ISOLATION AND PARTIAL CHARACTERIZATION OF BOUND FORMS OF INDOLE-3-ACETIC ACID IN ZEA MAYS

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Cesar Labarca

1965

THESIS

This is to certify that the

thesis entitled

ISOLATION AND PARTIAL CHARACTERIZATION OF BOUND FORMS OF INDOLE-3-ACETIC ACID IN ZEA MAYS

presented by

Cesar Labarca

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Plant Physiology

Robert S. Bandurski
Major professor

Date April 26, 1965

O-169

ABSTRACT

ISOLATION AND PARTIAL CHARACTERIZATION
OF BOUND FORMS OF
INDOLE-3-ACETIC ACID IN ZEA MAYS

by Cesar Labarca

Bound forms of indole-3-acetic acid (IAA) have been isolated from mature sweet corn kernels, and partially characterized. Aqueous acetone was used in the extraction procedure, following the method described by Berger and Avery (22). A water-insoluble and a water-soluble fraction, containing IAA in bound form, were obtained.

The water insoluble fraction was a zein-like compound, soluble in aqueous acetone and aqueous ethanol, with a high molecular weight, and similar in its properties to the fraction isolated by Berger and Avery. This fraction has 0.5 to 0.8 % IAA, which is released upon milk alkaline hydrolysis. Acid hydrolysis of this fraction yielded amino acids and, probably, reducing monosaccharides. This, and the content of nitrogen, of about 8 per cent, suggests that this compound could be a glycoprotein.

The water soluble fraction was further purified by chromatography on Sephadex G-25, and its components separated by paper chromatography. Four water soluble IAA complexes were obtained. Alkaline, acid, and enzymatic hydrolysis were used to study the composition of these complexes. In all of them IAA is conjugated with inositol, and is released by mild alkaline hydrolysis. The four complexes were numbered according to their decreasing mobility in paper chromatography in a n-butanol-water system. B_1 has 34.0 % IAA, and contains also inositol, and probably glucose; B_2 has 26.2 % IAA, inositol, and glucose; B_3 has 20.5 %

IAA, inositol, glucose, and arabinose; B_4 has 22.2 % IAA, inositol, glucose, and arabinose. The molar ratios of the components of each complex have not yet been determined.

No estimation has been made of the efficiency of the extraction procedure, and thus the present estimates of the total content of bound IAA may be low. There is a minimum of 45 mg of IAA in bound form per kilogram of mature sweet corn kernels. This amount is equal to about 10 micrograms of IAA in bound form per kernel, which is about 100,000 times more than the IAA which has been calculated to be present in one Avena coleoptile tip.

The conjugation of IAA with inositol, which is known to be a growth factor and to participate in cell wall metabolism is a new finding and could have physiological importance.

ISOLATION AND PARTIAL CHARACTERIZATION OF BOUND FORMS OF INDOLE-3-ACETIC ACID IN ZEA MAYS

by

Gabriel
Cesar^Labarca

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1965

ACKNOWLEDGMENTS

The author wishes to acknowledge his great indebtedness to Dr. Robert S. Bandurski for guidance and encouragement. I am also grateful for the help and critical reading of the thesis by the other members of my Guidance Committee, Drs. John E. Cantlon, Leo Mericle, and James Fairley.

I would also like to express my appreciation to Dr. Norman E. Good for his generous help and suggestions; to Drs. Alex Kivilaan and Lloyd G. Wilson for their help and encouragement.

I would also like to thank Dr. Peter Nicholls who first suggested inositol as a component of the indoleacetic acid complexes.

This work was done while the author was holding a fellowship from the International Atomic Energy Agency and the University of Chile to whom he wants to express his appreciation.

The support of the National Science Foundation, under research grant G-22069, is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

A. General considerations of bound forms of indoleacetic acid.

Although it is now evident that indoleacetyl compounds occupy an important place in the hormone physiology of plants, it is not yet known what their chemical form *in vivo* may be. It was first observed by Kögl et al. (79) that alkaline hydrolysis released auxin from seeds. Indoleacetic acid (IAA) has been isolated from corn meal and the endosperm of immature and mature corn kernels after alkaline hydrolysis (56, 22).

Recently the application of paper chromatographic techniques to the purification of plant extracts has made possible the detection of minute traces of active growth-regulating substances, present in too small an amount to be detected by the older classical chemical methods. By use of these techniques, IAA has been detected in a wide range of plant tissues. In some instances IAA was detected on the paper by both color reactions and biological activity, but many references report only biological activity in the zone where IAA was expected to occur. There are also available some reports (18) in which workers were unable to detect IAA in plant extracts, although it was carefully looked for, and often a great deal of material was used.

The evidence now suggests that although free IAA probably occurs in many plant storage tissues, its presence in growing plants can not be regarded as definitely and universally established. It has been evident for many years that auxins in plant tissues do not exist only as free molecules of IAA (18), and there is a growing body of evidence that it may be operative as a complex, that is, in a bound form.

Free IAA may be considered, operationally, as that which is readily and quickly extracted from the plant by methods which are designed to prevent its release from bound forms. These methods should involve rapid extraction with solvents that will inactivate enzymes.

Bound forms are regarded as consisting of a molecule of IAA linked in some way to another molecule. Only recently it has been realized how easily some of the bound forms or complexes yield free IAA, and thus how difficult any investigation of the physiological importance of the free and bound forms must be. This has led to a great deal of confusion in the interpretation of results. Some of the complexes appear to be so unstable that it is difficult to prevent the production of free IAA, for example by hydrolysis, during the processes of extraction and chromatography. Undoubtedly in most cases the free IAA reported in plant extracts is really an artifact due to its liberation from some bound form during the manipulations of extraction and purification.

It has been known for many years that even mildly alkaline conditions liberate IAA from some bound forms in plant tissues. More recently (163) it has been shown that solvents containing ammonia, which have been the most commonly used in chromatography of indoleacetyl compounds (133), lead to the breakdown of some of the bound forms of IAA and to the formation of free IAA and other artifacts. Unfortunately there are some workers who still rely, almost exclusively, on these methods in their extraction and purification procedures (72).

Various theories concerning the physiological significance of the bound forms of IAA have been proposed. Probably the most important problem is to know whether they are simply immobilized forms of IAA or are actively concerned in the growth process. Some of these bound forms

have been regarded as precursors of IAA (22), storage forms, detoxification products (2, 3, 165), and also as being without physiological importance (160).

From a consideration of both the chemical and physiological evidence on naturally occurring IAA complexes, Bentley (21) concludes that free IAA is not the auxin physiologically active in normal growth. It probably occurs free in many tissues, but only in very small amounts compared with non-ether soluble forms which are held to be the physiologically active forms. The evidence now is fairly strong that IAA and possibly also other indoleacetyl compounds are produced by breakdown of complex forms of some kind. Some of these naturally occurring complexes are present in relatively large amounts, especially in seeds, and only traces of some others have been found. Some of them have been detected after incubating plant tissues with rather large amounts of IAA. A few of these complexes have been identified and their structure is known, but there are still a large number of reports in the literature about compounds whose composition and physiological importance have not been established.

B. Ascorbigen and glucobrassicin.

The extraction of ascorbigen from Brassica was reported in 1939 by Pal and Guha (110) and Sen-Gupta and Guha (121). The compound liberates ascorbic acid upon acid hydrolysis, while on alkaline hydrolysis it yields IAA, indole-3-carboxylic acid, and other unidentified indoles (20). It is itself inactive in the Avena test. Bose and Guha (25) reported the presence of ascorbigen in eight different species of Brassica. Apparently it has been located only in the Cruciferae and is the only naturally occurring bound form of ascorbic acid so far isolated (20).

Bose and Guha (25) found that roots and stalks are usually poor in ascorbigen and ascorbic acid, while leaves and reproductive organs are especially rich. Kutacek et al. (84) studied the relationship between ascorbigen and growth in plants of the Cruciferae family and were able to demonstrate a very high ascorbigen content in the growing apex, and also in the young leaves, with a decrease with age in both tissues. This distribution is similar to that of native auxin in plant tissues. On chromatography in ammoniacal solvents, ascorbigen breaks down to a number of indole compounds, several of which have growth regulatory activity (20). These artifacts would explain the activity found in extracts of Brassica in chromatograms developed under ammoniacal conditions.

Kutacek (83) has recently reported that one of the principal indole compounds present in Brassica oleracea is glucobrassicin, and not ascorbigen as was reported before (84). Other indole derivatives, like ascorbigen and indoleacetonitrile are probably present, but in very small amount and he considers that most of them are degradation products of glucobrassicin. Gmelin (44) has also reported that glucobrassicin and neoglucobrassicin are the key substances for most of the hitherto described indole derivatives occurring in cabbage. The sensitivity of these compounds toward heat or hydrolytic conditions at acidic or alkaline pH, and their spontaneous cleavage by myrosinase, an enzyme present in the press juice of cabbage, makes it understandable that most of the earlier methods used for extracting and assaying this plant material, led to complex mixtures of artifacts. Enzymatic degradation at pH 7.0 produces glucose, sulfate, and probably indole-3-methylisothiocyanate, which decomposes immediately into thiocyanate and

3-hydroxymethylindole. The products of the enzymatic reaction at pH 4.0 are glucose, sulfate, sulfur, H_2S, and indole-3-acetonitrile. Degradation by chemical hydrolysis follows two pathways, the first yielding essentially the same products as the enzymatic degradation at pH 7.0, and the second leading to indole-3-acetonitrile as the first reaction product; indole-3-acetamide and indole-3-acetic acid appear gradually on prolonged hydrolysis. Depending upon whether conditions are alkaline or acidic, a number of byproducts are formed during hydrolysis, such as indole, skatole, indole-3-carboxylic acid, etc. The conditions of the process determine which of the degradation products mentioned above is formed preferentially in enzymatic or chemical degradation.

C. Indoleacetyl peptides and glycosides.

(1) Indoleacetylaspartic acid.

A study of the metabolism of IAA was first undertaken by Andreae and Good in 1955 (2, 5, 47, 48), and led to the isolation of indoleacetylaspartic acid. They showed that when a wide range of plant tissues were incubated with IAA, the presence of indole-3-acetylaspartic acid could be demonstrated. The presence of indoleacetylaspartic acid in tissues of IAA treated plants is well established now and its natural occurrence has been reported by Klämbt (77) in the extracts of tissues from a number of flowering plants, and by Row et al. (118) in tomato seedlings. Indoleacetylaspartic acid has been confirmed as the main metabolite of exogenous IAA in pea tissues by Zenk (164) and other authors. The evidence shows that the peptide linkage is between the carboxyl group of IAA and the amino group of aspartic acid.

Wightman (147) found indoleacetylaspartic acid in pea, tomato, and bean tissue, but not in wheat coleoptile, after incubation with IAA, indoleacetamide, and methylindole-3-acetate. Winter and Thimann (155) allowed radioactive IAA to be transported through pea stem sections and found that part of the activity remained in the sections after two hours as a bound form which was identified as indoleacetylaspartic acid. In similar experiments in Avena coleoptiles no bound forms were found. Fang et al. (34) found indoleacetylaspartic acid in pea shoots after treatment with radioactive IAA, but only a variety of compounds, unidentified after chromatography in ammoniacal solvents, were found in corn tissues. Bennet-Clark and Wheeler (17) found two major products of metabolism of applied IAA in potato tuber disks, and one of them was identified as indoleacetylaspartic acid. They suggest the formation of this compound in the tissue from bound IAA rather than free IAA. Thurman and Street (142) reported the formation of indoleacetylaspartic acid after incubation of excised tomato roots with IAA. The complex was metabolized when the roots were transferred to an IAA free medium.

Andreae, Good et al. (5, 48) have shown that indoleacetylaspartic acid predominates in the legumes and onion, but not in the grasses, where indoleacetamide, which later proved to be an artifact, was found. They found however, that indoleacetylaspartic acid is highly active in the oat coleoptile test, about equivalent to IAA, but is practically inactive in peas, where only 1/1000 of the activity of IAA was found when assayed in the slit pea curvature test or in pea stem elongation (2). These authors found that both indoleacetylaspartic acid formation and growth in pea epicotyls became maximal at the same concentration of applied IAA of about 0.6 x 10-4 M. With lower concentrations almost

all the IAA found in the tissues was present as indoleacetylaspartic acid, and only with the higher, growth inhibitory concentrations, did free IAA accumulate in the tissues to any extent. Following the metabolism of two micromoles of radioactive IAA in 20 ml of solution, Andreae et al. (4) found that 17 % of it was conjugated with aspartic acid after 24 hours. They also reported (6) that the tips of intact roots accumulated indoleacetylaspartic acid at a rate considerably greater than epicotyl sections, but excised roots almost entirely lost this ability unless supplied with sucrose and calcium.

(2) Other auxin peptides.

Incubation of pea epicotyls with radioactive naphthaleneacetic acid (NAA) for 24 hours, resulted in the conjugation of up to 95 % of the NAA taken up as naphthylacetylaspartic acid. The synthesis of the peptide proceeded via the free L-aspartic acid (164). The authors considered that the uptake of NAA in epicotyls represents an active process; the concentration of the acid in the tissue exceeding twice the concentration in the medium. The formation of naphthylacetylaspartic acid was found to be an adaptive process which could be induced by preincubation with NAA, IAA, 2,4-D, and benzoic acid. An adaptive (L)-aspartic-N-acylase had been postulated by Zenk (164).

Sŭdi (135) in a study of the induction of the formation of complexes between aspartic acid and IAA and NAA, reported an effect of pretreatment with IAA and six other compounds on the subsequent formation of indoleacetylaspartate and naphthylacetylaspartate. He found that IAA, NAA, 2,4-dichlorophenoxyacetic acid, 2,3,6-trichlorobenzoic acid, and S-carboxymethyl-N,N-dimethyldithiocarbamate, which are active as growth regulators, are all highly active as inducers. By contrast,

2,4-dichlorophenoxyisobutyric acid, and 3,5-dichlorophenoxyacetic acid, which possess little or no activity as growth regulators, were inactive.

The presence of malonyltryptophan has also been reported in plant tissues. It was first found by Good and Andreae (47) in spinach leaves incubated in a tryptophan solution, and later, as a naturally occurring compound in tomato, spinach, pea, and oats. The presence of D-tryptophan in this conjugated form has been reported (117,166, 77), and it has been shown to be present in the vegetative tissues and fruits of plants of 14 different genera (166). In fruits it is found that intoleacetylaspartic acid and malonyltryptophan are produced especially during maturation (165). This was first observed in apple fruits (117).

Thurman and Street (139) have reported the possible presence of indoleacetylglutamic acid in excised tomato roots exposed to IAA.

Andreae and Good (3) in a study concerning the metabolism of applied carboxylic acids, found that when pea epicotyl sections were incubated in solutions of indoleformic (indole-3-carboxylic) acid, indolepropionic acid, indolebutyric acid, benzoic acid, and 2,4-dichlorophenoxyacetic acid, all the corresponding aspartic conjugates, except indoleformyl-aspartic acid, were found in the tissues.

Zenk (164) has presented evidence suggesting that these metabolites are enzymatically formed from carboxyl activated auxin and L-aspartic acid. He also reported (162) on an ATP dependent formation of IAA-coenzyme A esters, catalyzed by octanoate-thiokinase, an enzyme from liver mitochondria. Indoleacetyl-AMP was an intermediate in the formation of indoleacetyl-CoA, and this was shown to be an intermediate product in the enzymatic formation of the indoleacetylglycine. The esterification of IAA with CoA, as a mechanism of action of the plant

hormone, has been previously postulated by Leopold (88).

Winter and Street (154) found a 5-hydroxytryptophan peptide, containing glutamic acid-arginine-alanine-valine-leucine, and 5-hydroxytryptophan, in "staled" tomato root culture medium. They isolated 2.2 mg of the peptide from 20 liters of culture medium after exhaustive extraction with ethylacetate. This peptide is highly active in promoting both the extension growth of coleoptile segments and the expansion of root cells. Street et al. (134) have reported the presence of indole compounds in extracts of seedling roots of Vicia faba and Phaseolus multiflorus which resemble the 5-hydroxytryptophan peptide in their R_f values in paper chromatograms, their movement in paper electrophoresis and their biological activity. They postulate that such compounds could be closer to the active growth regulating molecules than the free indole-containing compounds, and could arise directly from biologically active indole-containing proteins.

(3) Indoleacetylglucoside.

Zenk (163), in 1961, isolated and identified a conjugated form of IAA and glucose from leaves of Colchicum neapolitanum incubated in an IAA solution. The structure of the glucoside was 1-(indole-3-acetyl)-β-D-glucose. This conjugated form was also isolated by Klämbt (78) from wheat incubated with IAA and, together with the formation of indoleacetylaspartate, is probably a general mechanism of conjugation of exogenous IAA in plant tissues.

Zenk (165) has shown that indoleacetylglucose can be synthesized from IAA and uridinediphosphoglucose by a soluble enzyme system from lentiles similar to the one used by Jacobelli et al. (73).

The glucose-IAA bond, being a hemiacetal-carboxylic anhydride, is more reactive than an ester bond, and can donate the acyl group to ammonia, amines, hydroxylamine and alcohol (165). At pH 7.0 the reaction with ammonia and hydroxylamine leads to the formation of indoleacetamide and indoleacetylhydroxamate. Incubation of the glucoside with amino acids or ethanol at alkaline pH yields, in addition to IAA, the corresponding acetylated amino acids or the indoleacetic-ethyl ester. Reports in the literature of the occurrence of indoleacetamide are considered erroneous and probably due to artifacts of chromatography and isolation (165).

Andreae and Good (3) in a study of the metabolism of applied carboxylic acids, found that when pea epicotyl sections were incubated in solutions of indoleformic (indole-3-carboxylic) acid, indolebutyric acid, benzoic acid, or 2,4-dichlorophenoxyacetic acid, all the corresponding amides, except 2,4-dichlorophenoxyacetamide were present in the tissues. As was later shown by Zenk (163) the use of ammonia in the development of the chromatograms probably led to the formation of the amides from the glucose esters which almost certainly were the compounds actually present in the tissues.

A conjugation product of NAA, 1-(α-naphthylacetyl)-β-D-glucose, was isolated and identified by Zenk (164) from pea epicotyls incubated with radioactive NAA, and its structure was verified by synthesis. In a similar experiment Klämbt (78) found the glucose ester of benzoic acid in wheat.

In <u>in vitro</u> studies with polyphenol oxidase, Leopold (58) separated three pigments which seem to be addition products of IAA and quinones of catechol, chlorogenic acid, and caffeic acid.

(4) Indoleacetylarabinoside.

Analysis of several Ehrlich positive spots on paper chromatograms of corn fractions with growth promoting activities, led to the isolation by Steward et al. (123, 131) of a non-crystalline but chromatographically homogeneous substance. Its probably structure, based upon reactivity, hydrolysis products, and other properties, is 1-(L-arabinosyl)-indole-3-acetate. This compound was found to promote a somewhat greater degree of cell division in carrot tissue than did IAA, whereas in the promotion of elongation in the Avena mesocotyl it has only about 1 % of the activity of IAA.

(5) Physiological significance,

There has been some question as to whether the compounds formed after feeding IAA, or other synthetic auxins to plant tissues actually represent naturally occurring compounds. As Bentley (21) points out, it is not clear whether the application of large amounts of auxin to a tissue leads to reactions that would not normally occur with the much lower levels of endogenous auxins. In this respect, Müller (101) distinguishes between two groups of compounds: a) Derivatives which appear in the tissues after incubation in solutions of IAA and other indole compounds, and are broken down again by true enzymatic degradation. These can be considered as native in the tissues. b) Compounds which appear, probably because of non-physiological conditions, are not broken down again and accumulate in the tissues. In this second group he classifies the indoleacetylaspartic acid, the indoleacetamide, and other unidentified substances.

Andreae, Good, et al. (2, 3), and Zenk et al. (165) agree in

considering that the conjugated forms of IAA with glucose and aspartic acid found in their experiments are the products of detoxification processes which dispose of an excess of IAA. In this respect the mechanisms could be compared with the formation of indoleacetyl-L-glutamine and indoleacetylglucosiduronic acid, which appear to be detoxification products of IAA in humans (74, 75, 127). A large amount of internal indoleacetylaspartic acid apparently does not interfere with the elongation of roots (1).

Zenk (165) has studied the distribution of the principal conjugation systems in about 170 species by applying rather high IAA dosages. The two principal pathways found were conjugation with aspartic acid and with glucose. Conjugation with glucose seems to be the more primitive way, being present in Bacteria, Fungi, Bryophyta, and Sphenopsida. Lycopsida, and Pteropsida, including Filicinae, Gymnospermae and Angiospermae, still have the ability to conjugate IAA with glucose, but they also have a second system, the conjugation of IAA with aspartic acid. Among the Angiospermae, the Liliales seem to favor most the pathway of glucose conjugation. Most species of plants made use of both types of conjugation, but since there exist species which apparently form only indoleacetylaspartic acid or only indoleacetylglucose, it is unlikely that either conjugate is an essential part of the growth induction mechanism. Zenk therefore assumes that both conjugates are true detoxification products.

There is only qualitative data to date about malonyltryptophan, but since it has been identified as the detoxification product of D-tryptophan (166), Zenk has suggested the following hypothesis (165): At the point where growth stops in a fruit and IAA is no longer consumed,

it is converted to indoleacetylaspartic acid or indoleacetylglucose. Further, in order to block IAA production, some indole precursor of IAA is transformed to D-tryptophan, which is immobilized and detoxified as malonyl-D-tryptophan. This would lead to the accumulation of the malonyl derivative in mature fruits. Thus, according to Zenk, both the production and the action of IAA may be stopped in the fully developed organ.

Based on the information available about the induction of formation of indoleacetylaspartate after the application of IAA or other growth regulators to plant tissues (165), Zenk concludes that the formation of indoleacetylglucose in plants seems to be a mechanism for very rapid detoxification of aromatic carboxylic acids during a time when the conjugation system utilizing aspartic acid is being adapted in the plant. The glucose ester of IAA is not stable in the cell after the aspartic acid conjugating system has been adapted, but becomes eventually transformed into indoleacetylaspartic acid.

D. Macromolecular complexes.

(1) Protein conjugates.

It has been suggested in the literature that auxins exert their effect on cell elongation by combining with a protein, thus yielding an active enzyme complex (60, 148). Presumably the level of this enzyme then controls some aspect of growth. Consequently there has been a great deal of interest in IAA-protein complexes.

The existence of auxin-protein complexes was first reported by Wildman et al. (153). On the basis of extensive investigations on spinach leaf proteins, they arrived at the conclusion that IAA combines with one of the major leaf proteins, a fraction which has phosphatase activity. The IAA- protein presumably played a role in the transfer of

energy required for growth. IAA was not dissociable from the protein by repeated fractionation with ammonium sulfate and remained in the molecule after removal of the phosphorus containing substances (150). Yields of auxin on treatment with alkali or proteolytic enzymes (153, 53) approach the ratio of one mole of IAA per mole of protein of 200,000 molecular weight. Schoken (120) obtained optimum yields from this fraction by refluxing at 100°C in 0.1 N NaOH.

Gordon (49) found auxin associated with each of the five major proteins isolated from the wheat grain. Two types were obtained, acid labile-alkali stable, and acid stable-alkali labile. Only the first of them, or IAA type, was obtained upon alkaline hydrolysis. Avery et al. (11) obtained auxin yields of the same magnitude from wheat with high and low protein content. They found the highest yields at pH 9.3 to 10.5, and marked destruction occurred by autoclaving with 1 N NaOH, in contrast with the alkali stable auxin from corn endosperm. They postulate the presence of two precursors which are converted into two auxins upon alkaline hydrolysis and differ markedly in their stability toward alkali.

In 1944 Berger and Avery (22, 23) reported that 95 % of the total auxin activity in sweet corn kernels was attributable to a bound form of IAA, extractable with aqueous acetone, and which, in its purest form, had an IAA content of 3.7 %. This IAA-complex was insoluble in water and was readily hydrolyzed by mild alkaline treatment to yield IAA. It did not release IAA on treatment with proteolytic enzymes. The solubility properties, nondializability, and other characteristics of the purified fraction suggested a macromolecule, probably a protein, but the nitrogen content was low, about 4.7 %.

Attempts to obtain the conjugation of IAA with protein have been made *in vivo* and *in vitro* by applying rather high concentrations of IAA. Siegel and Galston (124) have claimed the *in vivo* binding of IAA in pea root apices, and an *in vitro* binding in the presence of ATP to a protein fraction of pea brei. The protein complex formed was stable to boiling, acid and alkali, and was not destroyed by IAA oxidase. The compound was rapidly formed during the initial hours of incubation and precipitated by trichloroacetic acid. The evidence for this protein conjugate however, has been questioned by Andreae et al. (7, 1), who found all the colorimetrically determinable IAA metabolites in IAA treated pea homogenates and tissues to be small molecules soluble in 70 % ethanol, and could find no evidence of a metabolically produced IAA-protein complex. They believe that the IAA-protein complex reported by Siegel and Galston is not a product of plant metabolism, but an artifact of the trichloroacetic acid precipitation procedure.

Zenk (165) has also reported the formation of an IAA-protein complex *in vivo* by applying carboxyl-C^{14}-labeled IAA to pea epicotyls. This IAA was not removed from the protein complex by Sephadex filtration, but a quantitative recovery of the radioactivity was obtained upon alkaline hydrolysis.

Skoog and Thimann (126, 141) observed that protein hydrolyzing enzymes were capable of producing auxins from various protein materials. The yield of auxin from *Lemna* was increased greatly by short incubation with chymotrypsin or trypsin. Crystalline trypsin brought about only a small yield, while chymotrypsin was highly effective. No auxin was liberated from *Lemna* either by extraction with water or alkaline hydrolysis in an autoclave. They concluded that auxin in *Lemna* is bound to

protein or to a protein-like substance. Wildman and Bonner (144) working with spinach proteins found that trypsin gave almost as high auxin yields as alkaline hydrolysis when the trypsin treatment was preceded by a treatment with pepsin. Muir (100) reported production of auxin by using pancreatin.

(2) Protein-tryptophan as a possible source of IAA.

The possibility that the auxin obtained upon alkaline treatment of proteins arises from tryptophan has been considered. In theory any protein containing tryptophan could produce IAA upon hydrolysis and oxidation. However there is little evidence correlating IAA release with tryptophan content. Kulescha (81) found that gelatin, agar, and gum arabic, compounds which do not contain tryptophan, yielded considerable quantities of auxin upon treatment with trypsin. Alkali treatment of tryptophan yields at the most very small amounts of IAA (9, 120, 30). Although traces of IAA may be produced from tryptophan in a number of proteins (9, 120), it has been calculated that the tryptophan-IAA conversion is less than 0.01 % (9). Treatments with various enzyme preparations have yielded equally small amounts (52, 149, 152). It is extremely unlikely that the mild conditions used in most of the alkaline hydrolysis of the auxin complexes were sufficient to break the peptide bonds in the protein molecule (23), yet under those conditions highly active indole auxins are obtained from indole-containing complexes.

Although protein materials may conceivably serve as an IAA source, auxin production has not been generally associated with protein breakdown in the plant. In fact, auxin production in growing tissue may more logically be associated with protein synthesis.

Some bacteria very readily convert tryptophan to IAA (132). Acetobacter xylinum cells incubated with DL-tryptophan produced IAA, and the yield was of the order of 5 % (87). IAA was also detected when tryptamine or tryptophan was added to the medium where the fungus Diplodia natalensis was growing (15). In the higher plants only a minute amount of active auxin appears to be produced from tryptophan or tryptamine, according to Stowe (132), much less than the amount found in plant extracts (160). Wildman et al. (152) infiltrated tryptophan into living spinach leaves, and found an increase in the ether extractable auxin after 3.5 hours. They also reported rapid conversion of tryptophan to auxin by a lyophilized, cell free extract of spinach leaves, and demonstrated the reaction to be enzymatic. In general though, work on the in vitro conversion of tryptophan to auxin has shown a very low yield (20, 103, 156). Gordon and Paleg (51) found that phenols, under conditions leading to their oxidation, react with tryptophan to form IAA. The reaction is catalized by the polyphenolase enzymes of mung bean, oat, and sunflower, and occurs spontaneously at an alkaline pH. They considered the reaction as possibly more important in wounded plant tissue, where lysis and activation of the phenolase system takes place, than in normal metabolism.

(3) RNA conjugates.

Meudt and Galston (96, 97) have reported the formation of a complex between RNA and IAA in shoots and roots of etiolated pea seedlings. Incubation of IAA with peroxidase yielded an IAA derivative of an unknown structure, which forms a complex with pruified RNA from pea shoots at pH values of 4.8 or below. Apparently the complex was stable at higher pH, and the IAA derivative was firmly bound to the RNA, when the

RNA was precipitated by trichloroacetic acid or by other means. The complex was not formed when IAA itself was added to RNA, or when RNA was first treated with RNAase. They also found an RNA fraction resembling the *in vitro* complex after dextran gel filtration of etiolated pea epicotyl homogenates previously exposed to substrate levels of IAA. They interpreted this as evidence of the *in vivo* occurrence of the process.

E. Other unidentified naturally occurring bound forms of IAA.

(1) In Avena coleoptiles.

Much interesting work has been carried out on the state of the auxin in *Avena* coleoptiles. There is evidence that the major part of the auxin probably exists in the form of a complex. Since the *Avena* coleoptile is so widely used as a bioassay for auxins, it is obviously important to know the nature of the native auxins of the coleoptile.

A group of German workers (42, 98) found that the auxin in *Avena* is released fron an antiauxin complex with inhibitory properties. This inhibitor-auxin complex was demonstrated to be present in seedlings of *Zea mays*, *Avena*, and tubers of *Solanum tuberosum*.

Terpstra (138, 139) has carried out chromatographic examinations of the auxins extractable from coleoptiles. She suggests that the free form in the intact tissues may be present in neglibile amounts and that its exact quantity would be of little physiological interest. She demonstrated that the auxin in extracts of ground coleoptile tips is present as a complex which is water soluble but ether insoluble. The complex is unstable and yields IAA very easily.

Raadts and Sõdung (116) carried out an extensive chromatographic

study of auxin obtained from coleoptile tips by diffusion. They found IAA and an inactive, labile substance that appears to be an addition compound of IAA with something else. It very easily breaks down, and they suggest that the IAA obtained in their extracts is actually an artifact arising from breakdown of this compound.

Goldsmith and Thimann (46) found considerable retention of radioactivity in Avena coleoptile sections after removal of the source of radioactive IAA. They think that the immobilized IAA is probably very tightly bound.

The intracellular localization of native auxin in the Avena coleoptile has also been studied by Yamaki and Nakamura (159, 103). Both free and bound auxin were localized in the cytoplasm and identified as indole compounds. There was about twice as much bound auxin as free auxin.

(2) Water soluble forms of IAA complexes.

Numerous water soluble, but relatively ether insoluble, auxin complexes, which yield IAA by treatment with acid or alkali, have been found. (IAA is very ether soluble and partitions almost quantitatively from water into ether.) These substances often appear in large quantities relative to the amount of free IAA in the tissue.

Avery et al. (10) reported that about 90 % of the total auxin content of dormant maize endosperm exists as a physiologically inactive compound, readily soluble in water but not in ether, and easily converted to auxin at alkaline pH. A water soluble precursor which gives IAA by ether extraction after mild heat treatment has been reported in a number of plants, especially maize seeds by von Denffer (32). This seems to be comparable with the IAA precursor reported by Housley and Bentley (71). Britton and Housley (27) found a bound form of IAA in the aqueous, ether

insoluble extract of immature corn kernels which possessed marked growth promoting activity. This compound was hydrolyzed by acid and alkali and by chromatography in ammoniacal solvents to give IAA.

Farrar et al. (35, 36) found only part of the auxin activity in the ether extract of immature corn kernels. The juice expressed from the ether extracted kernels was still highly active biologically, and most of the activity remained in the aqueous phase after exhaustive ether extraction under acidic conditions. A number of substances were found which gave a purple color with Salkowski reagent quite distinct from the red given by IAA. These substances decompose on paper chromatography in solutions containing ammonia, and one of the breakdown products is IAA. Farrar called them zeanins, and described them as "plant growth substances occurring in Zea mays giving a purple reaction with the Salkowski reagent, and breaking down on chromatography in ammoniacal solutions to give IAA". All the substances are more polar than IAA and run behind it in butanol-water chromatography. Farrar considers these substances different from native bound auxin and suggests the possibility that they are intermediates between free and native bound IAA.

Thimann and Skoog (140) found that water was essential for rapid and complete extraction of auxin in all the materials studied. From this they concluded that water was necessary for the hydrolytic liberation of auxin from its bound forms in the tissues. In Lemna they could obtain repeatable results by successive extractions with ether, but the process required several months to reach completion. Similar results have been obtained by other workers (109, 141). Avery et al. (14) in a comparative study of methods for single solvent ex-

traction of growth hormone from plant tissues, found very wide differences in yield depending on the solvent used and the extent of hydration of the tissue. Water gave the highest yields, and water and alcohol gave the most reproducible results. They proposed successive extraction with different solvents for assaying total hormone content.

Deverall and Daly (33) found that normal mature wheat leaves converted approximately 60 % of exogenous carboxyl-labeled IAA in six hours into four radioactive water soluble compounds which seem to be bound forms of IAA and were different from each other.

Britton et al. (28) in a paper chromatographic study of extracts of tomato roots, found three water soluble, ether insoluble compounds. Two of them, called X and Y, act as precursors or complexes, yielding the third, Z, and yet they are themselves biologically active and mutually interconvertible. They also found two interconvertible spots on chromatograms of water soluble extracts of immature corn kernels (27). A similar behavior has been noted with auxin obtained from algal extracts by Bentley (19), although in these extracts only components X and Z were present in quantity. Audus and Gunning (8) have also reported four interconvertible auxins in pea roots.

(3) Other bound forms of IAA.

Tandler (136, 137) has reported the presence in the giant unicellular alga Acetabularia, of intracellular crystals containing the indole ring. Alkaline hydrolysis of ethanol extracted algae liberated substantial amounts of indole compounds. Insolubility in acid media, in concentrated ammonia, in organic solvents, and in strong alkali, after formaldehyde treatment, probably indicates a high molecular weight compound. The crystalline derivative has also been found in

several other genera of the Dasycladaceae. It is localized mainly in the central vacuole.

Muir (100) has reported that large amounts of plant growth hormones are present in bound form in pollen grains of several species. It can be converted to an active form by alkaline hydrolysis. In dried ovary tissue of Nicotiana and Antirrhinus, hormones are present only in an inactive form which can be activated by incubation at pH 8.5 and in the case of Nicotiana, by a water extract of Nicotiana pollen in acid medium in vitro.

Srivastava (128, 129) has reported the extraction of auxin from corn kernals in the early milky stage with cold ether, or ether at 25°C followed by cold ethanol. On paper chromatographic separation, he obtained three indole compounds by ether extraction of neutral aqueous solutions of the corn extracts, six more by ether extraction of the acid solution, and another six indole compounds remained in the aqueous phase. The water soluble, ether insoluble compounds, gave IAA on autoclaving under alkaline conditions, and he suggested the presence of arabinose in one of them. Based only on biological activity he estimated the total amount of IAA in this plant material as more than 100 mg of IAA per kilogram of fresh weight. Apparently no further attempt was made to characterize these fractions, in spite of the very large amount of them available.

Vlitos et al. (144, 145, 146) could only detect IAA in barley and tobacco tissue after alkaline hydrolysis, which indicates the existence of an IAA complex. Köves and Sirokman (80) found two Ehrlich-positive radioactive compounds with the R_f of tryptophan and IAA in chromatograms of extracts of pea seedlings grown for 24 hours with radioactive phosphate.

Hamilton (58, 59) found IAA in 80 % ethanol extracts of corn kernels, as well as in sugar beet roots and leaves. He suggests that IAA may occur as a labile complex in the ethanol extracts of those tissues. An 80 % ethanol-soluble, water insoluble fraction from corn kernels was found to release IAA upon alkaline hydrolysis. It appears this fraction may be proteinaceous in nature, and probably similar to the complex reported by Berger and Avery (22).

Hamilton (59), investigating the presence of free IAA in growing tissues, extracted 11 kg of 5 day old etiolated corn shoots using the acid ether extraction procedure, and did not find any traces of IAA, unless the material was treated with alkali. In his experiments he added C^{14}-labeled IAA to permit recovery calculation.

In the studies of Kuraishi and Muir (82), the diffusible auxins of several plant species were shown to be different from free IAA, but had the same R_f as IAA on paper chromatograms developed with solvents containing ammonia; probably some labile complexes were broken down under these conditions.

Scott and Jacobs (121) reported in a very recent paper that the only compound which diffused into agar blocks from the apical shoot tips of Coleus Blumei was IAA. No bound forms or any other compounds with growth regulating activity were found. However, they extracted the agar cubes with ether, and furthermore, they used ammoniacal solvents for chromatography. Using such extraction procedures it would be possible to detect only those bound forms soluble in ether and resistant to breakdown by ammonia.

F. IAA complexes in corn.

Cereal seeds are a rich source of IAA in bound form. Hatcher (62,

63, 64) found that among the species of seed tested, corn had the highest auxin content, followed by oat, rye, wheat, and barley. Probably the best known naturally occurring auxin complex is that present in corn kernels. The nature of this complex was extensively studied by Berger and Avery (23), who reported a total concentration of IAA equal to about 140 mg per kilogram of sweet corn kernels. Of this they were able to isolate about 14 mg of IAA per kilogram.

There are marked fluctuations in the content of extractable auxin during the development of the corn kernel, and this has been studied by several authors (13, 50, 62, 63, 64, 91). Preceding pollination, the content of readily extractable auxin of the ovary is low, but starts rising immediately after fertilization, rising steadily as the embryo develops, to reach a maximum in from one to three weeks. Then the content of extractable auxin falls as the seeds ripen. Other authors (66) using different methods of extraction, reported complete disappearance of the auxin in fully ripened kernels. A similar pattern of change in auxin levels has been noted for many species, both monocotyledons and dicotyledons (12, 62, 63, 64, 91, 92, 104, 105). This marked decrease in maturing seeds has been ascribed to the conversion of free IAA, or an easily extractable water soluble form, to a more tightly bound form (130) subsequently available to the embryo upon germination (55).

Avery et al. (11) found that the bulk of the auxin was present as a "precursor" in corn kernels. Yamaki and Nakamura (160) found only negligible amounts of bound IAA in the embryo, but an abundance in the endosperm of corn. Hatcher (63) reports an increase in the amount of auxin as the grain ripens, and its location in the endosperm and aleurone layer in rye. Polevoi (113) has shown a bound to free auxin

ratio of 120:1 in dry corn kernels, and a similar ratio is reported by Zinovev (167).

During germination there seems to be a decrease in bound auxin in the endosperm and an increase of water soluble auxin in the corn embryo and seedling (14, 55, 65, 113, 167). Apparently the same phenomenon occurs in barley and rice (90, 125).

The fluctuations of the auxin content in corn kernels suggest that IAA is present in early stages of the development of the seed in a water-soluble bound form, easily extracted and hydrolyzed. Probably this is gradually converted to the high molecular weight, water-insoluble form, which is present in the mature kernel. Upon germination of the seed, the high molecular weight bound form may gradually liberate, as the endosperm tissue is used up, a water soluble form which is then found in the embryo and the seedling.

The fraction extracted with aqueous acetone by Berger and Avery (22), as well as the one extracted with aqueous ethanol by Hamilton (58), had many properties similar to zein, the water insoluble protein of corn kernels. Undoubtedly the fraction of both Berger and Avery, and of Hamilton, contained at least a high proportion of zein, since their methods of extraction were similar to those sometimes followed in the deliberate extraction of zein (95).

Zein constitutes about one third of the total protein of the mature corn kernels (40, 99) and is localized almost exclusively in the endosperm (156, 157, 158) where it accounts for about 40 to 60 % of the total nitrogen. There is no agreement about the molecular weight of this protein (99) but it seems to average about 35 to 40,000. Apparently there are at least three components in zein with different molecular

weights (45). There is no tryptophan in zein, but the contents of glutamic acid, leucine, proline and alanine are very high. According to Mosse (99) it could be assigned the approximate general formula of $Glu_2Leu_2Prol_1Ala_1X_4$, where X indicates other aminoacid residues. The elevated number of its non-polar residues and the exceptional scarcity of its ionic groups probably accounts for several of its peculiar properties: insolubility in water; weak hydration; solubility in a wide range of organic solvents; and resistance to denaturation, even at temperatures of 100 to 180°C.

The globulins and glutelins of the corn kernel appear to be synthesized at a relatively uniform rate throughout the growth period (161), but zein is nearly absent in the very immature corn kernel and is synthesized at a very rapid rate as the corn approaches maturity. The rapid increase in zein nitrogen to total nitrogen ratio, from about 3 to 40 %, is almost exactly paralleled by the decrease in water-soluble non-protein nitrogen. Sweet corn is apparently similar to field corn in zein content.

There seems to be a parallel between the synthesis of zein in corn kernels at the expense of the non-protein water-soluble nitrogen in the late stages of the development of the grain, and the formation of a water-insoluble bound form of IAA, with the simultaneous disappearance of a water soluble form.

The fraction isolated by Berger and Avery (23) was believed to be a protein but its 4.7 % nitrogen content is very low for a protein. From the studies of Zenk (163) and some less adequate reports (123, 128, 131) we know of the conjugation of IAA with sugars, and therefore the possibility exists that Berger and Avery were actually dealing with

a glycoprotein.

Recently Hochstrasser (67, 68, 69) in studying the glucoproteins of barley endosperm, has been able to isolate peptidyl-polysaccharides upon hydrolysis of barley albumin. These peptidyl-polysaccharides were found to contain aspartic acid as the principal aminoacid, and xylose, arabinose and glucose. He concludes that these compounds are probably involved in the bridging link between protein and carbohydrate. The investigation of similar glucoproteins in the endosperm of corn and other cereals and its relationship with IAA apparently has not been undertaken yet and it could prove to be extremely interesting.

Although there is now a good deal of information about the presence of bound forms of IAA in bound tissues, very little is known about the chemical structure of the numerous IAA complexes reported or, in many cases, only suggested. It may be that some, or even most, of the substances found differ in their properties as a result of the isolation procedures employed. Thus the possibility exists that many of these complexes are identical. As long as the chemical identity of these compounds remains unknown, the investigation of their physiological importance is difficult, and even the isolation procedures will remain empirical.

Mature corn kernels are one of the richest sources of IAA among plant materials and the prupose of the work described in the next section has been to characterize the bound forms of IAA present in this tissue. The fraction isolated by Berger and Avery (22) from sweet corn was selected as the starting material, since it is present in large amounts and it is readily extracted by a rather simple procedure.

EXPERIMENTAL

A. Materials and analytical methods.

Mature kernels of a white sweet corn, Stowell's Evergreen Hybrid, were supplied by the Lansing Farm Bureau.

A modified Salkowski reagent was employed for the colorimetric determination of IAA. The Salkowski reagent contained 15 ml of 0.5 M $FeCl_3$, 300 ml of concentrated H_2SO_4, and 500 ml of water. Three milliliters of this reagent and two milliliters of an aqueous IAA solution were mixed, and color intensity measured after 30 minutes using a #54 filter in a Klett-Summerson colorimeter, or in a Beckman spectrophotometer at 530 millimicrons.

The solvent used for chromatography of the IAA complexes was n-butanol:acetic acid:water, 5:1:2.2 (163). This solvent, and ethyl acetate:pyridine:water, 8:2:1, were used in the chromatography of sugars and oligosaccharides.

The detection of IAA and IAA complexes on paper chromatograms was made by using an Ehrlich dipping reagent, prepared by dissolving 2 g of paradimethylaminobenzaldehyde in 100 ml of concentrated HCl:acetone, 10:90. Sugars and oligosaccharides were detected on paper chromatograms with silver nitrate (143), aniline phthalate and p-anisidine (70, 111).

Glucose was determined by the glucose oxidase method (119), using a "Glucostat" reagent from Worthington Biochemical Corp.

The beta-glucosidase was from Sigma Chemical Corp., and Nutritional Biochemical Corp. The Snail Gut Juice (Suc d'Helix Pomatia) was from Industrie Biologique Francaise, S.A., Gennevilliers, France.

Throughout this work purity of compounds is calculated on the basis of the content of IAA obtained after alkaline hydrolysis.

B. Extraction procedure.

The extraction procedure described by Berger and Avery (22) was used to obtain the crude fractions.

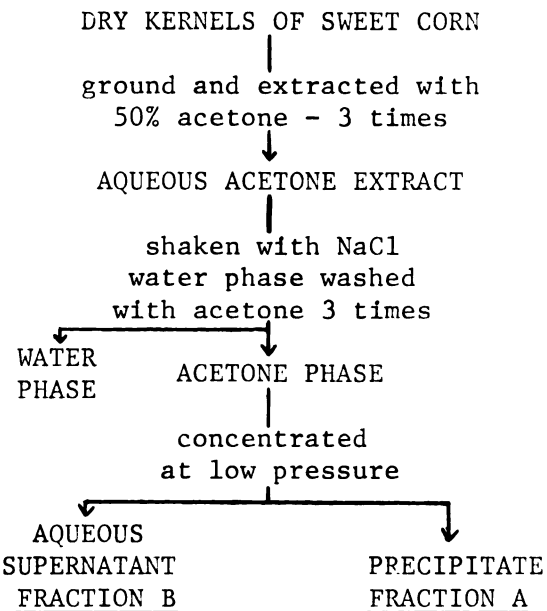

Figure 1. Schematic outline of the extraction procedure of IAA complexes from Zea mays.

The steps followed in the isolation are summarized in Figure 1, and were as follows: Corn kernels were ground and extracted with 50 % aqueous acetone, v/v, for 48 hours, using two liters of solvents per kilogram of corn, with occasional stirring. The suspension was filtered with suction to give a clear yellow extract, and the residue reextracted as above one or two more times. The combined acetone extracts were saturated with sodium chloride and shaken vigorously. Following separation of the two solvent layers, the upper (acetone phase) was saved and the water phase reextracted three times with a volume of fresh acetone equal to the volume of acetone removed. The combined acetone fractions were concentrated in vacuo in a flash evaporator to yield an aqueous supernatant solution and a zein-like precipitate. The precipitate is

called fraction A, and the aqueous supernatant fraction B.

C. Purification of fraction A.

The orange, gummy precipitate obtained upon concentration of the acetone fraction, was redissolved in 50 % acetone, and reprecipitated by pouring the solution into about 40 times its volume of cold 2 % NaCl solution. The resultant precipitate was collected on a thick filter cloth, the water squeezed out, and the precipitate dried in a desiccator over P_2O_5. The dry sample was ground in a mortar to a fine yellow powder.

Fraction A is insoluble in water, ether, acetone, and alcohol, but is soluble in aqueous acetone and aqueous alcohol. When this fraction was suspended in water, KOH added to pH 11.5 to 12.0, a clear yellow solution was formed, with simultaneous release of free IAA. After adjusting the pH to 3.0 with H_3PO_4 and separating the precipitate by centrifugation, the supernatant was extracted three times with peroxide-free ether. The ether extracts were reduced to dryness, and the IAA taken up in a small volume of water for colorimetric analysis using Salkowski reagent.

The amount of fraction A obtained was 3.8 g per kilogram of corn kernels in the first batch extracted, and 2.3 g per kilogram in the second. The first batch was extracted three times with aqueous acetone, and the second batch only two times. This could account in part for the difference, furthermore, the corn used in the second extraction was from a different batch, probably harvested a year later. The IAA content of fraction A from the first batch was 0.8 %, and from the second 0.53 %.

The purification of fraction A was difficult, due to its peculiar properties, especially its insolubility in water, which excluded the

application of mahy of the generally used fractionation methods. When fraction A was chromatographed on a column of Sephadex G-25 with Tris buffer pH 8.0, 0.002 M in 50 % acetone, a single peak was obtained, which suggested a large molecule, probably with a molecular weight higher than 3,000. The IAA content of fraction A after treatment with Sephadex was approximately the same.

Fifty milligrams of fraction A was hydrolyzed successively with increasing concentrations of sulfuric acid. The first hydrolysis was in 1 N H_2SO_4 for 6 hours in a boiling water bath. The sample did not dissolve completely and the clear solution was separated and neutralized with $BaCO_3$, the precipitate separated by centrifugation and the supernatant solution lyophilized. The process was repeated with 4 N H_2SO_4 being used in the second hydrolysis, and 6 N H_2SO_4 in the third hydrolysis. A brown precipitate still remained after the third hydrolysis. The first hydrolysis yielded 7.6 mg of a slightly yellow powder, the second hydrolysis 11.1 mg of an almost white powder, and the third hydrolysis 4.2 mg of a white powder. Samples of 0.5 mg of each hydrolyzate was used for a paper chromatographic analysis. The samples did not dissolve completely in 0.1 ml of water and after stirring for a few minutes, the undissolved material was discarded and the clear supernatant used. Whatman #1 chromatographic paper, and n-butanol:acetic acid:water, 5:1:2.2 as the developing solvent were used. One chromatogram was treated with silver nitrate and the other with ninhydrin. The result, shown in Figure 2, was as follows:

$\underline{AgNO_3}$: There are two faint spots on the chromatogram of the first hydrolyzate, with R_f 0.11 and 0.21; the second hydrolyzate shows the same spots, but fainter, and there are only traces of them in the third

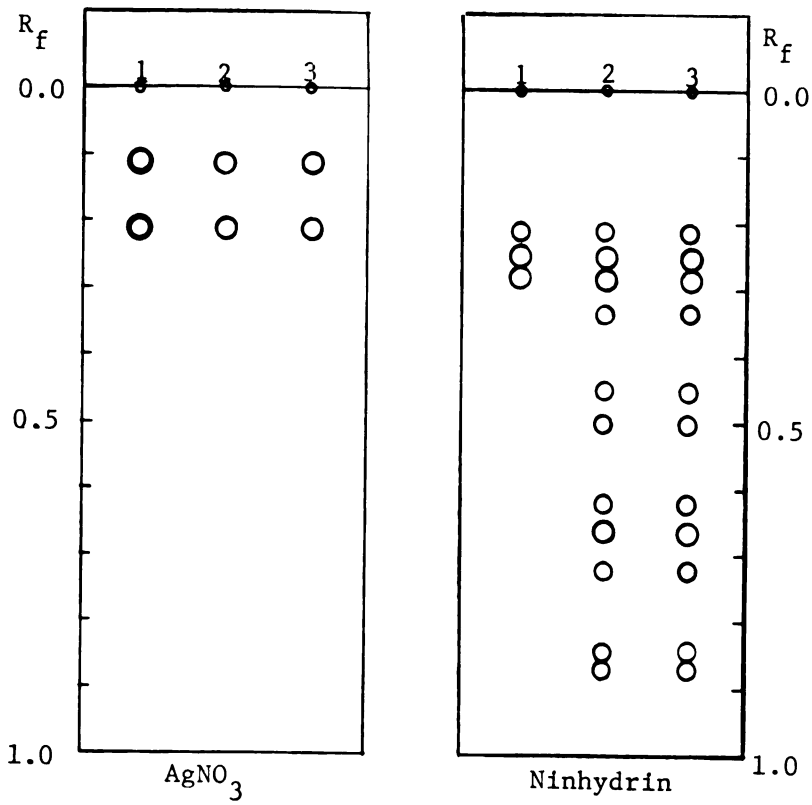

Figure 2. Diagram of the chromatograms of the first, second, and third H_2SO_4 hydrolysates of fraction A.

hydrolyzate. These spots suggest the presence of reducing carbohydrates, probably a monosaccharide at the higher R_f.

Ninhydrin: There are three ninhydrin-positive spots in the first hydrolyzate, one of them with the same R_f as one of the $AgNO_3$-positive spots. There are 11 spots in the second and third hydrolyzate, the color being more intense in the third hydrolyzate. This indicates the presence of amino acids. The nitrogen content of fraction A was 8 % and was determined by a Micro-Kjeldhal method (94). These results suggest that fraction A is, or contains, a glycoprotein.

D. Purification and partial characterization of fraction B.

(1) Purification of the crude fraction.

Fraction B is the aqueous supernatant solution obtained upon concentration of the acetone fractions. When the pH of this solution is adjusted to 12.0 with KOH, free IAA is released, which can be extracted by washing with ether after adjusting the pH to 3.0 with H_3PO_4.

Fraction B was lyophilized and a white powder, with a very high content of NaCl from the extraction procedure was obtained. The IAA content was between 0.06 and 0.13 %. The IAA-complexes were separated from the NaCl by using a column of Sephadex G-25. The column was prepared with 260 g of Sephadex, and had a diameter of 5 cm and a length of 50 cm. The bed volume was about 1000 ml. Fraction B was chromatographed on this column in batches of 150 ml of a 25 % solution. The separation between the IAA-complexes and the NaCl was good, but the IAA-containing compounds came out in the 1250 to 1600 ml fraction of the eluant, immediately after the NaCl, which came out in the 800 to

1250 ml fraction. This suggested that the IAA-complexes were rather small molecules, and that the separation on Sephadex was not due to molecular size, but rather due to adsorption. When two milliliters of a solution of fraction B containing about 50 micrograms of IAA was dialyzed against one liter of water for four hours, only traces of IAA were found in the dialysis bag. After purification with Sephadex and lyophilization, fraction B was a white and very light powder with an IAA content of 13.5 to 15.2 %. The recovery of this purification step was 43 %.

Fraction B gave a purple color with Ehrlich's reagent, but no color was formed with Salkowski reagent, unless the sample was previously hydrolyzed with alkali. At this stage of purification, fraction B is readily soluble in water and insoluble in ether. When a solution of this fraction was washed with ether, all the IAA-containing compounds were found in the water phase.

When fraction B was chromatographed on paper using n-butanol:acetic acid:water as developing solvent, it was shown that it contained at least four components, with R_f of about 0.40 to 0.60, and there was no free IAA present. IAA has an R_f of about 0.90 in this solvent, and the four IAA-containing compounds seem to be more polar than IAA.

When fraction B was chromatographed using either isopropanol: ammonia:water, 8:1:1, or n-butanol(sat.with water):ammonia, 7:1, the IAA complexes were broken down and the only spot which could be detected in the chromatograms after treatment with Ehrlich's reagent was due to free IAA.

Treatment of fraction B with concentrated ammonia at room temperature overnight resulted in the complete breakdown of the IAA complexes,

with formation of free IAA and indoleacetamide. Upon chromatography in three different solvents, using synthetic indoleacetamide as a standard, it was estimated that more than 50 % of the original compounds were ammonolyzed, and the rest hydrolyzed. This result indicates that IAA is bound to the other part of the complex molecule through the carboxyl group in a linkage more labile than the amide bond.

Fraction B was assayed for biological activity in the Avena coleoptile straight growth test and in the Cucumber root inhibition test (115). The concentration of fraction B was expressed in terms of its content of IAA, and on this basis had about 50 % of the activity of an authentic IAA standard. Thus, the complex is less active, per mole of IAA, than is free IAA.

(2) Separation of fraction B into its components.

Various attempts to separate the components of fraction B using column chromatography were unsuccessful. Sephadex G-25, Whatman cellulose powder, MN 300 cellulose powder, and finely ground Whatman #3 chromatographic paper were tried, and water, ethanol-water mixtures, and butanol-water mixtures were used as eluants. In all cases the IAA containing material appeared in the eluant in a single broad peak.

A sample of 140 mg of fraction B, in solution in 2 ml of water, were chromatographed in a 1.1 cm x 45 cm column of Sephadex G-25, with a bed volume of 45 ml. Water was used as eluant and the fractions collected were analyzed by paper chromatography. It was found that the first part of the single broad peak obtained was richer in the components with the lower R_f on paper. Many attempts to get a better resolution were not successful. The fractions richer in the components with the higher R_f on paper were collected separately and lyophilized,

and the same was done with the fractions containing mainly the components with the low R_f in paper. The IAA content of the mixture of components with the higher R_f on paper was 29.2 %, while the mixture of components with the low R_f contained only 15.2 % IAA. This suggests that the migration of the components of fraction B during chromatography with the butanol-water solvent depends on the IAA content. In other words, migration depends on the size of the residue attached to the IAA.

Since paper chromatography seemed to be the only method which gave a good separation of the components of fraction B, a survey was made of the different types of chromatographic paper and different solvents, in an attempt to improve the method. The best result was obtained with the system originally used, that is, n-butanol:acetic acid:water, 5:1:2.2 and Whatman #1 paper.

In preliminary experiments it was observed that when the components of fraction B were eluted from the chromatograms, they were heavily contaminated with UV absorbing substances. When a blank was analyzed by eluting chromatographic paper with water, considerable absorption was found between 200 and 300 millimicrons. When a 46 cm x 57 cm sheet of Whatman #1 was cut into small pieces, soaked in water overnight, and the eluant evaporated to dryness, 14 mg of a yellow powder, which gave a color reaction with anthrone reagent, was obtained. Several methods were tried to wash the sheets of paper free of this contamination. It was considered acceptable for the existing conditions, to wash the sheets of Whatman #1 with distilled water in a chromatography cabinet for 60 to 70 hours. No bacterial or fungal growth was apparent during the process.

A sample of 840 mg of fraction B, with an IAA content of 15.2 %

was chromatographed on 42 sheets of washed Whatman #1 paper, using 20 mg of sample per sheet. The chromatograms were developed for 24 hours with n-butanol:acetic acid:water. After this period of time the component with the highest R_f was near the front of the paper. The location of the desired compounds was accomplished by cutting narrow strips from the center and both sides of the chromatograms and treating them with Ehrlich reagent. The components were numbered from 1 to 4, B_1 being the component with the highest R_f, and B_4 the component with the lowest R_f. The areas of the chromatograms containing the different components were cut out, cut into small pieces, and eluted with water. Components B_1 and B_2 are very close and the area where they are almost in contact was eluted separately and rechromatographed in order to avoid cross-contamination. The chopped paper was eluted three times for two hours with distilled water and the eluants lyophilized.

The fractions were checked for contamination with the neighboring components using paper chromatography. The dry samples were white and light powders. The yields and IAA content of each is shown in table 1.

Since the IAA content of all the fractions was lower than the IAA content of the original fraction B, they were obviously contaminated with some material from the chromatographic paper. When 40 to 50 mg of the samples were suspended in 1 ml of water, a clear deep yellow solution and a grey-brownish precipitate resulted. The precipitate was removed by centrifugation and discarded. The supernatant fluid was chromatographed on a column prepared with 20 g of Sephadex G-25, 1.5 cm x 54 cm, and with a bed volume of 100 ml, in batches of about 40 to 50 mg in solution in 1 ml of water. The yellow colored material came out of the column ahead of the IAA-complexes, but traces of yellow color were pre-

sent in the eluant containing the IAA-complexes. The IAA content of the four components and the yield obtained are shown in table 1.

Table 1. Fractionation and purification of the water soluble IAA complexes from 15 kg of sweet corn kernels.

Fraction.	Dry weight.	IAA content.	Total IAA.
B	840 mg	15.2 %	126 mg
↓ paper chromatography ↓			
B_1	120 mg	15.0 %	
B_2	108 mg	15.5 %	
B_3	80 mg	11.5 %	
B_4	86 mg	9.5 %	
	394 mg		52 mg
↓ Sephadex G-25 ↓			
B_1	56 mg	34.0 %	
B_2	50 mg	26.2 %	
B_3	42 mg	20.5 %	
B_4	36 mg	22.2 %	
	184 mg		48 mg

(3) Partial characterization of the components of fraction B.

a.- Ultraviolet spectra.

The ultraviolet spectra of the four components differ from the ultraviolet spectrum of IAA. All of them show a broad peak of absorption at 320 millimicrons and much more absorption at 235 millimicrons than IAA. The absorption at 280 millimicrons is much more than could be accounted for by the IAA contained in them. The spectra of B_1 and B_2 are more similar to IAA than the spectra of B_3 and B_4, but the peaks at 288 and 274 millimicrons are sharper. B_3 in addition, has a broad peak at 264, and B_4 two peaks at 272 and 256 millimicrons.

A sample of B_1 contaminated with a larger amount of the yellow

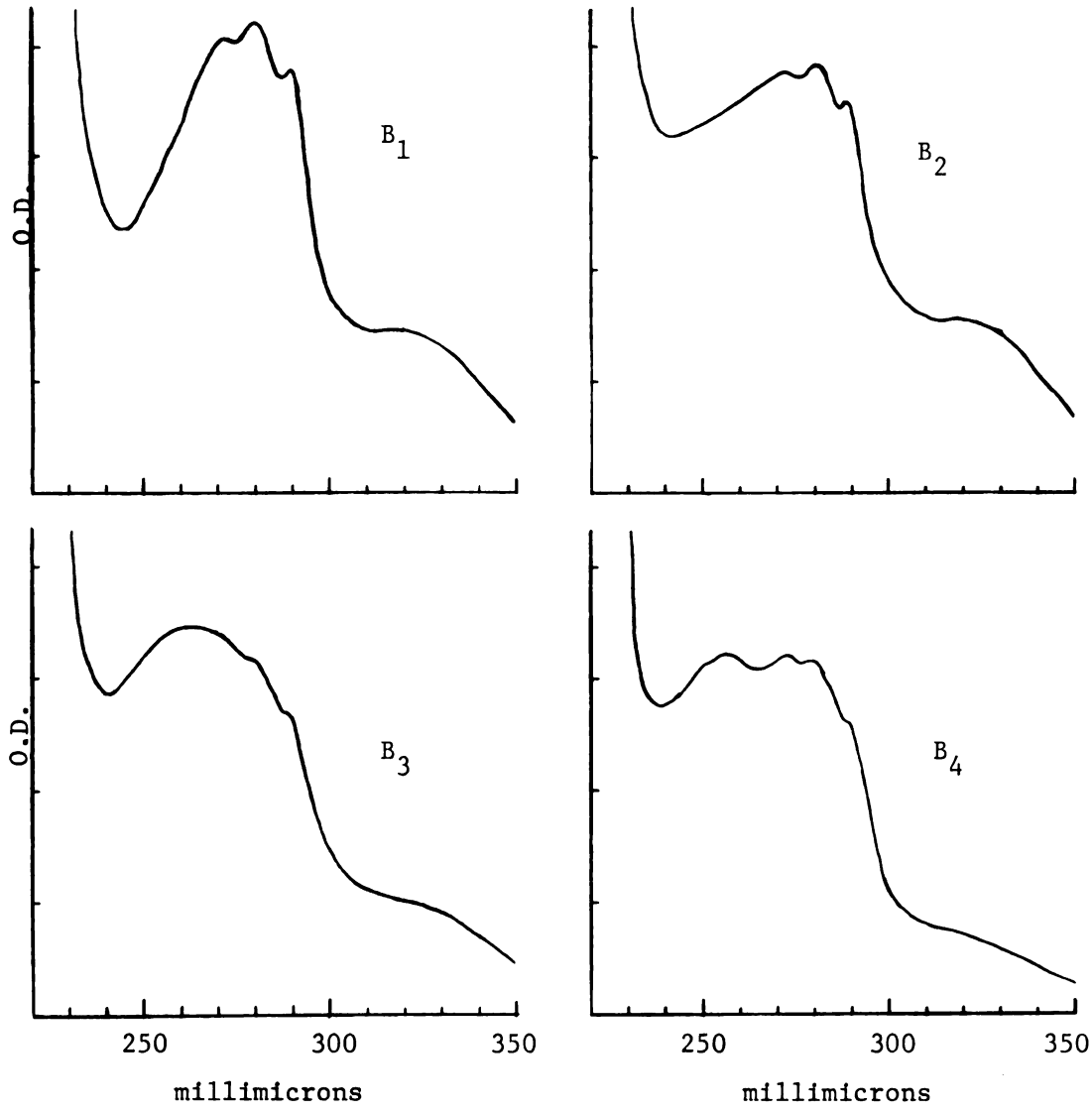

Figure 3. Ultraviolet spectra of the four components of the water soluble fraction B.

colored material mentioned above, shows fluorescence when observed under ultraviolet light on a chromatogram. When the sample is hydrolyzed with alkali and then chromatographed, the fluorescent spot is found again at the same R_f as B_1, even though the IAA-complex has been completely broken down and all the material giving a color reaction with Ehrlich's reagent is present now at the R_f of free IAA. The fluorescent spot was eluted and its ultraviolet spectrum showed two broad peaks at 320 and 295 millimicrons. These results suggest the presence of a contaminant with the same R_f as B_1 and a high extinction coefficient. A similar contaminant seems to be present in all four fractions. It probably came from the chomatographic filter paper and probably accounts for the absorption at 320 millimicrons and the increase in the absorption at 290 millimicrons.

b. Determination of amino acids.

Samples of 200 micrograms of B_1, B_2, B_3, and B_4 were hydrolyzed with 6 N HCl at 100°C for 12 hours. When a paper chromatogram of the hydrolyzate was sprayed with ninhydrin, no colored spots appeared.

c. Determination of phosphorus.

No phosphorus was found when B_1, B_2, B_3 and B_4 were hydrolyzed with 3 N HCl at 100°C for 6 hours and analyzed with ammonium molybdate reagent. The analysis was repeated by igniting the samples in the presence of KOH and analyzing with the ammonium molybdate reagent, and a negative result was also obtained. Glucose-1-phosphate was used as a standard.

d. Determination of reducing groups.

The four components of fraction B give a faint grey color when

treated with silver nitrate in a paper chromatogram. Since this method involves spraying with a NaOH solution, hydrolysis of the complexes could occur. Furthermore, IAA itself gives a faint grey color with this reagent. For these reasons, two non-alkaline reagents for reducing groups were used: aniline phthalate in aqueous butanol, and aniline oxalate in water (70, 111). A sample of 100 micrograms of each of the components was spotted on filter paper, and 25 micrograms of glucose, fructose, and arabinose, and 50 micrograms of cellobiose used as standards. After spraying and heating at 105°C, there was a strong positive reaction in all the standards, but no color was obtained with the samples. This result indicates that there are no reducing groups in the intact molecules of the IAA-complexes.

Samples of 100 micrograms of B_1, B_2, B_3, and B_4 were hydrolyzed with NH_4OH, spotted on filter paper, and treated with silver nitrate, aniline phthalate, aniline oxalate, and p-anisidine. The four complexes gave a black spot with silver nitrate, but no color was formed with any of the other reagents. This indicates that the IAA in the IAA-complexes is attached to a group which gives a strong positive reaction with silver nitrate.

e. Hydrolysis with ammonium hydroxide.

In order to simplify the nomenclature, the IAA complexes have been called IAA-X, X being the unknown moiety bound to the IAA. According to this B_1 is equal to IAA-X_1, B_2 is equal to IAA-X_2, and so on.

Samples of 0.1 ml of solutions, containing 100 micrograms of the complexes, were hydrolyzed by addition of one drop of concentrated NH_4OH and heating at 60°C for a few minutes. The hydrolyzed samples were chromatographed using Whatman #1 paper and butanol-acetic acid-water,

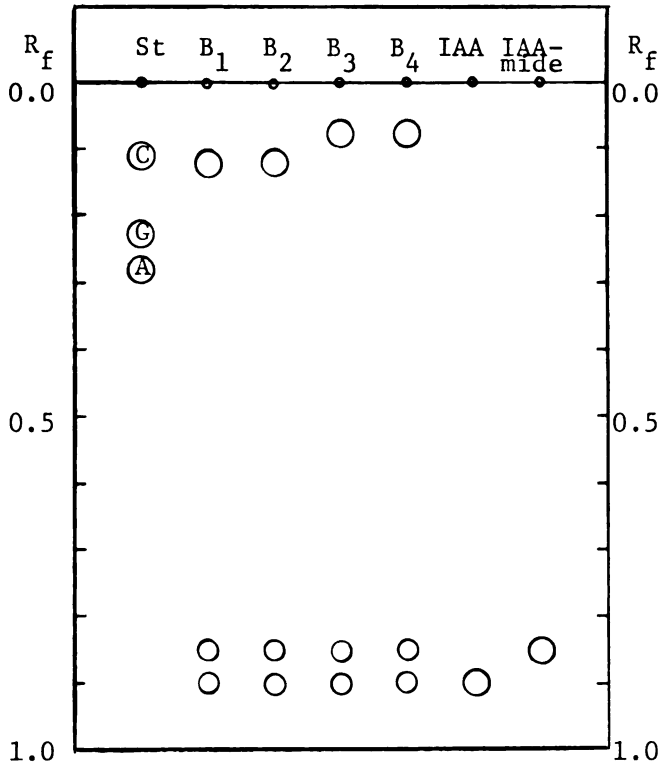

Figure 4. Diagram of a chromatogram of the four components of the water soluble fraction, B_1, B_2, B_3, and B_4, hydrolyzed with NH_4OH. Standards are: C = cellobiose; G = glucose; A = arabinose.

and the chromatograms treated with silver nitrate. Cellobiose, glucose, and arabinose were used as standards. A diagram of the chromatogram is shown in figure 4.

The R_f of X_1 and X_2 is the same, 0.12, and slightly higher than the R_f of the Cellobiose standard. The R_f of both X_3 and X_4 is the same, 0.076, but much lower than the R_f of X_1 and X_2. In order to see if there was some small difference between the migration of X_1 and X_2, and between the migration of X_3 and X_4, the four hydrolyzed samples were chromatographed again using the same solvent, but developing the chromatogram for 60 hours. The distribution of the spots and their position with respect to the standards was the same.

f. Electrophoretic analysis.

The carbohydrate nature of the X moieties was suggested by the chromatographic behaviour reported above. In order to obtain more evidence concerning this possibility, the behaviour of the four complexes during electrophoresis in borate buffer was studied.

Samples of 50 micrograms of each fraction on Whatman #1 paper were used for electrophoresis in 0.05 M borate buffer, pH 9.4, at 600 volts for 2 hours. The potential gradient was 11 volts/cm. The paper strip was treated with silver nitrate, and faint grey spots were obtained, showing the migration of the compounds toward the positive pole. The electrophoresis was repeated under the same conditions but for a longer time, 4.5 hours, and the paper strip was treated with Ehrlich reagent. Single spots and the same rate of migration was obtained for all the four complexes, indicating that there was no breakdown of the IAA-X molecules during the process. The IAA standard used migrates much faster than the IAA-complexes, as it is shown in figure 5. In order to

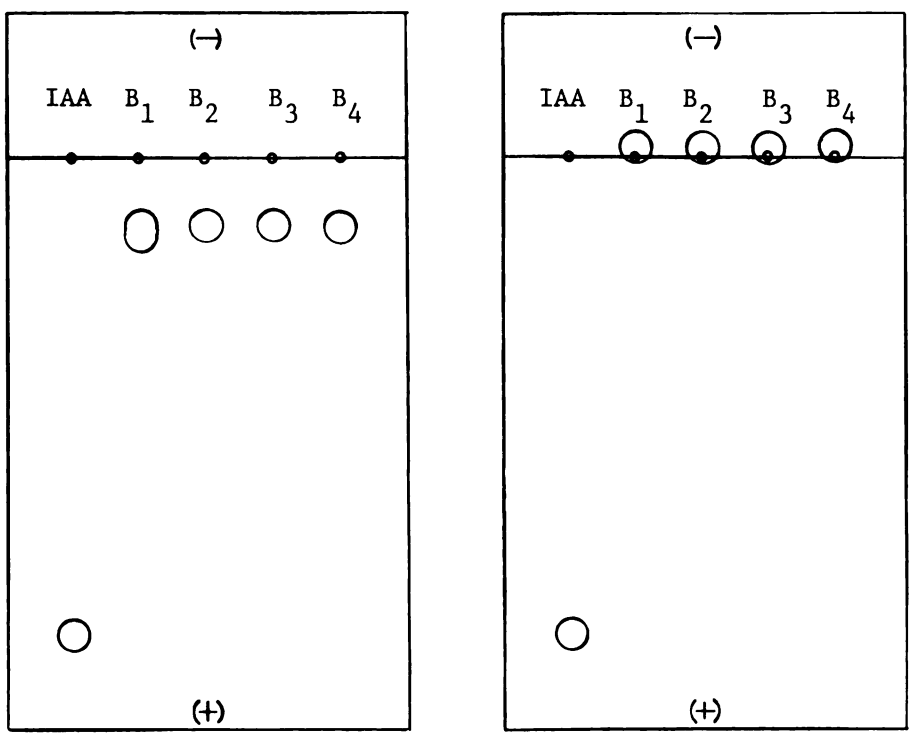

Figure 5. Diagram of the electrophoretograms of the four components of the water soluble fraction, B_1, B_2, B_3, and B_4 in: a) borate buffer pH 9.4; b) glycine buffer pH 9.4.

determine if the migration of the IAA-X molecules was due to the formation of a charged complex with borate, the electrophoresis was repeated using 0.05 M glycine buffer, pH 9.4, instead of borate buffer. There was no migration of any of the four fractions. The rate of migration of the IAA standard was the same as in borate buffer.

g. Hydrolysis with nitric acid.

In all the experiments involving acid hydrolysis, the IAA moiety of the IAA-X complexes was destroyed, as would be expected. The compounds detected on the chromatograms were the corresponding X part of the molecules and their hydrolysis products.

A sample of 50 micrograms of B_1 was hydrolyzed with 3 % HNO_3 in a boiling water bath for 0.5, 1, 2, and 4 hours. The samples were dried and chromatographed, and the chromatogram treated with silver nitrate. There was little hydrolysis of the X moiety, with formation of very small quantities of a compound with R_f similar to glucose. However most of the sample remained unhydrolyzed, and apparently there was no more hydrolysis after 4 hours than after 0.5 hours. The small amount of glucose could have resulted from a slight contamination of B_1 with B_2.

h. Hydrolysis with hydrochloric acid.

Samples of 25 micrograms of B_1, B_2, B_3, and B_4 were hydrolyzed with HCl at two concentrations, 1 N and 2 N, at 80°C for 6, 12, 24, and 48 hours, in sealed tubes. After hydrolysis the samples were dried, chromatographed, and treated with silver nitrate for detection of the spots. Very little hydrolysis of the X moieties was observed in all the treatments. There are traces of a compound with the R_f of glucose

in all the samples, and some traces, more abundant in B_4, of a compound with the R_f of arabinose. X_3 and X_4 have the same R_f as X_1 or X_2 after hydrolysis, suggesting partial hydrolysis of those compounds with conversion to compounds similar to X_1 or X_2.

i. Hydrolysis with sulfuric acid.

Samples of 30 micrograms of B_1, B_2, B_3, and B_4 were hydrolyzed with sulfuric acid at two concentrations, 2 N and 4 N, at 80°C for 6, 12, 24, and 48 hours, in sealed tubes. After hydrolysis the samples were neutralized with $BaCO_3$, the precipitate separated, and the samples chromatographed. Silver nitrate was used for the detection of the spots.

There is very little hydrolysis of X_1, with only traces of two spots with the R_f of glucose and arabinose. Hydrolysis products are present in much larger quantity in B_2, but most of the sample is present as X_2. There is a spot with the R_f of glucose, and traces of a spot with the R_f of arabinose. There are two spots present in B_3 and B_4, one with the R_f of glucose and the other with the R_f of arabinose. X_3 and X_4 have been converted to compounds with the same R_f as X_1 or X_2.

All the treatments gave about the same extent of hydrolysis. Apparently there is no difference between treatment with 2 N sulfuric acid or 6 hours and treatment with 4 N sulfuric acid for 48 hours in any of the four samples. Synthetic IAA was hydrolyzed as a control and no spots were obtained, since probably complete decomposition occurred.

The results of this experiment suggest that there is a glucose-like residue in X_2 which is easy to separate from the rest of the molecule. A glucose-like, and an arabinose-like residue seem to be easily

separated in X_3 and X_4, with conversion of these moieties to X_1. X_1 in turn seems to be very hard to hydrolyze.

j. Hydrolysis with HCl under mild and strong conditions.

In order to check the hypothesis advanced above about the behaviour of the X moieties on acid hydrolysis, these were hydrolyzed under very mild conditions: 1 N HCl at room temperature for 3 hours; and under strong conditions: concentrated HCl at 100°C for 4 hours. All the samples were previously hydrolyzed with NH_4OH to separate the IAA from the X moieties, and the NH_4OH was eliminated before the acid hydrolysis by concentrating to dryness. The acid hydrolyzed samples were chromatographed and $AgNO_3$ was used for the detection of the compounds. Samples hydrolyzed with ammonia but not with acid were used as controls. The results are shown in figure 6.

The position of X_1 does not change, and apparently there is very little hydrolysis with the strong treatment. There are traces of monosaccharides present only in the mild treatment. The hydrolysis of X_2 shows a similar result. Both X_3 and X_4 are partially converted to a compound with the same R_f of X_1 with the mild treatment, with simultaneous formation of two compounds with the same R_f of glucose and arabinose. The strong treatment produces the total conversion of X_3 and X_4 to the X_1-like compound, but there are no traces of monosacharide-like compounds, which are presumably destroyed.

The results of this experiment seem to confirm that the X_1 moiety of B_1, is very resistant to acid hydrolysis. This X_1 moiety is probably identical to the compounds arising upon very mild acid hydrolysis of X_2, X_3, and X_4.

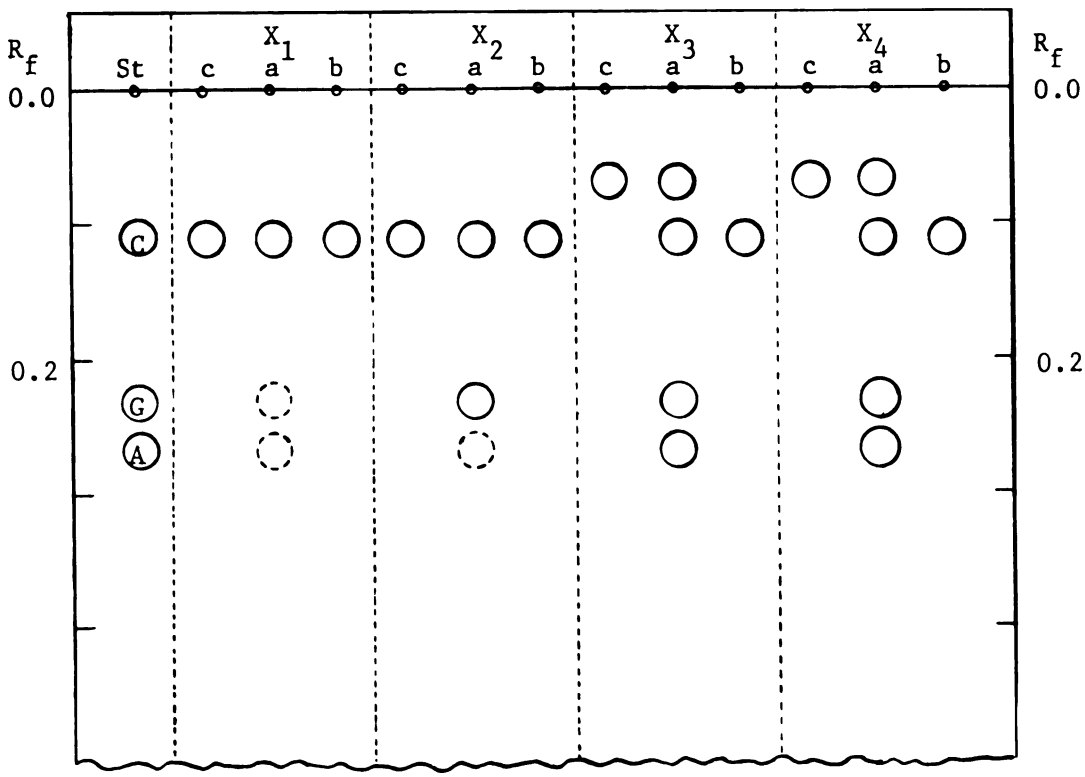

Figure 6. Diagram of chromatogram of X_1, X_2, X_3, and X_4 (obtained by alkaline hydrolysis of B_1, B_2, B_3, and B_4) c = control; a= hydrolyzed with 1 N HCl at room temp.; b = hydrolyzed with conc. HCl at 100°C. Standards are: C = cellobiose; G = glucose; A = arabinose.

k. Hydrolysis with 2.5 N sulfuric acid in an autoclave.

Fractions of 1 mg of each compound were hydrolyzed with 2.5 N sulfuric acid in the autoclave at 15 psi. (about 120°C) for 1.5 hours. After hydrolysis the solutions are brownish and there is some black precipitate. The solutions were neutralized by addition of a small amount of Dowex anion exchange resin in carbonate form and were kept at room temperature until a neutral solution was obtained. The samples were concentrated to dryness and chromatographed on paper for 24 hours using ethyl acetate: pyridine: water, 8:2:1 as the developing solvent. Galactose, glucose, mannose, arabinose, and xylose were used as standards, and the chromatograms were treated with silver nitrate, aniline phthalate, and p-anisidine. Silver nitrate gave much neater spots than the other two reagents. Diagrams of the chromatograms are shown in figure 7.

Both B_1 and B_2 yield a compound with the R_f of glucose upon hydrolysis. In B_3 and B_4 the hydrolysis products are two compounds with the R_f of glucose and arabinose. The spots with the R_f of glucose and arabinose give the same color as the glucose and arabinose standards with aniline phthalate and p-anisidine.

The unhydrolyzed compounds or the X moieties are not detected on these chromatograms. In order to find out if this was due to total hydrolysis or to partial destruction of the compounds, the hydrolysis was repeated for one hour under the same conditions, and the sames chromatographed using n-butanol-acetic acid-water as the developing solvent. The upper part of the chromatogram, from the origin to R_f 0.33 was treated with silver nitrate, and the lower part was treated with Ehrlich's reagent. The hydrolysis products are the same as before and the glucose

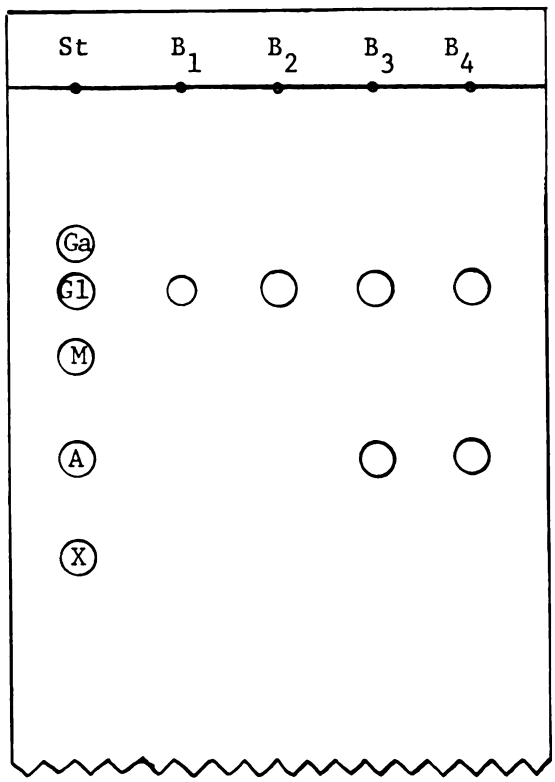

Figure 7. Diagram of a chromatogram of the four components of the water soluble fraction, B_1, B_2, B_3, and B_4, hydrolyzed with 2.5 N H_2SO_4 in autoclave at 15 psi for 1.5 hours. Standards are: Ga = galactose; Gl = glucose; M = mannose; A = arabinose; X = xylose.

and arabinose spots are very neat and have the same R_f as the glucose and arabinose standards. No Ehrlich positive compounds were found in the lower part of the chromatogram, and only some very diffuse spots at the R_f of the X moieties in the upper part. This suggests that there is partial destruction of the compounds during hydrolysis. The amount of glucose obtained from B_1 was very little, less than the amount produced upon hydrolysis of the other fractions.

l. Hydrolysis with beta-glucosidase.

The fractions were incubated with beta-glucosidase in 0.05 M acetate buffer pH 5.0 and at 30°C, for 20 hours. Although there was evidence of some hydrolysis, no conclusions were possible because the enzyme preparation was contaminated with carbohydrate material. When the enzyme was incubated without substrate and chromatographed, a small amount of glucose and arabinose were obtained. The enzyme was purified with Sephadex G-25, after incubation at 30°C for 6 hours to get hydrolysis of polysaccharide materials that could be present. When the samples were incubated with the purified enzyme, and with beta-glucosidase from a different source, the result was better, but there was still some carbohydrate material from the enzyme preparation in the chromatogram, interfering with the detection of the hydrolysis products and the method was discarded.

In one of the experiments with beta-glucosidase, fraction B_1 was incubated with buffer, as a blank, for 7 days. After this period of time there was no decomposition of B_1.

m. Hydrolysis with snail gut juice.

A sample of 1 ml of snail gut juice was diluted to 3 ml with 0.2 M phosphate buffer pH 6.0. The solution was centrifuged and the

precipitate discarded. The supernatant is a dark brown solution containing about 10 mg dry weight per ml. A solution of 1 ml of each fraction containing 1 mg of the IAA-complexes was incubated with 0.02 ml of the snail gut juice preparation for 48 hours at 30°C. After incubation, the tubes with the reaction mixtures were heated in boiling water for 5 minutes and stored in a freezer. The samples were chromatographed on paper using butanol-acetic acid-water as the developing solvent. The upper part of the chromatogram was treated with silver nitrate, and the lower part with Ehrlich's reagent. A diagram of the chromatogram is shown in figure 8.

The results of this experiment agree with the results of the acid hydrolysis. Since there is no breakdown of the IAA or the other units of the complexes, the chromatograms are very neat and it is possible to follow the change in the B fractions during hydrolysis by the change in position of the Ehrlich positive spot in the chromatograms.

There is a small amount of glucose produced from B_1, and much more from B_2, B_3, and B_4. Arabinose is present only in B_3 and B_4. The R_f of B_1 has not changed, and there is no clear change in B_2, but B_3 and B_4 are converted, upon release of the monosaccharide units, to a compound with the same R_f of B_1. There is no free IAA in the chromatogram, indicating that the bond between the IAA and the X moieties is not affected by the incubation with the enzyme preparation. The B_3 and B_4 controls show two spots each, suggesting partial interconversion, from B_3 to B_4 and, peculiarly, vice versa. Something similar could have happended in B_1 and B_2, which appear in the chromatogram with the same R_f. In addition, the spots are elongated and shaped a little like the figure 8, suggesting two components.

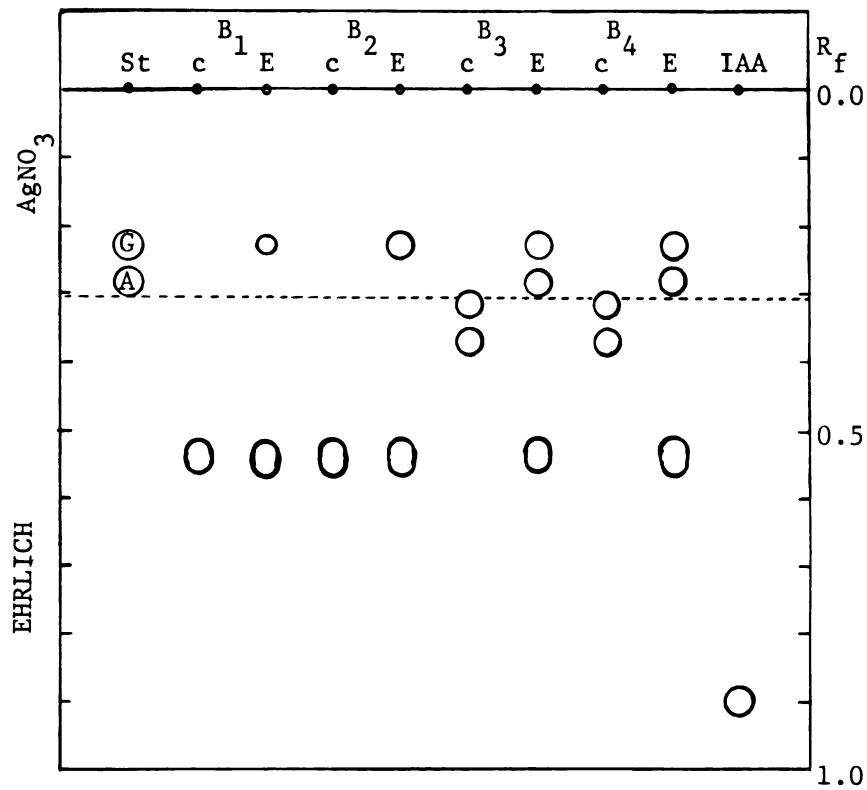

Figure 8. Diagram of a chromatogram of the four components of the water soluble fraction, B_1, B_2, B_3, and B_4, hydrolyzed with snail gut juice. c = control; E = incubated with snail gut juice. Standards are: G = glucose; A = arabinose.

n. Quantitative estimation of glucose.

There was evidence from paper chromatography that there was probably more glucose in the enzymatically hydrolyzed samples than in those that were acid hydrolyzed. Several attempts were made to estimate the amount of glucose in the hydrolyzate by using the Anthrone method, but the results were inconsistent. The Glucostat reagent, which uses glucose oxidase, is very sensitive and specific, but gave inconsistent results with the enzymatic hydrolyzate. In all analyses it was found that considerably less glucose was released from B_1 than from the other three fractions. After hydrolysis with 2.5 N H_2SO_4 in an autoclave for 1 hour, the amount of free glucose found in the hydrolyzate was about 8 % in B_1, and 25 % in B_2, B_3, and B_4.

o. Determination of inositol.

The X moieties obtained upon hydrolysis of the water soluble fractions do not give a positive reaction for reducing sugars but react strongly with silver nitrate and form a complex with borate, suggesting the presence of a polyhydroxy compound which is not a sugar. The possibility that the X moieties contained an hexitol was investigated.

B_1 was hydrolyzed with ammonium hydroxide and chromatographed on paper, with myo-inositol as a standard, and using four different solvents: n-butanol:acetic acid:water, 5:1.5:3.5; isopropanol:acetic acid: water, 5:1:7.5 (ascending); ethyl acetate:pyridine:water, 8:2:1; and phenol:water, 4:1.

As is shown in figure 9, X_1 and inositol have the same R_f in the four solvent systems used.

A sample of 1 mg of fraction B_1 was hydrolyzed with Rexyn AG4, a quaternary amine strong anion exchanger, by adding resin in small amounts

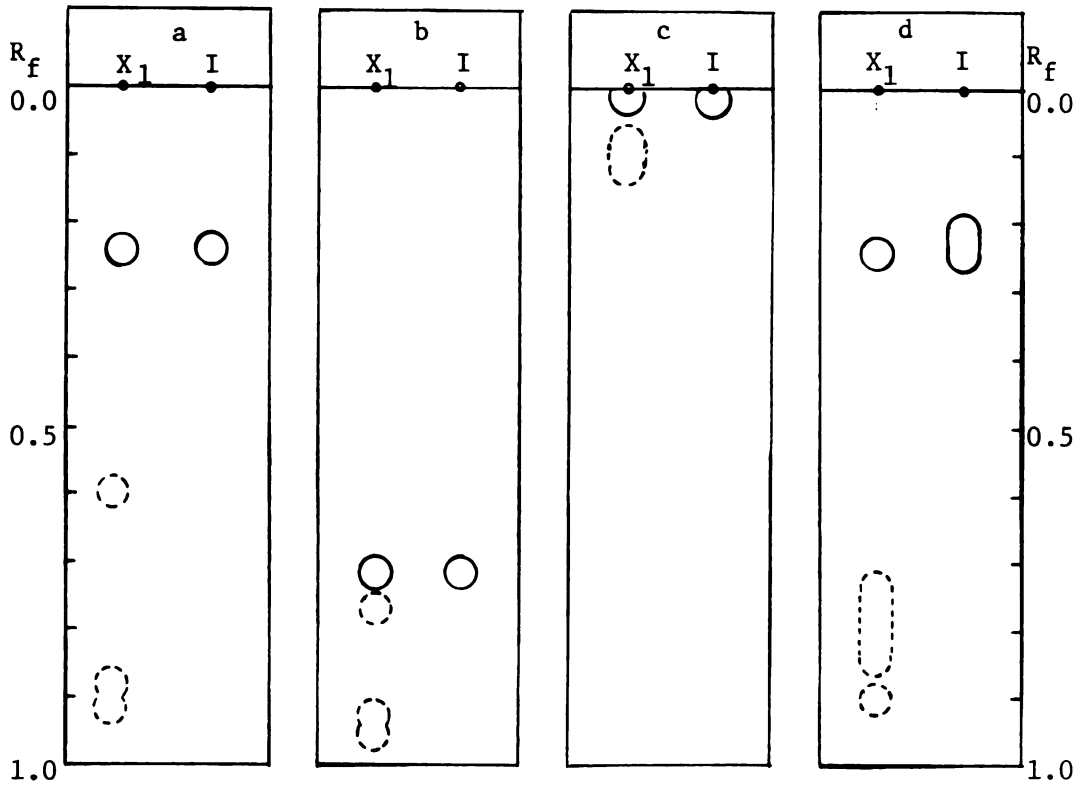

Figure 9. Diagram of chromatograms of X_1 and inositol.
a = n-butanol-acetic acid-water.
b = isopropanol-acetic acid-water.
c = ethyl acetate-pyridine-water.
d = phenol-water.

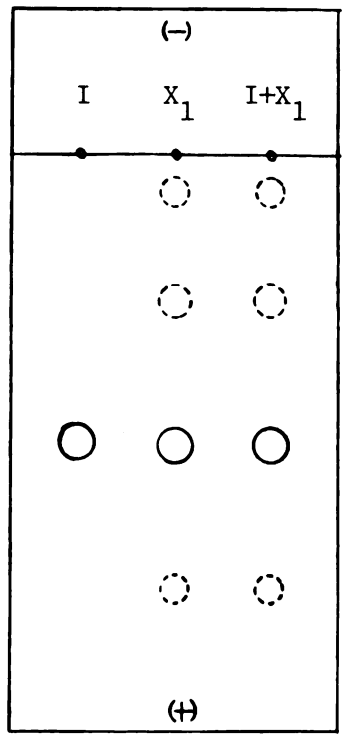

Figure 10. Diagram of an electrophoretogram of X_1 and inositol, in borate buffer pH 9.2 and 0.05 M.

to a solution of B_1 until the pH was constant at about 10.5. The hydrolyzate was analyzed for inositol by the Scherer test described by Feigl and Gentil (39). Three replicates of the sample and two standards of 25 and 50 micrograms of inositol were used. A pink color, indicating the presence of inositol, was obtained. A sample of the hydrolyzate was used for electrophoresis in borate buffer pH 9.2 and 0.05 M, at 600 volts for four hours, with a potential gradient of 11 volt/cm. A standard of myo-inositol was used and the electrophoretogram was treated with silver nitrate. X_1 has the same mobility as myo-inositol and when myo-inositol and X_1 were used a single spot was obtained, as shown in figure 10.

A sample of 10 mg of B_1 was hydrolyzed by treating it with Bio Rad AG-1 X10, a strong anion exchange resin, in a small column (bed volume about 0.5 ml). No IAA was detected in the eluant, which, after lyophilization yielded 1.1 mg of a white substance. The melting point of this fraction and myo-inositol was determined. A small amount of X_1, a small amount of myo-inositol, and a mixture of both were used. A tan color appeared in the sample of X_1 at about 175°C, turning light brown at 200°C. The same was observed in the mixture of X_1 and myo-inositol. The melting point of myo-inositol was 224-225°C, the melting point of X_1 was 221-223°C, and the mixed melting point was 221-225°C. Upon cooling, the samples recrystallized and the melting point was determined again, obtaining the same values.

The results of these analysis give good evidence of the presence of myo-inositol in X_1.

DISCUSSION

During the last three decades indoleacetic acid has been regarded as a plant growth hormone, and its participation in growth and morphogenesis in plants has been well established. However, although it has been generally accepted for many years that free indoleacetic acid is the active form of the auxin, there is actually no conclusive evidence to support this idea, and there is now a growing body of evidence which points to the importance of the bound forms. There are authors as, for example, Bentley (21) who think that the free form of IAA is not the auxin physiologically active in normal growth.

There has been a great deal of confusion in this field, owing mainly to the use of inadequate techniques of extraction and purification, and inadequate chemical characterization. Extraction of plant tissues with water or solvents that do not inactivate enzymes might lead to the breakdown of labile forms during the process. Even the solvent itself, as in the case of ethanol under alkaline conditions, can give rise to artifacts (165). Solvents containing ammonia have been used extensively in chromatography of indole compounds, because they give a good resolution for several simple indole derivatives, but the use of this method results in the breakdown of labile complex forms of IAA during the process. The use of ammoniacal solvents, as isopropanol-ammonia-water, has been responsible for the report of artifacts in a very large number of papers. In 1961 it was clearly shown by Zenk (163) that the indoleacetamide and the indoleacetic acid he found in chromatograms of plant extracts, were artifacts due to the use of ammonia in the developing solvent. In spite of this, there are still some authors who rely on this method for the analysis of plant extracts. Diffusion techniques, such

as diffusion from coleoptile tips into agar undoubtedly permit hydrolytic enzymes liberated at the cut surface to act.

Inadequate chemical characterization has been very common, and actually it is possible to say that only in a few instances has a careful characterization been tried. Many reports are based upon bioassay of crude extracts or upon bioassay, or chemical analysis, of crude fractions separated by a single chromatographic step.

Recently it has become evident that several of the derivatives with growth regulatory activity which have been reported, are breakdown products of more complex molecules. Glucobrassicin, recently reported as the principal indole compound in cabbage (83) has been found to give rise to a number of indole derivatives upon enzymatic and chemical hydrolysis. Some of the degradation products of glucobrassicin are (44): ascorbigen, indole-3-acetonitrile, 3-hydroxymethylindole, indoleacetamide, indoleacetic acid, indolecarboxylic acid, skatole, and indole. The sensitivity of glucobrassicin to heat or hydrolytic conditions and its spontaneous cleavage by myrosinase, which is present in cabbage tissues, explain the presence of some of these "artifacts" in extracts of cabbage. The fact that some of the degradation products of glucobrassicin are products of a reaction catalyzed by an enzyme present in cabbage tissue, indicates that some of them could be considered as naturally occurring compounds.

Apparently glucobrassicin has been reported only in Brassica, but this, or similar compounds, could be present in other plants. The possibility exists that IAA is present in plant tissues in a few bound, and very labile, forms, which could be the key substances for the hitherto described indole derivatives, including IAA, which have been

reported in plant tissues. The variety of indole derivatives which have been found could arise from these compounds. Different methods of extraction and purification could give rise to different derivatives.

Fraction A, the water insoluble fraction reported in this work, has been demonstrated to be a high molecular weight compound, similar in its physical properties to the fraction isolated by Berger and Avery (22), but with a lower content of IAA and a higher content of nitrogen. The fraction reported by Berger and Aver had 3.7 % IAA in its purest form, and 4.7 % nitrogen. Fraction A has between 0.5 and 1.0 % of IAA, and about 8 % nitrogen.

The isolation and partial characterization of the bound forms of IAA was restricted to two samples of corn of the same variety, but obtained in different years. This could explain, in part, some of the differences found between the fractions obtained from the two samples. The first sample, of 6 kg, was extracted three times with aqueous acetone, and the second sample, of 15 kg, was extracted only two times. The yield obtained in the first case was 3.78 g of fraction A, with 0.8 % of IAA, per kilogram of corn, this being equal to 30 mg of IAA in the water insoluble form per kilogram of corn. The yield from the second sample was 2.28 grams of fraction A, with 0.53 % IAA, per kilogram of corn, equal to 15 mg bound IAA in water insoluble form per kilogram of corn. The quantity of water soluble IAA complexes was larger in the second case than in the first, being equal to about 15 mg of IAA per kilogram, and to about 20 mg of IAA per kilogram of corn in the second. Thus, the total amount of bound IAA obtained was equal to about 45 mg per kilogram of corn in the first sample, and to 32 mg per kilogram in the second. If however IAA content is calculated as percentage of the

total weight of the complexes including water soluble and insoluble forms (weight of fraction A plus fraction B) the value obtained for the two samples is not very different, being equal to 1.2 % for the first, and 1.35 % for the second.

The peculiar solubility properties of fraction A made its purification difficult. Fraction A, like zein, is soluble at pH 11-12, but under these conditions, there is hydrolysis of the IAA-complex, with release of IAA. The fact that fraction A appeared in a single peak in the eluant after chromatography in Sephadex G-25, and with about the same content of IAA, does not indicate that the compound is pure, since it could be contaminated with high molecular weight compounds, which are not separated from the IAA-complex during gel filtration. Apparently zein has at least three components with different molecular weight (45) and the same could be true for fraction A. The main obstacle in the purification of this fraction was the lack of methods suitable for compounds with these solubility characteristics

Zein is almost certainly associated with fraction A. As was explained in the Introduction, there seems to be a correlation between the disappearance of water soluble forms of IAA and synthesis of zein in maturing corn kernels. Zein is a peculiar protein, this type of protein being present mainly in cereal seeds. It is probably not likely that bound forms of IAA similar to fraction A are very widely distributed in plants. Apparently the only fraction reported in the literature similar to the water insoluble fraction A found in this work is the "auxin precursor" described by Berger and Avery (22), and the fraction reported by Hamilton (58), extracted with 80 % ethanol from corn kernels.

The purification of the water soluble complexes was achieved by

paper and Sephadex G-25 chromatography. The four components isolated have very similar solubility properties and this makes their separation difficult, since they run very close to each other on paper chromatography, and on different adsorbents in column chromatography. A great deal of time was spent trying to find a suitable column chromatographic method to separate these compounds, but without success. The method used in this work permits a good separation, but the yield is poor, about 40 %, and there is a great deal of contaminating material coming from the paper. Fifty large sheets (46 x 57 cm) of Whatman #1 paper are necessary for the separation of the components of 1 g of fraction B, and the process is very time consuming. A better method, probably based on column chromatography, where larger amounts of material can be used and no contamination from the adsorbent is obtained, should be worked out.

The water soluble bound forms have not been completely characterized. There is information about the units present in the complexes, and about the IAA content of each of them, but the molar ratios of the different components have not yet been obtained. The study of the composition of the complexes was done by analyzing the hydrolysis products after alkaline, acid, or enzymatic treatment. Treatment of the samples with concentrated ammonium hydroxide yielded indoleacetamide, showing that the IAA is bound to the carbohydrate moiety through the carboxyl group. Very mild alkaline treatment hydrolyzed the complexes by breaking the bond between the IAA and the carbohydrate moiety, showing that the same type of bond (very labile to alkali) is present in the four water soluble fractions. In the water insoluble fraction A, IAA is linked to the rest of the molecule by a similar alkali-labile bond.

A preparation of snail gut juice split the water soluble complexes quantitatively and very neatly, presumably without breakdown of the individual components. Since however a mixture of enzymes is present in this crude preparation, no information was obtained about the nature of the bonds between the units.

As a working hypothesis and as a guide to the reader, the present concept of the composition and structure of these complexes is shown in figure 11.

B_1	(IAA - INOSITOL) X
B_2	(IAA - INOSITOL - GLUCOSE) X
B_3, B_4	(IAA - INOSITOL - GLUCOSE - ARABINOSE) X

Figure 11. Tentative composition of the water soluble complexes.

The available evidence shows that B_1 contains IAA and inositol. When B_1 was hydrolyzed with acid or enzyme, there was always a small amount of glucose in the hydrolyzate. The R_f of B_1 did not change when treated with snail gut juice, and the R_f of X_1 (B_1 minus the IAA) did not change when treated with acid. Something similar was observed when B_2 was hydrolyzed, as is explained below. Inositol, and inositol-glucose could have the same behaviour in paper chromatography and paper electrophoresis. The calculated IAA content for a molecule containing IAA, inositol, and a six-carbon carbohydrate unit is 35.1 %. The IAA content found in B_1 is 34.0 %. The small amount of glucose obtained from B_1 when hydrolyzed with acid or enzyme, indicated the possibility that this compound could be formed only by IAA and one unit of inositol. In

that case, however, about 33 per cent of the weight of this sample would be impurities.

B_2 contains IAA, inositol, and glucose, but the amount of IAA found in this complex was 26.2 %. The theoretical content of IAA in a compound containing IAA, inositol, glucose, and a six-carbons carbohydrate unit, is 26.5 %. A much larger amount of glucose is obtained from B_2 upon hydrolysis than from B_1. When enzymatically hydrolyzed, B_2 is apparently converted to B_1, with release of glucose. The difference between B_1 and B_2 seems to be the presence of one more glucose unit in B_2. The R_f of X_2 and X_1 are the same, and the R_f of X_2 does not change when treated with acid, in spite of the appearance of glucose in the hydrolyzate, showing that the R_f of inositol linked to at least one glucose unit is the same as the R_f of free inositol. If the hypothesis about the composition of B_1 and B_2 is correct, it would imply that the R_f of inositol and inositol-glucose is the same in the solvent system used.

B_3 and B_4 contain IAA, inositol, glucose, and arabinose. The IAA content is 20.5 % in B_3, and 22.2 % in B_4. They have a different R_f in paper chromatography, B_4 being more polar than B_3, and some evidence for interconversion between them was found. The theoretical IAA content for a molecule containing IAA, inositol, glucose, arabinose, and a six carbons carbohydrate unit, is 22.1 %. B_3 and B_4 are quantitatively converted to B_1 by incubation with snail gut juice, with release of glucose and arabinose. X_3 and X_4 have the same R_f in paper chromatography, but different from the R_f of X_1 and X_2. Both X_3 and X_4 are quantitatively converted to X_1 with mild acid hydrolysis, with release of glucose and arabinose.

In all the water soluble IAA complexes, the IAA is bound to inositol. This bond is very labile to alkali, but is not attacked by snail gut juice or beta-glucosidase.

There is a very close relationship between the water soluble IAA complexes. The hypothesis could be advanced that B_1 is a conjugate of IAA-inositol, plus X; B_2 IAA-inositol-glucose, plus X; B_3 and B_4 IAA-inositol-glucose- arabinose, plus X. The difference between B_3 and B_4 is not, as yet, clear.

The relative abundance of these forms is as follows: B_1 39 %, B_2 27 %, B_3 17 %, and B_4 17 %. This type of distribution would probably be obtained if B_1 is a "limit" product of the breakdown of a straight chain as long as B_3 or B_4 or longer, with very labile bonds between the units beyond the B_1 moiety. There is a possbility that the water soluble complexes were originally part of a high molecular weight compound like fraction A. The presence of amino acids, and some reducing spots with the R_f of monosaccharides and inositol after acid hydrolysis of fraction A, and its content of nitrogen of about 8 %, indicates that it is a glycoprotein-type compound in which IAA could be bound to the carbohydrate moiety, as it is in the water soluble fractions. There is evidence that the bond linking the IAA to the rest of the molecule is very similar in the water soluble and water insoluble fraction. Then, the possibility exists that there was originally a single compound whose components were IAA-inositol-glucose-glucose-arabinose-glycoprotein. A compound of this type, with labile glucose-glucose, glucose-arabinose, and arabinose-glycoprotein bonds, could yield, upon partial breakdown of these bonds, all the IAA-complexes found in this investigation. The straight chain structure was assumed for simplicity, but a

structure with inositol as a nucleus with the other components bound to it, or other arrangements would be conceivable.

Fraction A is very similar to the water insoluble fraction obtained by Berger and Avery (22) using the same extraction procedure. The main difference is in the content of IAA and nitrogen. Fraction A has 0.53 % IAA and 8 % nitrogen. The fraction of Berger and Avery had 3.7 % and 4.7 % N. Different varieties of sweet corn were used and that could explain the differences, but the fact that fraction A has a higher content of nitrogen and a lower content of IAA, suggests that there could be a correlation between IAA and N content. If we visualize a model in which IAA is bound to the carbohydrate part of a glycoprotein, a loss of carbohydrate chains with IAA attached to them, as in the water soluble fractions described in this work, would increase the content of nitrogen and decrease the IAA content of the remaining glycoprotein. In this work the IAA complexes were extracted with aqueous acetone at room temperature, and enzymatic activity, which could break up the original glycoprotein, is conceivable under those conditions.

The amount of IAA in bound form isolated during this work was up to 45 mg per kilogram of mature corn kernels. No estimation was made of the total amount of bound IAA present in this material, but undoubtedly the quantity actually present was larger. The weight of one kernel of the variety used is about 200 mg, that is, there are about 5000 kernels per kilogram. The amount of IAA in bound form in each kernel is then, at least, about 10 micrograms. Hamilton (58) based on information found in the literature, estimates that 10,000 oat coleoptile tips might contain about 1 microgram of IAA. It is clear then, that the amount of bound IAA found in sweet corn kernels is indeed a large amount

from the physiological point of view (about 100,000 times more than in an Avena coleoptile tip) and more than enough to account for the promotion of growth in the young seedling.

The crude fraction B assayed for biological activity was shown to be active in the promotion of growth, although less active than IAA. The fraction assayed was rather crude, and, as has been reported above, not homogeneous and thus it is possible that not all the components are active. The purified components have not been tested for growth promoting activity.

Hamilton (59) failed to find free IAA when he extracted, with ether, an acidified 80 % ethanol extract of growing (5 day old) corn shoots, but about 13 micrograms per kilogram of fresh weight were found when he first adjusted the pH to 8.0 for preliminary removal of neutral ether soluble substances, and then acidified and extracted with ether. He concluded that IAA could occur in growing corn shoots in the form of a very labile complex, from which it was liberated by the alkaline pH treatment. It should be emphasized that the alkaline treatment, which was not intended for hydrolysis and is almost routine in the extraction and purification of IAA, was extremely mild, and probably more IAA could have been obtained by using a somewhat stronger alkaline treatment. The complex suggested by Hamilton as present in growing corn seedlings could be very similar to the water soluble fractions found in this work.

Terpstra (138, 139) in a study of the extractable auxins from Avena coleoptiles, demonstrated that the auxin is present in extract of ground coleoptile tips as water-soluble, ether-insoluble complexes, which yield IAA very easily.

Many water soluble auxin complexes which yield IAA on hydrolysis have been found to be naturally occurring (10, 27, 28, 32, 35, 36, 71), but they have not been characterized. The "zeanins" found by Farrar (35) in immature corn kernels have some similarities with the water soluble fractions found in this work, as for example, similar behaviour in paper chromatography in a butanol-water mixture and lability to ammonia. The zeanins give a purple color with Salkowski reagent, and this does not occur with the water soluble forms reported in this work, which do not give a color reaction with Salkowski reagent, unless they are first treated with alkali.

Farrar (35) considers the zeanins as intermediate compounds between native bound IAA and free IAA. It is not known yet if the water soluble fractions found in this work are "artifacts" produced by breakdown of a larger water insoluble molecule, similar to fraction A, or if they are normally present in corn as an intermediate form, as has been suggested by Farrar for the zeanins.

The fluctuations in the auxin content in developing corn kernels suggest that IAA is present, in early stages of the development of the seed, in a water soluble bound form, which probably is gradually converted to a high molecular weight, water insoluble form which is present in the mature kernel. The "reserve materials" of the seeds, which are largely macromolecules, are metabolically broken down during germination and utilized by the young seedlings. The fate of the large amount of IAA present in corn, and probably other seeds, is not known. A mechanism for germination could be visualized where an active form of IAA were liberated from a glycoprotein-IAA complex by the action of enzymes activated upon absorption of water by the seed.

Myo-inositol is widely distributed in microorganisms, plants, and animals, and it is considered a growth factor for some microorganisms and for warm-blooded animals. It is known that inositol participates in some way in cell wall metabolism. Deficiency of inositol induces agregation of yeast cells, and a greater proportion of glucan in the cell wall (29). In a strain of Neurospora crassa inositol deficiency gives rise to colonial growth (29). The mode by which inositol brings about alterations in the polysaccharide composition of fungal cell walls is unknown, but definite effects in polysaccharide biosynthesis have been demonstrated. Inositol deficiency may also be induced by inositol antimetabolites, with consequent effects on morphogenesis. Myo-inositol has been shown to be present in, and to be characteristic of, three natural sources of the growth promoting "neutral fraction" from Cocos, Zea, and Aesculus (114).

It is not good scientific reasoning to assume that a conjugated form of IAA and inositol is physiologically important in growth regulation simply because it is present in large amounts. On the other hand, it would be a great mistake to disregard the possibility of some such role in view of the fact that both compounds are known to be involved in plant growth and morphogenesis, and three decades of work have not shown with any certainty that free IAA is present in plant tissues; the amount of "free" IAA reported has often been so small that is presence has been guessed rather than demonstrated.

The real problem to be faced now is to find out what is the active form of auxin in plant tissues. Definitive evidence about the nature of the native plant auxin will be difficult to obtain and will probably require several kinds of experimental approaches. In addition to the

difficulties involved in identifying the forms present, due to the labile nature of many of them which might lead to the production of artifacts and mistaken identifications, there is the problem of finding out what the physiological role of the naturally occurring forms is. Zenk (165) has advanced the hypothesis that the action and formation of IAA could be controlled by conjugation to form inactive compounds, probably implying that free IAA is the active form. The presence in animal tissues of conjugated forms of IAA which are considered detoxification products (74, 75, 127) and the fact that large amounts of exogenous IAA are "neutralized" by conjugation in plant tissues (4, 6, 164) have pointed to the possibility that a similar system could be operative in normal plant metabolism and give rise to bound forms. The high concentration of bound forms found in some seeds suggests that they are storage forms, which probably would be inactive as such. It is not known how IAA is transported in the plant, but a low molecular weight "neutral" compound, as a conjugate with sugars, would probably be a suitable form.

Final identification of an active auxin complex as "native" will most likely require an <u>in vitro</u> system which respons to the added complex. If in addition the complex is ubiquitous in nature and occurs in the right concentration in growing tissues, then it would probably be safe to conclude that the naturally occurring active form of auxin has been found.

CONCLUSIONS

1. Two indoleacetic acid containing fractions - one water-soluble and one water-insoluble - have been isolated from mature sweet corn kernels and partially characterized.
2. The water-soluble fraction was further fractionated into four components: one containing indoleacetic acid and inositol; one containing indoleacetic acid, inositol, and glucose, and two containing indoleacetic acid, inositol, glucose, and arabinose. The indoleacetic acid is released from these compounds upon very mild alkaline hydrolysis.
3. The water-insoluble, aqueous acetone-soluble fraction, is a high molecular weight compound, containing 0.5 to 0.8 % indoleacetic acid and about 8 % nitrogen. This fraction yields indoleacetic acid upon very mild alkaline hydrolysis, and amino acids, and probably sugars and inositol, upon acid hydrolysis.
4. There is a minimum of 45 mg of indoleacetic acid in bound form per kilogram of mature sweet corn kernels.
5. The conjugation of indoleacetic acid with inositol is a new finding and could have physiological importance.

LITERATURE CITED

1. Andreae, W. A. Auxin metabolism and root growth inhibition. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 123: 559 (1963)

2. Andreae, W. A. and Good, N. E. The formation of indoleacetylaspartic acid in pea seedlings. Plant Physiol. 30: 380 (1955)

3. Andreae, W. A. and Good, N. E. Studies on 3-indoleacetic acid. IV. Conjugation with aspartic acid and ammonia as processes in the metabolism of carboxylic acids. Plant Physiol. 32: 566 (1957)

4. Andreae, W. A., Robinson, J. R. and Van Ysselstein, M. W. H. Studies on 3-indoleacetic acid metabolism. VII. Metabolism of radioactive 3-indoleacetic acid by pea roots. Plant Physiol. 36: 783 (1961)

5. Andreae, W. A. and Van Ysselstein, M. W. H. Studies on 3-indoleacetic acid metabolism. III. The uptake of 3-indoleacetic acid by pea epicotyls and its conversion to 3-indoleacetylaspartic acid. Plant Physiol. 31: 235 (1956)

6. Andreae, W. A. and Van Ysselstein, M. W. H. Studies on 3-indoleacetic acid metabolism. V. Effect of Ca ions on 3-indoleacetic acid uptake and metabolism by pea roots. Plant Physiol. 35: 220 (1960)

7. Andreae, W. A. and Van Ysselstein, M. W. H. Studies on 3- indoleacetic acid metabolism. VI. 3-indoleacetic acid uptake and metabolism by pea roots and epicotyls. Plant Physiol. 35: 225 (1960)

8. Audus, L. J. and Gunning, B. E. S. Growth substances in the roots of Pisum sativum. Physiol. Plantarum 11: 685 (1958)

9. Avery, G. S. and Berger, J. Tryptophan and phytohormone precursors. Science 98: 513 (1943)

10. Avery, G. S., Berger, J. and Shalucha, B. The total extraction of free auxin and auxin precursor from plant tissue. Amer. J. Bot. 28: 596 (1941)

11. Avery, G. S., Berger, J. and Shalucha, B. Total auxin extraction from wheat. Amer. J. Bot. 29: 612 (1942)

12. Avery, G. S., Berger, J. and Shalucha, B. Auxin content of maize kernels during ontogeny, from plants of varying heterotic vigor. Amer. J. Bot. 29: 765 (1942)

13. Avery, G. S., Berger, J. and Shalucha, B. Extent of auxin precursor hydrolysis in different Avena assay methods. Bot. Gazette 105: 364 (1944)

14. Avery, G. S., Creighton, H. B. and Shalucha, B. Extraction methods in relation to hormone content of maize endosperms. Amer. J. Bot. 27: 289 (1940)

15. Bailey, G. B. and Gentile, A. C. Indole compound synthesized by Diplodia natalensis. Plant Physiol. 37: 439 (1962)

16. Bennet-Clark, T. A. and Kefford, N. P. Chromatography of the growth substances in plant extracts. Nature 171: 645 (1953)

17. Bennet-Clark, T. A. and Wheeler, A. W. The metabolism of 3-indolyl-acetic acid in plant tissues. J. Exp. Botany 10: 468 (1959)

18. Bentley, J. A. The naturally occurring auxins and inhibitors. Ann. Rev. Plant Physiol. 9: 47 (1958)

19. Bentley, J. A. Role of plant hormones in algal metabolism and ecology. Nature 181: 1499 (1958)

20. Bentley, J. A. Chemistry of the native auxins. Encyc. Plant Physiol. XIV: 485 (1961)

21. Bentley, J. A. The states of auxin in the plant. Encyc. Plant Physiol. XIV: 609 (1961)

22. Berger, J. and Avery, G. S. Isolation of an auxin precursor and an auxin (indoleacetic acid) from maize. Amer. J. Bot. 31: 199 (1944)

23. Berger, J. and avery, G. S. Chemical and physiological properties of maize auxin precursor. Amer. J. Bot. 31: 203 (1944)

24. Bohling, H. Paper chromatography of the growth-stimulating and growth-inhibiting substances of the oat seedling and the scutellum of maize. Planta 53: 69 (1959)

25. Bose, S. and Guha, B. C. Studies on ascorbigen. I. Survey of the occurrence and distribution of ascorbigen in plants. Ann. Biochem. and Exp. Med. 23: 149 (1963)

26. Bressani, R. and Mertz, E. T. Studies on corn proteins. IV. Protein and amino acid content of different corn varieties. Cereal Chem. 35: 227 (1958)

27. Britton, G. and Housley, S. Chromatography of water-soluble auxins in immature corn kernels. Phyton 16: 1 (1961)

28. Britton, G., Housley, S. and Bentley, J. A. Studies in plant growth hormones. V. Chromatography of hormones in excised and intact roots of tomato seedlings. J. Exp. Botany 7: 239 (1956)

29. Cabid, E. Carbohydrate metabolism. Ann. Rev. Biochem. 32: 321 (1963)

30. Carter, J. E. and Street, H. E. Studies of the growth in culture of excised wheat roots. IV. The activation of DL-tryptophan by autoclaving. Physiol. Plantarum 16: 347 (1963)

31. Cutler, H. G. and Vlitos, A. J. The natural auxins of the sugar cane. II. Acidic, basic, and neutral growth substances in roots and shoots from 12 days after germination of vegetative buds to maturity. Physiol. Plantarum 15: 27 (1962)

32. Denffer, D. von, Behrens, M. and Fischer, A. Papierchromatographischer und papierelectrophoretischer Nachweis des β-Indolacetonitrils und des β-Indolaldehyds in Extrakten aus Kohlpflanzen. Naturwissenschaften 39: 550 (1952)

33. Deverall, B. J. and Daly, J. M. Metabolism of indoleacetic acid in rust diseases. II. Metabolites of carboxyl-labeled indoleacetic acid in tissues. Plant Physiol. 39: 1 (1964)

34. Fang, S. C., Theisen, P. and Butts, J. S. Metabolic studies of applied indoleacetic acid-1-C^{14} in plant tissue as affected by light and 2,4-D treatment. Plant Physiol. 34: 26 (1959)

35. Farrar, K. The Zeanins: a group of growth-promoting compounds from immature maize kernels. Biochem J. 83: 220 (1962)

36. Farrar, K., Bentley, J. A., Britton, G. and Housley, S. Auxins in immature maize kernels. Nature 181: 553 (1958)

37. Fawcett, C. H. Indole auxins. Ann. Rev. Plant Physiol. 12: 345 (1961)

38. Fawcett, C. H., Wain, R. L. and Wightman, F. Beta-oxidation of omega-(3-indolyl)alkanecarboxylic acids in plant tissues. Nature 181: 1387 (1958)

39. Feigl, F. and Gentil, V. In Spot tests in organic analysis, Feigl, F. p. 429. Elsevier Publishing Co. (1960)

40. Foster, J. F., Yang, J. T. and Yui, N. H. Extraction and electrophoretic analysis of the proteins of corn. Cereal Chem. 27: 477 (1950)

41. Frey, J. K. The interrelationships of proteins and amino acids in corn. Cereal Chem. 28: 123 (1951)

42. Funke, H. and Söding. H. Über das Wuchstoff-Hemmstoffsystem der Hafer-Koleoptile und der Kartoffelknolle. Planta 36: 341 (1948)

43. Galson, A. W., Jackson, P., Kaur-Sawhney, R., Kefford, N. P. and Meudt, W. J. Interactions of auxins with macromolecular constituents of pea seedlings. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 123: 251 (1963)

44. Gmelin, R. Occurrence, isolation, and properties of glucobrassicin and neoglucobrassicin. Colloq. Intern. Centre. Natl. Rech. Sci. (Paris) 123: 159 (1963)

45. Goertner, R. A. and MacDonald, R. T. Studies on the fractionation of zein. Cereal Chem. 21: 324 (1944)

46. Goldsmith, M. H. and Thimann, K. V. Some characteristics of movement of indoleacetic acid in coleoptiles of Avena. I. Uptake, destruction, immobilization, and distribution of indoleacetic acid during basipetal translocation. Plant Physiol. 37: 492 (1962)

47. Good, N. E. and Andreae, W. A. Malonyltryptophan in higher plants. Plant Physiol. 32: 561 (1957)

48. Good, N. E., Andreae, W. A. and Van Ysselstein, M. W. H. Studies on 3-indoleacetic acid metabolism. II. Some products of the metabolism of exogenous indoleacetic acid in plant tissues. Plant Physiol. 31: 231 (1956)

49. Gordon, S. A. Auxin-protein complexes of the wheat grain. Amer. J. Bot. 33: 160 (1946)

50. Gordon, S. A. Occurrence, formation, and inactivation of auxins. Ann. Rev. Plant Physiol. 5: 341 (1954)

51. Gordon, S. A. and Paleg, L. G. Formation of auxin from tryptophan through action of polyphenols. Plant Physiol. 36: 838 (1961)

52. Gordon, S. A. and Sanchez-Nieva, F. The biosynthesis of auxin in the vegetative pineapple. II. The precursors of indoleacetic acid. Arch. Biochem 20: 367 (1949)

53. Gordon, S. A. and Wildman, S. G. The conversion of tryptophan to a plant growth substance by conditions of mild alkalinity. J. Biol. Chem. 147: 389 (1943)

54. Grenot, C. Growth substances (indole derivatives) in the milky endosperm of corn. Compt. Rend. Acad. Sci. 251: 771 (1960)

55. Guttenberg, H. von, and Lehle-Joerges, E. Uber das Vorkommen von Auxin und Heteroauxin in ruhenden und keimenden Samen.

56. Haagen-Smit, A. J., Dandliker, W. B.; Wittwer, S. H. and Murneek, A. E. The isolation of indoleacetic acid from immature corn kernels. Amer. J. Bot. 33: 118 (1946)

57. Haagen-Smit, A. J., Leech, W. D. and Bergren, W. R. The estimation, isolation, and identification of auxins in plant materials. Amer. J. Bot. 29: 500 (1942)

58. Hamilton, R. H. I. Studies on plant growth substances. II. Isolation of the precursor of 6-methoxybenzoxazolinone in Zea mays. Thesis. Michigan State University. (1960)

59. Hamilton, R. H., Bandurski, R.S. and Grigsby, B. H. Isolation of indoleacetic acid from corn kernels and etiolated corn seedlings. Plant Physiol. 36: 354 (1961)

60. Hansch. C., Muir, R. and Metzenberg, R. Further evidence for a chemical reaction between plant growth-regulators and a plant substrate. Plant Physiol. 26: 812 (1951)

61. Hansen, D. W., Brimhall, B. and Sprague, G. F. Relationship of zein to the total protein content in corn. Cereal Chem. 23: 329 (1946)

62. Hatcher, E. S. J. Auxin production during development of the grain in cereals. Nature 151: 278 (1943)

63. Hatcher, E. S. J. Studies in the vernalization of cereals. IX. Auxin production during development and ripening of the anther and carpel of spring and winter rye. Ann. Bot. 9: 235 (1945)

64. Hatcher, E. S. J. and Gregory, F. G. Auxin production during the development of grain of cereals. Nature 148: 626 (1941)

65. Hemberg, T. Studies on the balance between free and bound auxin in germinating maize. Physiol. Plantarum 8: 418 (1955)

66. Hinsvark, O. N., Houff, W. H., Wittwer, S. H. and Sell, H. M. The Extraction and colorimetric estimation of indole-3-acetic acid and its esters in developing corn kernels. Plant Physiol. 29: 107 (1954)

67. Hochstrasser, K. Uber die Bindungsart von Kohlenhydrat an Protein in Gerstenalbumin. Isolierung von 4-L-alanyl-D-xylopyranose als Bruckenglied. Z. Physiol. Chem. 324: 250 (1961)

68. Hochstrasser, K. Uber die Bindungsart von Kohlenhydrat an Protein in Gerstenalbumin. II. Nachweis von O-glucosidpeptidbindungen. Z. Physiol. Chem. 328: 61 (1962)

69. Hochstrasser, K. Uber die Bindungsart von Kohlenhydrat an Protein in Gerstenalbumin. III. Nachweis von Asparagylglucosidbindungen. Z. Physiol. Chem. 333: 99 (1963)

70. Hough, L. p-Anisidine HCl for sugars. Nature 165: 400 (1950)

71. Housley, S. and Bentley, J. A. Studies in plant growth hormones. IV. Chromatography of hormones and hormone precursors in cabbage. J. Exp. Botany 7: 219 (1956)

72. Isogai, Y., Okamoto, T. and Koizumi, T. Isolation of growth regulating substances from Phaseolus seedlings. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 123: 143 (1963)

73. Jacobelli, G., Tabone, M. J. and Tabone, D. La synthese enzymatique de l'ester beta-glucosidique de l'acide anthranilique: Ses relations avec les processus de synthese et de degradation de l'uridine diphosphate glucose. Bull. Soc. Chim. Biol. 40: 955 (1958)

74. Jepson, J. B. Indolylacetyl-glutamine and other indole metabolites in Hartnup disease. Biochem. J. 64: 14P (1956)

75. Jepson, J. B. Indolylacetamide: a chromatographic artefact from the natural indoles, indolylacetylglucosiduronic acid and indolepyruvic acid. Biochem. J. 69: 22 (1958)

76. Kefford, N. P., Kaur-Sawhney, R. and Galston, A. W. Formation of a complex between a derivative of the plant hormone indoleacetic acid and ribonucleic acid from pea seedlings. Acta Chem. Scand. 17: 5313 (1963)

77. Klämbt, H. D. Indole-3-acetylaspartic acid, a naturally occurring indole derivative. Naturwissenschaften 47: 398 (1960)

78. Klämbt, H. D. Induction of growth and the metabolism of growth substances in the cylinder of the coleoptile of wheat. II. Products of the metabolism of indoleacetic acid and benzoic acid. Planta 56: 618 (1961)

79. Kögl, F., Erxleben, H. and Haagen-Smit, A. J. Uber die Isolierung der Auxins a und b aus pflanzlichen Materialen. IX. Z. Physiol. Chem. 225: 215 (1934)

80. Köves, E. and Sirokman, F. Occurrence of indole compounds containing phosphorus in etiolated pea seedlings. Nature 200: 910 (1963)

81. Kulescha, Z. Remarque sur l'emploi de trypsines pour l'extraction des substances de croissance contenues dans les tissues vegetaux. Compt. Rend. Soc. Biol. 142: 931 (1948)

82. Kuraishi, S. and Muir, R. M. Paper chromatographic study of diffusible auxin. Plant Physiol. 39: 23 (1964)

83. Kutacek, M. and Prochazka, Z. Determination and isolation of indole compounds in Cruciferae. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 123: 445 (1963).

84. Kutacek, M., Valenta, M. and Icha, F. Untersuchungen uber den Ascorbigengehalt von Kohlrabi (Brassica oleracea v. gongylodes) wahrend der Vegetation und den Zusammenhang zwischen Ascorbigen und Wachstum bei den Pflanzen der Familie Brassicaceae. Experientia 13: 284 (1957)

85. Larsen, P. Formation, occurrence, and inactivation of growth substances. Ann. Rev. Plant Physiol. 2: 169 (1951)

86. Larsen, P. Growth substances in higher plants. In Modern Methods of Plant Analysis, Vol. III. p. 565-625. (K. Paech and M. V. Tracey, Ed.) Springer Verlag. (1955)

87. Larsen, P., Harbo, A., Klungsoyr, S. and Aasheim, T. On the biogensis of some indole compounds in Acetobacter Xylinum. Physiol. Plantarum 15: 552 (1962)

88. Leopold, A. C. and Guernsey, F. S. A theory of auxin action involving Coenzyme A. Proc. Natl. Acad. Sci. 39: 1105 (1953)

89. Leopold, A. C. and Plummer, T. H. Auxin-phenol complexes. Plant Physiol. 36: 589 (1961)

90. Linser, H. Zur Methodix der Wuchsstoffbestimmung. II. Die Extraktion von Pflanzenmaterial. Planta 29: 392 (1939)

91. Luckwill, L. C. The hormone content of the seed in relation to endosperm development and fruit drop in the apple. J. Hort. Sci. 24: 32 (1948)

92. Luckwill, L. C. Studies of fruit development in relation to plant hormones. I. Hormone production by the developing apple seed in relation to fruit drop. J. Hort. Sci. 28: 14 (1953)

93. Lund, H. A. Growth hormones in the style and ovaries of tobacco responsible for fruit development. Amer. J. Bot. 43: 562 (1956)

94. Ma, T. S. and Zuazaga, G. Micro-Kjeldahl determination of nitrogen. Ind. and Eng. Chem. 14: 280 (1942)

95. Mason, I. D. and Palmer, L. S. Preparation of white zein from yellow corn. J. Biol. Chem. 107: 131 (1934)

96. Meudt, W. J. and Galston, A. W. Binding of an indoleacetic acid metabolite to macromolecules of pea plants. Federation Proc. 21: 399 (1962)

97. Meudt, W. J. and Galston, A. W. Binding of an indole-3-acetic acid metabolite to the RNA of peas. Plant Physiol. (Suppl) 37: xiv (1962)

98. Meyer, E. Uber den Wuchsstoff-Hemmstoffgehalt gesunder und abbaukranker Kartoffelknollen. Planta 38: 213 (1950)

100. Muir, R. M. The relationship of growth hormones and fruit development. Proc. Natl. Acad. Sci. 33: 303 (1947)

101. Müller, F. Uber den Abbau von synthetischen Indolverbindungen durch Blumenkohlrosengewebe. Planta 57: 481 (1961)

102. Murakami, Y. and Hayashi, T. Conversion of tryptophane to indoleacetic acid by the sap of immature kernels of rice plants. Nippon Nogei-Kagaku Kaishi 31: 468 (1957)

103. Nakamura, T., Ishii, H. and Yamaki, T. Intracellular localization of native auxin in Avena coleoptile. Plant Cell Physiol. (Tokyo) 3: 149 (1962)

104. Nitsch, J. P. Growth and morphogenesis of the strawberry as related to auxin. Amer. J. Bot. 37: 211 (1950)

105. Nitsch. J. P. Plant hormones in the development of fruits. Quart. Rev. Biol. 27: 33 (1952)

106. Nitsch. J. P. and Nitsch. C. An artifact in chromatography of indolic auxins. Plant Physiol. 35: 450 (1960)

107. Oberbeek, J. van. A quantitative study of auxin and its precursor in coleoptiles. Amer. J. Bot. 28: 1 (1941)

108. Oberbeek, J. van. Hormonal control of embryo and seedling. Cold Spring Harbor Symp. Quant. Biol. 10: 126 (1945)

109. Oberbeek, J. van, deVazquez, E. S. and Gordon, S. A. Free and bound auxin in the vegetative pineapple plant. Amer. J. Bot. 34: 266 (1947)

110. Pal, J. C. and Guha, B. C. Combined ascorbic acid in plant foodstuffs. Part I. J. Indian Chem. Soc. 16: 481 (1939)

111. Partridge, S. M. Aniline salts for reducing sugars. Nature 164: 443 (1949)

112. Pilet, P. E. Les Phytohormones de Croissance. Masson et Cie., Ed. Paris. (1961)

113. Polevoi, V. V. Dynamics of 3-indoleacetic acid in ripening and sprouting corn grains. Doklady Akad. Nauk. S.S.S.R. 124: 695 (1959)

114. Pollard, J. K., Shantz, E. M. and Steward, F. C. Hexitols in coconut milk: Their role in nurture of dividing cells. Plant Physiol. 36: 492 (1961)

115. Putnam, A. R. Tests for biological activity of an indoleacetic acid complex. Unpublished report. (1962)

116. Raadts, E. and Söding, H. Chromatographische Untersuchungen uber die Wuchsstoffe der Haferkoleoptile. Planta 49: 47 (1957)

117. Raussendorff-Bargen, G. von. Indolderivate im Apfel. Planta 58: 471 (1962)

118. Row, V. V., Sanford, W. W. and Hitchcock, A. E. Indole-3-acetyl-D,L-aspartic acid as a naturally occurring indole compound in tomato seedling. Contr. Boyce Thompson Inst. 21: 1 (1961)

134. Street, H. E., Butcher, D. N., Handoll, C. and Winter, A. Natural regulators of root growth. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 123: 529 (1963)

135. Sudi, J. Induction of the formation of complexes between aspartic acid and indoleacetic acid or 1-naphthaleneacetic acid by other carboxylic acids. Nature 201: 1009 (1964)

136. Tandler, C. J. A naturally occurring crystalline indolyl derivative in Acetabularia. Naturwissenschaften 49: 213 (1962)

137. Tandler, C. J. Bound indoles in Acetabularia. Planta 59: 91 (1962)

138. Terpstra, W. Chromatographic identification of the growth substances extracted from Avena coleoptile tips. Proc. Koninkl. Ned. Akad. Wetenschap. 56: 206 (1953)

139. Terpstra, W. Extraction and identification of growth substances, with special reference to the Avena coleoptile tip. Thesis. Botanical Laboratory. Utrecht. (1953)

140. Thimann, K. V. and Skoog, F. The extraction of auxin from plant tissues. Amer. J. Bot. 27: 951 (1940)

141. Thimann, K. V., Skoog, F. and Byer, A. C. The extraction of auxin from plant tissues. II. Amer. J. Bot. 29: 598 (1942)

142. Thurman, D. A. and Street, H. E. Metabolism of some indole auxins in excised tomato roots. J. Exp. Botany 13: 369 (1962)

143. Trevelyan, W. E., Procter, D. P. and Harrison, J. S. $AgNO_3$ for reducing saccharides. Nature 166: 444 (1950)

144. Vlitos, A. J. and Meudt, W. The role of auxin in plant flowering. I. A quantitative method based in paper chromatography for the determination of indole compounds and of 3-indoleacetic acid in plant tissues. Contrib. Boyce Thompson Inst. 17: 197 (1953)

145. Vlitos, A. J., Meudt, W. and Beimler, R. The role of auxin in plant flowering. IV. A new unidentified naturally occurring indole hormone in normal and gamma irradiated Maryland Mammoth tobacco. Contrib. Boyce Tompson Inst. 18: 283 (1956)

146. Vlitos, A. J., Meudt, W. and Beimler, R. A new unidentified hormone in Maryland Mammoth tobacco. Nature 177: 890 (1956)

147. Wightman, F. Metabolism and biosynthesis of 3-indoleacetic acid and related indole compounds in plants. Can. J. Botany 40: 689 (1962)

148. Wildman, S. G. and Bonner, J. The proteins of green leaves. I. Isolation, enzymatic properties and auxin content of spinach cytoplasmic proteins. Arch. Biochem. 14: 381 (1947)

119. Saifer, A. and Gerstenfeld, S. The photometric microdetermination of blood glucose with glucose oxidase. J. Lab. Clin. Med 51: 448 (1958)

120. Schoken, V. The genesis of auxin during the decomposition of proteins. Arch. Biochem. 23: 198 (1949)

121. Scott, T. K. and Jacobs, W. P. Critical assessment of techniques for identifying the physiologically significant auxins in plants. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 123: 457 (1963)

122. Sen-Gupta, P. N. and Guha, B. C. Combined ascorbic acid in plant foodstuffs. Part II. J. Indian Chem. Soc. 16: 496 (1939)

123. Shantz, E. M. and Steward, F. C. The growth-stimulating substances in extracts of immature corn grain: a progress report. Plant Physiol. 32 (Suppl):viii (1957)

124. Siegel, S. M. and Galston, A. W. Experimental coupling of indoleacetic acid to pea root protein in vivo and in vitro. Proc. Natl. Acad. Sci. 39: 1111 (1953)

125. Sircar, S. M. and Das, T. M. Growth hormones of rice grains germinated at different temperatures. Nature 168: 382 (1951)

126. Skoog, F. and Thimann, K. V. Enzymatic liberation of auxin from plant tissues. Science 92: 64 (1940)

127. Sprince, H., Parker, C., Dawson, J. T., Jameson, D. and Dohan, F. C. Paper chromatography of urinary indoles extracted under alkaline and acid conditions. J. Chromat. 8: 457 (1962)

128. Srivastava, B. I. S. Ether-soluble and ether-insoluble auxins from immature corn kernels. Plant Physiol. 38: 473 (1963)

129. Srivastava, B. I. S. The auxins in immature corn kernels. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 123: 179 (1963)

130. Stehsel, M. L. and Wildman, S. G. Interrelations between tryptophan, auxin and nicotinic acid during development of the corn kernel. Amer. J. Bot. 37: 682 (1950)

131. Steward, F. C. and Shantz, E. M. The chemical regulation of growth. (Some substances and extracts which induce growth and morphogenesis) Ann. Rev. Plant Physiol. 10: 379 (1959)

132. Stowe, B. B. The production of indoleacetic acid by bacteria. Biochem. J. 61: ix (1955)

133. Stowe, B. B. and Thimann, K. W. The paper chromatography of indole compounds and some indole-containing auxins of plant tissues. Arch. Biochem. Biophys. 51: 499 (1954)

149. Wildman, S. G. and Bonner, J. Observations on the chemical nature and formation of auxin in the Avena coleoptile. Amer. J. Bot. 35: 740 (1948)

150. Wildman, S. G., Campbell, J. and Bonner, J. The proteins of green leaves. II. Auxin, adenine, pentose and phosphorus content of spinach fraction I. Amer. J. Bot. 35: 813 (1948)

151. Wildman, S. G., Campbell, J. and Bonner, J. The proteins of green leaves. II. Purine, pentose, total phosphorus and acid-labile phosphorus of the cytoplasmic proteins of spinach leaves. Arch. Biochem. 24: 9 (1949)

152. Wildman, S. G., Ferri, M. G. and Bonner, J. The enzymatic conversion of tryptophan to auxin by spinach leaves. Arch. Biochem. 13: 131 (1947)

153. Wildman, S. G. and Gordon, S. A. The release of auxin from isolated leaf proteins of spinach by enzymes. Proc. Natl. Acad. Sci. 28: 217 (1942)

154. Winter, A. and Street, H. E. A new natural auxin isolated from "Staled" root culture medium. Nature 198: 1283 (1963)

155. Winter, A. and Thimann, K. V. Bound auxin. Plant. Physiol. (Suppl) 39: xvii (1964)

156. Wolf, M. J., Buzan, C. L., MacMasters, M. M. and Rist, C. E. Structure of the mature corn kernel. II. Microscopic structure of pericarp, seed coat, and hilar layer of dent corn. Cereal Chem. 29: 334 (1952)

157. Wolf, M. J., Buzan, C. L., MacMasters, M. M. and Rist, C. E. Structure of the mature corn kernel. III. Microscopic structure of the endosperm of dent corn. Cereal Chem. 29: 349 (1952)

158. Wolf, M. J., Buzan, C. L., MacMasters, M. M. and Rist, C. E. Structure of the mature corn kernel, IV. Microscopic structure of the germ of dent corn. Cereal Chem. 29: 362 (1952)

159. Yamaki, T. Intracellular localization of native auxins and gibberellins. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 123: 319 (1963)

160. Yamaki, T. and Nakamura, K. Formation of indoleacetic acid in maize embryo. Sci. Papers Coll. Gen. Educ. Univ. Tokyo 2: 81 (1952)

161. Zeleny, L. The distribution of nitrogen in the seed of Zea mays at different stages of maturity. Cereal Chem. 12: 536 (1935)

162. Zenk, M. H. Enzymatische Aktivierung von Auxinen und ihre Konjugierung mit Glycin. Z. Naturforsch. 15: 436 (1960)

163. Zenk, M. H. 1-(Indole-3-acetyl)-β-D-Glucose, a new compound in the metabolism in indoleacetic acid in plants. Nature 191: 493 (1961)

164. Zenk, M. H. Aufnahme und Stoffwechsel von α-Naphthylessigsaure durch Erbenepicotyle. Planta 58: 75 (1962)

165. Zenk, M. H. Isolation, biosynthesis, and function of indoleacetic acid conjugates. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 123: 241 (1963)

166. Zenk, M. H. and Scherf, H. D-Tryptophan in hoheren Pflanzen. B. B. Acta 71: 737 (1963)

167. Zinovev, L. S. and Naumova, N. P. Dynamics of 3-indoleacetic acid and the activity of some enzymes in the germinating corn. Leningrad (1959) C. A. 55: 13560b (1961)