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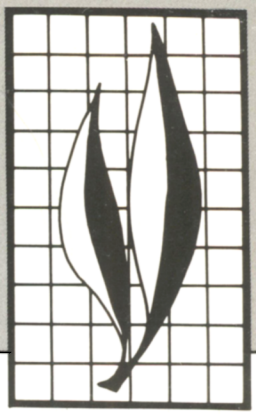

Methodology for Indole-3-Acetic Acid: Sample Preparation, Extraction, and Purification Techniques

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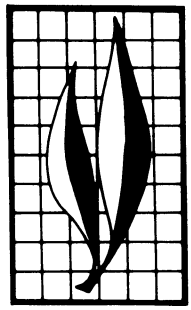

ABSTRACT

Indole-3-acetic acid (IAA) has been implicated as a regulating agent in numerous plant-growth phenomena. In an attempt to assess its role in plants, identification and quantification are necessary. Estimating the amount of IAA in plant samples is difficult because this molecule degrades rapidly during most extraction-purification systems. A method is presented which reduces IAA degradation to a minimum by drying the sample at about 10^{-5} torr of oxygen and subsequently extracting with anhydrous solvent. A review of earlier methods is included.

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In the spirit of admitting fallibility, even among scientists, this article is dedicated to the human condition as revealed by Hans Christian Anderson in his story, "The Emperor's New Clothes."

George C. Martin, R. Kammereck, and C. Nishijima

Methodology for Indole-3-Acetic Acid: Sample Preparation, Extraction, and Purification Techniques[1]

INTRODUCTION

IF INDOLE-3-ACETIC ACID (IAA) is one of the chemicals regulating plant growth, then its quantitative determination is a prerequisite to understanding IAA action in growth-regulating events. The chemical instability of IAA, as well as the inevitable physical losses inherent in extraction and isolation techniques, have made measuring it difficult. Early assay procedures, although seemingly crude, were simple and rapid, and may have been less prone to loss of IAA than modern procedures. Generally, plant parts were allowed to diffuse contents into agar blocks for later assay, using Avena curvature procedures, and cells were not homogenized (Avery, Burkholder, and Creighton 1937). In modern extraction-purification procedures, plant parts are homogenized and physiochemical methods are used to estimate the amount of isolated IAA. When this is coupled with the employment of isotope dilution techniques to correct for losses during purification, accurate results are made possible (Bandurski and Schulze 1974; Horgan 1981). These modern procedures may still lead to IAA losses, not accounted for, during the sample workup.

Problems in Determining IAA

Chemical instability and effect of oxygen

Early literature (Boysen-Jensen 1936; Gordon 1954) enumerated many chemical factors involved in the instability of IAA *in vivo* and during extraction from plant materials. These IAA degradative features are summarized by Galston and Hillman (1961), who cited literature showing IAA in water solution to be degraded by acid, ionizing radiation, ultraviolet (UV) light, visible light, peroxide, and peroxidase systems during homogenization. Further, they stated, "oxygen speeds degradation by all mechanisms."

Results of investigations of the mechanism and rate of IAA destruction by plant-derived oxygenase-peroxidase systems suggest that in the presence of oxygen (O_2) substantial amounts of IAA are lost during the initial stages of homogenization because of enzyme catabolism (Hinman and Lang 1965; Mann and Jaworski 1970; Nakajima and Yamazaki 1979; Raa 1971; Ricard and Job 1974). Common to all conventional extraction procedures is the homogenization of cell tissue in aqueous mixtures of polar organic solvents. This initial step in modern procedures, compared with diffusion techniques of the past, introduces the major problems of O_2 and loss of compartmentalization. Commonly, fresh plant material is extracted with methanol; water in the plant dilutes to about 60 percent aqueous methanol. At that concentration

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of methanol many hydrolases remain active, raising another question as to the magnitude of the contribution by IAA esters and peptides to the final level of IAA (Horgan 1981).

Efficiency of IAA extraction and measurement

Estimates of IAA with physicochemical methods are precise; however, corrections for extraction efficiency, as for example by isotope dilution, can yield valid results only from the moment when the internal standard is uniformly distributed and equilibrated in the endogenous IAA pool. Substantial errors in estimating IAA will occur, if before internal equilibration takes effect, endogenous IAA is destroyed by catabolism or induced degradation occurs through cutting or mechanical damage associated with sampling. Furthermore, any adding of IAA during the isolation process, as may occur through enzymatic splitting of IAA conjugates (Bandurski and Schulze 1974), or by oxydative conversion of indolepyruvic acid to IAA via indole-3-acetaldehyde (Schneider and Wightman 1974; Sweetser and Swartzfager 1978), cannot be distinguished without adding more internal standards and potentially distorts the estimate of original endogenous IAA levels.

A solution to these problems on efficiency of IAA extraction and IAA's instability in the presence of oxygen was offered by Link, Eggers, and Moulton (1941). They found that vacuum drying of frozen material prevented IAA destruction; they also reported that thawing of plant material during vacuum drying would initiate degradative processes. The presence of oxygen involves bacterial action that increases IAA in a plant extract (Libbert, Kaiser, and Kunert 1969). Studies revealed that sterile plants of maize, pea, and cucumber contain less IAA than do nonsterile ones. Further, higher extractable IAA content could be restored within 1 day by water soaking of sterile materials or by reinfection with epiphyte bacteria. Thus, some of the factors that induce IAA degradation or its synthesis have long been known and the most likely solution to these problems was presented more than four decades ago.

In previous studies of IAA oxidation (Fox, Purves, and Nakada 1965; Nakajima and Yamazaki 1979), O_2 was selectively excluded from reaction vessels by purging with nitrogen (N_2) and by adding such oxidation inhibitors as butylated hydroxytoluene (BHT). Another good method, which allows attainment and maintenance of sufficiently low O_2 concentrations over prolonged periods, is the physical evacuation of O_2 by vacuum pumping.

Development of a Method for IAA Extraction

The inherent, documented, unstable nature of IAA and the preponderant use of IAA extraction methods, previously shown to be unreliable, led us to reinvestigate extraction methods and devise alternative nondestructive sample workup procedures. Thus, the purpose of this article is to review the background literature and research which led to developing a method for determining IAA, to test this procedure with two plant materials, and to present the final sample preparation, extraction-purification schemes. In summary, this method follows: Plant parts are removed and immediately submerged in liquid nitrogen (LN_2), and the plant part is kept at $-5°C$ while lyophil-

ization takes place at about 10^{-5} torr of O_2 until the moisture content of plant tissue reaches about 0.02 percent of dry weight. At that point the plant tissue is processed in the presence of air with anhydrous solvents. Using this approach, we found substantially higher concentrations of IAA in corn epicotyls than previously reported. We believe these results are due to the inhibition of IAA oxidative catabolism during initial sample preparation and extraction.

BACKGROUND LITERATURE AND EXPERIMENTS IN EXTRACTION-PURIFICATION SYSTEMS

Review of IAA Methods Literature

Previous efforts directed toward the quantitative determination of *in vivo* levels of IAA have suffered from the extraordinary chemical lability of IAA, as well as the systematic physical losses inherent in isolation techniques. Procedures for measuring IAA concentrations have been much improved by applying the isotope dilution techniques of Bandurski and coworkers. Proper working of this method requires meeting several conditions, the most important of which are: accurate determination of specific activity at the end of analysis, homogenous mixing of tracer IAA with endogenous IAA, and knowledge of absolute quantity of tracer at the time homogenous mixing occurs; that is, the method can only account for IAA losses due to degradation from the moment a tracer has been homogenously mixed with the pool of endogenous IAA and equilibrium established with the IAA present in cell fragments and the IAA dissolved in the extraction solvent. If measurable quantities of tracer are lost before homogenous mixing with tissue is attained, IAA concentration will be seriously overestimated.

The procedure for exhaustive extraction of IAA from plants calls for homogenizing freshly harvested plant parts in acetone so that plant water dilutes the organic solvent to about 70 percent (Bandurski and Schulze 1977; Iino and Carr 1982). After mixing and homogenizing the plant organs to destroy cell aggregates and cell membranes, an internal standard in the form of an isotopic derivative of identical composition with the substance of interest is added while stirring. From that point, losses incurred in additional isolation steps and in purification of IAA for subsequent assay are accounted for by the internal standard. Numerous reliable procedures for isolating IAA exist, depending on availability of physicochemical detectors, plant size, type, age, isolate composition, pool size of IAA, etc. In most cases, some chromatographic technique, in combination with polar-nonpolar solvent extraction, precedes the final assay, followed by structural verification using various mass spectrometric methods. For a review of extraction-purification techniques in general see Yokota, Murofushi, and Takahashi (1980).

A large error resulting in substantial underestimates will occur if endogenous IAA is destroyed before the internal standard is uniformly distributed and in equilibrium with the IAA pool. The time involved in this first part of the isolation procedure may be several minutes, varying from laboratory to laboratory. Work by Nakajima and Yamazaki (1979), Ricard and Job (1974), Raa (1971), and Hinman and Lang (1965), as well as our studies, indicates that substantial losses of IAA occur during the

initial sample processing. These losses are due to oxidase-peroxidase catalyzed destruction and can occur before a balancing of the isotope in the homogenized plant sample is achieved.

Catabolism of IAA by oxidase-peroxidase systems was described in early literature (Ray 1958), but only recently have the mechanistic details become known so that this information can be used to modify extraction procedures for a meaningful determination of endogenous IAA levels. While degradation of IAA by oxidase- peroxidase systems is exceedingly complex, the outstanding features are recognized as follows:

(a) The reaction consumes approximately equimolar quantities of molecular oxygen and is capable of proceeding without exogenously-supplied hydroperoxide (Nakajima and Yamazaki 1979; Hinman and Lang 1965).

(b) Numerous reaction parameters influence the rate of the reaction without substantially altering the reaction products; these include electrolyte content, pH, certain transition metal ions, O_2 concentration, initial IAA concentration, and the concentration of oxygenase-peroxidase (Nakajima and Yamazaki 1979; Hinman and Lang 1965). Although peroxidases are ubiquitous in higher plant species and are found in all plant parts, their content and makeup vary considerably between and within species, as does their potency with regard to IAA catabolism. Schneider and Wightman (1974) indicate that peroxidases are highly compartmentalized in cells and are mostly attached to or are part of membrane structures. During mechanical rupture of cells, in wounding, and particularly through homogenization, peroxidases are released and the concentration of reactively available enzymes may increase several orders of magnitude.

(c) The important individual reaction steps in the overall scheme of destruction of IAA by this process are classified as free radical-type abstractions and rearrangements. This type of reaction is characterized as a chain reaction with an insensitivity to rate deceleration by lowering temperature.

(d) Numerous cofactors occur naturally that can be broadly divided into those that enhance IAA breakdown and those that act as protectors of IAA. Compounds, mostly of the phenolic type, promote IAA degradation (Lee et al. 1980).

Some uncertainty about the mechanistic details of the IAA catabolism reaction still exists, but those aspects relevant to the early fate of IAA are reasonably well understood. In the presence of dissolved molecular O_2, IAA reacts rapidly with oxygenase-peroxidase to form unstable intermediates that decay slowly toward an end product, usually 3-methylene-oxindole (MOI). At physiologically important IAA concentrations of 10^{-6} to 10^{-5} M and oxygenase-peroxidase concentrations of 10^{-6} to 10^{-7} M, IAA concentration decreases rapidly (Hamilton et al. 1976; Cohen and Bandurski 1978).

It is important to point out that, even though the very early reaction products may be closely related but unstable radicals of hydroperoxide derivatives of IAA, the reaction once begun is irreversible and produces compounds which exhibit no auxinlike behavior (Lau, John, and Yang 1978). Furthermore, where oxygenase-peroxidase concentrations are substantially higher than those studied here, as is likely when fresh plant tissue is subjected to massive mechanical degradation as in homogenization, reaction rates will be proportionately faster. Information on very early catabolism, as obtained by fast-scanning spectrophotometry under anaerobic conditions (Nakajima and Yamazaki 1979) and stopped-flow techniques (Fox, Purves, and Nakada 1965), as well as our own method of adding colorimetric reagent to frozen and anaerobically

prepared mixtures, clearly reveals the extent of degradation (table 1). These degradation events make determining endogenous IAA content difficult and sets IAA strongly apart from other growth regulators. The real situation exacerbates analytical problems and potentially leads to huge errors, and, thus, to misinterpretation of the physiological significance of the IAA levels found.

Due to these IAA-plant characteristics during sample processing, reactions cannot be substantially prevented by decreasing the temperature as long as the reaction medium is still in a liquid state. Quick-freezing of potentially reactive mixtures (e.g., the aqueous medium present in plants, by immersion in inert, liquid-cooling agents such as LN_2 or liquid helium), is very effective in converting mobile, liquid reaction mixtures into solid matrices. Quenching or formation of solids in micro-second time spans is highly effective in preventing radical-type reactions. In homogenous solutions adding a suitable antioxidant, such as ascorbic acid, dithiocarbamate, or BHT, can effectively inhibit destruction of IAA. Under heterogenous conditions, as when plant organs or complex cell aggregates are homogenized for subsequent extraction, the effectiveness of adding radical scavengers to the extraction solvent is questionable. As was pointed out earlier, radical scavengers can intercede with early stages of IAA catabolism only if there is direct molecular contact with the reacting species. In intact plant cells, however, the reactive peroxidase, O_2, and IAA are contained intracellularly, whereas exogenously applied scavenger remains extracellular, at least until substantial portions of the cells have undergone plasmolysis, a process that may take place for several minutes. Potential complications are added by the presence of large amounts of antioxidants in the subsequent isolation and assay steps, as when BHT confounds the mass spectrometry of IAA or dithiocarbamate interacting with stationary phases in high performance liquid chromatography (HPLC) work.

When gaseous substrates are essential reaction components in free radical-promoted reactions, then a facile route for preventing reaction progress presents itself in the physical removal of the gases. Nakajima and Yamazaki (1979) and Fox, Purves, and Nakada (1965) have excluded O_2 selectively from their reaction vessels by purging with N_2 and additional employment of such fast-scavenging O_2 preparations as ascorbate-ascorbate oxidase or glucose-glucose oxidase mixtures.

Another method that allows attainment of even lower O_2 concentration is the physical evacuation of O_2 by vacuum pumping (Link, Eggers, and Moulton 1941). Air pressures of 1×10^{-5} torr are attained without elaborate technical equipment, and at these pressures the concentration of O_2, assuming that evacuation started from ambient air mixtures (8.7×10^{-3} M in O_2) at 20°C, is about 1.2×10^{-10} M. Etiolated corn seedlings consisting of 93 percent water, when degassed to an air pressure of 1×10^{-5} torr, have an estimated (Henry's Law) dissolved O_2 concentration in equilibrium with the evacuated airspace of about 3.9×10^{-12} M. If a glass apparatus is frame sealed after evacuation, this concentration can be maintained for indefinite periods contrasting with flushing with precleaned N_2 and chemical O_2 removal, which for obvious reasons last only as long as the chemical supplied. The above O_2 concentration is assumed to be sufficiently low to effectively stop free radical reactions (Sheehan 1970) proceeding with rate constants of $10^6 M^{-1} sec,^{-1}$ as is the case in the enzymatic degradation of IAA (Nakajima and Yamazaki 1979). Procedures for manipulating and transfering solvents, gases, and dissolved solids in strictly anaerobic conditions under high vacuum are similar to the methodology

employed for work with O_2-sensitive, pyrophoric compounds, that is organo-alkali compounds employed in living anionic polymerization (Szwarc 1968; Morton, Kammereck, and Fetters 1971).

When solutions of IAA in buffered saline containing 2 percent sucrose at pH 6 are degassed to about 10^{-5} torr O_2 and a suitable quantity of horseradish peroxidase (HRP) equally diminished in O_2 concentration is added in vacuo, then the enzymatic reaction is completely inhibited (fig. 1). The course of the reaction was monitored by means of the absorbances at selected wave lengths at room temperature. No change in the absorbances (A) at 280, 262, or 253 nm was observed, and the absorption spectrum of the reaction mixture was identical to that of authentic IAA for as long as O_2 was excluded. Mixtures of IAA and HRP solutions were kept in this manner for several days at room temperature and exposed to incandescent light without noticeable change in the UV spectrum of IAA or the appearance of degradation products. Opening the reaction vessel to air leads to immediate changes in the UV spectrum of the mixture. Because of the necessity of removing the reaction vessel from the spectrophotometer to break seals and stir reaction vessel contents during O_2 admittance, a time gap of about 2 minutes occurs, during which no data are recorded. Thereafter, the absorbance at 280 nm (A_{280}) increases slightly but significantly and then begins to drop sharply until a steady state is reached after about 75 minutes (fig. 1). The small initial increase is ascribed to the formation of the IAA hydroperoxide intermediate described earlier (Nakajima and Yamazaki 1979). Because of the extinction of IAA at 280 nm (E = 6100), the drop in A_{280} indicates IAA disappearance, and its rate can be roughly estimated after subtracting the contribution of the degradation product MOI to A_{280}, using published data for E MOI at A_{253} and A_{280} (Hinman and Bauman 1964). The observed maximum slope of 0.0035 A per minute translates thus to 8.9×10^{-7} M of IAA per minute. This estimate is likely to be somewhat low because of the contribution of other intermediates absorbing at 280 nm which cannot be quantitatively accounted for. The attainment of zero slope of the absorbance trace at 262 nm represents an isosbestic point (Ray 1956) for IAA, and its formation coincides with complete consumption of initially present material (Hinman and Lang 1965). This observation was verified in our work by submitting aliquots of reaction mixtures progressively in time to destructive colorimetric tests for IAA. Under the conditions described in figure 1, this point was reached about 30 minutes after admittance of O_2. Formation of the end product, MOI, as represented by A_{253}, lags characteristically behind the disappearance of IAA, since final reaction sequences are comparatively slow, nonenzymatic steps as described previously (Hinman and Lang 1965). The UV spectrum of the reaction mixture after 160 minutes (fig. 1, insert *b*) showed essentially the features of MOI (A_{248} and A_{253}), although a comparison with the spectrum of authentic MOI (Hinman and Bauman 1964) indicates less than quantitative conversion at this time.

Degradation rates calculated from isosbestic point measurements, end-point analyses (Cohen and Bandurski 1978), and UV absorbance rate studies are approximately equal in value for comparable reaction conditions, but these techniques are inherently incapable of assessing the degradation rate at the first instant of reaction. Oxygen consumption analysis, as studied by Nakajima and Yamazaki (1979), indicates initial rates up to tenfold faster than rates at mid-course.

In work to be reported next we sought to test the competitive destruction of IAA by a strongly acidic, oxidative colorimetric reagent (Salkowski assay) and by O_2 dissolved in the reagent. Data indicate a substantial and statistically significant rate difference (table 1). Results suggest that mixtures of IAA and oxygenase in the presence of dissolved oxygen cannot be accurately assayed for IAA content, even when radical scavengers are employed.

Several authors (see Horgan 1981) have presented evidence that organic solvents used to extract IAA from biomaterial will affect the quantity of measurable auxin. Data shown in table 2 support the idea that certain organic solvents will decrease the rate of IAA degradation by oxygenase-peroxidase. A significant decrease in the rate of IAA loss was found with increasing concentration of methanol, but loss of IAA was not completely inhibited, even in nearly absolute methanol. More important, however, was the observation that HRP is not irreversibly inactivated by an aqueous

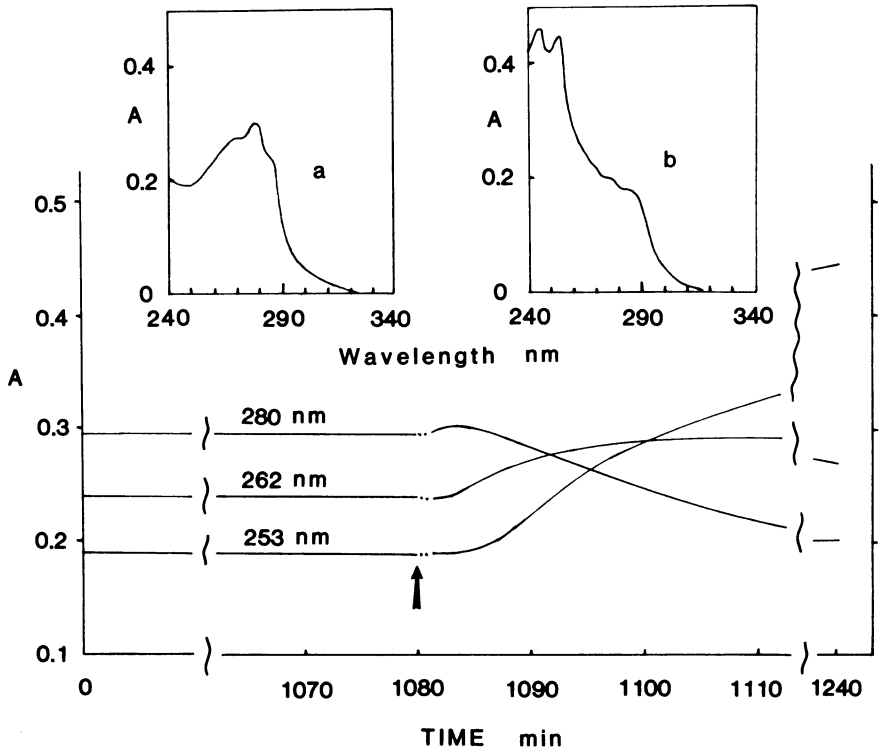


Fig. 1. Stability of IAA in a mixture of IAA and horseradish peroxidase (HRP) under anaerobic conditions and after admittance of O_2. IAA (49.2×10^{-6} M) was mixed with HRP (2.0×10^{-7} M, 8.5 purpurogallin units) in an atmosphere containing 10^{-10} M of O_2 and maintained for the indicated period of time in a flame-sealed glass vessel. Absorbances at 280 nm, 262 nm, and 253 nm were monitored over time. After 18 hours O_2 was admitted by opening the vessel to air (*arrow*). Inserts *a* and *b* show the spectra of the reaction mixtures immediately prior to admittance of O_2 and after 160 minutes, respectively.

TABLE 1. IAA LOSSES DURING INITIAL STAGES OF ENZYMATIC AEROBIC DEGRADATION WITH HRP[a]

Treatment	Initial (IAA) (μM)	Average (IAA) found (μM)	Loss (%)	• SE
A	45.8	37.3	*18.6	• 3.4
B	47.3	46.1	2.5	• 3.0

[a]Solutions of IAA in K-phosphate buffer, 2 percent sucrose, pH 6, were mixed anaerobically (10^{-5} torr) with degassed solutions of HRP. In *A*, the mixtures were cooled to LN_2 temperature and then the reaction vessel was opened to atmospheric O_2. Salkowski reagent (at room temperature and equilibrated with O_2) was then immediately layered on top of the frozen reaction mixture. Mixture occurred at the solid-liquid interface as the solid melted. In *B*, Salkowski reagent degassed to about 10^{-5} torr of O_2 was added *in vacuo* to the anaerobic reaction mixture and allowed to react for 5 minutes. The vessels were then opened to atmospheric O_2. Loss rates are averages of three and five determinations for *A* and *B*, respectively, and differ at the 0.05 level (*) as determined by the Student-T test for small populations. The loss rate in *B* is not significantly different from zero and the indicated standard errors (SE) in *A* and *B* are measures of the precision of the colorimetric method employed.

mixture of methanol and that IAA was destroyed at an accelerated rate once concentration of methanol was diluted with water. This effect has significance in extraction schemes where fresh biomaterial is extracted by adding organic solvents and the solvent is subsequently removed by evaporation, resulting in an essentially aqueous phase that is then subjected to more extractive manipulation.

General Parameters for IAA Extraction-Purification

Plant material used for developing the extraction method

Apple seedlings were grown in vermiculite in artificial light under typical temperature and nutrient conditions from 'Golden Delicious' seeds, stratified for 1000 hours at 5.5°C. After 8 weeks of vigorous growth, 200 plantlets were harvested by carefully freeing the root system from adhering vermiculite in a stream of water and then quick freezing the whole plantlets in LN_2. After freeze drying, the plants were ground and uniformly mixed in a Wiley mill equipped with a 40-mesh sieve. The dry powder was then divided into 2-g lots and stored in flame-sealed, evacuated vials at −20°C until used.

Method criteria

Criteria for ideally determining IAA in plants are discussed at length elsewhere (Reeve and Crozier 1980; McDougall and Hillman 1978) and are briefly repeated here. The method must (a) yield an unequivocal qualitative identification of IAA (accuracy); (b) determine endogenous IAA concentration existing in the plant part at time of sampling, that is, it must take into account the fragile nature of IAA toward degradation and prevent change in concentration by conjugation or deconjuga-

TABLE 2. INFLUENCE OF METHANOL ON THE RATE OF IAA DESTRUCTION BY HRP IN AIR[a]

IAA degradation rate ($M/min \times 10^8$)	Methanol content (%)
3.2	99
20.1	50
34.5	20
89.0	<1

[a]IAA (43.2 μM) in 99 percent methanol was mixed with 8.5 purpurogallin units of HRP in air and the rate of IAA loss monitored by UV spectrometry at 280 nm. After 60 minutes sufficient aqueous reaction medium (K-phosphate, pH6, 2 percent sucrose) was added to dilute the methanolic solution to the indicated concentration and a corresponding amount of HRP was added also to restore the initial HRP concentration. IAA concentration was estimated from tracings of A_{280} vs time approximately 7 minutes after each change of reaction condition. Slopes of A_{280} per minute were corrected for interference by A_{280} of MOI as described above.

tion; and (c) be an easily reproduceable procedure and less time consuming than existing conventional protocols. Furthermore, a measure of the precision of the method (reproducibility) must be obtained to help interpret comparative experiments on the physiological effects of IAA and to lay out and plan investigations.

The theoretical considerations for estimating the amount of a chemical in plant tissue are reviewed by Deming and Parker (1978) and Reeve and Crozier (1980). They advocate repetitive sample cleanup and measuring until the same quantity of chemical is measured each time. At this point the structural identity of the measured compound must be established by unequivocal methods, such as the mass spectrometer (MS), and the absence of undesirable contaminants accertained. Application of their procedure can be found in work by Sandberg and Dunberg (1982). The information presented by Reeve and Crozier (1980) is critically discussed by Scott (1982), and the ensuing debate about these procedures is good reading for hormone physiologists (Reeve and Crozier 1983; Scott 1983).

In our work the multi-channel, high-resolution MS was thought to provide the information needed to identify IAA. The MS is widely employed for other growth-regulating substances. It is important to point out that less selective analytical methods, such as UV spectroscopy, colorimetry, or spectrofluorimetry, may be employed for routine measurements when chemical purity can be established. In applied physiological studies chemical purity is rarely established and plant chemical constituents may change substantially during an investigation.

Internal standard and isotopic equilibrium

Isolation of single compounds from mixtures containing a variety of similar substances, of necessity, must involve a multitude of isolation steps. Recovery of the desired component in each step is less than 100 percent and overall recovery at the time of final assay may therefore be only a small fraction of the originally present mass of IAA (Reeve and Crozier 1980). To make matters even more difficult, the reproducibility of individual isolation steps is likely to fluctuate, depending on such

factors as reagent purity or operator skill, and the final precision is thus a composite of numerous errors. This dilemma is best solved by incorporation of an internal standard or marker at the beginning of the isolation process (Bandurski and Schulze 1974). This marker should ideally be indistinguishable in its chemical properties from IAA but possess a physical "handle," a property that can be independently measured without interference from the originally present IAA. It can be shown that adding such an internal standard compound in known quantity to the existing endogenous IAA will fix the quantity of endogenous IAA, regardless of the losses and fluctuations in recovery rates that may occur during isolation. It is merely required that the internal market be homogeneously mixed with the pool of endogenous IAA and that a sufficient amount of purified IAA and physical "handle" remain at time of assay to allow an unequivocal independent determination of the marked IAA concentration.

Since absolute recovery of IAA is of minor importance and recovery may vary from one analysis to another, design of a quick isolation procedure is made feasible. Repetitive isolation steps aimed at quantifying recoveries, such as multiple binary solvent distributions, may be reduced in number. This reduces the imprecision caused by multiple steps, and purification protocols can be perfected for qualitative identification of IAA because quantitation follows automatically. But most importantly, if the internal standard is uniformly distributed in the homogenized plant tissue being processed, it is not necessary to extract all of the IAA present. Anhydrous nonpolar, and thus more selective, solvents can be employed in the primary extraction step, instead of the commonly used polar aqueous mixtures which tend to extract a wider range of soluble biological compounds which then have to be painstakingly removed during the following isolation.

The physiologically active amount of plant-growth substance may exist in association with cell membranes, which contain the sites for a reversible temporary bonding (Stoddart and Venis 1980; Rubery 1981). Establishment of isotopic equilibrium requires, therefore, diffusion across membranes and exchange with partially bound ligands and is a time-dependent process, certainly not an instantaneous one. A crucially important task of a successful isolation procedure is thus to preserve the endogenous level of growth-regulating substance until isotopic equilibrium can be established.

The method of internal standardization described is known as isotope dilution because the physical "handle" referred to above is derived from incorporating into the molecule an isotope of one of the atoms comprising the molecule. If a radiating isotope is chosen, the method becomes a radiotracer dilution technique employed extensively in protein and amino acid biochemistry (Rittenberg and Foster 1940) and more recently in studies of plant-growth regulating compounds (Bandurski and Schulze 1974). In our IAA studies and those of others, a carbon-14 nuclide placed in position 2 was selected as the internal standard for reasons of ease of commercial availability and safety, since upon decomposition 1-^{14}C-IAA releases gaseous radioactive CO_2, which may be incorporated into many other chemical compounds.

Extraction media

Based on the foregoing discussion, we developed a model system for estimating IAA at low concentrations in plant tissue. Free IAA was assumed to exist in two pools. In one pool, the ionized free acid and alkali counterions exist unassociated

with cell membranes or proteins. Because of the preponderance of potassium (K) in plant tissue, it was assumed that upon drying free IAA would precipitate as its K salt. In the second pool, the free acid is reversibly bound by weak secondary chemical bonds to receptors. Because of these possible conditions we developed a procedure to extract both free IAA and IAA-K salts.

To minimize the overall mass of primary extract at the beginning of the isolation, solubility in and volume of the extraction medium should be kept as low as feasible, i.e., the solvent should just be capable of dissolving the minute amount of IAA present. Any excess solubility will not result in an increased extraction of IAA, but an increase in such undesirable compounds as proteins, sugars, oligosaccharides, chlorophyll, or secondary plant products such as phenols which are present in greater amounts. The equilibrium solubilities of IAA and its K salt were determined in various candidate solvent systems (table 3). The polar solvents methanol, acetone, acetonitrile, and water are excellent-to-moderate solvents for both IAA and IAA-K. Chloroform, benzene, and even petroleum ether should be considered adequate solvents for the amount of IAA under consideration but would be clearly inadequate for IAA-K. Ethyl acetate appears as the single most suitable nonaqueous solvent for both IAA and IAA-K. The solubility in any of the nonpolar solvents may be measured by adding several volume percent of the more polar solvents, but this will also drastically increase the quantity of undesirable solutes. Use of chloroform or petroleum ether/benzene mixtures would be of interest since bothersome and difficult-to-remove carbohydrates are practically insoluble in these solvents. Link, Eggers, and Moulton (1941) reported that IAA was not extracted from lyophilized tissue with dry ether; adding water to the ether resulted in IAA extraction.

To increase the solubility of these solvents toward IAA-K, adding a solubilizing agent of a different nature was investigated. Alpha-naphthalene acetic acid (NAA) is a synthetic auxin widely used in horticultural applications. Its physiological action has

TABLE 3. EQUILIBRIUM SOLUBILITY OF IAA AND IAA-K IN VARIOUS SOLVENT SYSTEMS

Solvent	Solubility[a] (mg/l)	
	IAA	IAA-K
Methanol	*[b]	*[b]
Acetone	*[b]	*[b]
Water (dist.)	1400 ± 42	*[b]
Chloroform	2214	<0.10
Benzene	425 ± 25	—
Petroleum ether	0.14	*[c]
Ethylacetate	>5000	34
20% benzene in petroleum ether	2.86	<0.20

[a] Equilibrium solubility was determined by stirring an excess of solute in 5 ml of solvent for 48 hours at room temperature followed by HPLC assay of IAA in the supernatant after filtration through 0.45μ fluorocarbon filters (Millipore).

[b] Solubility >10 g/l.

[c] Not detectable.

been under study for some time. It has a greater affinity to corn- and strawberry-derived receptors than IAA and thus displaces IAA as ligand in this *in vitro* system (Ray, Dohrmann, and Hertel 1977; Narayanan, Mudge, and Poovaiah 1981). In our work we found that adding small amounts of dissolved NAA to suspensions of crystalline IAA-K in chloroform or 20 percent benzene in petroleum ether increased the solubility of IAA in these nonpolar solvents (table 4). Data obtained in chloroform show a statistically significant direct correlation between NAA concentration in solution and solubilized IAA-K at a molar ratio of about 22:1 up to the equilibrium solubility of free IAA in chloroform. The solubility of free IAA in chloroform is not changed by adding NAA, indicating that some kind of molecular exchange takes place at the interface between solid IAA-K and NAA in solution. This contrasts with the increased solubility when the average polarity is increased by adding high dielectric solvents. Even in the benzene petroleum ether system, an enhanced IAA-K solubility is observed with addition of NAA. Potassium concentrations in solution were not measured and there are insufficient data at present to elucidate the mechanism of the effect observed. But for the purpose of finding a suitable extraction medium for IAA from the plant-derived materials, the quantities of NAA necessary to provide an adequate solubilizing effect are well within reasonable limits. Removing NAA in subsequent isolation steps presents no problem.

Adsorptive behavior of IAA toward poly-amid 6 (polycaprolactam, PA-6) and polyvinylpyrrolidone (PVP)

As noted earlier, IAA may exist in a pool of reversibly adsorbed ligand, as in the case of sterically favored adsorption on proteinlike receptors or weakly attached to the polar functional groups of carbohydrate constituents of cell membranes. Solubility of IAA in nonpolar solvents will therefore be reduced proportionately to the strength of interaction with the adsorbent. By employing an internal standard the reduction in solubility, i.e., temporary loss of extractable IAA, is inconsequential as long as certain provisions are maintained. The most important is based on the assumption that the

TABLE 4. EFFECT OF NAA ON THE EQUILIBRIUM SOLUBILITY OF IAA-K IN NONPOLAR SOLVENTS AT ROOM TEMPERATURE

Solvent	Added NAA ($\mu M \times 10^{-2}$)	Dissolved IAA-K[a] (μM)
Chloroform	0	0.2
	7.4	21.4
	61.3	217
	122.6	549
20% v/v benzene in petroleum ether	0	0.6
	7.1	5.6
	59.1	9.3

[a]Equilibrium solubility was determined by stirring an excess of IAA-K in 10 ml of solvent containing the indicated quantities of NAA for 20 hours at room temperature. Dissolved IAA-K was assayed in the supernatant as IAA by HPLC after filtration through 0.45μ fluorocarbon filters (Millipore).

adsorbate is reversibly bound and in a dynamic equilibrium with the nonadsorbed IAA present in solution so that if isotopic composition of the dissolved IAA is disturbed by adding very small amounts of an isotope tracer, attainment of isotopic equilibrium with adsorbed IAA will occur within a finite time span. The following series of experiments tested this assumption.

The adsorptive behavior of any compound dissolved in nonpolar solvents can be quickly approximated through its chromatographic property on the adsorbent in question (Pryde and Gilbert 1979). In thin layer chromatography (TLC) the R_f value is related to the capacity ratio k′ commonly used in HPLC for adsorptive or partition distribution systems, represented as follows:

$$R_f = \frac{1}{1 + k'}; \text{ or } k' = \frac{1}{R_f} - 1. \quad (1)$$

The capacity ratio is proportional to, but not identical with, the partition coefficient K obtained in a single-step equilibrium distribution of dissolved compounds between two immiscible phases. Thus,

$$k' \propto K = \frac{C_i^s}{C_i^L}; \text{ and } C_i^s = \frac{m_i^s}{m^s}, C_i^L = \frac{m_i^L}{m^L}, \quad (2)$$

where m = mass of total solute = $m_i^s + m_i^L$
C = concentration of solute i present at any given time in solid phase s or liquid phase L
m^s = mass of solid phase and
m^L = mass of liquid phase.
From this relationship the ratio of extractable to total solute, m_i^L/m, can be estimated by rearrangement

$$m_i^L/m = \frac{1}{k'q + 1}; \quad (3)$$

where q is the mass ratio of solid to liquid phases, and the proportionality constant for (2) is close to unity.

Interestingly, m_i^L/m is identical with the degree of dissociation which plays a prominent role in the theory of adsorption and phase equilibria. The expression obtained in (3) is attractive because an adsorbent such as a proteinlike plant cell constituent when arranged as stationary phase on a TLC plate can be readily tested for its adsorptivity toward a given solute, e.g. IAA, in any nonpolar solvent by simply measuring the compound's R_f. Conversely, the R_f value allows estimating the quantity of solute extractable from an adsorbent in a single step. Sedimentation fractionated proteins from apple tissue were not available in this investigation, so commercially available polyamino acids were used as model systems.

Poly-amid 6 (polycaprolactam, PA-6) is a synthetic polymer acid with the following structure:

$$[(CH_2)_5 - CO - NH]_n, n \geq 10$$

In TLC it is widely employed in powder form as a stationary phase for the chromatography of such acid-sensitive compounds as IAA (Railton 1972). With chloroform

as the eluant, IAA has an R_f value of 0.02 whereas in methanol or ethyl acetate the R_f is about 0.9. Therefore, if used in a single-step extraction, a tenfold excess of methanol or EtOAc would extract IAA almost quantitatively to about 99 percent of the total IAA present, but the same volume chloroform would extract only about 17 percent.

Polyvinylpyrrolidone (PVP) is a synthetic polymer with a poly amino acidlike structure which, when crosslinked, forms an insoluble powder employed in many extractive applications and in the chromatographic separation of phenols and other aromatic compounds (Clifford 1974). Chromatographed on PVP, IAA has R_f values of less than 0.01, 0.015, and 0.5 for chloroform, EtOAc, and methanol, respectively. Methanol is again a good eluant, yielding about 95 percent extracted IAA in a single-step extraction, but it is also apparent that PVP is a much stronger adsorbent for IAA than is PA-6. Chloroform would not extract adsorbed IAA in appreciable quantity and to achieve extractable quantities similar to the system PA-6/chloroform, the much more polar-solvent EtOAc is required. The time course of adsorption of IAA on PA-6 and PVP from chloroform and EtOAc solution was determined, and the results are depicted (fig. 2). Equilibrium was established after only 40 minutes, and the equilibrium concentration of IAA in solution was 3 to 5 percent of the original concentration, somewhat lower than the percentage calculated from R_f values, but still confirming the predictive quality of the chromatographic method.

To test whether a truly dynamic equilibrium situation existed, a small quantity of radioactive 2-^{14}C IAA with known specific activity was added to the equilibrium supernatant solution of IAA adsorbed on PA-6 and PVP. Small aliquots were withdrawn in time periods of increasing length, and the specific activity of the contained IAA was assayed by HPLC and scintillation counting. In the case of PA-6/chloroform,

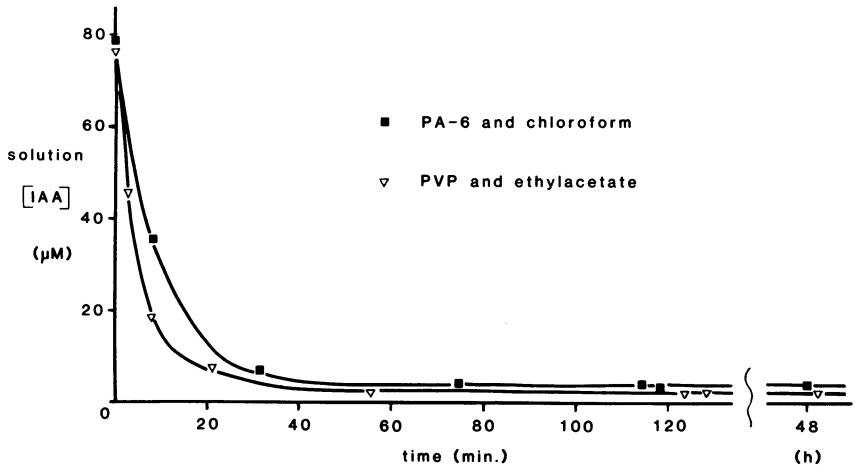


Fig. 2. Adsorption of IAA from solution on PA-6 and PVP adsorbents

the isotope dilution method was able to detect accurately the total quantity of IAA adsorbed plus dissolved in the supernatant only 12 minutes after addition of tracer.

Using PVP and ethyl acetate, isotopic equilibrium was established after 3 hours. In this case, the result was also verified by measuring the specific activity of adsorbed IAA. This was accomplished by filtering the solid support from the supernatant, rinsing with EtOAc, and then eluting the adsorbed IAA with methanol. Determination of the specific activity of the extracted IAA yielded, upon computation, the same results as that obtained from the supernatant alone, thus proving that isotopically labeled IAA had become uniformly distributed with adsorbed IAA, even though only about 5 percent of total IAA was in solution at any given time. The increase in time necessary for isotopic equilibration of PVP, as opposed to PA-6, is due to a greater activation barrier for the rate of exchange of IAA onto PVP and differing particle composition of the solid phases employed in these experiments.

Isolation and assay

In addition to proving the hypothesis that not all of the contained IAA need be extracted to determine its total quantity accurately, these experiments also suggest two separate strategies for sample isolation and assay. In the first strategy, dried plant tissue was pulverized and stirred for 18 hours in a poor solvent for IAA, such as chloroform or methylene chloride, along with 2-^{14}C-IAA. When isotopic equilibrium was attained, the supernatant was removed and IAA further purified by TLC and HPLC and finally assayed. Since the extract contained only a small fraction of the total IAA present, the original biomaterial could be extracted repeatedly and the efficiency of the isolation procedure tested by successive approximation. Even though each extraction sequence employed identical protocols, the accuracy and precision of IAA determination should increase with the number of extraction sequences, if the concentration of deleterious compounds removed from the biomaterial in each sequence decreases at a higher rate than IAA; therefore, the relative purity of IAA and the accuracy of assay are enhanced in each successive extraction sequence.

The data in figure 3 were obtained from extracting standard apple tissue equilibrated with 2-^{14}C-IAA, using methylene chloride as the sole extractant. Dry weight comparison revealed that at each sequence the solvent removed approximately 4 percent of the total IAA from the biomaterial. The data show that the identical isolation procedure employed for each sequence was clearly inadequate for at least the first extraction, that is, the first extract contained an unmanageable amount of undesirable compounds. In sequences 2, 3, and 4, the mean concentration of IAA obtained was statistically not different, but the variability of the assay in sequences 2 and 3 was unacceptably large. Therefore, a successful extraction protocol should discard the first three successively obtained extracts and employ only the fourth or higher sequences for isolation and assay. Alternatively, the selectivity of isolation procedures would have to be enhanced.

Interestingly, the measured IAA concentration decreased with increasing extract purity. This effect is associated with the choice of UV spectrometric absorbence measurement as the final assay and the mechanics of the isotope dilution method. This method requires determining a ratio of specific activities of IAA at the start and after dilution. Therefore, if at the final assay UV absorbances of foreign compounds are

added to the absorbance of IAA, the diluted specific activity appears too small and the computed IAA quantity is artificially inflated. Conversely, if unremoved spurious compounds increase the diluted specific activity of IAA, as in fluorescence quenching in spectrofluorometric assays, the IAA concentrations computed are underestimated.

The second strategy emerged from results of the adsorption study (fig. 2), which makes use of the high affinity of PVP for IAA. However, instead of multiple extractions with a weak solvent, it employs EtOAc as the medium for isotope dilution of endogenous IAA and a single-step, 18-hour extraction period. The raw, dark green colored tissue extract was then filtered to remove remaining solid particles. PVP was added to the filtrate for adsorption of IAA, and the PVP-IAA equilibrium took place in about 1 hour. The extraction with EtOAc removed approximately 60 percent of the total endogenous-free IAA from the standard apple tissue; thus, a greater quantity of measurable IAA was carried through the isolation process. At this point, the great bulk of extraneous dissolved substances had been removed by filtering the solution from PVP and rinsing the filter cake with portions of fresh ethyl acetate. The filter cake was then devoid of extract-derived discoloration and appeared as a nearly white powder. Adsorbed IAA was then easily eluted from PVP with small volumes of methanol.

Several subsequent purification steps, using one or two TLC treatments or TLC followed by HPLC, led to the most desirable results (fig. 4). The main problems with less rigorous extract treatment included erroneous and variable IAA estimation. The shortest isolation path (I-1) is clearly unsatisfactory and gives erroneous results. Introduction of an additional TLC step, in which IAA was moved to R_f 0.3 (II-1), somewhat improves the accuracy of the assay, but the method still did not yield an unequivocal IAA identification. Fractionation of the putative IAA peak, followed by

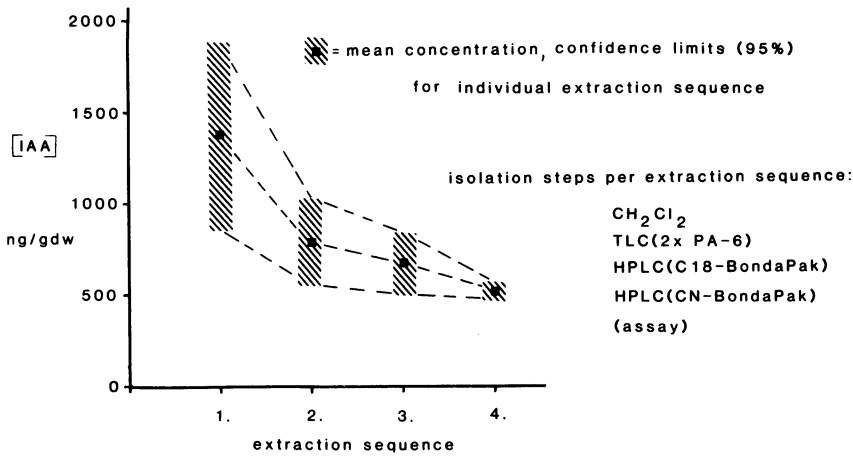
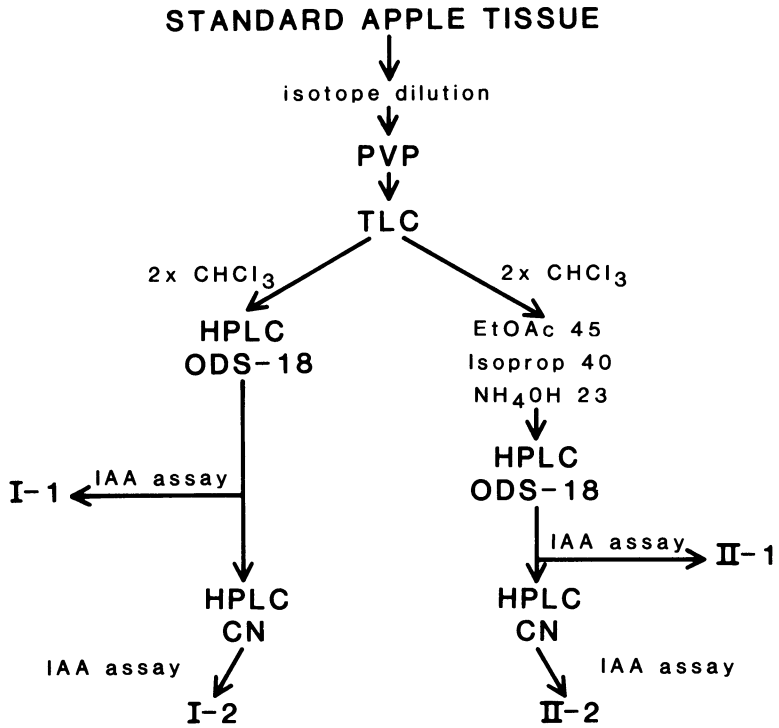


Fig. 3. Determination of IAA concentration in identical standard apple-tissue preparations by successive approximation and isotope dilution

reinjection onto a CN-Bondpak reverse phase HPLC column (I-2 and II-2), yielded single-peak chromatograms from absorbances at 280 and 254 nm, which were used for quantitative assay. Qualitative identification of IAA was obtained by the R_t of co-chromatographed authentic IAA, the characteristic absorbance ratio A-280/A-254 of IAA, and the high resolution mass spectrogram of putative IAA collected from the final chromatographic run. Details of the mass spectrometric analysis are given later. Method I-2 leads to very high variability; method II-2 is the method of choice for the biomaterial considered here. The mean IAA concentration obtained from I-2 is statistically not different from II-2. The values obtained in II-2 compared favorably with data of the earlier described multi-extraction method (sequence 4, fig. 3).



method	ng IAA/gdw ± S.E.(%)
I-1	2764 ±17.9
I-2	652 ±47.3
II-1	1203 ±12.7
II-2	515 ±6.9

Fig. 4. Variation in isolation schemes for IAA following prepurification of extracts with PVP

Concentration of IAA in standard apple tissue

After having established a procedure for determining free IAA quantities in standard apple tissue it was of interest to learn whether certain time consuming components of the procedure were amenable to change without infringing on the integrity of the overall method. It was also necessary to test whether the practice of isotope equilibration—by stirring of the dried tissue in anhydrous solvent in the presence of air—yielded different results from the treatment under strictly controlled O_2-free conditions as described in our study of the IAA content of corn seedlings. Environment devoid of reactable O_2 concentrations can be produced by conducting all extraction and equilibration operations in a sealed glass apparatus at partial O_2 pressures of less than 10^{-5} torr (Szwarc 1968). The data presented in table 5 (treatments *A* and *B*) indicate that there is no statistically significant difference of IAA levels between *in vacuo* and in-air equilibrated freeze-dried tissue. There is also no significant difference between IAA levels found when tissues were equilibrated for varying periods of time (treatments *C* and *D*). Apparently, an equilibration extraction time of 3 hours is sufficient, and at least under the conditions and tissue preparation employed here equilibrations for 18 hours are not necessary. The results also strongly indicate that oxidoreductases from vegetative apple tissue is sufficiently inhibited in the anhydrous state and in the presence of the moderately polar solvent EtOAc to permit attainment of isotope equilibrium before a measurable loss of endogenous IAA occurs. Similarly, since hydrolysis reactions require the presence of water, an erroneous inflation of the determined IAA concentration due to hydrolytic release of IAA-conjugate appears unlikely. Literature on plant-derived oxidoreductases amply documents the species' dependent variability in the reactivity of these enzymes (Schneider and Wightman 1974). The results ob-

TABLE 5. INFLUENCE OF EXTRACTION CONDITIONS ON THE CONCENTRATION OF FREE ENDOGENOUS IAA IN STANDARD APPLE TISSUE DETERMINED BY ISOTOPE DILUTION IN ANHYDROUS MEDIUM

Time in hr and treatment[a]	IAA concentration • SE (%)[b] (ng/g dry wt)
A: 18, air	515 ± 6.9[d]
B: 18, *in vacuo*	491 ± 3.0
C: 3, air	544 ± 4.1
D: 6, air	541 ± 4.4
E: 6, air[c]	33 ± 4.5

[a]Time allowed for isotope equilibration under stirring in anhydrous EtOAc in the presence of air (*A, C, D, E*) or in the absence of air (O_2 partial pressure $< 10^{-5}$ torr) (*B*). Isolation and assay procedures as in II-2 (fig. 4) for all determinations. Standard apple tissue storage conditions identical for all samples except in *E.*

[b]Mean concentration of IAA from three assays per treatment and standard error (SE) as percent of mean.

[c]Lyophylized apple tissue was stored in air at room temperature for one year in covered containers prior to analysis.

[d]Means of treatment *A* through *D* are not significantly different from each other ($F = 0.98$; $F = 0.05$, $(3,8) = 4.07$).

tained here can therefore not necessarily be applied to other plant species. Whether anhydrous extractions alone suffice to protect endogenous IAA levels must be decided on the merits of each case.

That plant samples, even in a dried state, cannot be kept indefinitely in air without serious loss in IAA is shown by data in treatment *E,* table 5. This sample lost approximately 94 percent of its free IAA after storage in air for 1 year. The data, however, do not permit concluding that degradation was entirely enzyme related since IAA is also known to slowly oxidize in air over extended periods (Sembdner et al. 1980). These findings are of practical interest because combining the much less time-consuming methodology for in-air extractions with shorter equilibration times allows a significant decrease in analysis time for applied physiological investigations.

Method performance parameters

The literature abounds with studies in which the parameters, as in IAA concentrations, are reported from a single determination of a complex variable and inferences are made regarding increase or decrease of this variable parameter as a result of horticultural treatment (Avery, Burkholder, and Creighton 1937; Galston and Hillman 1961; Goodwin 1978). These procedures were among the best available at the time, yet they contain possible error because of the omission of an important and perhaps controlling source of variance that originates in the inevitable propagation of error in measuring individual physical observations. The magnitude of this variance depends on many factors, but as a rule of thumb increases with the number of individual measurements and parameter manipulations.

Precision, defined as concordance between repeated measurements of the same quantity, may be expressed by any statistical quantity which describes deviation of populations from a mean value, that is, standard deviation, coefficient of variability, standard error, etc. For the method described here (fig. 4 and table 5), the main contributors to imprecision originate in the isolation scheme and in the determination of specific activities of IAA involving measurements of radioactivity and of IAA mass by indirect physical methods. An estimate of the precision of this method can be obtained from the data in table 5. Accordingly, the highest relative standard error in a range from 33 to 544 ng IAA/g dry weight was 6.9 percent.

The accuracy, defined here as concordance between measured and true value, may also be obtained from table 5. It depends mainly on the degree to which an isolation scheme is capable of purifying a target compound and the discriminatory power of a physicochemical method to establish the chemical structure and identity. By assumption, full accuracy is attained if successive purification efforts do not result in the change of a measurable quantity by more than the fluctuations allowed through imprecision (Reeve and Crozier 1980). As illustrated by an example of the IAA concentration in standard apple tissue, the values of IAA as shown using the scheme in figure 4, II-2 are both accurate and adequately precise. All other choices lacked either precision or accuracy or both. The true value of the free IAA concentration of the standard apple tissue employed here is with high probability within a range of ± 12 percent of 523 ng/g dry weight (confidence limit at 95 percent, data from table 5).

Data on the reproducibility of procedures are rarely reported in the literature but are necessary to evaluate results properly. The precision statement for method II-2 (table 5 and fig. 4) of ± 6.9 percent compares with values of ± 7.1 percent to ± 9.2 percent for methods using methyl indolo-pyrone fluorescence assays of IAA in *Pinus sylvestris* tissue published by Sandberg and Dunberg (1982). Thus, for duplicate assays, the least significant differences ($P = 0.05$) between treatment means is 51.4 percent which decreases to 27.1 percent if triplicate assays are performed. It is important to point out that these uncertainties arise solely from considerations of methodology and do not take into account any biological variability of interest to the horticulturist. Because precision is influenced by the complexity of composition of biomaterials it is likely that applying method II-2 (table 5, fig. 4) to single types of plant organs or vegetative plant parts, such as internodes, petioles, or buds of apple tissue, will result in a relative standard error of less than 6.9 percent.

The lower range limit of method II-2 can be estimated from the performance data of components of the isolation and assay procedure. From an observed minimum absolute detectability of 5 ng IAA by HPLC, using UV-absorption detectors, an overall recovery of about 20 percent, and for three assays per sample, the lowest detectable concentration of IAA can be calculated as 25 ng/g dry weight. An overall recovery of 21 percent was found experimentally, the result of losses incurred during isolation in addition to partial extraction of 60 percent of originally present and equilibrated IAA. An upper range limit for IAA concentrations has not been determined, but it can be estimated by inspection to be in excess of 100 μg/g dry weight, well within reasonably encountered IAA levels in biological tissue.

Preparation of endogenous IAA for mass spectrometry

Mass spectrometry is one of a few physicochemical methods capable of unequivocally identifying the structure of complex organic compounds such as IAA. In this investigation it was essential to verify the structure of the compound assayed in the final HPLC run as IAA. MS instrumentation is not affordable at most research laboratories, but the analytical service is available at designated specialized research centers. The amount of IAA necessary for high-resolution MS analysis varies with instrument insensitivity and 600 ng is often required—an amount higher than the quantity of endogenous IAA found in apple tissue routinely assayed but easily found in the etiolated corn to be described later. To validate the assay procedure employed here, methods were developed to isolate sufficient IAA from the mobile phase after the final HPLC run and to secure the isolated compound for analysis. A combined dry weight of 6.65 g of apple tissue was subjected to extraction and isolation procedures as described for routine samples, except that multiple extractions and HPLC runs were necessary to accommodate the larger amount of plant material. To isolate the IAA in a form suitable for MS, an additional chromatographic column was attached to the outlet of the HPLC apparatus. Thus, after passing through the previously mentioned ODS-18 column (fig. 4) and at the precise moment the final IAA peak was beginning to pass through the detection system, the IAA was collected on the aforementioned additional column. This column consisted of a 4.6 mm × 25 cm stainless steel tube packed with Bondapak C-18 on 36 to 75 micron porosil-B and was pretested for IAA retention properties.

After collecting the IAA peak, the collection column was disconnected and the mobile phase with a volume approximating that of the dead volume was expelled by applying a slight positive N_2 pressure. This was followed by loading the column. This operation had the effect of rinsing the column's stationary phase containing absorbed IAA with 1.5 ml of distilled water and then expelling the liquid again with N_2. Methanol of the same purity as used in the isolation work was then introduced and the first 0.2 ml emerging were discarded. All of the absorbed IAA was eluted with the following 2 ml of methanol. The solvent was evaporated in a stream of N_2 to a volume of 25 μL, of which 20 μL were transferred to a 1.5 × 100 mm glass capillary sealed at one end and the solvent slowly evaporated under reduced pressure. The capillary was then flame-sealed under vacuum and mailed to the MS laboratory in a shock-proof container for MS analysis of its content.

Prepared in this manner, IAA required no derivatization and the capillary content was analyzed by a direct-probe insertion technique. The mass spectrum of the IAA derived from apple tissue compares favorably with the control spectrum from authentic IAA employed for standard purposes (fig. 5); both were prepared as described above. The analysis confirms the condition of the IAA sample after undergoing collection and isolation from mobile phase constituents and gives evidence for the absence of impurities in the IAA sample submitted for MS analysis. Results of the MS provide additional evidence for the identity of IAA extracted and isolated from apple vegetative biomass. The mass fragments coincide with relevant aspects with those obtained from standard IAA as control, and agree with the MS of IAA published by Jamieson and Hutzinger (1970).

IAA from a Low Contaminant Tissue—Corn

Once the basic parameters for IAA sample processing were established and tested, the procedure was evaluated on corn epicotyls for comparison with published literature. Corn was selected because the epicotyls are relatively easy to work with, material can be generated quickly, and more quantitative literature for comparison exists than for other plant materials.

Using physicochemical methods and radio-labeled internal standards, recent estimates of IAA content of etiolated corn seedlings have been 15 ng/g fresh weight for 3-day-old mesocotyl tissue, 28 ng/g fresh weight for 3-day-old coleoptile tissue, and 24 ng/g fresh weight for 5-day-old whole seedling shoots (Bandurski and Schulze 1977). As determined from their materials and methods they may not have successfully prevented oxidative destruction of IAA from the instant the plant system was disturbed. In contrast, our method can inhibit completely the enzyme system responsible for the rapid degradation of endogenous IAA by quick-freezing the plant sample in LN_2. Also, when combined with an internal standard to estimate losses during purification for assay, the method is capable of inhibiting enzyme action until isotopic equilibrium between endogenous IAA and exogenous tracer is attained. Finally, by selecting an anhydrous extraction medium the probability of adding IAA through enzymatic hydrolysis of IAA conjugates was minimized and the complexity of the isolation process reduced.

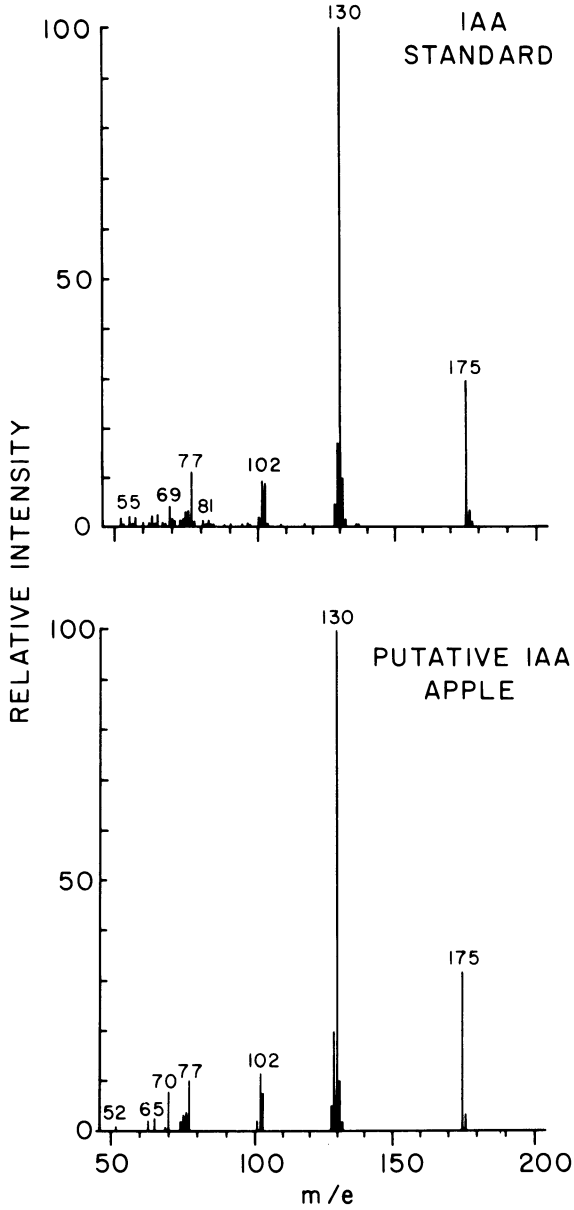


Fig. 5. Mass spectra of nonderavitized IAA standard and putative IAA from apple vegetative tissue obtained on a Kratos MS-50 instrument at the Midwest Center for MS, University of Nebraska, Lincoln, Nebraska. Electron impact, 70 eV direct probe insertion, probe temperature 90°C.

Plant material

Seedlings of *Zea mays* L. 'Stowell's Evergreen' hybrid sweet corn were grown in vermiculite at 25°C in the dark, as described by Bandurski and Schulze (1974). All aboveground tissue was harvested between 3 and 8 days after sowing and immediately immersed in LN_2. During lyophilization the plant sample remained frozen throughout the drying process. Processed samples may be stored indefinitely at this stage at temperatures of −20°C or below, preferably sealed in glass to avoid rehydration.

Procedure

All experimental techniques, including chemicals, apparatus, extraction, and purification procedures, and qualitative and quantitative assays were carried out as illustrated in figure 9. Both UV spectrophotometer and nitrogen-phosphorus-gas chromatography (NP-GC) were used for quantifying IAA (Martin, Nishijima, and Labavitch 1980).

Mass spectra

A purified corn epicotyl extract equivalent to 3 mg dry weight was inserted by direct probe for MS spectral characteristics. The putative Me-IAA sample from corn compares favorably with the Me-IAA standard (fig. 6). Both spectra show the characteristic base peak of m/e 130 for Me-IAA and the strong molecular ion at m/e 189 for Me-IAA. The corn sample shows a contaminant peak at 149 m/e.

IAA concentration in corn

Positive identification of IAA in corn was followed by estimation by UV and NP-GC. The concentrations of free IAA in shoots of etiolated corn seedlings with O_2 excluded during extraction ranged from 2943 to <2 ng/g dry weight (table 6). Methyl acetate, as sole extraction agent, was capable of extracting only 10.4 ng IAA/g dry weight (table 6, sample 4). The same sample, when extracted with methyl acetate but with NAA added as solubilizing agent (sample 3), yielded an IAA level of 296 ng/g dry weight. IAA levels in seedlings of the same age at harvest were similar when either CH_3CN (sample 10) or NAA (sample 9) were used as solubilizing agents. When biomass was insufficiently freeze dried and contained 19 percent moisture (sample 8), only 18.5 ng/g dry weight IAA was found in comparison with 106 ng/g dry weight with proper freeze drying (sample 7). Allowing a freeze-dried sample to rehydrate in air (sample 5) or allowing a frozen sample to thaw before freeze drying (sample 6) resulted in extremely low IAA levels of <2 ng/g dry weight.

Free IAA levels in corn seedlings 3, 4, and 4.5 days old were substantially higher than levels found in 6- and 7.5-day-old seedlings (table 6). We have employed a method capable of inhibiting completely the enzyme system responsible for rapid degradation of endogenous IAA from the moment an environmental stimulus is applied to the plant system (e.g., mechanical stress or excessive light intensity), and combined this method with an internal marker to estimate inevitable losses during the purification for assay. Results, as listed in table 6, indicate that the content of total free IAA in corn is considerably higher than previously reported estimates (Iino and Carr 1982;

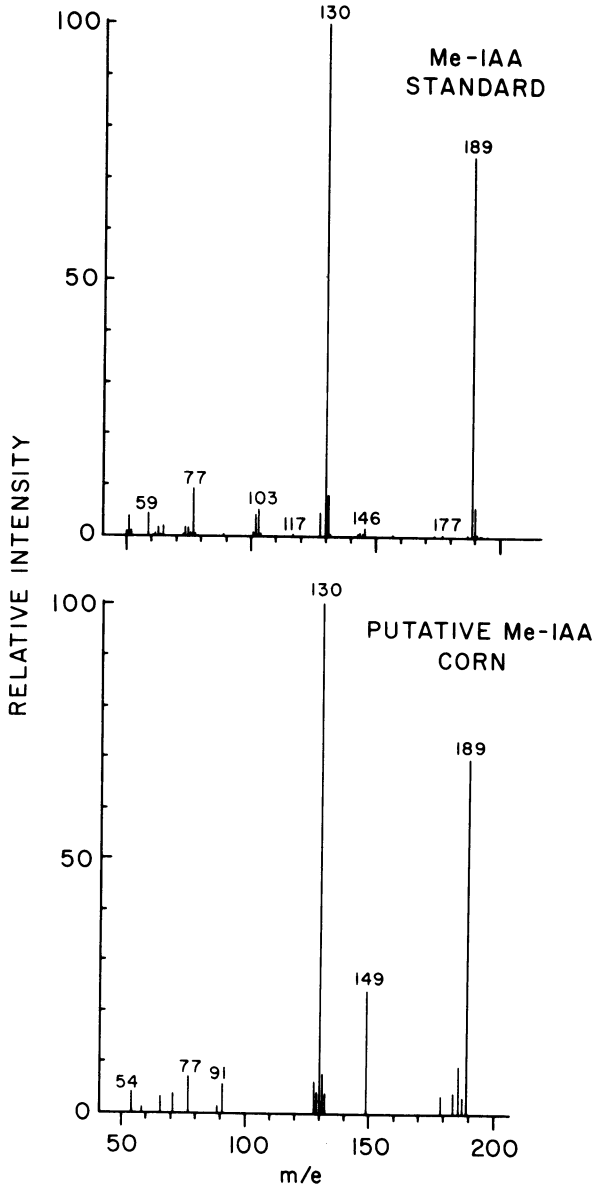


Fig. 6. Mass spectra of Me-IAA standard and putative Me-IAA from corn epicotyl with Finnegan instrument at UC Davis. Temperature program was 40° to 280°C, 10° per minute. He gas flow 28 cm per second, column DB-1 30 M × 0.25 mm OD, 70eV, scan speed 1.6 seconds per decade, and 60°C source temperature.

Iino, Yu, and Carr 1980; Bandurski and Schulze, 1977; see comparison in figure 7). The concentration of total free IAA in shoots of etiolated corn seedlings was found to decrease with age (fig. 7) and ranged from 2943 ng/g dry weight at 3 days (table 6, sample 1) to an average of 40.7 ng/g dry weight at 7.5 days (table 6, samples 9 and 10). This decrease in IAA may be associated with the shift in proportion of coleoptile to mesocotyl which takes place in the seedling at this time and suggests that a huge excess of IAA is located in the coleoptile part of the seedling during early growth stages. A similar interpretation may be given the studies of Weiler, Jourdan, and Conrad (1981) where diffusable IAA from the coleoptile tip was estimated with the enzyme-linked immunoassay.

Discussion

It is not the purpose of this review to discuss IAA physiology; yet, a point in sampling corn for IAA determination must be made. The samples taken at less than 4 days contain the highest concentrations of IAA (table 6). These samples reveal the crucial nature of handling protocol. Samples taken during a period of rapid change

TABLE 6. CONCENTRATION OF FREE IAA IN CORN SEEDLINGS[a]

Sample number	Age of seedlings at harvest (days)	Extraction additive	Free IAA • SE (%) (ng/g dry wt)[b]	Free IAA (ng/g fresh wt)[c]
1	3	CH_3CN	45270 ± 19.0	2943
2	4	CH_3CN	14986 ± 10.7	974
3	4.5	NAA	4553 ± 4.5	296
4	4.5	—[d]	160 ± 5.8	10.4
5	5	NAA	28[e]	< 2.0
6	5	NAA	20[e]	< 2.0
7	6	CH_3CN	1624 ± 10.7	106
8	6	CH_3CN	284 ± 1.4	18.5
9	7.5	NAA	568 ± 6.2	37.1
10	7.5	CH_3CN	680 ± 6.2	44.2

[a]All samples were lyophylized to <0.02 percent moisture before extraction, except sample 8 which had a water content of 19 percent w/w. In sample 5 the lyophylized sample was soaked in distilled water for ½ hour in air, then lyophylized again before extraction. The sample showed distinct brown coloring. In sample 6 the freshly harvested, quick-frozen sample was allowed to thaw and remain at room temperature in air for ½ hour before it was refrozen and lyophylized before extraction. The sample showed no evidence of browning. Plant material from sample pairs 3 and 4; 5 and 6; 7 and 8; 9 and 10 were derived from batches grown under identical conditions. Sample 1 was extracted with ethyl acetate and samples 2-10 with methyl acetate.

[b]Mean concentration of IAA from three to six assays per sample • standard error in percent.

[c]Mean concentration of IAA computed from ng/g dry weight, assuming a dry matter content of corn seedlings of 6.5 percent.

[d]None was added.

[e]Estimate only. IAA concentration at lower range limit of assay method.

will be difficult to manage; note the larger standard deviation at 3 and 4 days (table 6). We believe this deviation is not due to workup variance but to the stage of corn epicotyl development. We compared a single 3-day sample containing IAA at 48510 ng/g fresh weight and when analyzed by NP-GC on different days we found a standard deviation of 0.9 ng/g fresh weight. This result reveals the potential precision with NP-GC. The point remains that the dynamics of IAA in the corn epicotyl are in a rapid state of change at 3 and 4 days, and sampling for uniform comparison requires careful attention to protocol.

In plant samples of identical age, the possible loss of IAA caused by oxidation is seen by comparing the IAA content found in samples 7 and 8 (table 6). The latter sample was insufficiently lyophilized and retained enough cell water (19 percent) to

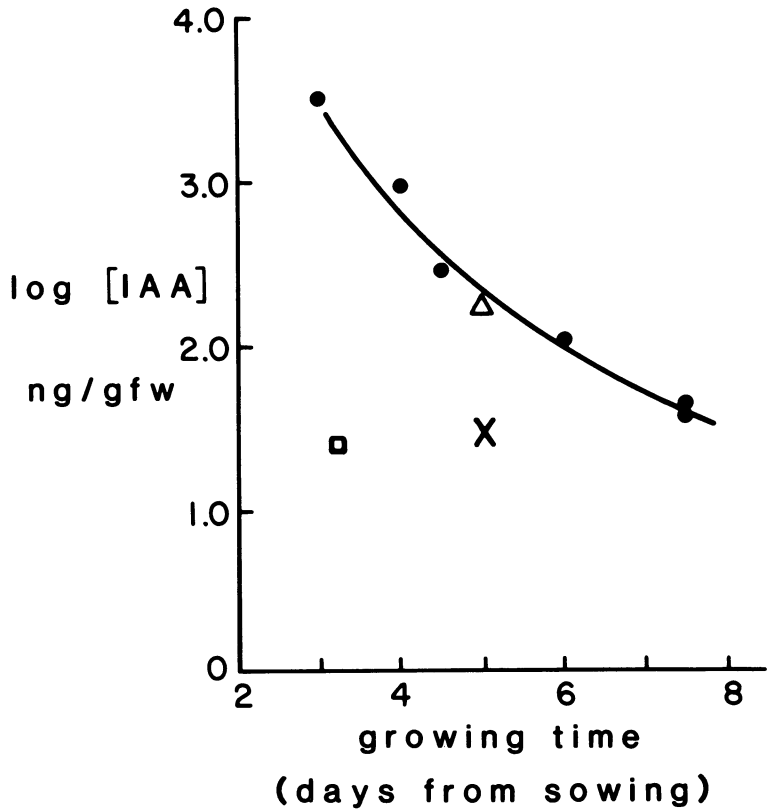


Fig. 7. Free IAA content in etiolated seedling shoots of several *Zea mays* L. cultivars as determined by extractive methods. Legend: • = 'Stowell's Evergreen' (present work; table 6, samples 1,2,3,7,9,10); X = 'Stowell's Evergreen' (Bandurski and Schulze 1974, 1977); □ = 'GH 390' (Iino and Carr 1982); and △ = 'Anjou 21' (Weiler, Jourdan, and Conrad 1981).

give favorable reaction conditions for IAA degradation. Cell damage induced by freeze-thaw cycles led to even greater depletion of free IAA (samples 5 and 6, table 6).

Methyl acetate as sole extractive agent was capable of dissolving only a fraction of the total free IAA in plant samples of identical age (sample 4, table 6). The same sample when extracted in the presence of 1 μM NAA/g dry weight, added as solubilizing agent (Walton and Ray 1981), yielded an IAA level of 296 ng/g dry weight. Considering the conditions under which these data were obtained we conclude that the reported free IAA level ranging from 10 to 25 ng/g dry weight in dark, grown corn seedlings is compartmentalized and partially protected from oxidation. While not tested, the suggestion by Trewavas (1981) that substantial quantities of IAA might accumulate temporarily on the surface of coleoptile tips, as a result of evaporative deposition from guttation fluid, offers a very attractive explanation for these IAA levels. In methods calling for freshly harvested seedlings to be homogenized in an organic extractive solvent, IAA so deposited could be dissolved first and stabilized with internal standard methods. On the other hand, the physiologically important IAA associated with receptors in the Golgi regions and the endoplasmic reticulum (Walton and Ray 1981) is very close to oxygenase-peroxidase enzymes found in the membrane folds of the endoplasmic reticulum (Waldrum and Davies 1981) and is therefore subject to uncontrolled degradation upon destruction of cell integrity. Results of *in vitro* work reported here and by others (Nakajima and Yamazaki 1979) clearly show that neither organic solvents nor low temperature nor the subsequent addition of antioxidants can stop or adequately decelerate IAA degradation once the enzyme system, in the presence of oxygen, has come in contact with the auxin.

IAA from a High Contaminant Tissue—Apple

The vacuum method tested well on corn epicotyls, a plant material which presents fewer problems in processing because of the nature of its chemical constituents. Other plant materials, particularly those containing polyphenols, present more workup problems and opportunities for oxidation-reduction reactions which interfere. Auxin is important to the hormonal regulation of growth events during vegetative development of perennial plants. Of particular interest to horticulturists is the influence of auxin derived from the growing points of aerial portions of fruit trees on such growth characteristics as branching habit (Abbas 1978; Phillips 1975), dwarfing (Jindal et al. 1974; Jindal and Dalbro 1977), and distribution patterns of leaf-accumulated photosynthate (Robinson and Schwabe 1977; Goodwin 1978). Because of the paucity of reliable data on seasonal endogenous IAA concentrations in apple vegetative tissue and this tissue's high concentration of polyphenols, we chose apple as a test material. An investigation was begun, utilizing the newly developed methodology for free IAA determination in terminal buds and shoots of 'Golden Delicious' trees.

Plant material

Samples were collected at approximately monthly intervals during the 1983 season from six randomly selected 12-year-old standard 'Golden Delicious' trees. Sample collection was made from randomly chosen, upright-growing, first-year branches in the

outer tree canopy. Eligible branch tips still attached to the tree were immersed in LN_2 and depending on growth state either the terminal bud or the last two internodes along with the corresponding petioles (but not their leaves) were collected. Because time limitations prevented analysis of all samples as they were collected, samples were treated and stored as described earlier and assayed in the first half of 1984. In growth stage I, (fig. 8), the terminal buds were in the bud break to silvertip stage of development. At this stage (March 16, 1983) 12 buds per tree were harvested. The same number of buds and growing points were obtained on March 30, 1983 and April 29, 1983 for the purpose of isolation on a larger scale for mass spectral identification of IAA.

In growth stage II, (fig. 8), comprising the growth flush during April, May, and June, six growing points per tree were harvested. The same number of buds per tree were obtained in stage III, (fig. 8), July onward. By that time all of the collected samples had set terminal buds on current year growth. The study's experimental design called for determining the average concentration of free, nonconjugated IAA obtained from the pooled samples of each tree, with emphasis on unaltered hormone concentration as it existed at the instant of harvest. The variability of intra-tree changes, as a source of variance in IAA concentrations, was calculated. Each sample was subjected to at least three assays.

Procedure

Experimental techniques were carried out as illustrated in figure 10.

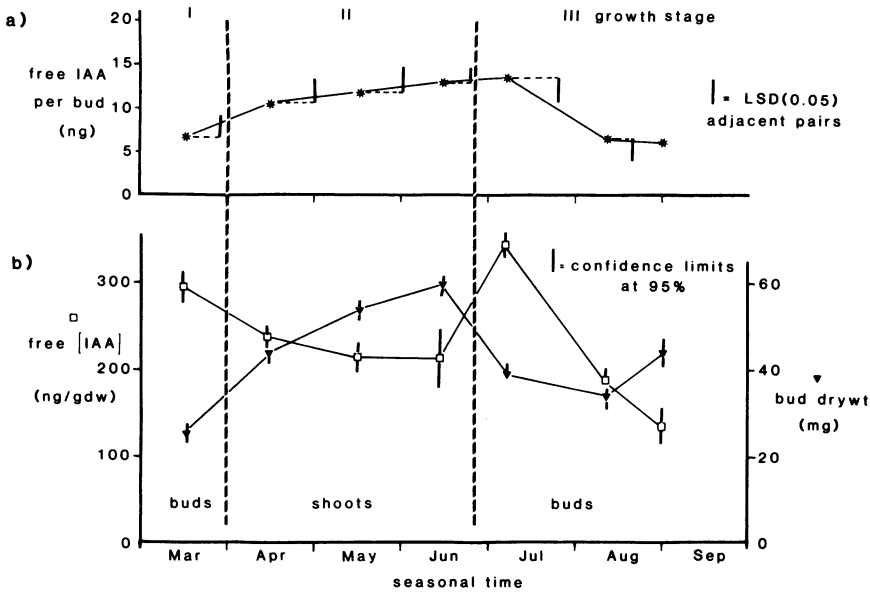


Fig. 8. Seasonal levels of free IAA in 'Golden Delicious' terminal buds and shoots

Mass spectra

The samples taken for MS were shown earlier to corroborate the procedure (fig. 5). The spectra are nearly identical and we assume the putative IAA in apple to be identical to IAA standard.

IAA concentration in apple

Although apple terminal buds and shoots contain more complex chemicals that need to be separated from the putative IAA than does corn epicotyl, the apple samples were processed with relative ease. The additional purification steps included for apple did not result in special problems and the anhydrous condition of the samples precluded enzymatic degradation of IAA. Each data point in figure 8*a* and *b* is the mean computed from the result of 18 assays. The data plotted in figure 8*b* show the confidence limit (at $p = 0.05$) for each point, and in figure 8*a* the least significant differences (at 95 percent) to neighboring points at indicated sampling dates are listed. Expressing IAA concentration in conventional fashion in units of mass of IAA per dry mass of host organ, the data (fig. 8*b)* suggest a decrease from about 300 ng/g dry weight at stage I (end of rest, beginning of bud break) to about 220 ng/g dry weight during the period of flush growth (stage II) with very little change throughout this important, expansive growth stage. This is followed by a sharp rise in IAA concentration, accompanied by formation of new terminal buds and subsequent decline to levels below that of the stratified terminal bud at the beginning of the season. The variation in concentration of IAA with time appears to be inversely proportional to bud weight, when the seasonal dry weights for each corresponding growing point are superimposed (fig. 8*b*). These data show a steady increase over time as dry matter is accumulated by photosynthesis, then a sharp decline upon entering summer dormancy, followed by a slight increase in late August to September. This slight increase may be explained by sink-loading, inasmuch as the newly formed terminal bud acts much like a fruit sink, accumulating stored carbohydrates instead of developing new organs.

Discussion

Combining the dry mass data with their associated IAA levels, one can calculate the endogenous-free IAA content per organ, bud, or internode-petiole and arrive at a different relationship. Calculated in this manner the total quantity of IAA is now seen to increase, rather than to decrease as is the case when data are expressed in mass concentrations; the reason for this reversal lies in the observed increase in dry matter (fig. 8*a*). A statistically significant difference in IAA content per bud occurs between stage I and stage II, and throughout the growth flush period there is a trend toward increasing IAA levels (fig. 8*a*). Since in apple only the last two internodes per petioles exhibit significant elongation, the third and higher (older) internodes being almost inactive, these data could possibly be correlated with the observed development of the new current-year branch, but only for stage I and stage II.

There is an additional distinct difference between data in figure 8*a* and 8*b*. Total IAA quantities in vegetative organs do not decline sharply upon entering summer dormancy, as suggested by mass concentration data, but maintain their level during

the first third of July and are statistically not different from the total quantity of IAA in growing tissue in mid-June. There is, however, a highly significant drop in IAA content of buds after terminals have set, suggesting that IAA amount is not causally related, at least by itself, in the induction of dormancy. If there were sufficient IAA for self enlargement and elongation of internodes during the growth flush, the presence of inhibitory compounds or a decrease in sensitivity to IAA would be necessary to explain the cessation of growth in early July. The level of IAA in dormant buds is unchanged from that in stratified buds after rest; that is, the amount if IAA is apparently not involved in the shifting hormone balances that are thought to be an important ingredient for breaking winter dormancy (Wareing and Saunders 1971). The total molar IAA concentrations during the growth flush can be estimated, assuming a 30 percent dry matter in elongating tissue, to be approximately 0.5 μM, a magnitude in reasonable agreement with the exogenously applied concentration of IAA which successfully stimulates rapid growth in other plant tissue (Penny and Penny 1978).

EXTRACTION-PURIFICATION SYSTEMS

This investigation has led to development of two systems for IAA extraction and purification. These systems reveal procedural choices based on perceived contaminants in plant material. Some plant materials (seeds, etiolated leaves, flowers, fruit parts, and vascular exudates) are easy to process as they contain fewer problematic chemical constituents. Other samples are more difficult to process as they contain many problematic chemicals such as polyphenols (vegetative and floral buds, bark, wood, and leaves). Either of the systems presented may require specific adjustments in solvents and chromatography for the plant part processed and for laboratory convenience. The system developed for etiolated, dark, grown corn epicotyls is less complicated as these plant parts contain fewer problematic chemicals (fig. 9). The system for apple tissue is more complex as it contains large quantities of polyphenols and oxidative reduction reactants (fig. 10).

1. Fresh sample into LN_2, 100-ml flask.
2. Vacuum at about 10^{-5} torr O_2; immerse flask in ethylene glycol maintained at $-5°C$; dry to $<0.02\%$ H_2O (brittle epicotyls).
3. Record dry wt; epicotyl to 30-ml Corex centrifuge tube; add 10 ml solvent (90% EtOAc, 10% CH_3CN); add 2-^{14}C-IAA internal standard; homogenize to fine particles.
4. Centrifuge 30′ at 10,000 RPM; re-extract pellet and centrifuge 2×.
5. Rotary Film Evaporation (RFE) in small vial.
6. Add 1 ml 20% methanol in 0.1 M acetic acid.
7. HPLC on ODS-18, 30′ linear gradient 20% to 100% methanol in 0.1 M acetic acid, ml/min, collect IAA R_t.
8. Partition against EtOAc, RFE EtOAc fraction.
9. Methylate

aliquots for counting,	NP-GC,	and GC-MS

Fig. 9. Outline for extracting IAA from corn coleoptile samples with low contaminant content

Low-Contaminant Presence

Dark, grown corn coleoptiles 3 to 8 days old are harvested in subdued light with immediate transfer to LN_2 in a 100 ml round-bottom flask. The flask is immediately attached to a preconditioned vacuum line at about 10^{-5} torr O_2. The round-bottom flask is immersed in antifreeze and kept at $-5°C$ while the sample is lyophilized to less than 0.02 percent of dry weight. Maintenance of the round-bottom flask at $-5°C$ is critical; warming of the plant material, particularly until O_2 is evacuated to about 10^{-5} torr, results in a return to the liquid state where oxidation of IAA will take place. Properly dried material will retain its color and will be brittle.

The sample's dry weight is recorded and an appropriate amount taken for IAA determination: 200 mg is sufficient for corn epicotyls. The sample is placed in a 30-ml Corex centrifuge tube to which 10 ml solvent (90 percent ethyl acetate [EtOAc] and 10 percent acetonitrile [CH_3CN], both degassed), BHT, and 2-^{14}C-IAA are added. The sample is homogenized (e.g., Polytron for about 1 minute), centrifuged for 30 minutes at 10,000 RPM at 0°C, and the pellet is re-extracted twice in similar fashion. The extracts are combined and taken to dryness in a small vial on a rotary film evaporator (RFE), solubilized with 1 ml 20 percent methanol (MeOH) in 0.1 M acetic acid, and injected into an ODS-18 semiprep column (10 × 150 mm) with a 30-minute linear gradient elution from 20 percent MeOH in 0.1 M acetic acid to 100 percent MeOH at 2 ml per minute. The eluate is collected at the approximate R_f for IAA, partitioned against EtOAc, and the EtOAc fraction dried on a RFE and methylated. The methylation is conveniently accomplished by adding 1 ml benzene to the sample, followed by 2 ml BCl_3/10 percent MeOH, and heated for 1 hour at 40°C. The sample is cooled, then partitioned against 1 ml water. The water phase is discarded, the benzene phase dried on an RFE, and the sample resuspended in the desired volume of MeOH.

1. Fresh sample into LN_2, 100-ml flask.
2. Vacuum at about 10^{-5} torr O_2; immerse flask in ethylene glycol maintained at $-5°C$; dry to $<0.02\%$ H_2O.
3. Record dry wt; transfer to Corex centrifuge tube; add solvent (90% EtOAc, 10% CH_3CN); add 2-^{14}C-IAA internal standard; homogenize.
4. Centrifuge 30′ at 10,000 RPM; re-extract pellet and centrifuge 2×.
5. RFE in small vial.
6. Add EtOAc and PVP; mix 1 hr filter PVP; rinse with EtOAc; discard both rinse and filtrate.
7. Elute IAA from PVP with small portions of methanol; collect eluate; RFE.
8. Apply on polyamide-6 layers and TLC (twice in chloroform, followed if necessary by EtOAc, isopropanol, ammonia) (45:40:23 v/v/v/).
9. HPLC of IAA fraction on reverse phase C-18 Bondapak; isocratic with 20% CH_3CN in aqueous 0.05 M $NH_4H_2PO_4$.
10. Collect at IAA R_t and HPLC on reverse phase CN Bondapak; isocratic with mobile phase in step 9.
11. Collect at IAA R_t; RFE; methylate.
12. Aliquots for counting, NP-GC, and GC-MS.

Fig. 10. Outline for extracting IAA from apple tissue samples with high contaminant content

Once the purified, methylated sample is prepared, several reliable methods for determining the IAA content can be employed, including NP-GC, gas chromatography-mass spectrometry (GC-MS), UV spectrophotometry, infrared spectrophotometry, and nuclear magnetic resonance. Whichever method is used, an additional aliquot is assayed by scintillation counting to account for workup losses.

High-Contaminant Presence

The fresh sample of apple tree tissue is harvested for immediate immersion in LN_2 and processed as given in figure 9 through step 5. Hereafter, additional purification steps are added that are necessary for satisfactory separation of IAA from interfering substances in apple tissue. The sample is solubilized in EtOAc and mixed with PVP for 1 hour. PVP is filtered from the sample, rinsed with EtOAc, and both the filtrate and rinse discarded. IAA is removed from PVP with methanol and the extract is further purified through a combination of TLC and HPLC steps to obtain a sufficiently pure final extract for assay by several available methods previously mentioned.

CONCLUSION

The general IAA processing systems presented (figs. 9, 10) are easily used and will allow later measurement of free IAA with confidence. Our investigations support the findings of others (Link, Eggers, and Moulton 1941; Galston and Hillman 1961; Hinman and Lang 1965; Nakajima and Yamazaki 1979; Ricard and Job 1974) that presence of O_2 during sample workup can lead to IAA destruction. Using vacuum drying at about 10^{-5} torr O_2 of frozen material precludes IAA destruction as peroxidases and oxidases are unable to function. While useful, adding chemicals to discourage oxidation is not sufficiently effective to prevent oxidation of IAA. A crucial feature of our procedure involves maintenance of the frozen condition during lyophilization, a factor made evident by Link, Eggers, and Moulton (1941) who showed that when frozen material thaws IAA oxidation can occur. Archbold and Dennis (1984) have shown IAA degradation during lyophilization. Their system used bottom heat to increase the vapor-pressure gradient (Archbold, personal communication); this type of IAA degradation, induced by thawing, was reported previously by Link, Eggers, and Moulton (1941). Thus, warming of frozen plant material provides a favorable environment for IAA destruction. Maintaining the drying flask at $-5°C$ decreases the vapor-pressure gradient and lengthens drying time.

Employment of an internal standard is an absolute requirement in any quantifying of IAA. Because the desired effects of internal standardization do not take place instantaneously, it is essential that *in situ* IAA and exogenous marker IAA be protected from oxidative catabolism by excluding oxygen or the creation of an anhydrous nonreactive environment until the exogenous marker becomes homogeneously mixed with the pool of endogenous IAA to be determined.

Since hydrolytic enzymatic activity requires the presence of water, extractive quantification of free IAA under anhydrous conditions will also prevent release of IAA

from conjugated forms during workup. Conjugated IAA is reported to constitute a major portion of total IAA (Bandurski and Schulze 1977) and its uncontrolled hydrolysis could significantly confound the determination of free IAA.

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