6-DCPA ON IAA INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 2,6-DCPA	Concentration of IAA mg./l. 0.00 Growth in absence of IAA	0.02	0.05	0.1	0.5	1.0	10.0
		Growth in presence of IAA minus growth in absence of IAA					
mg./l.	mm./section/12 hrs.	mm./section/12 hrs.					
0.0	0.44	0.61	1.14	1.53	2.18	2.46	2.12
0.1	0.41	0.53	1.01	1.41	2.10	2.43	2.14
0.5	0.41	0.44	0.85	1.22	2.00	2.40	2.12
1.0	0.43	0.35	0.69	1.08	1.92	2.34	2.05

TABLE 4

EFFECT OF 2,6-DCPA ON 2,4-D INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 2,6-DCPA	Concentration of 2,4-D mg./l. 0.00	0.02	0.05	0.1	0.5	1.0	10.0
	Growth in absence of 2,4-D	Growth in presence of 2,4-D minus growth in absence of 2,4-D					
mg./l.	mm./section/12 hrs.	mm./section/12 hrs.					
0.0	0.42	0.29	0.59	0.78	1.39	1.58	1.60
0.1	0.42	0.22	0.47	0.71	1.34	1.58	1.59
0.5	0.43	0.17	0.37	0.60	1.27	1.52	1.57
1.0	0.42	0.14	0.30	0.50	1.18	1.48	1.53

TABLE 5

EFFECT OF 2,4,6-TCPA ON IAA INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 2,4,6-TCPA	Concentration of IAA mg./l.						
	0.00	0.02	0.05	0.1	0.5	1.0	10.0
	Growth in absence of IAA	Growth in presence of IAA minus growth in absence of IAA					
mg./l.	mm./section/12 hrs.	mm./section/12 hrs.					
0.00	0.41	0.62	L.15	1.48	2.19	2.47	2.16
0.1	0.40	0.50	0.95	1.33	2.08	2.44	2.10
0.5	0.40	0.37	0.73	1.09	1.95	2.40	2.08
1.0	0.41	0.29	0.62	0.95	1.85	2.31	2.06

TABLE 6

EFFECT OF 2,4,6-TCPA ON 2,4-D INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 2,4,6-TCPA	Concentration of 2,4-D mg./l.						
	0.00	0.02	0.05	0.1	0.5	1.0	10.0
	Growth in absence of 2,4-D	Growth in presence of 2,4-D minus growth in absence of 2,4-D					
mg./l.							
0.0	0.42	0.25	0.53	0.73	1.40	1.57	1.61
0.1	0.41	0.20	0.43	0.65	1.30	1.53	1.57
0.5	0.43	0.14	0.31	0.55	1.14	1.47	1.59
1.0	0.42	0.10	0.23	0.39	1.00	1.40	1.54

TABLE 7

EFFECT OF 2,4-DCA ON IAA INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 2,4-DCA	Concentration of IAA mg./l.						
	0.00	0.02	0.05	0.1	0.5	1.0	10.0
	Growth in absence of IAA	Growth in presence of IAA minus growth in absence of IAA					
mg./l.	mm./section/12 hrs.	mm./section/12 hrs.					
0.0	0.42	0.67	1.17	1.54	2.20	2.48	2.10
1.0	0.42	0.50	0.96	1.37	2.12	2.36	2.11
5.0	0.41	0.33	0.65	1.04	1.96	2.27	2.05

TABLE 8

EFFECT OF 2,4-DCA ON 2,4-D INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 2,4-DCA	Concentration of 2,4-D						
	0.00	0.02	0.05	0.1	0.5	1.0	10.0
	Growth in absence of 2,4-D	Growth in presence of 2,4-D minus growth in absence of 2,4-D					
mg./l.	mm./section/12 hrs.	mm./section/12 hrs.					
0.00	0.41	0.33	0.56	0.80	1.35	1.60	1.62
1.0	0.41	0.22	0.45	0.68	1.21	1.44	1.54
5.0	0.42	0.11	0.25	0.43	1.07	1.31	1.56

TABLE 9

EFFECT OF 2,4,6-TCPP ON IAA INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 2,4,6-TCPP	Concentration of IAA mg./l.								
	0.00	0.02	0.04	0.08	0.16	0.32	0.64	1.28	100.0
	Growth in absence of IAA	Growth in presenc of IAA							
mg./l.	mm./section/12 hrs.	mm./section/12 hrs.							
0.0	0.42	1.11	1.49	1.87	2.22	2.51	2.83	3.01	1.59
0.1	0.39	1.08	1.48	1.90	2.23	2.57	2.78	2.96	1.64
1.0	0.40	1.08	1.44	1.82	2.24	2.46	2.78	2.98	1.62
10.0	0.38	0.81	1.02	1.32	1.77	1.96	2.34	2.37	1.31
50.0	0.31	0.44	0.55	0.71	0.89	1.03	1.16	1.21	0.77

TABLE 10

EFFECT OF 2,4,6-TCPP ON IAA INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration of 2,4,6-TCPP	Concentration of 2,4-D mg./l.							
	0.00	0.02	0.04	0.08	0.16	0.32	0.64	1.28
	Growth in absence of 2,4-D	Growth in presence of 2,4-D						
mg./l.	mm./section/12 hrs.	mm./section/12 hrs.						
0.0	0.41	0.65	0.83	1.00	1.33	1.69	1.90	2.17
10.0	0.37	0.41	0.52	0.61	0.84	1.07	1.29	1.39
50.0	0.30	0.31	0.33	0.35	0.43	0.52	0.61	0.65

TABLE 11

EFFECT OF MALEIC HYDRAZIDE ON 2,4-D INDUCED GROWTH OF AVENA COLEOPTILE SECTIONS.

Concentration maleic hydrazide	Concentration of 2,4-D mg./l.							
	0.00	0.02	0.04	0.08	0.16	0.32	0.64	1.28
	Growth in absence of IAA	Growth in presence of IAA minus growth in absence of IAA						
mg./l.	mm./section/12 hrs.	mm./section/12 hrs.						
0.0	0.41	0.27	0.43	0.67	0.97	1.29	1.48	1.65
1.0	0.40	0.18	0.33	0.60	0.90	1.24	1.46	1.64
10.0	0.38	0.11	0.25	0.51	0.87	1.17	1.44	1.62
50.0	0.33	0.04	0.13	0.33	0.64	1.03	1.41	1.62

TABLE 12

THE INTERACTION EFFECT OF IAA AND NAA IN THE GROWTH OF AVENA SECTIONS.

Relative concentration of NAA*	Relative concentration of IAA*						
	0	1	2	4	8	16	32
	Growth (mm./section/12 hrs.)						
0	0.43	1.19	1.50	1.95	2.22	2.50	2.80
1	0.74	1.15	1.40	1.76	2.15	2.42	2.71
2	0.93	1.28	1.50	1.77	2.13	2.43	2.69
4	1.18	1.43	1.59	1.86	2.06	2.39	2.60
8	1.47	1.62	1.75	1.89	2.10	2.34	2.52
16	1.72	1.76	1.88	2.00	2.12	2.32	2.45
32	1.93	1.94	2.01	2.08	2.10	2.26	2.39

* The relative unit of concentration, 1 = 1.460×10^{-7} M.

TABLE 13

THE INTERACTION EFFECT OF 2,4-D AND NAA IN THE GROWTH OF AVENA SECTIONS.

Relative concentration of NAA*	Relative concentration of 2,4-D*							
	0	1	2	4	8	16	32	64
	Growth (mm./section/12 hrs.)							
0	0.42	0.78	1.01	1.34	1.64	1.94	2.10	2.19
1	0.75	0.98	-	-	-	-	-	-
2	0.97	-	(1.15)**	-	-	-	-	-
4	1.25	-	-	1.61	-	-	-	-
8	1.54	-	-	-	1.88	-	-	-
16	1.81	-	-	-	-	2.04	-	-
32	1.96	-	-	-	-	-	2.15	-
64	2.10	-	-	-	-	-	-	2.22

* Relative unit of concentration, 1 = 1.426×10^{-7} M

** This value unexplanably low.

TABLE 14

THE INTERACTION EFFECT OF IAA AND TIBA IN THE GROWTH OF AVENA SECTIONS.

Relative concentration of TIBA*	Relative concentration of IAA*									
	0	1	2	4	8	16	32	64	128	256
	Growth (mm./section/12 hrs.)									
0	0.42	0.80	0.98	1.28	1.61	2.02	2.31	2.53	2.78	2.99
4	0.67	0.99	1.25	1.52	1.73	2.06	2.35	2.55	-	-
8	0.79	1.25	1.32	1.61	1.83	2.03	2.35	2.64	2.77	2.79
16	0.94	1.46	1.59	1.72	1.88	2.27	2.48	2.70	2.73	2.71
32	1.07	1.57	1.67	1.83	2.03	2.32	2.70	2.80	2.69	2.59
64	1.18	1.64	1.74	1.92	2.25	2.34	2.58	2.66	2.63	2.56
128	1.14	-	-	-	2.12	2.26	2.34	2.40	2.57	2.50
256	1.03	-	-	-	1.81	2.04	2.14	2.25	2.33	2.44

* Relative unit of concentration, 4 = 1.428×10^{-7} M.

TABLE 15

THE INTERACTION EFFECT OF IAA AND PBA IN THE GROWTH OF AVENA COLEOPTILE SECTIONS.

Relative concentration of PBA*	Relative concentration of IAA*						
	0	1	2	4	8	16	32
	Growth (mm./section/12 hrs.)						
0	0.41	1.33	1.65	1.99	2.31	2.53	2.79
2	0.40	-	1.32	1.69	2.13	2.43	2.72
4	0.42	-	1.19	1.57	2.01	2.37	2.63
8	0.44	0.95	1.23	1.67	2.06	2.37	2.68
16	0.47	1.09	1.35	1.74	2.15	2.45	2.70
32	0.52	1.23	1.50	1.85	2.25	2.50	2.78
64	0.60	1.30	1.65	1.97	2.33	2.56	2.80
128	0.71	1.42	1.73	2.05	2.41	2.59	2.84
256	0.87	1.56	1.78	2.14	2.43	2.59	2.75

* Relative unit of concentration 2 = 3.050×10^{-7} M.

APPENDIX II

CULTURE OF ISOLATED FIAX ROOTS

Introduction

It was originally planned that antimetabolite studies would be carried out with cultures of isolated roots. However, the work which has previously been presented in this investigation and that which is presented in Appendix III had priority over the root culture work. Nevertheless, some experiments were carried out with isolated flax roots and the results are presented in this part of the investigation.

For work with isolated roots, it is desirable to use root clones. Isolated flax roots grow at a rapid rate and produce short lateral roots. Upon subculturing, however, the excised lateral root tips fail to elongate and it is therefore impossible to obtain flax root clones by normal culture techniques. A culture method for obtaining flax root clones has been found and this method is described.

Experimental and Results

ESTABLISHMENT OF FLAX ROOT CULTURES. Flax seeds (unknown variety) were surface sterilized for ten minutes in 1% NaOCl and a pinch of detergent, rinsed in sterile water, and germinated in the dark on moistened filter paper in Petri dishes. After four days the primary root tips (1 cm. in length) of approximately 150 seedlings were excised aseptically and transferred to Petri dishes containing 20 ml. of sterile nutrient medium. The medium consisted of Bonner's salts (1), two per cent sucrose, ferric tartrate at a

concentration of 1.5 mg./l., and Pyrex redistilled water. Following the first week of growth, the root tips were subcultured into the above medium which contained, in addition, thiamin and pyridoxine at a concentration of 0.1 mg./l., and nicotinic acid, at a concentration of 0.5 mg./l. For the next six weeks, the primary root tips were subcultured weekly but following this period, all stock subcultures were made at intervals of nine to 12 days (usually ten days). In all root growth experiments, two roots were grown in each Petri dish and 20 roots or more were used per treatment. The roots were kept in the dark but not at constant temperature. The temperature variations were from about 20°C to 27°C.

DEVELOPMENT OF FLAX ROOT CLONES. During the course of ten days growth, many short lateral roots developed on the primary roots. Many hundreds of these lateral roots were subcultured during the first 90 days of flax root culturing but they failed to elongate. If, however, a short portion (1-2 cm.) of primary root bearing one to four laterals was transferred, occasionally a lateral root would elongate. The elongated lateral root resembled a primary root and, if the lateral root tip were subcultured, it subsequently grew and became indistinguishable from the primary roots. The above observation suggested that it might be possible to cause the lateral roots to elongate if more of the primary root tissue were present with the lateral roots. Or, alternatively, the lateral roots might elongate if the tip of the primary root were removed. Therefore, after the thirteenth passage of the original primary root tips, the primary

tips of several roots were excised and the remaining portion of the primary roots with their short laterals were transferred to fresh nutrient. During the next ten days, several laterals elongated and upon subsequent subculturing the tips of the lateral roots grew like the original primary root tips. By repeating this procedure, it was possible to obtain flax root clones. Several clones were started but after several passages all but one clone was discarded.

Since it was inconvenient to transfer primary roots, after excision of the tips, which were 25 cm. or more in length, it was decided to cut each primary root into two or three portions and to transfer each portion to separate Petri dishes. When this was done, lateral roots elongated as well as when the entire primary root (minus tip) had been transferred to fresh medium. It should be stated, however, that although all lateral roots did elongate to some degree when either of the above methods was employed, the excised lateral tips did not always elongate. It was found, however, that the excised lateral tips which were capable of growing had a whitish translucent appearance whereas those which were incapable of growing had a slight yellowish transparent appearance. It was quite easy therefore to select lateral root tips which would grow upon excision and to discard the remainder. There was considerable variability in the number of lateral roots which were capable of supplying usable tips. The number of usable lateral tips was seldom as high as 50 per cent of the total number of

laterals present. The fact that all lateral tips were not suitable for use made it necessary to employ large numbers of Petri dishes to maintain sufficient stock roots for experimental purposes. This then is an undesirable feature of the present technique for the maintenance of a flax root clone. The flax root clone which was used in all the experiments presented below was cultured for 780 days.

ISOLATED FLAX ROOT EXPERIMENTS. An experiment was set up to determine the influence of growth factors on the rate of elongation of the flax roots. Bonner and Devirian have shown that flax roots require only thiamin, in addition to sucrose and mineral salts, for continued rapid growth. The data presented in table 1 is very similar to that obtained by Bonner and Devirian (2). The data show that of the growth factors tested, vitamin B is as satisfactory or better than any of the other growth factors alone or in combination with thiamin. Furthermore, it is apparent from the data that neither pyridoxine nor nicotinic acid contribute to the elongation rate of the roots. At the conclusion of this experiment, vitamin B_1 only was supplied as growth factor to the root cultures.

Following the development of the method for obtaining a flax root clone the question arose as to why the excised tips of short lateral roots fail to elongate when subcultured. It was considered possible that further growth factors were required or that elongation was inhibited by excess auxin.

To test the latter possibility, excised lateral root tips were treated with compounds which have been described as antiauxins.

TABLE 1

INFLUENCE OF GROWTH FACTORS ON FLAX ROOT GROWTH

Added growth factors	Observation
none*	Growth declined to 67 mm./10 days after 70 days
B_1	Growth maintained at an average of 239 mm./10 days after 70 days
Nicotinic acid	Growth declined to 52 mm./10 days after 70 days
B_6	Growth declined to 90 mm./10 days after 70 days
B_1 + Nicotinic acid	Growth maintained at an average of 241 mm./10 days after 70 days
B_1 + B_6	Growth maintained at an average of 230 mm./10 days after 70 days.
Nicotinic acid + B_6	Growth declined to 81 mm./10 days after 70 days
B_1 + Nicotinic acid + B_6	Growth maintained at an average of 242 mm./10 days after 70 days

* Basal medium = Bonner's salts, ferric tartrate, and 2 per cent sucrose.

Excised tips from the short lateral roots were treated with TIBA and 2,4-DCPIB over a wide range of concentrations. The roots, however, did not elongate as a result of the antiauxin treatment and it is therefore doubtful that failure of these roots to elongate was due to excess auxin in the tissue. Normal growing lateral root tips were also treated with the above mentioned compounds. TIBA at a concentration of 0.1 mg./l. and higher was found to be inhibitory to flax root growth. That this was so is shown in table 2. That concentrations below 0.1 mg./l. had no significant effect on the growth rate is also evident from the data of table 2. (The least significant difference value of 16mm. is an average value calculated only for the roots growing at or near the maximum growth rate. This is true also for the values presented in succeeding tables.) An increase in the rate of flax root elongation was obtained with the antiauxin 2,4-DCPIB. The data of table 3 show that this was so. 2,4-DCPIB concentrations of 0.1 mg./l. and lower increased the growth rate of the roots whereas concentrations higher than 0.1 mg./l. inhibited growth. It is also apparent from the data of table 3 that the degree of inhibition increased with each successive passage while there was a tendency for 2,4-DCPIB induced growth rate to increase during the first three or four passages.

Although 2,4-DCPIB did not induce elongation of the excised short lateral root tips, it was considered possible that it might cause elongation of such laterals if primary root tissue were present. Portions of primary roots (minus tips) were therefore treated with

TABLE 2

INFLUENCE OF TIBA ON GROWTH OF ISOLATED FLAX ROOTS SUBCULTURED AT 10 DAY INTERVALS

Concentration of TIBA (mg./l.)	Passage Growth(min./10 days) 1	2	3	4	5	6	7
0.0	242	246	245	257	253	244	238
0.001	254	262	260	256	251	240	242
0.01	215	238	239	232	241	237	239
0.1	222	202	217	205	189	193	197
1.0	137	127	117	125	94	-	-
10.0	42	0	-	-	-	-	-
100.0	11	0	-	-	-	-	-

Least significant difference at 1 per cent probability level = 16 mm.

TABLE 3

INFLUENCE OF 2,4-DCPIB ON GROWTH OF ISOLATED FLAX ROOTS SUBCULTURED AT 10 DAY INTERVALS

Concentration of 2,4-DCPIB (mg./l.)	Passage Growth(mm./10 days) 1	2	3	4	5	6
0.0	232	241	246	235	243	242
0.001	255	280	285	308	283	280
0.01	253	284	289	322	281	286
0.1	249	284	279	294	274	287
1.0	109	68	47	13	0	-
10.0	14	0	-	-	-	-
100.0	5	0	-	-	-	-

Least significant difference at 1 per cent probability level = 18 mm.

2,4-DCPIB. Observations made after ten days revealed that 2,4-DCPIB did not cause elongation of the short laterals which normally would not grow if excised. 2,4-DCPIB did, however, increase the rate of growth of the laterals which, if excised, would be capable of growing.

Two experiments were carried out to determine whether or not isolated flax roots were suitable material for antiauxin investigations. The data of tables 4 and 5 show the effect of 2,4-DCPIB and 2,4-D or IAA, alone and in combination, on the growth rate of isolated flax roots. It is evident from the data of table 4 that a concentration of 2,4-DCPIB, which was of itself capable of increasing root elongation rate, alleviated 2,4-D induced inhibition of root growth at intermediate 2,4-D concentrations. That entirely similar results were obtained with 2,4-DCPIB and IAA alone and in combination, is apparent from the data of table 5. Further work was not done in this direction because the Avena coleoptile section test was found to be much more satisfactory for the antiauxin investigations.

The effect of the two diortho substituted phenoxyacetic acid antiauxins, 2,6-DCPA and 2,4,6-TCPA, on the growth rate of roots has not heretofore been reported. The data of tables 6 and 7 show the influence of 2,6-DCPA and 2,4,6-TCPA, respectively, on the growth of isolated flax roots. It is apparent from these data that, at a concentration of 0.1 mg./l., both compounds possess the property of increasing the rate of root growth.

TABLE 4

EFFECT OF 2,4-DCPIB AND 2,4-D ALONE AND IN COMBINATION ON GROWTH OF EXCISED FLAX ROOTS.

Concentration of 2,4-DCPIB (mg./l.)	Growth (mm./7 days) Concentration of 2,4-D (mg./l.) 0.0	0.0001	0.001	0.01	0.1	1.0	10.0
0.0	140	140	128	46	11	6	4
0.1	165	172	147	85	16	0	0
1.0	57	78	117	55	9	0	0

Least significant difference at 1 per cent probability level = 12 mm.

TABLE 5

EFFECT OF 2,4-DCPIB AND IAA ALONE AND IN COMBINATION ON GROWTH OF EXCISED FLAX ROOTS.

Concentration of 2,4-DCPIB (mg./l.)	Growth (mm./7 days) Concentration of IAA (Mg./l. x 10^{-3}) 0.0	0.01	0.1	1.0	10.0
0.0	134	132	127	48	7
0.1	161	159	138	101	29
1.0	59	80	92	51	22

Least significant difference at 1 per cent probability level = 10 mm.

TABLE 6

EFFECT OF 2,6-DCPA ON GROWTH OF ISOLATED FLAX ROOTS SUBCULTURED AT 10 DAY INTERVALS.

Concentration of 2,6-DCPA (mg./l.)	Passage Growth (mm./10 days) 1	2
0.0	200	190
0.1	245	254
1.0	111	119

Least significant difference at 1 per cent probability level = 19 mm.

TABLE 7

EFFECT OF 2,4,6-TCPA ON GROWTH OF ISOLATED FLAX ROOTS SUBCULTURED AT 10 DAY INTERVALS.

Concentration of 2,4,6-TCPA (mg./l.)	Passage Growth (mm./10 days) 1	2
0.0	190	197
0.1	249	262
1.0	115	108

Least significant difference at 1 per cent probability level = 17 mm.

REFERENCES

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(2) Bonner, J. and Devirian, P.S. Amer. Jour. Bot. (1939) 26:661. Growth factor requirements of four species of isolated roots.

APPENDIX III

EFFECT OF CERTAIN ANTIMETABOLITES ON THE GROWTH OF TOMATO PLANTS

Introduction

There is a large background of information on the economy of certain vitamins in plants (1, 2, 3). Plant vitamins are growth factors synthesized in certain parts of the plant and may be utilized in the cells which produce them or may be transported to particular sites of utilization. For example, in tomato plants, thiamine is synthesized in the leaves and is transported to the roots where it is required for root growth (4). Entire plants normally make all the vitamins necessary for growth. The deficiency symptoms of exogenously required substances for plant growth are well recognized. It is of interest, therefore, to discover whether or not there are circumstances under which a plant may have a deficient synthesis of a vitamin or any other endogenously synthesized substance and to determine how the deficiency symptoms would manifest themselves.

An approach to this problem is through the use of competitive metabolite antagonists. Many organic compounds have been found which inhibit growth of bacteria, fungi, and such animals as mice and rats. In the main, these organic compounds are vitamin, amino acid, purine and pyrimidine analogues. In many instances the analogues competitively inhibit the utilization of the corresponding vitamin, amino acid, purine or pyrimidine, by the organism.

It is the purpose of this investigation to present some preliminary experiments on the effects of certain recognized antimetabolites on the growth of young tomato plants grown under aseptic

conditions. Experimental difficulties which arose through the course of this investigation made it impossible to achieve the desired goal in the time available for the work. The original plans for the investigation were, first of all, to determine the concentration range over which the various antimetabolites showed visible effects upon growth of the plants, and secondly to try and overcome the antimetabolite-induced effects with an appropriate metabolite. Alleviation of the toxic action of the antimetabolite by the metabolite would be required in order to demonstrate that the toxic symptoms were in fact deficiency symptoms and not some, perhaps, quite unrelated non-specific action. In the first few experiments carried out, the tomato plants grew quite well under the experimental conditions employed. Subsequently however, and for some unknown reason, the plants grew very poorly. It was not possible, therefore, to obtain satisfactory experiments on reversals of the antimetabolite-induced inhibitions. The data obtained in some attempted reversal experiments are presented however, as well as data which show the effects of the antimetabolite on growth in height and dry weight of the plants.

The literature concerning the effects of antimetabolites is very voluminous (see, for example, 2, 3, 5, 6) and no attempt will be made to review it here. The literature relative to the action of a given antimetabolite, on bacteria, fungi or animals, is also quite extensive and the following brief survey of the antimetabolites used in this investigation is presented with the view of acquainting

the reader with their existence and is in no way intended to be a complete literature review.

The compounds, pyridine-3-sulfonic acid and 3-acetylpyridine, are nicotinic acid analogues which cause nicotinic acid deficiency symptoms. McIlwain (7) has shown that pyridine-3-sulfonic acid inhibits the growth of several strains of bacteria and that the inhibition was partially reversible with nicotinic acid. Nicotinic acid deficiency symptoms induced in mice by 3-acetylpyridine was abolished by administration of nicotinic acid (8) or tryptophan (9). Woolley (10) observed that animals which had received benzimidazole, one of the first purine antagonists utilized (11), had symptoms which were suggestive of biotin deficiency. Biotin did not however overcome benzimidazole growth inhibition of microorganisms; but inhibition of growth was overcome by the aminopurines, adenine and quanine. Klotz and Mellody (12) also studied the reversal of benzimidazole growth inhibition of E. coli and found that yeast nucleic acid reversed the inhibition, but the nature of the reversal was such that it could not be due merely to the adenine of the nucleic acid. The effects of the isomeric 2-, 3- and 4-fluorophenylalanines on the growth of young rats have been investigated by Armstrong and Lewis (13). The 2- and 4-fluorophenylalanines were toxic but much less so than the 3-fluorophenylalanine. It has also been shown (14, 15, 16) that phenylalanine alleviates fluorophenylalanine-induced inhibitions. Another important phenylalanine antagonist is thienylalanine. The inhibitory effects of β-2-thienylalanine on the growth of several microorganisms have been

reversed by phenylalanine (17-21) and by tyrosine (20). The important methionine antagonist, ethionine, is one of the earliest reported amino acid antimetabolites. Dyer (22) found that rats fed ethionine failed to gain weight, but if methionine was included in the diet, the inhibition of growth was alleviated. That ethionine inhibits the uptake of methionine into protein in animal tissues was shown by Simpson et al. (23) who also showed that the inhibition was relieved by methionine. It has been shown (24) that the inhibition of growth of E. coli induced by 2-chloro-4-aminobenzoic acid may be overcome by pantothenic acid; although 2-chloro-4-aminobenzoic acid is a growth factor for some microorganisms (25). 2-chloro-4-aminobenzoic acid may also completely antagonize the growth inhibitory action of sulfanilamide (26). Finally, pyrithiamine, the pyridine analogue of thiamin, has been found (27) to interfere competitively with the utilization of thiamin by S. Aureus. Woolley and White (28) have shown that organisms which require the intact thiamin molecule for growth are inhibited by much smaller concentrations of pyrithiamine than organisms which are able to utilize either the pyrimidine or thiazole portions of thiamin.

Material and Methods

Tomato seeds (var. Extra Early) were sterilized for ten minutes in a one per cent NaOCl solution containing a pinch of detergent and were then planted in 38 x 250mm. culture tubes (three seeds per tube) which had been prepared in the following manner. Approximately

five grams of vermiculite (sieved to remove small particles) and 30 ml. Hoagland's solution were added to each tube. The tubes were stoppered with cotton plugs and autoclaved for 15 minutes at 15 pounds pressure. The planting of seeds and all subsequent operations were carried out employing aseptic techniques. The plants were grown in the Earhart Laboratory. After germination, which took place in the dark at 26°C, the tubes were transferred to a shady area in the green day room (day temp. 23°C and night temp. 17°C). Two days after germination the seedlings were thinned to one plant per tube, and on the third day following germination, the plants were treated with 5 ml. solution containing the compound or compounds undergoing test. Ten plants were used per treatment in each experiment. At the time of treatment and at two day intervals, the height of each plant was determined. The experiments terminated ten days after treatment at which time the plants were harvested and oven dry weights of root, stem and leaf (including cotyledonary leaves) were obtained. In order to reduce the amount of water which accumulated through condensation on the inside of the stoppered tubes, the tubes were treated with Desicote, a water repellent. This precaution was not taken in the first experiment which was set up in one of the constant temperature rooms (17°C) under artificial light.

Results

The data of table 1 show the effect of pyridine-3-sulfonic acid on the growth of tomato plants. As mentioned above, the plants of this experiment were grown at a constant temperature of 17°C. and, furthermore, only six plants per treatment were used. It is apparent from the data of table 1 that treatment with pyridine-3-sulfonic acid resulted in an increase in the height of the plants as well as an increase in the dry weight, particularly the leaf weight, at a concentration of 0.1 mg./plant. Following this experiment, two further experiments with pyridine-3-sulfonic acid and nicotinic acid alone and in combination were carried out. It was found that the results obtained in these experiments were not in accord with the first experiment. Pyridine-3-sulfonic acid or nicotinic acid alone and in combination had no noticeable effect on the growth of the tomato plants. It is possible that the disagreement is a consequence of the different temperature and light conditions used in the two sets of experiments. It is equally possible that the growth differences obtained in the first experiment are not significant.

The influence of another nicotinic acid analogue, 3-acetylpyridine, on the growth of tomato plants is shown in table 2. It is evident from the data of this table that 3-acetylpyridine inhibits the growth of the plants at the relatively high concentration of 10.0 mg./plant. At the end of ten days, the leaves of plants treated at this concentration were dying. There was also an

TABLE 1

THE EFFECT OF PYRIDINE-3-SULFONIC ACID ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS

Concentration of pyridine-3-sulfonic acid mg./plant	Increase in Height (mm.)	Dry Weights (mg.)			
		Root	Stem	Leaf	Total
0.0	46	5.3	18.0	25.9	49.2
0.001	44	4.8	18.4	27.6	50.8
0.01	45	5.1	17.3	26.7	49.1
0.1	59	8.4	22.4	42.1	72.9
1.0	50	5.0	20.3	31.1	56.4
10.0	41	5.4	16.4	27.0	48.8

TABLE 2

THE EFFECT OF 3-ACETYLPYRIDINE ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS

Concentration of 3-acetylpyridine (mg./plant)	Increase in Height (mm.)	Dry Weights (mg.)			
		Root	Stem	Leaf	Total
0.0	34	9.2	23.3	33.1	65.6
0.01	33	9.1	22.3	32.7	64.1
0.1	33	8.4	22.2	33.5	64.1
1.0	29	10.0	22.3	33.7	66.0
10.0	22	7.2	21.8	22.5	51.5

abundant development of short adventitious roots which possessed many root hairs.

That the antagonist benzimidazole exerts a marked inhibitory action upon the growth of young tomato plants is evident from the data of table 3. The inhibition is, however, obtained only at fairly high concentrations of benzimidazole. At the end of ten days, plants treated with a benzimidazole concentration of 10 mg./plant were dead or dying. There was no stem elongation, the cotyledonary leaves turned black, and the stems appeared brown and desiccated. Attempts to reverse the benzimidazole-induced inhibition by concurrent application of adenine or guanine were not successful. Tables 4 and 5 present data from such an experiment. The data show that adenine, at the concentrations tested, did not alleviate benzimidazole-induced inhibition. It is suggestive, however, from the data of table 6 that guanine, at a concentration of 0.1 mg./plant, was able to cause slight reversal of the inhibition induced by a benzimidazole concentration of 10 mg./plant. It may be noted from the data of tables 4, 5, and 6 that growth of the tomato plants was considerably less than in the experiment of table 3. It may also be noted that in the latter experiments benzimidazole was not as inhibitory as in the former experiment. The reason for this behavior is not apparent.

The phenylalanine antagonists, 2- and 4-fluorophenylalanine, inhibit the growth of young tomato plants. That this is so is evident from the data of tables 7 and 8. The data of table 7 show

TABLE 3

THE EFFECT OF BENZIMIDAZOLE ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS

Concentration of Benzimidazole (mg./plant)	Increase in Height (mm.)	Dry Weights (mg.)			
		Root	Stem	Leaf	Total
0.0	63	13.2	47.7	58.6	119.5
0.001	64	13.4	48.4	59.9	121.7
0.01	62	13.9	45.9	59.7	119.5
0.1	64	13.6	48.3	61.3	123.2
1.0	54	6.0	36.8	67.7	110.5
10.0	-	0.8	5.2	8.5	14.5

TABLE 4

THE EFFECT OF BENZIMIDAZOLE AND ADENINE ALONE AND IN COMBINATION ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS.

Concentration of adenine (mg./plant)	Increase in Height (mm.) Concentration of Benzimidazole (mg./plant)			
	0.0	1.0	3.0	10.0
0.0	40	37	19	12
0.001	41	44	22	13
0.01	39	46	21	11
0.1	38	42	22	10

TABLE 5

THE EFFECT OF BENZIMIDAZOLE AND ADENINE ALONE AND IN COMBINATION ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS.

Concentration of adenine (mg./plant)	Total Dry Weight (mg.) Concentration of Benzimidazole (mg./plant)			
	0.0	1.0	3.0	10.0
0.0	65.5	57.1	45.4	33.7
0.001	72.6	65.2	51.9	30.9
0.01	65.2	66.7	40.0	28.8
0.1	66.1	63.6	53.0	33.8

TABLE 6

THE EFFECT OF BENZIMIDAZOLE AND GUANINE ALONE AND IN COMBINATION ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS.

Concentration of Guanine (mg./plant)	Increase in Height (mm.) Concentration of Benzimidazole (mg./plant)			
	0.0	1.0	3.0	10.0
0.0	40	36	22	10
0.001	40	37	21	12
0.01	33	33	23	14
0.1	36	41	23	17

that the inhibitory action of the 2-isomer is more pronounced than the inhibitory action of 4-fluorophenylalanine (data of table 8). Although treatment with 2-fluorophenylalanine caused marked growth inhibition at concentrations of 1.0 and 10.0 mg./plant, there was no indication that the plants would die. At these concentrations of the antagonist, the leaves were small, thick and dark green. There was a marked development of adventitious roots and the deep greenish-red appearance of the stems indicated an accumulation of anthocyanins. As mentioned above, the inhibitory action of 4-fluorophenylalanine was not so marked. The plants treated with this compound appeared quite normal but they were stunted. There was also considerable development of adventitious roots.

The data of table 9 show that another phenylalanine antagonist, β-2-thienylalanine, also inhibits the growth of young tomato plants at concentrations of 1 and 10 mg./plant. Plants treated with the high concentrations of β-2-thienylalanine developed adventitious roots even though, with the 10 mg./plant treatment, the plants, at the end of ten days, were dying at the base of the stems.

The effect of ethionine, a methionine antagonist, on the growth of young tomato plants was similar to the effect of β-2-thienylalanine. That this was so is evident from the data of table 10. Concentrations of 1 and 0.1 mg./plant induced a vigorous production of adventitious roots. The stems, however, were not noticeably injured at concentrations of 10 mg./plant.

TABLE 7

THE EFFECT OF 2-FLUOROPHENYLALANINE ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS.

Concentration of 2-fluorophenylalamine (mg./plant)	Increase in Height (mm.)	Dry Weights (mg.)			
		Root	Stem	Leaf	Total
0.0	50	18.6	56.5	68.3	143.4
0.001	52	18.2	60.1	75.1	153.4
0.01	51	19.6	57.5	71.8	148.9
0.1	54	19.6	57.8	68.6	146.0
1.0	31	17.1	42.1	50.7	109.9
10.0	11	10.3	33.5	33.0	76.8

TABLE 8

THE EFFECT OF 4-FLUOROPHENYLALANINE ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS.

Concentration of 4-fluorophenylalanine (mg./plant)	Increase in Height (mm.)	Dry Weights (mg.)			
		Root	Stem	Leaf	Total
0.0	43	6.5	29.5	29.3	65.3
0.01	43	7.5	31.0	31.7	60.2
0.1	38	6.7	26.7	25.0	58.7
1.0	24	7.1	22.9	23.0	53.0
10.0	12	6.9	19.1	18.2	44.2

TABLE 9

THE EFFECT OF β -2-THIENYLALANINE ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS.

Concentration of β -2-thienylalanine (mg./plant)	Increase in Height (mm.)	Dry Weights (mg.)			
		Root	Stem	Leaf	Total
0.0	37	15.2	31.1	43.5	89.8
0.01	35	15.9	26.0	42.3	84.2
0.1	34	23.5	26.0	36.1	85.6
1.0	18	13.8	21.7	23.4	58.9
10.0	4	4.1	15.2	13.5	32.8

TABLE 10

THE EFFECT OF ETHIONINE ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS.

Concentration of ethionine (mg./plant)	Increase in Height (mm.)	Dry Weights (mg.)			
		Root	Stem	Leaf	Total
0.0	46	7.1	31.8	31.1	70.0
0.01	46	7.5	31.3	28.5	67.3
0.1	30	6.8	25.6	25.0	57.4
1.0	15	6.7	21.9	16.6	45.2
10.0	10	3.6	18.3	13.7	35.6

That 2-chloro-4-aminobenzoic acid inhibits growth of young tomato plants is apparent from the data of table 11. Again, the higher concentrations of this compound induced the development of adventitious roots.

The data of table 12 show that the thiamine antagonist, pyrithiamine, at concentrations up to 1.0 mg./l. had no inhibitory effect on the growth of young tomato plants.

Discussion

The present experiments, because of their preliminary nature, were not designed to permit a statistical analysis of the data. It is not possible, therefore, to estimate the significance of small growth differences or even large growth differences when there appears to be considerable variability within an experiment. The results of the investigation, as presented above, do, however, show that several recognized purine, vitamin and amino acid antagonists inhibit the growth of young tomato plants. Furthermore, the data indicate the usefulness of the competitive antagonist approach to the demonstration of metabolic requirements and the symptoms of the deficiency of the metabolite. It is to be expected that further studies with antimetabolites, under improved cultural techniques, may provide information on the biochemistry of endogenously synthesized substances in intact higher plants.

A further consideration with respect to the effects of antimetabolites on the growth of higher plants is that all antimetabolites

TABLE 11

THE EFFECT OF 2-CHLORO-4-AMINOBENZOIC ACID ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS.

Concentration of 2-Chloro-4-amino-benzoic acid (mg./plant)	Increase in Height (mm.)	Dry Weights (mg.)			
		Root	Stem	Leaf	Total
0.0	37	15.2	31.1	43.5	89.8
0.01	38	14.9	28.0	36.3	79.2
0.1	34	15.0	26.9	33.1	75.0
1.0	34	16.5	26.0	42.3	84.8
10.0	13	20.9	19.9	24.0	64.8

TABLE 12

THE EFFECT OF PYRITHIAMINE ON THE GROWTH OF TOMATO PLANTS GROWN UNDER ASEPTIC CONDITIONS.

Concentration of pyrithiamine (mg./plant)	Increase in Height (mm.)	Dry Weights (mg.)			
		Root	Stem	Leaf	Total
0.0	56	18.0	44.8	60.1	122.9
0.001	56	16.3	43.4	58.6	118.3
0.01	55	16.3	43.0	59.3	118.6
0.1	53	17.4	42.6	59.8	119.8
1.0	57	16.9	44.5	60.0	121.4

are potential herbicides. It would appear, from the results of the present work, that the practical usefulness of the compounds studied in this investigation would be restricted because of the relatively high concentrations required to induce inhibition of growth. It is possible, however, that foliar application of this type of compound would be much more effective than application to the root system.

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