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THE PHYSIOLOGY AND ANALYSIS OF INDOLE-3-ACETIC ACID AND ITS *MYO*-INOSITOL ESTERS

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Jerry David Cohen

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Robert S. Bandurski
Major professor

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THE PHYSIOLOGY AND ANALYSIS OF INDOLE-3-ACETIC ACID AND ITS *MYO*-INOSITOL ESTERS

By

Jerry David Cohen

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ABSTRACT

THE PHYSIOLOGY AND ANALYSIS OF
INDOLE-3-ACETIC ACID AND ITS
MYO-INOSITOL ESTERS

By

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I. *The Bound Auxins: Protection of Indole-3-acetic Acid from Peroxidase-catalyzed Oxidation.* Indole-3-acetic acid (IAA) was oxidized by horseradish peroxidase, but ester and amide conjugates of IAA were not degraded. Addition of indoleacetyl-*myo*-inositol, indoleacetyl-L-aspartate, indoleacetylglycine, indoleacetyl-L-alanine, indoleacetyl-D-alanine, or indoleacetyl-β-alanine did not affect the rate of oxidation of IAA by horseradish peroxidase. Peroxidase preparations from *Pisum sativum* L. and *Zea mays* L. behaved similarly in that they rapidly oxidized IAA, but not conjugates found in the plant from which the peroxidase was prepared. These results indicate that conjugation could affect the stability of IAA in vivo.

II. *Photo-regulation of the Ratio of Ester to Free Indole-3-acetic Acid.* A light exposure, sufficient to cause a 30% reduction in growth rate of seedlings of *Zea mays*, causes a *decrease* of 40% in the concentration of free indole-3-acetic acid in the seedling and an *increase* in the content of esterified indole-3-acetic

acid. It is concluded that one mechanism for regulation of plant growth is alteration of the ratio of free to conjugated indole-3-acetic acid by environmental stimuli.

III. *Synthesis of ^{14}C-Indole-3-acetyl-myo-inositol.* Synthesis of the mixed isomeric ^{14}C-indoleacetyl-*myo*-inositols from carrier-free β[2-^{14}C]-indoleacetic acid (57.2 mCi/mmole) and inositol *via* an imidazolide intermediate is described. Radiological decomposition of the indolylic compounds was prevented by the use of a volatile thiol, dithioethane, and anthracene.

IV. *Automated Analysis of Indolylic Compounds in Plant Extracts.* An automated procedure for analysis of indolylic compounds has been developed utilizing a chromogenic reagent yielding stable chromogens with E^{m}_{cm} = 24,000. Coupling this detection procedure to an adsorption chromatographic system of sulfonated polystyrene divinylbenzene, such as used in automated amino acid analysis, permitted separation, detection and assay of indoles in crude extracts of kernels of *Zea mays*. Analysis of the major neutral and acidic indoles in crude extracts was accomplished in less than 3 hours, where previous methods required several days. Adaptability of this method to other tissues and column chromatographic systems is described. These methods describe the first versatile indole analyzer useable with crude plant extracts. The analyzer should have significant applications in assays of indoles in both plant and animal extracts.

V. *Double Standard Isotope Dilution Assay I. Quantitative Assay of Indole-3-acetic Acid.* Isotope dilution analysis for the quantitation of labile compounds has been limited by the amount of sample necessary to redetermine specific activity. A method is described for the analysis of radiolabeled compounds which allows the direct determination of specific activity by gas chromotography. It requires the availability of the radiolabeled compound to be analyzed and also requires a chemically-related radiolabeled compound. The method is illustrated by assaying indole-3-acetic acid in plant extracts using ^{14}C-indole-3-acetic acid and adding ^{14}C-indole-3-butyric acid at the final stage of analysis prior to gas chromatography. Used with a nitrogen specific thermionic detector the method is selective and is sensitive at the nanogram level. The synthesis of ^{14}C-[2-ring]-indole-3-butyric acid is also described.

To my parents, my brother, my sister, and my wife;

together we share troubled and joyful times.

ACKNOWLEDGMENTS

For his support, enthusiasm and friendship I will always fondly remember my major professor, Dr. Robert S. Bandurski. His constant drive to understand biochemical systems should serve as an inspiration to all scientists. Dr. Norman E. Good has, in addition to serving on my committee, provided helpful discussions, showed concern and offered advice for which I am indebted to him. The other members of my guidance committee, Drs. Robert P. Scheffer and Philip Filner, provided patient and scholarly advice for which I remain most grateful. Dr. Axel Ehmann provided an early autocatalytic introduction to indole chemistry which led to many of these investigations.

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Volker was both a pleasure and an honor. Aga Schulze, a true pioneer in the quantitative analysis of indole-3-acetic acid in plants, has served as my teacher, colleague and friend during this thesis work. I have also benefited greatly from knowing many graduate students, postdocs and faculty at M.S.U. and only regret that space does not permit listing them all by name.

I thank Bill Richards, Gail Ryder and all of the delightful people at the stores order desk for decreasing the time and effort required to complete this research.

Lastly, I wish to express my love and thanks to my wife Susan who has worked with me to complete this dissertation.

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INTRODUCTION

Indole-3-acetic acid (IAA) is the major plant growth hormone ("auxin" (Kögl and Haagen-Smit, 1931)) found in higher plants (cf. review by Gordon, 1954). Studies on auxins have been published for well over 100 years--predating even the first use of the word "hormone" (Starling, 1905) by 30 years--and yet its biosynthesis, degradation, and mode of action are still topics of research and debate. Indeed, the unequivocal demonstration of the presence of IAA in plant tissue occurred only in this decade (cf. Ueda et al., 1970; Greenwood et al., 1972) even though the total number of scientific papers on auxins probably now numbers nearly 10,000.

The bulk of the IAA in all plants appears to be linked by either an ester or amide linkage to other compounds (Bandurski and Schulze, 1977b), although few of these compounds have been identified. At the time this study was initiated (1974/1975) little was known about the function of these conjugates of IAA, although earlier workers in this laboratory had identified all of the compounds in kernels of *Zea mays* down to a level of 10 µg/kg dry wt. (cf. review by Ehmann, 1973). This study was initiated, therefore, with the objective of elucidating the function of the covalently linked conjugates of IAA in the normal hormonal physiology of corn seedlings. During the course of this work it became apparent that

continued progress in understanding hormonal biochemistry required faster and more sensitive methods for analysis with the requisite selectivity. Development of some of these methods, therefore, forms a major part of this research. The experimental work is in the form of five individual research papers, three of which have been published, while the other two are in manuscript form.[1]

[1]The second and third reprints are included only for the convenience of the reader. Parts I, IV, and V constitute the 'corpus' of the dissertation. As Albert Einstein has said in discussing university administrators (Anon., 1979), "Let elephants live."

LITERATURE REVIEW

I. Oxidation of Indole-3-acetic Acid

For a hormone to regulate growth it must be limiting and the maximum rate of growth is determined by the supply of the hormone. Thus, it is necessary for the plant to destroy IAA once it has performed its growth promoting act. These relationships have been anticipated previously (Bonner and Thimann, 1935; Galston and Hillman, 1961) and have formed the theoretical basis for a wealth of studies on the peroxidative oxidation of IAA (cf. reviews by Galston and Hillman, 1961; Hare, 1964).

Thimann (1934) provided the first evidence that plant tissues can inactivate auxin. He incubated *Vicia faba* and *Helianthus* leaf extracts in auxin solutions and showed decreased levels of auxins, while leaves of *Malva*, having lower levels of polyphenol oxidase, were less active in auxin destruction. Van Overbeek (1935) correlated auxin levels with the rate of which corn tissues degraded auxin. These early experiments led to a number of correlative studies which now predominate in the literature on auxin destruction (see review by Hare, 1964).

The first careful study of the nature of the auxin destroying enzyme in plant extracts was that of Tang and Bonner (1947), although Larsen (1936, 1940) earlier showed that the "auxin inactivating substance" was enzymatic and required oxygen for activity.

Tang and Bonner further characterized this activity and showed that the enzyme was an iron metalloprotein which oxidized IAA in a manner which retained the indole nucleus. They also established the specific nature of the side chain involvement by showing that indoleacetamide, indolepropionic acid, indolebutyric acid and tryptophan were not oxidized and showed that other auxins did not inhibit IAA oxidation. Galston et al. (1953) extended these studies to show that the enzyme from peas which Tang and Bonner described was in fact a peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) and that crystalline horseradish root peroxidase also catalyzed the oxidation of IAA in the presence of H_2O_2.

The enzymatic degradation of IAA by horseradish as well as other plant peroxidases has subsequently received considerable attention (see review by Ray, 1958) and a proposed pathway has been suggested by Hinman and Lang (1965) based on spectrophotometric and chemical evidence. The oxidation of IAA is concentration dependent (Hinman and Lang, 1965) and below 2×10^{-4} M the reaction yields predominately 3-methylene oxindole, whereas at higher concentrations the principal product is a neutral indole which has the properties of an ester between IAA and indole-3-carbinol. The formation of the 3-methylene oxindole is probably accomplished (Hinman and Lang, 1965; Yamazaki et al., 1977) by peroxidase acting as a one-electron oxidizing agent and forming indolenine hydroperoxide as the first intermediate. This is then converted to an indolenine epoxide which goes on to oxindole-3-carbinol. The carbinol then undergoes a slow conversion to 3-methylene oxindole. BeMiller and Colilla (1972)

proposed another mechanism in which the enzyme catalyzes a two electron oxidation and results in the formation of a cyclic 5-membered peroxide at ring carbons 2 and 3. The peroxide rearranges to the β-keto alcohol which converts to 3-methylene oxindole spontaneously. BeMiller and Colilla also propose a skatyl peroxide as an intermediate in the formation of the minor product, indole-3-aldehyde, while according to Hinmann and Lang (1965) this is formed by the addition of a proton to the epoxide. The Hinman and Lang (1965) proposal is more consistent with known indole chemistry since oxidation at the 2-position usually results in ring-opening and because attack at the electron dense 3-position should be favored. Both proposals involve as a first step a decarboxylation which would be inhibited by esterification or amide linkage at the carboxylic acid (Hamilton et al., 1976; Cohen and Bandurski, 1978). Hinman and Lang (1965) report IAA-ethyl ester oxidation at a very slow rate, but this could be due to an internal ester hydrolysis. By whatever route IAA is oxidized by peroxidase it represents a rare example of indole oxidation not involving cleavage of the hetero ring.

In the few studies in which the contribution of the decarboxylation route to IAA destruction has been studied *in vivo* it appears to be a minor route, accounting for only 15-30% of the IAA degradation (Davies, 1973; Epstein and Bandurski, 1978). The idea that peroxidase oxidation of IAA may simply be an artifact of cutting or homogenization (Briggs et al., 1955; Bonner, 1957) certainly cannot be excluded. The peroxidative route, however, probably becomes more important during injury (Haard and Marshall,

1976), tissue aging (Galston and Dalberg, 1954; Pilet and Galston, 1955), in tissue culture (Epstein et al., 1975) at cut surfaces and during tissue homogenization (Went, 1928), and when tissue is subjected to large amounts of exogenous IAA (Galston and Dalberg, 1954). It would appear that a peroxidative decarboxylation is the major degradative pathway for IAA provided to tissue cultures since Epstein et al. (1975) showed 90% decarboxylation in 4 hrs with 3 month old apple callus. Hamilton et al. (1976) demonstrated that 53% of the IAA supplied to cultures of *Parthenocissus* was decarboxylated within 48 hrs and they showed that labeled products of peroxidase degradation of IAA were found in the tissue and media. Substrates such as indole-3-propionic acid, indole-3-buytric acid, IAA-ethyl ester, tryptamine, and tryptophan are usually found to be relatively immune to peroxidative attack (Tang and Bonner, 1947; Hinman and Lang, 1965) and the prolonged efficacy of some synthetic auxins, as well as naturally occurring 4-chloro-IAA, has been attributed to this resistance (Marumo et al., 1974; Thimann, 1977). This may well be true in some systems like tissue culture and rooting, but the evidence in many studies is ambiguous since IAA is also more easily oxidized by chelate formation with metals (Hinman and Lang, 1965).

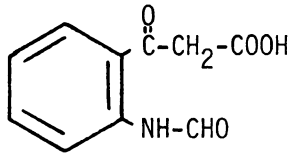
Whether peroxidase and "IAA oxidase" activity always reside on the same enzyme is still unresolved, although recent work with high resolution isoelectric focusing and electrophoresis has begun to answer this question. Hoyle (1977) has shown that commercial horseradish peroxidase preparations contain 42 isoenzymes, all with

both activities. It would appear, therefore, that all horseradish peroxidases are capable of IAA oxidation, although several earlier reports of plant peroxidase without IAA oxidizing activity have appeared (Endo, 1968; Yoneda and Endo, 1969; Sahulka, 1970; Frenkel, 1972). Are there enzymes, however, which oxidize IAA by a similar manner as peroxidase but which lack peroxidase activity? This is difficult to evaluate since much of the work on IAA oxidizing systems utilize methods which simply measure the loss of IAA without regard for the products produced. Several papers, however, have reported separation of IAA degrading activities which do not have peroxidative activity when tested with one or more model redox dye systems (Sequeira and Mineo, 1966; Van der Mast, 1969; Bryant and Lane, 1979). These observations may have their explanation in the differential sensitivity of the assays employed.

Aside from peroxidase catalyzed oxidation other possible degradative routes for IAA have not been closely examined. However, on purely chemical grounds (Remers, 1972; Smith, 1972), ring opening between $C_{(2)}$ and $C_{(3)}$ might be a likely route. Nair and Vaidyanathan (1964) have described an indole 2,3-dioxygenase (indole:oxygen 2,3-oxidoreductase E.C. 1.13.11.17) from leaves of *Tecoma stans* which adds O_2 across $C_{(2)}$ and $C_{(3)}$ of indole to give N-formyl-*o*-aminobenzaldehyde. This same enzymatic activity is also present in leaves of *Zea mays* (Chauhan et al., 1978) and is analogous to the reaction of tryptophan 2,3 dioxygenase (L-tryptophan:oxygen 2,3 oxidoreductase E.C. 1.13.11.11) which forms L-formylkynurenine from L-tryptophan (Tanaka and Knox, 1959)

and it is also analogous to the indoleamine 2,3 dioxygenase described by Hirata et al. (1977). Indole oxidizing enzymes of *Tecoma* and *Zea* are cuproflavoproteins whereas the tryptophan and indoleamine 2,3 dioxygenases are heme metalloproteins. Both the *Tecoma* and *Zea* enzymes appear to give anthranil (2,1-benzisooxazole) as the final product, suggesting the formation of *o*-aminobenzaldehyde from N-formyl-*o*-aminobenzaldehyde and the further oxidation to anthranil. Anthranilic acid was also identified as a product of the *Zea* enzyme although it apparently is not formed by the enzyme from *Tecoma*.

If IAA were metabolized in an analogous reaction to that catalyzed by indole-2,3-dioxygenase then one might expect a product such as N-formyl-*o*-aminobenzoylacetic acid:



This could be metabolized to provide entry into the C_6-C_3 phenylpropanoid pathways (cf. Geissman and Crout, 1969). A less likely route for IAA degradation would be removal of the side chain in a reaction similar to that catalyzed by tryptophanase (Happold, 1950) (L-tryptophan indole-lyase E.C. 4.1.99.1) to yield free indole which could proceed as recounted above. Kinashi et al. (1976) have

suggested another oxidation pathway which may operate in, at least, rice bran. This pathway, based on isolation of some of the intermediates, would involve oxidation to a dioxindole and its ring expansion to a β-acid. At some point hydroxylation at indole ring carbon 5 would take place and the amide, ethanolamide and free acid of the hydroxy β-acid have been identified. The relative contribution of this pathway to the degradation of the endogenous IAA in rice bran was not determined. Clearly the *in vivo* degradation of IAA will be an important subject of future research, especially those as yet poorly understood pathways which do not decarboxylate and which apparently account for the bulk of the IAA which is degraded.

II. Environmental Effects on Indole-3-acetic Acid Levels

The early work leading to the concept of hormone involvement in tropic behavior has been reviewed (Boysen-Jensen, 1936; Went and Thimann, 1937; van Overbeek, 1939) and recently reexamined (Ehmann, 1973). Duhamel du Monceau (1758), Knight (1806), De Candolle (1832) and Frank (1868, see also Rawitscher, 1932) observed and experimented with plant tropistic behavior in response to light and gravity. However, it was Ciesielski (1872) and later Darwin (1880) who began the detailed study of the nature of the tropic responses. Ciesielski's methods, that is the removal of the tip and its replacement with the test material, has been the basis for many of the studies on tropic behavior and these methods are still in use (cf. Vanderhoef and Briggs, 1978; Vanderhoef et al., 1979). Ciesielski showed that removal of the root tip prevented growth and geotropism and he showed that replacement restored these properties. It was Blaauw (1918), however, who showed that phototropic bending was a function of the energy received by the plant and he correctly interpreted the tropic response as a result of differential growth. Boysen-Jensen (1910, 1913) showed the same effects for the light stimulus in coleoptiles as Ciesielski demonstrated for geotropism and further demonstrated that insertion of a thin piece of mica into a cut on the illuminated side did not prevent curvature but that insertion on the opposite side inhibited the phototropic response. If a block of gelatin was used in place of the mica, however, normal tropic bending occurred. Paal (1919) confirmed these results and showed that the substance

which could diffuse through gelatin but not through the mica sheet was also important in normal straight growth. Paal postulated, based on these experiments, that a substance moves from the tip and produces growth in the tissue below.

In a series of 17 papers from 1918-1935 Cholodny (1924 and see review by Cholodny, 1935a) began to develop the concept of the mechanism of tropic behavior which, as refined by Went (1926), became known as the Cholodny-Went theory of tropisms. This theory stated that curvature of plant organs was a consequence of a light or gravity induced lateral diversion of auxin in the apical region of the organ. Since the amounts of auxin reaching the two sides of a rapidly elongating region would differ, the growth rates of the two sides would also differ and curvature would result. Evidence for the lateral diversion of auxin by geotropic induction was first provided by Dolk (1936), but whether a lateral redistribution of IAA is involved is still unclear.

Briggs (1964) reviewed four theories for phototropic curvature which have played an important role in the understanding of phototropism. These theories are: (1) light induced changes in the tissue's ability to grow (perhaps in response to auxin (Blaauw, 1918)); (2) the light induction of lateral transport (Cholodny, 1935a); (3) the inactivation of auxin (Galston et al., 1953; Reinert, 1953; Brauner, 1953); and (4) the inactivation of some component of the auxin-synthesizing system (Galston, 1950, 1959). These same basic arguments have also been applied to geotropism, although the relationship between the two tropic

responses is still uncertain. Support for lateral transport in photo-induced plants has been provided by Went (1928), van Overbeek (1933), Asana (1938) and Wildin (1939) who used bioassay techniques to quantitate the auxin transported into receiver blocks at the bissected base of coleoptiles. However, the effect of light shown by these researchers was not only a redistribution of auxin but also a net decrease in total amount, possibly due to the red component in the white light used for illumination (Briggs, 1964). Although subsequent repetition of this work by Briggs et al. (1957) and Briggs (1963a) with better defined conditions has resulted in a smaller difference, this decrease is still unexpected since Went and Thimann (1937, also see Cholodny, 1929) indicate that total growth was the same in tropic stimulated and unstimulated coleoptiles.

A more serious challenge to these early experiments came with the use of radiolabeled IAA to study tropism. Numerous groups (Bunning et al., 1956; Gordon and Eib, 1956; Reisener, 1957, 1958; Ching and Fang, 1958; Reisener and Simon, 1960; also see below) were unable to show a lateral redistribution of ^{14}C-labeled IAA following either geotropic or phototropic induction. Pickard and Thimann (1964) were able to demonstrate a lateral redistribution of ^{14}C-IAA both in agar receiver blocks and in the tissue, however only for the light exposures in the region of the first positive curvature were the data significant. Since the first positive curvature is morphologically limited and is a protracted response (Briggs, 1964) which is absent in coleoptiles of some species

(Asomaning and Galston, 1961), it is the region of the second positive curvature which is somewhat more interesting. In those experiments with the second positive curvature Pickard and Thimann showed only a 46.4% distribution on the lighted side for oat coleoptiles and 46.1% for corn coleoptiles. Since they report recoveries of ^{14}C-IAA of 98-106% it is safe to consider a redistribution of less than 8% to be insignificant. Shen-Miller and Gordon (1966) and Naqvi and Gordon (1967) used techniques similar to those employed by Pickard and Thimann except that they purified the IAA by solvent extraction and paper chromatography prior to counting and they bioassayed the radioactive fraction to confirm the distribution. The contribution of IAA resulting from hydrolysis of conjugates is difficult to estimate in these studies since they used cold ether extraction. However, the Shen-Miller and Gordon (1966) experiments show that, although the receiver blocks show a redistribution, an equal but opposite redistribution is found in the tissue. The sum of the IAA in the tissue and receiver block on the illuminated and shaded sides of the coleoptile is a constant. Thus, the results of these experiments are inconsistent with the theory of Cholodny-Went and suggest some mechanism which retards ^{14}C-IAA movement through the shaded side of the coleoptile.

Bruinsma et al. (1975) used the fluorescence of the indolo-α-pyrone derivative to study endogenous IAA in sunflower

seedlings.[2] Although this analytical method is as yet unverified by more rigorous methods, they were able to confirm earlier bioassay work which showed no lateral redistribution in this tissue (Blaauw, 1918; Went and Thimann, 1937). Wright et al. (1978) also used this analytical method to study IAA levels in grass nodes (see also early bioassay data of Schmitz, 1933). The findings of Wright et al. indicate that although a differential is noted in upper and lower halves, the same distribution is obtained when the segments are split prior to geostimulation. Thus, in this tissue contiguity is unnecessary (see also Gradmann, 1925; Firn and Digby, 1977) and they attribute the effect to "gravity-controlled input evoking changes in IAA metabolism within small groups of cells of the leaf sheath base: perhaps an increase in rate of synthesis or decrease in the rate of degradation or conjugation of IAA in the lower sides with decreased synthesis or increased degradation or conjugation in the upper sides"

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[2] *Helianthus* hypocotyls and, to a lesser extent grass nodes, have long been a source of data which conflicts with the Cholodny-Went theory of tropisms. Went and Thimann (1937, p. 161) note after describing the tropic behavior of *Helianthus* and nodes of grasses, that the result ". . . does not, however, agree with the Cholodny-Went theory . . ." and "It must be left for the present as an unexplained curiosity." Explanations for these inconsistencies involving gibberellins as the tropic hormone (Krass and Vardar, 1962; Phillips, 1972) have not been confirmed in subsequent work (Phillips and Hartung, 1976; Firn et al., 1977). It has been shown that only the outer cell layers of *Helianthus* hypocotyls respond to auxins (Mentze et al., 1977; Firn and Digby, 1977), which is not dissimilar from grass coleoptiles where the central cylinder is hollow. Many other dicotyledenous stems behave similarly (Cholodny, 1926; Iwami and Masuda, 1974; Mentze et al., 1977) and a hollow cylinder produced with a cork borer will exhibit normal tropic behavior.

(Wright et al., 1978). Clearly, while they were able to exclude the Cholodny-Went theory as an explanation they were unable, with the data they obtained, to distinguish between the synthesis and degradation theories (see above) and newer concepts involving the hydrolysis and formation of covalent linked conjugates for the control of IAA levels and growth as illustrated by Bandurski et al. (1977) using careful analytical techniques.

Wilkins' group used "point source" applications of 5-^{3}H-IAA to study redistribution of IAA during geotropic (Shaw et al., 1973) and phototropic (Gardner et al., 1974) induction. They found small redistributions in most experiments, but curvature was observed under conditions in which no lateral redistribution occurred. Also, they were able to confirm the observed light induced change in apparent rate of IAA transport reported by Shen-Miller and Gordon (1966). Phillips and Hartung (1976) failed to observe a gravity induced redistribution of ^{14}C-IAA in *Helianthus*, indeed they applied 2 μM of ^{14}C-IAA in agar to the upper half of a *Helianthus* stem and obtained geotropic curvature while the radioactivity was distributed 4:1 upper to lower (i.e. the ^{14}C-IAA concentration was higher on the slower growing top half). It would seem, therefore, that although lateral transport of IAA may occur during tropic response it does not appear necessary under all conditions or in all tissues. Digby and Firn (1976) have suggested that the lateral redistribution noted by many workers may be due to IAA carried in the water which flows as the

growing cells expand.[3] This hypothesis is supported by the experiments of Gardner et al. (1974) in which redistribution of ^{3}H-IAA could only be observed in cut segments. Cut segments represent a closed system for water since little uptake from the agar occurs. Experiments with intact seedlings failed to show a redistribution of ^{3}H-IAA, possibly because water was amply provided from below.

It should be pointed out however that this area of research is one in which the use of careful analytical technique is woefully lacking. In none of the experiments described above can it be stated with certainty that the compound isolated or reisolated from the plant or agar block was in fact IAA. Only a few authors even carry characterization through a single chromatographic step (cf. Shen-Miller and Gordon, 1966; Shaw et al., 1973; Gardner et al., 1974) and a single chromatographic step does not prove identity. It will be important that future work carefully determine the identity and amounts of the compounds involved in tropisms if the biochemistry of tropic behavior is to be resolved.

In addition to the tropic response, light affects plant growth several other ways. The most obvious of these is the change from the etiolated pattern of growth shown by seedlings raised in the absence of light to the light growth pattern. Characteristic of this change in growth pattern is inhibition of stem or coleoptile

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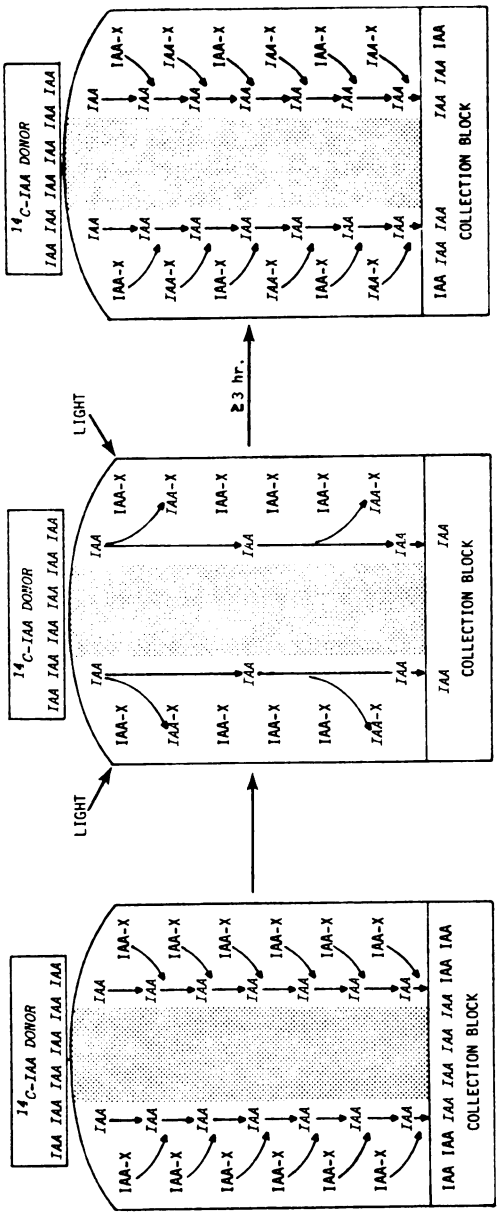
[3]Goswami and Audus (1976) have shown that tropic stimuli also lead to a redistribution of various ions. This redistribution appears to be part of the response process rather than of perception as suggested by Cholodny (1922).

growth, a response which is closely related to phototropism differing only in whether illumination is unilateral or equilateral (Elliot and Shen-Miller, 1976). This response has been found to be caused by very low intensities of blue light and has been defined as a light-growth response (Van Dilliwijn, 1925; also see Went and Thimann, 1937). Photo-inhibition of growth shows the same dose response curve as phototropism (Elliot and Shen-Miller, 1976; also see Briggs, 1960; Zimmerman and Briggs, 1963), the same change in sensitivity due to red light exposure (Elliot and Shen-Miller, 1976; also see Briggs, 1964) and similar spectral sensitivities (Elliott and Shen-Miller, 1976). In addition to these agreements between photoinhibition of growth and phototropism, a similar close correlative relationship exists between photoinhibition of growth and light inhibition of ^{14}C-labeled IAA transport (Shen-Miller et al., 1969; Thornton and Thimann, 1967). If one accepts the rather convincing evidence that these responses are related, then the results of Pickard and Thimann (1964) that show no effect of light on basipetal ^{14}C-IAA transport are in contradiction with those of Thornton and Thimann (1967), Shen-Miller et al. (1969), and, as already discussed, in disagreement with the phototropic studies of Gardner et al. (1974) and Shen-Miller and Gordon (1966). One possible explanation for an apparent decrease in rate of transport would be that light treatment results in an increase in the amount of conjugated IAA in the tissue. In time, the ^{14}C-IAA thus conjugated would be hydrolyzed and thus mask the apparent decrease in rate of ^{14}C-IAA transport noted in the shorter time experiments (Figure 1). That this explanation is

Figure 1. Diagram of photoinhibition of growth which accounts for discrepancies found in the literature.

In the control coleoptile (A) radioactive IAA moves from the donor block through the tissue where it is diluted with cold IAA formed from conjugate hydrolysis. The effect of the light could be conjugation of the free IAA in the tissue and this would include some ^{14}C-IAA. Experiments working with short time intervals would observe a decrease in the rate of basipetal transport as diagrammed in (B) due to the slower movement of the conjugated IAA. During prolonged transport experiments, however, the ^{14}C-IAA-conjugate would be hydrolyzed as the pools of conjugate reserve are used up (C) and the total radioactivity collected would be the same as the control (A). The same situation would occur in phototropic bending except (B) and (C) would only apply to the lighted side of the tissue.


^{14}C-IAA DONOR
IAA IAA IAA IAA IAA IAA
IAA-X
IAA
IAA IAA IAA IAA IAA IAA
COLLECTION BLOCK
LIGHT
≥3 hr.
A
INITIAL STATE (CONTROL)
EQUILATERAL LIGHT TREATMENT
B
PROLONGED INCUBATION AFTER
LIGHT TREATMENT
C

consistent with the experimental data is indicated by the somewhat longer incubation time and higher temperature used by Pickard and Thimann (1964) and by the demonstration that a growth inhibiting flash of light leads to an increase in conjugated IAA and a corresponding decrease in free IAA (Bandurski et al., 1977). This explanation is also consistent with the summation effect found by Shen-Miller and Gordon (1966, see discussion above), however additional experimental work is required to fully account for the discrepancies found among the studies reported by these groups.

Elongation of the mesocotyl in cereal grass seedlings and some dicot internodes is also a light controlled phonomenon. Elongation can be inhibited by exposing germinating seedlings to red or yellow light (Beyer, 1927; van Overbeek, 1936; Goodwin, 1941; Schneider, 1941; Blaauw et al., 1968). Such exposure also results in increased growth of coleoptile sections (Schneider, 1941; Hopkins and Hillman, 1965; Blaauw-Jansen and Blaauw, 1966). Similarly, mesocotyl growth can be inhibited by heat exposures (du Bay and Nuerbergk, 1929a, 1929b, 1930) such as 45 minutes at 48 C (van Overbeek, 1936). Coleoptile tips from growth inhibited plants contain less auxin, as measured by bioassay, than controls and this apparent auxin deficiency can be countered by application of an auxin paste to the coleoptile tip (van Overbeek, 1936). Red light exposure does not, however, change the optimum concentration for exogenous application of IAA (Kohler, 1978; Vanderhoef and Briggs, 1978). Red light exposure also alters the coleoptile's sensitivity to blue light, increasing the light requirement for the first

positive phototropic curvature (Curry, 1957) and increasing the light sensitivity of the second positive curvature (Briggs, 1964). Roots of corn seedlings require exposure to light to change their growth pattern from plagiotropic (horizontal orientation) to positively geotropic (Porodko, 1924; Scott and Wilkins, 1969; Shen-Miller, 1978). Light exposures also result in increased rates of root tip growth and altered optima for exogenous IAA application (Shen-Miller, 1974), although the effect of red and blue light appear profoundly different in this regard.

Most of the preceding discussion has dealt with tropic and growth behavior in response to either a gravity or light situation. There are, however, other ways in which the environment impacts on the plant to affect its growth rate or direction. Wounding has been known to cause a temporary redirection of growth (Darwin, 1880; Stark, 1917, 1921; Beyer, 1925; Bunning, 1927; Tendeloo, 1927; Weimann, 1929; Keeble and Nelson, 1935) and this has been ascribed either to interference in auxin transport (Went and Thimann, 1937) or to involve destruction of auxin by enzymes freed from the cut cells (Thimann, 1934). Thigmatic bending and reductions in growth rate also may involve changes in auxin levels (Stark, 1916; Boysen-Jensen, 1936; Jaffe, 1973). Boyer (1967), using bioassay, reports a complete absence of auxin in mechanically stimulated *Bryonia* plants, while detectable levels were found in controls. Similarly, electrolytic effects have been abscribed to auxin movement. Koch (1934) induced curvature toward the negative pole by placing a 4 volt potential across *Helianthus* hypocotyls

for 1 hour. Subsequent bioassay showed that the convex side had higher auxin activity than the concave side. Numerous other electropharmacological studies have been described which affect plant growth (cf. Lund, 1947) and, as already mentioned, a redistribution of inorganic ions accompanies geotropism (Goswami and Audus, 1976).

As expected, various chemical treatments cause changes in growth and tropic behavior, possibly by effects on auxin levels. Amlong (1933) reported tropic behavior with salt solutions placed laterally on *Vicia faba* roots. Treatment with IAA, other auxins and auxin antilogs have resulted in modified geotropic behavior, sometimes resulting in complete inversion where the root grows up and the shoot down (Rufelt, 1957; Robert, 1959; Guha et al., 1966). The morphactin, 2-chloro-9-hydroxyfluorene-9-carboxylic acid methyl ester, has been reported to abolish geotropism as well as polar auxin transport (Khan, 1967; Krelle and Libbert, 1968; Bridges and Wilkins, 1973; Clifford, 1978) and n-1-napthylphthalamic acid inhibits geotropism while growth is less affected (Ching et al., 1956). It has been suggested that ethylene affects auxin levels or transport (Kang and Burg, 1974a) and that this accounts for the inhibition of geotropic (Burg and Burg, 1966) and phototropic (Kang and Burg, 1974b) curvature noted after treatment with the gas.

Certainly there are many ways in which the environment modifies the growth rate of plants, possibly in many cases by localized changes in the level of IAA or in the ratio of free to bound IAA. Continued study utilizing modern analytical techniques

will undoubtedly provide information about the biochemical basis for these responses and may provide a common explanation for the multitude of these effects.

III. Covalently Bonded Hormones

A. Auxin Conjugates

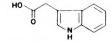
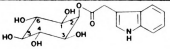
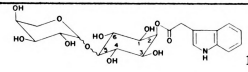
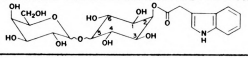
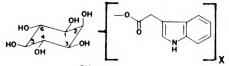
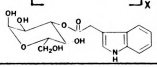
The first "commercial" use of plant growth regulators was probably the use of cereal grains to induce rooting. Farmers in Afghanistan would bury 15 to 20 barley seeds with cuttings of grape, poplar or citrus. Similar practices were also commonly used for centuries by Dutch gardeners (Weaver, 1972) who, unknowingly, were taking advantage of a rich natural source of auxin. Though the history of their use dates to antiquity, the modern study of seed hormones was begun by Cholodny (1935b) who showed that the endosperm of cereal grains was a rich source of growth hormone which could be liberated with fairly mild treatment. Pohl (1935), Laibach and Meyer (1935), Hatcher and Gregory (1941), Hatcher (1943, 1945) and Avery et al. (1940) continued these studies and showed that the seed hormone was probably the same as that found in the tip of the coleoptile and that the seed reserves of this hormone decrease during seed germination. Heyn (1935) and Skoog (1937) showed that removal of the endosperm from the seedling made it more responsive to exogenous auxin and prevented regeneration of the physiological tip. Skoog (1937) attributed this to the seed being a storage site for a hormone precursor which is converted to auxin after moving to the tip. Thimann (1934) was the first to suggest that some of the IAA in plants is "bound" and he also introduced the use of non-polar solvents for auxin extraction--a technique which caused confusion in the literature because of the

variable extent of bound hormone autolysis which results (see Hamilton et al., 1961). Thimann's experiments were extended by van Overbeek (1941) who showed that only 5% of the auxin exists as an "available" form and concluded that most of the IAA in a plant exists bound in the form of a precursor. That this bound form in cereals was alkali labile was shown by Kögl et al. (1934), Avery et al. (1941) and Haagen-Smit et al. (1942) and additional evidence of the ester nature of the complex was provided by Haagen-Smit et al. (1946).

The auxin compounds of the seeds of corn were first studied by Cholodny (1935b) and elaborated by the detailed studies of Berger and Avery (1944a,b). They described a yellow, gummy, water-insoluble material which liberated IAA after alkaline hydrolysis (pH 9.6 for 5 min.). These properties were confirmed by Stehsel (1950), however its further characterization was not accomplished until Piskornik and Bandurski (1972, see also Bandurski and Piskornik, 1973) described a cellulosic β 1-4 lipophilic glucan which contained between 7 and 50 glucose residues per IAA (Table 1). This compound decreases in amount during germination (Ueda and Bandurski, 1969) and is apparently synthesized 30 to 50 days after anthesis (Corcuera, 1967).

Yamaki and Nakamura (1952) compared the water soluble indole compounds of germinating corn by solvent extraction methods, but concluded that the "bound" IAA was of little importance and was probably an artifact of hydrolysis. This view was supported by reports of IAA production by alkali treatment of proteins (Schocken, 1949). However, water soluble compounds were obtained from corn seeds by

TABLE 1. Indole-3-acetic acid and its adducts in *Zea mays* kernels. References are cited in text.

COMPOUND	STRUCTURE	AMOUNT IN DRY SEED MG/KG		PERCENT OF TOTAL
Indole-3-acetic acid			0.5	0.8%
Indoleacetylinositols			10.1	15.2%
2-*O*-(indole-3-acetyl)-*myo*-inositol		7.0	10.5%	
1-DL-(indole-3-acetyl)-*myo*-inositol		3.1	4.7%	
Indoleacetylinositol-arabinosides			15.4	23.2%
5-*O*-ß-L-arabinopyranosyl-2-*O*-(indole-3-acetyl)-*myo*-inositol		11.7	17.6%	
5-*O*-ß-L-arabinopyranosyl-1-DL-(indole-3-acetyl)-*myo*-inositol		3.7	5.6%	
5-*O*-ß-L-galactopyranosyl-2-*O*-(indole-3-acetyl)-*myo*inositol			5.4	8.1%
Trace compounds			0.2	0.3%
Di-*O*-(indole-3-acetyl)-*myo*-inositol		0.08	-	
Tri-*O*-(indole-3-acetyl)-*myo*-inositol		0.03	-	
2-*O*-(indole-3-acetyl)-D-glucopyranose		0.02	-	
4-*O*-(indole-3-acetyl)-D-glucopyranose		0.02	-	
6-*O*-(indole-3-acetyl)-D-glucopyranose		0.05	-	
LOW M.W. COMPOUNDS -- TOTAL			31.2	47.6%
(indole-3-acetyl)-glucan	ß 1 4 cellulosic glucan with 7 to 50 glucose units per IAA		35.0	52.5%

von Denffer et al. (1952) and these yielded IAA after mild treatments which suggested that they were close precursors of IAA. Stowe and Thimann (1953, 1954) suggested that a water soluble compound could be indolepyruvic acid, but this was disputed by subsequent workers (Bentley et al., 1956; Britton and Housley, 1961) who showed that these compounds were alkali labile and chromatographically distinct from IAA and indolepyruvic acid. Additional chromatographic studies were carried out by Bennet-Clark and Kefford (1953) who described an "α-accelerator" which was apparently a neutral ester fraction which they extracted from corn as well as several other plants. Similar studies were made by Farrar et al. (1958) whose active fraction was probably the inositol esters of corn seed discussed below. This early progress has been reviewed by Stowe (1959) and Bentley (1958, 1961).

Hamilton et al. (1961) were the first to quantitatively show that the esters of IAA in corn kernels consisted of about half water soluble and half water insoluble (high molecular weight glucan, see above) compounds. Labarca et al. (1965) partially characterized a series of major water soluble esters of IAA from kernels of *Zea mays*. Four chromatographically distinct compounds were found (B_1-B_4), two (B_1 and B_2) which contained IAA and inositol and were interconvertible. The other spots (B_3 and B_4) contained arabinose and could be converted to the B_1 and B_2 esters by snail gut juice (mixed glucosidases). An earlier report of IAA-arabinose (Shantz and Steward, 1957) in corn seed was, probably, an incorrect identification of the B_3, B_4 compounds. The identity of the IAA-inositol

conjugates was confirmed by Nicholls (1967) who identified the B_2 compound as indole-3-acetyl-2-*O*-*myo*-inositol by comparison with a synthetic standard and by its characteristic downfield chemical shift (δ) in the nuclear magnetic resonance spectrum (Nicholls et al., 1971). Ueda et al. (1970) identified an additional ester as containing IAA, *myo*-inositol and galactose and Ueda and Bandurski (1974) established the structures of the IAA-inositol ester compounds in a comprehensive coupled gas chromatography-mass spectrometry (GC-MS) study. Ueda and Bandurski (1974) also showed that galactose and arabinose were 5-*O*-pyranosyl on the IAA-*myo*-inositol glycosides. Ehmann (1974a) established the identity of 2,4 and 6-*O* esters of IAA and glucose and Ehmann and Bandurski (1974) established the identity of IAA-*myo*-inositol esters with two and three molecules of IAA esterified to the cyclitol. Thus, all of the IAA containing compounds of *Zea mays* kernels present in amounts of 10 µg/kg or greater have been identified and their concentrations determined (Bandurski et al., 1969; Ueda and Bandurski, 1969; Ehmann, 1973) as summarized in Table 1. The formation of these compounds in developing kernels and their decrease in amount during germination has been described (Corcuera, 1967; Ueda and Bandurski, 1969). Piskornik (1975) has also shown that the indole compounds of corn kernels occur almost exclusively in the endosperm. The *in vitro* enzymatic synthesis of IAA-*myo*-inositol and IAA-glucose has been described (Kopcewicz et al., 1974), further characterized as to its cofactor requirements (Michalczuk and Bandurski, 1979)

and the chemical synthesis of ^{14}C-IAA-*myo*-inositol has been accomplished (Nowacki et al., 1978).

No other plant material has been so carefully studied as to its bound auxin content as *Zea mays* kernels, however several other esters of IAA have been partially described. Percival and Bandurski (1976) described an ester of IAA from *Avena* seed which accounted for about 80% of the IAA found in this seed. This ester was found to be a heterogenous glycoprotein of 5,000 to 20,000 daltons and the carbohydrate was a mixed β 1-3, 1-4 lichenin type glucan. *Avena* also contained small amounts of IAA which was liberated by 7 N NaOH at 100 C for 3 hrs and was presumably in amide linkage to some other compound. Although their analytical methods were inadequate, Kaglevic and Pokorny (1969) reported cochromatography of a compound from *Avena* coleoptiles with synthetic 1-*O*-indole-3-acetyl-β-D-glucopyranose and Shantz and Steward (1968) partially characterized an ester of IAA which yielded rhamnose and glucose which was isolated from immature fruits of *Aesculus woerlitzensis*. IAA and rhamnose were suggested by chromatography as the hydrolysis products of a compound isolated by Ganguly et al. (1974) from floral parts of *Peltophorum ferrugineum*. Several authors have reported chromatographic evidence for the amide linked conjugate indole-3-acetyl-L-aspartic acid as a natural product (Klambt, 1960; Row et al., 1961; Olney, 1968; Tillberg, 1974) but confirmation is still necessary by more rigorous physical techniques. Sircar and Das (1951, 1954) and Sircar and Chakravorty (1957) conducted early bioassay studies of the bound IAA of rice. Their

results (Sircar and Das, 1954) show a decline in bound IAA with time after germination similar to that shown by Ueda and Bandurski (1969) for corn using chemical methods. Removal of the embryo from the germinating grain prevented this decline in amounts. In agreement with the data of Piskornik (1975) with corn, bioassay data indicate that rice contains 80% of its total IAA in the endosperm (Sircar and Chakravorty, 1957). Recently, one of the bound forms of IAA in rice has been identified as indole-3-acetyl-*myo*-inositol by cochromatography of the intact compound and its hydrolysis products on thin layer, high pressure liquid, and gas chromatography as well as by combined GC-MS (Hall and Bandurski, 1979). Bandurski and Schulze (1974, 1977b) have quantitatively studied the IAA content of a number of plant tissues including rice and, based on the alkali lability of the IAA compounds, have classified them as free, ester and amide forms.

The biological activity of a variety of halogenated indoles has been examined (Stevens and Fox, 1948; Hoffmann et al., 1952; Sell et al., 1953; Porter and Thimann, 1965) and it was found that substitution with chlorine in the 4 position of IAA resulted in a compound with up to 700 percent of the biological activity of the parent compound. The molecular implications of this activity enhancement has recently been reexamined (Katekar, 1979) and the biological activity in several additional bioassay systems has confirmed the high activity of 4-chloro-indole-3-acetic acid (Marumo et al., 1974; Bottger et al., 1978b). The methyl ester of 4-chloro-IAA has been isolated and well characterized from

immature pea seeds by several groups (Marumo et al., 1968a; Gandar and Nitsch, 1967; Engvild et al., 1978) and the free acid has also been reported (Marumo et al., 1968b). The bulk of the 4-chloro-IAA is as the methyl ester (87 µg/kg) with the free acid accounting for only 14 µg/kg. The mono methyl ester of 4-chloro-indolyl-3-acetyl-L-aspartate has also been identified by rigorous chemical methods from immature pea seed and accounts for 9 µg/kg (Hattori and Marumo, 1972). Interestingly, Hattori and Marumo (1972) were unable to isolate IAA or IAA methyl ester from this plant material by methods which should have detected as little as 4 µg/kg. Although previous bioassay data had claimed IAA isolation (Housley and Griffiths, 1962), this was probably due to an artifact of the isolation methods which do not separate IAA from 4-chloro-IAA (Marumo et al., 1971). Mature pea seeds, however, contain IAA (93 µg/kg), an amide linked conjugation product (202 µg/kg), but no esterified IAA (Bandurski and Schulze, 1977b). This conversion from halogenated to primarily non-halogenated indole metabolism in the developing pea seed must be studied further.

Redemann et al. (1951) isolated a compound from immature corn kernels which appeared to be the ethyl ester of IAA. The use of ethanol in isolation makes this uncertain since esterification of the free acid can occur during extraction and the report was subsequently withdrawn (Fukui et al., 1957). If ester linked conjugates were present, transesterification to the alcohol could also result, as reported by Zenk (1964). A similar criticism could be applied to the methyl esters of the chlorinated compounds (see

above) since in all cases the investigators used methanol for extraction. Marumo et al. (1968b) did utilize an acetone control, but their analysis of this control by bioassay was insufficient evidence for the endogenous nature of these methyl esters. A recent report (Hofinger and Bottger, 1979) using alkaline buffer extraction, however, confirms the presence of the methyl ester. The methyl and ethyl esters of IAA are potent stimulators of parthenocarpy (Sell et al., 1953). Seeley et al. (1956) presented chromatographic evidence of methyl ester hydrolysis by several plant tissues.

Conjugates of IAA from non-plant sources have been reported. Zenk (1960) isolated an enzyme from liver mitochondria which synthesized indole-3-acetylglycine *via* an IAA-CoA intermediate. Indole-3-acetylglucosiduronic acid (Jepson, 1958) and indole-3-acetylglutamine (Jepson, 1956) have been isolated from abnormal human urine. A plant pathogenic bacterium, *Pseudomonas savastanoi*, produces indole-3-acetyl-ε-L-lysine (Hutzinger and Kosuge, 1968a) in large amounts (Hutzinger and Kosuge, 1968b). Also, numerous plant indole conjugates have been reported which are not IAA derivatives. For example, Ehmann (1974b) isolated N(*p*-coumaryl)-tryptamine and N-ferulyl-tryptamine from corn kernels and the corresponding 5-hydroxy compounds as well as the β-D-glucopyranoside have been isolated from *Carthamus tinctorium* (Sakamura et al., 1978). A stigmasteryl-β-D-glucoside has been reported from peanut plants and has a synergistic effect with IAA in *Avena* elongation bioassays (Kimura et al., 1975). The physiological significance

of these conjugates is unknown, however the urine compounds might be considered excretion products and the lysine conjugate could play a role in a pathogenic mechanism.

In addition to the natural products discussed above, a number of conjugates have been identified as products of exogenous auxin application. Andreae and Good (1955) identified indoleacetylaspartic acid as the major metabolite of pea stems and roots fed with large amounts of IAA. Andreae and Good (1957) extended these studies to a number of other indoles and auxins. Indolepropionic acid, indolebutyric acid, 2,4-dichlorophenoxyacetic acid (2,4-D) and benzoic acid gave products with properties identical to those of the respective synthetic aspartic acid derivatives. Subsequent reinvestigation however revealed that the benzoic acid derivative was benzoylmalic acid (Venis and Stoessl, 1969). Trace amounts of the aspartic acid conjugate were formed in tissue which had been pretreated with auxins, but none was produced in tissue not pretreated. Thus, this is an example of the errors possible when identification is based simply on chromatographic migration of impure extracts. Zenk (1962; also, cf. Schraufolf, 1971) reported the formation of the aspartic acid conjugate with the additional synthetic auxin, napthaleneacetic acid.

The ability of the plant tissue to make the aspartate conjugates is enhanced by pretreatment with a variety of active auxins (Andreae and Van Ysselstein, 1956; Sudi, 1964; Venis, 1964; Sudi, 1966; Venis, 1972) such that pretreatment with IAA will lead to increased ability to conjugate the synthetic auxins as well as IAA.

Aside from this apparent inducibility, this enzymatic activity has not been further characterized except for two reports of *in vitro* synthesis (Lantican and Muir, 1969; Higgins and Barnett, 1976) which have been disputed (cf. Venis, 1972). Although the work cited above was done with peas, Good et al. (1957) and Zenk (1964) showed that a large number of plant tissues would produce compounds which were chromatographically similar to the aspartate conjugate when exogenous IAA was applied.

Klambt (1961) and Zenk (1961) isolated a compound after exogenous application of IAA which appeared to be the glucose ester of IAA and to which they assigned the structure 1-*O*-indole-3-acetyl-β-D-glucose. Adequate published chemical documentation of the 1-*O* linkage is still lacking. Several years earlier the β-glucosidic ester of indole-3-propionic acid had been identified as a product of cultures of *Bacillus megatherium* (Tabone and Tabone, 1953). The presence of the glucoside in treated higher plants suggests that the indoleacetamide found on chromatograms by Good et al. (1956) was a product of ammonolysis with the solvents employed (Jepson, 1958). Synthetic 1-*O*-indole-3-acetyl-β-D-glucose has been reported to have a higher biological activity than IAA (Keglevic and Pokorny, 1969), however this may be a result of various factors such as uptake rate. Zenk (1964) speculates that the IAA-glucose compound is a transient form which is made prior to the appearance of the enzymatic activity which produces the aspartate conjugate. A comparison of the data of Good et al. (1956) with that of Bandurski and Schulze (1977b) indicates that those plants which make large amounts of ester

conjugates are those with a high endogenous amount of ester IAA (see also data of Feung et al., 1978 for 2,4-D). Therefore, it is more likely that the ratio of complexes formed from exogenous application is species related as proposed by Good et al. (1956). It is also difficult to envision how a complex multiproduct response as proposed by Zenk (1964) might evolve in plants when environmental exposure to large amounts of exogenous auxins was an unusual occurrence until the advent of modern growth regulators.

Feung et al. (1976) identified IAA conjugation products in tissue culture from *Parthenocissus* crown gall by chromatographic and mass spectral techniques. They identified the glycine, alanine, and valine conjugates as major products and the aspartate and glutamate conjugates were formed in lesser amounts. Feung et al. (1977) also studied the biological activity of 20 L-α-amino acid conjugates of IAA in *Avena* straight growth and in supporting growth of soybean cotyledon tissue culture. Recently, interest in these compounds for control of morphological development in tissue culture has developed (Peterson, 1978; Hangarter et al., 1979). Feung et al. (1974) also studied the biological activity of the 2,4-D amino acid conjugates and numerous other reports exist on various biological activities of one or more IAA conjugate (cf. Jerchel and Staab-Muller, 1954; Nicholls, 1967; Keglevic and Pokorny, 1969; Rekoslavskaya and Gamburg, 1976).

Because of their wide usage as herbicides the metabolism of the phenoxyacetic acids has been carefully studied. Holley (1952) found that a major metabolite of 2,4-D appeared to be a ring

hydroxylated glycoside. Thomas et al. (1964b) confirmed this and showed that the hydrolysis products cochromatographed with 2,4-dichloro-5-hydroxyphenoxyacetic acid and, as a minor component, 2,3-dichloro-4-hydroxyphenoxyacetic acid. In contrast to the results with bean plants, oat seedlings appeared only to produce the glucose ester (Thomas et al., 1964a). This earlier work on the carbohydrate containing auxin conjugates was the subject of a review by Hilton (1966). As discussed above, Andreae and Good (1957) found chromatographic evidence for the formation of 2,4-dichlorophenoxyacetylaspartic acid. This was confirmed, again only by chromatographic migration, by Klambt (1961) who also provided evidence for the glucose ester.

Hamilton et al. (1971) confirmed the presence of the hydroxylated phenoxyacetic acids in treated bean plants using multiple chromatographic systems as well as mass spectrometry. They found the major products of 2,4-D treatment to be 2,5-dichloro-4-hydroxyphenoxyacetic acid, while 2,3-dichloro-4-hydroxyphenoxyacetic acid was a minor metabolite. These compounds accumulated as the glycosides which could be freed to the aglycone by a commercial β-glucosidase. Results with soybean cotyledon callus tissue showed that they yielded the same products as well as a conjugate of 2,4-D with glutamic acid (Feung et al., 1971). Feung et al. (1972) identified the aspartic acid conjugate and confirmed this, as well as the structure of the glutamic acid metabolite, by mass spectral data. Arjmand et al. (1978) found the aspartate and glutamate conjugates to be the major products of soybean callus supplied with

2,4,5-trichlorophenoxyacetic acid. Additional studies (Feung et al., 1973b) characterized five amino acid derivatives of 2,4-D: alanine, valine, leucine, phenylalanine, and tryptophan. In addition, they found that callus fed with the glutamic acid conjugate of 2,4-D produced the aspartic acid conjugate in larger amounts than tissue supplied with the free acid. Davidonis et al. (1978) have shown that in very young root callus from soybean that the levels of free 2,4-D increased with amount added but that the levels of glycosides and amino acid conjugates increased only slowly. This was contrasted with the situation in more mature callus in which the free 2,4-D concentration remains at a constant, apparently regulated, level. This level is maintained by the tissue by increasing its rate of 2,4-D conjugation as the levels of added 2,4-D are increased. Thus, the older root callus appeared to regulate the level of free 2,4-D at about 4 nanomoles per gram of tissue, and this was primarily by formation of the amino acid derivatives. Feung et al. (1973a) published an extensive mass spectral and chromatographic study of twenty L-form amino acid conjugates of 2,4-D and Feung et al. (1975) published a similar study on amino acid conjugates of IAA. These will serve as important reference spectra for future work on these compounds.

B. Gibberellin Conjugates

Murakami (1961) found that cucumber leaf disks produced large amounts of gibberellin β-glucoside when floated on solutions of gibberellin A_3. Although the product was not well characterized,

this glucoside of A_3 had chromatographic and partition properties similar to the water-soluble, non-ethyl acetate extractable gibberellins described as natural products by later investigators (Murakami, 1962; Ogawa, 1963; Zeevaart, 1966). Hashimoto and Rappaport (1966) showed that developing bean seeds exposed to gibberellin A_1 converted most of it to water-soluble compounds and that the biological activity of the gibberellins in the solvent phase remained constant regardless of the dosage of applied gibberellin A_1. Barendse et al. (1968) showed using ^{3}H-gibberellin A_1 that a compound was formed in developing *Pharbitis* and pea seeds which would yield gibberellin A_1 upon mild acid hydrolysis and that this compound was also converted to ^{3}H-gibberellin A_1 during seed germination. Although Barendse et al. did not characterize the "X" products found in the seeds, Murakami (1968) showed that *Pharbitis* seeds contain a water-soluble, biologically inactive compound which could be converted to an active gibberellin by hydrolysis with 0.2 N H_2SO_4.

Tamura et al. (1968) isolated three water-soluble neutral gibberellins from *Pharbitis* seeds. The major components accounted for 50 µg/kg out of the total of 72 µg/kg of neutral gibberellins present. Based on the chemical properties of the hydrolysis products and the nmr spectra of the intact compound, it was assigned the structure 2-*O*-β-glucosyl-gibberellin A_3. Schreiber et al. (1967) identified the related 3-*O*-β-D-glucopyranosyl-gibberellin A_8 from seeds of *Phaseolus coccineus*. These two publications were the first reports on the isolation and characterization of endogenous glysosidic gibberellins. Schreiber et al. (1969) later confirmed

the identity of the glycoside by synthesis and thus established the structure of the compound they had described years earlier (Sembdner et al., 1964) as the "Phaseolus ε" compound based on solvent partitioning and chromatography.

Yokota et al. (1969) described additional glycosides from *Pharbitis* and their chemical characterization was covered in an extensive publication which appeared later (Yokota et al., 1971). These compounds, which accounted for most of the gibberellin in the seeds, were: 2-*O*-β-glucosyl-gibberellin A_3 (as above); 2-*O*-β-glucosyl-gibberellenic acid; 2-*O*-β-glucosyl-isogibberellin A_3; 3-*O*-β-glucosyl-gibberellin A_{26}; 3-*O*-β-glucosyl-gibberellin A_{27}; 3-*O*-β-glucosyl-gibberellin A_{29}; and 3-*O*-β-glucosyl-gibberellin A_8. The 3-*O*-β-glucosyl-gibberellin A_8 was also isolated from shoot tips of *Althaea rosea* (Harada and Yokota, 1970) where the relative levels of free gibberellin and glucoside appeared to correlate with shoot development.

Yamane et al. (1974) characterized a glucoside of gibberellin A_{35} from *Cytisus scoparius* which upon careful chemical analysis proved to be 11-*O*-β-D glucosyl gibberellin A_{35}. Hiraga et al. (1972, 1974b) isolated four glucosyl ester compounds which were identified as esters of gibberellins A_1, A_4, A_{37} and A_{38}. The ester forms were present in mature *Phaseolus* seeds but absent in immature seeds. Lorenzi et al. (1976) isolated and characterized the gibberellin A_9 glucosyl ester from *Picea sitchensis* and Yokota et al. (1975) published a mass spectral study of the glucosides and ester compounds. Evidence for a high molecular weight bound

gibberellin was provided by Halinska and Lewak (1978) although they did not characterize the intact compound. The 2-*O*-acetyl gibberellin A_3, the only non-glucose containing natural conjugates so far identified, was isolated from *Fusarium* (Schreiber et al., 1966).

The biological activity of these compounds has been studied (Yamane et al., 1973; Hiraga et al., 1974a; Sembdner et al., 1976) but other aspects of the biology of these compounds have only been suggested. Sembdner et al. (1968) state that these compounds may function as "depot" forms in the seed which are used during germination since conjugated gibberellins form during maturation of fruits and yield the free compound during germination (Sembdner, 1974). Likewise, they suggest a long distanct transport role based on the presence of conjugated gibberellins in the bleeding sap of *Acer platanoides* and *Ulmus glabra* (Sembdner et al., 1968). Also, several authors have suggested a role for these compounds in seed dormancy (cf. Halinska and Lewak, 1978) and the results of Hashimoto and Rappaport (1966) *suggest* the possibility of a system for maintenance of free hormone levels similar to that *shown* with IAA and 2,4-D (Bandurski et al., 1977; Davidonis et al., 1978). Further work on the physiology and biochemistry of these compounds should, therefore, lead to new insights into the regulation of gibberellin levels and how gibberellins regulate plant processes.

C. Cytokinin Conjugates

Cytokinins, like the phytohormones IAA and gibberellins discussed above, exist in plant tissue in both bound and free form. Zwar et al. (1963, 1964) isolated several active fractions of cytokinins from apple and from coconut milk and found that fraction IV could be converted to fraction II by mild acid hydrolysis. These results were confirmed by Loeffler and van Overbeek (1964) and Wood (1964) reported the partial characterization of a cytokinin glucoside from crown gall cultures of *Vinca rosea*. Kende (1965) showed a similar change in chromatographic behavior following acid treatment of a fraction from sunflower root exudates and suggested a role for these compounds in hormone translocation. Yoshida and Oritani (1972) isolated a putative zeatin glucoside from rice, partially characterized the compound and suggested it was zeatin-9-β-glucoside. This identification was, however, in error since synthetic zeatin-9-β-D-glucoside is not hydrolyzed by β-glucosidase (Parker et al., 1975) and their compound was labile to this enzymatic hydrolysis. Van Staden (1976) claimed the identification of a zeatin glucoside from coconut milk, however the procedures utilized were insufficient to establish the position of the linkage and the identification of the hydrolysis products is uncertain.

In experiments where cytokinins have been fed to plant tissue several products have been identified. For example, Deleuze et al. (1972) identified the 7-glucoside of 6-benzylaminopurine in several plant systems after treatment with the parent compound and, based on its mass fragmentation pattern, assigned it the

glucofuranose configuration. Additional metabolites have also been identified as benzylamino-9-β-D-ribofuranosylpurine and its 5'-monophosphate (Fox et al., 1972; Dyson et al., 1972) and the 3 and 9-glucosides of 6-benzyl-aminopurine (Wilson et al., 1974; Letham et al., 1975). Similarly, treatment of plant material with zeatin (6-(4-hydroxy-3-methylbut-*trans*-2-enyl-amino)-purine) produces metabolites identified as 7-glucosylzeatin, 9-glucosylzeatin, zeatin riboside and its 5'-monophosphate, and dihydrozeatin (6-(4-hydroxy-3-methylbutylamino)purine) and its riboside and riboside 5'-phosphate (Sondheimer and Tzou, 1971; Parker et al., 1972; Parker and Letham, 1973; Parker et al., 1973; Tzou et al., 1973; Parker and Letham, 1974; Duke et al., 1979). Several zeatin metabolites with a 4-glucosyloxy moiety have also been identified by Letham et al. (1976) as *O*-β-D-glucopyranosyl-9-β-D-ribofuranosyldihydrozeatin, *O*-β-D-glucopyranosylzeatin, *O*-β-D-glucopyranosyldihydrozeatin and *O*-β-D-glucopyranosyl-*cis*-zeatin. The formation of the nucleotides of the 6-furfuryl-, 6-methyl-, and 6-propyl-aminopurines was shown by Doree and Guern (1973) who also found none of the corresponding nucleosides. Recently, a detailed study of the chemical synthesis and properties of many of these compounds has appeared (Cowley et al., 1978) and also a preliminary report has been published on their enzymatic synthesis (Entsch and Letham, 1979).

Several endogenous cytokinin conjugates have been identified with a precise chemical structure. Miller (1965) and Letham (1966a, b) described the compounds 9-β-D-ribofuranosylzeatin and its 5'-monophosphate from immature sweet corn and the riboside was also

convincingly shown to be present in the liquid media of a culture of *Rhizopogon roseolus* (Miller, 1967). Hall et al. (1967) identified the *cis* isomer of zeatin riboside (6-(*cis*-4-hydroxy-3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine) from immature sweet corn and Horgan et al. (1973) isolated a novel cytokinin, 6(*O*-hydroxybenzylamino)-9-β-D-ribofuranosylpurine, from *Populus robusta* leaves. Wang et al. (1977) found 6-(4-*O*-β-D-glucosyl-3-methylbutylamino)purine, a dihydrozeatin glucoside, in leaves of *Phaseolus* and identified it by a variety of chemical and biochemical tests including combined GC-MS. Peterson and Miller (1977) and Morris (1977) elegantly identified two cytokinin glucosides from cultures of *Vinca rosea* crown gall which, earlier, Wood (1964) had studied but not fully characterized. These compounds were a glucosylzeatin (6-(4-*O*-β-D-glucopyranosyl-3-methyl-*trans*-but-2-enylamino)purine) and a glucosyl zeatin riboside (9-β-D-ribofuranosyl-6(4-*O*-β-D-glucopyranosyl-3-methyl-*trans*-but-2-enylamino) purine).

The naturally occurring cytokinin 6-(3-methyl-2-butenyl) aminopurine is converted to adenosine by an enzyme from tobacco tissue cultures (Paces et al., 1971) and *Lemna minor* metabolyzes 6-benzylaminopurine to adenine (Bezemer-Sybrandy and Veldstra, 1971). Similar reactions have been observed to be catalyzed by mammalian xanthine oxidase (E.C. 1.2.3.2) acting on 6-(furfurylamino)purine (Bergmann and Kwietny, 1958; Henderson et al., 1962) and the substrate specificity of this type of reaction catalyzed by an enzyme from immature *Zea mays* kernels has been studied by Whitty and

Hall (1974). The enzyme is equally active on naturally occurring cytokinins and their ribosides, but cytokinin analogs with saturated or bulky side chains do not react. Thus, Wang et al. (1977) have suggested that the function of the dihydrozeatin glucoside which they isolated from bean might be related to the compound's resistance to such oxidative reactions.

Although careful quantitative studies have not, apparently, been done on the relative amounts of the various cytokinin derivatives in plants, numerous studies based on bioassay indicate that the conjugates account for more than half of the cytokinin activity of various plant extracts. This value is probably low, however since the glucosides are not as active as the free compound in some bioassays (Van Staden and Papaphilippou, 1977) and because of the lability of the compounds, especially in extracts where enzymatic hydrolysis has not been carefully eliminated. A brief report on germinating seeds indicate that the bound forms may serve as seed reserves since the glucosides were only found in the endosperm and declined after germination (Davey and Van Staden, 1977). Julin-Tegelman (1979) found that zeatin ribotide decreased during germination in *Zea mays* but not in sufficient amounts to account for new cytokinin which appeared. Though Julin-Tegelman suggested that these results indicated *de novo* synthesis, the possibilities of other bound cytokinins such as the glucosides was not examined.

D. Abscisic Acid Conjugates

Osborne (1955) first published evidence for the existence of diffusable abscission-accelerating substances. Such a compound was isolated and characterized by Liu and Carns (1961), Ohkuma et al. (1963), and Cornforth et al. (1965) and the structure 3-methyl-5-(1'-hydroxy-4'-oxo-2'-cyclyhexen-1'-yl)-*cis*-2,4-pentadienoic acid was confirmed by synthesis by Cornforth et al. (1965) and Roberts et al. (1968). This compound has been given the trivial name of abscisic acid (Addicott et al., 1968) although early workers referred to it as abscisin, abscisin I, abscisin II and dormin.

Koshimizu et al. (1968) isolated (+)abscisyl-β-D-glucopyranoside from the immature fruit of *Lupinus luteus* and confirmed its structure by exacting physical methods including nmr and mass spectrometry, thus establishing the structure of the first naturally occurring bound form of abscisic acid. Milborrow (1968) found that the major metabolite of racemic ^{14}C-abscisic acid fed to bean petioles or *Acer pseudoplatanus* sections was a water soluble compound later shown (Milborrow, 1970) to be identical to that isolated by Koshimizu et al. (1968). The compound has now been synthesized by preparation of the 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl ester followed by an enzymatic deacetylation (Lehmann et al., 1975). Milborrow (1970) also found that *trans-trans*-abscisic acid was converted to its glucose ester 10 times faster than the *cis-trans* isomer and several laboratories have found that additional free absicsic acid is released by alkaline hydrolysis of methanolic

plant extracts (Milborrow, 1968; Rudnicki and Pieniazek, 1971; Osborne et al., 1972; Goldschmidt et al., 1973; Milborrow, 1974). Most of these reports find that the forms released by hydrolysis account for one third or less of the abscisic acid in the tissue. Again, these values must be viewed as lower limits since careful quantitative work has not been done and in at least one study (Goldschmidt et al., 1973) the "bound abscisic acid" exceeded the free acid by ten fold.

Leshem et al. (1974) showed a change in the amounts of free abscisic acid and its glucoside during bud dormancy break in almond. Using a gas-liquid chromatographic assay system they were able to detect a decrease in free abscisic acid, including the *cis-trans* and *trans-trans* isomers, and an increase in the glucoside over a 15 week period. These data were confirmed by the later work of Wright (1975) and Harrison and Saunders (1975) on dormant buds of various deciduous plants. Barthe and Bulard (1978) examined abscisic acid levels in dormant and after-ripened embryos of apple using both bioassay and gas-liquid chromatography. Previously, they had found abscisyl-β-D-glucopyranoside in apple embryos (Bulard et al., 1974) by gas chromatographic identification of the hydrolysis products. They found a spectacular decrease of the free abscisic acid level following release from dormancy and a parallel increase in the bound form which became the major form. These data extended the earlier data of Rudnicki (1969). Hiron and Wright (1973) found that plants subjected to water stress had increased levels of abscisic acid and that as the abscisic acid levels dropped during

recovery, increased amounts of conjugated abscisic acid could be detected. Thus, they postulated that conjugate formation was a slow adaptive mechanism which prevents the rapid reopening of stomates following water stress.

The conjugation of plant hormones appears to be a general phenomenon in plant tissue, both in the fact that all plants so far critically examined have been found to have one or more conjugated hormone and that all of the major plant hormones (auxins, gibberellins, cytokinins and abscisic acid) form one or more conjugate. Many similarities in physiology appear among the conjugates of various hormones, although work on many of them is still in its infancy. Several have been suggested as transport forms, as protectants against oxidative attack, as regulators of hormone levels, and as slow release forms. Clearly, no future work on plant hormones will be complete without considering this important aspect of hormone metabolism and studies which examine the interactions between multiple hormones, both bound and free, could provide new insight into the hormonal relationships of higher plants. A general review of plant hormone conjugates, with a more Germanic viewpoint, has appeared (Sembdner, 1974).

IV. Analytical Chemistry of Indole-3-acetic Acid and Its Adducts

Indole chemistry began in the mid-nineteenth century with investigations on the dye indigo, which had been highly valued since ancient times. Baeyer and Emmerling (1869) proposed the presently accepted formula of indole, a benzopyrrole in which the benzene ring is fused to the 2- and 3-positions of the pyrrole ring. Several chemical aspects of the structure of indoles have direct bearing on its analysis. Indoles have a planer structure with ten π-electrons free to circulate throughout the molecule, two of which originate from the nitrogen atom thus making them relatively unavailable for salt formation as compared to those of, for example, pyridine. Indoles belong to the group of heterocycles designated π-excessive heteroaromatics (Albert, 1959), since π-electron densities on indole carbons are greater than those of benzene. Thus, they are highly reactive towards electrophilic reagents, including acids and some oxidants (Remers, 1972).

The nitrogen of indole is relatively acidic with a pK_a of 17, such that anion formation occurs only in strong base (Remers, 1972). The anion increases reactivity of the $C_{(3)}$ to electrophiles, but the ring system itself is relatively base stable.

Indoles are strongly ultraviolet (U.V.) absorbing with IAA having peaks at 220-222 nm (log ε=4.51) and 279-282 nm (log ε=3.78) and shoulders at 273 nm (log ε=3.77) and 294 nm (log ε=3.71) (Hinman and Lang, 1964; Bandurski and Schulze, 1974). They are also strongly fluorescent with the excitation maximum for the IAA

anion at 292 nm, the emission at 362 nm and a quantum efficiency of 56.5% (Bridges and Williams, 1968). Adsorption of a photon results in a pronounced shift in the pK_a of the indole, calculated to be 7.5 units (Longworth et al., 1966), making the molecule more prone to oxidative attack. At 77° K in rigid ethanol glass IAA will exhibit phosphorescence with excitation at 285 nm and emission at 436 nm and a τ_e of 7.1 seconds (time required for a decrease in intensity to 36.8% of initial) (St. John et al., 1967).

These chemical properties place certain intrinsic constraints on the quantitative analysis of microsamples of IAA. Because of the ease of indole oxidation, especially when exposed to U.V. light, and the propensity of the planar ring to adhere to glassware, large and variable losses occur during isolation. Moore and Shaner (1967) found 30% losses each time paper chromatography was performed and Little et al. (1978) have reported losses of over 99% during isolation. Hamilton et al. (1961) found that recoveries of IAA were between 0 and 56%, with the lower yields occurring with plant tissue or radioactivity present. Mann and Jaworski (1970) studied methods for minimizing losses during IAA isolation and found, as expected, that oxygen, light and protracted extraction times resulted in lower yields. Under their conditions using low actinic glassware, antioxidants and nitrogen atmosphere yields of 60% were obtained, although they used plant material fortified with unphysiologically high amounts of IAA. Clearly, any method for the isolation of IAA from plant material which does not use an internal standard to measure losses during sample preparation is unreliable.

Unfortunately, such corrections are rarely used and the first use of isotope dilution methods (Rittenburg and Foster, 1940) for IAA analysis was not until 1960 (Turian and Hamilton, 1960) even though ^{14}C-labeled IAA was available much earlier (cf. Stutz et al., 1951).

Detection of IAA in plant tissues requires separation of an IAA containing fraction from the bulk of the cell debris. Early workers used five basic methods (Boysen-Jensen, 1936): (1) diffusion of growth substance into agar or dextrose agar, (2) diffusion into water, (3) extraction with alcohol, (4) extraction with chloroform and (5) extraction with water. Because of problems with autolysis of bound IAA in tissue extracted with highly non-polar solvents or with water (Hamilton et al., 1961), most workers now use aqueous alcohol (cf. Hamilton et al., 1961; McDougall and Hillman, 1978a) or acetone (cf. Ueda and Bandurski, 1969; Bandurski and Schulze, 1974) for extraction. For some special situations the use of 80% $(NH_4)_2SO_4$ (Atsumi et al., 1976) or alkaline buffer (Hofinger and Bottger, 1979) extraction have proven useful but the use of aqueous acetone is advantageous since problems with esterification during extraction, as discussed previously, are avoided. Methods of trace enrichment, as applied to replace extraction and solvent partitioning in the purification of the cytokinins (Kaiss-Chapman, 1977), have so far not been successfully applied to the indole hormones.

Chromatographic purification of IAA was first carried out by Yamaki (1950) who applied paper chromatography to study the auxins in plant material. Since that time a variety of paper,

thin layer, column, high pressure and gas-liquid chromatographic systems have been applied to the purification of IAA. Paper and thin layer chromatographic methods have been the most widely employed because of the sensitivity afforded and probably because the equipment required is minimal. Some of the common paper and thin layer chromatographic solvent systems employed for IAA purification are summarized in Tables 2 and 3. Unfortunately, paper chromatography for the purification of IAA is less well documented than thin layer and fewer than 30% of the authors cited who employed this technique provided relative mobility data and many did not even state the source of the paper utilized. Paper electrophoresis has also been applied to IAA by some workers (Hamilton et al., 1961; Hemberg, 1972). Although still useful, these methods have the problem of exposing the compound to large surfaces where oxidation may result. Because of its higher resolution, silica gel thin-layer chromatography minimizes this exposure, despite the fact the support is acidic. Thus, recoveries are generally low with these methods and for critical work at submicrogram levels they can only rarely be employed.

A variety of column chromatographic methods have been used for IAA isolation. Silica gel and diatomaceous silica found early appeal (Hamilton et al., 1961; Seeley and Powell, 1964; DeYoe and Zaerk, 1976) and still find utility where relatively large amounts of plant material are to be analyzed. Indoles chromatograph on a variety of polymeric materials, probably because of their planar structure. This property has allowed the use of various Sephadex

TABLE 2.--Some common paper chromatographic systems used for the separation of indole-3-acetic acid.

Support	Solvents	Composition	References
Paper	Benzene:acetic acid (glacial): water	6:7:3	Kosuge et al. (1966)
Whatman no. 1	Benzene:acetic acid:water	2:1:1	Davies et al. (1975)
Paper	Benzene:1-butanol:acetic acid: water	95:5:5:1	Feung et al. (1975)
Toyo no. 2	Butanol:acetic acid:water	12:3:2	Yamaki and Nakamura (1952)
Whatman no. 1	1-Butanol:acetic acid:water	5:1:2.2	Labarca et al. (1965)
Whatman no. 1	1-Butanol:ammonia (sp.gr. 0.88): water	100:3:18	Housley and Griffiths (1962)
Toyo no. 51	Butanol:ammonia:water	4:1:1	Marumo et al. (1971)
Paper	Carbon tetrachloride:water with carbon tetrachloride: acetic acid:water atmosphere	50:1 1:1:1	Riddle and Mazelis (1965)
Toyo no. 2	Ethanol (70%)	-	Yamaki and Nakamura (1952)
Paper	Ethanol (70%)	-	Hemberg (1958)

TABLE 2.--Continued.

Support	Solvents	Composition	References
Paper	Ethyl acetate:2-propanol: acetic acid:water	90:50:1:1	Feung et al. (1975)
Paper	Hexane with water saturated atmosphere	-	Hemberg (1958)
Toyo no. 51 or Whatman no. 1	Isobutanol:methanol:water	85:5:15	Ohwaki (1970)
Toyo no. 2	Ligroin saturated with water	-	Yamaki and Nakamura (1952)
Toyo no. 2	Methanol (70%)	-	Yamaki and Nakamura (1952)
Paper	Petroleum ether:diethyl ether: methanol:acetic acid:water	60:60:15:1:2	Feung et al. (1975)
Toyo no. 2	Phenol saturated water	-	Yamaki and Nakamura (1952)
Whatman no. 1 or 3 mm	2-Propanol:water	10:1	Kefford (1955)
	with 2-propanol:ammonia: water atmosphere	10:1:1	
Paper	2-Propanol:ammonia:water	10:1:1	Stowe and Thimann (1954); Shen-Miller and Gordon (1966)

TABLE 2.--Continued.

Support	Solvents	Composition	References
Whatman no. 1	2-Propanol:ammonia:water	21:1:3	Davies et al. (1975)
Paper	2-Propanol:ammonia (sp.gr. 0.91):water	100:14:6	Hemberg (1958)
Paper	2-Propanol:ammonia:water	85:15:5	Klambt (1960)
Whatman no. 1	2-Propanol:ammonia (sp.gr. 0.88):water	16:1:3	Housley and Griffiths (1962)
Whatman no. 1	2-Propanol:ammonia:water	8:1:1	Andreae and Good (1955); Hamilton et al. (1961); Labarca et al. (1965); Gillespie and Thimann (1963); Ohwaki (1970); Feung et al. (1975)
Toyo no. 51	2-Propanol:ammonia (28%): water	8:1:1	Ohwaki (1970); Marumo et al. (1971)
Whatman no. 1	Water	-	Housley and Griffiths (1962)
Toyo no. 51	Water	-	Marumo et al. (1971)

TABLE 3.--Thin layer chromatographic systems which have been used for the separation of indole-3-acetic acid. Systems are listed in order of the compound's increasing mobility relative to the solvent front.

Solvents	Composition	Layer	R_f IAA	References
Chloroform:methanol	93:7	SG	0.01	Kaldewey (1969)
Chloroform:methanol:ammonia (35%)	80:25:0.1	SG	0.01-0.07	McDougall and Hillman (1978a)
Benzene:acetone	90:10	SG	0.02	Kaldewey (1969)
Chloroform:ethyl acetate: formic acid	50:40:10	SG-G	0.05	Zimmermann et al. (1976)
Methanol:water:formic acid	37.5:60:2.5	Polyamide	0.13	Kaldewey (1969)
2-Propanol:ammonia (35%):water	10:1:1	SG	0.24-0.31	McDougall and Hillman (1978a)
2-Propanol:ammonia (35%):water	100:5:10	SG	0.24-0.34	McDougall and Hillman (1978a)
Chloroform:acetic acid	95:5	SG-H	0.26	Marumo et al. (1971)
Butanol:ethanol (abs.):water	76:19:5	SG-G	0.27	Kaldewey (1969)
Isobutanol:methanol:water	80:5:15	SG-G	0.28-0.33	Kaldewey (1969); Wakhloo (1965)
Benzene:acetone:pyridine	60:39:1	SG-G	0.31	Bandurski and Schulze (1974)
2-Propanol:methyl acetate: ammonia	35:45:20	SG-G	0.31	Kaldewey (1969)
Methyl acetate:2-propanol: ammonia (25%)	45:35:2	SG	0.32-0.42	McDougall and Hillman (1978a)
Chloroform:acetic acid (96%)	95:5	SG-G	0.33-0.43	Kaldewey (1969); Ohwaki (1970)
Ethyl acetate:2-propanol:water	65:24:11	SG	0.34 (tails)	Kaldewey (1969)
Chloroform:methanol:water	85:14:1	SG-F254	0.37	Piskornik and Bandurski (1972)

TABLE 3.--Continued.

Solvents	Composition	Layer	R_f IAA	References
2-Propanol:ammonia (7N): water	80:10:10	SG-G	0.37	Kaldewey (1969)
Isopropanol:ammonia (28%):water	85:5:15	SG	0.37	Ohwaki (1970)
1-Butanol:ammonia (25%):water	10:1:1	cellulose	0.41	Rekoslavskaya et al. (1974)
Multiple development with hexane:methyl ethyl ketone	65:35	SG-G buf. 5.2	0.42	Kaldewey (1969); Zimmermann and Rudiger (1976)
Propanol:methyl acetate: ammonia	45:35:20	SG-G	0.43	Kaldewey (1969)
2-Propanol:ammonia (conc.): water	8:1:1	SG (Supelcosil 12A)	0.43	Feung et al. (1975)
Methyl acetate:acetonitrile: ammonia (28%):water	40:10:5:4	cellulose	0.44	Ohwaki (1966)
Sodium chloride (8%) solution	-	SG	0.44-0.52	McDougall and Hillman (1978a)
1-Propanol:ammonia (25%):water	80:10:10	SG	0.45	Wakaloo (1965)
Methyl acetate:isopropanol: ammonia (28%)	45:35:20	SG	0.46	Ohwaki (1970)
Isopropyl ether:acetic acid	95:5	SG-H	0.48	Marumo et al. (1971)
1-Propanol:ammonia (34%)	7:3	SG	0.55	Hutzinger and Kosuge (1968b)
Chloroform:ethyl acetate: formic acid	35:55:10	SG	0.56	Kaldewey (1969); Feung et al. (1975)
1-Butanol:ethanol:water	80:20:15	SG	0.61	Wakaloo (1965)

TABLE 3.--Continued.

Solvents	Composition	Layer	R_f IAA	References
Chloroform:ethanol (abs.): acetic acid (96%):water	74:15:7:4	SG-G buf. 5.2	0.62-0.75	Kaldewey (1969); Zimmermann and Rudiger (1976)
Chloroform:methanol:acetic acid	75:20:5	SG (Supelcosil 12A)	0.65	Feung et al. (1975)
Chloroform:methanol:acetic acid (96%)	75:20:5	SG-G	0.73	Kaldewey (1969)
Butanol:acetic acid:ethyl ether:water	45:30:10:5	SG-G	0.75	Zimmermann et al. (1976)
1-Butanol:acetic acid:water	65:13:22	SG	0.78	Kaldewey (1969)
Methanol:water	1:3	cellulose	0.84	Ohwaki (1970)
Ethyl acetate:chloroform: acetic acid	50:25:1	SG	0.87	Ganguly et al. (1974)
Ethyl acetate:methyl ethyl ketone:ethanol:water	5:3:1:1	SG-60	0.90	Labarca et al. (1965)
1-Butanol:acetic acid (glacial):water	8:2:4	SG	0.90	Hutzinger and Kosuge (1968b)
Acetone:ammonia (25%)	100:1	SG	0.93	Kaldewey (1969)
Chloroform:ethyl acetate: formic acid	5:4:1	SG-G	-	Labarca et al. (1965)
Benzene:dioxane:acetic acid	50:50:25	SG buf. 5.2	-	Zimmermann and Rudiger (1976)
1-Propanol:ammonia (5M)	25:75	SG buf. 5.2	-	Zimmermann and Rudiger (1976)
Chloroform:ethyl acetate: acetic acid	20:10:1	SG	-	Yokota et al. (1974)

gels in a non-traditional adsorption mode for indole purification Raj and Hutzinger, 1970; Anderson, 1968; Ueda and Bandurski, 1969; Magnus et al., 1978) as well as the use of neutral and acidic polystyrene and polystyrene divinylbenzene copolymers (Reeve and Crozier, 1976; Ehmann and Bandurski, 1972; Niederwieser and Giliberti, 1971). The use of insoluble poly-N-vinylpyrrolidone (Polyclar AT) has been suggested (Glenn et al., 1972; McDougal and Hillman, 1978a; Percival, 1979) and its properties are also due to IAA retention to the polymer. Lipophilic Sephadex LH-20 has also been used for partial purification of IAA, both in the non-retentive mode with absolute ethanol (Steen and Eliasson, 1969) and more selectively by eluting with 50% aqueous ethanol (Bandurski and Schulze, 1974) or 80% methanol (McDougall and Hillman, 1978b). Ion exchange has usually been carried out on resins such as DEAE-cellulose in aqueous buffer (McDougall and Hillman, 1978a) or organic solvents (Bandurski and Schulze, 1974). Use of DEAE-Sephadex in aqueous alcohol combines both the ion exchange mode and the adsorption phenomenon. This method has been developed by Ms. Aga Schulze and is discussed in the experimental section of this thesis since it provides a powerful method for cleaning up samples prior to purification by high pressure liquid chromatography (see also Sweetser and Swartzfager, 1978).

High pressure liquid chromatography (HPLC) provides high ratios of support surface area to solvent and allows rapid separation with minimal solvent dilution. In this way HPLC provides a rapid and efficient chromatographic system. The number of theoretical plates of an HPLC column is five to ten times that of a thin-layer

chromatogram and recoveries of IAA from such columns are typically high. For these reasons HPLC has now become a most promising method for IAA analysis. Several columns have so far been found to be useful for HPLC analysis of IAA. Graffeo and Karger (1976) first applied C_{18}-reversed phase columns to IAA analysis and these columns have since been used by several groups (Trefz et al., 1976; Sweetser and Swartzfager, 1978; Krstulovic and Matzura, 1979; Anderson and Purdy, 1979; Cohen, this thesis; Brenner, personal communication). Use of anion exchangers such as Partisil 10 SAX and Permaphase AAX have been less popular but hold promise for future improvements (During, 1977; Sweetser and Swartzfager, 1978). Sample size for HPLC, of course, is dependent on the columns employed and currently available systems (1979) exist with columns of several centimeters in diameter for use in organic preparative work at the gram scale down to columns of less than a millimeter diameter for microanalytical work. Most popular are columns of about 4 mm diameter and 25 cm length which work well for purification of 0.1 to 10 μg of IAA. With the aid of suitable detectors they have been employed for direct analysis of partially purified extracts or, alternately, the eluent can be collected and analyzed by other methods as detailed in the experimental section of this thesis.

Gas-liquid chromatography (GLC) has been utilized for the purification of plant extracts which contain IAA and is usually, but not always (cf. Bandurski and Schulze, 1974), the terminal step in analysis. One investigator (Wightman, 1977; Phipps and Wightman, 1977) has claimed IAA analysis from a plant sample after simply

solvent partitioning, however this is unrealistic and a high degree of prior purification is essential. Common columns and derivatives used for GLC of IAA are given in Table 4. Four basic types of detectors have been used in the GLC of IAA. The flame-ionization detector is, by far, the most commonly used detector and its sensitivity limits are at about 100 ng for useful work. Unfortunately, the flame-ionization detector responds to all organic compounds and it is extremely difficult to remove interfering compounds from the plant extracts. Thus, while the flame-ionization detector has wide applicability, it is non-selective and its main usefulness is for qualitative analysis of highly purified extracts. The electron capture detector provides an increase of several orders of magnitude in sensitivity, but responds only to substances that readily capture electrons. With IAA it is usual to convert the IAA to a halogenated derivative such as the heptafluorobutyryl and trifluoroacetyl derivatives of IAA-methyl ester (Seeley and Powell, 1974) or the trichloroethyl ester of IAA (Bittner and Even-Chen, 1975). Since many of the impurities of plant extracts also derivatize with the reagents used, this method offers little improvement in selectivity. Recently substantial advances have been made in detectors which are specific for compounds which contain organic nitrogen or phosphorus (Brazhnikov and Shmidel, 1976; Kolb et al., 1977; Lubkowitz et al., 1977; Lubkowitz et al., 1978; Patterson and Howe, 1978; Rubin and Bayne, 1979). These thermionic detectors approach the sensitivity of electron capture, but respond to the nitrogen of the indole ring directly. Thus they are selective as well as sensitive and can be

TABLE 4.--Gas-liquid chromatographic methods used for the separation and analysis of indole-3-acetic acid.

Derivative[1]	Derivatizing Reagent[2]	Oven Temperature	Column	Detector[3]	References
bis-TMS-IAA	BSA	100-250 (3/min)	SE-30 WCOT	FID	Seyedin (1979)
bis-TMS-IAA	BSTFA	155/210	5% SP-2401	FID/MS	Bandurski and Schulze (1974)
bis-TMS-IAA	BSTFA	165	5% SP-2401	FID	Hall and Bandurski (1978)
bis-TMS-IAA	BSA	170	3.6% XE-60	FID	DeYoe and Zaerr (1976)
bis-TMS-IAA	BSA	195	SE-33	MS	Hall and Medlow (1974)
bis-TMS-IAA	BSA	200	5% OV-101	FID	McDougall and Hillman (1978b)
bis-TMS-IAA	BSA	210	3% OV-1	MS	Little et al. (1978)
IAA-Me	DZMe	95-310 (3/min)	3% OV-17	FID	Wightman (1977)
IAA-Me	DZMe	180	3.6% XE-60	FID	DeYoe and Zaerr (1976)
IAA-Me	DZMe	180	3% OV-17	MS	Rivier and Pilet (1974)
IAA-Me	DZMe	200	2.5% Hi-Eff-8-BP	FID/MS	DeYoe and Zaerr (1976)
IAA-Me	DZMe	220	3% OV-17	FID	Wightman (1977)
IAA-Me	DZMe	220	3% OV-225	FID	Wightman (1977)
IAA-Me	DZMe	230	7% Versamid 900	AI	Powell (1964)
IAA-Et (+0-3 Cl)	EtOH or Cl-EtOH + TSA	190	1.5% QF-1	EC	Bittner and Even-Chen (1975)
IAA-Et (+0-3 Cl)	EtOH or Cl-EtOH + TSA	190	1.5% SE-30	EC	Bittner and Even-Chen (1975)
TFA-IAA-Me	DZMe, TFAA	140	2% SE-30	EC	Seeley and Powell (1974)
TFA-IAA-Me	DZMe, TFAA	170-240 (10/min)	1.5% OV-101	MS	Caruso et al. (1978)
TFA-IAA-Me	DZMe, TFAA	180	15% SE-30	EC	Brook et al. (1967)
HFB-IAA-Me	DZMe, HFBI	140	2% SE-30	EC	Seeley and Powell (1974)
HFB-IAA-Me	DZMe, HFBI	150	5% OV-17	MS	Bertilsson and Palmer (1972)
HFB-IAA-Me	DZMe, HFBI	150	3% XE-60	MS	Bertilsson and Palmer (1972)

[1]Abbreviations: Ethyl ester = Et; N-Haptafluorobutyryl = HFB; Methyl ester = Me; N-Trifluoroacetyl = TFA; Trimethylsilyl = TMS.

[2]Abbreviations: N,O-Bis-(trimethylsilyl)-acetamide = BSA; N,O-Bis-(trimethylsilyl)-trifluoroacetamide = BSTFA; Mono-, di- or trichloroethyl alcohol = Cl-EtOH; Diazomethane = DZMe; Ethanol = EtOH; Heptafluorobutyrylimidazole = HFBI; Trifluoroacetic anhydride = TFAA; ρ-Toluene sulfonic acid = TSA.

[3]Abbreviations: Argon ionization detector = AI, Electron capture detector = EC; Flame ionization detector = FID; Mass spectrometer = MS.

used with simple derivatives such as the methyl ester of IAA. This method has been used for qualitative work with little purification (Swartz and Powell, 1978) and, after purification by HPLC or affinity chromatography (Schulze and Bandurski, 1979), it has been used for quantitative isotope dilution analysis (Cohen et al., 1978; Cohen, this thesis). Although not as selective as the mass spectrometer, the thermionic detector should play an important role in routine assays for IAA. After all, the number of gas chromatographs in use in the world must now greatly exceed the 60,000 estimated in 1969 (McNair and Bonelli, 1969), such that access to this analytical equipment is now widely available. The use of a thermionic detector for detection of nitrogen-containing compounds on thin layer chromatograms (Ritchie et al., 1978) and with HPLC (Compton and Purdy, 1979) has also been reported.

Now, forgetting cost and availability, the most selective detector for GLC is the mass spectrometer. In addition to being the most selective, it is also the only detector which will simultaneously provide both amounts of the endogenous IAA and the amount of the isotopically labeled internal standard. The sensitivity of the coupled GC-MS is, in the selective ion monitoring mode, comparable to that of the nitrogen thermionic detector and several papers have compared these methods for analysis of compounds other than IAA (cf. Boutagy and Harvey, 1978; Davisson, 1978). A comprehensive review of quantitative mass spectrometry is available (Millard, 1978) and several reviews cover the use of stable isotopes for quantitative GC-MS (Gaffney et al., 1971; Knapp and Gaffney, 1972; Gordon and Frigerio,

1972). Several authors have used stable isotope labeled IAA for isotope dilution analysis with mass spectral detection (Magnus and Bandurski, 1978; McDougall and Hillman, 1978a; Caruso et al., 1978; Allen et al., 1979). A detailed study of the mass spectral properties of trimethylsilyl-indoles has been published (Ehmann et al., 1975). An as yet unexploited method for mass spectral quantitation of IAA would be by negative ion mass spectrometry. A recent report describes the use of negative ion chemical ionization mass spectrometry for the detection of dopamine, amphetamine and Δ^9-tetrahydrocannabinol at the attomole (10^{-18}) level (Hunt and Crow, 1978).

In addition to the detection methods for IAA used for GLC analysis, several other methods have been used with other analytical procedures. For column and high pressure liquid chromatography IAA has been detected directly by its U.V. absorbance, its fluorescence (cf. Graffeo and Karger, 1976), by counting the radioactivity of added standard ^{14}C-IAA and by the compound's electrochemical properties (Sweetser and Swartzfager, 1978; see also Buchta and Papa, 1976; Ponzio and Jonsson, 1979). For some situations U.V. examination has also proved useful for IAA detection on thin layer and paper chromatograms. Light based detection methods and electrochemical methods can be very sensitive (detection limits approximately 1 ng), however at submicrogram levels they are destructive and with plant extracts they offer poor selectivity.

Numerous sensitive biological assays for IAA exist. They are important since they detect biological activity, but they are generally time-consuming, have poor precision, are not specific for IAA

and are influenced by other compounds in plant extracts. Nevertheless, biological activity is an important parameter in hormonal physiology and much of our early understanding of hormonal relationships was based on such semi-quantitative techniques. The first bioassay for plant auxins was performed at 3:00 a.m. April 17, 1926 by F. W. Went (Went, 1928, 1974). Went measured the curvature produced by asymmetric application of auxin in agar blocks to decapitated *Avena* coleoptiles. After Went's report several improvements were made, the most important of which was the removal of the seed endosperm from the seedling which improved the sensitivity of the procedure (Skoog, 1937). Bonner (1933) described a simplified bioassay which measured the straight growth of *Avena* coleoptile cylinders floated on solutions of the test substance. These two methods, and the variations using coleoptiles of other seedling grasses, have been the most common methods for IAA determination. Thimann and Schneider (1938) introduced the first bioassay using dicotyledonous plants when they devised the split pea test and recently Meudt and Bennett (1978) introduced a new bioassay utilizing light grown bean internode sections which can be performed under normal laboratory lighting conditions. Other methods which have been used are, unfortunately, even more tedious but two especially clever methods are worthy of mention. Cholodny (1930) developed an elegant method for measuring growth in which a coleoptile was mounted in a micropotometer consisting of a fine capillary tube filled with water. Since the coleoptile base was sealed to the capillary, increases in water content of the tissue could be measured by a change in the position of the meniscus. Bose

(1906, 1927) and Korngsberger (1922) described high resolution growth recorders which were applicable to auxin problems; however newer apparatus, which are of equal sensitivity, are more convenient (see review by Evans, 1974).

Chemical modification of IAA to produce colored or floures-cent derivatives is a useful and popular technique for analysis and for visualization of IAA on thin layer and paper chromatograms. Salkowski (1885) provided the first analytical method for analysis of IAA by the reaction of IAA with an oxidant in strong mineral acid. An improvement in the Salkowski method was described by Tang and Bonner (1947) and, although not as sensitive as other methods, its reactivity toward IAA, to the exclusion of many other indoles, makes it an important technique. The Salkowski reagent has been used as a colorimetric reagent, especially for measuring IAA decomposition, and as a spray reagent for silica gel thin layer chromatography. The product of indoles reacted with the Salkowski reagent is not stable and the products' colors change quickly to non-diagnostic brown tones. Further, owing to the strong acidity of the reagent it is generally not suitable for carbohydrate type chromatography supports. Some of these problems are relieved by the modifications of Chrastil (1976). The use of *p*-dimethylaminobenzaldehyde or *p*-dimethylamino-cinnamaldehyde in acid with an oxidant (Ehrlich, 1901; van Urk, 1929; Hartley-Mason and Archer, 1958) are the most specific chromogenic reagents for indole derivatives, but color development is slow and the chromophores are unstable. The benzaldehyde derivative is 3-8 times more sensitive than the *p*-dimethylaminocinnamaldehyde reagent

(Ehmann, 1973), contrary to a previous report which found the *p*-dimethylaminocinnamaldehyde reagent superior (Hartley-Mason and Archer, 1958). Several cinnamic acid derivatives also react with these reagents as do isoindoles (Remer, 1972), however the cinnamic acids respond only at much higher levels. These Ehrlich type reagents work well with paper or cellulose thin layer chromatograms since they do not contain the strong mineral acids of the Salkowski or Ehmann (1977) sprays. An improvement in colorimetric determination of indoles was provided by Ehmann (1977) who described a reagent composed of a 1:3 mixture of van Urk and Salkowski reagents with which stable colors are produced in less than 10 minutes. This reagent is useful both for colorimetry and silica gel thin layer detection. Colors produced by this reagent with different indoles cover a wide spectral range and when coupled with migration on the chromatogram provide important diagnostic information.

Several yellow fluorescing indole condensation products have been used for IAA detection and are among the most sensitive reagents available. However, the presence of many naturally yellow fluorescing compounds in extracts of plant material make them of limited use. Fluorescing products of IAA are produced by formaldehyde-HCl and *o*-phthalaldehyde-HCl (see Seiler and Demisch, 1977) as well as with the Plieninger reaction (Plieninger et al., 1964). The Plieninger reaction has been studied as a quantitative assay for IAA in crude plant extracts by several groups of investigators (Stoessl and Venis, 1970; Knegt and Bruinsma, 1973; Eliasson et al., 1976; Kamisaka and Larson, 1977; Bottger et al., 1978a; see also Brieskorn and Mechtold,

1972). Eliasson et al. (1976) found that multiple purification steps were required before the assay was usable with a variety of plant materials, however when these precautions were taken then the results were similar to those obtained by bioassay. Unfortunately, bioassay is also subject to errors such that the methods are as yet unverified. At the level of purification used by Eliasson et al. (1976) other more exacting physical methods could have been employed, such that the laborious procedure of the fluorescence assay appears less attractive. The reaction is also sensitive to reaction conditions, extractable impurities, and the final product is unstable. Various substituted indole acids also react with this reagent and could be a source of difficulty (Bottger et al., 1978a). Other methods suggested for use with crude plant extracts such as densitometry of thin layer chromatograms sprayed with Ehrlich's reagent (Zimmermann and Rudiger, 1976; Zimmermann et al., 1976) and the use of radioimmunoassay (Pengelly and Meins, 1977) are subject to similar problems and remain unverified. The need for simple, rapid and inexpensive assays for IAA in plant extracts is very great. In the experience of this laboratory, no such assays (which have been critically verified) are as yet available.

The Ehmann reagent, as well as the reagents of Ehrlich and Salkowski, can also be used for the detection of the bonded forms of IAA (Andreae and Good, 1955; Labarca et al., 1965; Ehmann, 1977). The fluorometric methods, which rely on the reactivity of the side chain, are not suitable for this purpose unless hydrolysis is performed prior to detection. Chromatographic conditions for

purification of amino acid conjugates of IAA are reviewed by Feung et al. (1975) and Sephadex LH-20 chromatography was proven useful for purification of synthetic preparations (Peterson, 1978). In general, methods for purification of these compounds are similar to those used for IAA and many of the solvent systems shown in Tables 1 and 2 are useful for this purpose. The high molecular weight compounds (Piskornik and Bandurski, 1972; Percival and Bandurski, 1976) offer unique challenges as detailed in the original publications. The inositol ester compounds in corn seeds are well studied and the chromatographic behavior of those neutral compounds in many systems has been detailed. Column chromatography on Dowex 50 or partially sulfonated Dowex-type resin (Ehmann and Bandurski, 1972), on Sephadex G-10 or LH-20 (Ueda and Bandurski, 1969; Ehmann, 1974a), on polyvinylpyrrolidone (Percival, 1979), on amino acid analyzer resin Beckman PA-28 (Cohen and Bandurski, 1977) and on C_{18}-reverse phase HPLC (Brenner, personal communication) all provide good separation of the corn esters, although they differ in the time required for separation and the amount which can be purified. Thin layer chromatography (Labarca et al., 1965) and gas liquid chromatography of the fully trimethylsylilated compounds (Ueda and Bandurski, 1974) has been well described. Isotope dilution analysis of these compounds is generally accomplished by hydrolysis and using isotopically labeled IAA as the standard (cf. Bandurski and Schulze, 1974). Direct quantitative analysis of IAA esters using synthetic ^{14}C-indole-3-acetyl-*myo*-inositol should now be possible (Nowacki et al., 1978). Conditions for alkaline hydrolysis of both ester (1 N NaOH at 25 C for 1 hr)

and amide (7 N NaOH at 100 C for 3 hr) conjugates have been studied and used for isotope dilution analysis of free, ester and amide IAA in a variety of plant species (Bandurski and Schulze, 1977b).

Although many excellent methods are now available for the purification, detection and quantitation of IAA and its conjugates, the required analysis times are still quite long. Methods in use in this laboratory when this work was begun (1974/1975) required over a week to analyze IAA. Now we can perform this analysis with newer techniques in slightly longer than one day. Similarly, sample size has dropped from 1 kg to as low as 10 g. However, even 10 grams of some materials can be very difficult to collect and accurate methods which will allow multiple assays per day on very small amounts of plant material would be a valuable future improvement.

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The Bound Auxins: Protection of Indole-3-acetic Acid from Peroxidase-catalyzed Oxidation

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Abstract. Indole-3-acetic acid (IAA) was oxidized by horseradish peroxidase, but ester and amide conjugates of IAA were not degraded. Addition of indoleacetyl-*myo*-inositol, indoleacetyl-L-aspartate, indoleacetylglycine, indoleacetyl-L-alanine, indoleacetyl-D-alanine, or indoleacetyl-β-alanine did not affect the rate of oxidation of IAA by horseradish peroxidase. Peroxidase preparations from *Pisum sativum* L. and *Zea mays* L. behaved similarly in that they rapidly oxidized IAA, but not conjugates found in the plant from which the peroxidase was prepared. These results indicate that conjugation could affect the stability of IAA in vivo.

Key words: Auxin (bound) – Auxin oxidation – IAA oxidase – Peroxidase – *Pisum* – *Zea*.

Introduction

The plant hormone indole-3-acetic acid (IAA) is found in the seeds and seedling shoots of all higher plants examined (cf. review by Gordon, 1954). Most of the IAA found in plant tissues is covalently linked to some other compound (Bandurski and Schulze, 1977). The predominant forms of IAA in leguminous plants are amide conjugates while esterified IAA is found in monocotyledonous plants (Bandurski and Schulze, 1977). The metabolic significance of chemical conjugation of IAA is only now being studied but it has been shown that conjugates act as reserve forms for the homeostatic control of hormone concentrations (Berger and Avery, 1934; Bandurski et al., 1969) and more recently it has been shown by J. Nowacki (see Bandurski, in press) that IAA conjugates are much more rapidly transported from seed to shoot than IAA. To these two functions, homeostasis and transport, we wish to suggest a third possible function of conjugation – the protection of IAA against oxidation by peroxidase. This hypothesis is based upon our demonstration that all of the IAA conjugates tested are resistant to peroxidase-catalyzed oxidation. Conjugation may be important in preventing oxidation of the hormone while en route since 98% of the free IAA that migrates from the seed to the shoot is metabolized before reaching the shoot (Hall, 1977; Hall and Bandurski, in press; see also review by Bandurski, in press).

Peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) can act as an IAA oxidase (Galston et al., 1953; Hinman and Lang, 1965; Gove and Hoyle, 1975) and it has been proposed that enzymatic oxidation is the major degradative pathway for the hormone in vivo (Waygood et al., 1965; see review by Galston and Hillman, 1961). The degradative pathway for IAA in vivo may still be unknown, but *if* peroxidase does destroy IAA in vivo then the resistance of IAA conjugates to peroxidative destruction is important. In this study we show that horseradish peroxidase will degrade IAA but not IAA conjugates, either amide or ester. In addition, we show that peroxidase activities from *Zea* and *Pisum* also will degrade IAA but not the IAA conjugate native to the plant from which the activity was isolated.

Materials and Methods

Reaction Mixture

The standard reaction mixture contained 0.125 μg/ml horseradish peroxidase (Type I; Sigma Chemical Co., St. Louis, Mo., USA), 0.1 mM 2,4-dichlorophenol (Eastman Organic Chemicals, Rochester, N.Y., USA), 0.1 mM $MnCl_2$, 10 mM sodium phosphate, pH 6.1, and IAA or IAA conjugate as indicated. The final volume of the reaction mixture was 800 μl and all reactions were carried

Abbreviation: IAA = Indole-3-acetic acid

out at 25° C. In experiments with *Zea* and *Pisum* peroxidase preparations, buffer and enzyme composition were varied as noted.

IAA was from Sigma and indole-3-acetyl-L-aspartic acid, dicyclohexylammonoum salt, was obtained from Calbiochem (San Diego, Cal., USA). Other IAA-amino acid complexes were gifts from Mr. M. Peterson and Dr. N.E. Good (Michigan State University). Indole-3-acetyl-*myo*-inositol was synthesized on a preparative scale using the method of Nowacki et al. (1977) and purified on a Silica-gel 60 (E. Merck, Darmstadt, Germany) column with elution with methyl ethyl ketone-ethyl acetate-ethanol-H_2O (3:5:1:1, v/v). Silica-gel column chromatography was followed by chromatography on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) with 50% 2-propanol-water (v/v) as the mobile phase, and finally by preparative high-pressure liquid chromatography (Cohen and Bandurski, 1977). The identity of the product was confirmed by combined gas liquid chromatography-mass spectrometry. Determination of axial and equatorial substitution was made according to migration on Silica-gel thin-layer chromatograms (Ehmann and Bandurski, 1972).

Peroxidase Activity

One unit of peroxidase activity was defined as that amount of enzyme decomposing 1 µmole of peroxide per minute at pH 6.0 and 25° C. This was determined by adding 10 µl of enzyme to a 3 ml reaction mixture buffered at pH 6.0 with 10 mM sodium phosphate and containing 0.88 mM H_2O_2 (Superoxol, Merck & Co., Rahway, N.J., USA) plus 3.5 mM 3,3'-dimethoxybenzidine (Sigma) as the hydrogen donor. The reaction was followed by measuring the rate of color development at 460 nm. Under these conditions the horseradish peroxidase used in these experiments had an activity of 531 units/mg.

Enzyme Preparations

Corn kernels (*Zea mays* L. cv. Stowell's Evergreen; Vaughan's Seed Co., Oxid, Mich., USA) were grown in the dark on moist paper towels for 5 d at 25° C as previously described (Hall and Bandurski, in press). Shoots were removed from the seedlings and 280 g collected in an ice-chilled beaker. The shoots were placed in a 4-l model CB-6 Waring blendor (Waring Products, New Hartford, Conn., USA) and covered with 2 l of acetone previously chilled to −20° C by the addition of dry ice. After grinding for 2 min the slurry was filtered by suction and washed with an additional 1.5 l of −20° C acetone. The cold residue was washed with 500 ml diethyl ether at 4° C and placed in a ceramic dish inside a desiccator under vacuum for 14 h. The off-white powder was ground with a pestle and dried in vacuo over P_2O_5 for 24 h.

The dry residue (10.2 g) was suspended in 250 ml of 0.1 M potassium phosphate, pH 6.5, by stirring for 16 h and then centrifuged at 10,000 × *g* for 20 min at 4° C. Protein in the amber supernatant fluid was precipitated by a series of ammonium sulfate (Special Enzyme Grade, Mann Research Labs., New York, N.Y., USA) fractionation steps at 4° C. The supernatant fluid was brought to 35, 50, and 80% of saturation. After each addition of ammonium sulfate the suspension was centrifuged at 10,000 × *g* for 20 min and the pellet collected, resuspended in 20 ml 0.1 M potassium phosphate, pH 6.5, and assayed for IAA-oxidase activity. The 50–80% pellet was found to be enriched in activity. The resuspended 50–80% pellet was again centrifuged at 10,000 × *g* and the supernatant fluid used as the *Zea* enzyme in the experiments described.

Two peroxidase isoenzymes isolated from 7-day-old *Pisum sativum* L. cv. Alaska 2B (Asgrow Mandeville Co., Cambridge, N.Y., USA) stems were obtained from Ms. D. Gibson and Dr. E.H. Liu (University of South Carolina, Columbia, S.C., USA). These isoenzymes, designated by Dr. Liu as isoenzymes "1" and "5", had different physical properties. Isoenzyme "1" migrated to the anode during starch electrophoresis at pH 8.3 while isoenzyme "5" moved toward the cathode. As discussed later the ratio of peroxidase to IAA-oxidase activity for the two isoenzymes also proved to be different.

End-point Analysis

The reaction mixtures were incubated in a water bath for 1 h with shaking. Reactions were terminated by heating the tubes to boiling, a treatment found to prevent further oxidation of IAA by peroxidase. The reaction mixture was dried at 45° C in vacuo and the residue made to 100 µl with 50% 2-propanol-water (v/v). This resuspended reaction mixture was used for both qualitative and quantitative assays.

Twenty µl of the resuspended reaction mixture was applied to a Silica-gel thin-layer plate (5610; E. Merck, Darmstadt, Germany), then developed in the solvent described for Silica-gel column chromatography, and sprayed with Ehmann's reagent (Ehmann, 1977). This qualitative examination showed only a small amount of reaction products that reacted with the color reagent, and thus established that the colorimetric assay was a valid measurement of IAA destruction.

Quantitative data were obtained by incubation of 50 µl of the resuspended reaction mixture with 200 µl of Ehmann's reagent at 45° C. After 45 min the reaction was terminated by the addition of 600 µl of glass-distilled water and A_{615} measured on a Gilford 240 (Gilford Instrument Labs., Oberlin, O., USA) spectrophotometer (Percival and Bandurski, 1976; Ehmann, 1977). Although no residual IAA was detected in the horseradish peroxidase reaction mixture after 1 h incubation, a small absorbance was obtained. This resulted from the tail of a broad absorption band with a peak at 520 nm, and was probably the same compound observed above as a pink spot at the solvent front on thin-layer chromatograms.

Kinetic Analysis

The oxidation of IAA by peroxidase was monitored at 25° C by the change in absorbance at 251 nm (Ray, 1956; Meudt and Gaines, 1967) with a Cary 15 (Applied Physics, Monrovia, Cal., USA) spectrophotometer against a blank without enzyme. In early experiments ultraviolet scans were utilized to study spectral shifts during the peroxidase-catalyzed reaction. This method was not, by itself, adequate to study degradation of the conjugates since their oxidation products might differ from those of IAA oxidation. Complete oxidation of the 0.1 mM IAA in the 800 µl reaction mixture resulted in a change in A_{251} of about 0.62 and occurred in 45–75 min, depending on the enzyme preparation used.

Results

Experiments with Horseradish Peroxidase

Table 1 shows that horseradish peroxidase is unable to catalyze the oxidation of IAA when IAA is conjugated either through an ester bond to inositol, or by an amide linkage to an amino acid. In confirmation of numerous previous studies (cf. review by Hare, 1964) IAA was degraded by peroxidase at a rapid

Table 1. The oxidation by horseradish peroxidase of IAA and IAA conjugates incubated for 1 h at 25° C

The mixture contained 0.1 mM 2,4-dichlorophenol, 0.1 mM $MnCl_2$, and 10 mM sodium phosphate, pH 6.1. IAA, IAA conjugates and horseradish peroxidase included as noted. Total volume was 800 µl and the values given are the average of duplicate trials which agreed within ± 3%.

Compound added	Horseradish peroxidase	Indole compound recovered	
	(µg/ml)	nmol[a]	% of control
Indole-3-acetic acid	0	91.6[b]	
	0.125	3.6	3.9
Indoleacetyl-*myo*-inositol	0	59.0	
	0.125	59.6	101.0
Indoleacetyl-L-aspartate	0	47.9	
	0.125	49.1	102.5
Indoleacetylglycine	0	97.0	
	0.125	96.8	99.8
Indoleacetyl-L-alanine	0	82.7	
	0.125	82.5	99.8
Indoleacetyl-D-alanine	0	88.3	
	0.125	87.4	99.0
Indoleacetyl-β-alanine	0	69.6	
	0.125	66.3	95.3

[a] Calculated from A_{615} using the relationship $A_{615} \times 9.2 = \mu g$ of IAA

[b] Recovery was quantitative and, therefore, control values indicate initial amount

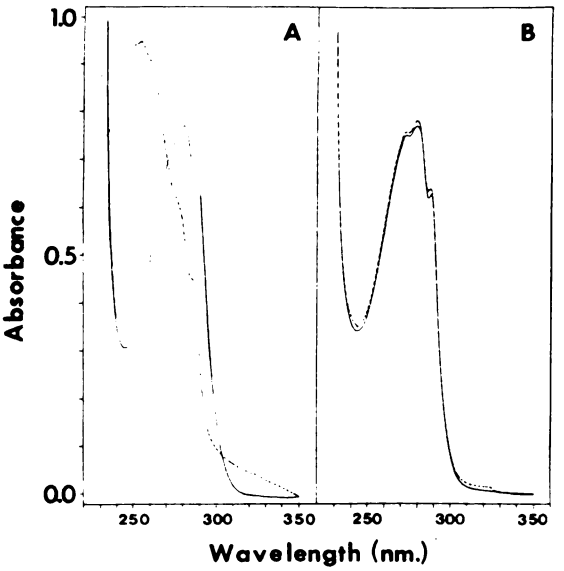

Fig. 1 A and B. Change in absorption spectra of standard reaction mixtures (see text) containing horseradish peroxidase and either IAA (**A**) or indoleacetyl-*myo*-inositol (**B**). The spectra shown by the dashed lines were taken 45 min after the reactions were begun; the solid lines show spectra at zero time. The product with the absorption at 251 nm has been reported to be 3-methyleneoxindole (Hinman and Lang, 1965)

rate (Table 1). These results are confirmed in Figure 1 which shows the marked spectral shift, the result of oxidation (Ray, 1956), in the IAA-containing reaction but very little spectral change after 45 min in the indoleacetyl-*myo*-inositol reaction mixture.

Inactivity of an enzyme toward a substrate can be based on, at least, three reasons. First, the compound may be so different from the normal substrate that an enzyme-substrate complex cannot be formed. Second, the compound may bind to the enzyme but not react; in this case, the compound should act as a competitive inhibitor. Third, a contaminant in the reaction mixture might inhibit the enzyme-catalyzed reaction. These possibilities were investigated by determining the rate of peroxidase degradation of IAA in the presence of various IAA complexes (Table 2). In initial studies with indoleacetyl-*myo*-inositol which had been purified only by Silica-gel and Sephadex LH-20 chromatography, a marked inhibition of IAA oxidation was noted (with an apparent K_i of 2×10^{-5} M). The reason for this inhibition was not determined, but inhibition diminished upon storage and was not observed with preparations of indoleacetyl-*myo*-inositol after purification by high-pressure liquid chromatography. At the concentrations tested none of the IAA complexes inhibited the rate at which peroxidase degraded IAA (Table 2). Because of the complex kinetics of the peroxidase-catalyzed oxidation of IAA (Hinman and Lang, 1965) competitive inhibition cannot be studied in the usual manner. However, these results demonstrate that the compounds tested were free from contaminating inhibitors and that at the concentrations tested they did not interfere with the binding of IAA by peroxidase.

A CoASH-, ATP- and Mg^{2+}-dependent enzyme system for the formation of indoleacetyl-*myo*-inositol has been described (Kopcewicz et al., 1974) and more recent data of A. Schulze (see Bandurski, in press) indicate that only the equatorial esters are synthesized by this enzyme system. The axial ester most likely results from acyl migration (Ehmann and Bandurski, 1972). A preparation of indoleacetyl-*myo*-inositol containing only the equatorial isomers was tried in the experiment shown in Table 2, but no difference

Table 2. Failure of IAA conjugates to inhibit the rate of free IAA oxidation by horseradish peroxidase (as measured by the change in A_{251})

The reaction mixture contained 0.125 µg/ml horseradish peroxidase, 0.1 mM dichlorophenol, 0.1 mM $MnCl_2$, 10 mM sodium phosphate, pH 6.1, 0.1 mM IAA, and IAA conjugate as noted. Values are the average of duplicate trials

Compound added	mM	ΔA_{251} /min[a]	ΔA_{251} after 45 min[b]
Control (0.1 mM IAA only)	–	0.025[c]	0.62
Experimentals (0.1 mM IAA + IAA conjugate)			
Indoleacetyl-*myo*-inositol (mixed axial and equatorial substitution)	0.12	0.023	0.60
(equatorial isomers only)	0.24	0.024	0.59
Indoleacetyl-L-aspartate	0.10	0.024	0.61
Indoleacetylglycine	0.10	0.026	0.64
Indoleacetyl-L-alanine	0.10	0.026	0.62
Indoleacetyl-D-alanine	0.10	0.027	0.62
Indoleacetyl-β-alanine	0.10	0.026	0.62

[a] Maximum slope obtained during reaction

[b] Indicates the extent of the reaction. Incubation longer than 45 min resulted in no further change in A_{251}

[c] Values were normalized by correcting for changes in control rates. The value 0.025 is the mean for 8 separate determinations, with actual values ranging from 0.021 to 0.026

in the rate of oxidation because of the isomeric form used was noted.

Experiments with Peroxidase from Pisum and Zea

Indoleacetyl-*myo*-inositol was isolated from and identified as a naturally occurring compound in *Zea* (Bandurski et al., 1969) and indoleacetyl-L-aspartate has been shown to be formed in *Pisum* seedlings after application of exogenous IAA (Andreae and Good, 1955). Table 3 shows the results obtained with peroxidase enzymes isolated from *Zea* and *Pisum*, and confirms the results obtained with horseradish peroxidase. Peroxidases isolated from *Zea* and *Pisum* oxidize IAA but are ineffective against the conjugate found in or formed by the respective plant.

The peroxidases tested exhibited different ratios of IAA oxidase to peroxidase activity. Equivalent IAA oxidation rates were obtained with 0.053 units of horseradish peroxidase (Table 2), 0.27 units of *Pisum* isoenzyme "1", 0.56 units of *Pisum* isoenzyme "5" and, by extrapolation, 0.84 units of *Zea* enzyme (Table 3). Also, the *Zea* enzyme had an acidic pH optimum for IAA oxidation while its peroxidase activity was higher at a more neutral pH.

Table 3. The oxidation of IAA and IAA conjugates by enzyme preparations from *Zea* and *Pisum*

Conditions are as in Tables 1 and 2 except as noted. Amount of enzyme is expressed in units of peroxidase activity (see text) added to the 800 µl reaction mixture. Values are the average of duplicate determinations.

A. End-point analysis

Compound added	Amount enzyme added (units)	Indole compound recovered	
		nmol[a]	% of control
Zea enzyme[b]			
Indole-3-acetic acid	0	113.5[c]	
	0.64	11.9	10.5
Indoleacetyl-*myo*-inositol	0	92.4	
	0.64	92.9	100.5
Pisum enzymes			
Indole-3-acetic acid	0	86.4	
Isoenzyme "1"	0.27	9.8	11.3
Isoenzyme "5"	0.56	11.7	13.5
Indoleacetyl-L-aspartate	0	111.5	
Isoenzyme "1"	0.27	110.9	99.5
Isoenzyme "5"	0.56	107.0	96.0

B. Kinetic analysis

Compound added	ΔA_{251} /min[d]	ΔA_{251} after 75 min[e]
Zea enzyme (0.64 units)[b]		
0.1 mM Indoleacetyl-*myo*-inositol only	0.001	0.04
0.1 mM Indole-3-acetic acid only	0.019	0.68
0.1 mM Indoleacetyl-*myo*-inositol +0.1 mM IAA	0.018	0.66
Pisum enzymes		
0.1 mM Indoleacetyl-L-aspartate only		
0.27 units isoenzyme "1"	0.000	−0.01
0.56 units isoenzyme "5"	0.000	0.00
0.1 mM Indole-3-acetic acid only		
0.27 units isoenzyme "1"	0.025	0.59
0.56 units isoenzyme "5"	0.026	0.62
0.1 mM Indoleacetyl-L-aspartate +0.1 mM IAA		
0.27 units isoenzyme "1"	0.023	0.55
0.56 units isoenzyme "5"	0.023	0.59

[a] Calculated from A_{615} using the relationship $A_{615} \times 9.2 = \mu g$ of IAA

[b] In assays with the *Zea* enzyme 50 mM sodium acetate, pH 3.5, replaced the 10 mM sodium phosphate pH 6.1 buffer

[c] Recovery was quantitative and, therefore, control values indicate initial amount

[d] Maximum slope obtained during reaction

[e] Indicates the extent of reaction. Incubation longer than 75 min resulted in no further change in A_{251}

Discussion

Galston and Hillman (1961) reviewed the methods used for study of IAA oxidation and in the present work we utilized the two spectrophotometric methods which they found most reliable. We also employed an end-point analysis using the indole reagent of Ehmann (1977). The Ehmann reagent is superior to the Salkowski reagent of Tang and Bonner (1947), being more specific for indoles and giving characteristic colors according to the position of substitution of the indole ring (Ehmann, 1977). Further, the colored product is stable and the reagent can be used for colorimetry or for chromatographic detection. This last-named feature allowed us to monitor by thin-layer chromatography for products which might interfere with colorimetry.

Inability of IAA-oxidase preparations to oxidize indoleacetylaspartate has been reported before (Andreae and Good, 1955; Rekoslavskaya et al., 1974; Hamilton et al., 1976). Two of these reports were however without specific data, and the experiments described by Rekoslavskaya et al. (1974) were performed with relatively impure enzyme and indoleacetylaspartate preparations, a confirmation of their result thus being necessary. To our knowledge, no previous study has been made of the resistance of the ester compounds to peroxidase oxidation nor has the susceptibility to peroxidase oxidation of amino acid conjugates of IAA other than indoleacetylaspartate been determined.

Rekoslavskaya et al. (1974) suggested conjugation as a means by which plants protect IAA from peroxidative decomposition and later liberate IAA for hormonal regulation of cell activity. Whether peroxidase does, in fact, play an in vivo role in the regulation of hormone concentration is uncertain but what does appear necessary is 1) that the plant protect itself against excess IAA, and 2) that once IAA has performed the growth-promoting act it be destroyed so that the hormone remains limiting and, thus, regulating. This system was anticipated by Bonner and Thimann (1935). Thus, without regard as to whether it is peroxidase – or a functionally related, but, possibly, more specific enzyme – we utilize our demonstration that IAA conjugates are immune to peroxidative attack together with other new knowledge to present a working hypothesis as to how IAA controls growth rates. The conversion of tryptophan to IAA is essentially inoperative in etiolated *Zea* seedlings (Hall and Bandurski, in press) and thus, the IAA conjugates serve as the sole source of IAA for the seedling. Further, conversion of IAA to its conjugate, hydrolysis of the conjugate to yield free IAA, and sensitivity of this system to light have been shown for *Zea*, both in vivo and in vitro (Hamilton et al., 1961; Kopcewicz et al., 1974; Hall and Bandurski, in press; Hall, 1977; Bandurski, in press; Bandurski et al., in press). To this system for varying or maintaining concentrations of free IAA we wish to propose, as is shown in Figure 2, a system for the destruction of excess hormone and a system for destruction of IAA once it has exerted its growth controlling effect. It is our belief that testing of this working hypothesis will lead to increased knowledge concerning how auxin mediates growth.

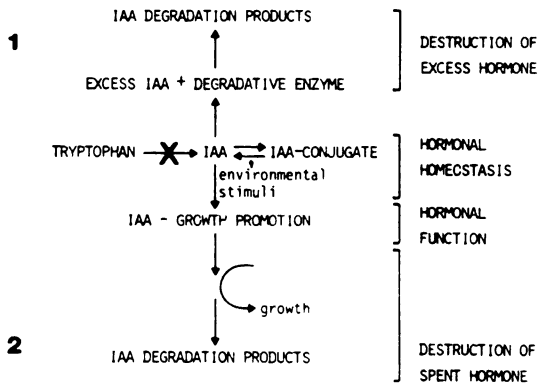

Fig. 2. A scheme for 1) the destruction of excess IAA, and 2) the destruction of IAA once it has performed its growth-promoting function (see discussion in text)

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Vol. 79, No. 4, 1977 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

PHOTO-REGULATION OF THE RATIO OF ESTER TO FREE INDOLE-3-ACETIC ACID

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SUMMARY: A light exposure, sufficient to cause a 30% reduction in growth rate of seedlings of *Zea mays*, causes a *decrease* of 40% in the concentration of free indole-3-acetic acid in the seedling and an *increase* in the content of esterified indole-3-acetic acid. We conclude that one mechanism for regulation of plant growth is alteration of the ratio of free to conjugated hormone by environmental stimuli.

Studies from this laboratory demonstrated that seeds and vegetative tissues of monocotyledonous and dicotyledonous plants have most of their indole-3-acetic acid (IAA) as ester or amide IAA (1, 2, 3). The esters are varied in structure ranging from IAA esterified to *myo*-inositol (3), or *myo*-inositol glycosides (4), or glucose (5), to high molecular weight compounds with IAA esterified to a glucan (6) or to a glucoprotein (7). Examples of amide IAA are indoleacetylaspartate (8) and indoleacetyllysine (9). An enzyme system for ester synthesis has been studied *in vitro* (10) and *in vivo* (11). Amide IAA has been demonstrated to be formed upon application of IAA to plants (12). IAA ester hydrolysis has been observed in autolyzing plant tissue (11) but IAA amide hydrolysis has not been studied. These data on the occurrence, complexity of structure, synthesis and hydrolysis of IAA derivatives led us to postulate a system for the control of hormone levels involving an equilibrium between free and ester or amide IAA (10). We now present data for control of the hormonal system, and thus the growth rate, by an environmental stimulus. To our knowledge, this is the first demonstration of a system for modification of hormonal homeostasis involving formation and hydrolysis of a covalently-bonded hormone conjugate.

MATERIALS AND METHODS

Seedlings of *Zea mays*, sweet corn, var. Stowell's Evergreen hybrid, were grown in a dark room at 25 C and 90% humidity using, when necessary, a phototropically inactive green light as previously described (1). The content of IAA, and IAA esters labile to hydrolysis by 1 N alkali, was determined by isotope dilution (1) except that purification was by solvent partitioning, DEAE cellulose, lipophyllic Sephadex, DEAE-Sephadex and thin layer chromatography with omission of purification by gas chromatography. Specific activity was determined by measuring radioactivity with scintillation counting in ACS solution (Amersham) and amounts of IAA with the Ehmann assay (13). Light exposure was for 27 sec with a 250 watt quartz-iodine lamp at a distance of 2 m and with a mirror at a 45° angle above and behind the trays of seedlings to provide uniform illumination.

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TABLE I
PHOTOINHIBITION OF GROWTH

Growth of corn seedlings following a 27 sec light flash as compared to a dark control.

	Dark	Light	Δ	%
	mm/90 min		mm	
	4.2	2.9	−1.3	−45
	2.8	1.9	−0.9	−32
	3.9	2.9	−1.0	−26
Mean	3.6	2.6	−1.1[a]	−34

[a] F value for difference between light and dark = 79;
F value at 5% level = 19. F value at 1% = 98.

The incident energy was 400 Kergs·cm² (in the region of the second positive phototropic curvature (14)) and between 600 to 2000 g fresh weight of seedlings were used for each sample. Estimation of seedling growth rate was made by photographing the seedlings against mm ruled graph paper at 0 to 90 min following the light flash using a phototropically inactive green safe light for illumination and Kodak Tri-X film with development in Ethol UFG. "Dark" controls were photographed similarly and, by projecting the resultant slides, growth of fracitons of a mm were measured.

RESULTS

Table I shows growth rates for the dark and light exposed seedlings. Growth reduction by light was 34% and was significant at the 5% level. Table II shows the content of free IAA in similarly treated seedlings. The reduction in concentration of free IAA following the light treatment was 10 μg/kg or 42%. Table II also shows a photo-induced increase in concentration of free plus ester IAA of 9 μg/kg. Thus, a light flash that causes a *loss* of 10 μg/kg of free IAA causes an *increase* in the amount of free plus ester IAA of 9 μg/kg. The loss of free IAA is significant at the 5% level. Labeled IAA ester was not available and so the exact concentration of free plus ester IAA could not be determined by isotope dilution, but that the values for free plus ester IAA for the dark and light treatments are different and that there is an increase in the light is significant at the 1% level. The isotope dilution assay used requires several kg of tissue and one week of time so it was impossible to do paired samples for both free and ester IAA and for this reason we report free and free plus ester IAA. Our isolation procedure has been shown to yield pure IAA by UV analysis and mass spectrometry (1). IAA and ester IAA measurements were made on seedlings harvested 90 min after the light flash. We have not detected amide IAA in *Zea mays* (1, 2).

DISCUSSION

This study shows that a light flash, sufficient to cause photoinhibition of growth, causes a decrease in concentration of free IAA and an increase in ester IAA. We interpret this result to mean that light affects the enzyme systems making and hydrolyzing IAA esters so the ratio of free to ester IAA is decreased in the light and increased in the dark with concommitant changes in growth rate.

TABLE II

PHOTO-INDUCED CHANGE IN FREE AND FREE PLUS ESTER IAA

Concentrations of free and free plus ester IAA in corn seedlings 90 min after a 27 sec light flash compared to a dark control.

	Dark	Light	Δ	%
	μg/kg			
FREE IAA				
	25	12	−13	−52
	22	14	− 8	−36
	21	13	− 8	−38
Mean	23	13	−10[a]	−42
FREE PLUS ESTER IAA				
	74	80	+ 6	+ 8
	60	66	+ 6	+ 8
	64	72	+ 8	+11
	69	82	+13	+16
	74	84	+10	+12
Mean	68	77	+ 9[b]	+11

[a] F value for difference between light and dark = 34; F value at 5% level = 19, F value at 1% = 98.

[b] F value for difference between light and dark = 42; F value at 5% level = 8, F value at 1% = 21.

Muir (15) earlier demonstrated that light-grown pea seedlings contain an indoleacetylaspartate conjugate, while dark-grown seedlings contained none. Conjugate formation was an all, or none, response and was not shown to be a system for control of hormone levels. Our results, together with those of Muir, and the knowledge that conjugates of giberellic acid, abscisic acid and cytokinins are known (cf. 16, 17, 18, 19), lead us to postulate, as a generalized working hypothesis, that the environment controls the rate of plant growth by altering the ratio of free to conjugated hormone.

The data presented here may permit reinterpretation of the mechanism of phototropic curvature. According to the Went-Cholodny theory for phototropic curvature, unilateral illumination of a seedling causes auxin to migrate from the illuminated to the darkened side (20). There is strong evidence that lateral migration of IAA can occur (cf. 21), but a study by Elliott and Shen-Miller (22) showing that photoinhibition of growth and phototropism are related physiological phenomenon—differing in whether illumination is uniform or unilateral—made lateral transport of IAA as an explanation for phototropic curvature less attractive. The Elliott and Shen-Miller experiment is diagrammed in Fig. 1. As can be seen on the right side of Fig. 1, the Went-Cholodny theory required that cells on the illuminated side of the coleoptile became hormone-exporter cells (e), while those on the dark side became hormone-importer cells (i). If the light moved 180° to the left side

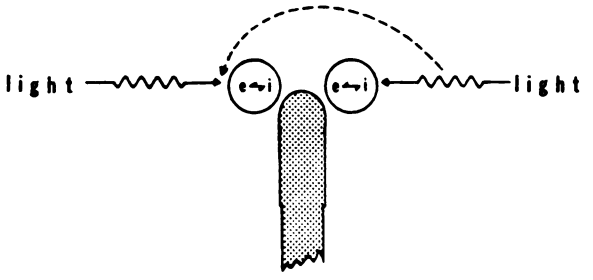

Fig. 1. A diagrammatic interpretation of the experiments of Elliott and Shen-Miller (22) and Shen-Miller *et al.*, (27) indicating the difficulty in explaining phototropism as a consequence of lateral transport of IAA if phototropism and photoinhibition of growth are mechanistically related phenomenon.

of the seedling of Fig. 1, the former hormone-exporter cells would become hormone-importer cells and *vice versa*. If then the light was moved rapidly, and rotated about the seedling, then *all* cells must simultaneously be both exporter *and* importer cells, and this is not a reasonable hypothesis. The data of this paper showing an effect of light on the ratio of free to ester IAA provides an alternative to lateral transport. However, it must be emphasized that our experiments do not differentiate between blue and red light photoreactions, nor inhibition of coleoptile and mesocotyl growth, nor explain the need for contiguity of tissue for tropic curvature (cf. 23, 24) and thus an overall explanation may not yet be possible.

Other early experiments can be interpreted in terms of a decrease in the ratio of free to ester IAA in light-exposed tissue. For example, van Overbeek (25) observed a 32% decrease in auxin diffusing from *Avena* seedling tips following an exposure to yellow light and, as much as an 80% decrease in auxin diffusing from *Zea* seedling tips following heating to 48 C. Both experiments are understandable if, as is commonly believed (cf. 26), it is free IAA which is transported downwards and the light, or heat, treatment decreased the ratio of free to ester IAA. Shen-Miller and Gordon (14) using ^{14}C-labeled IAA applied to *Zea* coleoptile tips, confirmed reduced diffusion of label from the illuminated side (cf. 20) but found that the sum of radioactivity that diffused out, plus the radioactivity remaining in the tissue, was the same for both sides. Possibly the greater radioactivity which remained in the illuminated tissue was ester IAA while that which diffused out was free IAA. Shen-Miller *et al.*, (27) observed photoinhibition of basipetal transport of auxin in *Avena* coleoptiles and, again, this can be interpreted to mean that light reduced the amount of free IAA available for basipetal transport.

The observation of an effect of light on the ratio of free to esterified IAA provides the first evidence of a system for control of hormone levels involving synthesis and hydrolysis of a covalent bond and may provide insight into the phenomenon of phototropism, photoinhibition of growth and photoinhibition of hormone transport.

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SYNTHESIS OF ^{14}C-INDOLE-3-ACETYL-*MYO*-INOSITOL

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SUMMARY

Synthesis of the mixed isomeric ^{14}C-indoleacetyl-*myo*-inositols from carrier-free β[2-^{14}C]-indoleacetic acid (57.2 mCi/mmole) and inositol *via* an imidazolide intermediate is described. Radiological decomposition of the indolylic compounds was prevented by the use of a volatile thiol, dithioethane, and anthracene.

Key Words: Dithioethane, Indole-3-acetic Acid, Indole-3-acetyl-*myo*-inositol, Inositol

INTRODUCTION

Esters of the plant growth hormone indole-3-acetic acid (IAA) and inositol or inositol glycosides comprise the bulk of the water soluble IAA found in seeds of corn (*Zea mays*) (1). These complexes are synthesized by a CoASH and ATP dependent enzyme system (2) and are hydrolyzed to free IAA by autolyzing plant tissue (3). The hormonal conjugates play a role in hormonal homeostasis (4,5), protection against peroxidase oxidation (5 and J. Cohen, unpublished), and transport of hormone within the plant (5 and J. Nowacki, unpublished). Further studies of these metabolic functions would be facilitated by the availability of isotopically labeled esters.

Labeled indoleacetyl-*myo*-inositol would also serve as a standard for quantitative studies of IAA-esters by isotope dilution (6). Free IAA is not the ideal internal standard for determination of total plant IAA since the cereals, for example, contain 90% ester IAA (7). Free IAA and esterified IAA differ in stability in plant extracts since IAA is oxidized by peroxidase while the ester forms are resistant (5 and J. Cohen, unpublished). The ester and free forms of IAA may also exhibit different chemical stability since, for example, Felker (8) has shown that free tryptophan was more stable than peptidically linked tryptophan during acid hydrolysis of protein. For these

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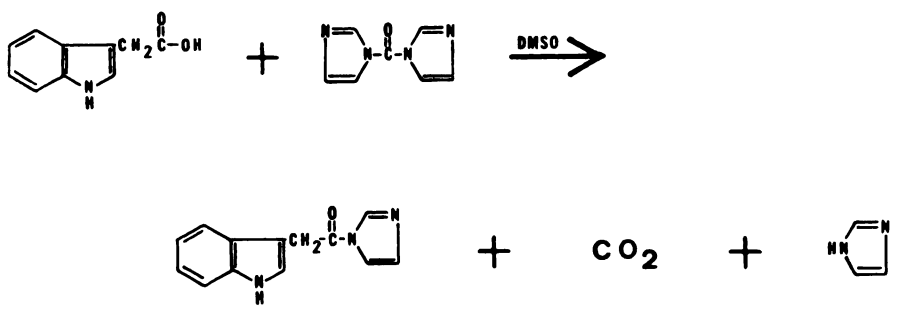
reasons, isotopically labeled indoleacetyl-*myo*-inositols would be a good internal standard for the quantification of either total plant IAA or ester IAA.

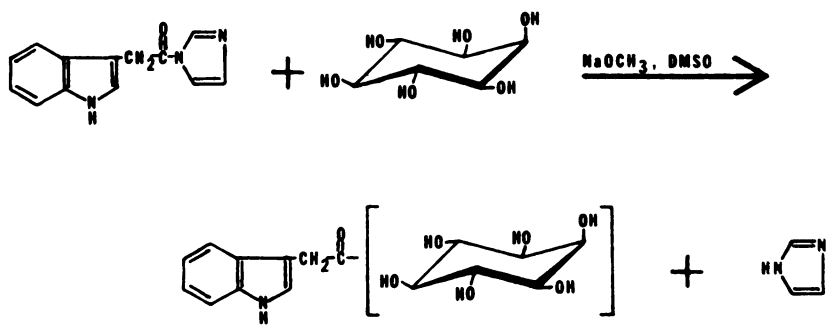
DISCUSSION

The reactions used to synthesize the indoleacetyl-*myo*-inositols are shown in scheme 1 (reactions 1 and 2). This is essentially the generalized method for ester synthesis described by Staab (9) involving the formation of the imidazolide intermediate. It was chosen over more conventional methods because of the mild reaction conditions used. No attempt was made to synthesize a particular axial or equatorial ester since under our conditions of usage acyl migration rapidly produces all four of the chemically resolvable indoleacetyl-*myo*-inositols (10).

1.



2.



β[2-^{14}C]-Indoleacetic acid, specific activity 57.2 mCi/mmole, was obtained from New England Nuclear and used without isotopic dilution. The ^{14}C-IAA was purified prior to use by chromatography on a 2 x 22 cm column of Sephadex LH-20 with elution by ethanol-water (1:1). The volatile thiol reagent, dithioethane (Aldrich Chemical Co., Milwaukee, Wisc.), was added (10 μmole/μmole ^{14}C-IAA) to protect the ^{14}C-IAA from oxidation (cf. 8). The ^{14}C-IAA fraction was evaporated to dryness under nitrogen in the presence of 0.56 μmole of anthracene (J. T. Baker,

Phillipsburg, New Jersey) per μmole of ^{14}C-IAA. Anthracene acted to protect the radioactive IAA during the drying step and its presence had no effect on subsequent reactions. Attempts at purification without use of dithioethane and anthracene resulted in almost complete loss of the ^{14}C-IAA. Purification of ^{14}C-IAA prior to use was obligatory.

Stoichiometric, rather than catalytic, amounts of sodium methoxide were found to be necessary, possibly because of the difficulty in abstracting a proton from the cyclitol. Sodium methoxide was prepared under dry nitrogen by adding freshly cut sodium metal to anhydrous methanol (11). Methanol was twice redistilled from magnesium activated with iodine (12) onto heat activated Linde 4 A (Supelco, Bellefonte, Pennsylvania) and used immediately. Residual methanol was removed from sodium methoxide *in vacuo* at 60°C and then by drying for 18 hr at 200°C under high vacuum. This product was stored *in vacuo* over P_2O_5 in a dry nitrogen flushed desiccator. Even small amounts of water resulted in the formation of enough NaOH to hydrolyze the esters as they were made. It was necessary to add sodium methoxide to the reaction as a dry powder since solutions of sodium methoxide in dimethylsulfoxide (DMSO) were ineffective.

myo-Inositol (Sigma Chimical Co., Saint Louis, Missouri) was dried *in vacuo* in an Abderhalden apparatus at 100°C for 18 hr. DMSO was freshly distilled *in vacuo* onto Linde 4A. DMSO was stored in sealed 1 ml vials over Linde 4A and the solvent was obtained through a teflon septum as needed. These vials were stored over anhydrous $CaSO_4$ and discarded once the septum had been perforated. All glassware was dried at 105°C overnight prior to use. Manipulations involving sodium methoxide were carried out in a glove box in a dry nitrogen atmosphere with P_2O_5 present as desiccant.

The identity of the radioactive product was established by a number of criteria. First, a parallel synthesis was run on a larger scale using unlabeled IAA and the identity of the products of this synthesis was confirmed as follows. The synthetic products had the same R_f values on thin layer chromatograms (E. Merck, Darmstadt, silica gel plates developed in methyl ethyl ketone-ethyl acetate-ethanol-water (3:5:1:1)) as did the naturally occurring mixture of axial and equatorial IAA-inostitols (1). They gave positive reactions with Ehmann's reagent (13) as did the indoleacetyl-*myo*-inositols isolated from corn seeds. Ammonolysis in 14% NH_4OH for 30 min at 45°C yielded two products, one of which co-chromatographed with authentic IAA and the other with indoleacetamide. The products had the characteristic elution volume on a high pressure liquid column (14) and the correct retention times when subjected to gas liquid chromatography (2). Combined gas chromatography/mass spectrometry of any of the four resolvable fully trimethylsilyated derivatives (10) using a LKB 9000 mass spectrometer yielded a molecular ion at m/e = 769 and a fragmentation pattern identical to that previously published for the axial and equatorial esters (10,15). The ^{14}C compounds were then compared with the unlabeled compounds. The ^{14}C compounds yielded labeled IAA and indoleacetamide upon ammonolysis. Their R_f values on thin layer chromatograms and their

elution volumes from a high pressure liquid chromatographic column were identical to those of the unlabeled isomeric indoleacetyl-*myo*-inositols.

EXPERIMENTAL

After chromatography on Sephadex LH-20, β[2-^{14}C]-indoleacetic acid (210 μg, 1.2 μmoles, 68.6 μCi) yielded 175 μg (1 μmole) of purified product. The pooled fractions were evaporated to a small volume on a rotary evaporator and 100 μl hexane containing 100 μg of anthracene was added. The mixture was dried under nitrogen in a 300 μl microflex tube (Kontes, Vineland, New Jersey).

1,1′-Carbonyldiimidazole (Sigma; 324 μg, 2 μmoles) in 10 μl dry DMSO was added to the dry residue through the septum of the microflex tube and mixed vigorously. Three 1 mm glass beads (B. Braun, Melsungen) were added to aid in dissolving the dry residue. After 30 min of incubation at 25°C, inositol (540 μg, 3 μmoles) in 60 μl DMSO was added with a syringe. The tube was then placed in a nitrogen-flushed dry box and a small amount of sodium methoxide (about 1 μmole) was added as a dry powder. The reaction mixture was mixed vigorously for 1-2 min and the reaction stopped by adding 100 μl of ice-cold 2-propanol-4 M acetic acid (1:1).

Immediately after termination of the reaction the 170 μl of reaction mixture was applied to a 0.9 x 17 cm high pressure column of sulfonated styrene-divinylbenzene copolymer (Beckman PA-28) and separated using 2-propanol-water (1:1) as the mobile phase (14). Fractions containing significant radioactivity eluted as expected for the isomers of indoleacetyl-*myo*-inositol and these were pooled. Alcohol was removed *in vacuo* and the water phase lyophilized onto 100 mg of cellulose powder. The products were eluted from the cellulose with 1 ml ethanol-water (1:1).

The yield was calculated from a 5 μl sample of the final solution based on the specific radioactivity of the sample. The yield was 76 μg of ^{14}C-indoleacetyl-*myo*-inositols (13 μCi, 23%) for this synthesis.

ACKNOWLEDGEMENTS

We thank Dr. W. H. Reusch for advice and Dr. C. C. Sweeley for the use of the mass spectrometer facility (PHS RR-00480). This work was supported by National Science Foundation grant PCM 76-12356 and is journal article 8250 from the Michigan Agricultural Experiment Station.

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EXPERIMENTAL IV

AUTOMATED ANALYSIS OF INDOLYLIC COMPOUNDS IN PLANT EXTRACTS

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Running Title: Indole analyzer

Received:

Abstract

An automated procedure for analysis of indolylic compounds has been developed utilizing a chromogenic reagent yielding stable chromogens with E^{m}_{cm} = 24,000. Coupling this detection procedure to an adsorption chromatographic system of sulfonated polystyrene divinylbenzene, such as used in automated amino acid analysis, permitted separation, detection and assay of indoles in crude extracts of kernels of *Zea mays*. Analysis of the major neutral and acidic indoles in crude extracts was accomplished in less than 3 hours, where previous methods required several days. Adaptability of this method to other tissues and column chromatographic systems is described. These methods describe the first versatile indole analyzer useable with crude plant extracts. The analyzer should have significant applications in assays of indoles in both plant and animal extracts.

Introduction

Derivatives of the indole nucleus are important in the nutritional and hormonal biochemistry of all higher organisms (cf. Bandurski, 1978; Lovenberg and Engelman, 1971) and automated analysis of indolylic compounds would be important in assays of the plant growth hormone, indole-3-acetic acid (IAA); in nutritional studies of tryptophan concentration; and in studies of biogenic amines in body fluids and in plants. Automated analysis has previously been difficult owing to the lack of a stable-colored indole derivative with sufficiently rapid formation for automation. In addition,

previous chromatographic procedures which were capable of selectively concentrating and separating indole compounds in crude plant extracts (Ueda and Bandurski, 1969; Ehmann and Bandurski, 1972; Magnus et al., 1978) were very slow and this protracted elution restricted attempts at automation.

In plant tissues most of the IAA exists not as the free acid, but bonded via an ester or amide linkage to other compounds (Bandurski and Schulze, 1977). In *Zea mays* about half of the IAA is in the form of low molecular weight esters conjugated with inositol or inositol-glycosides (Bandurski et al., 1969). These conjugates play important roles in the hormonal physiology of the young seedling (Bandurski, 1978) and new methods for analysis of these compounds are needed.

This work describes a system for the automated analysis of indoles using a modification of the Ehmann (1977) indole reagent. In addition a new column for the analysis of neutral and acidic plant indoles by absorption chromatography is described. The column method offers a purification superior to previous low pressure aqueous column methods and provides a 25-fold reduction in analysis time. Together, these methods comprise the first automated indole analyzer suitable for use with plant extracts. An abstract of this work has previously appeared (Cohen and Bandurski, 1977).

Materials and Methods

Reagents

Indole-3-acetic acid was from Sigma, indole-3-acetyl-L-aspartic acid was a gift from R. Hangarter and N. E. Good (Michigan State University) and the isomeric indole-3-acetyl-*myo*-inositols were synthesized by the method of Nowacki et al. (1978). The inositol derivatives were purified as previously described (Cohen and Bandurski, 1978). Ehmann's reagent was identical to that reported (Ehmann, 1977), a 1:3 mixture of Van Urk (1929) and Salkowski (Tang and Bonner, 1947) reagent freshly mixed from stocks of each. The Van Urk reagent was prepared from *p*-dimethylaminobenzaldehyde (Aldrich) decolorized by refluxing 200 g with 1 g norite in 500 ml absolute ethanol and recrystallized from ethanol-water to yield large off-white leaflets which were dried in partial vacuum over P_2O_5 for one week. Reagent *p*-dimethylaminobenzaldehyde (Sigma) is suitable, although sensitivity is reduced by 30%. The Van Urk reagent was stable for about one month and was prepared by dissolving 1 gm *p*-dimethylaminobenzaldehyde in 50 ml of reagent grade HCl (specific gravity = 1.18) and adding 50 ml of absolute ethanol which had been redistilled after mixing with 5 g/l of norite for 18 hours. Salkowski reagent was prepared by dissolving 2.03 g $FeCl_3 \cdot 6H_2O$ (Baker) in 500 ml of glass distilled H_2O and adding 300 ml reagent grade H_2SO_4 (specific gravity = 1.84). Water used in reagents and for dilution in the analyzer was distilled, deionized and redistilled so that the conductance was below 1 μmho.

Plant Material

Corn kernels (*Zea mays* L. cv Stowell's Evergreen, Vaughan's Seed Co., Ovid, Mich.) were ground in a mechanical hammermill to about 20-50 mesh and 100 gram aliquots were extracted with 3 portions of 300 ml of 80% aqueous acetone (v/v) during 16 hours. The combined extracts were reduced to 150 ml and stored 16 hours at 4 C to permit oligosaccharide retrogradation and precipitation. Filtration through Whatman 1 and 42 paper yielded a clear yellow solution which was then reduced in volume to 50 ml and applied to a 4.5 cm x 4 cm bed of Na^+ form Dowex 50X2-400 (Sigma). The Dowex was eluted by successive washing with 100 ml aliquots of 50% 2-propanol/water (v/v) using vacuum to aid filtration. The first 200 ml were discarded since they contained mainly saccharides and little or no indole compounds detectable by tlc (see below). Purification by Dowex 50 was essential to protect the analyzer resin from irreversible binding of damaging compounds and to remove sugars so the sample could be reduced to 1200 µl, the maximum column loading volume.

Thin Layer Chromatography

Thin layer chromatography for the separation of indole derivatives was on silica-gel 60 plates (5610; E. Merck, Darmstadt) using ethyl acetate, methyl ethyl ketone, ethanol, water (5:3:1:1) as solvent (Labarca et al., 1965). Indoles were visualized by spraying with the Ehmann reagent and with color development at 100 C for five minutes. Washing the developed plate in distilled

water removed acidic residues and permitted storage of the plates with spot color retention (Ehmann, 1977).

Column Regeneration

PA-28 resin was regenerated by treatment with 70% H_2SO_4 for 5 hours at 70 C. The acid-resin was diluted with 4 volumes of water and washed with water in a buchner funnel until neutral. Next the resin was mixed with 0.1% ethylenediaminetetraacetic acid (EDTA) disodium salt (Sigma) in 1 N NaOH and again washed with water until neutral. This procedure was repeated with 2 N HCl and finally with 1 N NaOH. The resin was then washed with 50% 2-propanol/water (v/v) until the eluent was colorless.

The Partisil ODS column was eluted with several bed volumes of absolute ethanol for regeneration and then stored with absolute ethanol to avoid dissolution of the silica gel support matrix. At least 5 bed volumes of solvent was passed through the column prior to a chromatographic separation.

Column Chromatography

Chromatographic separations were on a 0.9 x 17 cm column of spherical sulfonated polystyrene divinylbenzene resin designed for amino acid analysis (Beckman PA-28). A pressure of 10 atm was provided by a Beckman Accuflo pump yielding a flow rate of 0.3 ml/min with 50% 2-propanol/water (v/v) as the eluting solvent. Sample application was with a teflon Altex isolated loop type injection valve (Figure 1). Plumbing was with 1.5 mm ID teflon tubing except for the injection valve which was internally plumbed with 0.8 mm ID

tubing. Injection valve loops of 20 µl, 500 µl, and 1200 µl were used.

A second column used in these investigations was a 4 mm x 25 cm 10 µ Partisil ODS column (Whatman) or a "micro-bondapak C_{18}" column (Waters) operated at 150 atm by a Milton Roy 396 minipump equipped with a Rheodyne 7120 injection valve and a pulse damping system. Elution was isocratic with 20% ethanol/water (v/v) containing 1% acetic acid.

Samples eluting from these columns were "split" with a nylon "T" union (Value Plastics, Inc., Loveland, Colo.) on the 0.8 mm ID teflon column end tubing so that approximately 20% entered the autoanalyzer and 80% was collected in 1 ml volumes on a Gilson FC80K microfractionator. During initial column development the autoanalyzer was not available so the standard samples were simply collected and analyzed by measuring ultraviolet (U.V.) adsorption at 282 nm on a Gilford 240 spectrometer using 1 ml far U.V. quartz cuvettes.

Autoanalyzer

A diagram of the analyzer is shown in Figure 2. For use with the PA-28 column the sampling rate was 67 µl/min through 0.25 mm ID tubing. Other tubing sizes were adjusted such that the ratio of sample:air:reagent:water was 1:4:16:42.5. Critical values for proper analysis were: (1) The air bubble must be 1/2 to 1/5 the volume of reagent. Too many bubbles results in poor mixing and increases loss during "debubbling." (2) Sample volume must not be

greater than 25% of reagent volume, with less than 10% preferred. (3) Water should be about twice the reagent volume. Less water results in excessive background absorbance and larger amounts of water unnecessarily dilutes the color. The only modification necessary for use with the Partisil ODS column was an increase in sample size to 270 µl/min because of the higher column flow rates.

The colorimeter used during initial development was the standard fixed wavelength (570 nm) Technicon colorimeter. This was found to be unsatisfactory, however, since a loss of sensitivity resulted from the non-optimal wavelength and because with this colorimeter a stable baseline was difficult to obtain. Superior performance was obtained with a 3 channel colorimeter (M.E.R. Chromatographic, Mountain View, Calif. 94040, model 1020 with a model 1050 long-path channel add-on) operated at 5.0 volts by a 0-7.5 volt 10A regulated power supply (Model IP-2730, Heath Co., Benton Harbor, Mich. 49022). The colorimeter was equipped with 6.6 mm and 20 mm flowcells with 610 nm filters as well as a 6.6 mm flowcell at 800 nm with a near infrared photocell (M.E.R. Chromatographic, Model 25). The indole product from Ehmann's reagent has no absorbance at 800 nm, so this channel provided a measurement for turbidity--the major interference found in our work with this reagent. This colorimeter proved to be extremely stable and, in cost, represented a considerable savings over even the single channel Technicon unit. Output was to a Honeywell Electronik 15, 3 channel 5 mv multipoint recorder using 1000 ohm 10 turn helipots (M.E.R. Chromatographic, Model H-1000) for zero control.

Results and Discussion

Chromatography on PA-28 Resin

Beckman PA-28 resin is a spherical sulfonated polystyrene-divinylbenzene copolymer which, in 50% 2-propanol/water (v/v), has an average diameter of 23.2 ± 5 μm as measured with a microscope equipped with an ocular micrometer. The indoles used in this study were acidic or neutral and do not bind ionically to the strongly acidic resin. Adsorption of these compounds is attributable to non-electrostatic attraction due to hydrophobic or Van der Walls-London forces (Niederwieser, 1971). These forces also influence binding of amino acids (Neiderwieser, 1975), but the binding forces for indoles on polystyrene are much stronger than those for the amino acids (Niederwieser and Giliberti, 1971). Tryptophan exhibits the highest affinity for polystyrene of any of the amino acids (Niederwieser, 1971), however its maximum absorption coefficient is only one tenth as high as that for indole or indole-3-acetic acid (Niederwieser and Giliberti, 1971). The retention of IAA-*myo*-inositol and the glycosides of IAA-*myo*-inositol may be attributable to coplaner structures and also because *myo*-inositol and simple sugars, as well as the indolylic moiety, are retained by the column. Free rotation should allow both ring systems to interact with the resin. Separation of these compounds on similar resins using conventional liquid chromatography was described by Ehmann and Bandurski (1972).

The elution profile of a mixture of IAA and synthetic mixed isomeric IAA-*myo*-inositols is shown in Figure 3. The first peak is

IAA and the slower eluting double peaks are the IAA-*myo*-inositols. The inositol esters coelute with only some separation of the isomeric forms. These compounds exhibit acyl migration during preparation so that purification and concentration as a group is advantageous. Based on thin layer migration (Ehmann and Bandurski, 1972) it is possible to show that the first peak is a mixture of axial and one or more equatorial isomers and that the later shoulder is devoid of the axial isomer. As can be seen (Figure 3) flow rates faster than 14 ml/hr resulted in zone spreading and a decrease in retention time. Further reduction of flow rates to 8 ml/hr did not improve separation.

Increasing column temperature from 25 C to 50 C markedly changes the elution profile (Figure 4). The retention of IAA is increased slightly and the IAA-inositols elute earlier. Although slight tailing is still observed, the characteristic double peak is lost and the compounds elute in a smaller volume. This change in elution pattern could be advantageous for some separations where multiple passages through the column may be required.

Recovery of indoles from the PA-28 column is a function of the amount applied. Recovery averages 70% or better with samples of IAA of 3 μg or above whereas with samples of 1 μg the recovery drops to 30%. Addition of thioglycerol to the solvent and purging the solvent with N_2 had no effect on the recovery. The resins were EDTA treated so it is unlikely that metal contaminants accounted for this reduced yield. The loading capacity of the column is high and samples of synthetic IAA-*myo*-inositols in excess of 25 mg have

been purified on this column. The useable range for this column is, therefore, 1 to 25,000 μg.

Chromatography of Indoles on a C_{18}-Reverse Phase Column

The purification of simple indoles by HPLC on a C_{18}-reverse phase column has been previously reported for samples of animal (Graffeo and Karger, 1976; Balandrin et al., 1978; Anderson and Purdy, 1979) and plant origin (Sweetser and Swartzfager, 1978; Brenner, personal communication) using relatively nonspecific methods of detection. Graffeo and Karger (1976) described a system for indole analysis in urine using fluorometric detection which has also been applied to samples from cerebrospinal fluid, brain, and plasma (Anderson and Purdy, 1979). Other authors have detected indoles using U.V. and electrochemical detection (Sweetser and Swartzfager, 1978) and while these methods are very sensitive, they are not applicable to crude plant samples because of the presence of vast excesses of flourescent and U.V. absorbing phenolic acids.

A study of recovery from reverse phase columns has been conducted by A. Schulze of this laboratory. Using ^{14}C labeled IAA she found that recovery averages about 70% with submicrogram samples. Recovery falls to about 50% when plant samples prepurified on DEAE-Sephadex are analyzed. Interestingly, as the column ages the recovery of standards remains high while the recovery of IAA from the plant samples drops percipitously to about 25%. These low recoveries are probably due to the 50% of the available surface area of the support matrix that is not occupied by the C_{18} groups.

We believe that minute amounts of indoles are subject to oxidative attack by many compounds when absorbed to acidic supports.

Autoanalyzer

Automated assay is based on the Ehmann (1977) reagent, which is a sensitive and specific chromogenic reagent for indoles. Many indoles substituted on $C_{(3)}$ give products with a spectrum such as shown in Figure 5 for IAA. The extinction coefficient for this product at 615 nm is 24,000, or four times the extinction for indoles at 282 nm. Spectra and extinctions identical to that of IAA are obtained with IAA-*myo*-inositol and several amino acid amide conjugates of IAA (Cohen and Bandurski, 1978). The spectrum for the product of Ehmann's reagent with tryptophan has an optimum at 590 nm and an extinction of 17,000. However, the spectrum is broad and readings at 570 or 610 nm are within 8% of the absorbance at maximum. Numerous other indoles react, as described by Ehmann (1977), and could be analyzed using this reagent. This reagent is superior to the Salkowski reagent of Tang and Bonner (1947), since it is more selective for indoles and because the chromophore color develops rapidly and is stable. Compounds which interfere with the reagent are (1) those compounds which, in acid, yield insoluble products which impart turbidity, (2) large excesses of phenolic compounds (Ehmann, 1977), and (3) reducing agents.

The chromophore formed by reaction of the Ehmann reagent with IAA obeys Beer's law and is a linear function of amount of IAA over the interval 0.5 to 10 μg. These experiments were done by

direct injection of IAA into the analyzer inlet stream (Figure 6) using a 6.6 mm flow cell. With a 20 mm flow cell, samples as small as 100 ng are measurable with a 10:1 signal to noise ratio and fall on the extrapolated calibration line. Zone spreading within the analyzer is small as is indicated by plate number calculations. With 2 µg sample of IAA assayed by U.V. absorbancy at 280 nm, the plate number for the reverse phase column was 1120 (see Saunders, 1975 for calculations); whereas when 10 µg was injected onto the column and 20% of the eluent autoanalyzed, the plate number was 900. Since some of this loss of efficiency is due to increased column loading, zone spreading due to the autoanalyzer must be less than 20% of peak width.

The utility of the autoanalyzer is detecting indole compounds in crude samples is shown in Figure 7 and 8. A thin layer chromatogram of fractions eluted from the PA-28 column after application of a crude corn extract is shown in Figure 7. The plate was sprayed with Ehmann's reagent (Ehmann, 1977) after solvent development. A large amount of charable material was eluted in early fractions. These compounds lead to turbidity and a general rise in baseline is seen between 30 and 80 min in Figure 8. Some phenolic compounds yielding a pink color and some unidentified faint blue colored spots result in early peaks. The column retains IAA longer with crude extracts than when standards are chromatographed, probably because acidic compounds in the extract protonate the IAA and the undissociated IAA is more strongly adsorbed to the resin matrix (Niederwieser and Giliberti, 1971; Ehmann and

Bandurski, 1972). IAA-*myo*-inositols elute in a sharper peak from crude plant extract than from standards (compare Figures 7 and 8 with Figures 3 and 4), and this could be owing to lipodial plant compounds affecting entry of indoles into the stationary phase.

The indole analyzer is also useful for the detection of indole-3-acetyl-L-aspartic acid, which elutes slightly before the IAA-*myo*-inositols with a 14 ml elution volume. IAA-aspartic acid is another important IAA conjugate which has been identified after feeding *Pisum sativum* seedlings large amounts of exogenous IAA (Andreae and Good, 1955), however its presence as a natural product has not been demonstrated by chemically rigorous procedures.

Coupling the PA-28 column to the Ehmann assay provides the first automated system for the analysis of indoles in crude plant extracts. In this laboratory these methods have proved useful for the preparative scale purification of IAA esters from corn, the rapid purification of synthetic IAA-*myo*-inositols (both high specific activity ^{14}C labeled (Nowacki et al., 1978) and preparative scale purification (Cohen and Bandurski, 1978)), the identification of IAA-*myo*-inositol formed by enzymatic synthesis (Michalczuk and Bandurski, 1979), and the first isolation of IAA-*myo*-inositol from rice (Hall and Bandurski, 1979). The analyzer is not limited to use with the PA-28 column and can easily be adapted to other columns, such as the C_{18} column also used in this study. This versatility makes the analyzer useful for the analysis of at least 80 different indoles which react with the reagent (Ehmann, 1977). This method offers greater sensitivity for tryptophan assays than

a previously reported method (Amaya-F et al., 1977) and methods for the rapid separation of numerous tryptamine (Balandrin et al., 1978; Villanueva and Adlakha, 1978) and indole (Graffeo and Karger, 1976; During, 1977; Sweetser and Swartzfager, 1978; Anderson and Purdy, 1979) derivatives should be easily adapted to the analyzer.

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Legends for Figures

Fig. 1 Diagramatic representation of the PA-28 chromatographic system. Splitting the effluent after the column permits 20% of the sample to be analyzed and the remainder to be collected in the fraction collector (F.C.).

Fig. 2 Automated analyzer for the determination of indoles with the Ehmann reagent. Reagent and sample are drawn into a mixing manifold where an air bubble is introduced. The solution is mixed and then heated at 65 C for 5 min by passing through 3 meters of 1.35 mm ID teflon tubing in a water bath. After cooling in a condensor, water is added and the solution is again mixed. The air bubble is removed prior to measuring the absorbance at 610 nm. Pump tubing is tygon except for the reagent line which is "acidflex." The glass coils and manifolds are from Technicon, all other tubing is teflon.

Fig. 3 The effect of the rate of solvent flow on the elution profile of a mixture of IAA and mixed isomeric IAA-*myo*-inositols from the PA-28 column as measured by U.V. absorbance.

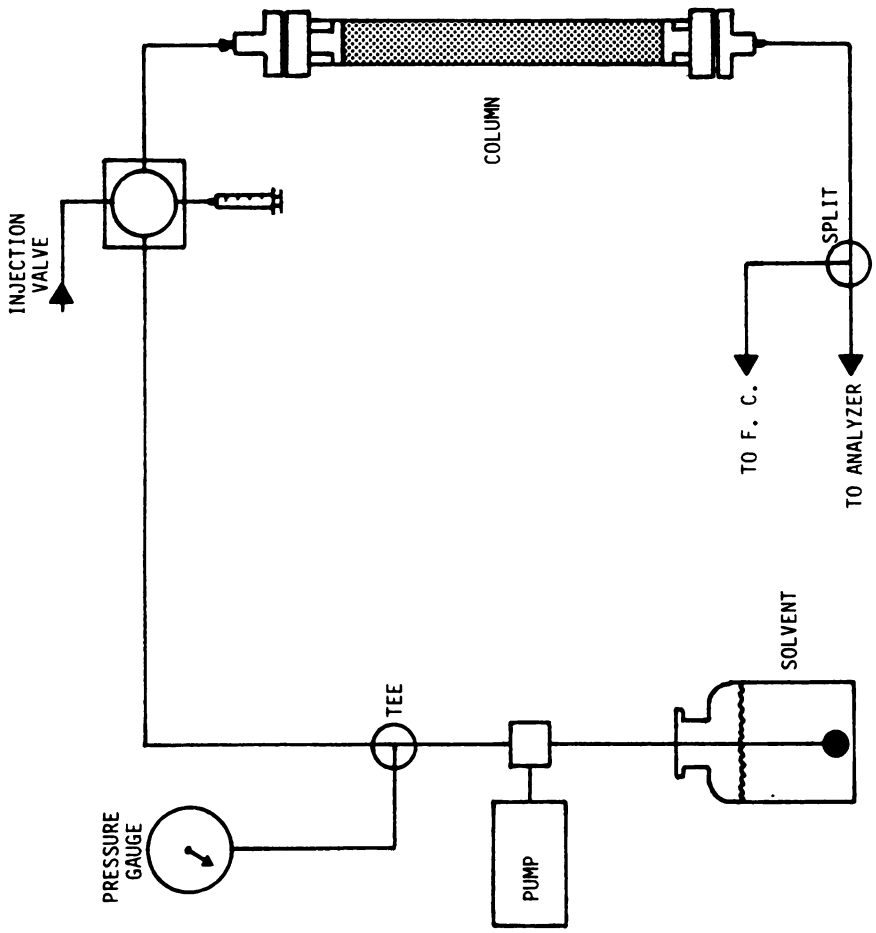
Fig. 4 The effect of column temperature on the elution profile of a mixture of IAA and mixed isomeric IAA-*myo*-inositols from the PA-28 column. Column temperature was controlled by circulating water through a water jacket on the column.

Fig. 5 Absorbancy of the Ehmann product of IAA as a function of wavelength using a Cary 15 spectrophotometer.

Fig. 6 Calibration of the autoanalyzer by injection into the sample stream and using 6.6 mm flow cells. Units refer to area under the peak.

Fig. 7 Thin layer chromatogram of fractions from the PA-28 column of a crude extract of *Zea mays* kernels. Details are as indicated in the text and areas indicated by letters correspond to regions in Figure 8. The compounds in later fractions are: R_f = 0.90, IAA; R_f = 0.39 and 0.35, IAA-*myo*-inositol; R_f = 0.23, 0.18, and 0.13, IAA-*myo*-inositolglycosides.

Fig. 8 Recording of the elution profile as visualized with the analyzer for a crude extract from *Zea mays* kernels. Regions indicated by letters correspond to those shown in Figure 7.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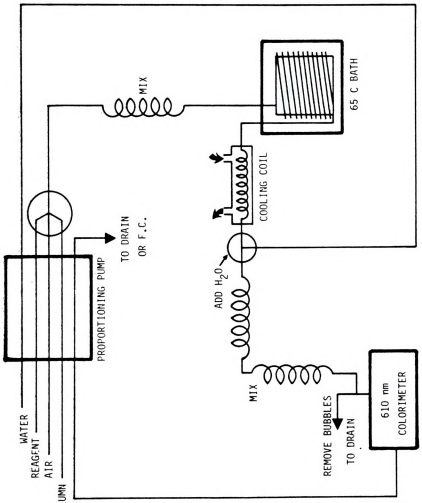
INJECTION VALVE
SPLIT
TO F. C.
TO ANALYZER
SOLVENT
TEE
PRESSURE GAUGE
PUMP


WATER
REAGENT
AIR
FROM COLUMN
PROPORTIONING PUMP
TO DRAIN
OR F.C.
MIX
65 C BATH
COOLING COIL
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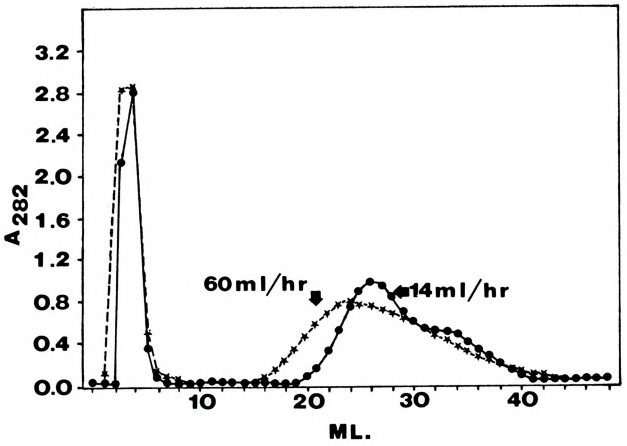
MIX
REMOVE BUBBLES
TO DRAIN
610 nm
COLORIMETER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2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
A 282
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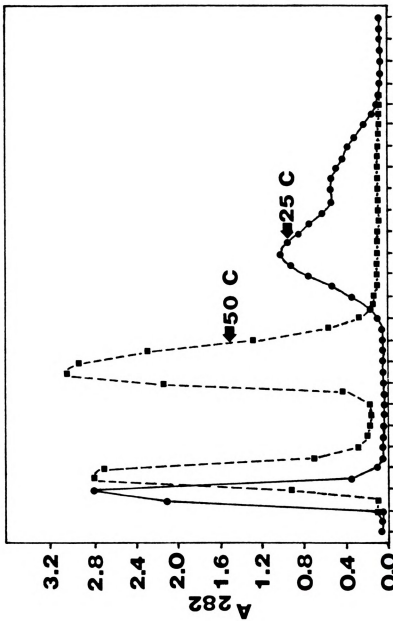
14 ml/hr
10
20
30
40
ML.


50 C
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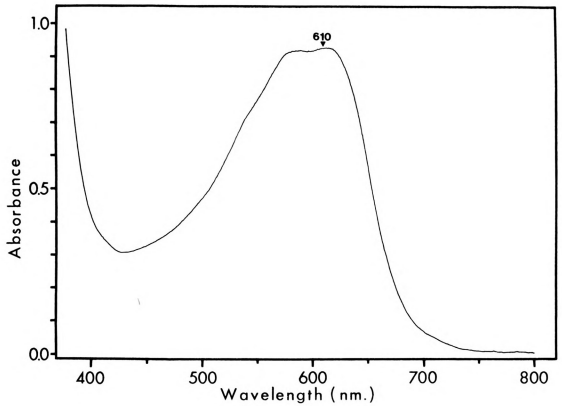
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600
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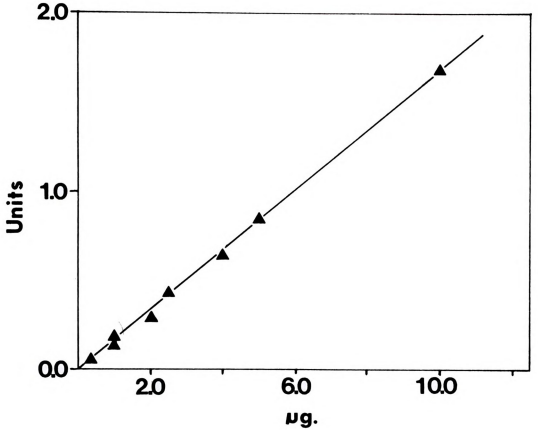
Wavelength (nm.)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6.0
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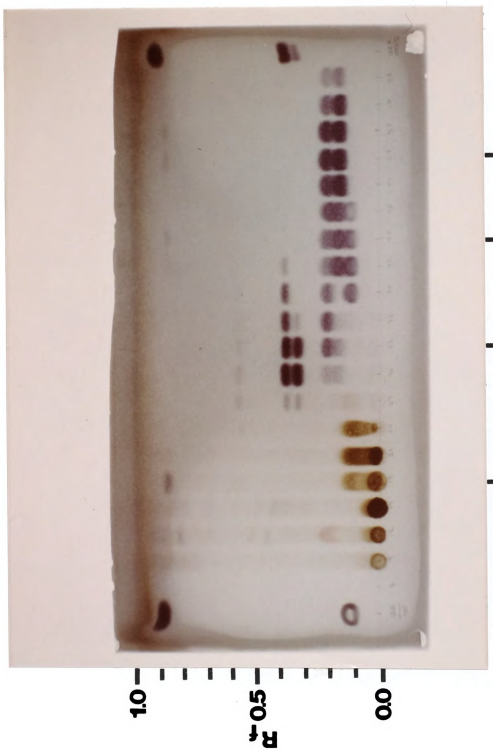
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b
c
d
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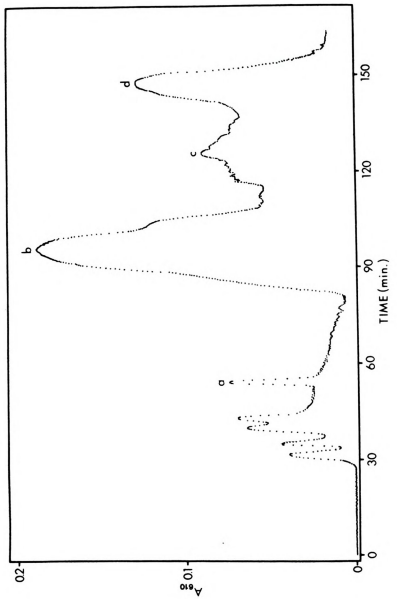
R_f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0
A 365
0
30
60
90
120
150
TIME (min.)
a
b
c
d

EXPERIMENTAL V

DOUBLE STANDARD ISOTOPE DILUTION ASSAY

I. QUANTITATIVE ASSAY OF INDOLE-3-ACETIC ACID

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Running Title: Double standard assay

Received:

Abstract

Isotope dilution analysis for the quantitation of labile compounds has been limited by the amount of sample necessary to redetermine specific activity. A method is described for the analysis of radiolabeled compounds which allows the direct determination of specific activity by gas chromatography. It requires the availability of the radiolabeled compound to be analyzed and also requires a chemically-related radiolabeled compound. The method is illustrated by assaying indole-3-acetic acid in plant extracts using ^{14}C-indole-3-acetic acid and adding ^{14}C-indole-3-butyric acid at the final stage of analysis prior to gas chromatography. Used with a nitrogen specific thermionic detector the method is selective and is sensitive at the nanogram level. The synthesis of ^{14}C-[2-ring]-indole-3-butyric acid is also described.

Double Standard Isotope Dilution Assay I. Quantitative Assay of Indole-3-acetic Acid

Isotope dilution analysis, described in 1940 by Rittenberg and Foster (1), can be used with stable or radioactive isotopes and is the method of choice for quantitative analysis of labile natural products. Use of this method requires knowledge of the initial specific activity of the applied labeled compound and determination of the final specific activity after dilution of the isotope by the endogenous compound being assayed. Ability to accurately assay radioactivity rarely limits the sensitivity of the method since high specific activity compounds are generally available. It is the accuracy with which the *amount* of compound recovered can be assayed by chemical means that limits sensitivity. In this work we describe a technique that quantifies gas-liquid chromatography using highly sensitive detectors and thus brings the sensitivity of the chemical determination to approximately the same level as that of the radioisotope assay. The method, which we have named "double standard isotope dilution assay," should have general applicability. It requires the availability of the isotopically labeled compound to be assayed and that a chemically-related, isotopically-labeled compound also be available. This second, chemically-similar labeled compound permits determination of the specific activity of the compound being assayed using gas-liquid chromatography. We illustrate the method by assay of indole-3-acetic acid (IAA)--a labile (cf. 2) growth hormone present in plant tissues in minute amounts (3). Nanogram sensitivity is attained by use of the double standard method and the nitrogen specific thermionic detector for gas-liquid chromatography.

Materials and Methods

The Double Standard

The assay procedure involves purification of endogenous and added ^{14}C-labeled IAA to the step just prior to gas-liquid chromatography. Then, ^{14}C-labeled indole-3-butyric acid (IBA) is added to the IAA in approximately equivalent amounts. The mixture is then derivatized and analyzed with a suitable gas chromatography detector. Knowing the peak area of IBA and the amount of IBA added, the amount of IAA in the sample at the time of derivatization may be calculated by the relationship:

$$\frac{\text{IAA (peak area)}}{\text{IBA (peak area)}} \times \text{amount of IBA added} = \text{amount of IAA in sample at time of derivatization}$$

Similarly, if a replicate injection is made and the radioactivity in the IAA and IBA collected then the radioactivity in IAA at the time of derivatization may be calculated:

$$\frac{\text{IAA (}\mu\text{Ci collected)}}{\text{IBA (}\mu\text{Ci collected)}} \times \text{IBA (}\mu\text{Ci added)} = \mu\text{Ci of IAA in sample at time of derivatization}$$

Then knowing the amount of IAA in the sample and the radioactivity:

$$\frac{\text{IAA (}\mu\text{Ci in sample)}}{\text{IAA (amount in sample)}} = \text{specific activity of IAA}$$

This is the diluted specific activity of the ^{14}C-IAA originally added to the plant extract and thus the isotope dilution equation of Rittenberg and Foster (1) may be applied:

$$Y = \left(\frac{C_i}{C_f} - 1 \right) x$$

where Y = amount of compound in sample, C_i = the initial specific activity of the applied labeled compound, C_f = the final specific activity, as determined above and x = the amount of ^{14}C-IAA initially added.

In practice it is convenient to add 2-3 times as much ^{14}C-IBA as IAA anticipated in the unknown. Similarly we adjust the specific activity of the IBA to be approximately equal to that of the IAA as reisolated. These precautions, while not essential, enhance the accuracy of the method. Likewise, the removal of an aliquot from the sample prior to the addition of the ^{14}C-IBA provides a sample for use in checking for compounds which might coemerge with the IBA peak during gas-liquid chromatography.

Determination of Specific Activity

Determination of peak areas and radioactivity can be accomplished in two separate injections or in one injection with an effluent splitter. If the single injection system is used it is advisable to use an annular splitter at the collection port since this splitter maintains a constant split ratio even with changing mass load (see 4 for details of splitter and sample collection). If two injections are used then, during one injection, the flame is extinguished and glass tubes placed over the jet for collection at the correct retention times. We have found this method of collection to be better than 98% efficient. After collection, the

samples are rinsed into vials containing ACS scintillation fluid (Amersham) and counted on a Packard 3003 liquid scintillation counter. From the other injection, or from the recording of the split injection, relative peak areas can be determined. From these two pieces of experimental data, the relative peak areas and radioactivity, and thus the specific activity of the IAA can be determined as described above.

Preparation of ^{14}C-indole-3-butyric acid

The synthesis of ^{14}C-[2-ring]-indole-3-butyric acid (Figure 1) was by a microscale modification of the industrial process of H. E. Fritz (5). Indole (Eastman) was recrystallized from water-ethanol and 12 mg of the dry crystalline leaflets were added to a 1 ml size (4 ml capacity) freeze-drying tear bulb (A. H. Thomas 5136-610) fitted with a condenser collar. To this was added 10 µl of 1,2-ethanedithiol (Aldrich) (4,6) and 50 µCi ^{14}C-[ring 2]-indole (50 mCi/mmole, ICN Pharmaceuticals). The 1,2-ethanedithiol destroys peroxides and free radicals permitting the ^{14}C-indole to be dried without decomposition (6). The hexane solvent and 1,2-ethanedithiol (stench) were removed under a stream of dry N_2. To the dry residue was added 0.75 g of freshly broken NaOH pellets and 1.5 ml of γ-butyrolactone (Aldrich). The vessel was then placed in a thermostated sand bath and the temperature of the sand was brought to 220 C at the rate of 2 C/min. A slow reflux was maintained for an additional 23 hours and the reaction terminated by the addition of water. The solidified reaction mixture was dissolved in the water

by warming and the vessel rinsed repeatedly so the final volume was 50 ml.

The water solution was shaken twice with an equal volume of chloroform and the chloroform discarded. The water phase was adjusted to pH 2.5 with 5 N HCl and this extracted twice with chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered and brought to near dryness *in vacuo* at 45 C. The residue was dissolved in 2 ml of 50% 2-propanol/water (v/v) and applied to a 3 x 30 cm Sephadex LH-20 column equilibrated with 50% 2-propanol/water (v/v). IBA was eluted with 50% 2-propanol/water (v/v) at 244 to 276 ml. Yield, based on indole or radioactivity, was 36%. Purity of the product was checked by gas-liquid chromatography of the trimethylsilyated derivative on a 4 ft., 2 mm ID column of 5% SP-2401 on 100/120 Supelcon AW-DMCS (Supelco) equipped with an annular effluent splitter. Collection of the radioactivity showed that less than 0.4% of the radioactivity emerged prior to the IBA peak, 93.3% was contained in the peak at the retention time of authentic bis-TMS-IBA and the remainder of the counts, 6.3%, were in a small shoulder at the retention time expected for the mono-TMS-IBA.

Product identity was established using several criteria. First, the putative IBA behaved exactly as the authentic IBA in its chromatographic behavior on Sephadex LH-20, and during silica gel 60 thin layer chromatography (chloroform, methanol, water; 85:14:1 and detection with Ehmann's reagent (7)). Gas-liquid chromatography of the trimethylsilyl derivative showed a retention time of 10.4 min

for both authentic and putative IBA. The Cary 15 (Applied Physics) U.V. spectrum of the product showed peaks at 282 and 222 and shoulders at 274 and 289 (Figure 2) as for authentic IBA. The 70 eV mass spectra (combined gas-liquid chromatography/mass spectrometry on an LKB 9000) of the methyl ester showed a molecular ion at 217 and base peak at 130 (Figure 3) as for authentic IBA.

Gas Chromatography

Gas-liquid chromatography was on a Hewlett-Packard model 402 equipped with a flame ionization detector (FID) with an annular effluent splitter and a Tracor 702 nitrogen-phosphorus specific thermionic detector (N_2T). Nitrogen and helium were used as carrier gas for the FID and N_2T detectors, respectively. The columns used during these investigations were (1) 4 ft x 2 mm ID glass column packed with 5% SP-2401 on 100/120 Supelcon AW-DMCS (Supelco) for use with TMS derivatized samples, (2) 4 ft x 2 mm ID glass column packed with 3% OV-17 on 100/120 Gas Chrom Q (Applied Science) for methyl esters, and (3) a 10 ft x 2 mm ID glass column packed with 3% OV-225 on 80/100 Chromosorb W-HP for gas chromatography-mass spectrometry analysis of plant samples on a Hewlett Packard 5985a instrument.

Several methods were utilized for the formation of volatile derivatives for gas-liquid chromatography. For the addition of trimethylsilyl groups the sample was dried under a nitrogen stream at 50 C, closed with a rubber septum and resuspended in pyridine (approx. 0.5 µl/µg IAA). An equal volume of N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS)

(Pierce) was added and the mixture reacted for 30 min at 45 C. For the formation of the methyl ester of IAA the most satisfactory method was with the use of diazomethane as described by Schlenk and Gellerman (8) for fatty acids. Use of 10% methanol in the reaction solution and the use of freshly redistilled, peroxide free ether was found to be obligatory if high yields were to be obtained. Finally, the N-heptafluorobutyryl-indole-3-acetic acid methyl ester was formed by reacting the IAA-methyl ester with 10 µl heptafluorobutyric anhydride (Pierce) and 2 µl of a solution of 0.1% dimethylaminopyridine in tetrahydrofuran. The reaction mixture was heated for 5 min at 100 C and blown dry with N_2. This N-acylation procedure was carried out in a nitrogen flushed dry box (9) with P_2O_5 present as a desiccant. The use of the dimethylaminopyridine acylation catalyst (10) substantially improved yields when working at the submicrogram level.

Plant Material

Corn kernels (*Zea mays* L. cv. Stowell's Evergreen; Vaughan's Seed Co., Ovid, Michigan) were grown in the dark in moist paper towels for 5 days at 25 C as previously described (11). Shoots were removed from the seedlings and samples of 50 g ground in 100 ml 80% aqueous acetone (v/v) in a Waring blender. Immediately after grinding, 0.039 µCi of (2-^{14}C)-indole-3-acetic acid (New England Nuclear, 57.2 mCi/mmole) was added to the homogenate. After gravity filtration through Whatman #1 filter paper the residue was made to 1 N with NaOH and held for 1 hr at 25 C. The filtrate was acidified with H_2SO_4 to pH 2.5 and extracted with 50 ml chloroform three

times. Chloroform phases were pooled, dried over anhydrous sodium sulfate and reduced to dryness *in vacuo* at 45 C. The residue was dissolved in 250 µl of 50% ethanol/water (v/v) and placed on a 0.7 x 12.5 cm DEAE-Sephadex-acetate A-25 (Sigma) column equilibrated with 50% ethanol/water (v/v). After washing with 24 ml of solvent, the IAA was eluted with a linear gradient of 0-2.5% acetic acid in 50% ethanol/water (v/v) starting with 100 ml of 50% ethanol/water (v/v) in the mixing flask and 100 ml of 50% ethanol/water containing 5% acetic acid (v/v/v) in the reservoir flask. IAA was eluted between 40 to 46 ml after beginning the gradient.

The IAA containing peak was pooled, dried, dissolved in 0.5 ml water and applied to a 1 x 15 cm column of bovine serum albumin coupled to Sepharose. This column was prepared by reacting 30 ml (settled volume) of Sepharose-4B with 3.5 g of cyanogen bromide at pH 11.5 and then reacting the activated Sepharose with 750 mg of the serum albumin (12). The column was eluted with 60 ml 0.01M potassium phosphate, pH 5.5, then with buffer saturated with benzene. A more complete report of the preparation and use of this column will be the subject of a future report from this laboratory. IAA eluted after approximately 20 ml of the benzene buffer.

An additional method of purification useful in place of, or in conjunction with, the BSA-Sepharose column in C_{18}-reverse phase high pressure liquid chromatography on a 25 cm x 4 mm Whatman 10 µ Partisil ODS column with isocratic elution with 20% aqueous ethanol containing 1% acetic acid.

Results and Discussion

The use of indole analogs as internal standards for quantitative determination of IAA have been previously described. For example, 5-methyl-IAA (13,14) IBA (15) and indole-3-propionic acid (16) have been used to correct for losses of IAA during isolation. These methods are unsatisfactory since isolation of IAA is a multistep procedure and these compounds are fractionally lost at several steps during purification (17). To alleviate this difficulty, isotopically labeled IAA is employed as an internal standard and ^{3}H (18), ^{14}C (3,19) and ^{2}H (side chain, 2d (20,21) or ring, 4d and 5d (22)) labels have all proved useful. Deuterated internal standards however require routine access to a mass spectrometer.

The method here described increases the sensitivity of the radioisotope method to about the sensitivity obtained with GC-MS-SIM (selected ion minotoring)--that is to 1-10 ng for reproducible quantitation of IAA (20, 21 and A. Schulze, unpublished). GC-MS-SIM is, of course, of variable sensitivity depending on machine design and source cleanliness. The use of the ^{14}C-IBA eliminates the four major sources of error in quantitative GC: (1) The volume used for assay of microliter samples of volatile solvents is difficult to control and evaporative losses of microliter samples occur even from "sealed" vials. (2) Precise control of injection volumes in the microliter range is difficult and although an attempt should be made to control injection volume for this method (23,24), errors resulting from even large changes in injection volume are small. (3) Derivatization of sample is always variable and incomplete and the extent of

derivatization with standards may differ from that with natural product extracts. (4) Finally, detector response on gas chromatographs change with each run. This is true for FID (4) and N_2T (25) detectors. For these reasons, the use of a ^{14}C-IBA internal standard added at the last step of the assay is superior to previously described methods.

The synthesis of ^{14}C-IBA was accomplished by treatment of indolyl sodium with γ-butyrolactone (5) yielding the $C_{(3)}$ substituted indole acid. Probably two different reactions occur. First, during the slow initial hearing period, alkylation should be $N_{(1)}$ directed (26) with subsequent intramolecular rearrangement to the $C_{(3)}$ substitution as temperatures increase. Second, unreacted indole, at reflux temperatures, is directly alkylated at the $C_{(3)}$ position (26). Although slow heating improves yield, the predominant pathway is apparently via the direct $C_{(3)}$ route since attempts by Fritz (27) to isolate 1-indolebutyric acid were unsuccessful. Specific activity of the reaction product was 5545 dpm/μg or 508 μCi/mmole, somewhat below the 833 μCi/mmole expected based on the manufacturers stated specific activity for indole. This activity was adequate for determination of IAA at the level of 50 ng or greater, but for more sensitive methods higher specific activity ^{14}C-IBA would be required. Modifications of the reactions described in order to increase the specific activity of the product should be possible since the γ-butyrolactone is already in great excess. Substitution of β-propiolactone for γ-butyrolactone would yield ^{14}C-indole-3-propionic acid (26,27). This could serve as an

internal standard for use with plant materials which contain compounds which interfere with ^{14}C-IBA.

Table 1 shows the results of assays of known amounts of IAA after derivatization of the IAA-IBA mixture with BSTFA + 1% TMCS and analyzed with an FID detector and an effluent splitter. The value obtained for IAA is low by 7%, probably due to the non-linearity of the FID detector (23). This problem with FID detectors has been studied (23,24), but for the purposes of this report, a simple correction factor suffices. Multiplying the IAA peak area by 1.07 brings it to agreement with the radioactivity collected, both in amount and relative standard deviation (Table 1). This difficulty with relative detector response for two (even closely related) compounds points to the need to intersperse known samples with the biological material. However, in all cases, during a three month period, while varying sample injection volume by 300% and with sample loading varied from 0.1 to 10 μg the 7% difference in relative detector response was observed. That this difference was, indeed, the result of detector response was confirmed in three ways. First, the amounts of radioactivity yielded the accurate ratios. Secondly, collecting the peaks and analyzing by $U.V._{282}$ absorbance confirmed the correct ratios and thirdly, this disparity was not seen in work with the N_2T detector. Table 1 also shows the high degree of reproducibility of the method. In practice, multiple injections were always used since the volumes necessary for satisfactory derivatization exceed injection volumes by, at least, 5 fold.

Figure 4 shows the recorder tracing resulting from a 2 μl injection of a methylated sample containing 19.7 ng IAA and 29.2 ng IBA per μl and analyzed with the N_2T detector. The ratio of IAA to IBA by weight is 0.675 and the molar ratio is 0.783. The peak area for IAA in Figure 4 is 22.8 units and for IBA 29.0 units for an IAA/IBA ratio of 0.786, which agrees with the calculated molar ratio. Since the N_2T detector is believed to respond to the cyanide ion formed in the hydrogen plasma (28) and since one cyanide ion results per indole nucleus, the equal molar response observed is as expected.

A plant extract was purified by solvent partitioning, DEAE-Sephadex and BSA-Sepharose and used to compare the relative selectivity and sensitivity of the two detectors (Figure 5). Recovery following the purification steps was 30% and the total IAA of corn seedlings after alkaline hydrolysis was found to be 350 ng/g (3, 29). The injection for FID analysis contained 3 μg of IAA as compared to the injection for N_2T detection which contained 60 ng. The FID sample shows an interfering peak which obscures the IAA peak and a large peak with a retention time somewhat longer than IBA. These interfering peaks are not nitrogen containing and do not interfere with analysis with N_2T detection (Figure 5). The N_2T detector also proved superior in that it is insensitive to the injection solvents, thus eliminating the solvent tail which made it difficult to integrate peaks on the sloping baseline. Also, the lack of a solvent response allowed the use of increased electrometer sensitivity with these chromatographic conditions. Thus, the N_2T detector allows quantitation of samples which are difficult to

analyze with an FID detector and offers a 100 fold increase in sensitivity (30 and this paper).

By the use of halogenated derivatives of IAA and IBA this method should be adaptable to electron capture (EC) detection. Previously EC has been used for the detection of halogenated derivatives of IAA but these methods have not fully corrected for losses and incomplete derivatization (9) or were not useable with plant samples (31). The advantage of EC detection would be a large increase in sensitivity although with loss of selectivity, since any compound which can be acylated after methylation would be detected by EC. We have studied the possibility of using the double isotope dilution method with halogenated derivatives by using repetitive scan gas chromatography/mass spectrometry. We find that the N-heptafluorobutyryl-indole-3-acetic acid methyl ester can be resolved from impurities in plant extracts after purification by solvent partitioning, DEAE-Sephadex and C_{18}-reverse phase HPLC. The separation requires a 10 ft column and a double derivatization procedure, both of which increase analysis time and reduce sample yield. Impurities in the samples are mainly hydroxy and methoxy cinnamic acids, which are also difficult to resolve from IAA by conventional techniques (29). The presence of large amounts of various lignin acids are serious problems with plant samples and it would appear that the N_2T detector, although less sensitive than an EC detector, has advantages in that it requires less sample preparation and is selective to only organic nitrogen or phosporus containing compounds.

Several methods for the quantitative analysis of IAA in crude extracts have been proposed. These methods include densitometry of thin-layer chromatograms (32), GLC after solvent partitioning (33), radio-immunoassay (34), and fluorescence of a chemical derivative (35,36). None of these methods have been compared to chemically rigorous methods of analysis so the reliability of these techniques remain unproven. The method based on the Plieninger reaction (37) is particularly troublesome since it has enjoyed wide usage, apparently without adequate documentation. We have examined this method of analysis using the procedure of Stoessl and Venis (35), the method of Knegt and Bruinsma (36), and a modification we developed using 1 M methanolic KOH as the reaction termination reagent. Our experimental material was 5 day old corn seedling tissue--tissue which has been extensively studied and which has a high level of endogenous IAA. Measurements were made with an Aminco-Bowman model 4-8202 spectrofluorometer and our standards yielded comparable sensitivity to that reported (36). We utilized the method of quench correction recommended and repeated all assays 4-6 times. One troublesome problem was the reverse addition blank recommended by both Stoessl and Venis (35) and Knegt and Bruinsma (36) which we found increased in fluorescence with time while the samples decreased in fluorescence. This necessitated producing two extrapolation curves for correction to zero time and increased the uncertainty of the final result. By these methods we found at least 1.41 mg/kg of "IAA" in corn seedlings following base hydrolysis. This is over four times the total IAA concentration in corn

seedlings when assayed with isotope dilution techniques, either on a large scale (3,29), by the double standard method, or using d_4-IAA as the internal standard (Hall and Schulze, unpublished) with GC-MS-SIM detection.

The method we have described using a "double standard" for loss corrections provides an accurate and reproducible method for the quantitation of labile organic compounds when a suitable labeled second standard is available. However, as with any system relying on chromatographic migration for identification, the method must be substantiated by additional physical characterization, such as that provided by the mass spectrometer, before application to an unknown biological sample.

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Legends for Figures

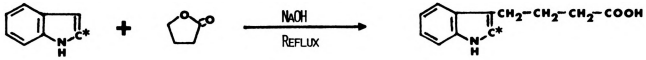
Fig. 1 Reaction scheme for the synthesis of ^{14}C-[2-ring]-indole-3-butyric acid from ^{14}C-[2-ring]-indole and γ-butyrolactone in strong base.

Fig. 2 Ultraviolet absorption spectrum for the synthetic ^{14}C-[2-ring]-indole-3-butyric acid after Sephadex LH-20 chromatography.

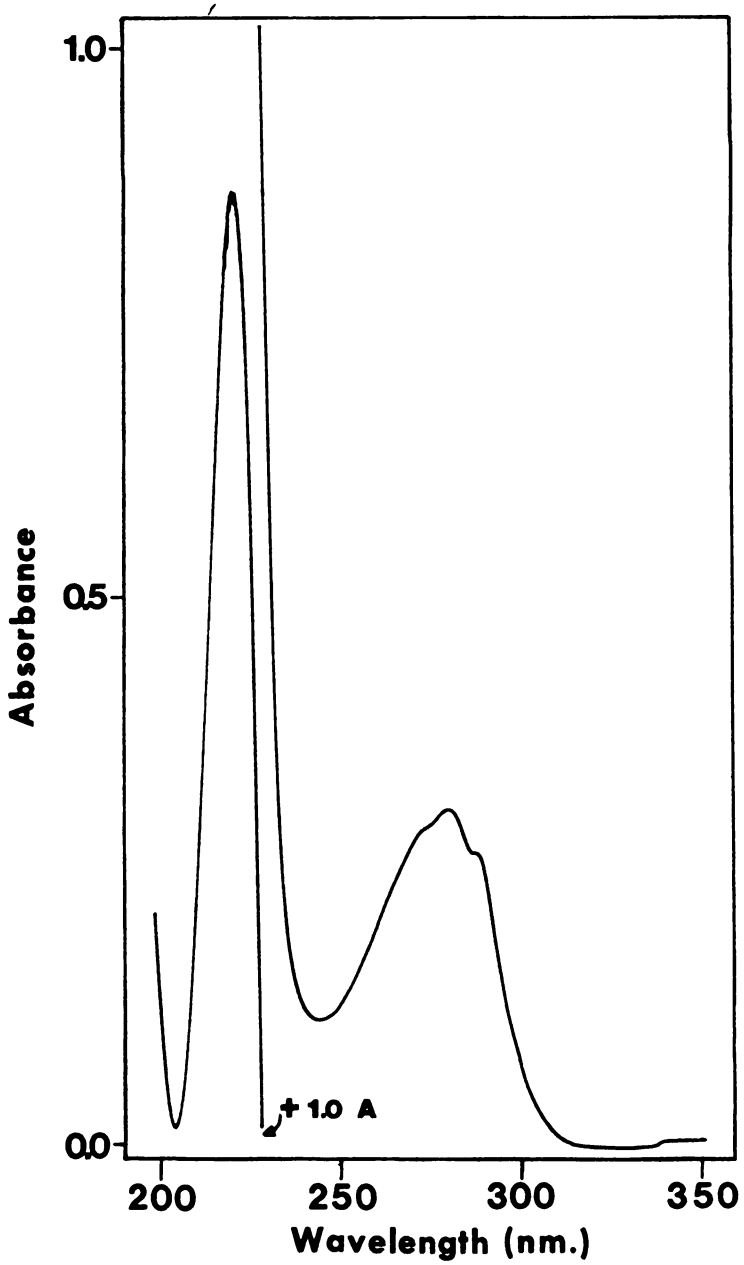
Fig. 3 70 ev electron impact mass spectrum of the synthetic ^{14}C-[2-ring]-indole-3-butyric acid methyl ester. Evident is the molecular ion at 217, the m-31 from loss of [-O-CH_3] at 186, the m-74 with loss of [-CH_2-$COOCH_3$ + H] at 143, and base peak at 130 from the β-cleavage fragment.

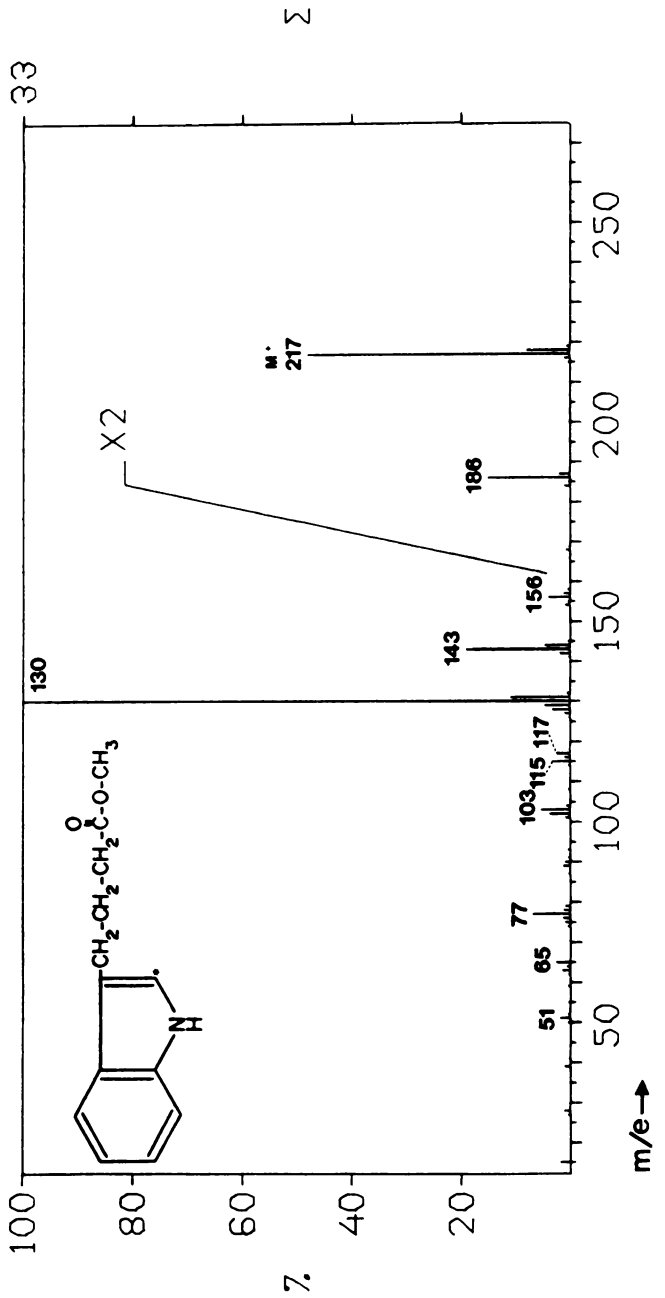
Fig. 4 Nitrogen specific thermionic detector recorder tracing of a mixture of IAA and IBA as the methyl esters. The injection volume was 2 μl and contained 19.7 ng IAA and 29.2 ng IBA per μl.

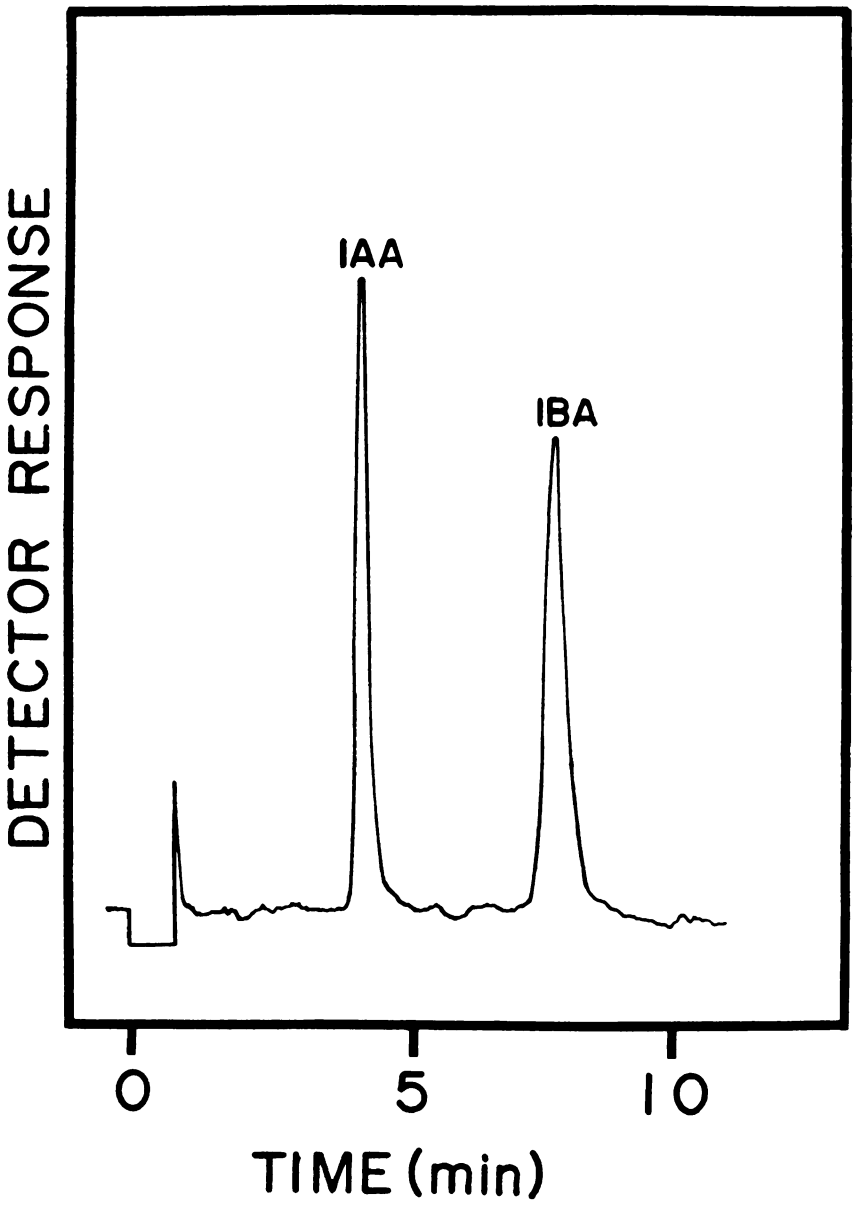
Fig. 5 Methylated sample from corn seedlings analyzed with the flame ionization detector (FID) and the nitrogen specific thermionic detector (N_2). The injection for the FID contained 50 times the sample load as was injected for the thermionic detector analysis.



1) Over a period of 1-1.5 hrs. heat slowly to reflux (sand bath = 220° C).
2) Reflux for an additional 23 hrs.
3) Add 50 ml. H_2O to stop the reaction.


1.0
0.5
0.0
Absorbance
+ 1.0 A
200
250
300
350
Wavelength (nm.)


Σ
33
X2
M·
217
186
156
143
130
103
115
117
77
65
51
CH2-CH2-CH2-C-O-CH3
O
N
H
100
80
60
40
20
%
50
100
150
200
250
m/e→


DETECTOR RESPONSE
IAA
IBA
0
5
10
TIME (min)

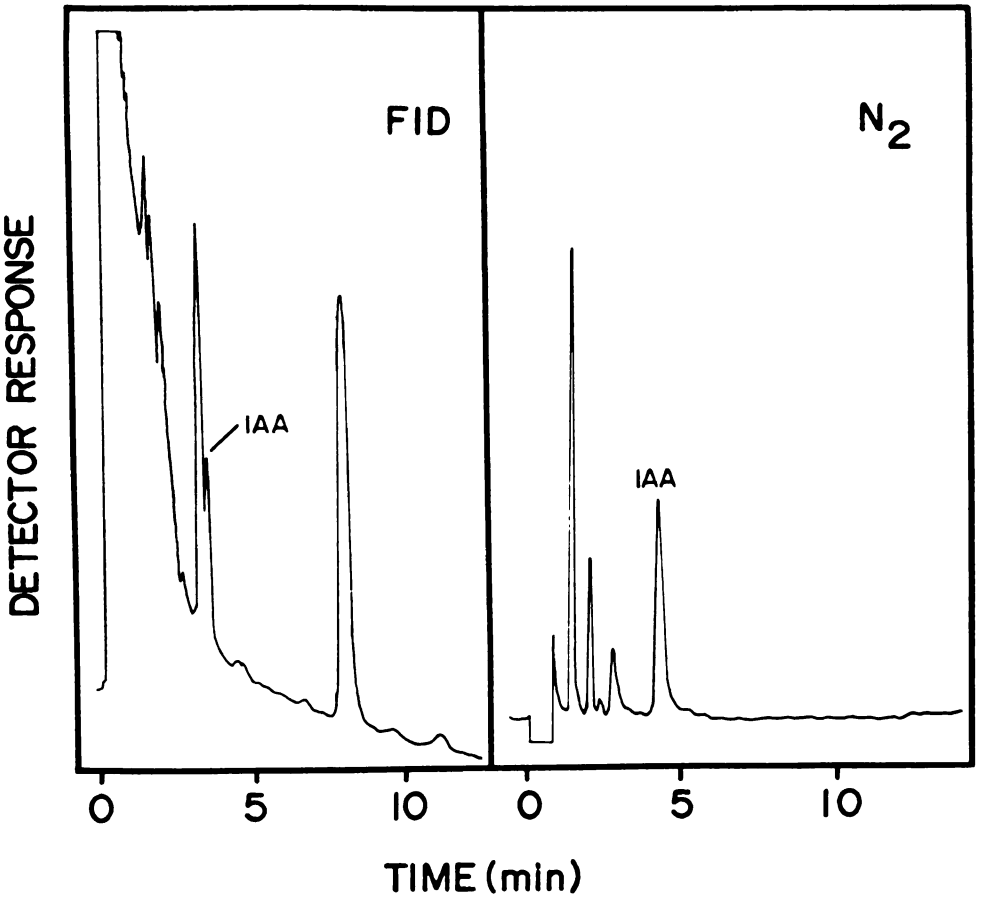

FID
N_2
IAA
IAA
DETECTOR RESPONSE
0
5
10
0
5
10
TIME (min)

TABLE 1.--Experimental values obtained with repetitive injections using derivatization with BSTFA + 1% TMCS and flame ionization detection.

Experiment Number	DPM		Ratio	Area		Ratio	Calculated Values	
	IAA	IBA	IAA/IBA	IAA	IBA	IAA/IBA	Amount IAA (μg)	DPM IAA
1	463	2127	0.218	47.9	73.9	0.648	18.9	32391
2	635	2808	0.226	59.5	94.2	0.632	18.4	33650
3	432	2119	0.204	43.7	69.5	0.629	18.4	30336
4	550	2494	0.221	59.4	91.2	0.651	19.0	32815
5	558	2696	0.218	50.4	86.0	0.586	17.1	32454
6	576	2592	0.222	48.5	75.6	0.642	18.7	33067
Mean Values			0.218			0.631	18.4 ± 0.63	32452 ± 1035
Standard Deviation as % of Mean							3.4%	3.2%
Amount Added	31,816	148,803	0.213	19.7 μg	29.2 μg	0.675	19.7	31,816
Difference from Amount Experimentally Determined							-7%	+2%

CONCLUSIONS

Hall and Bandurski (1978) found that when radioactive IAA was applied to the endosperm of corn seedlings radioactivity would move to the shoot, but only 2% of the radioactivity was in IAA or IAA esters. Most of the IAA which survived movement to the shoot was esterified. These results were extended by Nowacki (see Bandurski, 1978) who showed that ^{14}C-indole-3-acetyl-*myo*-inositol migrated from the seed to the shoot in much larger amounts than labeled IAA and that very little destruction occurred even though the ratio of free and esterified ^{14}C-IAA was identical to the endogenous compliment. These results suggested a protective role for the IAA conjugates which could be important for the hormone while in route. Likewise, other hormones are found as conjugates in the transport stream or have been shown to be transported as conjugates (Sembdner et al., 1968; Van Staden and Dimalla, 1978) and they may also therefore be protected from degradation (see discussion in Wang et al., 1977). In addition to protection, the bound compounds could also play an important role in defining the destination of the transported hormone--a kind of biological "zip code" where, as in the postal service, lack of the "zip code" results in loss of the hormone.

Although the contribution of peroxidative degradation to overall auxin destruction is probably only a fraction of the total, it does serve as a convenient model and could be especially important in transport since at least some peroxidases are localized in the cell wall region (see review by Lamport, 1970). Thus, the demonstration that conjugation protects the hormone from oxidative degradation (Cohen and Bandurski, 1978) may be important in understanding the function of these compounds.

Early notions that conjugation was a detoxification mechanism (Gordon, 1954; Andreae and Good, 1955; Aberg, 1957) may be incorrect since no degradation route is known that does not first release the free hormone. For a compound to be a detoxification product it must be secreted, sequestered, or destroyed and these compounds do not seem to function in this manner. A more likely role is for these compounds to function as a regulatory mechanism for the control of free hormone levels. This contention is supported by the observation that conjugate levels do not remain static during germination, as would be expected for sequestered detoxification products, but decline at a linear rate (Ueda and Bandurski, 1969; Cohen, unpublished). Similar utilization of bound forms has been shown for other hormones (cf. Hiron and Wright, 1973; Sembdner, 1974; Davey and Van Staden, 1977). Also, as the bound auxins decline in amount, the levels of free hormone remain constant, at an apparently regulated level (Ueda and Bandurski, 1969; Cohen, unpublished). In a similar manner, but in reverse, mature

soybean callus cultures control the level of free 2,4-D by conjugation (Davidonis et al., 1978).

The regulatory role of the IAA esters was demonstrated most convincingly by our results (Bandurski et al., 1977) which showed that the ratio of bound to free IAA can be perturbed by environmental inputs such as a light stimulus. This is the first instance in biology for which we have evidence for hormonal levels being controlled by the formation and hydrolysis of a covalent bond. In addition it is the first experiment on light effects on growth and tropisms in which the hormone has been identified by accurate physical methods. It is, therefore, an important step in understanding in precise chemical terms the nature of the light-growth process and may lead to new insights which will explain the discrepancies between the experimental data from several groups and the Cholodny-Went theory of tropisms (see literature review).

The experiments on the effects of light on hormone levels required 600-2000 grams of tissue for each determination using the techniques then available. In order to study more complex biological phenomena better methods had to be developed. The chemical synthesis of indole-3-acetyl-*myo*-inositol was an important step in this process since it will allow us to study the conjugation process as well as transport and environmental effects. The unlabeled product is important for reverse isotope dilution analysis where radiolabeled conjugates might be formed by tissue or enzyme extracts (cf. Michalczuk and Bandurski, 1979) in small amounts. The availability of high specific activity ^{14}C-indole-3-acetyl-*myo*-inositol has

already been used for transport studies as indicated above and for studies of metabolic turnover (Epstein and Bandurski, 1978). A prior synthesis of the 2-*O*-indole-3-acetyl-*myo*-inositol isomer was accomplished by Tate (cited in Nicholls, 1967) by condensation of pentabenzyl-*myo*-inositol-2-ol with IAA followed by hydrogenation; however the yields were very poor.

Chemical synthesis of other hormone conjugates has been previously reported. The 1-*O* isomer of the glucose ester of IAA was synthesized by Keglevic and Pokorny (1969) by reacting 2,3,4,6-tetra-*O*-benzylglucopyranosyl chloride with the silver salt of IAA followed by hydrogenation. Glucose esters of other hormones have been accomplished by similar procedures or by using the peracetylated sugar (Schreiber et al., 1969; Hiraga et al., 1974a; Schneider, 1974; Lehmann et al., 1975; Cowley et al., 1978). Synthesis of the amino acid conjugates has been accomplished by several methods (see Weiland and Horlein, 1955; Good, 1956; Weller and Sell, 1958; Armstrong et al., 1958; Hart et al., 1970; Mollan et al., 1972; Hattori and Marumo, 1972; Lischewski et al., 1974). However, our report on the synthesis of ^{14}C-indole-3-acetyl-*myo*-inositol (Nowacki et al., 1978) is the first chemical synthesis of a radiolabeled bound plant hormone and is one of the rare examples of a successful high specific activity radiochemical synthesis at the micromole scale. Previously, radiochemical methods have been used to follow enzymatic synthesis *in vitro* and this has resulted in small amounts of radiolabeled products which were used to monitor the reaction (Hutzinger and Kosuge, 1968b; Kopcewicz et al., 1974; Muller et al.,

1974; Entsch and Letham, 1979) but these methods produced yields too low to allow the product to be isolated and used in subsequent investigations.

Although it appears that an equatorial isomer of the inositol esters is produced in the enzymatic synthesis (see Bandurski, 1978; Michalczuk, personal communication), a mixture of isomers is found in corn seeds even when relative mild extraction conditions are used and acyl migration occurs rapidly even at neutral pH (Nicholls, 1967). This, as well as the knowledge that inositol esters substituted at multiple positions are found (Ehmann and Bandurski, 1974), suggests that the plant has found some way to deal with multiple isomeric forms. How this is accomplished must await characterization of those enzymes responsible for release of the free hormone.

The rapid method for the analysis of the inositol esters has resulted in improved procedures for evaluation of products produced by enzymatic reaction and has resulted in the identification of indole-3-acetyl-*myo*-inositol from rice when older, slower methods had failed (Michalczuk and Bandurski, 1979; Hall and Bandurski, 1979). The use of nitrogen selective gas chromatograph detectors and the double standard method will bring the ability to carefully quantitate IAA to laboratories which do not have routine access to mass spectral equipment. A modification of this method using electron capture detection is now being used (Epstein, personal communication). It is hoped that these new methods will spur

additional research into the physiology of the bound hormones and lead to new discoveries about this important dimension of hormonal metabolism.

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