A B S T R A C T

I.
STUDIES ON PLANT GROWTH SUBSTANCES

A particulate fraction from corn seedlings was found to convert tryptophan to indoleacetic acid (IAA) in minute amounts. None of the co-factors examined appeared to increase the activity, and a considerable portion of the activity was lost upon freezing. The activity sedimented in the so-called mitochondral fractions, but the microsomal fraction also contained some activity. All attempts to solublize the activity were unsuccessful. When tryptophan-2-C^{14} was used as substrate, IAA-1-C^{14} was isolated with no dilution of specific activity.

In order to determine if the <u>in vitro</u> system is actually of significance, direct labeling experiments were attempted. It was shown that little IAA is present in the seedlings, although the free tryptophan content was about 3 mg per kilogram. It appeared desirable, therefore, to develop an isolation method which would be suitable with larger amounts of corn seedling tissue. This work made it apparent that not more than the order of 10 µg of IAA was present per kilogram of rapidly growing etiolated corn seedlings.

A second phase of experiments in the realm of plant auxins concerns studies of the effect of N-1-naphthylphthalamic acid on the growth and the geotropic response of seedlings, and a study of the activity of tetrazole analogues of IAA and 2,4-dichlorophenoxyacetic acid (2,4-D). N-1-naphthylphthalamic acid (NPA) was shown to inhibit the geotropic response of seedlings disproportionally as compared to growth.

Studies on the tetrazoles demonstrated that a 5'-tetrazole ring may replace the carboxyl group of IAA, with the retention of growth promoting activity in the Avena section test. The 2,4-D analogue, on the other hand, was an inhibitor of endogenous growth, IAA induced growth, and 2,4-D induced growth. Though the inhibition of IAA induced growth was non-competitive, the inhibition of 2,4-D induced growth indicated a strong competitive component. This led to the conclusion that IAA and 2,4-D act at different growth inducing sites, or that there was competition at a transport or uptake site. Additional work on intact bean plants indicated that an uptake site may be involved.

II.

ISOLATION AND PARTIAL CHARACTERIZATION OF THE PRECURSOR OF 6-METHOXYBENZOXAZOLINONE IN ZEA MAYS

The work on a naturally occurring sweet compound in corn seedlings was the result of the difficulty encountered in separation of this compound from IAA in extracts of corn seedlings. A compound ($C_9H_9O_5N$) was isolated in crystaline form, and found to constitute about 0.5% of the dry weight of the seedlings. It appeared to be identical to that isolated by R. J. Suhadolnik (Ph.D. Thesis, Pennsylvania State University. 1956), who proposed the structure 2,5-dihydroxy-3,4-methlenedioxyphenylacetamide. Present studies indicated that the proposed structure was incorrect, and finally that the compound was readily converted to 6-methoxybenzoxazolinone with a loss of formic acid, as recently reported by Wahlroos and Virtanen (Suomen Kemistilehti B32:139-140. 1959). These authors suggest the structure 1,3-dihydroxy-2-keto-6-methoxy-1,4-benzoxazine, and our data appear to fit this structure.

Approved: Robert S. Bandurski

I. STUDIES ON PLANT GROWTH SUBSTANCES

(and)

II. ISOLATION AND PARTIAL CHARACTERIZATION OF THE PRECURSOR OF 6-METHOXYBENZOXAZOLINONE IN ZEA MAYS

By

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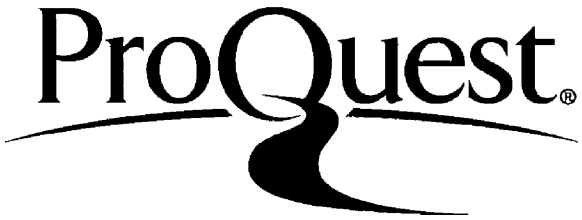

I should like to express my appreciation to Professor R. S. Bandurski for his many suggestions and assistance during these studies. I would also like to thank Dr. Te May Ching and Dr. Aleks Kivilaan who contributed so much to the studies on synthetic plant growth substances. It is also a pleasure to acknowledge the many suggestions of Drs. L. G. Wilson, W. H. Reusch and Professor R. M. Herbst.

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I N T R O D U C T I O N

The hormonal control of plant growth has been investigated by a large number of plant physiologists, particularly since the discovery by F. W. Went of a quantitative experimental approach. In spite of the array of interesting physiological experiments, much chemical and biochemical work remains. It may be, however, that the present state of chemical technique is not adequate to deal with problems on the hormonal level. Questions such as the following are not presently easy to deal with experimentally. What are the hormones operating in the various tissues of a plant at various stages of its life cycle? Are all hormones, like the vitamins, to be regarded as co-enzymes? If not co-enzymes, how may the hormones initiate the chain of reactions they do indeed trigger? How can one be certain that compounds isolated from plant extracts act as hormones in vivo? Why do diverse synthetic compounds have similar biological activity, at least in a qualitative manner?

The experimental work to be presented will be in the form of five papers plus one additional portion on the biosynthesis of 3-indoleacetic acid (IAA). The latter portion, and two papers concerning isolation of IAA from mature corn stalks, tumors and corn seedlings, constitute Part A of Section I on auxins. Part B of Section I will concern synthetic auxins, to be presented in the form of two published papers which deal with the effect of N-1-naphthylphthalamic acid on growth and the geotropic response in seedlings, and the biological activity of tetrazole analogues. Section II is concerned with isolation and characterization of a sweet phenol-like compound from corn seedlings.

A general review on auxins is presented first because each paper contains a rather specific and limited review.

I.
STUDIES ON PLANT GROWTH SUBSTANCES

A.
Occurrence and Biosynthesis of Indoleacetic Acid

In the present work an effort was made to avoid the crucial, but nebulous, questions enumerated in the introduction. Nonetheless, difficulties were encountered. It was intended to investigate a well-defined problem of restricted scope (the biosynthesis of indole-3-acetic acid from tryptophan), but soon questions arose about the assumptions on which the problem was based. First, it would be desirable to prove that indole-3-acetic acid (IAA) was, in fact, snythesized from tryptophan <u>in vivo</u>. Secondly, it is apparent that one must be able to measure initial and final IAA concentration in the seedlings if one is to determine specific activities of IAA synthesized from tryptophan.

A particulate fraction from corn seedlings was found to convert tryptophan to IAA in minute amounts. None of the co-factors examined appeared to increase the activity, and a considerable portion of the activity was lost upon freezing. The activity sedimented in the so-called mitochondral fraction, but the microsomal fraction also contained some activity. All attempts to solublize the activity were unsuccessful. When tryptophan-2-C^{14} was used as substrate, IAA-1-C^{14} was isolated with no dilution of specific activity.

In order to determine if the <u>in vitro</u> system is actually of significance, direct labeling experiments appeared to be essential. Other work in this laboratory indicated little radioactivity could be obtained in IAA and tryptophan by feeding $C^{14}O_2$ to etiolated corn seedlings with indole. In these experiments, about 25 grams of seedlings

were fed about 1 mc of $C^{14}O_2$, and the isolation of IAA and tryptophan facilitated through addition of carrier non-labeled IAA and tryptophan.

In the present work it was also shown that little IAA is present in the seedlings, although the free tryptophan content was about 3 mg per kilogram. It appeared desirable, therefore, to develop an isolation method which would be suitable with larger amounts of corn seedling tissue. This made it apparent that not more than the order of 10 µg of IAA was present per kilogram of rapidly growing etiolated corn seedlings.

B.
Synthetic Plant Growth Substances

A second phase of experiments in the realm of plant auxins concerns studies of the effect of N-1-naphthylphthalamic acid on the growth and the geotropic response of seedlings, and a study of the activity of tetrazole analogues of IAA and 2,4-dichlorophenoxyacetic acid (2,4-D). N-1-naphthylphthalamic acid (NPA) was shown to inhibit the geotropic response of seedlings disproportionally as comparted to growth. This appeared to conflict with the classical theory of Went and Cholodny, or to indicate that lateral polarity or redistribution of auxin was prevented. Studies on the tetrazoles demonstrated that a 5'-tetrazole ring may replace the carboxyl group of IAA, with the retention of growth promoting activity in the Avena section test. The 2,4-D analogue, however, was an inhibitor of endogenous growth, IAA induced growth, and 2,4-D induced growth. Though the inhibition of IAA induced growth was non-competitive, the inhibition of 2,4-D induced growth indicated a strong competitive component. This led to the interesting conclusion that IAA and 2,4-D act at different growth inducing sites, or that there was competition at a transport or uptake site. Additional work on intact bean plants indicated that an

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I.
STUDIES ON PLANT GROWTH SUBSTANCES

A.
Occurrence and Biosynthesis of Indole-3-Acetic Acid in Plants

1. Work on the Existence and Nature of Plant Hormones

Realization that a localized area at the tips of shoots and roots was concerned with geotropic and phototropic responses, may be attributed to Darwin (34). He observed that the coleoptile of canary grass, Phalaris cauariensis, was particularly sensitive to light and gravity. Even though the region of bending was below the tip, removal of the tip prevented the response. The seedling would not respond to light even if the tip was merely covered prior to unilateral illumination. Boysen-Jensen (24), working with oat coleoptiles, was able to show that continuity of living protoplasm was not essential for transmission of the stimulus. A transverse cut did not affect the curvature response and, in fact, replacement of an excised tip allowed the curvatures to develop. Paál (117) demonstrated that when the excised tip was replaced with only one side of the stump covered, the covered side grew faster; resulting in a curvature not unlike normal geo- or phototropic curvature. The stimulus could pass through a layer of gelatin, but not mica or platinum foil. Paál was first to suggest a hormone was responsible for geo- and phototropisms, while Söding (140) showed that replacement of the excised tip restored most of the growth lost by decapitation.

Fitting (41) was the first investigator to apply the term hormone in plant physiology. He applied the term to a substance extracted from the pollinia of tropical orchids which induced swelling of the ovary when

applied to the stigma. About twenty years later, Laibach (87) found these extracts contained auxin, and orchid ovaries responded to heteroauxin applications. Haberlandt (57) observed isolated potato tuber parenchyma did not divide unless a small bit of phloem tissue was present, and that the phloem could still induce division when separated from the parenchyma by agar. Crushed cells were also effective in inducing the response. Haberlandt concluded two hormones were required for the response; one a wound hormone coming from the cut surface, and the other a phloem hormone.

The classical studies of F. W. Went (177,178) resulted in development of a quantitative bioassay for the growth hormone of Avena coleoptile tips. Went found that if the excised coleoptile tip was placed on a very small block of agar, some of the active substance could be collected in the agar. If the block was placed asymetrically on a decapitated stump, a curvature developed. Under a specified set of conditions, the curvature was found to be directly proportional to concentration (number of tips allowed to diffuse, or time of diffusion) over a limited range. This assay made possible chemical work on the nature of the active substance, and also permitted many interesting physiological experiments. Went was further able to measure the rate of diffusion of the substance through agar and thus to estimate the molecular weight to be about 376. The active substance was stable to boiling and light. Thus the isolation and chemical structure of the active substance became an attractive problem, and it was intensively investigated, especially by Kögl, Haagen-Smit, and co-workers (79,80,81,82,83).

Nielsen (112) had already found in 1928 that the culture medium from Rhizopus suinus was rich in substance causing Avena curvatures. He later found (113) that the substance was ether soluble but readily inactivated by peroxides normally present. Furthermore, Nielson achieved a partial

purification such that 0.02 µg gave a curvature. Dolk and Thimann (36) were able to determine a pK of about 4.75 for the active substance from Rhizopus culture filtrate by shaking with ether at various buffered pH values. The substance seemed to be sensitive to oxidation and warm acids, but stable to warm alkali. At about the same time, Kögl and Haagen-Smit reported preliminary purification of acid auxins from Rhizopus reflexus, yeast, and E. coli (80).

Kögl, Haagen-Smit and Erxleben (82) also found human urine to be a very rich source of active material and isolated 40 mg of active crystals from about 33 gallons. This represented about a 50,000 fold purification as measured by the Avena curvature test. The formula for the active substance, called auxin a, was $C_{18}H_{32}O_5$ and the structure appeared to be 2,4-diisobutyl-1,5-cyclopenteneyl-α,β,δ-trihydroxyvaleric acid. Later they isolated from corn oil and barley malt a second active substance called auxin b (79). Auxin b could be converted to auxin a by dehydration of the α hydroxyl group, giving rise to the β keto derivative (79).

Still another compound (81) was present in the urine collected at a hospital from a particular 18-year-old youth. This isolated compound was called heteroauxin, and it was found to be identical to indole-3-acetic acid, originally identified from urine by Salkowski (129), and synthesized by Ellinger (37).

Thimann (157), in later work on the active material from Rhizopus sp., was able to obtain a minute amount of crystaline material by vacuum sublimation which appeared identical in all respects to indole-3-acetic acid (IAA).

Auxins a and b have not been subsequently isolated. Wieland, et al. (181) have recently followed the method of Kögl, Haagen-Smit, and Erxleben

(82) for the isolation of auxin a from urine and obtained only the methyl ester of indole-3-acetic acid (IAA). This was an artifact produced by boiling in methanolic HCl which was thought to lactonize auxin a. Brown, Henbest, and Jones (26) have prepared 1,5-cyclopentenyl-β-keto-δ-hydroxyvaleric-δ-lactone. This close analogue of auxin b appears to exist only as the lactone in aqueous solution with the β-keto group readily enolizing. Other differences were also found between this derivative and auxin b lactone. The accumulating evidence has slowly led to the opinion that auxin a and auxin b do not in fact exist. The early work, however, seemed to indicate that auxin a was probably the auxin of higher plants. These conclusions were based on determination of the molecular weight by the diffusion technique, and the partial acid stability.

Larsen (92) has reviewed the methodology and results of various investigations using the diffusion technique to estimate the approximate molecular weight of auxin extracted or diffusing from higher plant tissues. There are many difficulties, not the least of which is the presence of freely diffusible inhibitors and/or more than one active substance. Most recent determinations have resulted in values more closely corresponding to IAA than to auxin a or b. Terpstra (153) has pointed out that attributing the acid stable portion of the activity to auxin a may be in error. She found that the residual alkali and acid stable activity corresponded, after paper chromatography, to an Rf of IAA.

2. Detection of Indole-3-Acetic Acid in Higher Plants

The case of IAA as a natural plant auxin was much bolstered by the isolation of IAA after alkaline hydrolysis of corn seed (17,55) and the isolation of "free" IAA from immature corn seed (54). The physiological significance of the very high concentration of IAA in corn seed is unknown.

Holley et al. (67) separated the auxins of cabbage by means of counter current distribution using diethyl ether and pH 6.0 phosphate buffer. They found two active compounds, one of which had the same distribution coefficient as IAA, and another much more water-soluble. Since the development of paper chromatographic technique, many reports of detection of IAA (and other growth promoting and inhibiting substances) may be cited. The compiled list (Table I) makes it apparent that IAA and other indole compounds have been detected in a number of plants. Caution should be employed with regard to reports in which IAA is reported on the basis of biological activity at the proper Rf after unidirectional chromatography in one solvent. Salkowski and Ehrlich reagents are not, however, sensitive enough to allow detection of IAA at concentrations detectable in the various bioassays. The most popular bioassay has been the Avena section test (13), though Avena curvature (189), Cress root inhibition (108), pea root inhibition (10), wheat sections (98), Avena first internode (115), and inhibition of Coleus abcission (99) are some of the bioassay techniques used.

It appears that the new spray reagent p-dimethylaminocinamaldehyde proposed by Hartley-Mason and Archer (59) will find wide use. This aldehyde gives colors with many indole compounds and is about 10 times as sensitive as p-dimethylaminobenzaldehyde (Ehrlich's reagent). The specificity and reliability of color reagents for IAA leaves much to be desired, especially from the quantitative standpoint. Even aside from this, Ehrlich and Salkowski reagents give colors with a number of phenolic substances (142,146). Also, Salkowski reagent is not reliable in the presence of reducing substances (120). Both Ehrlich and Salkowski reagents were found to give colors at the Rf of IAA on paper chromatograms

TABLE I

Reports of Occurrence of Indole-3-Acetic Acid and Related Indole Compounds in Various Higher Plant Tissues

Plant Tissue & References	Compounds Found	Type of Extract	Bioassay	Color Detection
Black current-seeds, fruits[188]	IAA, IAN	Acid Ether	Wheat Sect.&	Yes
Gooseberry-seeds, fruits[188]	IAA	" "	Coleus	?
Apple seeds[188]	IAA	" "	abcission	?
Avena coleoptile tips[132]	IAA	Diffusate	Oat Curvature	No
Avena coleoptile tips[153]	IAA	ET_2O & H_2O	Oat Curvature	No
Corn seed (immature)[54]	IAA	Isolation	-	-
Corn seed[55]	IAA	Hydrolysis	(isolated)	
Corn seed[17]	IAA	"	"	
Corn seed[145]	IAA, +Unk.	Aq.acetone		Yes
Corn seed[30]	IAA	Ether	Wheat Sect.	Yes
Corn seed[170]	IAA	Ethanol	Oat Sect.	Yes
Corn seed[189]	IAA, IAAld	Ether	Oat Curvature	Yes
Corn pollen[43]	IAA	Ether	Oat Sect.	Yes
Pea seed & seedlings[30]	IAA, I Car.	Ether	Wheat Sect.	Yes
Grape[115]	IAA	Ether	Oat Sect., Oat Internode	No
P.tricuspidata crown gall[115]	IAA	Ether	Oat Internode	No
Tomato ovary[115]	"	"	" "	"
Immature bean seed[115]	"	"	" "	"
Citrus flowers[146]	IAA	Ether	Oat Sect.	Yes
Corn endosperm[146]	IAA, 2 unk.	"	" "	"
Datura ovule tumors[128]	IAA	Ether	Oat Curvature	Yes
Broccoli leaves[98]	IAA	Ether	Wheat Sect.	No
Broccoli leaves[96]	IAA	Ethanol	Oat (paste)	Yes
Asparagus fruit[98]	IAA	Ether	Wheat Sect.	No
Strawberry[114]	IAA	Ether	Oat Sect.	No

TABLE I
(continued)

Plant Tissue & References	Compounds Found	Type of Extract	Bioassay	Color Detection
Potato peel[19]	IBA	Ether	Oat Sect.	No
Cabbage[97,39]	IAA,IAN	Ethanol	Oat (paste)	Yes
Cabbage[73]	I. Car.	Ethanol		Yes
Cabbage[73,171,40]	IAld	Ethanol		Yes
Cabbage[74]	IAN	(Isolated & identified)		
Cabbage[97]	Skatole	Ether		Yes
Cabbage[67,172]	IAA	Ether	Oat Sect.	
Sunflower tumor[131]	IAA	?	?	Yes
Corn endosperm[131]	"	?	?	"
Tomato crown gall tumor[32]	IAA,I.Car.	Ether		Yes
Normal tomato stem[32]	I.Car.	Ether		Yes
Hevea leaves[20]	IAA	Ether	Oat Sect.	No
Tobacco ovarys[101,102]	IAA,IAN	Ether	Oat Curvature	No
Tobacco[169]	Unk.indole	Ethanol	Oat Sect, Pea-Tomato	Yes
Tobacco[78]	IAA	Ether	Oat Sect.	No
Sunflower shoots[13]	IAA & unks	Ethanol	Oat Sect.	No
Bean & pea roots & shoots[13]	" "	"	" "	"
Corn roots[13]	" "	"	" "	"
Potato peel[77]	IAA,IAN	Ethanol	Oat Sect.	No
Potato shoots [77]	IAA	"	" "	"
Potato tubers[118]	IAA	Hydrolysis		Yes
Cacao & bananas[111]	IAA	Ether	Wheat Sect.	No
Wheat roots[95]	IAA	Ether	Oat Sect.	No
Pea shoots[93]	IAAld	Ethanol	Oat Sect.	Yes

I. Car. = indole-3-carboxylic
IAld = indole-3-aldehyde
IAN = indole-3-acetonitrile
IAAld = indoleacetaldehyde
IBA = indolebuteryic

of the acidic ether-soluble material from cocao and bananas (111). In addition, biological activity was found at this Rf. By suitable selection of solvents, the component giving color tests could be separated from the growth promoting compound which, nonetheless, appeared to be IAA.

The best sources of indole compounds (Table I) would appear to be seeds, fruits, tumor tissue, and some crucifers (cabbage, cauliflower, broccoli, etc.). The physiological significance of IAA in these tissues is not at all clear. It is interesting that various lines of corn differ significantly in regard to the IAA content in the seed (143), and it is known that high IAA and tryptophan levels are linked to the sugary gene (151). It had been suggested that high IAA levels in immature corn seed were associated with low nicotinic acid levels, due to competition for tryptophan, which was thought to be the precursor of both. However, recent work makes it appear this is not the case, since labeling experiments indicate that tryptophan is not the precursor of nicotinic acid in corn (65). It should be mentioned that the IAA and tryptophan levels rise to a maximum in the immature corn seed at about 18 to 24 days after pollination, and then the levels fall with approaching maturity (143,151). The physiological significance of high levels of indole compounds in crucifers is unknown. Linser (96) has questioned any possible physiological role of high levels of IAN and other indole compounds in cabbage.

In rapidly growing seedlings, where the growth may be demonstrated to be under hormonal control by various physiological experiments, it is extremely difficult to detect IAA. It may be the minute amount present makes detection impossible except by sensitive (but nonspecific) bioassays; but, on the other hand, as Bentley (14) points out, sweeping statements are not yet prudent.

3. The Existence of Indole-3-Acetic Acid in Avena Coleoptiles

It is of interest to return to the Avena coleoptile and summarize the evidence available on the native hormone in this organ. It is obvious that where auxin is isolated by allowing it to diffuse from the excised tips into agar blocks, that one is measuring production per unit time. It also appears that some sort of steady state must exist between production and inactivation. The latter may involve non-specific binding, enzymatic destruction, or some sort of inactivation at the active site during the growth process. Though it is stated that cold ether extraction is a measure of free auxin in the tissue, the evidence is not convincing. Wildman and Muir (185) could demonstrate production of IAA (Avena curvature test) from tryptophan during wet ether extraction of fertilized tobacco ovarys. The process has a low temperature optimum of about 13°C, and though they state there was no production of IAA at 0°C in 16 hours, their data indicate a slight production of IAA even at this low temperature. Terpstra (153), also using the Avena curvature test, found not all the acid auxin could be extracted in one day at 4°C from Avena coleoptile tips, and after 7 days of extraction, a considerable quantity of neutral auxin was found. Much more auxin was found after incubating Avena tips 1 to 6 days in water. After freezing and thawing the coleoptile tips 4 times, little auxin could be detected, and also grinding fresh tips reduced the auxin yield to a similar extent. If frozen or fresh-ground coleoptiles were incubated at 23°C, more auxin was found, but addition of 0.01 ppm of diethyldithiocarbamate inhibited this increase. It was suggested that diethyldithiocarbamate inhibits both formation and destruction since in experiments with whole tips extracted with water at 23°C, it inhibited destruction of auxin. Thus one can conclude that only very short term,

low temperature extractions with ether are valid as estimates of the "free" IAA content.

At least three estimates have been made of the auxin content of the Avena tip (1-5 mm). Larsen (91) estimated 1.7×10^{-4} µg "IAA equiv.", Thimann (157) suggested about $1\text{-}1.5 \times 10^{-4}$ µg "IAA equiv.", while Wildman and Bonner (183) found about 1.6×10^{-4} µg "IAA equiv.". These estimates, which are in remarkable agreement, are either based on cold ether extraction or short term diffusion experiments. More recently, it has been estimated that the tip supplies an equivalent of about 0.075 µg/ml of IAA (6,7). Since a 1 mg tip (2 mm) is about 0.001 ml, this would correspond to about 7.5×10^{-5} µg per each tip, which is in excellent agreement with the other estimates. Thus about 10,000 tips might be anticipated to contain about 0.75 to 1.0 µg of IAA.

Wildman and Bonner (183) collected 23,000 lyophylized Avena tips (about 2.3 gm dry weight), and extracted the material with wet ether in the cold. The ether extracts contained 2.3 µg of "IAA equiv." as measured by the Avena curvature test. A Salkowski assay was positive, and indicated about 1.8 µg of "IAA" was present. They also allowed about 20,000 tips to diffuse for 3 hours in a small volume of water and estimated 1.9 µg of "IAA" by the Avena curvature test, but in this case the Salkowski test was pink instead of the usual deep red color. The coleoptile tip was found to be 6 times as effective as the remainder of the coleoptile in converting tryptophan to IAA, and there appeared to be regeneration of the converting system after decapitation of the tip. The molecular weight of the diffusible auxin was found to be about 306, but after extraction into ether, the molecular weight was estimated to be 206. This discrepancy was assumed to be due to the elimination of diffusible inhibitors by

ether extraction.

Terpstra (153) paper chromatographed the water, total ether, and acidic ether fractions from Avena coleoptile tips. The activity as determined by the Avena curvature test corresponded in each case to the Rf of IAA. Shibaoka and Yamaki (132) allowed 600 Avena tips to diffuse for 3 hours at 25°C on agar. The agar was extracted with ether and then water. Both the water and ether fractions were made up into agar blocks containing 5×10^{-3}M $FeSO_4$. The latter sensitized the Avena curvature test, resulting in a linear range from 0 to 0.1 µg. Only the ether fraction was active, so the remainder of it was chromatographed in 3 solvents, and in each case the Rf corresponded to an Rf of IAA. Thus from the available evidence, the case would appear strong that the auxin in Avena coleoptile tips is IAA.

4. Lack of Redistribution of Exogenous Indole-3-Acetic Acid Following Geo- or Phototropic Stimulation

Gordon and Eib (51) reported that Avena coleoptiles treated with IAA-2-C^{14} transported it in a polar manner at a velocity of 1mm/min. When the coleoptile was illuminated unilaterally, no difference in radioactivity could be detected between the light and dark side, but more auxin did diffuse from the dark side as measured by the Avena curvature test. At the same time, Bünning et al. (28) reported similar results. More recently, Ching and Fang (31), as well as Reisener and Simon (127), have reported lack of redistribution of radioactive IAA in geotropically stimulated roots and coleoptiles. If one accepts IAA as controlling geotropic and phototropic response, then one must assume that: (1) There is an effect on production or release of endogenous IAA as a

result of unilateral geotropic or phototropic stimulation; (2) The internal pool of IAA is separate from the externally supplied pool; or (3) The supplied IAA saturated the transport mechanism, leading to failure to redistribute a detectable quanitity of IAA-C^{14}.

An examination of these results indicates that in at least some cases the third suggestion may not apply, so perhaps the second suggestion is most attractive. This might imply some complexed or bound form of IAA is redistributed. Though several bound or complexed forms of IAA are known, the physiological significance of them is unknown.

5. Bound or Conjugated Forms of IAA

It was first observed by Kögl, Erxleben, and Haagen-Smit (79) that alkaline hydrolysis released auxin from seeds. Several proteins also release micro amounts of auxin following enzymatic alkaline hydrolysis (50,130) as measured by the Avena curvature test. Though Siegal and Galston (133) have demonstrated a complexing of IAA to pea root homogenate protein, no critical study of this complexing has been conducted. Many tissues will liberate auxin into ether for an extended period of time (161), and though this was formerly regarded as bound auxin, it is now apparent this liberation represents production from tryptophan (185).

The best known auxin complex is that occurring in corn seed and the nature of this precursor was extensively studied by Berger and Avery (18). These workers found that aqueous acetone extracts of Country Gentleman sweet corn seed contained a yellow, gummy, aqueous-insoluble material from which, after alkaline hydrolysis (pH 9.6 for 5 min.) crystaline IAA contaminated with a carbonyl containing compound could be isolated. The material was relatively soluble in aqueous ethanol, methanol, dioxane or dilute alkali, but it was relatively insoluble in ether, hot acetone, or

dilute acid. It was dialyzable (40% in 12 hours) in crude preparations, but after several reprecipitations, only 5-7% was lost upon dialysis. The precursor contained only about 4.5% nitrogen, so either it was impure or not a protein. Stehsel (143) has examined the nature of IAA releasing material from immature sweet corn seed. This "auxin complex" was extracted by aqueous methanol, dialyzable, and had an Rf of 0.25 in butanol/HCl/H_2O (60:25 ml, 1N HCl). The molecular weight as determined by agar diffusion was estimated to be 300 to 500. Conversion took place readily upon heating with 0.02 to 0.003 N NaOH for five minutes even under nitrogen. The complex was found to give a positive Salkowski color, but differed somewhat from that given by IAA. In confirmation of Avery and Berger, the "auxin complex" was inactive in the Avena curvature test.

Söding and Raadts (141) reported a water-soluble substance in oat coleoptiles which appears to yield IAA (detected by bioassay) upon paper chromatography. Terpstra (153) found a water-soluble complex in Avena coleoptiles which yielded IAA after alkaline hydrolysis. Housley and Bentley (69) reported that the water-soluble, ether-insoluble fraction in cabbage gives rise to an auxin having the same Rf as IAA in isopropanol/ammonia/water solvent, but a lower Rf in n-butanol/ammonia/water. It should be mentioned that early physiological experiments by Skoog (136) using the deseeded Avena test, and later experiments by Raadts (123), indicated a presursor of auxin was translocated from the seed to the coleoptile tip. This precursor had been suggested by Wildman and Bonner (183) to be tryptophan, but indoleacetaldehyde has also been suggested as the transported precursor (123). Pohl (121) and Tegethoff (152) have studied a growth inhibitor in corn scutellum which is converted to auxin by crude homogenates of Avena coleoptile tips (152).

The inhibitor appears to inactivate IAA by combining with it (152). It would be attractive to suppose that IAA in the endosperm could be converted to a transported complex which may be again converted to IAA (or another complex) at the coleoptile tip.

In addition to proteins and the above unknown substances which release IAA upon mild alkaline hydrolysis, several amino acid conjugates of IAA are known. It has been known that IAA is excreted in urine, partly as the glycine complex (60), and recently the glutamine complex has been detected (72). Investigations by Andreae, Good and associates (3,4,5,45) have shown that feeding IAA to seedlings results in formation of the aspartic acid conjugate primarily, though some of the IAA was converted to the amide. Jepson (71) found the latter could be formed, due to ammonia in the chromatography solvent. Andreae et al. expressed the view that these reactions may be involved in detoxification.

A compound called ascorbigen has been detected in various crucifers by Procházka (122). This compound yields IAA upon alkaline hydrolysis, or ascorbic acid after acid hydrolysis. It is of interest to know if this compound is one of the unknown complexes discussed above. In the suggested structure, the 3-indole-1,2-propylene group is linked 1,2 through oxide bonds to ascrobic acid in the 2,3 position.

6. Other Indole Compounds Occurring in Plants

Jones and co-workers (74) have isolated 3-indoleacetonitrile (IAN) from cabbage and it has since been detected in a number of other species (19,30,101). Indoleacetonitrile is very active in the Avena test, and Thimann (158) has demonstrated its conversion to indole-3-acetic acid.

Larsen (89,90,93) has detected 3-indoleacetaldehyde in pea seedlings

and Yamaki and Nakomura (189) report it to be present in corn seedlings. Gordon (49) has also detected it indirectly in pineapple leaf homogenates and mung bean seedlings.

The isolation of ethyl IAA from immature corn seed by Redemann, Wittwer and Sell (124) has been suggested to be an artifact (74) owing to the use of ethanol during isolation. Likewise, Luckwell and Powell (100) have questioned Teubner's (154) detection of the ethyl ester in apple endosperm.

The detection of indolepyruvic acid was reported by Stowe and Thimann (145). However, it has been shown that synthetic indolepyruvic acid breaks down completely under the alkaline conditions of chromatography used (15,76). A characteristic pattern of about six breakdown products are obtained upon chromatography in isopropanol/ammonia/water. These breakdown products have been studied by Kaper and Veldstra (76) who find 3 neutral compounds having an Rf greater than IAA; and 3 acidic compounds, one of which appears to be 3-indoleglycolic acid.

Though Fischer (39) has reported detection of indoleglycolic acid in cabbage, Bentley (14) found upon chromatography of a synthetic sample, two spots were obtained. The minor spot did have an Rf about like that reported by Fischer, while the major spot had a much lower Rf. Fischer and co-workers (40,171,172) as well as Jones and Taylor (73) have reported the occurrence of 3-indolealdehyde in cabbage. The existence of 3-indolecarboxylic acid in cabbage has been reported by the latter authors. Cartwright, Sykes and Wain (30) have also found this acid, along with IAN, in chromatograms of extracts of germinating peas. Clark, Dye and Wain (32) found IAA and indolecarboxylic acid in crown gall tumors of tomato, and a trace of the latter in normal stems. Fawcett et al. (38) have shown that upon feeding

IAN to pea seedlings, 3-indolealdehyde as well as 3-indolecarboxylic acid are found as breakdown products. It is suggested that α-oxidation occurs with the nitriles in contrast to β-oxidation of acids and amides. It is of interest that IAN is not converted to IAA in pea seedlings (150) as measured by either growth response or enzymatic conversion. Its detection in this tissue and lack of conversion to IAA, suggest strongly that IAN can not be considered a precursor of IAA. The occurrence of large amounts of IAN and its breakdown products, 3-indolealdehyde and 3-indolecarboxylic acid, in cabbage suggest a similar situation in this tissue.

Various other indole compounds are known to occur in at least certain plant species. Their physiological significance is unknown, but some of them could be degradation products of tryptophan and IAA. Compounds reported to occur are indole (146), N-methyl-tryptophan (44,146), 5-hydroxy-N,N-dimethyltryptophan (147), 5-hydroxytryptamine (119), 5-hydroxyindole-acetic acid (163), and skatole (104). It is of interest that hydroxylation of the ring also occurs upon feeding phenoxyacetic acid to plants (182).

7. Biosynthesis of Indole-3-Acetic Acid

Thimann (157) suggested quite early that IAA was synthesized from tryptophan in Rhizopus suinus. He had observed that tryptophan containing peptone and strong aeration was required for good yields of IAA from this fungus. Other indirect evidence that tryptophan might be converted to IAA in Avena coleoptiles comes from the early work of Skoog (136) who observed that tryptophan and tryptamine gave a delayed curvature in the deseeded Avena curvature test. The studies of Stehsel (143) and Teas, Cameran and Newton (151) on tryptophan levels and IAA levels in developing corn seed tended to indicate a correlation between free tryptophan content and IAA levels. Both reached a maximum 24 to 28 days after pollination,

and fell with approaching maturity of the seed. Nitsch (114) has likewise found a correlation between free tryptophan and IAA in the developing strawberry. Lund (102) has found an inverse correlation between free tryptophan and IAA in pollinated tobacco styles. He suggests the pollen tubes convert free tryptophan of the style to IAA. Other evidence for a correlation *in vivo* has been presented by Yamaki and Nakamura (189). These authors report a correlation exists between free tryptophan and IAA during germination of corn embryos. In endosperms, a very little IAA could be detected, which is in contrast to results of Cartwright, Sykes and Wain (30); Housley, Booth and Phillips (70); and the present investigator. Additional evidence that tryptophan is converted to IAA comes from the work of Skoog (136) and Tsui (162) who found that zinc deficiency results in a low level of auxin and tryptophan. Tsui showed the tryptophan level appeared to be affected first, and that the tryptophan-IAA converting system was just as active in the deficient tomato plants. Skoog has found auxin production to be sensitive to X-irradiation (135) and Weber and Gordon (175) have found the tryptophan converting system *in vivo* is quite sensitive. The *in vivo* evidence for a correlation between tryptophan and IAA is fairly good. On the other hand, the levels of free tryptophan are frequently two or three orders of magnitude higher than IAA levels. Thus it is surprising that IAA levels appear dependent on free tryptophan levels.

The *in vivo* tryptophan feeding experiments furnish addition evidence that tryptophan may be converted to IAA. The fact that tryptophan (as well as tryptamine) gives a growth response in the deseeded Avena curvature test (136) and is converted to IAA by Rhizopus (157) has been

mentioned. It has also been shown (186) that U. zeae myceleum as well as Agrobacterium tumefaciens (176) convert tryptophan to IAA. Wildman, Ferri and Bonner (184) showed that tryptophan-infiltrated spinach leaf disks had a higher IAA content that uninfiltrated leaf fisks. Indolepyruvic acid also was converted to IAA, but tryptamine was not. Lund (101) has reported that feeding L-tryptophan to tobacco pollen results in an increase in the amount of IAA in the pollen as detected by paper chromatography.

Dannenburg and Liverman (33) demonstrated conversion of tryptophan-2-C^{14} to labeled IAA in immature watermellon slices. Five radioactive Ehrlich positive acidic compounds were detected, one of which was IAA. It was suggested one of the others was some residual indolepyruvic acid which failed to breakdown following alkaline chromatography. Two basic compounds were found, one of which was suggested to be tryptamine; and two neutral compounds were detected, one of which appeared to be IAN.

In addition to the above in vivo feeding experiments, Wildman and Muir (185) have demonstrated conversion of tryptophan to auxin during ether extraction of tobacco ovarys.

The existence of 3-indoleacetaldehyde, a supposed intermediate in the tryptophan-IAA conversion, in at least some plants, has already been discussed. Though indolepyruvic acid has not yet been detected directly, ammonical chromatography breakdown products observed after feeding tryptophan to watermelon slices (76), closely resemble breakdown products of 3-indolepyruvic acid. Tryptamine also occurs naturally in some plants (180).

There have been many in vitro demonstrations of conversion of tryptophan to IAA by various plant tissues (49,63,102,183,184,189). Wildman, Ferri and Bonner (184) found conversion in spinach leaf homogenates of

L-tryptophan to IAA. The pH optimum was 7.5, oxygen was required, and conversion was sensitive to cyanide or bisulfite. The latter suggested that an aldehyde might be an intermediate. Gordon and Sanchez (47,48) found homogenates of pineapple leaf bases could convert tryptophan, tryptamine, 3-indolepyruvic acid, or 3-indoleacetaldehyde to IAA. Gordon (49) also reported that mung bean homogenates convert indolepyruvic acid, but not tryptamine, to IAA. Wolf (186) failed to find conversion of tryptamine by Ustilago zeae cultures. Yamaki and Nakumura (189) reported conversion of tryptamine by corn embryo preparations, though it could not be deomonstrated in the present work that corn seedlings could convert tryptamine to IAA. These latter workers also found conversion of indoleacetaldehyde to IAA by corn embryo homogenates. Henderson (63) has detected tryptophan-IAA conversion in sunflower stem and crown gall homogenates. The latter had a higher converting activity. When normal stem preparations were mixed with crown gall preparations, the activity of the latter was depressed. Since there appeared to be no difference in destruction, the presence of an inhibitor in normal stem tissue was suggested. The pH optimum was between 7.0 to 8.0. Wildman and Bonner (183) have reported Avena coleoptile tips convert tryptophan to IAA six times as effectively as basal portions of coleoptiles on a fresh weight basis. Also, there appeared to be regeneration of the converting ability in decapitated coleoptiles. Gordon has studied the intercellular localization of the tryptophan-IAA converting system in mung bean seedlings (46) and reports all the activity resides in the soluble fraction. He found this was true when the grinding medium was isotonic sucrose-phosphate, water or isotonic Carbowax 4,000. He also found the so-called Fraction I pro-

tein was inactive, whereas the converting activity resided in the heterogeneous cytoplasmic proteins. In this study, IAA was isolated by paper chromatography of an acid ether extract of the incubation mixture. The isolated IAA was assayed by the Salkowski and Avena curvature. These results are in contrast to those of the present study with corn seedlings.

Bonner (21) comments that at least some tryptophan-IAA converting activity is found in most tissues, even though on physiological grounds one would expect none. Direct in vivo labeling experiments showing tryptophan as the only source of IAA have not yet been possible. However, it can be concluded that tryptophan may be converted in vivo to IAA.

In summary, the biosynthesis of IAA from tryptophan insofar as it is known in plants (oxygen requirement and CN sensitivity), would correspond to an amino acid oxidase. Krebs (86) observed D- and L-amino acid oxidase activity in liver and kidney preparations from a number of animals. He demonstrated separation of D- and L-amino acid oxidase activity, and found the L-amino acid oxidase was sensitive to cyanide. The D-amino acid oxidase was found to be an FAD flavoprotein. More recently (134), the L-amino acid oxidase has also been shown to be a flavoprotein containing FMN. Tryptophan is one of the amino acids most readily oxidized by enzymes from various animal or microbial sources. The keto acid formed as an intermediate is decomposed by the H_2O_2 formed from reaction of the reduced flavin with oxygen. However, amino acid oxidase activity in higher plants has not yet been detected (148), unless the tryptophan-IAA system is in fact such a system. It would be of interest to know if the tryptophan-IAA system could also convert other amino acids.

B.
Synthetic Plant Growth Substances

Many diverse synthetic compounds have been found to have activity as growth substances in various biological tests. Kögl and Kostermans (83) reported in 1935 that the methyl IAA and indolepyruvic acid were active, but indolelactic acid was inactive, in the Avena curvature test. Haagen-Smit and Went (56) also reported the activity of indolepyruvic at about the same time. Thimann (156) found indene-3-acetic had slight activity but comural-2-acetic acid had no activity in the Avena section test.

The work of Zimmerman and associates both broadened the scope of active compounds and introduced the first practical use for a synthetic auxin. In 1935, Zimmerman and Wilcoxon (193) and Hitchcock (66) reported phenylacetic, γ-phenylpropionic, indolepropionic and naphthylene acetic acids were active in inducing rooting of cuttings, as well as inducing other formative or morphological responses. In the same year, Thimann and Koepfli (159) reported IAA was active in the rooting of cuttings. Thimann and Went (161a) had already studied the nature of the root forming hormone and concluded it was identical to auxin. Zimmerman and Hitchcock (190,191,192), using the morphological and epinastic responses of the intact plant as a primary criteria of activity, soon added halogenated-phenylacetic, -benzoic, and -phenoxyacetic acids to the list of synthetic growth regulators. Many compounds showed activity on intact plants even though no activity was shown in classical growth tests. Even ethylene was active in producing typical epinastic and "formative" responses of intact plants. It was already apparent that various tests led to different estimates of the activity of the same compound.

Gustafson (53) found in 1936 that synthetic "auxins" such as indolepropionic acid, indolebutyric acid and phenylacetic acid induced parthenocarpic fruit in tomato. Zimmerman and Hitchcock (191) observed increased fruit set in tomato after treatment with a number of synthetic "auxins." At the present time, commercial use of synthetic growth substances for fruit set and parthenocarpic fruit is limited to the fig (144), where normal pollination is accomplished by a small wasp which ovulates in male flowers produced by a separate male tree.

Another property of auxin was found by Laibach (88) in that auxin seemed to play a role in abcission. LaRue (94) demonstrated delayed abcission in coleus by synthetic growth substances, and the litereature in the field has been reviewed by Addicott and Lynch (2). Commercial use in preventing pre-harvest drop, especially in apples, is fairly wide spread.

Although the above uses of synthetic plant growth substances are considerable, the selective herbicidal effects of these compounds are of enormous economic importance in agriculture. The selective phytotoxicity of synthetic "auxins" and other organic compounds was investigated in England by Slade, Templeman and Sexton (139) as well as Nutman, Thornton and Quastel (116); and by a research team led by Kraus, Norman, Minarik and others (85) in the United States. The finding that important grain crops tolerated quantities of these materials which killed many dicotyledonous species, made them extremely valuable. In addition, many other phytotoxic organic chemicals have now been placed in commercial use as a direct result of the intensive work stimulated by the initial discovery.

In addition to the practical aspects, the synthetic growth substances have been of great theoretical interest to plant physiologists. In this

regard, great effort has gone into attempts to define the growth inducing site in terms of the specific structural requirements necessary, or to correlate *in vivo* activity with some model system. These types of studies have led to finding activity in diverse, unexpected groups of compounds; but on the other hand, within a related group of compounds, completely inactive analogues are common. It is now apparent that many secondary factors operate in the *in vivo* tests. A compound may not be taken up, or it may be inactivated by physical adsorption, metabolic conjugation, or destruction. At least the potentially active compound does not reach the active site. Conversely, an inactive compound may be converted to an active derivative within the plant (174). The unresolved problem of qualitative differences in activity in different bioassays may in part be due to these secondary factors, but could also be due to more than one active site. A more detailed discussion of competitive inhibition, as well as inhibition of tropisms by synthetic plant growth substances, will be given in the introduction to the papers on these topics.

EXPERIMENTAL

I.
STUDIES ON PLANT GROWTH SUBSTANCES

A.
Occurrence and Biosynthesis of Indole-3-Acetic Acid

1. In Vitro Experiments on the Biosynthesis of IAA from Tryptophan

In preliminary experiments, Aspergillus niger, Rhizopus nigricans and Penicillum chrysogenum were examined for their ability to produce IAA from tryptophan. It was hoped that a highly active source of the converting system could be found.

The above fungi were grown on liquid potato dextrose media for ten days in one liter flasks. The mats were then cut in half and transferred to 200 ml 0.005 M potassium phosphate buffer pH 7.0 plus or minus 0.5 mg/ml DL-tryptophan. The solutions were vigorously aerated for 12 hours, and 2 ml samples examined by Salkowski assay (149). A test for Salkowski positive substances was given by Rhizopus nigricans and Penicillum chrysogenum, with the former showing the most activity. Some additional experiments with Rhizopus mats indicated a maximum color (Salkowski assay) of about 17.5 µg of "IAA equivalents" was reached in about 24 hours.

A crude homogenate was prepared by grinding 5 grams (fresh weight) of frozen Rhizopus mat in 30 ml of 0.01 M potassium phosphate buffer pH 7.0 at 1°C using a glass homogenizer. After pouring through several layers of cheese cloth, the filtrate was centrifuged at 1°C in an angle centrifuge at 2,000 x G for five minutes. The supernatant liquid was used as a source of the crude enzyme.

An assay mixture consisted of 2 ml crude enzyme, 2 ml of 0.005 M tryptophan (10 µM), 2 ml of 0.005 M sodium pyruvate, and 0.5 ml of 0.01 M $MgCl_2$. A 2 ml sample was deproteinized after 0, 75 and 210 minutes with

1 ml of 5% TCA. Salkowski assay on 2 ml of deproteinized supernatant indicated that a very small amount of IAA could be detected after 75 and 210 minutes. Boiled enzyme and no-tryptophan controls were negative. Previous incubation of the mat on tryptophan-containing media gave no evidence for adaptive formation of the "enzyme". The omission of $MgCl_2$ or pyruvate separately had no effect on activity. Although visually detectable pink color appeared in the above assays after $1\frac{1}{4}$ to $3\frac{1}{2}$ hours incubation at 30°C, only after 18 to 20 hours was the Salkowski color readily measurable with the photoelectric colorimeter.

After prolonged incubation, IAA could be detected by paper chromatography (isopropanol/ammonia/water, 10:1:1 ascending) of the ether extracts of acidified deproteinized supernatants. However, when TCA was used for deproteinization, a yellow spot having the Rf of IAA appeared on paper chromatograms when sprayed with Ehrlich's reagent (1% p-dimethylaminobenzaldehyde and 8.5% HCl in 95% ethanol). Subsequently, deproteinization by boiling indicated the presence of IAA. However, when indole-3-acetonitrile and tryptamine were used as substrates, little if any IAA could be detected by direct Salkowski assay on paper chromatography as described above. In several experiments, small samples of the supernatants were also chromatographed on paper. There appeared to be a possible inter-conversion of tryptophan and tryptamine in several of the experiments. Also, there was a marked disappearance of IAN.

Corn seedlings (W23 x Oh51A) were found to be as active a source of the tryptophan converting crude enzyme as Rhizopus, so further studies were with this tissue. For these assays, 5 gm (fresh weight) of corn was ground in 30 ml of 0.01 M potassium phosphate buffer. The preparation and assay are as previously described for Rhizopus. It was found that activity in

corn was sensitive to freezing (Table I) and that roots were as active as shoots.

TABLE I

Optical Density at 525 mμ of 2 ml Deproteinized Supernatant and 8 ml Salkowski Reagent After 14 Hours Incubation of Tryptophan with Frozen and Unfrozen Corn Homogenate Preparations

Homogenate	Substrate	O.D. 525
4-day-old shoots	10 μM tryptophan	0.086
4-day-old shoots	0	0.005
4-day-old roots	10 μM tryptophan	0.046
4-day-old roots	0	0.002
Roots frozen 2X	10 μM tryptophan	0.000
Roots frozen 2X	0	0.001

Some corn homogenate prepared as previously described was centrifuged 1 hour at 25,000 x G. The original enzyme, the supernatant, and the residue (resuspended in the original volume) were compared. All the activity was found in the residue, so it appeared the converting "enzyme" was particulate in nature.

The above two observations (particulate nature and sensitivity to freezing) made it desirable to examine the particulate fractions under isotonic conditions. The procedure of Millerd *et al.* (107) was used. Thirty grams of tissue (F.W.) was ground at 1°C in a mortar with 10 gms of analytical grade sea sand plus 40 ml of 0.1M potassium phosphate buffer pH 7.0, and 0.4M sucrose isolation solution. The debris was strained through 8 layers of cheese cloth, the filtrate centrifuged at 500x G for 5 minutes, and the residue resuspended in 35 ml of isolation solution. The supernatant liquid was centrifuged at 10,000 x G for 15 minutes and the residue (mitochondrial fraction) resuspended in 20 ml of

isolation solution. The resuspended residue was centrifuged again for 15 minutes at 10,000 x G and the precipitated material resuspended in 3.5 ml of isolation solution.

The results of several experiments are summarized in Table II. In all cases, the residue and the supernatant were relatively inactive, but it must be pointed out 5.7 ml of supernatant would be required to be equivalent to 0.5 ml of mitochondria or residue (1/7 of the total volume) on a fresh weight basis. Also, the supernatant developed a red-brown color with Salkowski reagent, making detection of less than 5-10 µg IAA tube impossible by the direct Salkowski assay. Sodium pyruvate, α-ketoglutarate and pyridoxal phosphate do not stimulate the activity.

TABLE II

Activity of Residual, Mitochondrial, and Supernatant Fractions From 4-Day-Old Corn Shoots[1]

Fraction	Exp. No.:	Salkowski Color Complex O.D. at 525 mµ[2] 23	24	30	31	32	33
1. Residue (500 x G)		0.038	0.033	----	----	0.033	0.018
2. Mitochondria (10,000 x G)		0.084	0.094	0.103	0.104	0.163	0.150
3. Supernatant		0.040	0.000	----	----	0.020	0.010
4. Mito. +α-ketoglutarate instead of pyruvate		0.098	----	0.098	0.114	0.125	0.164
5. #4 plus pyridoxal phosphate[3]		----	----	0.101	----	0.114	0.118
6. #2 plus pyridoxal phosphate[3]		----	----	0.103	----	0.148	0.124
7. #2 minus pyruvate		----	----	----	----	0.144	0.144

[1]The assay mixture consisted of 2 ml of 0.005 M tryptophan; 2 ml of 0.005 M sodium pyruvate; 0.5 ml of 0.1 M $MgCl_2$; 0.5 ml of residue or mitochondria or 2.0 ml supernatant; and sucrose (0.4M) phosphate (0.1 M) buffer to bring the total volume to 6.5 ml. The incubation period was 8 hours at 30°C.

[2]O.D. with tryptophan minus O.D. without tryptophan.

[3]0.05 µM.

Two other co-factor experiments were run using a smaller volume assay mixture (Table III).

TABLE III

Effect of Various Factors on the Tryptophan Converting System of 4-Day-Old Corn Roots and Shoots[1]

	"Enzyme"	Substrate Factors Missing	Estimated IAA Equiv. µg/tube (2.5 ml) 1	2
1.	Shoots Mitochondria	0	13.3	12.0
2.	Shoots Mitochondria Boiled	0	0.0	0.0
3.	Shoots Mitochondria	Pyruvate, Succ.	16.7	8.0
4.	Shoots Mitochondria	ATP	16.7	29.0
5.	Shoots Mitochondria	ATP, Pyr., Succ.	23.4	29.0
6.	Shoots Mitochondria	Sucrose	25.0	18.0
7.	Shoots Residue	0	16.3	5.0
8.	Supernatant	0	----	----
9.	Roots Mitochondria	0	26.5	28.0
10.	Roots Mitochondria Boiled	0	0.0	0.0
11.	Roots Mitochondria	Pyr., Succ.	26.5	29.0
12.	Roots Mitochondria	ATP	11.6	36.0
13.	Roots Mitochondria	Pyr., Succ., ATP	16.7	30.0
14.	Roots Mitochondria	Sucrose	18.3	30.0
15.	Roots Residue	0	40.0	5.0
16.	Roots Supernatant	0	----	----

[1]Assay consisted of 0.5 ml "enzyme", 0.2 ml ATP 10^{-3} M, 0.25 ml $MgCl_2$ 0.01 M, 0.25 ml sodium pyruvate 0.005 M, 0.25 ml potassium succinate 0.001 M, and 0.5 ml tryptophan 0.005 M. Sucrose was added to bring molarity of mixture to 0.5 M and the total assay volume was 2.5 ml.

It is apparent that ATP, pyruvate, succinate or solutions made isotonic with sucrose are not stimulatory. The high activity in the residue fraction (500 x G, 5 min.) in Experiment 1 probably indicates a higher centrifugation speed, bringing about deposition of some mitochondria in this fraction.

Since it appeared that none of the co-factors included were essential, other assays of the supernatant, residue and mitochondrial fractions were run on an equivalent fresh weight basis (3 gms per tube), omitting everything except $MgCl_2$ and tryptophan. IAA was added to no-tryptophan control tubes either before incubation or after incubation and deproteinization. These results are shown in Table IV.

TABLE IV

The Relative Salkowski Activity of the Whole Homogenate Supernatant, Mitochondrial and Residual Fractions From Corn Shoots When Incubated with Tryptophan, or When IAA was Added[1]

Fraction	OD[2]	OD[2] for IAA Added to No-Tryptophan Controls					
		Initially	After Incubation 8 Hours 30°C				
		100 µg	5 µg	10 µg	25 µg	50 µg	100 µg
Whole Homogenate	.095	.058	.010	.009	.073	.209	.546
Supernatant	.064	.050	.024	.016	.075	.208	.537
Mitochondrial (10,000 x G)	.062	.223	----	.035	.264	.492	.801
Residue (500 x G)	.031	.188	----	----	----	----	----

[1]Assay mixture = 4 ml enzyme (3 gms - 37 shoots), ± 1 ml of L-tryptophan (5 µM), ± 1 ml water, + 0.5 ml of 0.01 M $MgCl_2$. Two ml was deproteinized by boiling and assayed with 8 ml of Salkowski reagent.

[2]In each case, the optical density of a no-tryptophan control without added IAA has been subtracted.

The data presented in Table IV illustrates the impossibility of direct Salkowski assay for small amounts of IAA in the presence of high concen-

trations of supernatant or whole homogenate fractions. The increase in the optical density of the whole homogenate and supernatant fractions over the no-tryptophan controls suggests considerable activity might reside in these fractions also. Destruction or inactivation of IAA also may have been a factor in these tests since when 100 µg IAA was added before incubation, considerable apparent destruction occurred. This apparent destruction was of the same order of magnitude (about 80%) in all fractions considering the insensitivity of the Salkowski assay with the whole homogenates and supernatant fractions.

Roots and imbibed kernels were fractionated as above and the equivalent of 15.6 gm of roots or kernels incubated with 10 µM of L-tryptophan in a total volume of 8 ml (Table V).

TABLE V

Converting Activity of Various Fractions of Corn Roots and Kernels when Incubated for 8 Hours With and Without Tryptophan

Fraction	Roots O.D.			Imbibed Kernels O.D.		
	+Tryptophan	-Tryptophan	Diff.	+Tryptophan	-Tryptophan	Diff.
Whole Homogenate	.272	.260	.012	.272	.240	.032
Supernatant	.201	.174	.027	.149	.097	.052
Residue (500 x G)	.076	.041	.035	----	----	----
Mitochondria (10,000 x G)	.190	.061	.129	.377	.248	.129

Again in this experiment it would appear that the mitochondrial fraction contains most of the converting activity. However, only in the kernel supernatant was the no-tryptophan optical density fairly low. The disappearance of IAA during incubation, and the ability to detect IAA with Salkowski

reagent in various fractions, was not determined. Since activity in the whole homogenate is low, it is probable the supernatant is either destroying IAA or precluding detection.

In the above experiments employing the Salkowski assay procedure to deproteinized incubation mixtures, it was impossible to decide whether the mitochondrial fraction accounted for most of the IAA biosynthesis from tryptophan. It is however certain that the mitochondial fraction is active in the conversion of tryptophan to IAA.

It was of interest to know how the whole homogenate and supernatant inactivates or destroys IAA. In two experiments, the supernatant fraction from corn shoots was incubated with 40 µg of IAA at 30°C, either with or without added catalase. (The catalase was obtained from Worthington and 170 units used per tube, 1 unit = 1 mg H_2O_2/Min). Samples were deproteinized and examined by Salkowski assay at 0, 2, 4 and 8 hours. The results indicated about 75% of the IAA disappeared even when the tubes were immediately deproteinized. The remaining IAA did not disappear upon incubation, and the addition of catalase had no effect. Several experiments were run in which, after addition of 5, 10, 20 and 40 µg of IAA to the various fractions, the assay mixtures were immediately deproteinized by saturation with ammonium sulfate. The IAA was isolated by paper chromatography of the acid ether fractions and the chromatograms dipped in Ehrlich's reagent. An attempt was made to elute the purple spots with 2 ml of 50% dioxane plus 4 drops of HCl. Even though this method did not lead to strict quantitative results, it indicated relatively great losses when IAA was added to whole homogenates of corn shoots, roots or kernels. Thus it was concluded that IAA appeared to be adsorbed on the protein of the crude whole homogenate or supernatent fractions, making it impossible to directly compare the

relative activity of these fractions with the mitochondrial fraction, from which recoveries were higher. The adsorption of IAA to protein of pea shoot homogenates has been reported to occur by Mârré (163) without the presence of ATP as was reported previously by Siegel and Galston (133) for crude pea root homogenates. In addition, Brian (25) has discussed the adsorption of 2-methyl-4-chlorophenoxyacetic acid to filtered crude homogenates of various plant species, using monolayers in a Langmuir trough. It is interesting to note that corn leaf bases showed fairly high relative adsorption in these tests.

In view of the above difficulties, further work was confined to the mitochondrial fraction. The enzyme dependency and the effect of freezing is shown in Figure 1. Each 0.1 ml of mitochondria is equivalent to 0.86 gms of corn shoots (fresh weight). The incubation mixture volume was 2.5 ml and it contained 12 µM sodium pyruvate, 2.5 µM sodium succinate, 0.1 µM ATP, 5 µM $MgCl_2$, 2.5 µM of L-tryptophan and sucrose to make the assay isotonic. It may be seen that 0.6 ml gave about the maximum activity, and that freezing twice reducedthe activity greatly. The highest activity would be equivalent to about 50 µg of IAA, or about 10% conversion in 8 hours at 30°C. It was apparent, however, that other Salkowski reactive products were probably present. For one thing, the rate of color development was much faster than for synthetic IAA added to the incubation mixtures. Some indole-3-pyruvic acid was obtained through the courtesy of Dr. H. Sell, and this sample also gave very rapid color development with Salkowski reagent. In addition, if phenylhydrazine hydrochloride was added to the deproteinized incubation mixtures and allowed to stand 30 minutes, a slight turbidity formed. After removing the turbidity by centrifugation, the supernatant was assayed with Salkowski assay. Color development in this

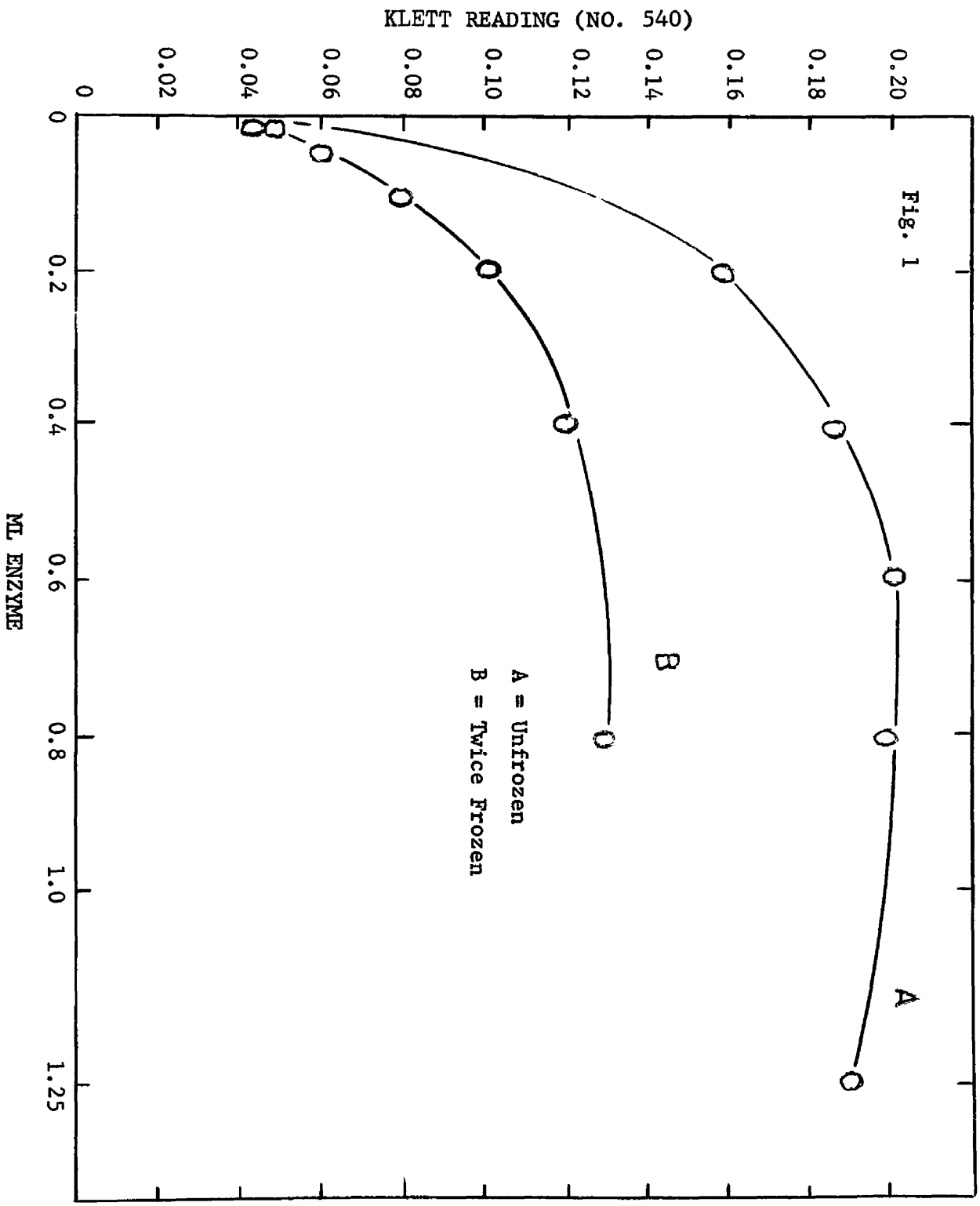

KLETT READING (NO. 540)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0
0.2
0.4
0.6
0.8
1.0
1.25
ML ENZYME
A
B
A = Unfrozen
B = Twice Frozen

Fig. 1

case was slow and more typical of IAA. When phenylhydrazine hydrochloride was added to a solution of 4 mg indolepyruvic acid in 2 ml of 50% ethanol, a crystaline precipitate was obtained.

The IAA formed from tryptophan by incubation with corn mitochondria was identified by ether extraction, followed by paper chromatography (isopropanol/ammonia/water, 8:1:1) of the acid ether fraction. When large amounts of incubation mixture were chromatographed, several spots appeared, with either Ehrlich or Salkowski reagents, in addition to the IAA spot. In all cases, the IAA detected was much less than estimated from direct Salkowski assay. The other unknown indole compounds appeared to correspond to the breakdown products of indolepyruvic acid when it was chromatographed, and will be considered below in connection with experiments using labeled DL-tryptophan as a substrate.

Other experiments in this laboratory had cast some doubt upon tryptophan being the main precursor of IAA in excised corn shoots. Therefore, experiments were devised to find if IAA really does come normally from tryptophan in the plant. It is known that in Avena coleoptiles the tip is the sole source of auxin; and if the tip is removed, growth will stop completely, or at least for an hour or so until there is so-called regeneration. This is the reason for double decapitation in the Avena curvature test. It was thought that the tip should have the system for converting tryptophan to IAA if this is the major pathway, and the basal portion of the coleoptiles would be unable to convert any tryptophan to IAA. However, it took about one hour to harvest the 320 coleoptiles; thus some of the basal segments could have acquired the ability to convert tryptophan to IAA. DL-tryptophan-2-C^{14} (specific activity - 0.43 $\mu c/\mu m$) was used so that there would be no

question about the IAA produced coming from tryptophan. Three hundred and twenty 3-day-old oat coleoptiles (grown in the dark at 25° and 95% relative humidity) were decapitated (5 mm from tip). The tips as well as the remaining coleoptile stumps were harvested. The weight of tips was 0.95 grams while the stumps weighed 6.2 grams. The mitochondrial fractions were isolated from both, as previously described, and resuspended in 1 ml of isolation solution. The enzyme consisted of 0.5 ml of mitochondria and boiled enzyme controls were run. The assay mixture contained 12 μM sodium pyruvate, 2.5 μM sodium succinate, 0.1 μM ATP, 5 μM $MgCl_2$, 2.5 μM DL-tryptophan and sucrose to make the assay isotonic in a volume of 1.75 ml. After 8 hours of incubation at 30°C, mixtures were deproteinized with NH_4SO_4. The acid fractions were chromatographed (ascending) on paper (isopropanol/NH_3/H_2O). The chromatogram was sprayed with Ehrlich reagent and radioautographed. Both the tips and the coleoptiles were able to convert labeled tryptophan to labeled IAA, but the tips were probably far more active, keeping in mind that the fresh weight of the stumps was about 6.5 times that of the tips. On radioautograms, an indole derivative was detected just below IAA in both the boiled and unboiled extracts. On paper it gave an Ehrlich's reaction, and the tryptophan sample was found to contain this substance as a contaminant.

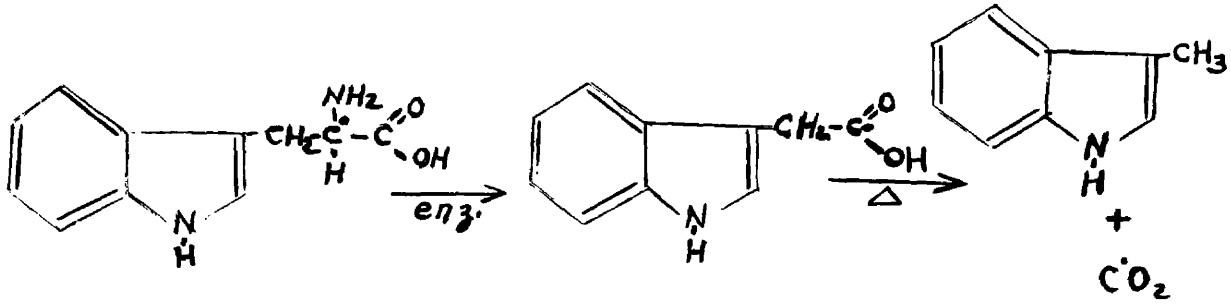
Another approach used was to compare the formation of IAA from other substrates with the amount formed from tryptophan, using the corn homogenate. It was thought that the whole homogenate should be used in these experiments because the supernatant might be able to convert other substrates to IAA even though it does not appear to convert tryptophan to IAA. Though this was tried, no IAA could be detected, probably due to the previously discussed difficulties. Another experiment was tried using only the

mitochondria and the following substrates: tryptophan, indole and serine, indole and glycolic acid, indole and glycine, indole and acetate, indole and fructose-1,6-diphosphate, and indole alone. Paper chromatography indicated traces of IAA were formed with indole and serine, indole and glycine, and perhaps indole and glycolic acid. However, no substrate was as good as tryptophan.

The possibility existed that tryptophan was degraded to indole by the mitochondria and then indole and some 2-carbon fragment such as glycolate was converted to IAA. The labeled IAA (formed from tryptophan) would still have been possible, but the side chain of tryptophan would be expected to be incorporated into many other components, and some $C^{14}O_2$ might even be lost. This is based, of course, on the assumption of the presence of a transammidase or an oxidative deamidation. In either case, a readily metabolizable organic acid would contain the label.

The overall reaction to alanine and indole has been found in E. coli and is called tryptophanase (187). Of course, if this system was operating, one might expect to find some indole on the chromatograms.

It was possible to see if the mitochondrial conversion was direct or indirect by using tryptophan-2-C^{14}. All the radioactivity should be in the carboxyl group of IAA and the specific activity of IAA should be comparable to that of tryptophan. The degradation of IAA by thermal degradation (129) should lead to inactive skatole and $C^{14}O_2$.



The above experiment was conducted four times with mitochondrial systems and twice using the whole corn shoots. The first two experiments were not carried to completion due to the small amount of IAA obtained in the first case, and the inability to recrystalize to constant activity in the second case. The third and fourth experiments were completed. The whole corn shoot experiments failed due to the small amount of activity in the IAA (700 counts total) isolated. Carrier IAA had been added.

In the third experiment, 102 grams of 4-day-old corn shoots were ground in 70 ml of 0.1 M phosphate 0.4 M sucrose buffer pH 7.0 at 1°C. The washed particles (prepared as previously described) were resuspended in a total volume of 12 ml in the phosphate sucrose buffer. This is a concentration of 0.87 gms fresh weight per ml of mitochondrial suspension. The incubation mixture used was 125 μM sodium pyruvate, 25 μM sodium succinate, 12.5 μM $MgCl_2$, 25 μM ATP, 5.0 μM tryptophan-2-C^{14}, and 6.0 ml of mitochondria in a total assay volume of 25 ml.

The incubation period was 8 hours at 30 degrees C. Salkowski estimates on the supernatant at this time indicated an apparent IAA concentration of about 10 μg per ml. The mitochondrial particles were spun off, the pH adjusted to 3.0 with tartaric acid and the supernatant extracted 3 times with ether. The ether was evaporated off under N_2 with a bath temperature of 57°C. The residue was partitioned between n-hexane and acetonitrile and the n-hexane discarded. The acetonitrile was then evaporated off as above, and the residue taken up in 10 drops of EtOH. The alcohol solution was chromatographed as a 3-inch strip on washed Whatman 1 paper (isoproponal/ammonia/water 8:1:1 v/v). The paper was equilibrated for 4 hours and run 16 hours at 1°C.

There was a small amount of IAA present as indicated by fluorescence

and p-dimethylaminobenzaldehyde (PDMABA) test of a $\frac{1}{4}$ inch strip in the center of the 3-inch band. The latter test indicated 4 compounds were present in addition to IAA (Table VI). The re-chromatography of spots A, C and D indicated approximately the same Rf values.

The IAA was removed from the paper moistened with 0.1M tartaric acid pH 3.0 by extraction 3 times with ether. The ether was concentrated, evaporated on a glass counting planchet, and it counted 19,630 CPM. The IAA was washed from the planchet with 2 ml alcohol. The alcohol was evaporated under N_2 at 57°C and a Salkowski test indicated 5 µg of IAA was present, or 10 µg total. The ratio of the molecular weights of tryptophan and IAA is 0.875 and the ratio of the counts per 10 µg is 0.860. (Under these conditions, 10 µg tryptophan = 16,850 cpm). The specific activity of the IAA isolated was in good agreement with the specific activity of the tryptophan fed.

The fourth experiment was carried to completion and both the specific activity of the IAA and the position of the label would suggest direct conversion of tryptophan to IAA. This experiment is summarized in Table VII.

TABLE VI

Results of Incubating Tryptophan-2-C^{14} with the Mitochondrial Fraction from 5-Day-Old Etiolated Corn Shoots. Substances Detected on Paper Chromatograms of the Acid Ether Extract.

Substance	Rf[2]	Approximate Radioactivity	Color With: Salkowski	Ehrlich	UV Light
A	0.90	50	Yellow	Pink	F. Lt. Blue
B	0.58	640	Pink	--	--
IAA	0.43[1]	1058	Red	Purple	F. Lt. Blue
C	0.31[1]	640	Red	Pink	F. Lt. Blue
D	0.24[1]	846	Red	Red	F. Lt. Blue

[1]Streaking in this area of chromatogram
[2]Isopropanol/ammonia/water (8:1:1)

TABLE VII

Purification and Degradation of Radioactive IAA Formed From Incubation of Corn Shoot Mitochondria with Tryptophan-2-C^{14}

IAA-C^{14} Eluted + 50 mg IAA			CPM/10 µM IAA	CPM/10 µM Skatole[2]	CPM/10 µM $BaCO_3$[3]
Recrystalization[1]		1	217.6		
"	"	2	187.7		
"	"	3	173.4		
"	"	4	209.2	0.4	189.7

[1]Recrystalization from 50% ethanol
[2]After one resublimation
[3]Corrected for thickness

Isolation of 3-Indolylacetic Acid from Ustilago zeae Tumors*

by

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Smut infections cause an increase in the auxin content of the host tissues[1]. This was first demonstrated in Ustilago zeae tumors by Moulton[2], using the Avena curvature test. Wolf[3] reported that four strains of U. zeae produced auxin, identified as indolyacetic acid (IAA) in a medium containing tryptophan. Von Guttenberg and Stutz[4] and Hirata[5] also detected auxin by bioassay in U. zeae cultures and in the corn tumors. Turian's observation[6] of high catalase levels in U. zeae tumors and the activation of acid phosphatase by tumor extracts[7], also supported the occurrence of a high IAA level. However, upon chromatography[8] of an extract from small quantities of tumors, no IAA could be detected with Salkowski reagent. Turian thus concluded that the IAA concentration (if present at all) must have been lower than 10^{-6}M. These results, together with the absence or at the most a very low concentration of IAA in healthy corn seedlings[9,10], suggested that chemical detection of the pathological hyperauxiny in the tumorized tissues would require

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Contribution No. 2574 from Michigan Agricultural Experiment Station

extraction of much larger amounts of tumor than previously used. Since the low concentration of IAA and the high concentration of interfering substances preclude direct chemical detection, it is necessary to use multi-step purification procedures. These procedures result in great loss of IAA during purification; so a small amount of IAA-2-C^{14} was added to permit correction for these losses. The radioactive IAA is also extremely useful in locating and following the IAA during the isolation.

Three main experiments were performed: the first with 1500 gms of maturing tumors (early-chlamydospore stage), the second with 1200 gms of younger tumors (mainly pre-chlamydospore stage), and the third with 1840 gms of uninfected corn stalks (early-tassel stage, collected in the field at the same time as the younger tumors.) The materials were stored at -20°C until used. The data and the following technical description correspond to our isolation of IAA from younger tumors, and serves to illustrate the general methods used.

Tumors were ground in a Waring blendor with sufficient 95% ethyl alcohol to make the final alcohol concentration about 80%. After filtration, 1.65 µg of IAA-2-C^{14} (specific activity of 9.18 x 10^4 d/m/µg[11] or, under our counting conditions, 34,500 c/m/µg), 7 grams of ethylenediaminetetraacetic acid (EDTA), and sufficient $NaHCO_3$ to make the ethanolic extract alkaline were added. The ethanol was removed <u>in vacuo</u> at 37°C by use of a steam-driven "spray dry" apparatus, and the resulting 1.5 liters of extract further concentrated to about 700 ml with a rotating film evaporator at 50°C and 17 mm Hg. The concentrate (pH about 8.5) was filtered through glass wool and extracted with two volumes of peroxide-free diethyl ether, and the ether was discarded. The aqueous portion

was adjusted to pH 3.5 with 10 N H_3PO_4 and extracted with 3 volumes of ether. The combined ether fractions were concentrated in vacuo to about 100 ml and extracted twice with 50 ml of 8% $NaHCO_3$. The combined bicarbonate extracts were adjusted to pH 4.0 with 10 N H_3PO_4 and extracted 3 times with equal volumes of ether. The combined ether extracts were dried over anhydrous sodium sulfate at 1°C, and then concentrated in vacuo to a dark reddish-brown "oil". This residue was diluted to 20 ml with ether, and a 0.2 ml aliquot was counted on an etched glass planchet (2.69 sq cm area) by using a thin-window gas flow G.M. tube. The total readioactivity in this fraction was 36,320 cpm, or about 64% of the IAA-2-C^{14} added.

The "oil" contained considerable acidic lipoidal material, possibly hydrolytic products of the glucolipid ustilagic acid[12]. In an effort to separate IAA from this lipoidal material, the "oil" was partitioned 2 times (after removing the ether) between 5 ml of acetonitrile and 5 ml of ligroin. The ligroin layers (light yellow) were discarded (less than 200 counts; about 0.2% of the IAA is lost with each extraction). After removal of the acetonitrile in vacuo, column chromatography was attempted by using 25 gm of diatomaceous silica (analytical-grade Celite, Johns-Manville Co., U.S.A.) moistened with 20 ml of 0.1 M phosphate pH 6.50 as the stationary phase, and ether saturated with the same buffer as the mobile phase. 26 fractions of 3.5 ml were collected and radioactivity was estimated by counting 0.2 ml aliquots from each tube. Fractions 6-18 were combined and dried over anhydrous sodium sulphate, and the ether was then removed in vacuo. The residues were paper chromatographed for 14 hours on Whatman 3mm paper (previously washed with alkaline EDTA and 0.1 N HCL) in isopropanol/ammonia/water (8:1:1) as

the ascending solvent. The IAA runs just above, but does not separate completely from a dark-brown pigment; so this entire area was eluted with 50% ethanol. The eluted sample was rechromatographed as above on a 6-inch strip but with a slightly more aqueous solvent (7:2:1). Upon examination of the chromatogram under UV light and monitoring for radioactivity, it appeared that some separation of IAA from the pigment had been achieved so that they could be eluted separately. After elution and counting as above, the dark-brown-pigmented fraction contained 8,350 cpm, while the light-yellow fraction had 22,780 cpm. Thus at this stage, total IAA recovery was about 55%. Since 63% of the IAA was in the light-yellow fraction, this was further purified by paper electrophoresis at 1°C on Whatman 3 MM paper (washed as above) by using 0.1 M citrate buffer of pH 5.25. The mobility of IAA under these conditions as judged by the application of known IAA to one edge of the electrophoretogram, is $2.94 \times 10^{-5} cm^2/volt\text{-}sec$. After drying, the IAA was located by means of a UV light, by radioactivity, and by spraying a narrow test strip containing known IAA with Ehrlich's reagent (1% p-dimethylaminobenzaldehyde plus 8.5% HCl in 95% ethanol.) The pigment-free IAA area was eluted with 50% ethanol, and the elution volume was adjusted to 1.5 ml. Measurements of the recovery of radioactivity on duplicate 0.05 ml samples indicated a recovery of 10,737 cpm, or 18.8% of the IAA-2-C^{14} added. For the quantitative estimation of the IAA recovered, a 0.3 ml sample was assayed with 1.2 ml of Salkowski reagent[13] by using the Klett microcolorimeter with a No. 54 filter. The rate of color development, followed at 10-minutes intervals for 40 minutes, was about the same in both standards and sample. The IAA concentration was 4.0 µg per 0.3 ml. Thus a total of 20 µg of IAA was isolated.

Since about 0.3 µg of this IAA was the IAA-2-C^{14} added, the total non-radioactive IAA isolated would be 19.7 µg and the total IAA present in the tumor tissue was 105 µg.

Results of the three isolations are summarized in the following table.

The IAA Content
of Ustilago zeae Tumors and Corn Stalks
Based on Isolation and Colorimetric Detection
of IAA and Corrected for Recovery of Added IAA-2-C^{14}

Tissue	Fresh Wt. (gms)	µg IAA Isolated[a]	Percent Recovery of IAA-2-C^{14}	µg IAA/Kilo of Fresh Weight Corrected For Recovery (M. Conc.)
Maturing[b] tumors (early chlamydospore stage)	1500	5.85	18.5	21.0 (1.2×10^{-7})
Younger tumors (mainly pre-chlamydospore stage)	1200	19.70	18.8	87.5 (5.0×10^{-7})
Corn stalks[c] (early tassel)	1840	1.10	14.3	4.2 (2.4×10^{-8})

a. Corrected for recovery of IAA-2-C^{14}. Amount added was for maturing tumors 0.825 µg, for younger tumors 1.65 µg and for corn stalks 3.30 µg.

b. After column chromatography, half of the combined IAA fraction was paper chromatographed in isopropanol/ammonia/water (8:1:1), and n-butanol/ammonia/water (10:1:1) successively. The eluent from the latter paper chromatogram was recombined with the remaining one-half IAA fraction from the column. The combined fractions were again column-chromatographed, paper-chromatographed as above (isopropanol/ammonia/water), and electrophoresed.

c. After column and paper chromatography (isopropanol/ammonia/water), the IAA-containing fraction was electrophoresed twice. The IAA concentration was estimated in this case on the final electrophoretogram by spraying it with Ehrlich's reagent and comparing it with IAA standards. Recovery of IAA-2-C^{14} was estimated just before the final electrophoresis on duplicate aliquots, and a radioautogram indicated that all the radioactivity on the final electrophoretogram was IAA.

The IAA concentrations have been determined in Ustilago zeae tumors in two stages of development, and in vegetative corn stalks (early-tassel stage). The values should be considered roughly quantitative, being limited by the colorimetric methods used. It should be noted that the partition column was not a satisfactory purification step in this study, though it has been employed satisfactorily in the case of corn seedlings.[9] It appeared that the proper partition was prevented by the residual oil-like residue obtained upon concentrating the acid ether fractions.

The only comparable results from a quantitative bioassay are those of Moulton[2]. He failed, however, to find proportionality between the amount of tumor extracted and the curvature in the Avena curvature test, and suggested the presence of an inhibitor. He also found continual production or release of auxins for several days by cold-ether or water extractions of fresh or lyophylized tumor. In one experiment, in which he used water extraction at 2°C for 1 hour, he found a curvature equivalent to approximately 15-30 µg of IAA/gm fresh weight. This huge discrepancy between our values, based on isolation and detection, and Moulton's values, based on bioassay, may suggest the presence of additional diffusible auxins. However, von Guttenberg and Strutz[4] report that the auxin activity in an alcoholic extract of tumor is inactivated by crude pea IAA oxidase. In any case, young Ustilago zeae tumors are characterized by an IAA level much above that of the uninfected stalk tissue.

We wish to thank Dr. S. A. Gordon, who kindly supplied the IAA-2-C^{14}, and Dr. R. S. Bandurski for his encouragement and suggestions.

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Studies on Isolation of Indole-3-Acetic Acid From Corn Kernels and Etiolated Corn Seedlings

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INTRODUCTION

Indole-3-acetic acid (IAA) has been commonly accepted as an important, if not the principal, plant auxin (5,15,21). Grain of corn (Zea mays) is a rich source and milligram quantities have been isolated by alkaline hydrolysis of the mature kernels (3,17), or by direct extraction from immature kernels (16). A large number of reports have also appeared concerning the occurrence of IAA in vegetative portions of plants; but in many cases diethyl ether has been used as the extracting solvent in a manner which can allow the enzymatic conversion of tryptophan to IAA (43). In a great many other cases, the existence of IAA is presumed from the bioassay of chromatograms run in a single solvent. Thus proof that free IAA is generally distributed in growing plant tissue is not yet available (2).

In studies reported elsewhere (38), relatively large amounts of indole-3-acetic acid (IAA) have been detected in U. Zeae tumors, and smaller amounts in healthy, early-tassel-stage corn stalks.

The techniques here reported permit handling of large amounts of tissue and quantitative evaluation of losses during isolation. They are

designed to measure free IAA (that is, IAA present when the tissue is quickly killed by homogenization in ethanol) and to permit physical characterization of the IAA so extracted. Using this method, it was not possible to isolate any free IAA from 80% ethanol extracts of corn seedlings if the pH of the aqueous concentrate was acidified to pH 4.0 and extracted with ether. Recovery of IAA added to filtered ethanol extracts or ethanol homogenates from kilogram quantities of corn shoots is discussed. It is suggested that IAA may complex with ethanol soluble substances from the corn shoots, and the percentage loss is greater with 2-3 µg than with 100 µg. By adjusting the aqueous concentrate to pH 8.0 with sodium bicarbonate prior to ether extraction, the recovery of 2-3 µg IAA-2-C^{14} was somewhat increased and IAA could then be detected in etiolated 5-day-old corn shoots. Possibly, therefore, IAA <u>in vivo</u> exists as a complex that is very easily hydrolyzed by mild alkali.

Free IAA could be isolated from corn kernels, but much more could be released following alkaline hydrolysis of a gummy, yellow-orange precipitate resulting from concentration of the 80% ethanol extracts. This precursor appeared identical to that reported by Berger and Avery (4), and may be an alcohol soluble protein.

METHODS

Michigan 350 hybrid corn was soaked in water for 4 hours and germinated on wet absorbent paper in plastic trays for 5 days in the dark at 25°C and 90% relative humidity. The shoots were harvested by cutting off the coleoptile plus the first internode. They were immediately homogenized in a blendor with sufficient 95% ethyl alcohol to make the final concentration 80% ethanol. After 1-4 hours, the ethanol homogenate was filtered and the residue re-extracted once or twice with 80% ethanol.

The combined ethanolic extracts were concentrated at 55°C *in vacuo* with a rotating evaporator or in later experiments with a vacuum spray drier at 35°C. In experiments where IAA-2-C^{14} was added to permit recovery calculation, it was added to the ethanol filtrate or to the homogenate prior to filtration, with identical results. The aqueous concentrate was adjusted to pH 4.0 with 5N H_2SO_4 or H_3PO_4 and the precipitate removed by filtration through glass wool. In later isolations (where noted in text), the pH of the aqueous concentrate was adjusted to 8.0 with $NaHCO_3$. The aqueous concentrate was extracted 3 times with peroxide-free diethylether (shaken with solid $FeSO_4$ before distillation). In the former case (extraction pH 4.0), the ether was partitioned with 8% $NaHCO_3$ 3 times, the combined bicarbonate fraction acidified to pH 4.0 (5N H_2SO_4) and extracted into ether 3 times. In the latter case (extraction pH 8.0), the ether was shaken once with 8% $NaHCO_3$. After acidification of combined aqueous bicarbonate fractions (as above), they were extracted with ether three times. The combined ether fraction was again bicarbonate partitioned as described above. The acid ether fractions were dried over anhydrous Na_2SO_4 at 1°C and the ether removed *in vacuo*. The residue was washed 3 times with 5 ml of ether and the combined ether fractions reduced in volume to about 3 ml. This fraction was applied to a buffered ether-water partition column (see below) and eluted with ether. The IAA containing fractions were pooled and dried over anhydrous sodium sulphate at 1°C. The dry ether was evaporated and the residue was taken up in a few drops of ethanol for chromatography or electrophoresis.

Fractions of sufficient purity could then be further fractionated by two dimensional electrophoresis-chromatography. In this procedure, the

sample was spotted toward one corner of a T-shaped piece of buffer-saturated paper. The flap (of the same length and width as the electrophoresis bed) was folded over and insulated by a plastic sheet. After electrophoresis, the paper was dried and used for chromatography at right angles to the direction of electrophoretic migration. Paper electrophoresis was conducted in a closed strip, solid support, plexiglass chamber using Whatman 3MM paper, 0.1 M citrate buffer, pH 5.25, 200 or 250 volts, and a temperature of 1°C.

In experiments involving more than a few hundred grams of corn shoots, the elctrophoresis-chromatography procedure could not be used, as the IAA-containing column fractions were too impure to be applied as a spot to the buffer saturated paper. Under these conditions, continuous flow electrophoresis proved to be valuable. The continuous flow electrophoresis unit was constructed after that of Durrum (11) except that the electrode compartment was baffled, and carbon electrodes were used. Whatman 3MM paper was washed successively with ethylenediaminetetraacetate at pH 8.0, 0.1 N HCl, and distilled water. Electrophoresis at pH 6.55 with 0.025 M phosphate buffer at room temperature (in the dark) was found to be satisfactory. Under these conditions, the IAA migrated half way toward the positive electrode when the wick was one inch from the negative edge, while the pigments did not migrate.

Although paper electrophoresis resulted in the best separation of IAA from the acidic pigments, paper chromatography was somewhat more convenient and was used unless otherwise stated. Whatman 3MM or Whatman 1 paper was used for ascending chromatography using isopropanol, ammonium hydroxide, water solvent (8:1:1, v/v) in most cases. The sample was usually applied as a 5 cm band with known IAA spotted at one end so as

to overlap the unknown slightly. Chromatography was conducted in the dark at 1°C for 20 to 30 hours, after equilibration for 8 to 12 hours. IAA was located on the paper by a light-blue fluorescence under short-wave, ultra-violet radiation (2537 A) or by spraying (with Ehrlich's reagent) a strip cut from the edge of the band containing the co-spotted IAA. The Ehrlich's reagent contained 1 gm p-dimethylaminobenzaldehyde dissolved in 91.2 ml of 95% ethanol and 8.8 ml of concentrated HCl. Chromatograms were eluted with 50% ethanol, and Salkowski reagent (35) was used for the detection of eluted IAA. The red color complex was measured with a colorimeter using a wide band, 540 mμ filter; standard concentration of IAA were included in each determination. The modified Avena section straight growth test of McRae et al. (25) was used for the bioassays.

The IAA-2-C^{14} used in these studies had a specific activity of 9.18×10^4 d/m/μg (34). Due to possibility of radio-decomposition, the sample was purified by column chromatography and paper chromatography twice during the course of these investigations. The stock solution was kept as a very dilute solution in ethanol and stored at -20°C. After the initial purification, no decomposition products were noted when the IAA-2-C^{14} was chromatographed.

Sugar beets, green peas and cucumbers were field grown on the Michigan State University farm and harvested immediately before use. Potatoes and bananas and sweet corn were purchased locally, while etiolated pea shoots (Alaska) were grown for 6 days at 25°C and 90% relative humidity. As carrier IAA-2-C^{14} was not used in most of these isolations, no recovery estimates were possible. In some of these isolations, as noted, initial alkaline ether extraction was used.

EXPERIMENTAL

In early experiments, it became apparent that corn seedlings contained high concentrations of a yellowish-brown phenol-like pigments*. Chromatograms of corn seedling extracts were very colored and it was impossible to detect µg amounts of IAA by fluorescence under ultra-violet radiation, or by Ehrlich or Salkowski reagents. These difficulties have also hampered the isolation of IAA by workers using other plant materials (1,8,27,40). Column chromatography was used in the isolation of IAA by Kögl et al. (22), and other columns have been described by Linser (24) and Fischer and Behrens (12). However, none of those columns appeared to be entirely satisfactory under our particular conditions.

The several columns tried in this study were cellulose, a styrene type polyamine resin (Dowex AG 3-X4), neutral alumina (Woelm), N,N-diethylaminoethylcellulose, and water-ether partition using analytical grade diatomaceous silica (Celite, Johns-Manville) as the inert base. The cellulose column channeled even with careful packing while wet. The neutral alumina and the Dowex AG 3-X4 appeared to bind the IAA very tightly and it could not be readily eluted. N,N-diethylaminoethylcellulose made a very satisfactory column. The IAA was adsorbed on the column from 0.005 N phosphate buffer at pH 7.0 and could be eluted by increasing the salt or buffer concentration. The column chosen for use was a true partition column employing a buffered aqueous phase adsorbed on Celite

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*The principal pigment was collected as a precipitate after concentration of the acid ether fraction prior to column chromatography. This substance was recrystalized from acetone-ligroin yielding light brownish-pink to white monoclinic sweet tasting crystals ($C_9H_9O_5N$) melting at 160-161°C (dec.). The identification and characterization of this substance will be reported elsewhere.

and ether as the mobile phase. This partition was similar to that used by Holley et. al.(19) except these workers used a Craig apparatus. Using this type of column, the IAA could be eluted in a small volume of ether and easily concentrated. Analytical grade Celite was thoroughly ground in a mortar with 0.8 times its weight of phosphate buffer. Portions of the damp Celite were slurried in the column with buffer saturated ether and the slurry packed with a glass rod. A flow rate of 0.5 to 2.0 ml per minute was obtained with 3 to 5 pounds pressure per square inch of nitrogen. The column was eluted with ether saturated with the same buffer used on the column. Figures 1 and 2 indicate the effect of molarity and pH of the phosphate buffer on the elution of IAA from a 2.5 cm column containing 10 g of Celite. High buffer concentrations depress the solubility of IAA in the aqueous phase. Over the small range of hydrogen ion concentrations tested, there was an almost linear relationship between pH and rate of movement of IAA through the column.

Maximum separation of IAA and the corn pigments was obtained with 0.1M phosphate buffer at pH 6.5. Under these conditions, with a 1.8 cm diameter, 20 g column, IAA came off in the 40 to 60 ml fractions; with a 2.5 cm, 30 g column, the IAA came off in the 50 to 70 ml fractions; and with a 5 cm, 100 g column, the IAA came off in the 200 to 500 ml fractions. The highest concentration of IAA was found just after the first tube or two containing it, indicating that the IAA band trailed upward on the column. Usually 2-3 ml of ether were collected per tube, and the IAA located by adding 4 ml of Salkowski reagent and shaking. The color development is somewhat slower than in the regular Salkowski assay.

The pK of IAA is 4.65 (23) and at pH 6.5, IAA is about 10% undissociated, that is, the ratio of dissociated to undissociated molecules

is about 9.0. The undissociated molecules theoretically enter the flowing-ether phase, move some finite distance down the column, and then dissociate at an aqueous-phase boundary. This cycle corresponds to a theoretical plate as defined in fractional distillation column theory. Martin and Synge (26) have shown that movement of solute on a partition column per volume (ΔV) of solvent passed, is defined by a binomial expansion. If the cross sectional areas of the mobile phase A_m, stationary phase A_s, the excursion value R (solute movement/solvent movement) and the total area, A, are known, then α, the partition coefficient, may be calculated from the formula:

$$\alpha = \frac{A}{RA_s} - \frac{A_m}{A_s}$$

Using a 20 g, 1.8 cm diameter column with 0.1 M phosphate buffer at pH 6.5, the value for R was estimated to be 0.227 and the value for α was 9.8.

RESULTS

Isolation and Recovery Experiments Employing Unlabeled Carrier IAA

Recovery experiments are essential if estimates of the amount of IAA present in a tissue are to be made. One hundred µg of IAA was added to 1 liter of 95% ethanol and carried through concentration, ether extraction, column and paper chromatography. The IAA was located on the paper chromatogram by UV fluorescence, and eluted with 50% ethanol. The ethanol was removed *in vacuo* at 55°C, 2 ml of water added, and the amount of IAA estimated by Salkowski assay. This experiment was repeated twice with overall recoveries of 50 and 56%. Other similar experiments, where losses for individual steps were evaluated, indicated losses were small for each operation, but cumulative.

The isolation of IAA from 5-day-old corn shoots was next investigated. Eight experiments without added IAA were conducted using from 178 g to 1000 g of shoots. No IAA could be detected in these experiments. Three recovery experiments with known amounts of added IAA were next conducted. In the first experiment, 100 µg of IAA was added to the 80% ethanol extract of 1000 g of corn shoots. Since some loss might be expected owing to adsorption by the residue, another 100 µg of IAA was mixed with the residue and the residue washed again with 2 liters of 80% ethanol. Recovery in this experiment was 25% in both cases. In the second experiment, 120 µg of IAA was added to 316 g of 5-day-old corn shoots before homogenization in a blendor. In this experiment, 27% of the added IAA was recovered. In the third experiment, 200 µg of IAA was added to 700 g of corn shoots and the recovery in this experiment was 30%. It thus appears that recovery for the overall isolation procedure using 100 µg per kilogram of corn shoots is 25 to 30%.

An attempt was made to isolate IAA from 11.3 kilogram of 5-day-old etiolated corn shoots. Five lots of corn shoots were grown over a 30-day interval. Each individual lot was harvested, extracted with 80% ethanol, and worked up to the partition column stage. This acid ether fraction was concentrated to dryness in vacuo and stored at -20°C. The pooled lots were dissolved in ether and the ether concentrate divided into 3 portions. Each portion was partitioned on a 5 cm diameter, 100 g column, and the IAA fractions pooled. The pooled eluant was electrophoresed in two runs using the continuous flow unit. No IAA could be detected by Salkowski assay. When 26 µg of known IAA were placed on the 5 cm diameter column, recovery was 77%; and when 10 µg of IAA was run on continuous flow electrophoresis, recovery was 70%.

Isolation experiments using IAA-2-C^{14}

Since the above recovery experiments might not be valid due to the addition of relatively large amounts of IAA, low level recovery experiments using IAA-2-C^{14} were carried out. The use of trace amounts of high activity IAA-2-C^{14} was valuable in several ways. Recovery data could be obtained during the actual isolation experiments; 1-3 µg could be added and detected by radioactivity, following isolation, even if losses were 90%; and the IAA could be followed during isolation by its radioactivity. The results of these isolation experiments are summarized in Table I.

Corn grain was a rich source of free and bound IAA in confirmation of numerous reports of the occurrence of IAA in the grain (3,8,16,32,33, 40,44). The grain was soaked 4 hours in tap water and allowed to germinate in the dark at 25°C and 95% relative humidity for 0, 12 or 24 hours. The samples were ground in a meat grinder, then extracted 3 times with 1 to 3 liter portions of 80% ethanol. A gummy, yellow-orange precipitate was obtained upon concentration of the ethanol extracts. Following washing with water, the residue was hydrolyzed successively with two 150 ml portions of 1 N KOH for 1 hour at 100°C. The hydrolysates were then carried through the described IAA isolation procedure. The results are summarized in Table II.

The free IAA and IAA released by hydrolysis were characterized by electrophoresis, and by paper chromatography in 4 solvents. The Rf values of the synthetic and isolated IAA were identical in all solvents (Table III), and there was no separation of the co-spotted natural and synthetic IAA. The estimate of concentration, obtained by the Salkowski assay, was verified by direct spectrophotometric assay of chromatograms

sprayed with Ehrlich's reagent, and by Avena section bioassay (Tables IV and V). The immature sweet corn kernels contained so much IAA that if 1 g was ground with 1.6 ml of water, the deproteinized solution gave a strong Salkowski test.

The origin of the IAA released upon hydrolysis is of interest. Other workers (13,29,40) have suggested that IAA may arise from proteins containing tryptophan. Though zein is low in tryptophan, it is the major alcohol soluble protein. A sample of pure zein A was hydrolyzed under the conditions used for the corn grain residue, but no IAA was found. Berger and Avery (4) also reported that their precursor was not zein. Similar alkaline hydrolysis of 100 mg of tryptophan itself yielded no IAA. To further determine whether the precursor was protein, about 500 g of corn meal was extracted three times with 1 liter portions of 0.01 M $MgCl_2$. The combined extracts were cooled to 1°C and the protein precipitated by saturation with ammonium sulfate. The precipitated protein was collected by filtration and washed with 80% ethanol. The ethanol was removed <u>in vacuo</u> at 60°C. The ethanol soluble and ethanol insoluble proteins were then hydrolyzed for 1 hour in 1 N KOH at 100°C. No IAA was found in the hydrolysate of the ethanol insoluble protein, but a considerable amount was obtained from the ethanol soluble fraction.

A number of other plant tissues have been examined qualitatively for the presence of IAA and other Ehrlich reactive substances (Table VI). It was interesting that vegetative sugar beet roots and leaves, and possibly pea shoots contained IAA in sufficient quantities to be detected.

DISCUSSION

The results presented in this study illustrate some of the difficulties in detection of IAA in vegetative plant tissues. Housley, Booth and

Phillips (20) reported failure to find IAA in corn seedlings, and our preliminary studies confirmed this finding. Reinert and Forstman (27) obtained similar results, and postulated some sort of binding of IAA in a manner perhaps similar to that investigated by Tegethoff (36) in corn scutellum. It is interesting that recovery of 1-3 µg of IAA is only the order of 12 to 14%; while recovery of 100 µg is about 25%. The increased recovery when the aqueous concentrate was made alkaline suggests some reversible binding. It is doubtful if actual destruction of IAA at pH 4.0 is a factor. Brian (6) has found binding of 2-methyl-4-chlorophenoxy acetic acid is greater at low pH and that crude corn extracts bind comparatively large amounts. One may question whether any free IAA exists in vivo. Though IAA has been detected in Avena coleoptiles (30,37), experiments with radioactive IAA indicate no redistribution under the influence of geo- or phototropic stimulation (7,9,14,28). From the present work, the occurrence of appreciable free IAA in ethanolic extracts of corn shoots must be considered doubtful, since added IAA (1-3 µg) is largely lost. One could conclude that free IAA does occur in vivo, but is bound to some ethanol soluble component upon grinding the tissue. A more attractive possibility is that all the IAA occurs in the form of a very labile complex. Alkaline ether extraction led to an estimate of 13.3 µg per kilogram. Using this value, one may estimate that about 2 µg should have been detected (at least qualitatively) in the experiments using acid ether extraction. This was not the case, however. Therefore, it is probable that endogenous IAA was liberated from some sort of complex in the case of alkaline ether extraction. The presence of a complex could explain the experiments on tropisms demonstrating a lack of redistribution of radioactive IAA.

Vlitos and Meudt (40) have also failed to find appreciable amounts of free IAA in ethanol extracts of several plants. The greater ease with which IAA is detected by wet ether extraction may be due in part to its ability to dissociate the bound IAA. In this respect, it is interesting to note that ether can prevent polar auxin transport (39). However, Wildman and Muir (43) have also demonstrated IAA production from tryptophan during wet ether extraction, and their production had a temperature optimum of 13°C.

In the present work, IAA could be detected in corn shoots or roots by wet ether extraction for 3 hours at 4°C. Furthermore, subsequent 70% ethanol extraction of the residue resulted in detection of IAA. However, no IAA could be detected in ethanol or subsequent ether extracts when the initial extraction was with 80% ethanol. There is now no doubt that IAA can be derived from tryptophan in vitro by preparations from various tissues (10,15,32,42,44). It must be mentioned that the production of IAA from tryptophan in vitro by a particulate fraction from corn seedlings was also observed (18). When tryptophan-2-C^{14} was used as substrate, carboxyl labeled IAA was obtained. The acid ether soluble products found were similar to those reported by Dannenburg and Liverman (10). However, in contrast to the results of these workers, little labeled IAA could be isolated when excised corn shoots were fed tryptophan-2-C^{14}.

In contrast to the growing shoot, corn kernels were found to contain considerable quantities of free IAA as has been reported (3,8,16,32, 33,40,44). In addition, a water-insoluble, ethanol-soluble fraction, presumably identical to that of Berger and Avery (3,4), was present. This substance might be a protein or polypeptide since it appears to be

precipitated with ammonium sulfate. It should be noted, however, that Stehsel (32) found the alkali labile IAA complex in immature sweet corn seed was dialyzable.

It has been suggested that tryptophan-containing proteins in general yield IAA upon alkaline hydrolysis. Gordon (13) and Schocken (29) found that when proteins were hydrolyzed with alkali, a substance, presumably IAA, causing stimulation in the Avena curvature test, could be isolated. The amount of stimulating substance obtained was correlated with the tryptophan content of the protein. On the other hand. Wildman and Bonner (41) found a specific fraction of spinach leaf protein contained bound IAA. In the present experiments, neither tryptophan itself nor the alcohol-insoluble, water-soluble protein of corn grain formed IAA upon mild alkaline hydrolysis; but the alcohol-soluble, water-soluble fraction did.

A number of Ehrlich reactive spots were detected in other plant tissues, but it may not be concluded that all these are indole compounds. As has recently been pointed out, a number of phenols give purple, red and pink colors (31).

It was found that the described ether-water partition column sometimes failed as a good purification step when the alkaline ether extraction constituted the first purification step. This was also noted during isolation of IAA from Ustilago Zeae tumors (38). In these cases, all the IAA-2-C^{14} appeared just behind the front in highly pigmented fractions. Since it was found that failure was not due to any change in the pH of the aqueous phase, it is probable that polar ether-soluble materials prevented the proper partition. A prior partition between n-hexane/acetonitrile did not prevent this difficulty. In these cases, it was usually necessary to chromatograph one or two times as a 20 to 40 cm

streak, and finally electrophorese the products as a 5 to 10 cm streak, as described above.

SUMMARY

Methods found useful in the isolation of IAA from kilogram quantities of plant tissue are described. A buffered ether-water partition column, electrophoresis-chromatography, and continuous flow electrophoresis techniques were developed. The use of trace amounts of IAA-2-C^{14} was especially valuable during the isolation, and in evaluating the recovery. Free IAA was detected in 80% ethanol extracts of corn kernels and shoots, as well as vegetative sugar beet roots and leaves. The difficulties of detection of IAA in corn shoots are discussed and it is suggested IAA may occur as a labile complex in ethanol extracts of this tissue. An 80% ethanol-soluble, aqueous-insoluble fraction from corn kernels was found to release IAA upon alkaline hydrolysis. It appears this fraction may be protein in nature.

We should like to take this opportunity to thank Dr. S. A. Gordon, who supplied the IAA-2-C^{14}; and Dr. H. Sell for use of the vacuum spray dry apparatus. It is a pleasure to acknowledge the assistance of Dr. H. Fukui in the ether extraction experiments.

TABLE I

The Recovery of IAA-2-C^{14} from Etiolated Corn and Pea Shoots and the Detection of IAA in Corn Shoots

Tissue	Fresh Wt.	µg IAA-2-C^{14} Added	% Recovery of IAA-2-C^{14}	µg IAA Detected/ Kg by Salkowski Assay Corrected for Recovery
Corn shoots	1000	1.89	0?	0
Pea shoots	950	0.95	10.7	Trace?
Corn shoots	1000	1.89	14.5	0
Corn shoots	1000	2.84	12.4	0
Corn shoots	1000	1.89	12.0	0
Corn shoots	1000	2.84	16.1	0
Corn shoots*	844	2.84	26.0	0
Corn shoots*	3000	1.65	28.1	13.3

*Alkaline extraction of aqueous concentrate with ether initially.

TABLE II

The Amount of Free IAA Isolated per Kilogram of Corn Grain, and the Amount of IAA Obtained by Hydrolysis of the 80% Ethanol-Soluble, Water Insoluble Residue for Two Successive One Hour Periods with 1 N KOH at 100°C

Experiment[1]	µg of IAA/Kilogram		
	Free	1st Hydrolysate	2nd Hydrolysate
1	82	186	98
2	115	509	63
3	171	---	--
4	330	---	--
5[2]	16,800	---	--

[1]In experiments 1-4, Michigan 350 grain was soaked for 4 hours. In experiment 1, it was ground after soaking; in 2 and 3, it was germinated 12 hours at 25°C; and in 4, germinated 24 hours at 25°C.

[2]Market stage yellow sweet corn kernels purchased locally.

TABLE III

The Rf of Isolated and Synthetic IAA in 4 Solvents, and its Migration after Electrophoresis in 0.1 M Citrate Buffer pH 5.0[1]

Solvent	Rf	Migration cm^2/Volt-Sec.
Isopropanol; NH_4OH; H_2O (8:1:1)	0.37	
Pyridine; NH_4OH (4:1)	0.62	
n-Butanol; Ethanol; H_2O (4:1:1)	0.85	
70% Ethanol	0.78	
(Electrophoresis)		2.94×10^{-5}

[1]10 µg of isolated IAA

TABLE IV

The Area of Ehrlich Reactive Spots Following Chromatography of Synthetic and Isolated IAA[1]

Amount and Source of IAA	Area (Sq. Inch)
10 µg Synthetic	1.32
10 µg Isolated[2]	1.41
5 µg Synthetic + 5 µg Isolated[2]	1.55
10 µg Synthetic	1.63
10 µg Isolated 1st Hydrolysate[2]	1.30
5 µg Synthetic + 5 µg 1st Hydrolysate[2]	1.24

[1]Plot was 0.1 inch per 0.1 inch on filter paper, and 0.1 inch per 2% transmission on vertical axis. Beckman DU Spectrophotometer, strip drive, 600 mµ.

[2]Salkowski estimates

TABLE V

Growth Promoting Activity of the Isolated "Free" IAA from Experiment 1 (Table I) Compared to That of Known IAA in the Avena Section Test[1]

Amount and Source of IAA	Inc. in Elong. (mm)	No. Measured
1 ppm isolated	2.97	24
1 ppm synthetic	3.20	21
0.1 ppm isolated	3.26	25
0.1 ppm synthetic	2.91	26
0.01 ppm isolated	1.94	26
0.01 ppm synthetic	1.77	25
Control	0.99	21

[1]Victory oats, initial section length 6.5 mm, floated on 0.01 M phosphate buffer pH 4.5 with 2% sucrose at 25°C for 10 hours.

[1]Victory

TABLE VI

Ehrlich Reactive Substances Detected on Paper Chromatograms Following Column Chromatography of the Acid Ether Fraction. Four 30 ml Fractions Were Collected From the Column and Chromatographed Separately on Paper

Plant	Fresh Wt.	Substances Found Rf & Color with Ehrlich's Reagent*
Sugar beets, roots**	920	IAA[2] (3.8 µg as est. by Salkowski)
Sugar beets, leaves**	1400	Blue 0.326[4], IAA (trace)[2,3], pink 0.76[2].
Sugar beets, flowering tops**	1100	Pink 0.50[3,4].
Banana	1000	No Ehrlich reactive spots
(Aq. hydrolysate after Et_2O ext.)		Lt. blue 0.27.
Cucumber, fruit	1500	No Ehrlich reactive spots.
Potatoes	1000	Blue changing to green 0.33.
Pea shoots, etiolated	950	Pink to purple 0.32[2,3], pink fading 0.69-0.89[2,3].
Green peas, market stage**	1000	Blue 0.367[2], pink 0.43[3], pink 0.88[3,4].
(Aq. hydrolysate after Et_2O ext.)		Blue 0.51[2], pink 0.43[3], pink 0.88[3,4].

* 1,2,3 and 4 are consecutive 30 ml column fractions starting with the front, and Rf values are for isopropanol/ammonia/water (8:1:1, v/v).

** A preliminary alkaline ether extraction was used in these cases.

Figure 1

The effect of molarity of the potassium phosphate buffer (pH 6.5) on the migration of IAA and corn shoot pigments on a 10 gram Celite column 25 mm in diameter. The boundary lines indicate qualitatively where elution of IAA-0-0, and pigments -x-x begin and end.

Figure 2

The effect of pH on the migration of IAA and corn shoot pigments on a 10 gram Celite column 25 mm in diameter. The boundary lines indicate qualitatively where elution of IAA-0-0, and pigments -x-x begin and end.

Fig. 1

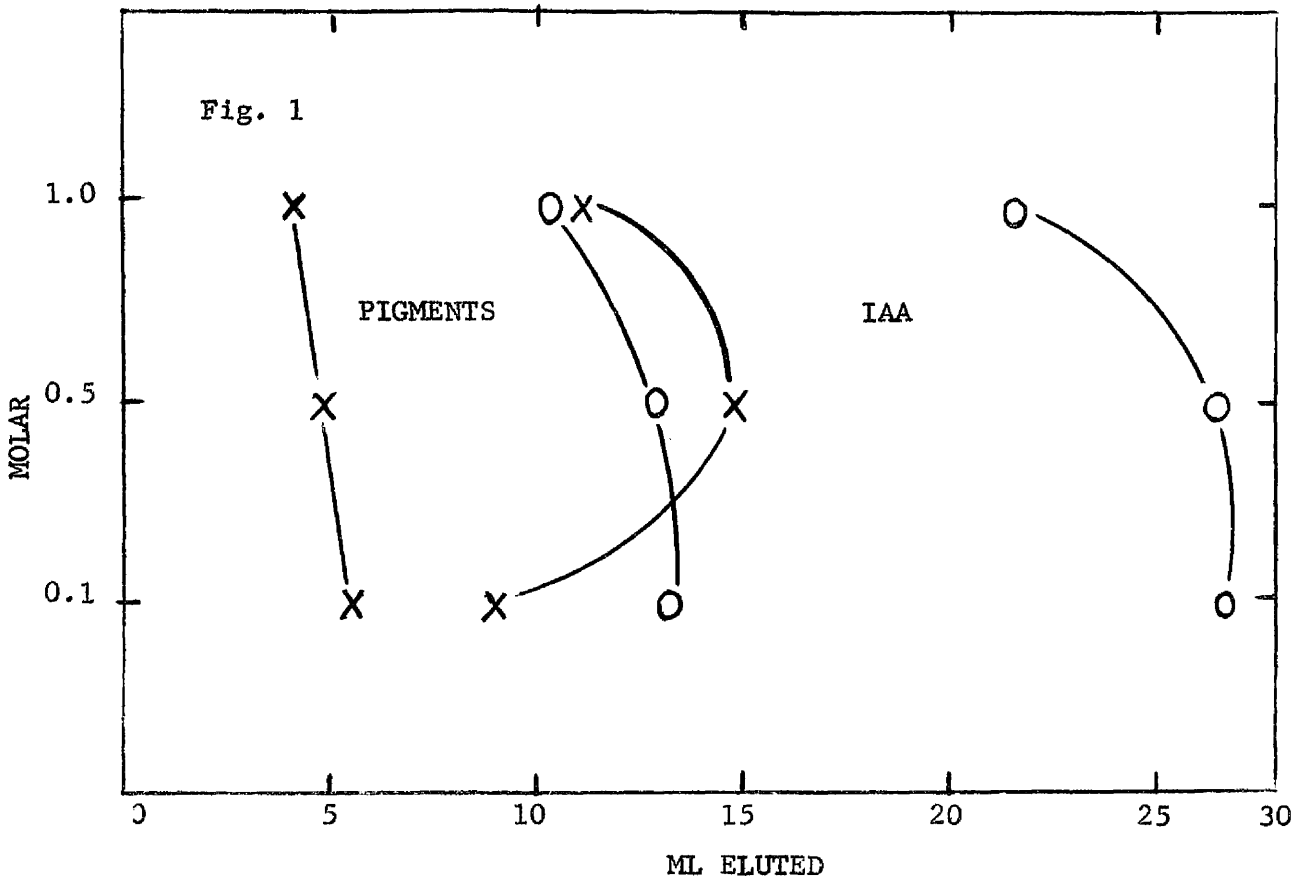
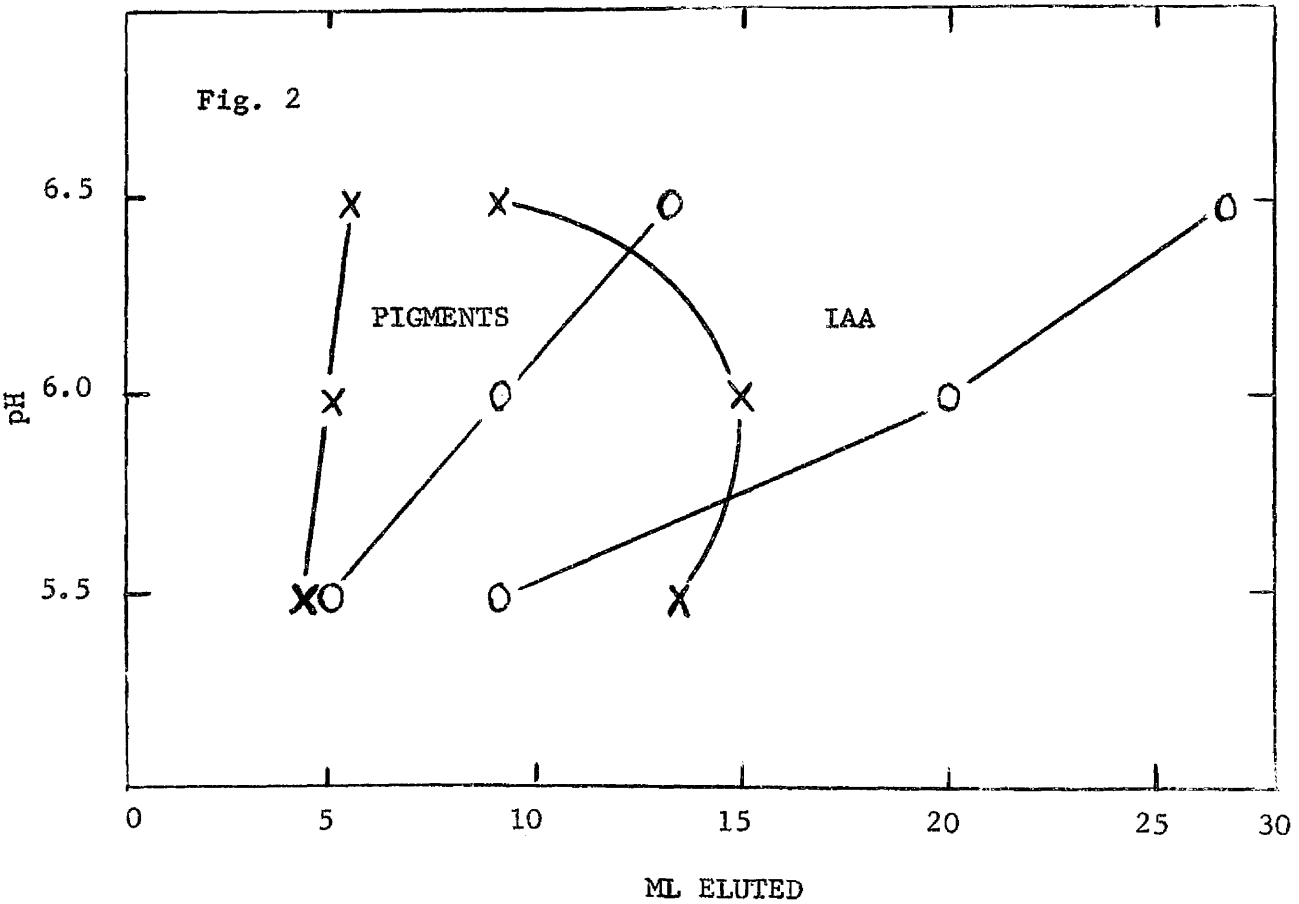

1.0
0.5
0.1
MOLAR
PIGMENTS
IAA
0
5
10
15
20
25
30
ML ELUTED

Fig. 2


6.5
6.0
5.5
pH
PIGMENTS
IAA
0
5
10
15
20
25
30
ML ELUTED

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3. In Vivo Labeling Experiments on the Biosynthesis of IAA

Though the isolation work indicated that corn shoots contained a minute amount of IAA, it was considered that IAA might be a fleeting intermediate and never accumulates in rapidly growing tissue. Thus the only possibility of demonstrating its presence would be to trap the IAA in some manner. It was thought that corn seedlings should be able to fix CO_2 in the dark via the phospheonolpyruvate carboxylase (11) or some other carboxylating reaction. In either case, the carbon is fixed into organic acids which are very active metabolites. It was hoped that by such feeding of $C^{14}O_2$ that the side chain of IAA would become labeled. In an attempt to get accumulation of the labeled IAA, unlabeled IAA was used as a trap. In the first experiment, 200 grams of corn shoots were fed 1 millicurie of $C^{14}O_2$ and 100 µm of IAA during 8-hour period in the dark. The shoots were washed and the IAA isolated. There was no radioactivity in the isolated IAA, and thus CO_2 was either a poor precursor, or no IAA was synthesized during the experiment. In considering the latter alternative, it was thought that the added IAA might have prevented its synthesis in vivo. A second experiment using 500 grams of corn shoots and feeding 100 µM of indole as well as 1 mc of $C^{14}O_2$ was run for 4 hours in the dark. Thus in this experiment a precursor of IAA was fed in an attempt to stimulate its synthesis. IAA was added as carrier prior to isolation. The isolated IAA had 8 counts per minute above background after chromatography once. It was then run on the continuous flow electrophoresis unit, but was lost due to poor pH adjustment before running.

Tryptophan was isolated by adsorption on an IR 120 exchange column in mixed Na and H form at pH 3.1, and elution with 0.1 M NH_4OH. For paper

chromatography, the volume was reduced in vacuo, and the sample spotted on Whatman 1 paper using isopropanol/ammonia/water (8:1:1) solvent ascending. The tryptophan area was eluted and rechromatographed using 77% ethanol as the solvent. The tryptophan was assayed by the quantitative ninhydrin reaction of Mooreand Stein (109). The specific activity of the tryptophan so isolated was 157 cpm/μM. The total free tryptophan isolated was 625 micrograms as estimated by ninhydrin assay (3.06 micromoles). Since the free tryptophan has such low activity (less than 1 cpm/μg) the activity in IAA would not have been detected, assuming a direct conversion. Only a trace of IAA would be expected to be present and its specific activity would be expected to be less than tryptophan due to loss of the terminal carbon. Thus under these conditions, $C^{14}O_2$ is not a suitable precursor for either tryptophan or IAA.

A final $C^{14}O_2$ labeling experiment was tried in the light. Eight hundred and twenty grams of 5-day-old corn shoots were fed 1 mc $C^{14}O_2$, 10^{-3} M indole, and 10^{-4} M IAA for 6 hours under diffuse window light. After flushing the $C^{14}O_2$ out of the modified desiccator into alkali, the corn shoots were washed and ground in sufficient 95% ethanol to make the final concentration about 80%. The IAA and tryptophan were isolated and their radioactivity compared by counting the dried 50% ethanol eluants from paper chromatograms. The usual isolation procedure for IAA was employed, but the isolated IAA was rechromatographed twice to constant specific activity. Tryptophan, isolated as described above, was also rechromatographed twice in an attempt to reach constant specific activity.

Under these conditions (Table I), the activity of IAA is 9.54 cmp compared to a final activity of the tryptophan of 0.382 cpm/μg.

Assuming the IAA and tryptophan were of about the same specific activity this would mean that 24.6 µg of IAA must have been present. Since this was not possible, the IAA may have a much higher specific activity than the tryptophan, in which case it could not have come from the tryptophan.

A tryptophan-2-C^{14} feeding experiment was next conducted in order to determine if significant amounts of IAA could be obtained from labeled tryptophan. A total of 3,730,000 cpm of tryptophan-2-C^{14} (specific activity = 0.42 µc/µM) was fed for 6 hours to 500 g of corn shoots. The total activity in the IAA fraction was 49.5 cpm. Since the tryptophan had a specific activity of 1785 cmp/µg, only 0.023 µg of IAA was formed from the labeled tryptophan under these conditions. However, the shoots contain 1 or 2 mg of free tryptophan, and since perhaps only 10% of the tryptophan-2-C^{14} entered the shoots, the internal tryptophan pool might have had a specific activity of about 0.1 or 0.05 that of the tryptophan fed. Thus the IAA concentration may have actually been 0.23 to 0.46 µg.

It is apparent that insignificant amounts of IAA are formed from either tryptophan or CO_2 even though an enzymatic converting system for tryptophan may be demonstrated <u>in vitro</u>.

In view of the extremely small amounts of IAA in the corn shoots, and the relatively great losses during isolation, it is not surprising that these <u>in vivo</u> labeling experiments were not entirely successful.

TABLE I

Radioactivity of Isolated Tryptophan and IAA From 820 Grams of Corn Shoots Fed 10^{-3} M Indole, 1 mc $C^{14}O_2$, and 10^{-4} M IAA for 6 Hours in the Light

	Counts/ Min.	Counts/ Min./µg	Total µg Isolated
IAA	13.4	0.061	220
Rechromatography 77% ethanol	6.0	0.043	140
Rechromatography - pyridine, ammonia (4/1)	2.6	0.043	60
Tryptophan	6,013.8	1.47	4100
Rechromatography - acetone, chloroform, ammonia, water (150/25/20/1)	1,785.9	0.853	2091
Rechromatography - pyridine, ammonia (4/1)	213.6	0.382	558

B.
Synthetic Plant Growth Substances

1. The Effect of N-1-Naphthylphthalmic Acid on the Growth and Geotropic Response of Seedlings.

Koepfli, Thimann and Went (34), using the pea test, formulated the specific structural requirements of a ring with a double bond, a side chain with a carboxyl group, at least one carbon atom removed from the ring, and the carboxyl group having a particular space relation to the ring. Many exceptions of active compounds not meeting these requirements are known. Veldstra (167), in investigating polaroigraphic reduction of the ring double bond of auxin found a suppression of the oxygen maximum. This indicated some sort of selective surface adsorption to the mercury drop. High surface activity in this test correlated fairly well with the beet-root section permeability for chlorophenoxy acetic acid derivatives. Booij and Veldstra, as cited by Veldstra (168), later investigated interface accumulation between oleic acid water. In a general way, the hydrophylic lipophilic balance is important for activity. This is proposed to be due to the adsorption of the lipophylic ring to the lipoidal cellular membrane. Veldstra (168) has said "surface activity comes to stand more and more for the specific adsorption affinity for the sites involved in growth."

Skoog, Schneider and Malan (138) developed the view of specific antagonism. They were able to demonstrate γ-phenylbutyric acid (though a weak auxin) inhibits activity of IAA in the Avena curvature test, and this inhibition could be reduced by increasing IAA concentration. Similar antagonisms could be established for transcinnamic acid (166), phenoxyisobutyric acids (29), and 1-naphthyl sulfide propionic acid (1)

as well as others. McRae, Bonner and Foster (42,105) introduced the use of classical enzyme kinetics for describing competitive inhibition in the Avena section test. Foster, McRae and Bonner (42) considered growth inhibition at high concentrations of IAA to be due to a requirement for two point attachment. The experimental data do fit the calculated kinetic expression for this case fairly well. The most important utility of this method, however, was an analysis of the nature of inhibition as well as estimating maximum velocity and K_s of a given growth substance. The basic assumption in enzyme kinetics is the formation of a dissociable enzyme substrate complex which can break up irreversibly into product plus enzyme. The total amount of enzyme (free and bound) being constant during the experiment, and the amount of substrate being in excess (i.e. amount bound insignificant and constant, or steady state assumption), it can be visualized that under these conditions the rate of reaction will be enzyme limiting and linear with time. Several problems arise in considering application to the in vivo system. Endogenous substrate would lead to non-linear reciprocal plots (125), but in general these are not found experimentally for several active growth substances. In the living system, enzyme or site synthesis is also possible. It has been found that a linear time course is not always obtained (12,68) and this was also the case in the present study. Housley, Bentley and Bickle (68) report that appreciable amounts of auxin enter through the cut ends of floating Avena sections, and thus translocation is a factor. In addition, they report permeability can also be a factor on the basis of indirect tests.

It has been pointed out by Bottelier (22) that the reciprocal form of the enzyme kinetic equation is formally the same as Langmuir's ad-

sorption isotherm. He suggests the growth of coleoptile sections is better fit by log G = A + b log C, where G = growth and C = concentration. Other growth tests were better fit by other empirical equations, and no one equation was satisfactory for all cases. However, it would appear that the enzyme kinetic concept still gives the most interesting physiological evaluation of the data in cases where it is valid. In the present study on tetrazole analogues, the presence of two sites of action appears indicated due to the fact the 2,4-D analogue acted competitively toward only 2,4-D induced growth. In addition, the curvature in the reciprocal plot of this data appears similar to the case for two enzymes discussed by Reiner (125).

Reiner (125) points out, however, that combination of the inhibitor with the substrate or co-enzyme so as to lower its concentration or result in an inhibitory complex, also results in curvature in the reciprocal plot.

2. Biological Activity of Tetrazole Analogues of IAA and 2,4-D

It has now been established that a number of synthetic growth substances can selectively inhibit both phototropism and geotropism in seedlings. Such effects were first observed by Mentzer and Nétien (106), but it appeared necessary in our work to establish its relation to inhibition of growth. The important work of Jones, Metcalfe and Sexton (75) indicated a number of compounds have these effects. Henderson and Peterson (64) have found 100-1000 ppm 2,4-D to inhibit the phototropic and geotropic response of oat coleoptiles. They assume no inhibition of straight growth, but present no evidence on this point. Vander Beek (164) failed to find an effect with 2,4-D in his oat shoot

test at lower concentrations. He found 2,3-6-trichloro-; 2,3,5-triodo-, and 2,6-dichlorobenzoic acid active in inhibition of both phototropic and geotropic response, whereas a number of other analogs were inactive in both tests. The evidence appears strong that certain synthetic compounds are especially effective in selective inhibition of phototropic or geotropic responses.

The present work has been discussed by Morgan and Söding (110) who report NPA (N-1-naphthylphthalamic acid) is active under their conditions in the Avena section test. In intereaction with low concentrations of IAA, it further stimulated growth. However, in the curvature test or cylinder tests, the NPA inhibited the IAA growth response. It was suggested NPA, though a weak auxin, inhibits polar transport of IAA. Thus in cylinder growth tests, stimulation induced by IAA (or NPA alone) was limited to first 1 or 2 mm, with the more basal zones showing inhibition. These authors suggest all chemicals selectively inhibiting tropisms may disturb polar transport of IAA. The lack of lateral redistribution of IAA-C^{14} during geotropic or phototropic curvature, however, has already been discussed. Morgan (private communication) has indicated this makes a lateral redistribution theory unattractive.

Anker (6,7) has presented evidence to show that geotropism has an optimum equivalent to the maximum acceleration of growth and that concentrations inducing maximum growth causes inhibition of geotropism. In his tests, decapitated coleoptiles were placed in aqueous solutions of the growth substances. Anker (6,7,8) in a series of studies, showed that IAN, IAA and NAA were all active (in descending order of activity), in inducing geotropic curvature in decapitated coleoptiles. Anker (6) assumes that inhibition of geotropism by high concentrations is due to

permeation of the substance through the cuticle rather than the tip, resulting in "flooding." DeWit (35), using Anker's technique, found that IAA had to be present during geotropic stimulation. "Flooding" occurred when 1 mg per 1 ml was presented to non-decapitated coleoptiles. Curvatures continued, when IAA was removed, for about 30 minutes; indicating depletion of the IAA. Bottelier and Roosheral (23) in a short paper, used Anker's technique to test our assumption that inhibition of growth and therefore geotropism, has to be proportional. They used diethyl ether and found the percent of inhibition of geotropism was more sensitive than percent inhibition of growth. They therefore suggest an inhibition of lateral transport, which was also one of our suggestions. Though the Dutch workers uphold the classical viewpoint of lateral polarity of auxin translocation, other explanations are possible, and these have been mentioned already.

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Selective Inhibition of the Geotropic Response by *n*-1-Naphthylphthalamic Acid[1]

By

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(Received June 1, 1956)

Introduction

According to the theory of Went and Cholodny (1937), the geotropic response of plant seedlings is due to differential growth following an internal redistribution of auxin. Since geotropic curvature results simply from unequal growth, a direct correlation between growth rate and rate of geotropic curvature is to be expected and an inhibition of growth should result in a proportional inhibition of curvature. In previous studies from this laboratory (Grigsby *et al,* 1954) it was found that treatment of pea seedlings with *n*-1-naphthylphthalamic acid (NP) resulted in a 70 percent reduction of straight growth and a complete loss of geotropic sensitivity. Nétien and Conillot (1951) had earlier made a similar, although qualitative, observation. This disproportionality of growth and geotropic response, if significant, would seem to require a modification of the classical theory of the tropic mechanism. A study was therefore initiated to examine these observations quantitatively and to extand them to several additional plant species.

Hoffmann and Smith (1949) have observed growth regulating effects of derivatives of phthalamic acid. They stated that at 0.1 ppm, NP induced

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[2] Agent of Field Crops Research Branch, Agricultural Research Service, United States Department of Agriculture.

leaf-rolling in tomato plants; at 0.31 ppm, epinasty was observed; and at 20 ppm, stem swelling. Mentzer, Molho, and Pacheco (1950) tested twenty-five related compounds on lentil roots, and concluded that a close spatial relationship of the peptide bond and a carboxyl group was required for inhibition of geotropism. Mentzer and Nétien (1950) studied the effect of NP on the geotropic response of pea, lentil, crucifer, tomato, sunflower, and cucumber seedlings, and found that 10^{-3} to 10^{-6} *M* concentrations caused an apparent negative geotropism. Treated tap roots were somewhat thicker and shorter than the control. In a study of the effect of NP on germination and early stages of growth, Nétien and Conillot (1951) claimed that the chemical retarded the germination of some species and inhibited straight growth in most species tested.

Materials and methods

Commercial grade NP was recrystallized from acetone-petroleum ether. For use, the recrystallized material (m.p. 191°) was converted to the potassium salt by the addition of a stoichiometric amount of potassium bicarbonate in aqueous-acetone solution. The salt was recovered by evaporation of the solvent in vacuo at 37° C with ebullition of dry nitrogen gas. Spectophotometric analysis yielded a molar extinction coefficient of $E_{284} = 1.595 \times 10^4$.

The standard Avena coleoptile section test (McRae, Foster and Bonner, 1953) was used to determine the interaction of indoleacetic acid (IAA) and NP. The standard error of each test was less than 10 percent, and there was no significant difference between replicates.

For straight growth and geotropic curvature tests, intact seedlings were used. Victory oats (*Avena sativa* var. Siegeshafer) were obtained through the courtesy of Dr. J. Bonner. Hybrid corn (*Zea mays*, W23×Oh51a, a single cross hybrid) was kindly supplied by Dr. E. Rossman, Department of Farm Crops, Michigan State University. Garden pea (*Pisum sativum* var. Alaska) was obtained from the Ferry Morse Seed Company.

Seeds were germinated in the dark at 25° C and all subsequent operations were conducted in subdued red light. Seedlings with straight tap roots and shoots of a desired length were selected for the experiments. Avena coleoptiles and roots of corn and pea were harvested at 2.0 ± 0.5 cm. while shoots of corn and pea were used at 1.25 ± 0.25 cm. Seedlings were pretreated with NP or other compounds by immersion of the seedlings in a solution of the compound dissolved in 0.005 to 0.01 molar phosphate buffer at pH 4.5. Control plants were similarly treated in the buffer. Pre-treatment time for oat coleoptiles was 30 minutes and 60 minutes for both roots and shoots of corn and pea. A longer pre-treatment time was used for corn and pea seedlings since the response subsequent to 30 minute treatment was not uniform. After pre-treatment the seedlings were divided into two groups: one for the geotropic test, and the other for the straight growth test. For the geotropic test, plants were held in a horizontal position by means of an Avena holder or by means of pressure sensitive tape with adhesive on both sides. The tape was mounted

on a moist blotter paper and the seeds covered with moist tissue paper. For the straight growth test, plants were held in a vertical position by the same means. Both sets were incubated at 25° C and 90 percent relative humidity.

Geotropic curvature was measured from shadowgraphs. Ten to sixteen plants were used per treatment and each experiment repeated one or more times. Straight growth was measured by one of two methods; 1. direct measurement of the difference between the initial and final total length; or 2. measurement of the increase in length of a zone extending from the tip to a charcoal-petroleum jelly mark placed 6 mm. from the tip following chemical treatment. The second method was preferred since the measured growing region then coincided with the zone responding to the geotropic stimulus. The first method was employed only for the shoots, since the elongation zone extends over a considerable length. In the case of corn shoots, two growth centers are involved; the coleoptile tip, and the rib meristem below the apical meristem.

Results

Interaction of NP and IAA

Over the range of 2.86 to 11.4×10^{-7} moles of IAA per liter and NP concentrations of 10^{-5}, 10^{-4}, and 5×10^{-4} *M*, there was no competitive interaction between IAA and NP (Figure 1) in Avena section growth. At high IAA concentrations (5.72 to 115×10^{-6} *M*) and 5×10^{-4} *M* NP, competitive inhibition was observed (Figure 2). Lower concentrations of NP were not tested competitively owing to their ineffectiveness in inhibiting the geotropic response. The inhibition of pea root section growth caused by 2.86 to 28.6×

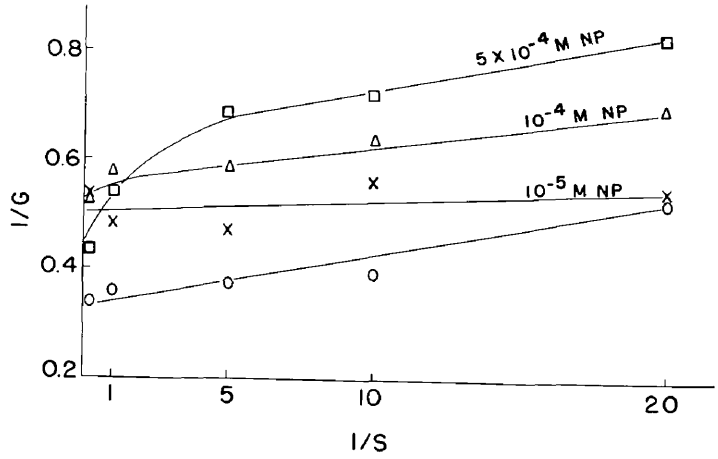

Figure 1. *Interaction in Avena coleoptile section growth test of IAA and NP.* 1/S=1/IAA concentration in mg/l. 1/G=1/growth in mm/section/12 hr.

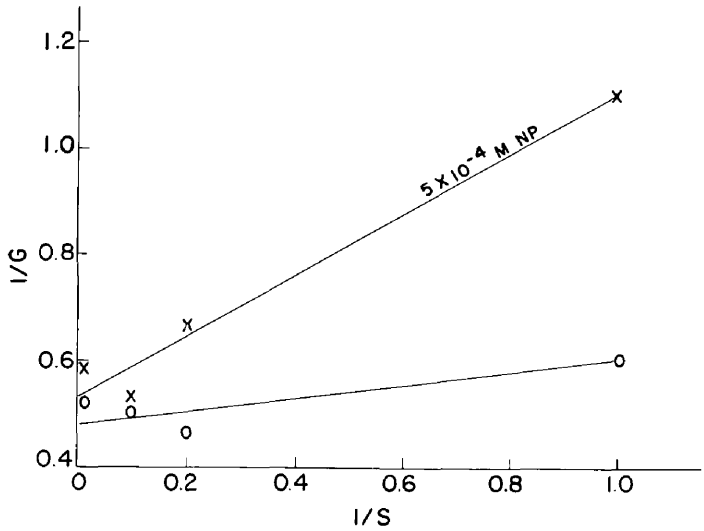

Figure 2. *Interaction of IAA and NP in Avena coleoptile section growth test at high IAA concentrations.* 1/S=1/IAA concentration in mg/l. 1/G=1/growth in mm/section/12 hr.

10^{-7} *M* IAA was not relieved by 5×10^{-4} *M* NP. Epinastic effects have been observed in tomato plants treated with 10^{-7} *M* NP (Hoffmann and Smith). Thus NP cannot unambiguously be classified as a growth promoting substance or as a competitive inhibitor of IAA action.

It should be pointed out that the competitive inhibition data may not hold true for shorter time intervals. A striking example of this is the 2,6 substituted phenoxy acetic acids (Osborne *et al*) which were active in extension growth when measurements were made after short time intervals.

Time course of straight growth and geotropic curvature

The time course of straight growth and geotropic curvature in control materials was studied in order to select a minimal incubation period on the linear portion of the time-response curve and of easily measurable magnitude. Results for the several plant materials used are summarized in Figures 3—7. In all the plant materials tested, growth is a linear function of time for at least four hours. The geotropic curvature reaches a maximum at 4 hours (earlier in corn roots), then remains constant or sometimes decreases as the bending zone becomes mature. These results indicate that under the conditions employed, a direct correlation between rate of straight growth and rate of geotropic curvature exists.

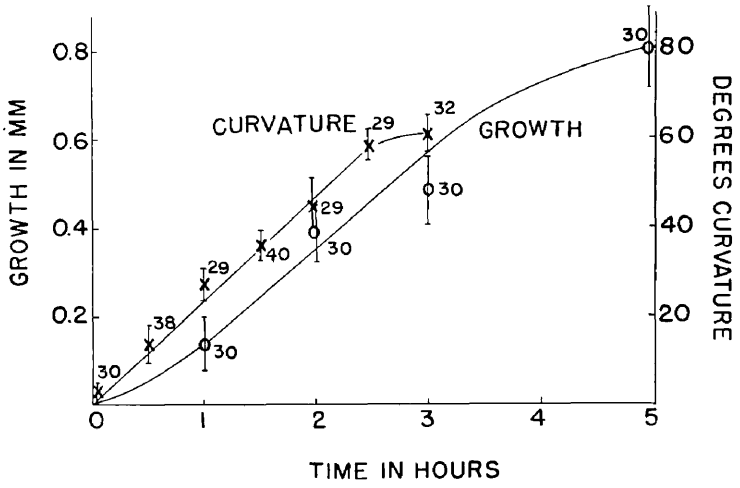

Figure 3. *Time course of growth and geotropic curvature of Avena coleoptiles.* Vertical lines crossing each point represents ± 2 X the standard error. The number of plants used is shown for each point.

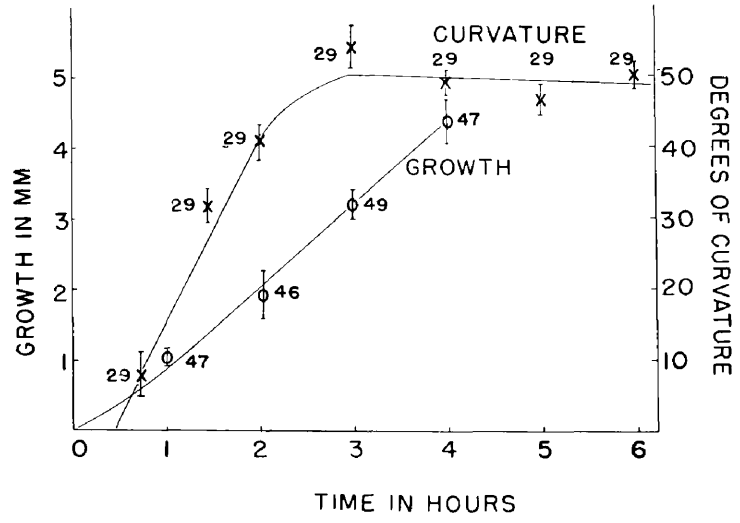

Figure 4. *Time course of growth and geotropic curvature of corn roots.*

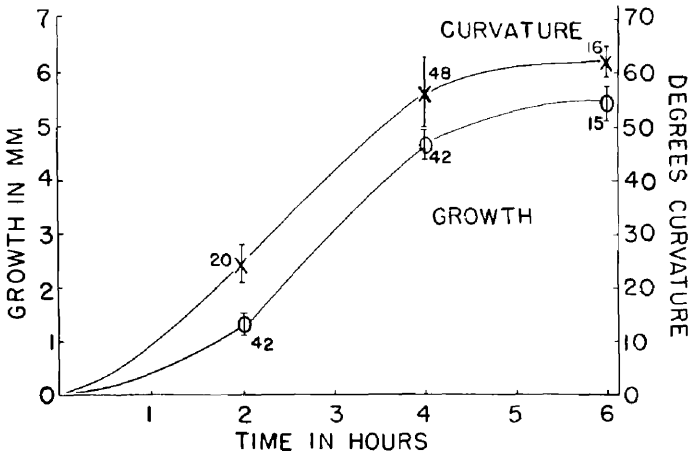

Figure 5. *Time course of growth and geotropic curvature of corn shoots.*

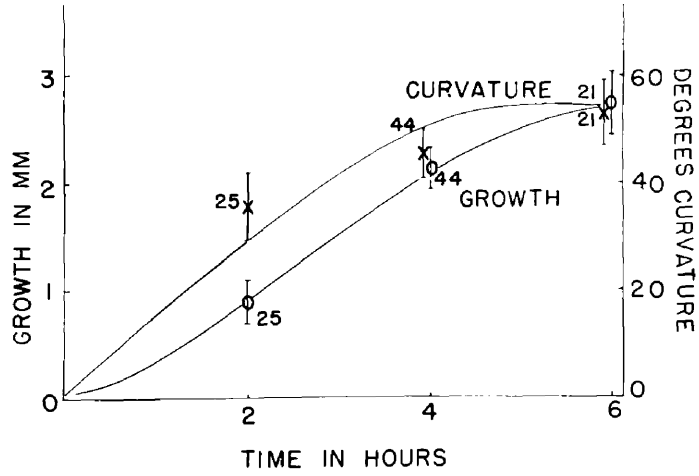

Figure 6. *Time course of growth and geotropic curvature of pea roots.*

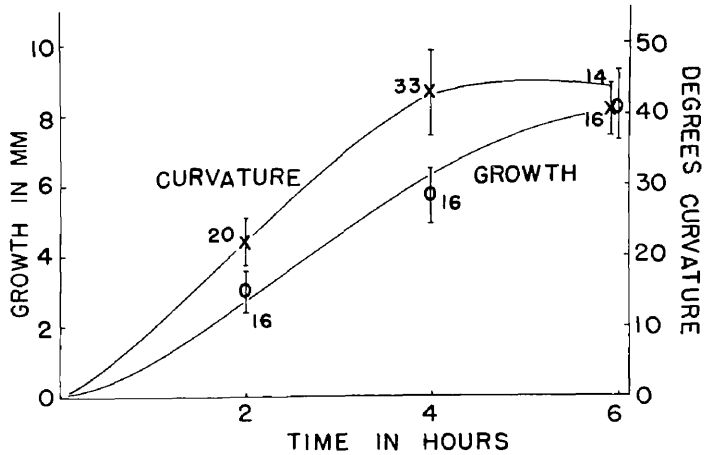

Figure 7. *Time course of growth and geotropic curvature of pea shoots.*

Disproportional inhibition of straight growth and geotropic curvature of shoots and roots of corn and pea

Straight growth and geotropic curvature of shoots and roots of corn and pea were determined after a 4 hour incubation following a one hour pretreatment of the plant material with the various concentrations of NP. The experimental data for each plant material tested are calculated as percent of the control and plotted in Figures 8—11. Low concentrations of NP, e.g. 10^{-5} *M*, inhibit both geotropic curvatures and straight growth of shoots

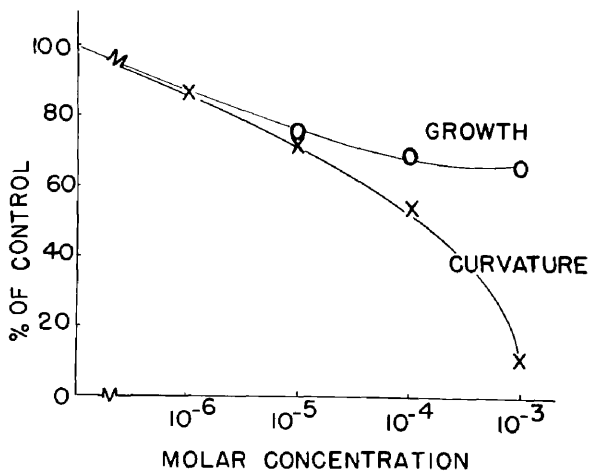

Figure 8. *Straight growth and geotropic curvature of NP pretreated corn shoots expressed as percent of the buffer treated control.*

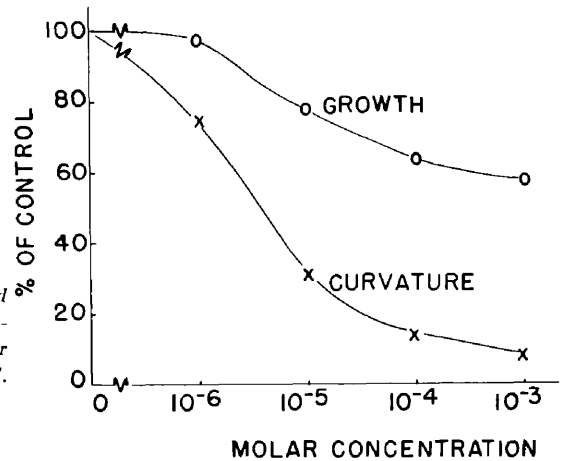

Figure 9. *Straight growth and geotropic curvature of NP pretreated corn roots expressed as per cent of the buffer treated control.*

(Figures 8 and 10) by approximately 30 percent. At 10^{-4} and 10^{-3} M a marked disproportionality is manifested; 10^{-3} M causing an almost total inhibition of the geotropic curvature of shoots and only a 40 percent inhibition of straight growth. A somewhat similar situation characterizes the curvature and straight growth of root tissue (Figures 9 and 11) except that the disproportionality becomes evident at lower inhibitor concentrations. A 70 to 80 percent inhibition of the tropic response is caused by 10^{-5} M NP, whereas straight growth is inhibited only 20 to 20 percent.

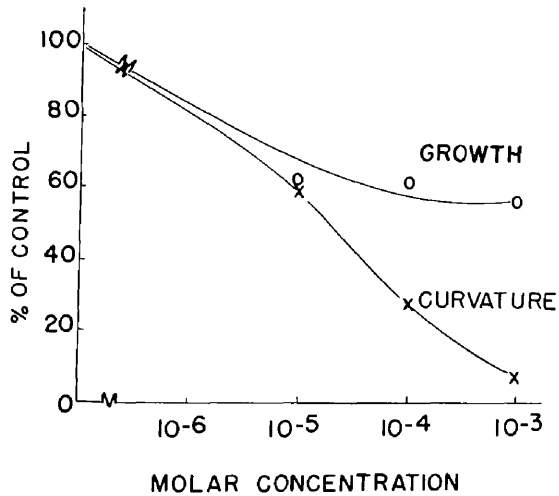

Figure 10. *Straight growth and geotropic curvature of NP pretreated pea shoots expressed as per cent of the buffer treated control.*

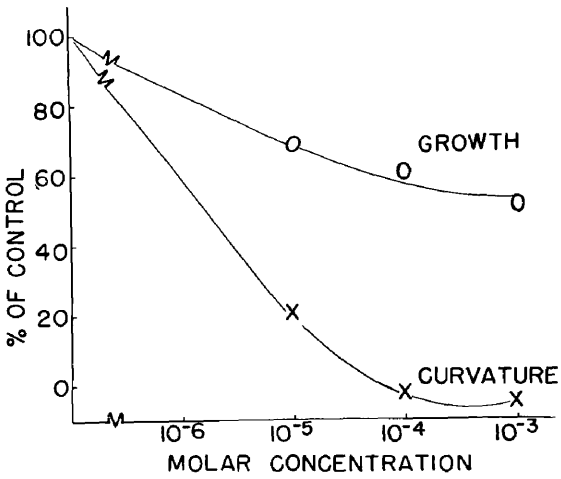

Figure 11. *Straight growth and geotropic curvature of NP pre-treated pea roots expressed as per cent of the buffer treated control.*

The concentrations inducing 50 per cent inhibition of each response for each plant organ, as estimated graphically, are summarized in Table 1. This peculiar, "uncoupling" of growth and geotropic curvature by NP occurs in root and shoot tissues of both monocotyledonous and dicotyledonous plants.

Proportional inhibition of straight growth and geotropic curvature of the Avena coleoptile by NP

Since Avena coleoptiles are very sensitive to growth regulators, and their physiological behavior is well known, earlier experiments were performed using intact Avena coleoptiles. It was found that NP inhibition was proportional to the concentration of the chemical used, and for a single concentration, inhibition increases with increasing time of incubation. Data for a 90 minute incubation following a 30 minute pretreatment are plotted in Figure 12.

Table 1. Concentrations in *M* of NP and DCIBA required for one-half inhibition of straight growth and geotropic curvature in different plant organs.

Material	Chemical	Growth	Curvature
Corn roots	DCIBA	1×10^{-4}	1×10^{-4}
Avena coleoptile	NP	1×10^{-4}	5×10^{-5}
Corn roots	NP	$>1\times10^{-3}$	4×10^{-6}
Corn shoots	NP	$>1\times10^{-3}$	1×10^{-4}
Pea roots	NP	1×10^{-3}	$<1\times10^{-5}$
Pea shoots	NP	$>1\times10^{-3}$	2×10^{-5}

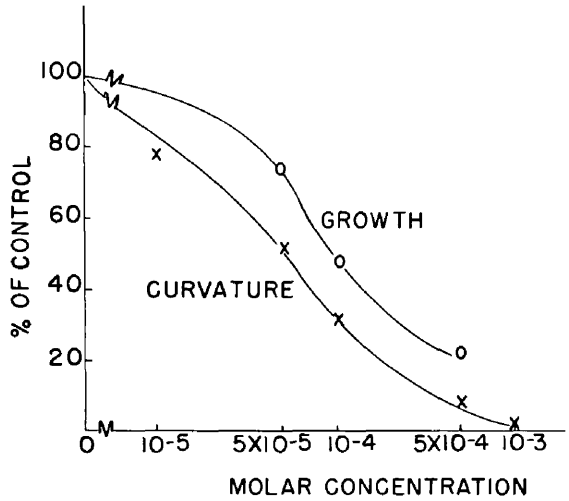

Figure 12. *Straight growth and geotropic curvature of NP pre-treated Avena coleoptiles expressed as per cent of the buffer treated control.*

There is a direct parallelism between inhibition of straight growth and inhibition of geotropic curvature in this tissue. Avena coleoptile tissue thus responds differently from the other plant materials tested.

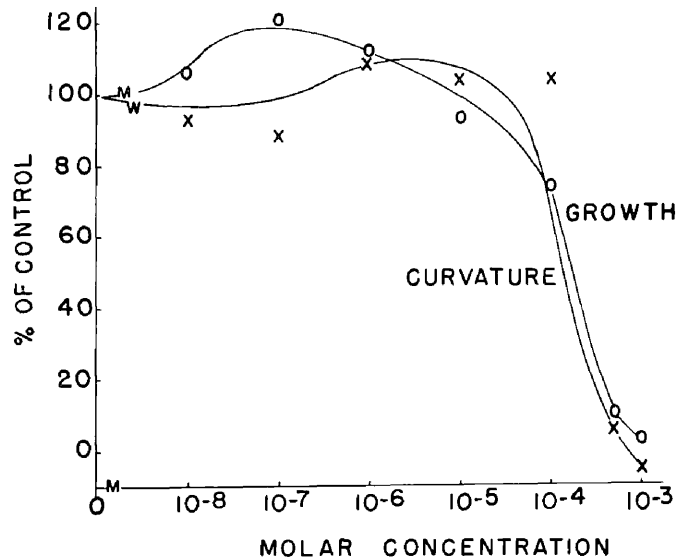

Figure 13. *Straight growth and geotropic curvature of DCIBA pre-treated corn roots expressed as per cent of the buffer treated control.*

Proportional inhibition of growth and geotropic curvature of corn roots by DCIBA and IAA

For comparison with the results of NP-treatment, 2,4-dichlorophenoxyisobutyric acid (DCIBA) was tested for inhibition of both growth and geotropic curvature. DCIBA has been reported to stimulate wheat root growth (Burström, 1951) and to competitively inhibit IAA action in the Avena section test (McRae and Bonner 1953). DCIBA, thus, has some of the properties associated with antiauxins. The experimental data are plotted as percent of the control in Figure 13. A direct correlation of straight growth and geotropic curvature is clearly obtained. Proportional inhibition apparently is not a general property of antiauxins, since Brumfield (1955) observed a disproportional inhibition of growth and geotropic curvature in timothy roots following treatment with another antiauxin, 2,4,6-trichlorophenoxyacetic acid.

The effects of IAA on growth and geotropic curvature of corn roots were examined, and in confirmation of results recently presented by Brumfield, IAA treatment results in proportional inhibition of growth and geotropic curvature.

Discussion

The geotropic response of plant organs may be visualized as occuring in three steps: *1.* the perception of the stimulus; *2.* the transport of the stimulus or a product resulting from that stimulus; and *3.* the growth response. Either the elongation zone or the tip may serve as the perception zone as shown by the experiments of Keeble, Nelson and Snow (1929) and more recently by Anker (1954). Loss of geotropic sensitivity following decapitation is due, apparently, to removal of the source of growth substance. There is no general agreement as to the mechanism of the gravitational perception or the transport of stimulus subsequent to the perception. It will suffice for the present purposes to consider the theory proposed by Went and Cholodny. These authors believe that gravity affects a displacement of growth substances, and that the resultant asymmetric distribution causes differential growth of the upper and lower sides of the plant organ; thus bending occurs. A direct correlation between growth and geotropic curvature may thus be expected and, as a first approximation, inhibition of straight growth should always result in proportional inhibition of curvature. This expectation is experimentally realized in the case of DCIBA and IAA inhibition of root growth and curvature and is also apparent from the correlation between growth and geotropic curvature in untreated control material.

The selective inhibition of geotropic curvature by NP is difficult to

explain. It is further difficult to understand the dissimilarities in behaviour of Avena coleoptile tissue, which shows proportional inhibition of straight growth and curvature, and the roots and shoots of corn and peas, which show disproportional inhibition. Our present data are insufficient to determine the mechanism of the selective tropic inhibition by NP. Three possibilities present themselves assuming the validity of the Went-Cholodny theory: *1.* An effect of NP upon the lateral transport of endogenous auxin; *2.* An effect of NP upon the perception of the gravitational stimulus; or *3.* An effect of NP upon only that fraction of growth associated with curvature and assuming two components contributing to growth.

We believe that experiments recently reported by Anker cast some doubt upon the importance of a lateral redistribution of auxin as a mechanism for the geotropic bending. Anker found that the geotropic response of decapitated Avena coleoptiles could be restored by incubation of the decapitated coleoptile sections in solutions containing IAA, indoleacetonitrile and naphthyl acetic acid. He did not interpret his results as being at variance with the Went-Cholodny theory, and further, did not determine internal auxin concentrations. It would appear that redistribution of auxin by coleoptiles immersed in a homogenous solution of IAA is improbable, and therefore an effect of NP upon internal auxin redistribution would not be a likely explanation.

An effect of NP upon the perception mechanism is similarly open to objection. NP has been reported by Nétien and Conillot to inhibit the phototropic response. These authors did not present quantitative data to show that the inhibition was not due simply to an inhibition of growth by NP. In view of the results reported here, however, it would seem that NP may inhibit growth and the phototropic response disproportionally. Since the perception of the photostimulus and the perception of the geo-stimulus probably involves dissimilar mechanisms, it seems unlikely that NP acts to inhibit both sensory mechanisms.

The possibility was entertained that straight growth consists of two components, and only one of these growth components is effective in producing curvature. If then NP were to inhibit only that fraction of growth normally associated with curvature, a disproportional inhibition of straight growth and curvature would be observed. This explanation appears unlikely because NP at 10^{-6} *M* significantly inhibits curvature with no inhibition of straight growth (Figure 9).

We are therefore left without a suitable explanation of the mode of NP action. Our current concept is simply that NP prevents the local acceleration and/or inhibition of growth caused by gravity. The elucidation of the mechanism of this action remains for future studies.

Summary

1. Over the time interval for geotropic bending to reach a maximum, a direct correlation exists between the rate of straight growth and geotropic curvature in untreated Avena coleoptiles and the young roots and shoots of corn and pea.

2. A disproportional inhibition of growth and curvature was found in *n*-1-naphthylphthalamic acid (NP) treated roots and shoots of corn and pea, but no disproportionality was observed in NP treated Avena coleoptiles.

3. Corn roots treated with 2,4-dichlorophenoxyisobutyric acid showed a proportional inhibition of straight growth and geotropic curvature.

4. The close correlation between straight growth and geotropic curvature to be expected on the basis of the Went-Cholodny theory was not obtained.

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BIOLOGICAL ACTIVITY OF TETRAZOLE ANALOGUES OF INDOLE-3-ACETIC ACID AND 2,4-DICHLOROPHENOXYACETIC ACID

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BIOLOGICAL ACTIVITY OF TETRAZOLE ANALOGUES OF INDOLE-3-ACETIC ACID AND 2,4-DICHLOROPHENOXYACETIC ACID[1, 2, 3]

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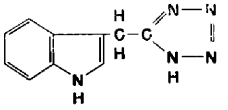
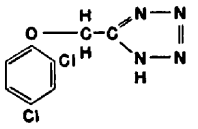
The present report is concerned with the biological activity of two growth substance analogues in which the tetrazole group has been substituted for the carboxyl group. The structure of these compounds, 5-(3′-indolemethyl)tetrazole (IMT) and 5-(2′, 4′-dichlorophenoxymethyl)tetrazole (2,4-DT) is as shown below:





An extensive program of synthesis and testing of tetrazoles has been conducted by Herbst and associates (2, 3, 5, 7, 15). Tetrazoles substituted only in the ring carbon ($R\text{-}CN_4H$) are acids having one dissociable hydrogen. When R is alkyl, the derivatives are slightly weaker acids, while when R is aromatic the derivatives are somewhat stronger acids than the corresponding carboxyl compounds (5, 11, 15). Herbst proposed the use of these derivatives as analogues of biologically important carboxyl compounds. The plant growth regulating activity of these compounds is of interest since only a few growth substances without a carboxyl group (or group convertible to a carboxyl) are known. Veldstra (12, 13) reported activity for several sulfonic, phosphonic, and phosphonous acids. Growth promoting activity of some tetrazole derivatives was reported by Van de Westeringh and Veldstra (11), subsequent to completing the present studies. A brief preliminary report of the present work has appeared (4).

It will be shown that IMT promotes elongation of *Avena coleoptile* sections and antagonizes growth induced by indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) in a manner expected for a weak auxin. By contrast, 2,4-DT does not promote growth in the Avena assay and inhibits both 2,4-D and IAA induced growth. Additional data suggest that the inhibition of 2,4-D induced growth by 2,4-DT may be partially explained by interference of 2,4-D uptake by 2,4-DT.

METHODS

IMT and 2,4-DT were synthesized by reaction of the nitrile with aluminum azide and sodium azide respectively. The details of synthesis and characterization are to appear elsewhere (7). The purity of IMT was examined by chromatography of 1 mg quantities of IMT on Whatman No. 1 paper using isopropyl alcohol/ammonia/water (8 : 1 : 1); pyridine/ammonia (4 : 1); and 77 % ethanol as ascending solvents. In the last two solvents IMT ran slightly above IAA and when sprayed with Ehrlich's reagent (1 % *p*-dimethylaminobenzaldehyde plus 8.5 % conc. HCl in 95 % ethanol) gave a purple spot changing to yellow. No IAA or indole-3-acetonitrile was detected as contaminant of the IMT. Since, under these conditions, 0.2 μg of IAA or the nitrile could have been detected, these compounds could not account for the growth promoting activity of IMT.

Victory oats were husked, soaked in tap water, and placed embryo up on the edges of plastic blocks covered with moist filter paper. The seedlings were exposed to continuous low intensity red light at 25° C and 95 % humidity for about 60 hours. Coleoptiles 25 to 35 mm in length were selected and one 6.5 mm section was cut 2 mm from the tip. In some experiments, the primary leaf was removed, but in most experiments it was not. The sections were floated in Petri dishes on 10 ml of 0.01 M KH_2PO_4 buffer pH 4.5 containing 2 % sucrose. Concentrated stock solutions of the growth regulating acids were prepared by dissolving the acid in a slight molar excess of alkali. Growth was measured at 5, 10, and 20 hours on the same lot of sections by means of a binocular microscope with an ocular micrometer. The 5 and 10 hour measurements were made as rapidly as possible using illumination from a 7.5 watt red safelight. Experiments indicated that sections measured

[1] Received June 12, 1959.

[2] Journal Article 2392 from the Michigan Agricultural Experiment Station.

[3] Supported in part by the National Science Foundation.

at 5 and 10 hours had not elongated significantly less at 20 hours than sections measured after 20 hours only. All data represent the average of at least two or three experiments using about 25 sections per treatment dish.

Seeds of bean (*Phaseolus vulgaris*, var. Wax Pencil Pod) were planted in soil in the greenhouse and used at the primary leaf stage just before the terminal bud unfolded. Solutions of growth substances used for treatment were in 95 % ethanol plus 0.1 % Tween 20, and treatments made by application of a 10 μl drop to one primary leaf.

Results

5-(3'-Indolemethyl) Tetrazole: The growth rate of Avena sections used in these studies was not a linear function of time but tended to decrease slightly at the longer incubation times. The buffer used, pH changes during incubation, successive measurement, or the particular lot of seed used did not account for the non-linear growth. The time course (fig 1) represents the average of a number of experiments. Although the deviations from linearity were not large, they were consistent. A low growth rate for the control sections, and to some extent for the 2,4-D treated sections, was noted during the first 5 hours. Only growth measurements obtained after a 10 hour incubation are here presented since these data are representative but less subject to error and non-linearity than the 5 or 20 hour measurements. As is shown in figures 1, 2, and 3, IMT promotes elongation of *Avena coleoptile* sections. Thus 10^{-4} M IMT is equivalent as a growth promoter to 2.8×10^{-7} M IAA (fig 2), or to 2.25×10^{-6} M 2,4-D (fig 3). By contrast, the effect of IMT in combination with IAA (fig 2), or 2,4-D (fig 3) may be *stimulatory*, *inhibitory* or *nil* depending on the concentration of 2,4-D or IAA. These results are interpretable in terms of a weak growth substance competing with a stronger one. At higher concentrations of IAA or 2,4-D, the less active IMT could occupy a number of binding sites thus blocking out the more active IAA or 2,4-D and resulting in inhibition of the IAA or 2,4-D induced growth.

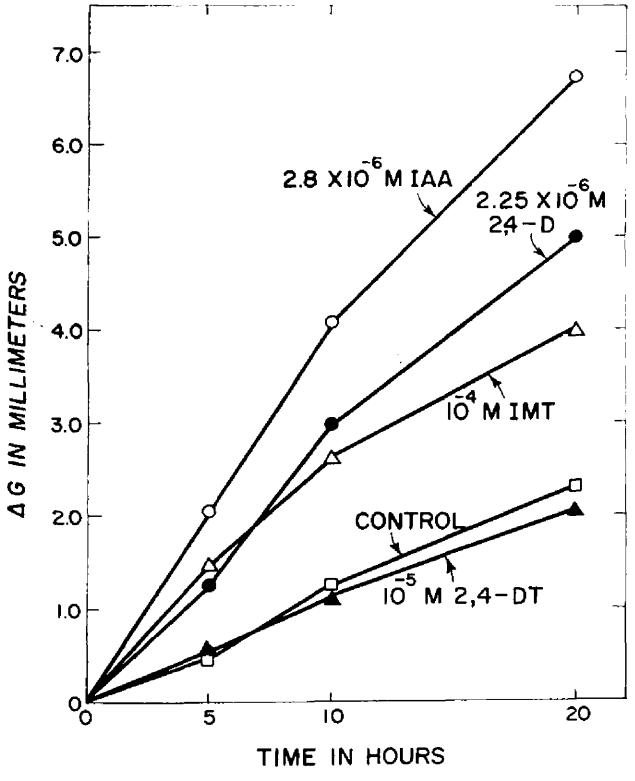
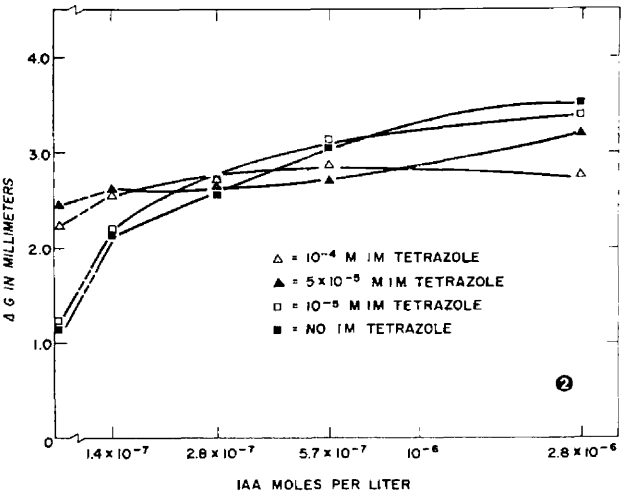


Fig. 1. Time course for growth of *Avena coleoptile* sections when floated in solutions containing IAA, 2,4-D, 2,4-DT, IMT or no growth substance. Vertical lines in figures represent ± 2 × standard error.



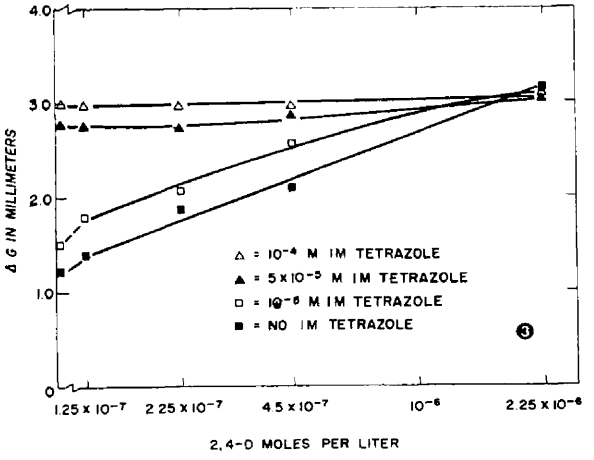


Fig. 2. Growth of *Avena coleoptile* sections after 10 hrs in three concentrations of IMT alone, and in combination with various concentrations of IAA.

Fig. 3. Growth of *Avena coleoptile* sections after 10 hrs in three concentrations of IMT alone, and in combination with various concentrations of 2,4-D.

TABLE I

EFFECT OF 2,4-DT ON IAA OR 2,4-D INDUCED ELONGATION OF AVENA COLEOPTILE SECTIONS

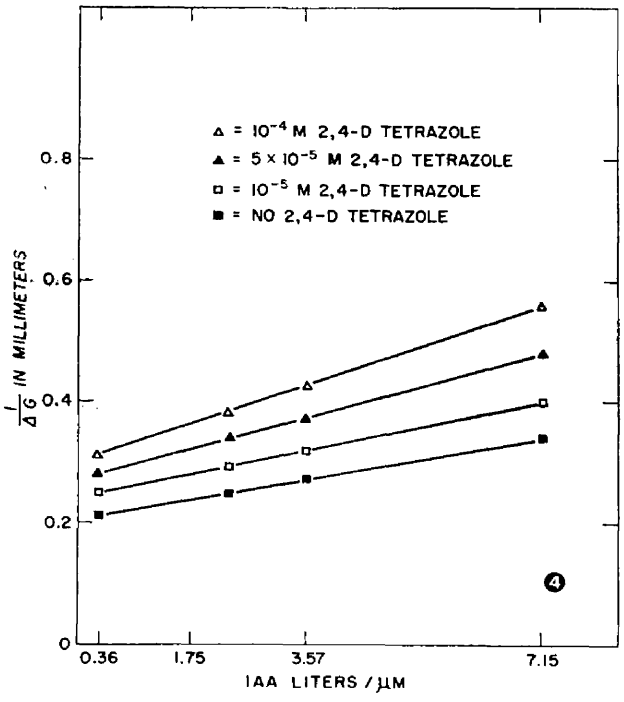
AUXIN MOLAR CONC			% INHIBITION 2,4-DT MOLAR CONC		
		0**	10^{-5}	5×10^{-5}	10^{-4}
IAA*	0	1.54 ± 0.054	27	38	51
	1.4×10^{-7}	2.74 ± 0.010	1	21	35
	2.8×10^{-7}	3.56 ± 0.15	7	17	35
	5.7×10^{-7}	3.89 ± 0.15	12	19	30
	2.8×10^{-6}	4.32 ± 0.11	2	11	27
2,4-D*	0	1.08 ± 0.052	24	44	67
	1.25×10^{-7}	1.09 ± 0.058	24	50	69
	2.25×10^{-7}	1.50 ± 0.064	27	49	71
	4.50×10^{-7}	2.07 ± 0.10	29	56	71
	2.25×10^{-6}	3.26 ± 0.14	15	37	51

* Average of three experiments with 2,4-D and three with IAA. Section length measured after 10 hrs

** Growth in millimeters ± 2 × standard error

5-(2', 4'-DICHLOROPHENOXYMETHYL) TETRAZOLE: The data of table I show that 2,4-DT did not promote elongation of *Avena coleoptile* sections at any of the concentrations tested and was, in fact, measurably inhibitory to growth at concentrations greater than 10^{-5} M. The interactions of 2,4-DT with 2,4-D and IAA were rather complex; the nature of the interactions is most clearly shown by a double reciprocal plot of the data. Thus 2,4-DT acted as a weak and non-competitive inhibitor of IAA induced growth (fig 4), but the interaction of 2,4-DT and 2,4-D (fig 5) is quite different, and a competitive component is noted. The nature of the interactions is also shown in a less striking manner in table I. Thus, the slight inhibition by 2,4-DT is scarcely influenced by IAA concentration. On the other hand, 2,4-DT inhibits 2,4-D induced growth to a greater extent and the highest 2,4-D concentration tends to overcome this inhibition.

APPARENT INHIBITION OF 2,4-D ABSORPTION BY 5-(2,4-DICHLOROPHENOXYMETHYL) TETRAZOLE: An experiment was designed to test the hypothesis that 2,4-DT might antagonize the absorption of 2,4-D by a plant tissue. For this experiment, the primary leaf of greenhouse-grown bean plants was used with either separate or coincident application of 2,4-D and 2,4-DT. The treatments (see fig 6) were as follows: 1) control, no treatment, 2) 2,4-DT alone, 3) 2,4-D alone, 4), 5), 6) 2,4-DT and 2,4-D both applied and to different areas of the leaf, 7) 2,4-D and 2,4-DT applied coincidently. After 24 hours the treated leaf was removed, and the curvature of the stem measured. As a convenient measure of formative response, the first trifoliate leaf was removed and weighed after two weeks. It is apparent from a typical experiment



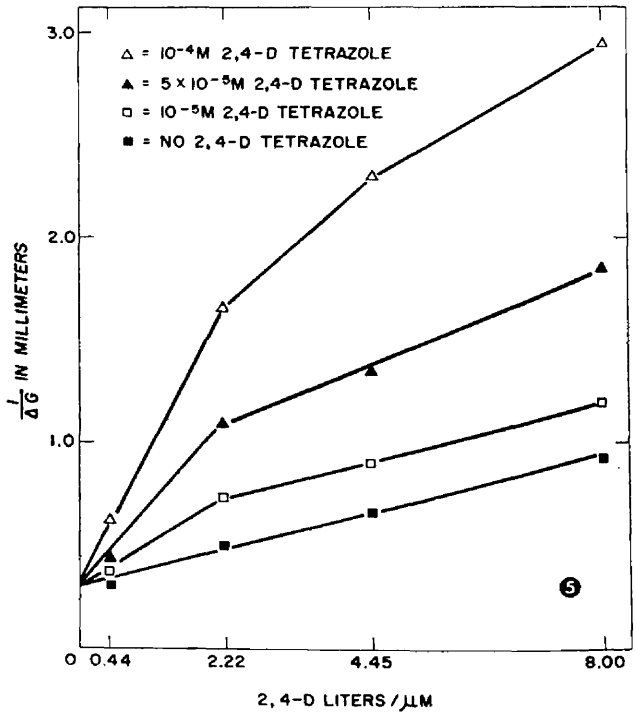


FIG. 4. Reciprocal of growth of *Avena coleoptile* sections after 10 hrs in presence or absence of 2,4-DT plotted as a function of the reciprocal of various concentrations of IAA.

FIG. 5. Reciprocal of growth of *Avena coleoptile* sections after 10 hrs in presence or absence of 2,4-DT plotted as a function of the reciprocal of various concentrations of 2,4-D.

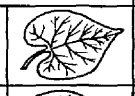
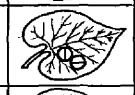
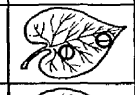
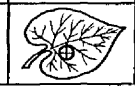
Location of treatment ⊖=2,4-D ⊕=2,4-DT ⊕=2,4-D plus 2,4-DT		2,4–D (µgm)	2,4–DT (µgm)	Stem curvature (degrees)	Trifoliate leaf (gm)
1		0	0	0	1.08
2		0	50	0	1.04
3		2.5	0	61	0.60
4		2.5	50	50	0.51
5		2.5	50	49	0.63
6		2.5	50	73	0.57
7		2.5	50	0	1.08

FIG. 6. Average curvature after 24 hrs, and average fresh weight of first trifoliate leaf after 14 days when 2,4-D and 2,4-DT were applied to one primary leaf of bean plants.

(fig 6) that 2,4-DT prevented 2,4-D induced curvature and formative effect *only* when the two substances were applied coincidently (that is, the 2,4-D and 2,4-DT dissolved in the same solution and applied to the leaf). If the same amounts were applied separately to different areas of the leaf there was no reduction of 2,4-D induced curvature or formative effect. In one experiment co-application of 10 µg of 2,4-DT completely prevented the curvature response induced by 2,4-D and only a slight formative response was observed.

DISCUSSION

The nature of the acidic group for growth promoting activity is important, but acid groups other than the carboxyl may confer at least some activity. Thus Veldstra (12, 13) lists the following compounds as active: 3-indolemethane-, 1-naphthalenemethane-, 1-naphthalene-, and 3-indolesulfonic acids. Vlitos and Hitchcock (14) have described in detail the growth promoting and herbicidial activity of 3-indolemethanesulfonic acid. In the present work, as in that of Van de Westeringh and Veldstra (11), it has been demonstrated that the tetrazole group may replace the carboxyl group of IAA with a retention of growth promoting activity.

Substitution of the tetrazole group for the carboxyl group of 2,4-D similarly leads to a biologically active substance. In this case, however, the analogue shows no growth promoting activity and acts as a strong, partially competitive, inhibitor of 2,4-D induced growth, but as a weaker non-competitive inhibitor of IAA induced growth. It is surprising that a single inhibitor should have a different interaction with IAA and 2,4-D since presumably both IAA and 2,4-D have similar modes of action and react with the same growth inducing site. It also seems possible that 2,4-DT may have two different mechanisms of antagonism. That is, it could be a non-competitive antagonist of both IAA and 2,4-D at the growth inducing site and, in addition, a competitive and specific antagonist of 2,4-D absorption. This possibility is bolstered by the bean leaf experiment here reported, and by previously reported experiments on the absorption of IAA and 2,4-D. Thus, Johnson and Bonner (6) have concluded that the major phase of 2,4-D uptake is specific and metabolic in nature. They also found no competitive effect of IAA on the uptake of 2,4-D. Earlier work with relatively high concentrations of IAA (1, 10) had indicated that IAA uptake appeared to be by the process of normal diffusion. In any case, it would appear that the uptake site of 2,4-D may be different from that of IAA. Direct experiments testing the antagonism by 2,4-DT of the absorption of C^{14} labeled 2,4-D and IAA would be desirable.

SUMMARY

The tetrazole group is shown to serve as an analogue of the carboxyl group in 2,4-D and IAA. The IAA analogue 5-(3′-indolemethyl)tetrazole was found to be a weak auxin in the *Avena coleoptile* section test, and to compete with both IAA and 2,4-D induced growth. The 2,4-D analogue, 5-(2′, 4′-dichlorophenoxymethyl)tetrazole is an antagonist of both 2,4-D and IAA induced elongation of *Avena coleoptile* sections. The antagonism of IAA is weak and non-competitive and that of 2,4-D is stronger and partially competitive at high 2,4-D concentrations. Tests with intact bean plants indicated that 5-(2′, 4′-dichlorophenoxymethyl)tetrazole may prevent the uptake of 2,4-D.

ACKNOWLEDGEMENTS

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II.

ISOLATION AND PARTIAL CHARACTERIZATION OF THE PRECURSOR OF 6-METHOXYBENZOXAZOLINONE IN ZEA MAYS[1]

R. H. Hamilton, R. S. Bandurski, and W. H. Reusch[2]

A sweet compound with phenolic properties has been isolated from corn seedlings. It yields 6-methoxybenzoxazolinone as a degradation product and appears to be the cyclic hydroxamate, 1,3-dihydroxy-2-keto-6-methoxy-1,4-benzoxazine.

Introduction

Etiolated corn seedlings were noted to have a very sweet, saccarin-like taste. The compound responsible for the taste, here designated as corn sweet substance (CSS), has been isolated and partially characterized and a structure proposed. CSS was found to be identical to a substance previously isolated from corn seedlings by R. J. Suhadolnik (1), who suggested the structure 2,5-dihydroxy-3,4-methylenedioxphenylacetamide (I). Our data indicate this assignment was incorrect.

Smissman, LaPidus, and Beck (2) and Virtanen, Hietala, and Wahlroos (3) have reported the isolation of 6-methoxybenzoxazolinone from corn seedlings, and the latter authors also from wheat seedlings. Recently, Whitney and Mortimore (4) have also reported isolation of this derivative from corn stalks. Virtanen and Hietala (5) had previously reported the isolation of benzoxazolinone from rye seedlings.

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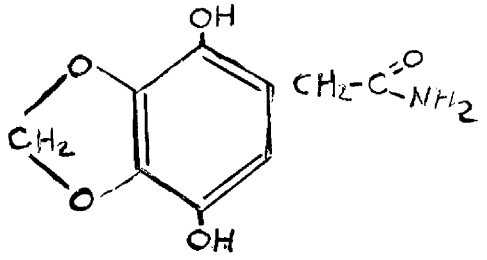
[2]United States Department of Agriculture, Agricultural Research Service, Crops Research Division; and Department of Botany and Plant Pathology and Department of Chemistry, Michigan State University, East Lansing, Michigan.

These benzoxazolinone derivatives were of interest because of their fungicidal activity (4,5), and their activity in inhibition of the European corn borer (2). Grasses of the genera Coix have been reported to contain a benzoxazolinone derivative, coixol, by Koyama, Yamato and Kubota (6). Methylation of coixol leads to a mixture of N-methyl-6-methoxybenzoxazolinone and the isomeric O-methyl derivative, suggesting that coixol may be 6-methoxybenzoxazolinone and its isomeric enol. Recent work by Virtanen and co-workers (7,8,9,10,11) indicates, however, that the benzoxazolinone derivatives isolated from corn, wheat and rye are artifacts formed from precursors during the isolation, owing either to enzymatic activity of the crushed seedlings or to heat degradation in aqueous solution. The precursor from corn seedlings existed as a monoglucoside which could be converted to the aglucone upon treatment with press juice from the seedlings (9). This aglucone is identical to our sweet compound, CSS. Both CSS and its glucoside do occur in corn seedlings since in our studies, employing ethanol as the extractant (which would inactivate glycosidases), CSS yields of 0.5% of the seedling dry weight were obtained. The glucoside may be detected by paper chromatography of seedling extracts. Virtanen and Hietala (11) have proposed a structure (II) for the aglucone from rye seedlings; and work to be presented here indicates that the sweet compound, CSS, may be the analogeous 6-methoxy derivative (III). It is of con-



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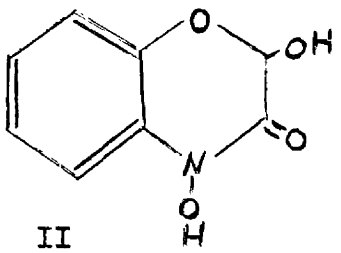

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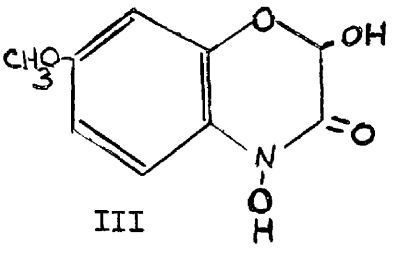


III

siderable interest that the antibiotics Aspergillic acid (12,13), and Nocardamin (14) are cyclic hydroxamates, and that antibiotic activity appears to be associated with this structure (15). A considerable number of cyclic hydroxamates have now been synthesized and found to have antibiotic activity (16,17,18,19). Recently, in vivo N-hydroxylation of 2-acetylaminofluorene has been demonstrated (20).

Discussion

After isolation from corn seedlings and purification by two or more recrystalizations from acetone-ligroin, light tan to white monoclinic, sweet-tasting, anisotropic crystals melting at 160-161°C (decomp.) uncorrected, were obtained. The crystals were somewhat soluble in methanol, ethanol, butanol and acetone and were insoluble in water, ligroin, benzene and dry ethyl ether. A 2.0% solution in ethanol showed no detectable rotation of polarized light.

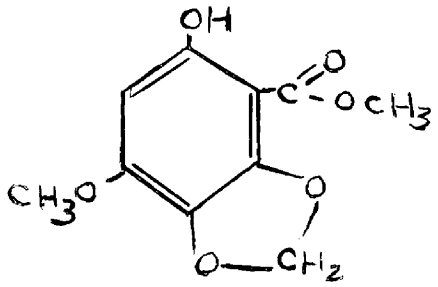
Paper chromatography in 4 solvents, and column chromatography on buffered celite revealed only one component as detected by $FeCl_3$ (blue), UV fluorescence (yellow-orange under 253 mμ hand lamp), or the yellowish-brown color obtained after exposure of chromatograms to ammonia. Paper chromatography of crude corn extracts (n-butanol-acetic acid-water, 50:40:1) also indicated the presence of another compound (Rf 0.50) giving the same ferric chloride color as the sweet compound (Rf 0.55). Urban (21) also detected these two compounds on chromatograms of corn seedling extracts in a similar solvent.

The solubility in 5% $NaHCO_3$ indicated an acidity greater than most phenols, and titration in 50% ethanol indicated a pK of 7.0 and an equivalent weight of about 195. The average of 4 molecular weight

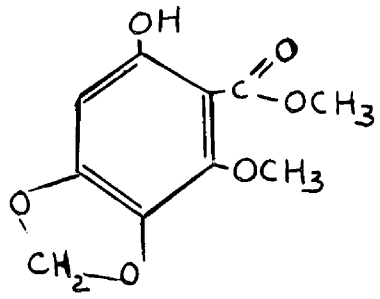
determinations using the method of Childs (22) was 212, and the average of 3 determinations using freezing-point depression of t-butanol was 199. Qualitative tests after sodium fusion indicated no sulpher or halogens, and analysis of C, H, and N indicated a formula of $C_9H_9O_5N$. Zeisel's HI cleavage indicated one methoxyl, and a C-methyl determination indicated 2.13% (theory 7.11%). Active hydrogen by $LiAlH_4$ reduction was 1.09% (theory for 2H: 0.95%). CSS gave a striking deep blue ferric chloride test and therefore was first assumed to be a phenol. It reduced Fehling's solution in 0.5 min., and Tollen's solution immediately in a boiling water bath, and gave immediate decolorization of $KMnO_4$. It gave negative tests for an aldehyde group with Shiff's reagent, and did not form a bisulfite addition product. It formed a phenylhydrazone upon heating with 2,4-dinitrophenylhydrazine in acid solution, but not in neutral solution.

A consideration of the above results indicated a reducing activity much greater than to be expected for a monohydric phenol, and yet titration had indicated only one acidic group. The ferric chloride color is very much like that of salicylic acid or salicylamide, but the latter derivative had little or no reducing activity in the above tests. The ultraviolet-absorption spectrum (Fig. 1) showed a long wave spectral shift of the 265 mμ acid peak as the pH was varied. The pK was 6.9 to 7.0. There was, in addition, a short wave shift in the ultraviolet spectra at pH values above pH 8.5, and a considerable increase in optical density. The shift which occurred at pH values above 8.5 was immediate, and CSS could be reisolated by acidification and extraction with ether. It was at first assumed that CSS might be a salicylamide derivative containing a methoxyl and a methylenedioxy group. This latter

group could not be detected by formaldehyde evolution during heating in strong acid (23). Positive tests were obtained only when an excess of chromotropic acid, phloroglucinol, or gallic acid was heated with CSS. Since methyl-6-hydroxy-4-methoxy-2,3-methylenedioxybenzoate (IV) and the corresponding 2-methoxy-3,4-methylenedioxybenzoate (V) had been synthesized by Wagner et al. (24), it was of interest that these derivatives gave good tests for formaldehyde evolution upon heating with strong acids (25).



IV



V

Comparison of the infrared absorption spectra (25) indicated that these derivatives were quite different from CSS in that they showed intense bands in the 1240 to 1260 cm^{-1} ether region, whereas the sweet compound had a single, moderate band at 1278 cm^{-1} (Fig. 2). Conclusive evidence that no methylenedioxy group was present was obtained from the NMR spectra (Fig. 3A).

Attempts to prepare the methyl ether of CSS, using dimethyl sulphate, and an acetate derivative, with acetic anhydride, resulted in neutral derivatives in small yield which had apparently lost C,2H,2O. The ether derivative still had only one methoxyl as determined by Zeisel's method. An alkaline hydrolysis of CSS resulted in a third derivative which had lost C,2H,2O; and the infrared spectra of all three derivatives showed a strong carbonyl band at about 1780 to 1800

cm^{-1} (Fig. 5 and 6). Since no carbon dioxide was lost on alkaline hydrolysis, it appeared one of the products might be formic acid. Formation of a volatile acid was demonstrated by steam distillation of an hydrolysate. The acid was identified as formic acid by paper chromatography of the ammonium salt, and by reduction to formaldehyde using Mg and HCl (26). Another product of the hydrolysis was about 0.5 equivalents of ammonia. Since this seemed to be about the maximum yield of ammonia obtainable after prolonged alkaline hydrolysis, it appeared unlikely that CSS could be a simple amide.

Since the alkaline hydrolysis product had a carbonyl group, an almost neutral nitrogen (slightly soluble in NaOH), and an unaccounted for oxygen, it appeared possible that it might be a benzoxazolinone derivative. However, the red color, and the known instability of benzoxazolinone under alkaline conditions, cast some doubt upon this conclusion. The report of Wahlross and Virtanen (9) that the precursor of 6-methoxybenzoxazolinone in corn had the same formula and melting point, and was converted to 6-methoxy-benzoxazolinone with loss of formic acid, made it appear that the red alkaline hydrolysis product was indeed 6-methoxybenzoxazolinone. A comparison with synthetic 6-methoxybenzoxazolinone revealed that the ultraviolet spectra, infrared spectra (Fig. 5) and melting points were identical (2).

An examination of the ether derivative by nuclear magnetic resonance (NMR) (Fig. 4B) clearly showed an N-methyl and an O-methyl peak at 198 and 225 cps, respectively, in addition to a multiplet at 408 cps for the ring protons. Confirmation that the ether derivative is N-methyl-6-methoxybenzoxazolinone is that the melting point (107°), ultraviolet maxima (234 mμ, 290 mμ), and the carbonyl band in the infrared spectra

(Fig. 6A) correspond closely to the reported values of Koyama et al. (6). The melting point (27), and the absence of an N-H stretch, as well as the lack of a strong band in the 1200 cm^{-1} region, and two carbonyl bands at 1805 cm^{-1} and 1720 cm^{-1} (Fig. 6B), indicate that the acetate derivative of the sweet compound is N-acetyl-6-methoxybenzoxazolinone.

The benzoxazolinone derivatives resulting from alkaline hydrolysis, methylation, or acetylation indicated that not more than one hydroxyl could be attached to the ring.

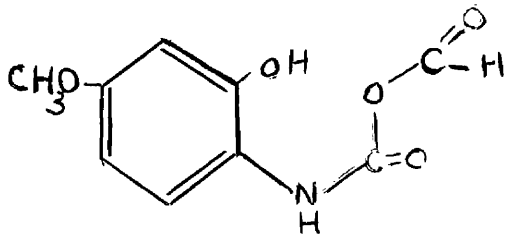
All attempts to prepare the dinitrobenzoate, the α-naphthylurethane and the aryloxyacetic acid derivatives failed. It has already been mentioned that CSS had much more reducing activity than would be expected of a monohydric phenol. Mass thermal degradation of CSS in vacuo resulted in products of mass 195, 193, 165, 150, and 140. The product with mass 195 appeared to have lost oxygen; a rather unique degradation product. The loss of oxygen upon heating aspergillic acid under N_2 with cupric chromate has been reported (12). An attempt was made to detect an ultraviolet shift associated with formation of a borate complex as reported for hydroxamates by Green (28), but no such shift was observed. A depression of the λ max. 292 and the λ min. 245 in borate buffer compared to Tris buffer at pH 9.04 was, however, observed.

It appeared desirable to prepare a nitro derivative of CSS in order to change the acidity of the weakly acidic hydroxyl group. A yellow, impure sublimable derivative was prepared by treatment of CSS with HNO_3. Surprisingly, a similar yellow, sublimable derivative was prepared when CSS was treated with cold nitrous acid in strong sulphuric acid. Both these derivatives had similar ultraviolet spectra and showed

UV-spectral shifts corresponding to a pK of 7.0. An infrared spectra of the latter derivative showed neither a typical nitroso or nitro band.

In summary, the corn sweet substance (CSS) has one acidic hydroxyl, a possible second weakly acidic hydroxyl (ultraviolet shift, NMR), one methoxyl, an aromatic ring, and a carbonyl (IR). The nitrogen is not basic, but some ammonia is evolved upon alkaline hydrolysis (up to a maximum of 45% of theory in 5 hours).

It would appear that the data are in accord with the conclusion of Virtanen and Hietala (11) that the phenol-like substances isolated from cereals are hydroxamates (Structures II & III). An alternative structure (VI), which had some value as a working hypothesis, seems excluded by the failure to observe by NMR a formyl proton below the 500 cps region.



VI.

The loss of oxygen upon mass thermal degradation would correspond to the reported loss of oxygen from the hydroxamate, Aspergillic acid, under similar conditions (12). All attempts to substitute the hydroxyl group have failed, and CSS is a much better reducing agent than most mono-hydroxy phenols.

Experimental

Ultraviolet spectra were measured with a Beckman DU spectrophotometer, and infrared spectra with a Beckman IR-5 spectrophotometer. All

melting points are uncorrected. Paper chromatography was with Whatman 3MM paper, ascending. Molecular weights were determined by the isothermal distillation method of Childs (22) or freezing point depression of t-butanol as noted. Micro analyses for C, H and N were conducted by Micro-Tech Laboratories, 8000 Lincoln Avenue, Skokie, Illinois. Methoxyl, C-methyl, N-methyl and active hydrogen analyses were by Schwarzkopf Laboratories, 5619 37th Avenue, Woodside 77, New York.

Isolation

Five-day-old Michigan 350 etiolated corn seedlings (3.6 Kg fresh weight) were homogenized in a Waring blendor with sufficient 95% ethanol to make the extract 80% ethanol. After filtration, the bright yellow ethanol extract (5 gallons) was concentrated in vacuo at 37° in a spray dry appartus. The reaction of the aqueous concentrate was adjusted to pH 4.0 with 5 N H_3PO_4, and extracted with three volumes of ethyl ether. The combined ether fractions were reduced in volume to 500 ml and then partitioned with 2 volumes of 8% $NaHCO_3$ (100 ml) under hydrogen. The combined bicarbonate fractions were acidified as above and extracted with 3 volumes of ether. The combined acid ether extracts were dried several hours at 1° over anhydrous Na_2SO_4, and then evaporated to dryness in vacuo. The resulting light tan semi-crystaline residue was washed twice with ether (5 ml), and recrystalized from acetone-ligroin yielding 2.50 g of light buff crystals. Upon recrystalization from the same solvent, 1.85 g of almost-white anisotropic crystals, m.p. 160-161° (decomp.) resulted. Analysis calculated for $C_9H_9O_5N$: C, 51.30; H, 4.27; N, 6.64; OCH_3, 14.65; C-methyl, 7.11; 2 active H, 0.95%; m.w. or neut. equiv., 211. Found: C, 51.34; H, 4.43; N, 6.73; OCH_3, 14.05; C-methyl, 2.13; active hydrogen ($LiAlH_4$ in N,N-dimethylmorpholine), 1.09%; m.w. 212

(22) or 199 (freezing point depression of t-butanol); neut. equiv., 195. Paper chromatography was in water, n-butanol-27% AcOH (1:1), m-cresol-water-AcOH (50:2:48), or 73% phenol. The Rf values are respectively 0.90, 0.95, 0.75, and 0.88-0.92 (observed with a 253 mμ ultraviolet light, or brown spots developed after exposure to ammonia). Column chromatography (20 ml 0.1 M potassium phosphate buffer, pH 6.5, adsorbed on 25 g Celite in a 1.8 cm diameter column using buffer saturated ether as the mobile phase) yielded one peak eluted in the 18 to 27 ml fractions as detected by $O.D._{265}$ and $FeCl_3$ color.

Formation of 6-methoxybenzoxazolinone, formic acid and ammonia upon alkaline hydrolysis

CSS (50 mg) was heated with 1 N NaOH and 0.001 N ethylenediaminetetraacetic acid under hydrogen at 75° for one hour. Upon acidification (1 N HCl) and cooling, 15 mg of red needles were obtained, m.p. 153-154°. The product was recrystalized from hot water yielding 9.2 mg, m.p. 153-153.5°. It was not sweet, ferric chloride negative, slightly soluble in dilute alkali, and insoluble in dilute acid. During the hydrolysis, about 20% of theory of ammonia was evolved, and with prolonged hydrolysis a maximum of about 46% theory of ammonia could be obtained in 5 hours. When the filtrate was steam distilled, about 1.3 equiv. of volatile acid was obtained which was identified as formic acid by chromatography as the ammonium salt (ethanol-ammonia-water, 80:4:16) and by reduction to formaldehyde (26). The latter was detected with chromotropic acid (26). The red needles had λmax. 233 (ϵ, 10,100), λ max. 2.90 (ϵ, 5,675), and λmin. 255. Calculated for $C_8H_7O_3N$: C, 58.18; H, 4.24; N, 8.48%; m.w., 165. Found: C, 57.85; H, 4.31; N. 8.73%; m.w., 214. The isolated sample was compared by infrared spectra, ultraviolet

spectra, and mixed melting point to synthetic 6-methoxybenzoxazolinone (2), and found to be identical.

N-acetyl-6-methoxybenzoxazolinone

CSS (77.5 mg) was dissolved in dry pyridine (3 ml), and acetic anhydride (7.5 ml) was added (1). The large test tube was plugged with cotton and heated in a boiling water bath for 10 minutes. After chilling, about 30 ml of cold distilled water was added and grey-white needles were obtained. These were collected on a Pregl filter, washed with cold water, and recrystalized from aqueous ethanol. After this recrystalization, 15.4 mg of white crystals, m.p. 147-148° were obtained. They were insoluble in 1 N $NaHCO_3$, and 1 N HCl, but were very soluble in methanol or ethanol. Analysis calculated for $C_{10}H_9O_4N$: C, 57.97; H, 4.35; N, 6.76%; OCH_3, 14.97%; m.w., 207. Found: C, 57.85; H, 4.29; N, 6.97; OCH_3, 14.97%; m.w., 215.

N-methyl-6-methoxybenzoxazolinone

Dimethyl sulfate was shaken with solid potassium carbonate and redistilled *in vacuo*. Methylation was carried out by two procedures, both yielding the same product. In the first method, 325 mg of CSS was dissolved in methanol (20 ml) and equivalent amounts of saturated methanolic KOH and dimethyl sulfate were added under nitrogen with stirring. The solution was brought to reflux, cooled and a sample then tested negative with ferric chloride. The solution was made alkaline, and after standing 90 minutes, 80 ml of water was added. The solution was cooled to 1° and allowed to sit 3 hours. The light-brown crystals were collected on a filter, washed with cold water, and dried at 60° *in vacuo*. The yield was about 161 mg, m.p. 105-107°. The product was recrystalized from aqueous methanol, yielding 100 mg of light tan

platelets, m.p. 105-107°. The product was insoluble in water, dilute HCl, and dilute alkali; but was soluble in methanol, ethyl ether, or chloroform.

In the second method, 105.5 mg of CSS was dissolved in acetone (40 ml). Solid potassium carbonate (4 g) and an excess of dimethyl sulfate (3 equiv.) were added to the solution under reflux, which resulted in a greenish color changing to a pale, yellow-brown. After refluxing for 1 hour, another portion (3 equiv.) of dimethyl sulfate was added. After an additional hour, the solution was poured into two volumes of 1 N NaOH and allowed to stand overnight a 1°. After removal of the acetone in vacuo, the solution was extracted with 2 volumes of ethyl ether. The ether was dried over anhydrous Na_2SO_4 at 1°, and the decanted ether concentrated to dryness in vacuo. A light-brown, aromatic, ferric chloride negative residue was obtained. The residue was recrystalized from aqueous ethanol, yielding 44 mg of light-brown platelets, m.p. 96-99°. The infrared spectrum of this preparation was the same as that of the first preparation, except for weak bands at 3200 cm^{-1} and 3450 cm^{-1}. By sublimation at 110° @ 25 mm Hg, white crystals were obtained from both preparations, m.p. 107-108°. Anal. calculated for $C_9H_9O_3N$: C, 60.40; H, 5.03; N, 7.83; OCH_3, 17.31; NCH_3, 8.38%; m.w., 179. Found: C, 60.31; H, 5.18; N, 7.42; OCH_3, 17.07; NCH_3, 2.78%; m.w., 217. The ultraviolet spectra indicates λmax. at 235 and 290 mμ, and λmin. at 255 mμ, and no spectral shifts when run in 0.05 N HCl or NaOH.

Acid hydrolysis of corn sweet compound

Fifty mg of CSS were dissolved in ethanol (2 ml) and 1 ml of 5 N H_2SO_4 added to the ethanolic solution in a Thunberg tube. One ml

of carbonate free alkali (5 N NaOH) was added to the side arm and the tube evacuated. The tube was heated in a boiling water bath for one hour, and allowed to stand over night. The carbonate in the side arm was precipitated by addition of barium carbonate (1 ml saturated), and the precipitate filtered, washed and weighed. About 0.66 equivalents of CO_2 was lost during the acid hydrolysis. The reddish-brown solution was extracted with 3 volumes of ether, which after drying and removal of the ether yielded a bright red precipitate. The precipitate was crystalized from aqueous methanol, yielding 10.1 mg of very fine brownish-red needles, m.p. 152-153°. The product was insoluble in dilute acid and water, but was soluble in 1 N $NaHCO_3$. It gave negative tests with Shiff's reagent, Fehling's reagent, and ferric chloride.

Reaction of corn sweet compound with nitrous acid

Solid sodium nitrite was slowly added to cold 90% H_2SO_4 until Na_2SO_4 precipitated. CSS (50 mg) was dissolved in 1 ml of hot acetone and 1 ml of distilled water added to the warm solution. After chilling in an ice bath, portions of the cold nitrous acid reagent were added. The yellow-orange solution was stirred from time to time with a glass rod and a yellow precipitate formed. After 2 hours at 1°, the solution was slowly diluted with 6 ml of ice water, filtered, and the yellow precipitate washed with ice water. The precipitate was dissolved in a 5% Na_2CO_3 (5 ml), and the solution slowly acidified with 5 N HCl. The reprecipitated product was collected on a filter, dried *in vacuo* at 60°, and recrystalized from aqueous ethanol. The yellow needles were collected on a Pregl micro-filter and dried *in vacuo* at 60°. The yield was 8 mg, m.p. 92.0-92.5°. Anal. calculated for $C_8H_8O_5N$: C, 48.48; H, 4.04; N, 7.07%. Found: C, 49.91; H, 4.33; N, 7.29%. At

acid pH, λmin. 260 mμ and λmax. 345 (ϵ, 21,810). At alkaline pH, λmin. 270, λmin. 360, λmax. 320 (ϵ, 12,500) and λmax. 410 (ϵ, 15,690). The pK estimated from the ultraviolet shift is 7.0. The product sublimes upon heating in vacuo (90° @ 0.05 mm Hg). Treatment of a yellow solution of the product in 50% ethanol with zinc and 1 drop of 6 N HCl yields a colorless solution.

Nitration of corn sweet compound

About 50 mg of CSS was dissolved in 1.5 ml of acetone and treated with 0.1 ml of concentrated HNO_3. There was an immediate formation of a brownish-red color so the solution was diluted with 4 ml of distilled water and cooled. The orange crystals obtained were recrystalized from aqueous ethanol, but had no definite m.p. The product was sublimed in vacuo (120° @ 0.05 mm Hg) and yellow crystals collected on the cold finger, leaving a red residue. The yellow product had no definite melting point. The ultraviolet maxima, minima and the spectral shift with pH were identical to the product obtained upon treating with nitrous acid.

Preparation of an azo dye

The preparation was after that of Tedder and Theaker (29) on a 50 mg scale. When the diazonium salt was coupled to resorcinol, an immediate deep-red color was observed. The azo dye was recrystalized from aqueous methanol, yielding about 16 mg of orange crystals, m.p. 221-231°. An analysis for C, H and N indicated the product was impure. Paper chromatography in ethanol-ammonia-water (10:1:1), isopropanol-ammonia-water (10:1:1), and n-butanol-ammonia-water-ethanol (44:1:20:20) revealed a contaminante giving brownish-pink color when sprayed with 0.01 N NaOH, and running just above the red dye. The Rf of the dye was respectively

0.50, 0.28, and 0.37. The dye was recrystaled from acetone-ligroin, but when chromatographed on paper was still found to be impure. The pK of the yellow-red indicator-like change of the dye was about 8.5.

Oxidation of corn sweet compound with neutral permanganate

The permanganate reagent consisted of 0.05 M $KMnO_4$ containing 5% $MgSO_4$ (30). About 0.1 mM (21.1 mg) of CSS was dissolved in 1 ml of acetone. The permanganate was added in 0.05 ml portions (0.05 mM) and upon loss of the purple color, another aliquot was added. It was necessary to centrifuge down the MnO_2 to observe the color change. Approximately 5 equivalents of permanganate were consumed. The excess permanganate was decomposed with ammonium oxalate. The solution was acidified with 1 N H_2SO_4 until all the MnO_2 went into solution, extracted with 2 volumes of ether, and the combined ether fraction dried over anhydrous Na_2SO_4 at 1°. The decanted ether layer was shaken with 3 ml of 1 N NH_4OH. A 0.2 ml sample was chromatographed on paper in ethanol-ammonia-water (80:4:16). Six ultraviolet absorbing or fluorescent spots were noted, none of which gave a $FeCl_3$ color. Two acids (Rf 0.14, 0.23), not matching any of the ultraviolet spots, were found upon spraying with bromphenol blue (0.5 mg per ml plus 2 mg/ml citric acid). The acid, Rf 0.23, was rechromatographed in 80% phenol and had an Rf in these two solvents very close to glycolic acid. The other acid had an Rf of 0.18 in this latter solvent and an Rf of 0.16 when chromatographed in n-butanol-formic acid-water (10:2:15).

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Figure 1: Ultraviolet spectrum of the corn sweet substance (CSS) as a function of pH. Buffers used were 0.1 M potassium phosphate (4.30, 6.50, and 7.05), trishydroxyaminomethylmethane (7.75 and 8.45). A solution of 0.005 N NaOH was used for a pH value of 11.60 and a solution of 0.1 N KOH for pH value of 13.05.

Figure 2: Infrared spectrum of the isolated sweet substance from corn. A composite spectra determined on a mull in Nujol "mineral oil" and hexachlorobutadiene.

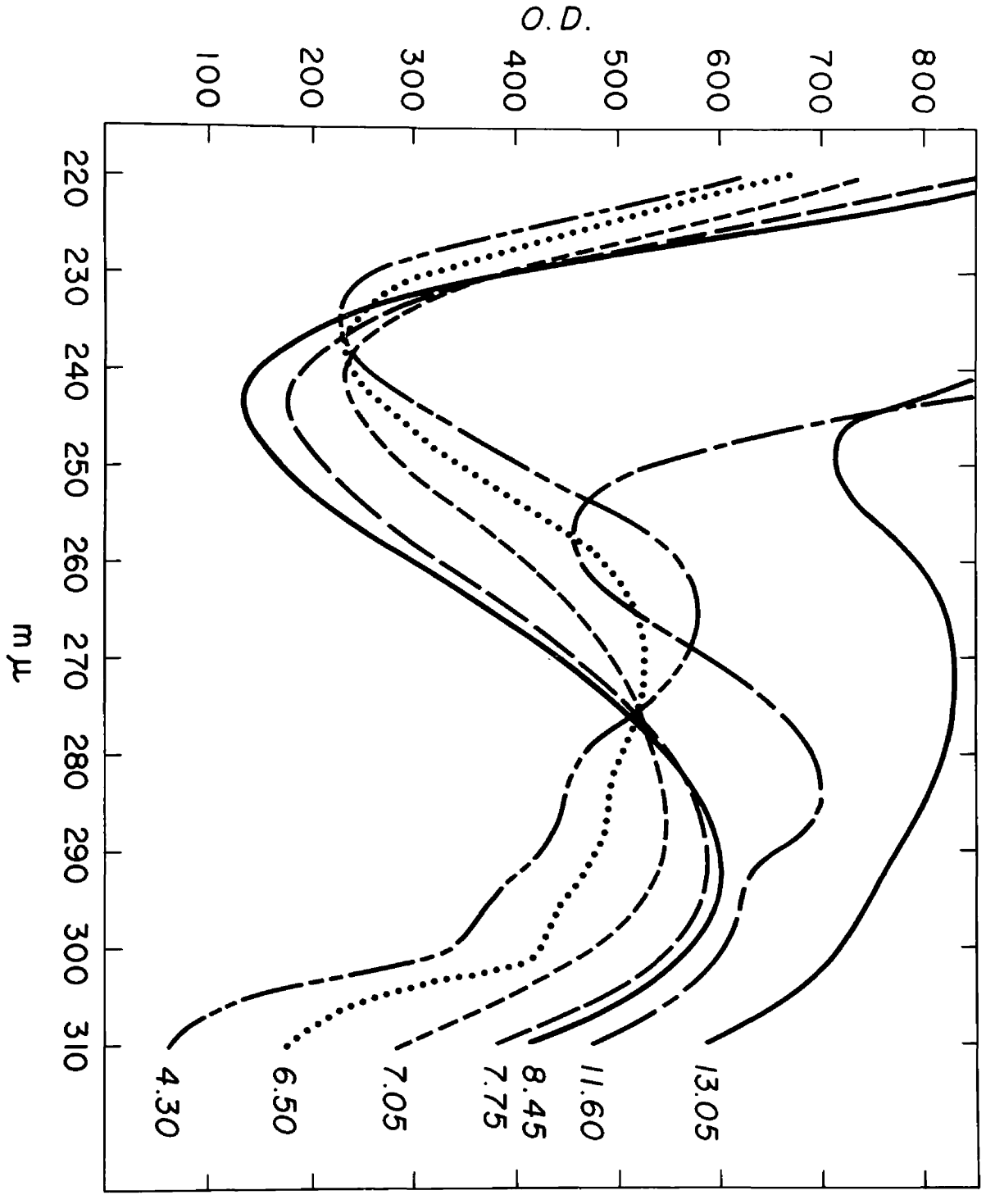
Figure 3A: Infrared spectrum of the isolated corn sweet substance in various solvents as indicated. 3B: An infrared spectrum of the product of reaction of CSS with nitrous acid. (Solvent was CS_2 except the 1400 to 1700 cm^{-1} region in $CHCl_3$).

Figure 4A: Nuclear magnetic resonance spectrum of corn sweet substance in acetone. 4B: A nuclear magnetic resonance spectrum of N-methyl-6-methoxy-benzoxazolinone in deuterated chloroform.

Figure 5A: Infrared spectrum of synthetic 6-methoxybenzoxazolinone. 5B: Isolated 6-methoxybenoxazolinone, both in chloroform.

Figure 6A: Infrared spectrum of N-methyl-6-methoxybenzoxazolinone in CCl_4. 6B: N-acetyl-6-methoxybenzoxazolinone in CCl_4.

Figure 1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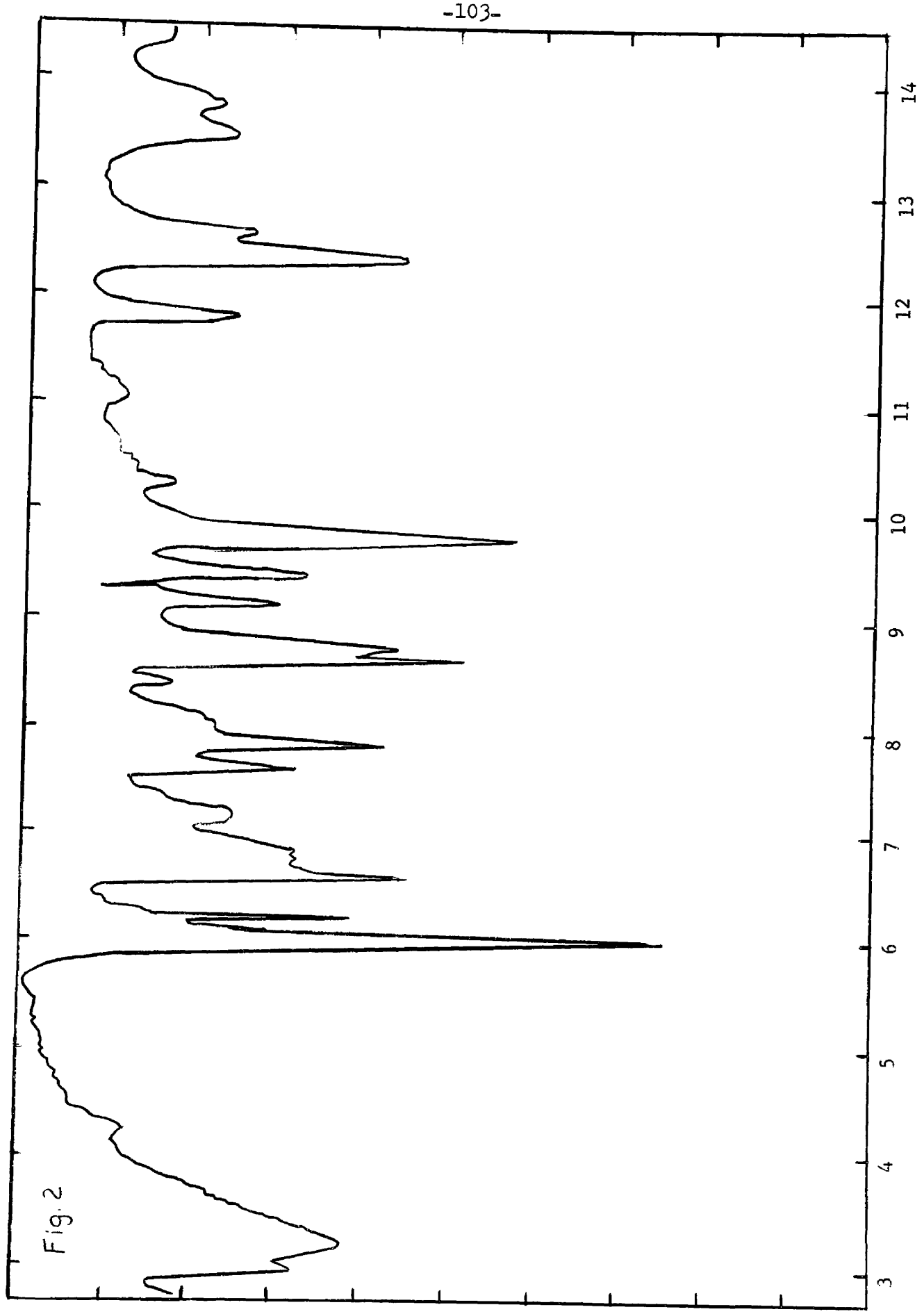

Fig. 2

Figure 3

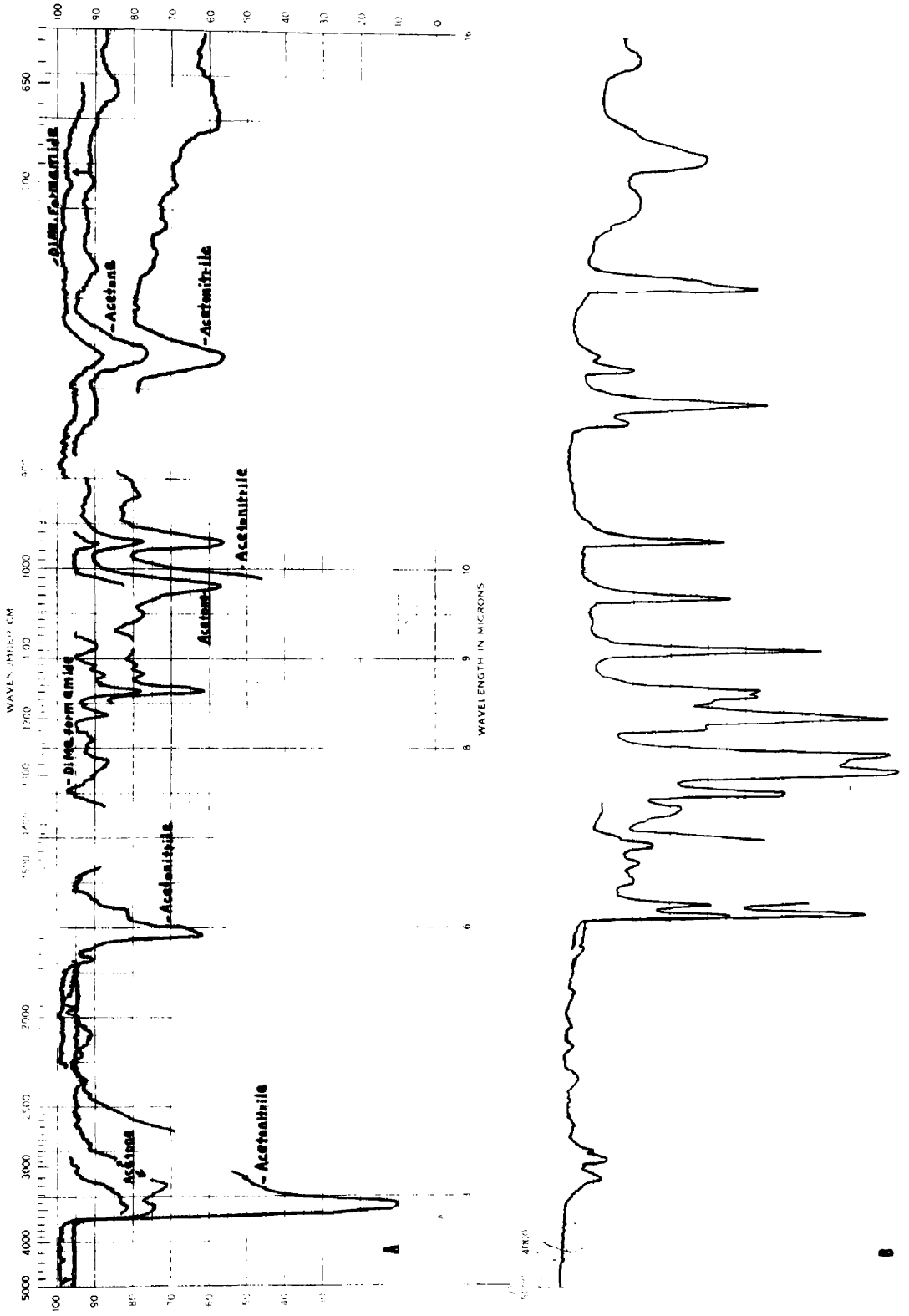
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Figure 4

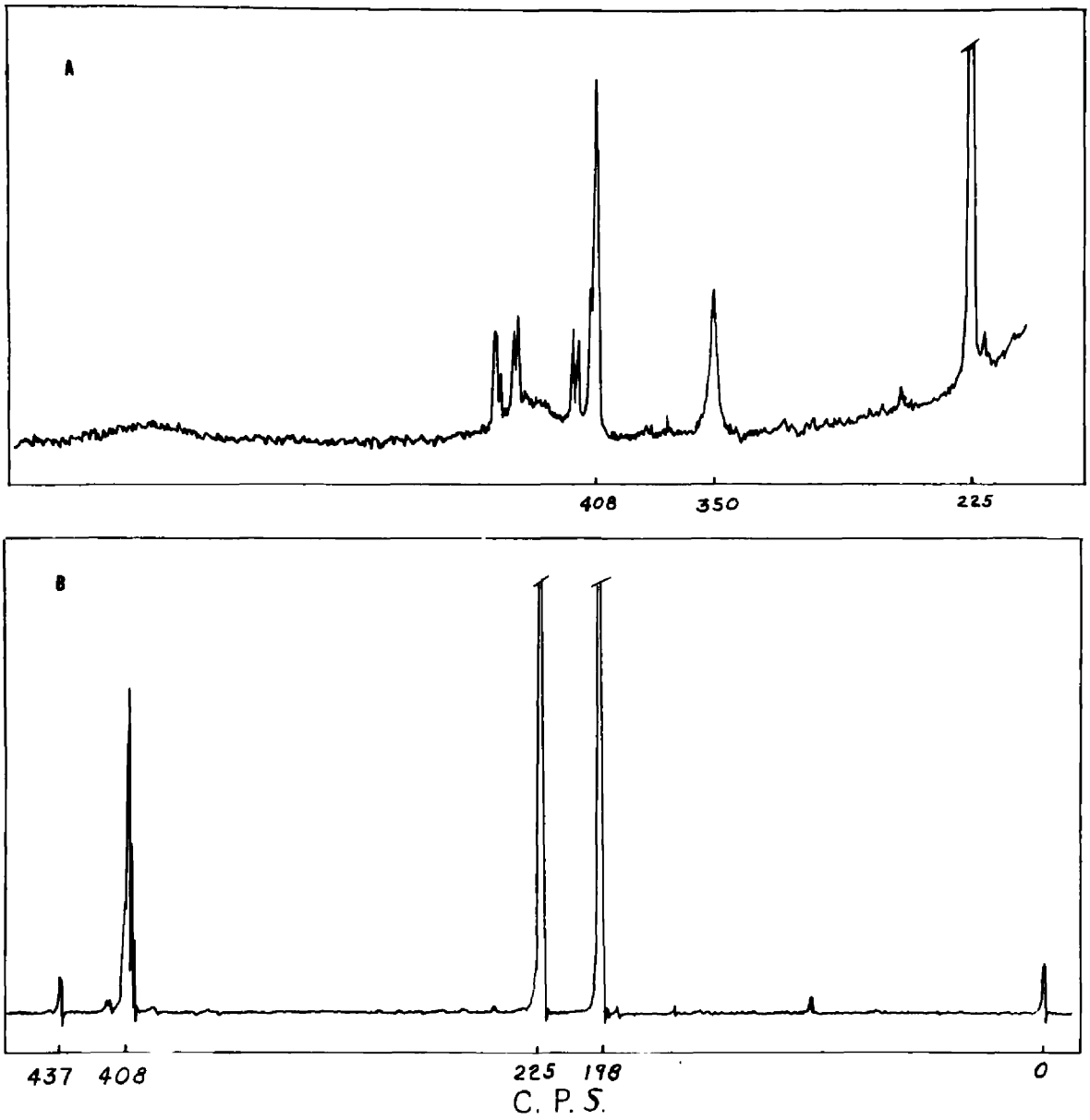
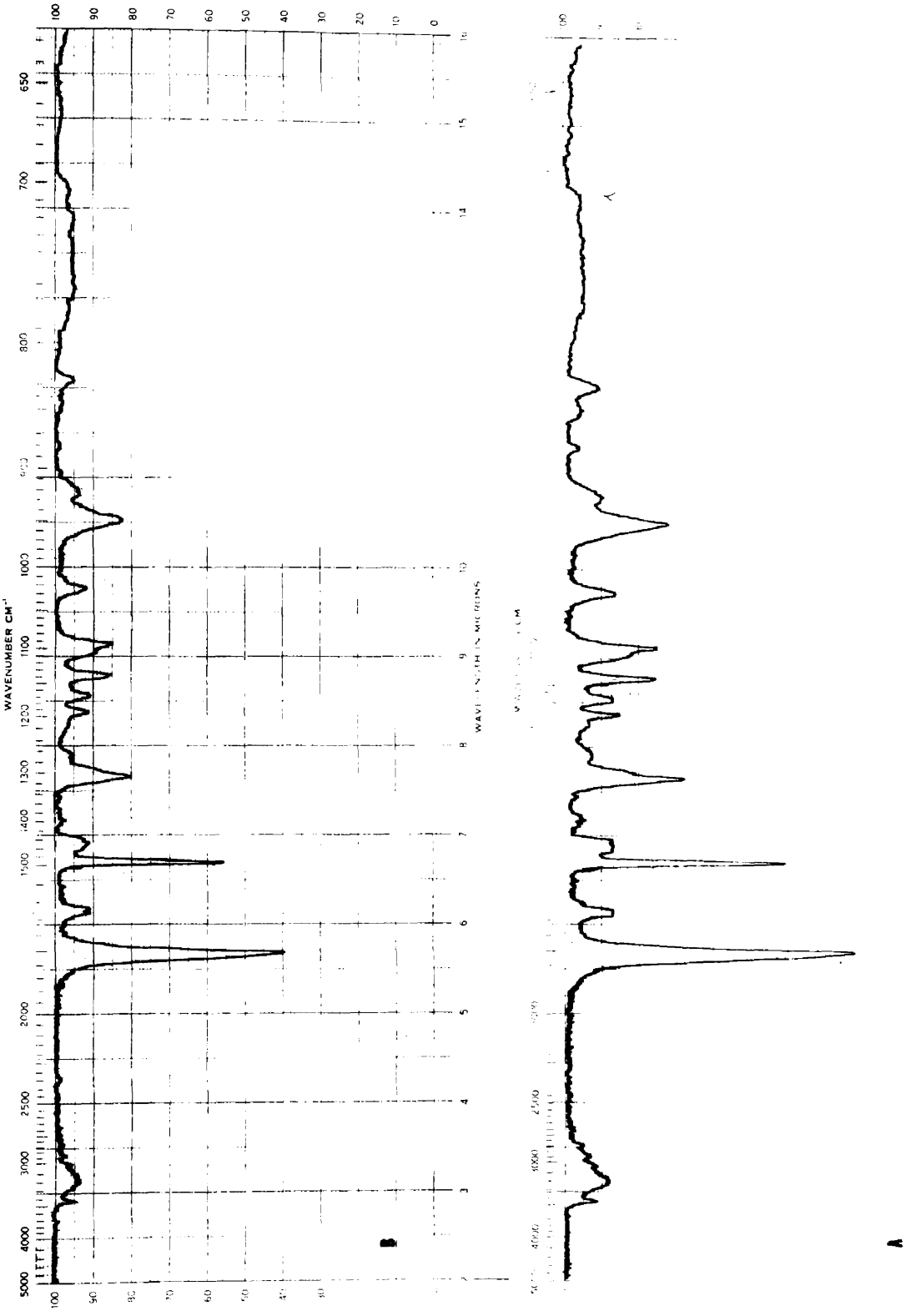
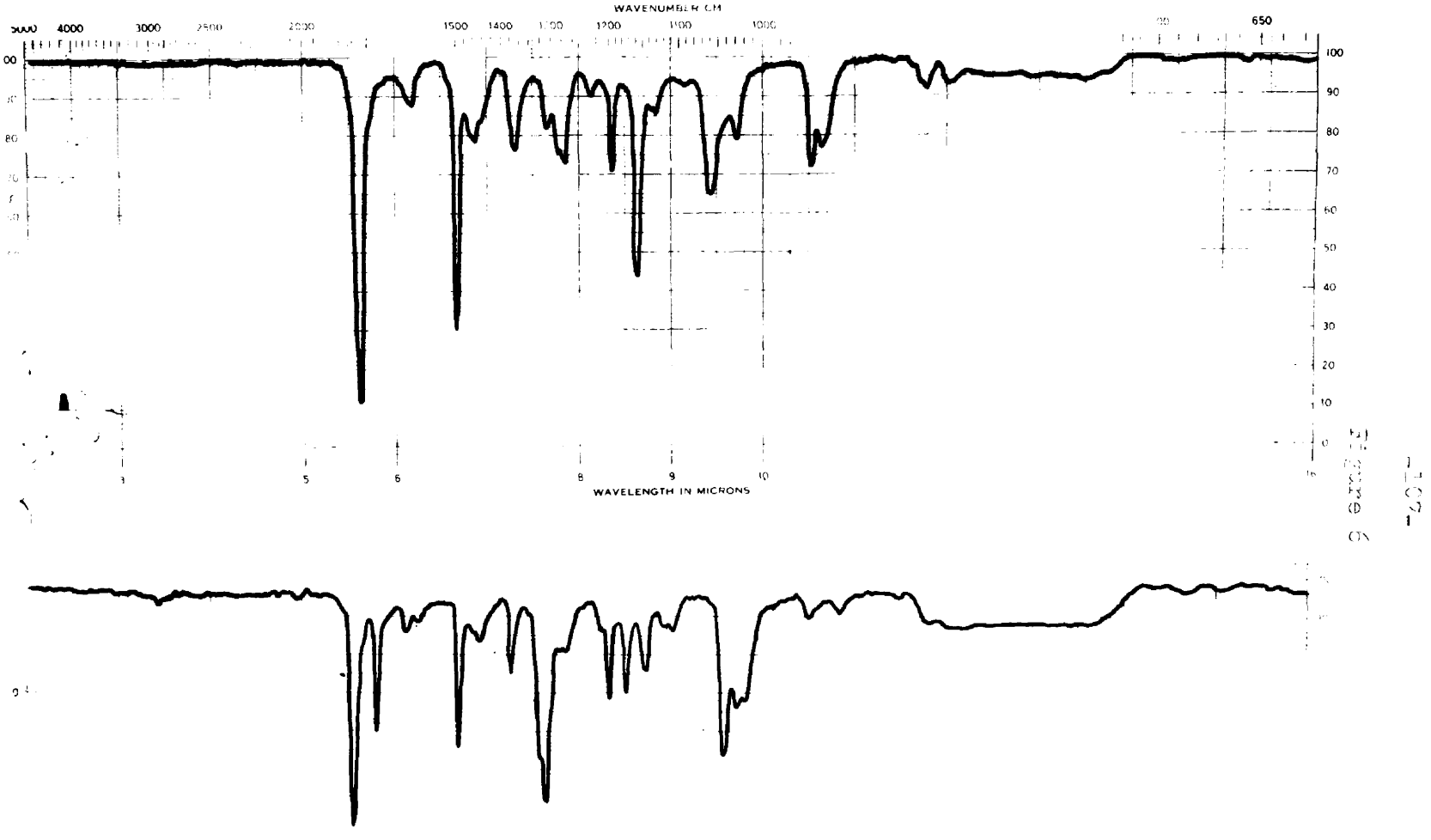


Figure 5

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WAVELENGTH IN MICRONS

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