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THE BIOSYNTHESIS OF

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THE BIOSYNTHESIS OF INDOLE-3-ACETIC ACID IN *ZEA MAYS*

BY

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A DISSERTATION

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ABSTRACT

THE METABOLISM OF INDOLE-3-ACETIC ACID IN *ZEA MAYS*

BY

Philip J. Jensen

The onset of *de novo* synthesis of indole-3-acetic (IAA) and tryptophan in seedlings of *Zea mays* was examined using deuterium labeled water as a general precursor. IAA and tryptophan synthesized via the shikimic acid pathway would contain deuterium in one or more positions. We have demonstrated, by GC-MS-SIM, that 7 day old, dark grown seedlings grown on 30% D_2O incorporate deuterium into tryptophan, but not into IAA. Protium and deuterium NMR spectra indicate at least the six position in the ring structure of tryptophan is labelled.

Kernels of *Zea mays* accumulate large amounts of IAA during development. A single endosperm may contain as much as 0.5 μmol of esterified IAA. The origin of the IAA has never been demonstrated. To answer that question GC-MS assays of free IAA and ester IAA were made throughout the course of development. Ester IAA appears at day 10 and at day 45 reaches a maximum value of 0.5 μmol kernel^{-1}. Free IAA never accumulates so we conclude that either IAA

biosynthesis in the kernel or transport of IAA esters, and not esterification, is the rate limiting step.

Zea mays endosperm cells can grow *in vitro* on media containing no growth hormones. This suggests the endosperm is the site of IAA biosynthesis in the developing kernel. A time course study using endosperm cells in suspension culture has provide clear evidence that these cells are indeed synthesizing IAA. There was an increase of nearly 10 μg of total IAA per 20 gm fwt. in 6 days. This system will be ideal for studying the biosynthesis of IAA and its regulation.

The endosperm of *Zea mays* most likely contains all the enzymes required to biosynthesize IAA *de novo*. To determine the feasibility of this system for examining the ability of putative IAA precursors to sustain IAA synthesis we have examined the conversion of ^{14}C-anthranilic acid to IAA in isolated endosperm. Anthranilic acid was used because it is common to all proposed pathways yet occurs early enough so as not to presuppose one pathway over another. Data relating to the rate of IAA synthesis from anthranilic acid and proof as to the identity of the IAA will be presented.

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ABBREVIATIONS

^{14}C	carbon 14
DAP	days after pollination
^{2}H	deuterium
DEAE	diethylaminoethyl
GC-MS	gas chromatography-mass spectrometry
GC-MS-SIM	gas chromatography-mass spectrometry-selected ion monitoring
HPLC	high pressure liquid chromatography
IAld	indole-3-acetaldehyde
IAA	indole-3-acetic acid
IPyA	indole-3-pyruvic acid
m/z	mass to charge ratio
MHz	mega Hertz
^{15}N	nitrogen 15
NMR	nuclear magnetic resonance
2,4-D	2,4-dichlorophenoxy acetic acid
^{3}H	tritium
TNH_2	tryptamine
TRP	tryptophan

HORMONES AND GERMINATION

Webster's New World Dictionary defines germinate as: 1) to sprout or cause to sprout, as from a spore, seed or bud, 2) to start developing or growing. Among plant scientist there is a debate over the definition of germination which revolves around the question of when germination ends. All would agree that as soon as the radicle has emerged the seed can be considered germinated; but the endpoint of germination is unclear.

In general, germination has been divided into two phases (Berrie and Drennan, 1971, Sen and Osborne, 1974). Phase I includes those events leading up to the emergence of the radicle. Desiccation during this stage does not result in the death of the seed. Phase II includes the subsequent events relating to seed reserve dependent seedling growth. Desiccation during this phase results in death. Based on these phases, germination ends when the seedling becomes autotrophic. The majority of research done on hormone relations in germinating seeds involves only phase I. There is a conspicuous lack of data during phase II of germination.

In contrast, the growth of cereal seedlings has been divided into three stages (Derwyn *et al.*, 1966) Stage I consists of the heterotrophic period beginning with the imbibition of water and ending with the commencement of photosynthesis. Stage II is the transition period when hydrolysis of reserves and photosynthesis occur simultaneously. Stage

III is the autotrophic period when the seedling is totally reliant on photosynthesis for its nutrition.

Function and origin of hormones during germination

During the stages of germination, extensive elongation occurs. This elongation must be under hormonal control. Seeds are very rich sources of plant growth substances (Bandurski and Schulze, 1977). The role of these compounds in germination is not well understood. Most of the research done in this area has concentrated on the release of seeds from dormancy and phase I of germination. The best understood hormonal aspect of germination is the promotion of α-amylase production in the aleurone cells of cereals in response to gibberellins (GA) released by the embryo. Additional research has shown exogenously applied GAs can promote germination in dormant seeds. Though it is known that GA breaks dormancy, the exact mechanism is still uncertain. There is only a limited amount of evidence however showing that GAs are synthesized during exposure to dormancy breaking treatments (Jones and Stoddart, 1977). During germination, in contrast, the *de novo* biosynthesis of GAs during germination has clearly been demonstrated (Jones and Stoddart, 1977).

Abscisic acid (ABA) is widely believed to be responsible for seed dormancy. ABA appears to act by suppressing the production of specific enzymes while inducing the pro-

duction of others (Ho *et al*. 1989). In cereals, ABA is involved in the prevention of precocious germination but is not involved in maintaining dormancy (for review see Zeevart and Creelman, 1988). During germination the only putative role for ABA is acting as an antagonist of GA promoted events during germination (Walton, 1977, Chrispeels and Varner, 1967b, Khan and Downing, 1968).

Cytokinins are best known for their role in differentiation of callus tissue in culture. They have also been show to function during germination. Cytokinins can affect the permeability of membranes, thus regulating the release of GAs from the germinating cereal embryo (Thomas, *et al*., 1975). Cytokinins also play a role in the transcription and translation of DNA but a mechanism for this has not been elucidated. Stimulation of ethylene production by cytokinins has been proposed as a factor in bringing about germination in dormant peanuts (Ketring and Morgan, 1971).

Ethylene can break dormancy in many seeds including clover, cockleburr, lettuce and celery (Ketring, 1977). In the case of the peanut, the ethylene involved in dormancy breaking is believed to be synthesized in the embryo (Ketring and Morgan, 1969) During growth of etiolated peas ethylene appears to act as an inhibitor of elongation in response to an increase in auxin levels. Auxin itself is not an inhibitor of elongation in peas (Burg and Burg, 1966). In etiolated corn and oat coleoptiles, ethylene production

does not continuously increase with increasing auxin concentrations as in etiolated peas. Ethylene formation and growth reached maximum rates around 10^{-5} M IAA and declined as more auxin was added (Burg and Burg, 1966).

Luckwill (1952) was the first to suggest that dormancy is regulated by a balance of endogenous inhibitors and promoters. Cytokinin application can overcome dormancy induced by exogenous abscisic acid (ABA) (Aspinal *et al.*, 1967, Khan, 1967). In contrast, GA cannot overcome this induced dormancy (Khan and Waters, 1969). Recently, Karssen and Lacka (1985), working with GA and ABA mutants of *Arabidopsis thaliana*, concluded the two hormones never act simultaneously in the seed. ABA induces dormancy during seed development and GA stimulates germination. This is in contrast to the conclusions of Walton (1977) but may represent a real difference between *Arabidopsis* and cereals.

Role of auxins in dormancy and germination

Seeds contain abundant stores of IAA (Table 1). Even though it is present in large amounts, it is generally believed that IAA plays an insignificant role in the breaking of dormancy. To date only Nikolaeva (1970), working with *Acer*, and Sircar (1967), working with rice, have presented data suggestive of a role for IAA in the physiological events leading to the induction of germination. Both investigators theorized that high IAA

Table 1. Amounts of free and conjugated IAA in variuos seeds and other plant tissues (from Bandurski and Schulze, 1977).

Species	Tissue	IAA Content		
		Free IAA[a] $\mu g/kg^{-1}$	Ester-IAA[b] $\mu g/kg^{-1}$	Amide-IAA[c] $\mu g/kg^{-1}$
Cereals				
Avena sativa	Vegetative tissue	16	5	69
Avena sativa	Seed	440	7,620	n.d.[f]
Hordeum vulgare	Seed (milled)	40[e]	329	–[g]
Oryza sativa	Seed	1,703	2,739	–
Panicum miliaceum	Seed	633	3,198	–
Triticum aestivum	Seed	123	511	–
Zea mays	Vegetative tissue	24	328	60
Zea mays	Seed	500 to 1,000	71,600 to 78,500	–
Legumes				
Glycine max	Seed	4	50[e]	524
Phaseolus vulgaris	Seed	20[e]	30[e]	136
Pisum sativum	Vegetative tissue	35	5	43
Pisum sativum	Seed	93	n.d.	202
Others				
Cocos nucifera	Liquid endosperm	0	905	–
Fagopyrum esculentum	Seed	40	127	25
Helianthus annuus	Seed	30[f]	110[f]	–
Lycopersicum esculentum	Fruit	Trace	Trace	–
Saccharomyces cerevisea	Packed cells	290	n.d.	–

[a] No alkaline hydrolysis.
[b] IAA after hydrolysis with 1 N alkali minus the free IAA.
[c] IAA after hydrolysis with 7 N alkali minus the free and ester IAA.
[d] Seedlings and fruits are fresh weight, seeds are air dry and yeast cells contain 30% dry matter.
[e] A visual estimate of IAA was made on a TLC plate since colorimetry was precluded by contaminants.
[f] n.d. (not detectible). Where the ester content is high, small amounts of IAA escape detection.
[g] A dash (–) indicates the assay was not done.

levels inhibit germination and only after these levels have decreased can the seeds germinate. Once dormancy has been broken, the embryo can begin germination. In *Zea mays* the initial step of germination is cell elongation, which is then followed by cell division (for review see Berlyn, 1972). Since elongation occurs first it is reasonable to assume IAA is required during the initial stages of germination.

In addition to its well known and studied role in elongation, IAA has also been suggested as having some function in regulating α-amylase levels during germination . MacLeod and Palmer (1969) found that IAA can stimulate α-amylase formation when applied 12 hours prior to adding GA. No stimulation was found when the two hormones were applied simultaneously. Detipping of the coleoptile also leads to a reduction in α-amylase production. Working under the assumtion the coleoptile tip was the site of auxin production addition of IAA to detipped coleoptiles resulted in normal levels of α-amylase, confirming their hypothesis. Verbeek *et al.* (1973) found IAA could reverse the decrease of α-amylase activity induced by kinetin. They also found a correlation between α-amylase activity and coleoptile length as both reach their maximums at the same time.

Auxin dynamics during germination

Perhaps the best physiological study of IAA levels and the various events occurring during germination has been done by Sircar (1967) working with rice. Immediately after imbibition, free auxin levels increase in both scutellum and endosperm. Total auxin levels were not measured. Auxin in the endosperm adjacent to the scutellum disappears first. This disappearance is under the control of the embryo. When the embryo and epithelial layer were removed after sprouting, there was no further decrease in IAA in the endosperm. Free IAA levels increase for the first 72 hours in both the embryo and endosperm as does the availability of respirable materials, including amino acids. After 72 hours, there is a general decline in the levels of all these compounds. Asparagine is the predominant amino acid in the endosperm after 24 hours. By 48 hours the four amino acids, arginine, tyrosine, ornithine and tryptophan, which are all absent in the endosperm, arise by transamination in the embryo. They believe the increase of tryptophan between 48 and 96 hours to be due to synthesis from serine condensed with indole. The indole moiety was believed to have come from IAA which disappears during germination.

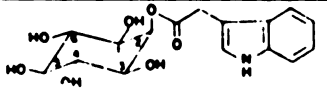
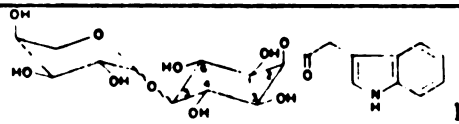
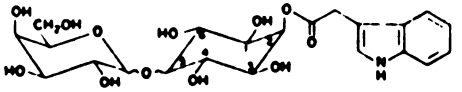
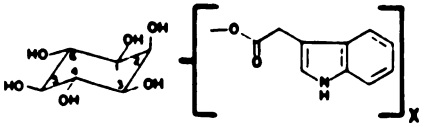
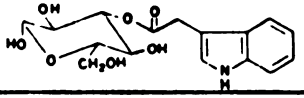
The most complete biochemical study of IAA levels during gemination has been done on *Zea mays*. Ueda and Bandurski (1969) determined not only the levels of free IAA during germination but also the levels of ester IAA, including 1-O

and 2-O indole-3-acetyl-*myo*-inositol, and their arabinosides. All of the various IAA esters declined at a relatively uniform rate during germination. The total amount of IAA in the whole seedling decreased nearly 90% in the first 96 hours of germination. Free IAA levels rose during the first 24 hours and then declined at a constant rate. During this study, the occurrence of five new IAA compounds was also shown. The breakdown of IAA compounds in *Zea mays* kernels is shown in Table 2.

Metabolism and transport of IAA during germination

Free IAA and its potential precursor tryptophan are not transported from the seed in sufficient quantities to satisfy the needs of the shoot (Hall and Bandurski, 1978, Nowacki and Bandurski, 1980). Indole-3-acetyl-*myo*-inositol (IAInos) is the auxin transported from the seed to the shoot in *Zea mays* (Nowacki and Bandurski, 1980, Komoszynski and Bandurski, 1986, Chisnell and Bandurski, 1988).
Also working with *Zea mays*, Whitehouse and Zalik (1967) concluded free IAA was not transported from seed to shoot. Additionally, they found that tryptophan is not actively transported through tissue segments. This agrees with the findings of Schrank and Murrie (1962) who found that tryptophan is not translocated to any great extent in oat coleoptiles. Jackson and McWha (1983) have found that like

Table 2. A summary of the structures and amounts of indole-3-acetic acid and its conjugates in *Zea mays* kernels.

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*-β-L-arabinopyranosyl-2-*O*-(indole-3-acetyl)-*myo*-inositol		11.7	17.6%	
5-*O*-β-L-arabinopyranosyl-1-DL-(indole-3-acetyl)-*myo*-inositol		3.7	5.6%	
5-*O*-β-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	β 1-4 cellulosic glucan with 7 to 50 glucose units per IAA		35.0	52.5%

in *Zea*, free IAA is not transported from seed to shoot in *Avena* seedlings.

In a study of 4 day old *Zea mays* seedlings, Epstein *et al.* (1980) found that IAA in the seed is rapidly turning over, most being metabolized to unidentified products. Free IAA appears in the endosperm through ester hydrolysis, not through synthesis from some precursor. The IAA-*myo*-inositols however turn over more rapidly than the rate at which free IAA appears. It is possible that free IAA is being released from an IAA-glucan and not IAA-*myo*-inositols (Piskornik and Bandurski, 1972). It was postulated that the IAA-*myo*-inositol glycosides play a role in glycosylation and/or in regulating the concentration of IAA-*myo*-inositol.

Pengelly and Bandurski (1983) studied the IAA metabolism in *Zea mays* seedlings using deuterium oxide as a tracer. Their data indicated that IAA was being made *de novo* in 4 day old light or dark grown seedlings. The *de novo* biosynthesis accounted for 25% and 9.6% of the IAA in the shoots and roots respectively. The remainder is from seed reserves. The IAA of the shoots was labeled at a greater rate suggestive of different biosynthetic pathways operating in shoots and roots. In beans, seed stores appears to play a minor role in supplying the developing seedlings with IAA (Bialek and Cohen, 1991) The IAA required for growth is apparently synthesized *de novo* as germination proceeds. This

may represent a fundeamental difference in IAA metabolism between corn and beans, and possibly monocots and dicots.

The origin of building blocks during germination

During germination the food reserves stored in the seed begin to break down. Hydrolysis of these reserves provide the seedling with the nutrients required for growth. The compounds released may be used directly to build new macromolecules, or may be further metabolized and serve as precursors for other compounds. There exists great variation among seeds in the mechanisms and timing of these events. These differences are especially evident when comparing monocots, dicots, and gymnosperms. In some dicot seeds the cotyledon is the major storage tissue, in others it is the endosperm. Additionally the major storage compound can be starch, protein or lipid. All of these factors affect the synthesis of new compounds.

The starch stored in the endosperm of cereals is broken down to hexoses which are transported into the scutellum where they are converted to sucrose (James, 1940) for transport throughout the seedling. The pentose phosphate pathway in particular is very active in metabolizing carbohydrates in germinating seeds. This pathway provides the embryo with NADPH (NADH is provided via glycolysis and the TCA cycle)(Roberts, 1969). The pentose phosphate pathway also

produces precursors of the aromatic amino acids and lignin (Higuchi and Shimada, 1967)

Hydrolysis of storage proteins results in the release of amino acids. The metabolic fates of these amino acids vary. Most are transported intact to the seedling (Oaks, 1965). Others, including glutamic, aspartic, their corresponding amides, and proline are extensively metabolized (Oaks, 1965). The amino acids in the fat storing seed of castor bean are extensively used in gluconeogenesis (Stewart and Beevers, 1967). The amino acid metabolism during germination in corn differs from that in oats and barley. When removed from their food source, embryos of oat and barely grow feebly (Harris, 1954) while those of corn appear normal (Dure, 1960). This may represent a difference in capacity for *de novo* synthesis of amino acids. Oaks and Beevers (1964) found that while the corn embryo is capable of synthesizing amino acids, the supply of organic nitrogen from the endosperm in some way curtails endogenous synthesis within the embryo. Excised embryos were able to synthesize glutamic and aspartic acids from glucose and inorganic nitrogen. Synthesis therefore appears to occur when seed supplies are not sufficient to meet the demands of the seedling. Thus the rate limiting sequence of reactions are those involved in the further conversion of these key amino acids. They also reported that tryptophan levels were low, while leucine and proline were present in large amounts suggestive that

zein is the principle donor of amino acids. This suggests the possibility that low reserves of tryptophan in corn may enhance its de novo synthesis at a comparatively young age with respect to the other amino acids. The biosynthesis of amino acids in 6 day old barley seedlings has been studied by Mitra *et al.* (1976) using deuterium oxide. All of the amino acids studied, including phenylalanine and tyrosine, became deuterated to some extent indicating their biosynthesis. This indicates that by six days of age the seed can no longer meet the amino acid demands of the seedling.

Nucleic acids and nucleotides stored in the endosperm and scutellum of the corn kernel are of minor importance as a reserve for the developing embryo (Ingle and Hageman, 1965). Synthesis of nucleotide material therefore most likely occurs in the developing embryo.

The transition from heterotrophy to autotropy

At some time the seedling must switch from a heterotrophic existence to an autotrophic one. The age at which this occurs varies not only with the type of plant studied but also with the compound being studied. Some compounds are synthesized *de novo* immediately at the beginning of germination, others are not synthesized *de novo* until the seed reserves have been depleted. The transition to autotrophy in *Zea mays* seedlings with respect to overall carbon metabolism has been determined by Deleens *et al.* (1983, 1984).

Using CO_2 depleted in ^{13}C they found seedlings to be totally dependant on seed carbon reserves for the first 7 days. After 7 days, carbon from photosynthesis begins to appear. The seed continues to supply carbon to the seedling for up to 4 weeks. The autotrophic carbon is preferentially used for respiration while carbon from the seed is used for building leaf matter. The age of transition was also shown by observing the enzyme parameters of phosphoenolpyruvate carboxylase which change depending on the carbon source.

Very little is currently known about the transition to autotrophy with respect to tryptophan, IAA, or the other plant hormones. This is undoubtably one of the most important events during a plant's life. Failure to establish a self sufficient nutrition will ultimately result in death. Despite the importance of this transition little is known about the timing, signals, etc. that bring about this change. Altering IAA metabolism during this critical period may prove to be an effective means of regulating the subsequent growth of the plant. This could be accomplished by manipulating conjugate hydrolysis, transport and/or *de novo* synthesis. Before this can become of practical value however, more research on IAA dynamics in germinating seeds is needed.

DEUTERIUM EFFECTS IN BIOLOGICAL SYSTEMS

Deuterium is an isotope of hydrogen containing two neutrons. The existence of deuterium oxide (heavy water) was first shown by Urey in, 1932. After an initial burst of experiments upon its discovery, the number of experiments using deuterium in biological studies decreased. A decrease in price made possible by new separation technologies led to a reawakening of the field which proliferated in the, 1960's.

Besides its larger size there are several other important physical characteristics of deuterium oxide with respect to biological systems. The viscosity of D_2O is 31% and 18% greater than that of H_2O at 5^0 and 38^0C respectively. The rate of diffusion is therefore slower in D_2O than in H_2O. The ionization constant is approximately one-fifth that of H_2O, altering the pH of systems in D_2O. Long and Bigeilsen (1959) have shown that the rates of acid-base catalyzed reactions may be quite different in H_2O as compared to D_2O.

At the biochemical level, deuterium exhibits primary isotope, secondary isotope and solvent effects. Primary isotope effects are those due to the difference in reactivity between deuterium and hydrogen bonds to carbon, nitrogen, oxygen, etc. Bonds to deuterium are more stable and thus require a greater activation energy to break them. In partially deuterated systems, deuterium bonded to nitrogen

and oxygen will rapidly exchange with the hydrogen of water. Deuterium bound to carbon does not exchange freely. These deuterium however can be exchanged as a result of keto-enol tautomerism. Secondary isotope effects are those which occur due to deuterium located in positions other than the reaction site. Solvent effects are those due to the physical differences between D_2O and H_2O. In actuality it can be very difficult to distinguish one effect from the others in biochemical reactions.

The strength of intermolecular hydrogen and hydrophobic bonds is greater in D_2O than H_2O (Kresheck *et al.*, 1965). Proteins dissolved in D_2O exhibit a more stable helical structure as a result of altered H-bonding (Tomita *et al.*, 1962.) In contrast non-exchangeable deuterium in amino acid side chains decreases protein stability (Hattori *et al.*, 1964). The alteration of the equilibrium between random coil and ordered helix forms of DNA has been suggested as a primary effect of deuterium oxide (Henderson and Dinning, 1962).

A major problem in the use of labeled precursors for metabolic studies is compartmentalization. Applied precursors may fail to enter the pool of interest. This problem can be avoided by using a compound, such as deuterium oxide, that distributes itself relatively uniformly throughout the organism. Plants do have some selective capabilities for the uptake of H_2O vs D_2O. *Lemna* grown on partially deuter-

ated medium was able to keep the deuterium content inside the plant 10 to 15% less than that of the media over a two year period (Cope *et al.*, 1965).

The effects of deuterium on growth is dependent to a large extent on enzymatic events. Although no single reaction is significantly altered at low D_2O concentrations, the sum of all reactions probably results in a metabolic imbalance capable of producing severe functional disturbances. Metabolism may also be greatly effected as a result of decreased penetrability of substrates to specific pools. In the presence of 95-100% D_2O the overall reaction rate of succinic dehydrogenase was inhibited by about 35%. In 15% or 30% D_2O the inhibition was only 5% (Thomson and Klipfel, 1960). Deuterated compounds are not metabolized as rapidly as their non-deuterated counterparts (Erlenmyer *et al.*, 1936, Frei and Aebi, 1958) Based on all of the research done on deuterated enzyme systems it appears evident that (1) deuterated substrates and coenzymes are more tightly bound than are the corresponding normal compounds and (2) the presence of deuterated reactant does not influence the binding of non-deuterated reactant (Thomson, 1963)

The effects of deuterium oxide on plants appears to be that of a nonspecific chaotropic agent (Table 3). Deuterium oxide effects every level of plant organization. Crane *et al.* (1964) and Blake *et al.* (1964) have studied the effects

Table 3. Deuterium effects reported in plants.

Table 3. Growth responses to Deuterium oxide

Author	year	species	Results
Bhattacharya *et al.*	1969	various seeds	Decrease in respiration rate, higher RQ initially, lower later.
Uphaus *et al.*	1975	*Nicotiana tobaccum* whole plants	Marked reduction in size, flowering, and extensive necrosis at high D_2O levels. Heightened transpiration rates, and a decrease in alkaloid production and fractionation of carbon isotopes during photosynthesis.
Crane *et al.*	1967	*Atropa belladonna*	In 70% D_2O growth was abnormal and unpredictable, in 50% and 60% D_2O abundant floweres were produced, most were small and did not become fertilized. Many abnormal flower parts and berries were seen and seeds were small and infrequent.
Uphaus *et al.*	1965	*Atropa belladonna*	Plants in 30% D_2O appear nearly normal except for a slight inhibition of elongation. In 50% D_2O shoot length was 60% of the controls. In 60% D2O plants were very poorly developed.
Stein and Forrester	1964	*Zea mays*	Seedling roots stop growth when placed in 80% D_2O. The pericycle recovers slower than the apical meristem. Swellings appear above the root meristem. Cell enlargement and mitosis are also inhibited.
Crumley and Meyer	1950	Clover, Radish Tobacco, and Kentucky bluegrass	All species could germinate in 99.9% D_2O however the germination time was increased. The delay increased with increasing D_2O concentration.

of D_2O on *Mentha piperita*. Cuttings were grown in nutrient solutions containing up to 100% D_2O. At the morphological level they found that above 20% D_2O there was a proportional reduction in growth with increasing concentrations. The roots and shoots appeared normal up to 50% D_2O except for a reduction in length. No inflorescence primordia were seen at any D_2O concentration. Histologically the volume of the cells was increased resulting in greatly thickened leaves. The number of vascular elements was decreased and the amount of collenchyma increased in order to provide greater support. Cellulose production was normal, however, the amount of calcium-pectate produced was greatly reduced. All of the effects were more pronounced in differentiating tissue. Partially deuterated atropine was produced in *Atropa belladonna* grown in 30% and 50% D_2O but in smaller amounts than the controls (Uphaus *et al.*, 1963).

There appears to be a high degree of selectivity in sensitivity of seeds to D_2O. Large seeds germinate more readily than small ones, likely as a result of the greater hydrogen content of the food reserves (Blake *et al.*, 1968). This inhibition may be due to a repression of hydration in D_2O impairing GA-independent α-amylase synthesis resulting in altered germination as seen in barley (Bhandarkar, 1970). Among all of the seeds tested for germination and grown in D_2O, rye has shown the least deleterious effects (Siegel *et al.*, 1964). Stein *et al.* (1963) working with *Pisium* and *Zea*

found that submersion of roots in 80% D_2O led to an eventual reduction in the growth rate to zero. The time required to reach the zero rate, as well as the recovery time in H_2O, varied with the plant type. Monocots appeared to be more sensitive than dicots and differed in their response to D_2O.

High levels of D_2O have been shown to increase the permeability of beet roots (Waber, 1984). This effect was theorized to be due to an alteration in metabolic processes required for membrane maintenance and not to direct action by D_2O. This was based on the fact that the use of specific protein synthesis inhibitors resulted in a similar response.

The rate of protein turnover in *Lemna* fronds was increased 50% in D_2O due to an inhibition of protein synthesis and an increase in protein degradation (Cooke *et al.*, 1979). The increase in protein degradation was found to be the result of an influx of vacuolar proteolytic enzymes due to altered tonoplast properties.

The use of D_2O in studying metabolism offers several advantages over other labels. (1) D_2O evenly distributes itself throughout the cell and organism. (2) D_2O is a more efficient labeller of compounds. (3) D_2O can easily be fed to plants *in vivo* and does not require any manipulations which might result in artefacts. The use of low D_2O concentrations does result in a alteration of metabolism. This alteration however is of a non-specific nature resulting only in a lag of growth. Therefore, D_2O would be an ideal label

to use for determining if a system makes IAA. The rates of incorporation can be used to determine the relative importance of *de novo* biosynthesis versus conjugate hydrolysis during seedling development.

INDOLE-3-ACETIC ACID BIOSYNTHESIS IN DEVELOPING SEEDS

The site of auxin production in the developing cereal seedling was originally thought to be the coleoptile (for review see Went and Thimann, 1937). This conclusion was based on the fact that auxin would diffuse out of excised coleoptile tips into agar blocks, but not out of the stump (Skoog, 1937). Additionally, removal of the tip stopped growth but growth would resume after regeneration of a "physiological tip" (Dolk, 1930). Today we believe the shoot is the site of hydrolysis of esters transported from the seed (Sheldrake, 1973, Cohen and Bandurski, 1982). Seventy years ago, however, there was no knowledge of IAA conjugates or even auxin "precursors" and the coleoptile tip was widely accepted as the site of auxin biosynthesis.

The presence of growth hormone in *Avena* endosperm was originally shown by Cholodny (1935b), Laibach and Meyer (1935), and Pohl (1935). Cholodny obtained growth curvature by applying blocks of endosperm moistened with water to the side of the coleoptile. When moistened with alcohol there was no curvature, most likely the result of enzyme inactivation. Pohl depleted seeds of hormone and observed a decrease in growth which led him to conclude that growth hormone is not produced in the coleoptile but is transported up from the seed and activated by the tip. Went and Thimann believed this hypothesis was incorrect. They believed that some precursor, not growth hormone itself, is transported

from the seed to the coleoptile where it is converted to auxin. Our current level of knowledge shows that both Pohl and Went and Thimann were correct in some respects. Growth hormones are transported to the shoot from the seed as Pohl concluded, but in an inactive conjugated form (Nowacki and Bandurski, 1980, Komoszynski and Bandurski, 1986, and Chisnell and Bandurski, 1988). The apparently logical conclusion by Went and Thimann that the transported compound was a precursor has lead to some confusion. The "precursor" which we now know to be IAA conjugates are synthesized FROM indole-3-acetic acid, not the other way around.

In the course of conducting a morphological study on the development of the rye seed Nutman (1939) made several conclusions about the production of growth hormones. Correlations were found between antipodal and nucellar pillar degeneration and growth of the aluerone and embryo sac. The embryo stops growth when the antipodals have disintegrated, and resume growth when the aleurone layer is initiated (concomitantly with nucellar pillar disintegration). Based on these observations Nutman concluded that growth substance production in the seed is associated with the degeneration of certain tissues. This hypothesis was later supported by Luckwill (1948) who believed IAA was produced via TRP released from proteins upon autolysis. Several other researchers have proposed the seed/ovary as the site of hormone production. Muir (1942) working with tobacco found

more growth substance in the ovary than can be explained by the contribution from the pollen and pollen tube. This was contrary to the views of Gustafson (1939) who felt growth substances from the pollen were responsible for ovary development. Based on his results, Muir concluded, "growth substances found in the style and ovary are not transferred from the pollen but are produced *in situ* as the result of enzymatic hydrolysis of proteins." In addition it was hypothesized that growth hormones move out of the ovary and play a role in the development of the conducting elements in the pedicel and other portions of the ovary/inflorescence. In support of this was the work by Soding (1926) who found that removal of the flower buds greatly decreases growth of the flower stalks. Soding (1926) and Uyldert (1928) suggested that the growth acceleration is due to growth promoting hormones produced within the flower bud and young fruit. This suggestion was latter reaffirmed by Soding (1932), Snow (1932) and Kostytschew (1931).

Laibach and Meyer (1935) found hormone levels highest during flowering, fruit setting, and germination and lowest during vegetative growth and flower formation of *Helianthius*. Fertilization was shown to be required to obtain a large increase in auxin in the egg and young fruit by Soding (1936), Dollfus (1936) and Judkins (1941). Gustafson (1939) found that while auxin content increased rapidly in seeded fruit, it decreased in seedless fruit. Katunsky (1936a,b) working

with *Papaver* and *Crepsis* found auxin maximas associated with the development of the nucellus and rapid cell division after synapsis. It was concluded that developing ovules were the centers of hormone production. In doing now classical strawberry experiments, Nitsch (1950) concluded that the achenes supply the developing receptacle with auxin. It was well known by this time that auxins were capable of inducing parthenocarpy. Britten (1950) observed that parthenocarpic kernels most often occurred adjacent to fertilized ones. To explain the development of parthenocarpic maize kernels it was concluded that large amounts of stimulating agents were produced by the fertilized kernels and diffusion of these to adjacent unfertilized embryos induced parthenocarpy.

In coniferous seeds it is not known if hormone are produced *in situ* or translocated into the seed as it develops (Sandberg and Ernsten, 1987). For angiosperms it seems that the old work and theories proposing the seed as a site of auxin production have largely been either forgotten, ignored, or both. There has been virtually no auxin biosynthetic research conducted with reproductive tissues. Based on the high concentrations of hormones in seeds (relatively speaking), developing seeds or seed tissues should prove to be a valuable system for studying IAA biosynthesis.

AUXIN DYNAMICS DURING SEED DEVELOPMENT

The levels of IAA during seed development have been determined *in Zea mays* (Avery and *et al.*, 1942, Teas and Newton, 1951, Jensen and Bandurski, 1991), rye (Hatcher and Gregory, 1941 Hatcher, 1943 Hatcher, 1945), wheat (Wheeler, 1972), apple (Luckwill, 1948, 1953), strawberry (Nitsch, 1954), peach (Miller *et al.*, 1987) and bean (Bialek and Cohen, 1989). All seeds examined so far, except for beans, generally have an auxin peak at some developmental stage followed by a rapid decrease as the seed matures. Hatcher and Gregory (1941, 1943, 1945) working with rye and using bioassays were the first to study, and have perhaps given the best account of, the physiology of IAA accumulation in developing seeds . Their first paper showed that no hormone was produced until 14 days after anthesis at which time it underwent a rapid and large increase. Most of the hormone was found in the endosperm. After reaching a maximum, hormone levels rapidly decreased, concurrent with the desiccation of the seed, to a concentration approximately 12% that of the maximum. Avery *et al.* (1941) discovered that large amounts of IAA could be formed upon alkaline hydrolysis of water extracts of *Zea mays* kernels from what they called an auxin "precursor". Using alkaline hydrolysis they conducted a comparative study on the auxin content in developing *Zea mays* kernels of varying heterogenic vigor (1942). While the total auxin content of the dry seeds from the different lines did not vary signifi-

cantly the maximum attained during development varied by as much as 50%. They found no absolute relationship between the amount of precursor and free auxin, but free auxin never amounted to more than 12% of the total. Prematurely harvested kernels lost less auxin upon drying compared to those allowed to dry on the plant. They found that 50% to 70% of the decrease in auxin concentration during maturation could not be explained by destruction during drying. This raises the possibility of some physiological reason for the loss, which to this day remains unknown. They proposed the "precursor" might be a simple ester. However, both methyl and ethyl esters of IAA give a growth response in bioassays while the precursor substance did not. Another possibility was that of an acid amide, possibly of IAA.

Armed with the then new technique of alkaline hydrolysis Hatcher completed a new study of auxins in rye and found 20 times more auxin than found with plain water extraction (1943, 1945). Unlike the findings of Avery *et al*. (1942) for maize, in rye no precursor substance was formed until after the auxin concentration had reached its maximum. The levels of the precursor continued to increase even in detached seeds suggestiong it is synthesized in the seed. The ratio of the alkali to water extracted auxin remained relatively constant. As the water soluble auxin declined so did the alkali soluble auxin. This decrease was shown not to be due to translocation but occurred *in situ*. In order to account for

the stability of the ratio it was hypothesized that: (a) an equilibrium exists between precursor and free auxin during development or (b) the two substances are produced independently at different rates. Seventy percent of the auxin was found in the aleurone layer in the vicinity of the embryo which itself was found to be lacking auxin. Based on these results it was concluded that the "precursor" is a derivative of already synthesized auxin, and auxin inactivation occurs *in situ*. Additionally they concluded auxin is not translocated from the mother plant but is produced in the seed from the cytoplasm of disintegrating cells during development.

Teas *et al*. (1951) used a colorometric method to determine IAA levels in developing *Zea mays* seeds. The general shape of the curve they obtained is very similar to that obtained by Avery *et al*. Unfortunately both groups failed to sample up to the time of maximum fresh weight, and most likely missed the true maximum in IAA content which occurs just before the onset of desiccation (Jensen and Bandurski, 1991).

During apple seed development two hormone peaks were observed by Luckwill (1948). The first peak corresponded with the formation of the endosperm and suggests the endosperm as the site of hormone production. The second peak occurred after the embryo had finished growth. Luckwill concluded that the hormone plays some part in embryo devel-

opment and that high hormone levels and seed numbers are involved in preventing premature fruit drop. Very similar results have been obtained in developing peach seeds by several other researchers (Stehly and Thompson, 1959, Powell and Pratt, 1966, Miller *et al.*, 1987).

The auxin level dynamics of developing bean seeds however, is different from those discussed previously. In all of the developing seeds discussed so far the auxin levels undergo a rapid increase in both free and bound auxin to a maximum and then decrease rapidly as the seed matures. The timing of these stages varies with the type of seed. In beans, the level of total IAA remains stable throughout the developmental period. During development free and ester IAA gradually decrease to a level approximately three times less than that present initially, while amide IAA gradually increases to a value five times the initial. Recent work suggests IAA stored in bean seeds may play only a minor role in supplying IAA to the developing seedling. Instead IAA required by the seedling for growth is produced *de novo* (Bialek and Cohen, 1991).

Since the mid fifties little research has been done on the auxin levels in developing seeds. This decline in research on auxins during seed development may be the result of a lack of any proven function for IAA during seed development or during the breaking of dormancy. On top of this,

other hormones have been identified which definitely function during these times and have proven easier to study.

Other areas of IAA research have not been so stagnant. Our knowledge of auxin function, metabolism in other tissues, and under various stimuli has since then increased dramatically. The true nature of the "precursor" auxin found by Avery *et al.* (1941) has since been determined. Large amounts of IAA are bound to various sugars and amino acids. IAA exogenously applied to plants tissues was found to be converted to IAA-aspartate (Andreae and Good, 1955) and to IAA-glucose (Zenk, 1961). The identity of many other conjugates of IAA has also been determined (for reviews see Cohen and Bandurski, 1982, Bandurski *et al.*, 1987). This conjugation of IAA is important in storage, transport, hormonal homeostasis, and protection from peroxidative attack.

Developing kernels of *Zea mays* have proven to be a valuable tissue for studying the enzymatic conversion of IAA to its various conjugates (Leznicki and Bandurski, 1988, Kowalczyk and Bandurski, 1990, 1991, Kesy and Bandurski, 1990). The similarity in auxin level dynamics between different seeds during development is noteworthy. Avery *et al.* (1942) believe the decrease during maturation is due to some physiological reason and is not an artefact of desiccation. The decrease in auxin during maturation has not been studied

in detail and such a study may lead to valuable new information on IAA regulation and function. The *in situ* production and relatively high auxin levels make developing seeds an attractive system for studying auxin biosynthesis and its regulation.

INDOLE-3-ACETIC ACID METABOLISM IN TISSUE CULTURES

The advent of tissue culture techniques has brought about the ability to manipulate the genetic makeup of cells in an attempt to modify the plant in a beneficial manner. A major obstacle in this endeavor is the inability to regenerate whole plants, from not just transformed cells, but cells in general. This is especially true of most major crops including wheat, maize, barley, oats and soybean. Removal of this obstacle will most likely come through a better understanding of hormonal regulation of differentiation. IAA most undoubtedly plays a major role in this respect.

Most tissue culture systems require an external auxin source, usually 2,4-dichlorophenoxy acetic acid (2,4-D) or napthalene acetic acid (NAA), for sustained growth to occur. IAA is not as effective as its analogs in this respect. Externally applied IAA is rapidly taken up and metabolized by cells in culture. The rate of inactivation is much slower in autonomous (non auxin requiring) cultures (Rekoslavskaya, 1975). Synthetic IAA analogs are inactivated at a slower rate (Andrae and Good, 1957). These analogs however do not simply serve as a replacement for endogenous IAA. It has been proposed (Lee, 1972, Negutiv *et al.*, 1979) that the role of 2,4-D is to alter the levels of endogenous auxins. It has been shown that normal (auxin requiring) cultures produce IAA at levels commensurate with that of whole plant tissues, and the levels change with the different stages of

the culture cycle (Maloney *et al.*, 1983). Michalczuk *et al.* (in press) have reported exogenously applied 2,4-D causes a significant change in endogenous IAA levels in carrot cell cultures. The 2,4-D somehow confers upon the cells the ability to undergo somatic embryogenesis when transferred to hormone free media.

The most striking aspect of IAA levels in tissue cultures is the variability seen not only between different species, but even between cultures of the same species (Tables 4 & 5). This makes it difficult to draw many general conclusions and suggests a greater diversity in IAA metabolism than previously believed. Conclusions can be drawn within a culture type, but care must be taken when making conclusions between different species. Each species in culture (or whole plant) likely has its own particular level of free IAA and maintains that level in a way unique to that culture (or plant). Undoubtedly there exist some general trends, but data from only a small number of cultures (and plants) has been collected. Free IAA is considered to be the form of physiological importance, but of equal importance is its ratio to the conjugated forms. The regulation of these two are most certainly intertwined. Currently only one paper has appeared presenting in depth data on the relationship between free and conjugate IAA in a tissue culture system (Michalczuk *et al.*, 1992).

Table 4. IAA levels reported in culture

species	tissue type	IAA (ng/g fwt)	year	author
Soybean	T	62-11	1988	Owen *et al.*
	H	12		
	N	8-20		
Carrot	N	31*	1979	Sung
	-2,4-D	128*		
	5MTR	352*		
	-2,4-D	5145*		
Pennisetum purpureum	N	8	1987	Rajasekaran *et al.*
Sunflower	T	45	1979	Chirek
	N	31		
Tobacco	T	100		
	N	35		
Sugar Beet	N	1400	1981	Keevers *et al.*
	H	1250		
Discorea deltoidea	N	149	1981	Kutacek *et al.*
Helianthus annus	T	10-13	1980	Atsumi
	N@	0.2-4		
Tobacco	T	100	1988	VanOnckelon *et al.*
	T	20		
	N	<5		
Soybean	N	2-13	1988	Wyndaele *et al.*
	N	2-30		
	H+Kn	7.5-15		
	H	24-31		
	T	2-16		
	T	2		

N= normal (auxin added), N@= mutanagized cells, T= *Agrobacterium* infected, H= habituated, H+Kn= habituated + kinetin, 5MTR= 5-methyltryptophan resistant

* value approximated from ng/g dry wt assuming 90% moisture

Table 5. Maximum IAA levels reported during culture cycle

species	tissue type	free IAA (ng/g fwt)	year	author
Nicotiana rustica	T	17	1982	Mousdale
	N	5		
Daucus carota	T	90		
	N	6		
Helianthus annus	T	23		
	N	3		
Kalanchoe daigremontiana	T	6		
	N	2		
Parthenocissus tricuspidata	T	2		
Daucus carota	EN	40+ 672t	1992	Michalczuk *et al.*
	NEN	40+ 603t		
Panax ginseng	N	1200	1976	Bekker *et al.*
Polyscias filicifolia	N	1500		
Acer pseudoplantanus	N	27*	1982	Moloney & Elliot
Acer pseudoplantanus	N	40	1981	Moloney *et al.*

continued on next page

Table 5. (cont.)

species	tissue type	free IAA (ng/g fwt)	year	author
Soybean	T	3.5	1985	Wyndaele
	N	3.5		
Tobacco	N	190	1986	Russian
Tobacco	T	800	1981	Nakajima
Tobacco	N	6++	1987	Koves &
	H	4++		Szabo
Tobacco	T	110	1987	El Bahr
	T	130		*et al.*
	N	75		
Tobacco	T	125	1984	El Bahr
	N	70		*et al.*

N= normal (auxin added), T= *Agrobacterium* infected, H= habituated, 5MTR= 5-methyltryptophan resistnat, EN= normal embryogenic, NEN= normal nonembryogenic, t= total IAA

* value approximated from ng/g dry wt assuming 90% moisture
+ estimated from graph
++ estimated based on 20 g of tissue/flask

In most cases the level of IAA (free) in tissue cultures rises to a maximum prior to the period of rapid mass increase and then declines through the exponential growth phase to a low level as the cultures enter the stationary phase. This holds true for both normal and autonomous cultures. The peak in IAA level appears to be associated with the initiation of cellular division and suggests some type of involvement in this regard. This is supported by the fact that the peak in IAA occurs before the mitotic index reaches its maximum (Nishinari & Yamaki, 1979). In addition it has been shown (Gamborg, 1982) that IAA is required during the growth phases of the cell cycle of tobacco cells in culture.

The large decrease in IAA coincides with the period of rapid increase in mass. There are of course exceptions to this trend. A number of researchers have reported on cultures where the maximum level of IAA occurs during the late exponential or stationary phase (Atsumi & Hayashi, 1978, El Bahr *et al.*, 1984, El Bahr *et al.*, 1987). These cultures however were of a tumorous type and it is not known how this may affect IAA relations with respect to the culture cycle. There has also been a report of random variations in IAA levels with no correlation to the cell cycle (Wyndaele *et al.*, 1985).

The most studied aspect of IAA in tissue culture has been the role of IAA levels in the autonomous growth of

crown gall infected and habituated tissue cultures. The T-DNA inserted into the host's genome from the Ti plasmid of *Agrobacterium tumefaciens* encodes for proteins responsible for the production of IAA from tryptophan via Indole acetamide (IAM) (Kosuge *et al.*, 1966, Inze *et al.*, 1984, Schroeder *et al.*, 1984, Thomashaw *et al.*, 1984). The presence of this alternate pathway diminishes the usefulness of these cultures in studying IAA biosynthesis as it occurs normally in plants. Habituated cells appear as a result of stably inheritable changes in the gene expression which occur at a frequency several orders of magnitude greater than that of normal mutations (Meins, 1981).

In general tumorous tissues produce more IAA than non-tumorous ones (Tables 4 & 5) which apparently enables them to initiate the phase of rapid growth. This has been proposed, and is generally accepted, as the reason for auxin independence (Beiderbeck, 1977). In a comparison of IAA levels from a number of different species in normal versus infected tissues with respect to time, Mousdale (1982) observed that only the normal cultures failed to exhibit a rapid increase in IAA content. It was suggested that the rapid growth phase is triggered by an increase in endogenous IAA above a critical threshold level. In addition to auxin overproduction, auxin independence may be the result of an alteration in sensitivity to endogenous auxin, or the cells could no longer be subject to auxin control of rate-limiting

steps in cell growth and division (Mousdale, 1982). An altered sensitivity may explain the results of Keever *et al.* (1981) and Kutacek *et al.* (1981) who both observed little difference between normal and habituated cultures.

A number of researchers have examined the relationship between tryptophan and IAA levels in tumorous and in non-tumorous tissue cultures. Most of the data indicate no relationship between tryptophan and IAA content (Atsumi, 1980, Kutacek *et al.*, 1981, Maloney and Elliot, 1982, El Bahr *et al.*, 1984). This agrees with the findings of Allen and Baker (1980) who found no relationship in the leaves of *Ricinus communis*. In *Discorea deltoidea* cultures, auxin-habituated cultures possessed a higher anthranilate synthase activity than did normal cultures (Kutacek *et al.*, 1981). The habituated cell line was also more resistant to L-tryptophan feedback inhibition, yet the level of IAA, which was high, was similar to that of the normal cell line. This led them to assume that the tryptophan and IAA pools are relatively independent. Widholm (1972) selected a number of 5-methyl-tryptophan resistant cultures for experimentation. Ten of the cultures accumulated high levels of tryptophan but only five were auxin-autotrophic. Therefore tryptophan accumulation alone is not sufficient for auxin-autotrophy. IAA levels in these cultures have not yet been measured. Alternatively, Sung (1979) examined an autonomous 5-methyl-tryptophan resistant carrot culture and found it contained high levels of trypto-

phan and also contained high levels of IAA. This finding is supportive of a relationship between tryptophan, IAA, and autonomous growth. Maloney and Elliot (1982) measured the levels of tryptophan and IAA in *Acer* cell culture and found that IAA reached its maximum level well before tryptophan did. This evidence goes against the suggestion by Sheldrake (1973) that IAA levels are regulated by the availability of tryptophan. Additionally no relationship was found between cell autolysis (as a source of tryptophan) and IAA levels. El Bahr *et al.* (1987) examined the activities of two enzymes of the indole pyruvate (IPyA) pathway for IAA in tumorous and non-tumorous tobacco cultures. They found no relationship between the activities of tryptophan amino transferase (TAT), Tryptophan dehydrogenase (TDH) and IAA levels. They concluded the increase in IAA seen in the tumorous line was not the result of increased biosynthesis via the IPyA pathway. Black and Hamilton (1976) observed conversion of ^{14}C-tryptophan into IAA in soybean callus tissues. They also found that cell free medium could convert ^{14}C-tryptophan to IAA. Since exogenously applied tryptophan has been shown to enter a pool apparently separate from that of endogenous tryptophan (Sung, 1975) they concluded that either excess tryptophan leaked out of the cell and into the medium and was converted to IAA or the tryptophan might have entered a pool were conversion can occur.

Maize tissue cultures as model systems

Maize is an ideal plant for studying in tissue culture systems. The biochemistry and genetics of maize has been very well characterized and many genetically well defined lines exist. Cultures initiated from endosperm during the period of high meristematic activity (9-12 DAP) have been used for a number of physiological and biochemical studies. Maize endosperm in culture appears to retain a number of characteristics of the intact tissue. Maize endosperm cultures exhibit autonomous growth on media containing mineral salts, sucrose, asparagine, and thiamine (Shannon, 1982). Endosperm cultures of *Ricinus communis* also require no exogenous hormones for autonomous growth. Cultures of endosperm have the ability to synthesize and store starch, but not to the extent of the intact kernel (Chu and Shannon, 1975). Zeins are also produced (Shimamoto, 1983). This is interesting since at the age from which the cultures were initiated, synthesis of zein begins has yet to begin (Randolph, 1936). In endosperm cultures zein is being produced in actively proliferating tissue, while in intact kernels zein deposition begins at the time when division is nearly complete. Anthocyanins are also produced in cultures initiated from anthocyanin containing endosperms (Straus, 1960, Racchi, 1985).

At the ultrastructural level, several differences from intact kernels are apparent (Felker, 1988). In addition to

normal storage cells there are meristematic cells and an abundance of highly vacuolate cells. Many cells have an abundant SER, a feature not associated with intact maize endosperm. It could not be concluded whether the differences were associated with an altered state induced by the culture technique or represents an amplification of normally minor cellular activities. Miernyk (1987) assayed the medium from maize endosperm cultures and found an abundance of acid hydrolases. Enzyme activities were similar when compared to those from intact endosperm except for a substantial increase in β-galactosidase activity in the cultures. The abundance of SER and the data of Miernyk supports the findings of Wink (1984) who suggested the medium serves as an extracellular lytic compartment in tissue culture systems.

Maize kernels can be grown *in vitro* resulting in viable seeds (Gegenbach, 1977). *In vitro* grown kernels offer a unique opportunity to regulate the nutrient supply to the developing kernels for research purposes. Shimamoto and Nelson (1981) examined thiamine transport and found uptake very limited compared to that of sucrose. Since thiamine is a necessary component in most culture media the difficulty in taking up supplemental nutrients may explain the difficulty in obtaining auxotrophic mutants. Using ^{35}S-methionine, Cully *et al.* (1984) compared protein synthesis in developing kernels grown *in vitro* to those grown in the field. They found the *in vitro* grown kernels to be comparable to that of the

field grown kernels thereby demonstrating the appropriateness of the system for future study. Singletary and Below (1989,, 1990) and Singletary *et al.* (1990) have used developing kernels to study the affects of different levels of carbon and nitrogen on the production of storage products and associated enzymes.

Maize kernels grown *in vitro* do not require hormones in the media. Therefore it is reasonable to conclude the kernels have the capacity to synthesize the hormones they need. The production of cytokinins by *in vitro* grown kernels has been demonstrated (Reinicke and Rubenstein, 1991S). In light of the large amounts of IAA produced in developing maize kernels (Jensen and Bandurski, 1991S), *in vitro* kernel culture should be an excellent system for studying IAA biosynthesis.

Tissue culture systems are a valuable tool in studying plant hormones. The production of each of the major hormones has been demonstrated in a variety of culture systems (Nickel, 1958, MacKenzie and Street, 1970, LaRue and Gamborg, 1971, Nakajima *et al.*, 1981, Wyndaele, 1985, Rajasekasan *et al.*, 1987, Koves and Szabo, 1987). The specific activities of enzymes extracted from tissue cultures can be significantly greater than those from differentiated tissues (Gamborg, 1966). A major difficulty in studying plant hormones has been their presence in small amounts. Those cell lines which exhibit high hormone levels should be particularly valuable in this regard. In culture systems

the biosynthesis of the hormones can easily be demonstrated and quantified. Thus, in metabolic studies, the amount and rate of production from labeled precursors can be calculated and the relative significance of that conversion determined. For the study of IAA, those cultures which are autotrophic, yet non tumorous, should prove to be the least complicated and most valuable to study.

INDOLE-3-ACETIC ACID BIOSYNTHESIS RESEARCH: WHERE IS IT HEADED?

Since the discovery and identification of indole-3-acetic acid (IAA) as a major plant hormone many scientists have conducted experiments in an attempt to elucidate the biosynthetic pathway. Thimann (1935) first suggested tryptophan (TRP) as a precursor of IAA based on feeding experiments with the fungus *Rhizopus suinus*. Wildman, Ferri, and Bonner (1948) were the first to present evidence for the conversion of TRP to IAA in higher plants.

At various times over the past 50-60 years reports have occasionally appeared suggesting TRP is not a precursor of IAA in higher plants. Working with *Avena* coleoptile tips Winter (1966) and Thimann and Growchowska (1968) failed to observe elongation when supplied with TRP. On this basis they concluded TRP was not a precursor. Another possible reason for a lack of conversion is that this tissue does not normally synthesize IAA. Black and Hamilton (1971) obtained low rates of conversion of ^{14}C-TRP to ^{14}C-IAA in *Avena* coleoptiles. They believe their low rate of conversion was due to the fact that exogenously applied TRP does not fully equilibrate with the free TRP pool important in IAA biosynthesis.

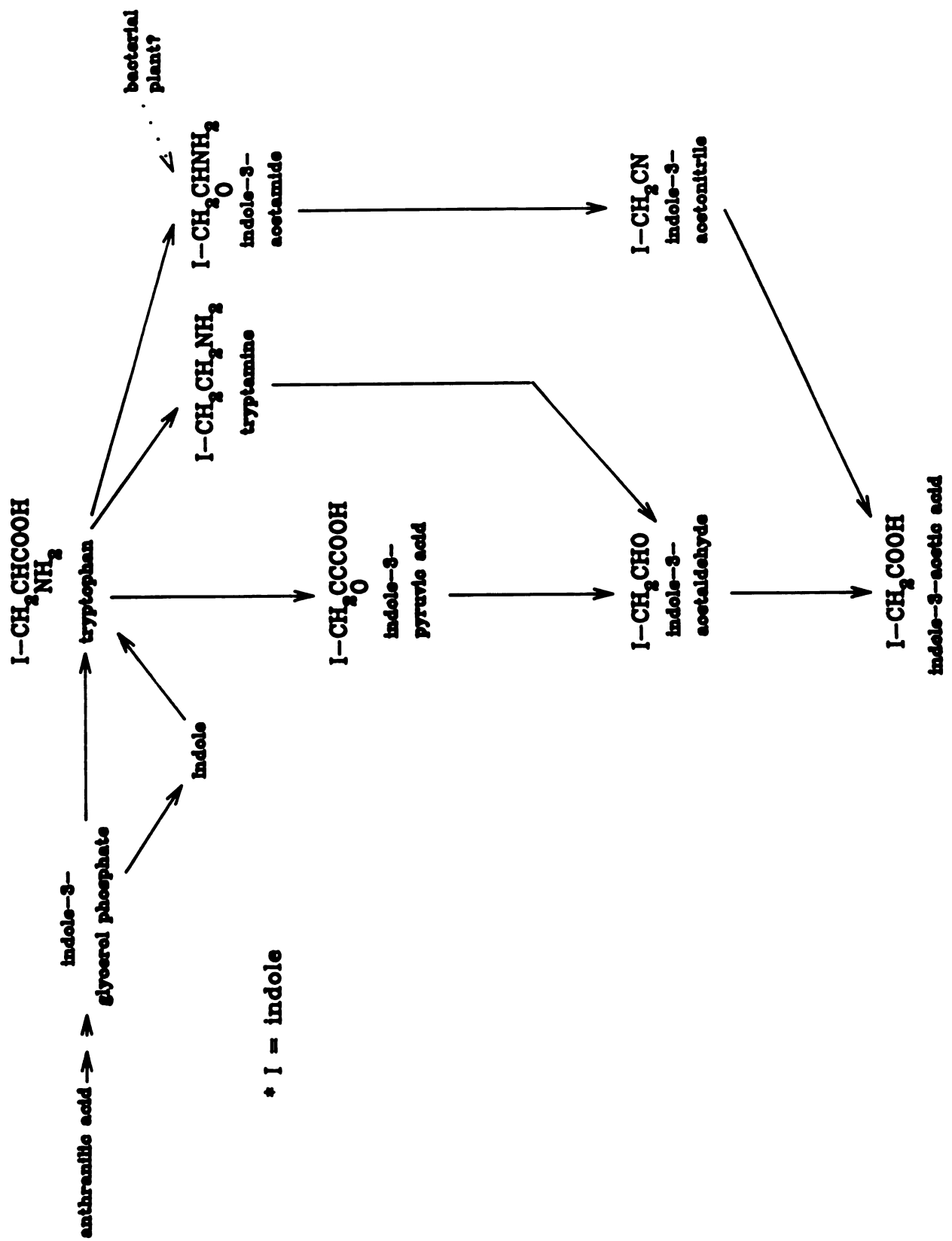
More recently the role of TRP as a precursor has been seriously questioned. Working with a corn mutant containing a defective TRP synthetase Wright *et al.* (1991) have demonstrated conclusively that TRP is not a precursor of IAA in

this plant. When seedlings were grown on media supplemented with (^{15}N-indole)-TRP there was no incorporation of the ^{15}N into IAA even though IAA levels in the mutant were 10 times that of the control. This indicates there may be at least two biosynthetic pathways, one having a branch point somewhere before TRP. Multiple pathways for IAA biosynthesis have been proposed before (Wightman, 1968), but they have always included TRP as a precursor. This coupled with the continued inability to produce unequivocal data substantiating any of the proposed TRP to IAA pathways has slowed advancement in all areas of IAA research compared to that of the other plant hormones. The purpose here is not to criticize the volumes of work done over the years but to highlight the steps which need to be taken to unequivocally elucidate the IAA biosynthetic pathway(s).

Proposed biosynthetic pathways

Several pathways from TRP to IAA have been proposed and studied. A diagram of the most widely accepted pathways is shown in Figure 1. The most evidence and the greatest support is for the indole pyruvic acid (IPyA) to indole acetaldehyde (IAld) to IAA pathway. A number of putative enzymes of the IAA biosynthetic pathway have been partially purified but they generally are of a relatively unspecific nature (Wightman, 1968, Muir, 1968, Kutacek, 1985). IPyA is

Figure 1. Proposed pathways for indole-3-acetic acid biosynthesis in plants


bacterial
plant?
I-CH2CHNH2
O
indole-3-
acetamide
I-CH2CN
indole-3-
acetonitrile
I-CH2CH2NH2
tryptamine
I-CH2CHCOOH
NH2
tryptophan
I-CH2CCCOOH
O
indole-3-
pyruvic acid
I-CH2CHO
indole-3-
acetaldehyde
I-CH2COOH
indole-3-acetic acid
indole-3-
glycerol phosphate
anthranilic acid
indole
* I = indole

produced in plants as a result of deamination of TRP by a multispecific aromatic amino acid transaminase (Gamborg, 1965). Transaminases that have been demonstrated in plants have a specificity for TRP significantly lower than that of phenylalanine and tyrosine (Truelson, 1972, Forest and Wightman, 1972, 1974). Additional evidence has come from the promotion of catalysis by adding pyridoxal phosphate and α-keto glutarate, both typical promotors of transamination (Wightman, 1968, Muir and Lantican, 1968). The presence of IPyA has been shown by isolating the 2,4-dinitrophenylhydrazone derivative of this highly unstable compound (Wightman and Cohen, 1968, Gibson et. al, 1972,). Positive mass spectral identification of IPyA in plants has been made by Cooney and Nonhebel (1989). A TRP dehydrogenase has been found which can convert TRP to IPyA (Ebeid *et al.*, 1985). The back reaction was found to be more active and it was proposed that this would be a possible way to regulate the levels of IPyA and IAA biosynthesis.

No enzyme has yet been found in plants which can convert IPyA to IAAld. An IPyA decarboxylase has been demonstrated in yeast (Sukanya *et al.*, 1971). Due to its highly unstable nature IPyA can spontaneously break down to form IAA. Circumstantial evidence for enzymatic decarboxylation comes from the stimulatory effects of thiamine pyrophosphate and lipoic acid (Gordon, 1961) known cofactors of oxidative

decarboxylations. Gibson *et al.* (1972) has also presented qualitative evidence for the decarboxylation of IPyA.

The conversion of IAAld to IAA has been demonstrated by a dehydrogenase (Wightman and Cohen, 1968) and an oxidase (Rajagopal, 1971). Both enzymes were found to be mainly cytoplasmic however they had different pH optima. Kutacek (1985) suggest IAAld oxidase is important in regulating IAA levels.

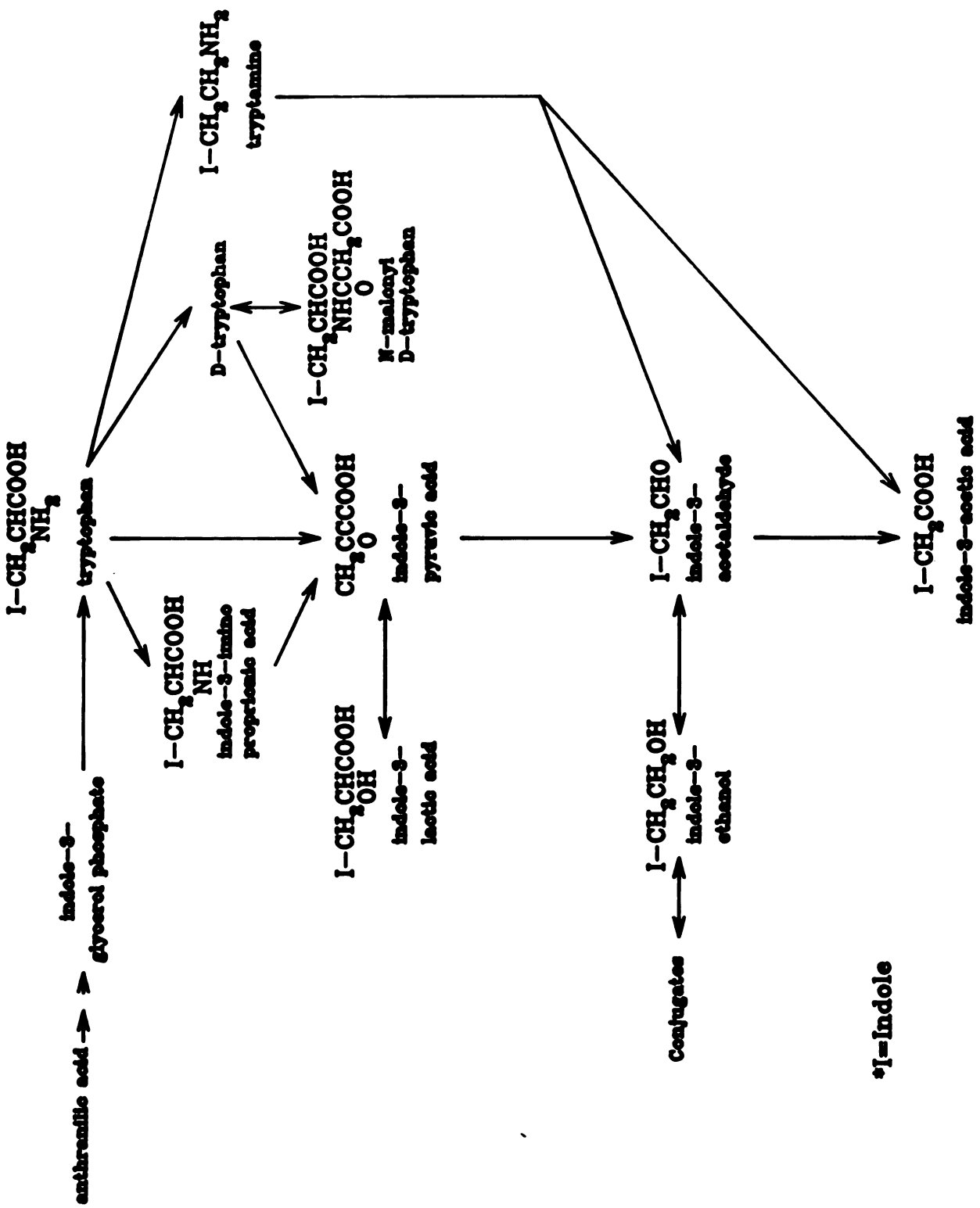
The possible role of tryptamine (TNH_2) as an IAA precursor was first shown by Skoog (1937) who observed coleoptile curvature with TNH_2 after a lag period. The existence of TNH_2 has been demonstrated in some plants but has not been found in a number of common plants (Schneider *et al.*, 1972). Enzymes which decarboxylate TRP to form TNH_2 have been found in a number of plants (Phelps and Sequira, 1968, Sherwin, 1970, and Gibson *et al.*, 1972a,b). The next step in this pathway is deamination to from IAAld. A number of amine oxidases have been examined in plants (Mann, 1954, Yamazaki *et al.*, 1970).

The indole acetamide (IAM) pathway has been demonstrated in *Pseudomonas savastanoi* (Kosuge *et al.*, 1966) and *Agrobacterium tumefaciens* (Thomashaw *et al.*, 1984, Inze *et al.*, 1984) The Ti-plasmid of *A. tumefaciens* contains genes which code for enzymes that convert TRP to IAM and IAM to IAA (Thomashaw *et al.*, 1984, Inze *et al.*, 1984). The plasmid is inserted into the hosts genome during infection. This results in an

increase in IAA levels and subsequent tumor formation. Kutacek *et al.* (1987) have demonstrated both the IAM and IPyA pathways to be operative in infected tobacco cells in culture.

Modifications to the pathways in Figure 1 are indicated in Figure 2. The pool of TRP in plants is large compared to that of IAA and this presents a regulatory problem. How can the small amount of TRP needed for IAA biosynthesis be regulated? To get around this it has been proposed that D-TRP is a more immediate precursor than L-TRP (Law, 1987, McQueen-Mason and Hamilton, 1989, Tsurusaki *et al.*, 1990). L-TRP is first racemized to the D form resulting in a greatly reduced pool size. D-TRP is then further metabolized via IPyA to IAA. The presence of N-malonyl-D-TRP in plants is well established (Good and Andreae, 1957, Aenk and Scherf, 1963) and may serve as a storage form of TRP (Rekoslavskaya, 1986) as well as a detoxification product (Rosa and Neisch, 1968). N-malonyl-D-TRP accumulates in water stressed plants and decreases upon removal of stress which is accompanied by a large increase in IAA levels (Gamborg *et al.*, 1991). Indole ethanol (IEt) has been identified as a normal constituent of plants (Rayle and Purves, 1967, Sandberg, 1984) and may served as a storage form of precursor. IEt also reversibly forms glycosidic conjugates which may serve as further storage forms (Magnus, 1973)

Figure 2. IAA biosynthetic pathway variations and regulation mechanisms.

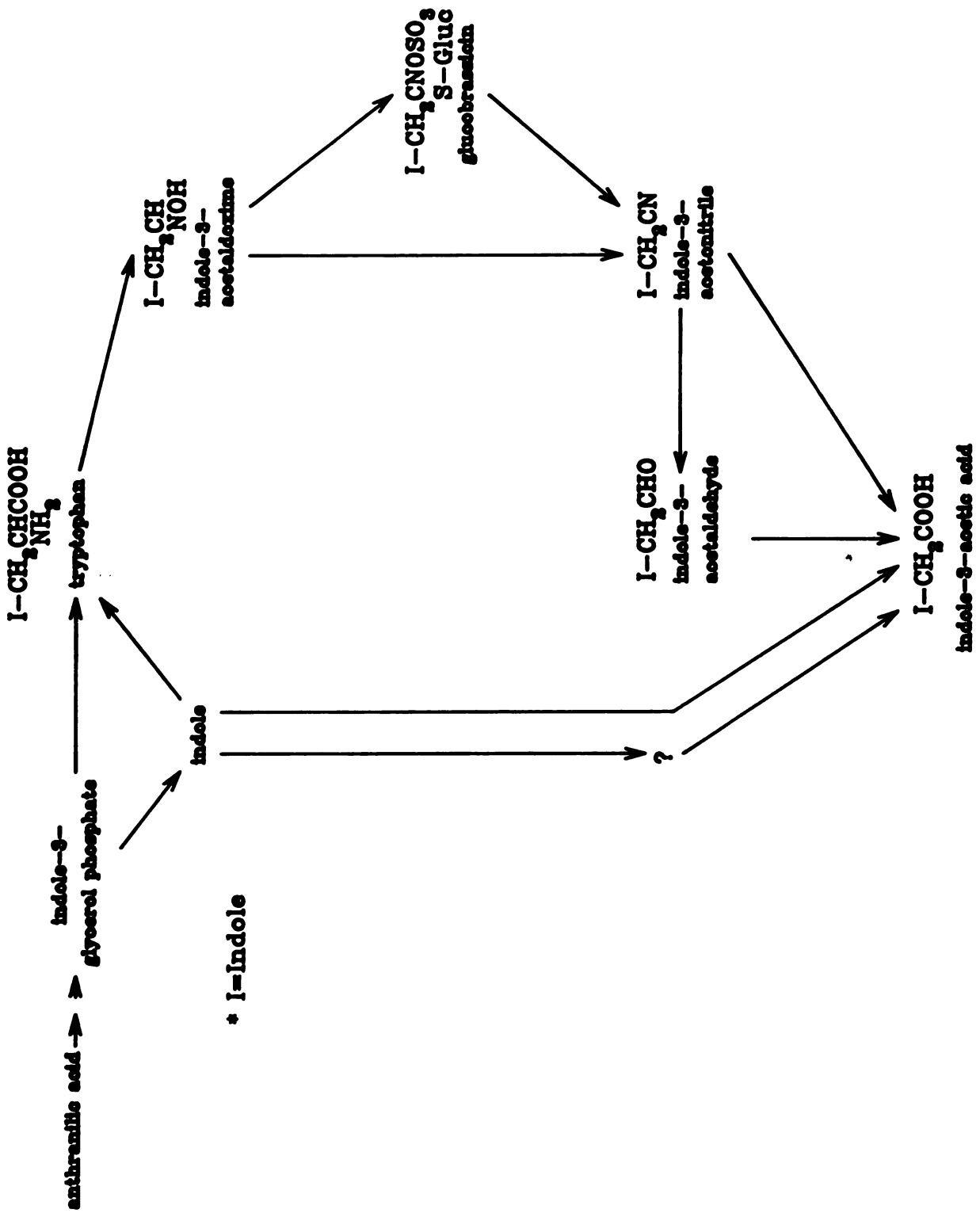

anthranilic acid
indole-3-glycerol phosphate
I-CH2CHCOOH NH2 tryptophan
I-CH2CH2NH2 tryptamine
D-tryptophan
I-CH2CHCOOH NHCCH2COOH O
N-malonyl D-tryptophan
I-CH2CHCOOH NH
indole-3-imino propionic acid
CH3CCOOH O
indole-3-pyruvic acid
I-CH2CHCOOH OH
indole-3-lactic acid
I-CH2CHO
indole-3-acetaldehyde
I-CH2CH2OH
indole-3-ethanol
I-CH2COOH
indole-3-acetic acid
Conjugates
*I=Indole

Finally Figure 3 shows other pathways which have been studied or proposed. The role of indole acetaldoxime (IAOX) and glucobrassicin as an IAA precursor has been shown in various members of the Brassica family (Kutacek and Kefeli, 1968), and cereals (Bentley and Housley, 1952, Rajagopal and Larsen, 1972, Helming *et al.*, 1987) but is not believed to be widespread in the plant kingdom.

Steps toward definitive answers

A major problem in the study of IAA is its presence in small amounts, generally 1-100 ng/g fwt. When working with such small amounts it is difficult to distinguish between artifact and actuality. It has been demonstrated that radiolabelled TRP can be converted to IAA with a 30% yield merely by drying (Epstein *et al.*, 1980). Another compounding aspect to this problem is the amount of labelled and/or unlabeled potential precursors which has been fed to the conversion system being examined. Care must be taken to insure that only physiological levels are used. In their work with *Lemna*, Baldi *et al.* (1991) obtained stable isotope labelled IAA from intact plants grown on agar containing ^{15}N-L-TRP. The amount of L-TRP added in order to obtain conversion however was 400 to 1600 times greater than its pool size. They were unable to obtain conversion with D-TRP at any concentration. Even though they did obtain conversion of TRP to IAA, this conversion was determined to be physiologically not

Figure 3. Proposed alternate pathways for indole-3-acetic acid biosynthesis.


anthranilic acid
indole-3-glycerol phosphate
indole
I-CH2CHCOOH NH2
tryptophan
I-CH2CH NOH
indole-3-acetaldoxime
I-CH2CNOSO3 S-Gluc
glucobrassicin
I-CH2CN
indole-3-acetonitrile
I-CH2CHO
indole-3-acetaldehyde
?
I-CH2COOH
indole-3-acetic acid
* I=Indole

important under normal conditions. These results led them to conclude TRP is not a precursor of IAA in this system. Most of the feeding experiments conducted to date used large amounts of labelled and/or unlabeled potential precursors. The conversion rates obtained with ^{14}C-TRP in most experiments are below 0.05%. There is no doubt that these experiments have demonstrated the conversion of TRP to IAA. The weakness of these studies is the lack of quantitative evidence as to the significance of the conversion. As a result, definitive proof of TRP as an important precursor has not been demonstrated. This shows the need to know the pool sizes of the precursors and products such that the relative conversion of the precursors can be determined and checked for significance.

Catabolism and the formation of IAA conjugates are important processes in the regulation of IAA levels (Cohen and Bandurski, 1983). Free IAA is the form active in growth but the conjugates represent the bulk of the IAA in plants (Schulze and Bandurski (1977). Black and Hamilton (1976) and the recent work in Cohen's lab are to date the only ones to examine booth free and conjugate IAA in their systems. Free IAA is readily conjugated and by not examining the total IAA it is difficult to determine the significance of any observed conversion.

There has been rapid development in separation technologies over the past 15 years. The majority of the data

collected on IAA biosynthesis was completed prior to the recent upsurge in new technology. Identification of putative intermediates and IAA has been based largely on paper chromatography Rfs and response to colorometric tests. Data of this nature does not represent conclusive proof as to the identity of a compound. The presence of interfering or contaminating compounds cannot be excluded. Definitive proof can be obtained by purifying to a constant specific activity and demonstrating that the specific activity remains constant when subjected to additional purification procedures. Positive identification of the molecule then needs to be made by MS, NMR, or IR analysis. The latter two are impractical due to the large amounts required for analysis. Conclusive evidence can also be obtained with the use of stable isotopes. Proof that one compound has been converted to another is easily obtained by demonstrating the presence of the label in the product upon mass spectral analysis. This method is the most reliable, unfortunately the cost and availability of labelled compounds can be prohibitive.

Many different tissues have been used to study IAA biosynthesis. Often missing is the evidence that the tissue being used is one that normally produces IAA. The coleoptile of cereals has been a popular experimental tissue. There is however data suggesting that in some cereals the seedling does not biosynthesize IAA but is totally reliant on the seed stores of the hormone (Pohl, 1935, Sheldrake,

1973, Jensen and Bandurski, 1990). IAA is generally believed to be synthesized in the meristematic regions of plants (for reviews see Sembdner, 1981, Sheldrake, 1973). Definitive proof that a system is producing IAA adds strong support to the results and conclusions arrived at using that system. Proof that a system is producing IAA can be obtained by measuring IAA levels over time (free and conjugate) and checking for an accumulation. Additionally the incorporation of stable isotopes from the feeding of general labels such as D_2O or ^{13}C-glucose can be used to demonstrate that IAA biosynthesis is occurring.

Greater emphasis needs to be placed on how biosynthesis is occurring *in vivo*. The use of extracts or tissue sections can lead to misconceptions about what occurs in an intact plant. Cells contain a variety of different compartments and as a result there are enzymes and substrates that normally do not come in contact with one another. Disruption of the cells may result in two materials coming together resulting in a reaction not normally seen. Several researchers have demonstrated the conversion of TRP to IAA when incubated with catechol at alkaline pH or in the presence of phenolase at lower pH (Gordon and Paleg, 1961, Whitmore and Zahner, 1961). Both researchers doubted the importance of this reaction *in vivo* but warned of its possible importance when working with plant extracts. The use of *in vitro* experiments is still an important tool for studying the

IAA biosynthetic pathway. Initial *in vitro* results however need to be followed up with carefully set up *in vivo* experiments using intact plants.

Wounding initiates a wide range of responses in tissues including a variety of free radical catalyzed reactions. In experiments using cuttings care must be taken to distinguish between normal and wounding related pathways. The likelihood of two possible pathways is an important consideration at this point. One pathway, which normally accounts for only a minor amount of the total biosynthesis may play a more important part in wounded or infected tissues. This can be misleading when trying to determine the significance of a particular pathway. The difficult task for the future is to distinguish between major, minor and artifactual pathways.

Plants can be colonized by many species of bacteria (Libbert, 1966). These bacteria can produce IAA (Libbert, 1966, and Wichner and Libbert, 1968) and non-sterile plants were found to contain more IAA than sterile ones (Libbert *et al.*, 1968). It has been suggested that the production of IAA observed in many earlier experiments was the result of contamination. This series of experiments by Libbert's lab have shown the importance of insuring that any experimental system used be checked for and maintained under sterile conditions.

Since it's discovery, many different aspects of IAA biology have been studied. Several basic aspects of IAA metabolism however remain unresolved. There is clearly much we do not know about IAA. There is a high potential for pratical applications of IAA related research. Recent papers indicate a definitive biosynthetic pathway for IAA has not been demonstrated. Even though IAA is widely believed to be synthesized in meristematic zones there is little conclusive data to back this up. The timing, regulation of production, and IAA levels in various tissues throughout plant development is poorly understood at best. Resolution of these questions will enhance our ability to regulate plant growth.

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EXPERIMENTAL I

INCORPORATION OF DEUTERIUM INTO INDOLE-3-ACETIC ACID AND TRYPTOPHAN IN *ZEA MAYS* SEEDLINGS GROWN ON 30% DEUTERIUM OXIDE.

ABSTRACT

The onset of *de novo* synthesis of IAA and tryptophan in seedlings of *Zea mays* sweet corn, var. Silver Queen was examined. Deuterium labeled water as a general precursor was used to minimize assumptions as to the biosynthetic route. Protium in positions 2,4,5,6, & 7 of the indole ring are non-exchangeable (Magnus *et al.*, 1980). IAA and tryptophan synthesized via the shikimic acid pathway would contain deuterium in one or more of these positions. Protium on the indene nitrogen, the carboxyl, the amino group (TRP only), or the protium alpha to the carboxyl (IAA only), exchange readily and were removed by base catalyzed exchange prior to GC-MS analysis. We have demonstrated, by means of gas chromatography-selected ion monitoring mass spectrometry, that 7 day old, dark grown seedlings grown on 30% D_2O incorporate deuterium into tryptophan, but not into IAA. Randomization of deuterium in the indole ring during mass spectrometry makes difficult to localize the deuterium to the tryptophan ring. Deuterium NMR is used for the determination of deuterium incorporation in tryptophan. Data will be presented on the localization of the deuterium in the ring structure.

INTRODUCTION

The endosperm of corn kernels contain large amounts of bound indole-3-acetic acid (IAA) (Haagen-Smit *et al.*, 1942, Avery *et al.*, 1944, Ueda and Bandurski, 1969, Piskornik, 1975) which is used by the seedling during germination. At what time does the seedling stop relying on these reserves and begin *de novo* biosynthesis of IAA? The determination of this time is important in the study of the kinetics of IAA levels and transport in seedlings. While the seedling is relying on its IAA reserves, the metabolism of IAA may be considered as a closed system. In a closed system the only inputs of IAA that need be considered are conjugate hydrolysis and transport, allowing for a less complicated picture of IAA metabolism in seedlings. An open system with respect to IAA is difficult to study owing to the lack of a clear demonstration of a definitive biosynthetic pathway and data regarding synthetic rate.

The onset of *de novo* biosynthesis of IAA and tryptophan can be determined by growing seedlings on deuterium enriched water and monitoring the incorporation of deuterium into the molecules. The use of deuterium oxide (D_2O) as a tracer removes many of the problems associated with uptake of applied precursors such as compartmentation, specific changes in metabolism (for review see Thomson, 1963) and wounding responses. During *de novo* biosynthesis involving the formation of the indole rings of IAA and tryptophan, there would be

the opportunity for deuterium incorporation through keto-enol tautaumerization. Proton exchange of partially purified samples insures any deuterium present are of enzymatic origin. The presence of heavy isotope abundancies greater than that naturally occurring indicates *de novo* biosynthesis of the compound has occurred. Tryptophan was chosen to be analyzed because of its structural similarities to IAA and possible common biosynthetic pathway. In this paper we present data that as of 7 days of age there is *de novo* biosynthesis of tryptophan but not of IAA.

MATERIALS and METHODS

Plant Material

Corn kernels *Zea mays* cv. Silver Queen (W. Altee Burpee Co.) were surface sterilized for 10 minutes in a 0.5% NaOCl (1:10 dilution of commercial bleach) solution. The kernels were then rinsed a minimum of four times with sterilized distilled water. All materials were sterilized prior to use. Approximately 25 kernels were placed into each of two beakers containing 20 ml of H_2O or 30% D_2O. The beakers were covered with aluminum foil and placed on a shaker for 24 hours. After this time the kernels were rolled in paper towels, seven kernels per roll, and placed into 500 ml graduated cylinders, three rolls per cylinder. Fifty milliliters of H_2O or 30% D_2O was added to the graduated cylinders which were then plugged with a cotton ball to

maintain sterility and allow gas exchange. All kernel manipulations were carried out in a laminar flow hood with the use of sterile gloves. The kernels were allowed to grow for seven days in the dark at 25°C and 80% relative humidity. After seven days the seedlings were harvested, shoot lengths taken, and then either used immediately or frozen at -20°C for later analysis. Plants grown on 30% D_2O show a growth inhibition of 23%. The plants appeared normal except for a slower rate of development. The fresh weight to dry weight ratios were similar in H_2O and 30% D_2O grown plants (data not shown).

Purification of indole-3-acetic acid and tryptophan.

Approximately 2.5 gm of shoots were ground in sufficient acetone to give a final concentration of 70%. At this time approximately 50,000 cpm of [5-^{3}H] IAA and 50,000 cpm of [5-^{3}H] tryptophan were added to the extract to aid in peak identification. The mixture was allowed to set for one hour at room temperature, after which time the extract was filtered, the residue being saved for a dry weight determination. The filtrate was dried under vacuum at 50°C until nearly dry then redissolved in approximately 2 ml of 50% ethanol. The sample was then loaded onto a DEAE Sephadex column (acetate form, 3 ml bed volume, Pharmacia) and washed with 50 ml of 50% ethanol. The tryptophan is not retained by the DEAE, washing off in the first 10 ml of solvent directly onto a Dowex 50 column (H^+ form, 100 mesh, 10 ml bed

volume,). The IAA is eluted from the DEAE with a 0 to 2.5% (v/v) linear gradient of acetic acid in 50 % ethanol. The IAA fractions were pooled, dried under vacuum and dissolved in solvent appropriate for HPLC. The sample was chromatographed on a HPLC (LDC/Milton Roy model CM400 multiple solvent delivery system with a model 3000 spectro Monitor variable UV detector set at 280 nm) equipped with a C18 column (5 micron, 250 X 4.6 mm ID, Val-U-Pak, Regis). The mobile phase was 25% aqueous acetonitrile containing 1% acetic acid. The IAA fractions were pooled, dried under vacuum, dissolved in 100 μl methanol, and methylated with 250 μl of an ethereal solution of diazomethane for 5 minutes at 20^{o}C. The methylated sample was dried under a stream of N_2, dissolved in 300 μl of solvent and chromatographed on a C18 HPLC column using 45% aqueous acetonitrile. The IAA fractions were pooled, dried under vacuum, and taken up in a small volume of acetonitrile for subsequent GC-MS analysis.

The Dowex column containing the tryptophan was washed with 20 ml of 50% ethanol and the sample was eluted with 2N NH_4OH. The tryptophan fractions were pooled, dried under vacuum and dissolved in 1.5 ml of chromatography solvent and chromatographed under low pressure on a silica gel 60 column (15 ml bed volume) using ethyl acetate:isopropanol:water:NH_4OH (20:20:25:1). The tryptophan fractions were pooled, dried under vacuum, and taken up in solvent for HPLC analysis. The sample was chromatographed

on a C18 HPLC column using 0.01M sodium acetate buffer (pH 5.1):Acetonitrile (95:5). The tryptophan fractions were pooled, dried under vacuum, then acetylated with acetic anhydride:pyridine (4:1) at 25^{o}C for five minutes. The reaction mix was then dried at 35^{o}C under a stream of N_2, dissolved in 100 μl methanol, and methylated with 250 μl diazomethane. The sample was then dried at 35^{o}C under a stream of N_2, and the monoacetyl, methyl ester derivative of tryptophan was dissolved in 30% aqueous acetonitrile and chromatographed on a C18 HPLC column using the identical solvent. Sample recoveries varied from 3% to 25 % and averaged 12%. The fractions containing the derivatized tryptophan were pooled, dried under vacuum, and taken up in a small volume of acetonitrile for subsequent GC-MS analysis.

Purification of preparative amounts of tryptophan for NMR analysis was done as above except for a few simple modifications. The bed volume of the Dowex 50 column was increased to 20 ml and washed with an additional 20 ml of 50% ethanol. HPLC purification was done on a Phenomenex Bondclone C18 column (300 X 7.8 mm, 5 micron) at a flow rate of 3 ml/minute. Purified tryptophan samples were dissolved in CD_2Cl_2 for ^{1}H-NMR analysis or in CH_2Cl_2 for ^{2}H-NMR analysis. Spectra were obtained using a Varian VXR 500 MHz NMR spectrometer.

GC-MS analysis

Mass Spectral data was generated with a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970 MS. Samples where separated on a DB-17 fused silica capillary column (15 m X 0.25 mm) purchased from J & W Scientific. Analysis of the data were carried out using MS-MSD 59974J software (Hewlett-Packard).

The methyl ester of IAA (MeIAA) was chromatographed using a temperature program consisting of a 1 minute hold at 100 ^{o}C, then 100^{o}-230^{o}C at 30^{o}C/min. MeIAA had a retention time of 5.1 minutes. The helium carrier gas was at 2 ml/min. Each major mass spectral fragment and it's associated isotope peaks through m+5 were analyzed using separate injections and selected ion monitoring.

The monoacetyl methyl ester of tryptophan was chromatographed using a temperature program consisting of a 1 minute hold at 100 ^{o}C, then 100^{o}-260^{o}C at 30^{o}C/min. The monoacetyl methyl ester of tryptophan had a retention time of 6.5 minutes and the spectral data was collected in a manner similar to that of IAA. The relative abundancies for the isotope peaks for each fragment were compared to those of the control to determine the amount of deuterium incorporation.

RESULTS and DISCUSSION

The mass spectrum of MeIAA consists of a molecular ion at m/z 189, base peak at m/z 130 and additional fragment ions at m/z 103, and 77 (Figure 1a). The mass spectrum of the tryptophan derivative consists of a molecular ion at m/z 260, base peak at m/z 130 and additional fragment ions at m/z 201, 103, and 77 (Figure 1b).

The purification schemes for IAA and tryptophan produced samples which gave single peaks upon GC-MS analysis (Figure 2a,b). The lack of additional peaks makes it possible to rule out the possibility of contamination causing abnormally high abundancies for the isotope peaks. A partial spectrum for tryptophan from D_2O grown seedlings is shown in Figure 3b. A difference in isotope abundancies from the controls (Figure 3a) indicates the presence of deuterium. The isotope abundancies for the tryptophan from D_2O grown seedlings are clearly and significantly different from those of the control. The IAA from treated plants is unchanged.

Table 1 summarizes the mass spectral analysis of tryptophan and IAA isolated from H_2O and 30% D_2O grown plants. Natural isotope abundancies account for the approximately 10.0% of the molecules from the control plants with an m/z of 131-135. With the IAA there was no difference in the percent of molecules containing heavy isotopes for H_2O or 30% D_2O grown seedlings. This indicates there has be no

deuterium incorporation into IAA, and hence, no *de novo* biosynthesis by seven days of age. With tryptophan however, there was a substantial difference in the amount of heavy isotope containing molecules present. In plants grown on 30% D_2O, 43.8% of the tryptophan molecules contained heavy isotopes compared to 10.2% for those grown on H_2O. Correcting for natural isotope abundancies, 32.0% of all tryptophan molecules contain at least one heavy isotope as a result of the experimental conditions. We have clearly demonstrated deuterium incorporation into tryptophan by seven days of age. This however does not constitute definitive proof of *de novo* indole ring synthesis.

Preliminary IAA samples were subjected to a base exchange to remove any deuterium that might be of non-enzymatic origin. Previous work done in this lab by Magnus *et al.* (1980) has shown that the 2,4,5,6,and 7 positions are stable to base exchange in 14% KOH at 120^{o}C. After no deuterium incorporation into IAA was found in preliminary samples this step was dropped from the purification scheme. Using tryptophan synthetically deuterated in the side chain (D_3 2,3,3), the base exchange procedure was found to be ineffective at removing the deuterium from the molecule (Table 2). Deuterium in the side chain positions are stable, unlike that in IAA, so the base exchange step was dropped from the tryptophan purification scheme. Definitive proof of *de novo* biosynthesis is indicated by the presence of deuterium in

the indole ring. As a result of the non-exchangability of the side chain protons in tryptophan, the mere presence of incorporation is insufficient to indicate *de novo* biosynthesis. The tryptophan could be synthesized from some preformed indole ring with all of the deuteriums in the side chain.

Mass spectral data from these samples can not be used to determine the location of the deuteriums in the tryptophan molecules. Mass spectral analysis of synthetically side chain deuterated tryptophan (D_3 2,3,3) revealed that there is a rearrangement of deuteriums upon ionization such that the deuterium ended up in the benzene ring fragment (Table 3). In the plant samples, the presence of deuterium in this fragment could potentially be from the side chain. The use of mass spectrometry can not conclusively demonstrate that *de novo* synthesis has occurred. Location of the deuteriums was therefore accomplished through NMR analysis.

Excellent resolution of the ring protons of tryptophan was obtained with the 500 MHz NMR spectrometer (Figure 4a) A ^{2}H-NMR spectrum of tryptophan isolated from seven day old seedling grown in the dark on 30% D_2O revealed the presence of deuterium in the 2 and 3 positions of the side chain and the 6 position of the indole ring (Figure 4b). Additional ring positions may be labelled but the resolution is such that this may only be hypothesized. In any case there is definitely deuterium present in the indole ring of trypto-

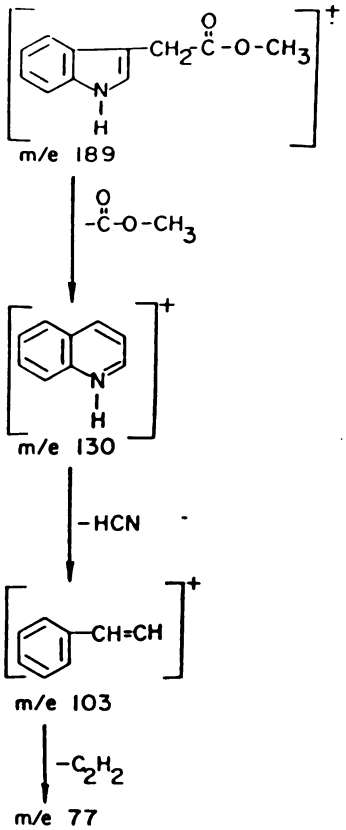
phan, therefore we have conclusively shown that tryptophan is synthesized *de novo* by 7 days of age.

Based on the mass spectral and NMR data, we conclude that as of 7 days of age *Zea mays* cv. Silver Queen seedlings have yet to begin *de novo* biosynthesis of IAA. At this point in growth, all of the IAA used by the seedling must come from the hydrolysis of IAA conjugates or from a preformed indole ring. The *de novo* synthesis of tryptophan, however, has begun by day 7, as indicated by the high level of deuterium incorporation into the molecules. The lack of *de novo* synthesis for IAA may be the result of the high level of IAA stored in the seeds (Haagen-Smit *et al.*, 1942, Avery *et al.*, 1944, Ueda and Bandurski, 1969, Piskornik, 1975), and small pool size and slow turnover rate. In view of this data, the metabolism of IAA may be viewed as being a closed system in young seedlings. Tryptophan on the other hand has a very large pool, and the turnover rate is very rapid, increasing the likelihood of deuterium incorporation. Most of the amino acids released by storage proteins during germination are transported to the seedling intact (Oaks, 1965). There is evidence however that the aromatic amino acids are synthesized *de novo* (Sircar, 1967, Mitra *et al.*, 1976). This is further supported by the high activity of the pentose phosphate pathway which provides the precursors for aromatic biosynthesis (Higuchi and Shimada, 1967).

Based on a turnover rate of 5 pmol/hr (Bandurski, 1989) there is enough IAA present in *Zea mays* seeds to meet the seedlings needs for upwards of 6 weeks. The transition to autotrophy in *Zea mays* seedlings with respect to overall carbon metabolism has been determined by Deleens *et al.* (1983, 1984). Using CO_2 depleted in ^{13}C they found seedlings to be totally dependant on seed carbon reserves for the first 7 days. After 7 days, carbon from photosynthesis begins to appear. The seed continues to supply carbon to the seedling for up to 4 weeks. This further supports our findings that the seedling is totally reliant on seed stores of IAA during early seedling growth.

Figure 1. Mass spectral fragmentation patterns for methylated IAA (A) and methylated and acetylated tryptophan (B)

A


m/e 189
-C-O-CH3
m/e 130
-HCN
CH=CH
m/e 103
-C2H2
m/e 77

B

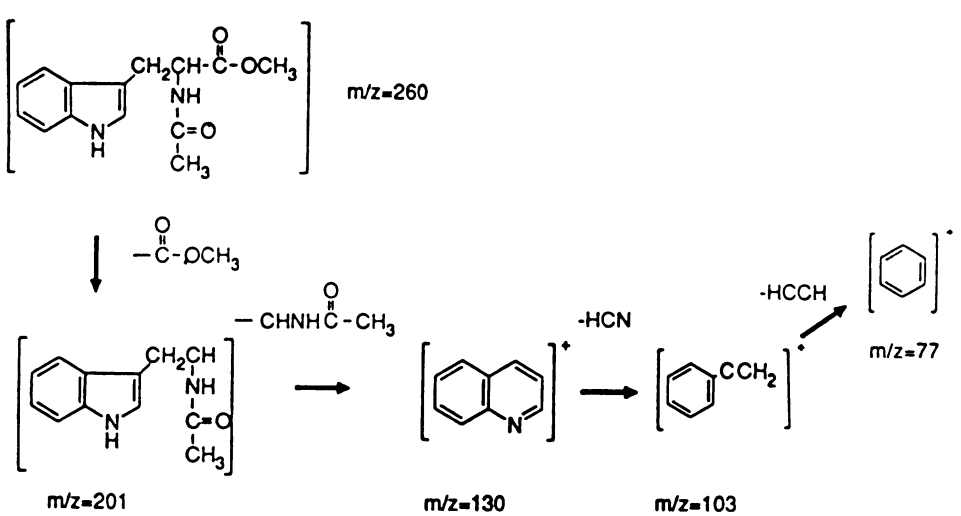

m/z=260
-C-OCH3
-CHNHC-CH3
-HCN
-HCCH
m/z=77
m/z=201
m/z=130
m/z=103

Figure 2. GC-MS total ion chromatograms of methylated IAA (A) and methylated and acetylated tryptophan (B) isolated from *Zea mays* seedlings grown in 30% D_2O for 7 days in the dark.

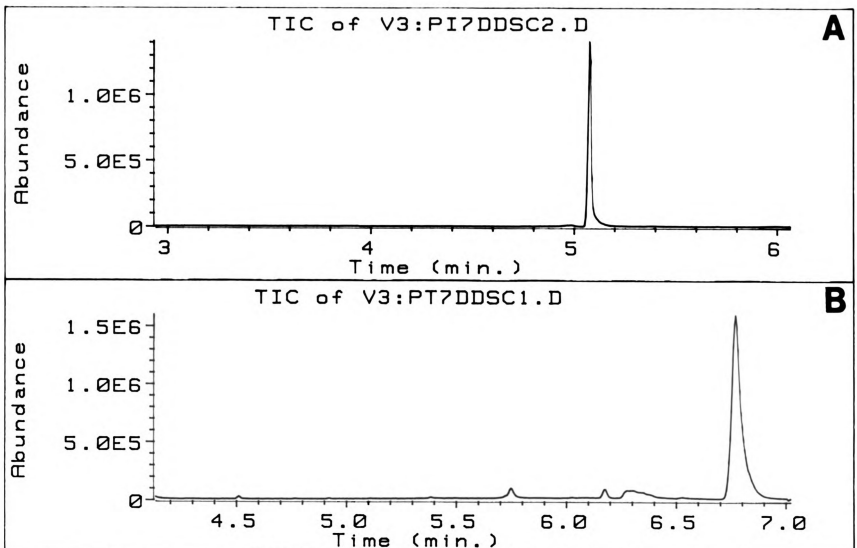

TIC of V3:PI7DDSC2.D
A
Abundance
1.0E6
5.0E5
0
3
4
5
6
Time (min.)
TIC of V3:PT7DDSC1.D
B
Abundance
1.5E6
1.0E6
5.0E5
0
4.5
5.0
5.5
6.0
6.5
7.0
Time (min.)

Figure 3. Partial mass spectrum showing the quinolinium ion and its associated isotope peaks from control (A) and methylated and acetylated tryptophan (B) isolated from *Zea mays* seedlings grown in 30% D_2O for 7 days in the dark. An increase in isotope abundancies for the isotope peaks compared to the controls is evidence for the incorporation of deuterium.

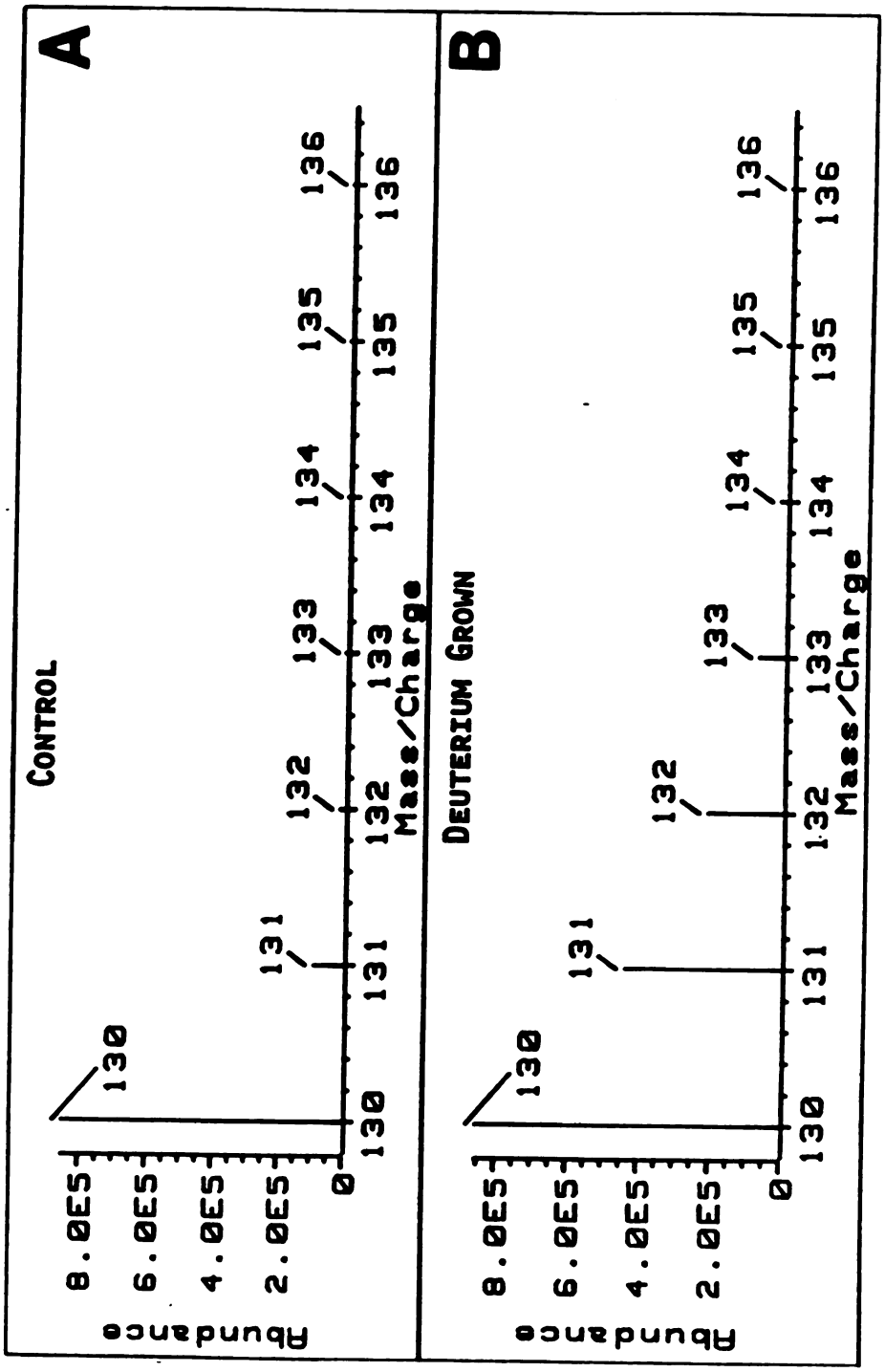

A
Control
Abundance
8.0E5
6.0E5
4.0E5
2.0E5
0
130
131
132
133
134
135
136
Mass/Charge
B
Deuterium Grown
Abundance
8.0E5
6.0E5
4.0E5
2.0E5
0
130
131
132
133
134
135
136
Mass/Charge

Table 1. Incorporation of deuterium into indole-3-acetic acid and tryptophan by *Zea mays* grown on H_2O or 30% D_2O for 7 days in the dark

Abundancies of the isotope peaks for the Base Peak (percent of total)

	Control	30% D_2O
	Tryptophan	
m/z 130	89.0 ± 0.1	56.2 ± 1.6
131	10.19 ± 0.3	26.1 ± 1.6
132	0.59 ± 0.07	12.4 ± 1.5
133	0.04 ± 0.03	3.9 ± 0.7
134	0.07 ± 0.05	0.9 ± 0.2
135	0.06 ± 0.07	0.4 ± 0.04
136	0.09 ± 0.07	0.08 ± 0.06
	IAA	
m/z 130	88.8 ± 0.1	88.4 ± 0.2
131	10.27 ± 0.3	10.04 ± 0.1
132	0.59 ± 0.09	0.90 ± 0.35
133	0.12 ± 0.12	0.22 ± 0.08
134	0.04 ± 0.04	0.07 ± 0.05
135	0.06 ± 0.03	0.21 ± 0.09
136	0.15 ± 0.09	0.12 ± 0.07

Table 2. Percent abundancies of the isotope peaks for the MS fragment at m/z 130 for D_3 2', 3', 3', Tryptophan before and after base exchange treatment

m/z	% abundance standard	exchanged
130	1.8	1.8
131	4.9	5.1
132	77.1	82.6
133	9.5	9.7
134	4.9	0.7
135	1.8	0.0

Table 3. Percent abundancies of the isotope peaks for the MS fragment at m/z 77 for D_3 2', 3', 3', tryptophan showing movement of side chain deuterium into the benzene ring fragment.

m/z	% abundance
77	23.4
78	42.9
79	29.0
80	4.1
81	0.5

Figure 4A. ^{1}H-NMR spectrum of methylated and acetylated tryptophan isolated from *Zea mays* seedlings grown in 30% D_2O for 7 days in the dark.

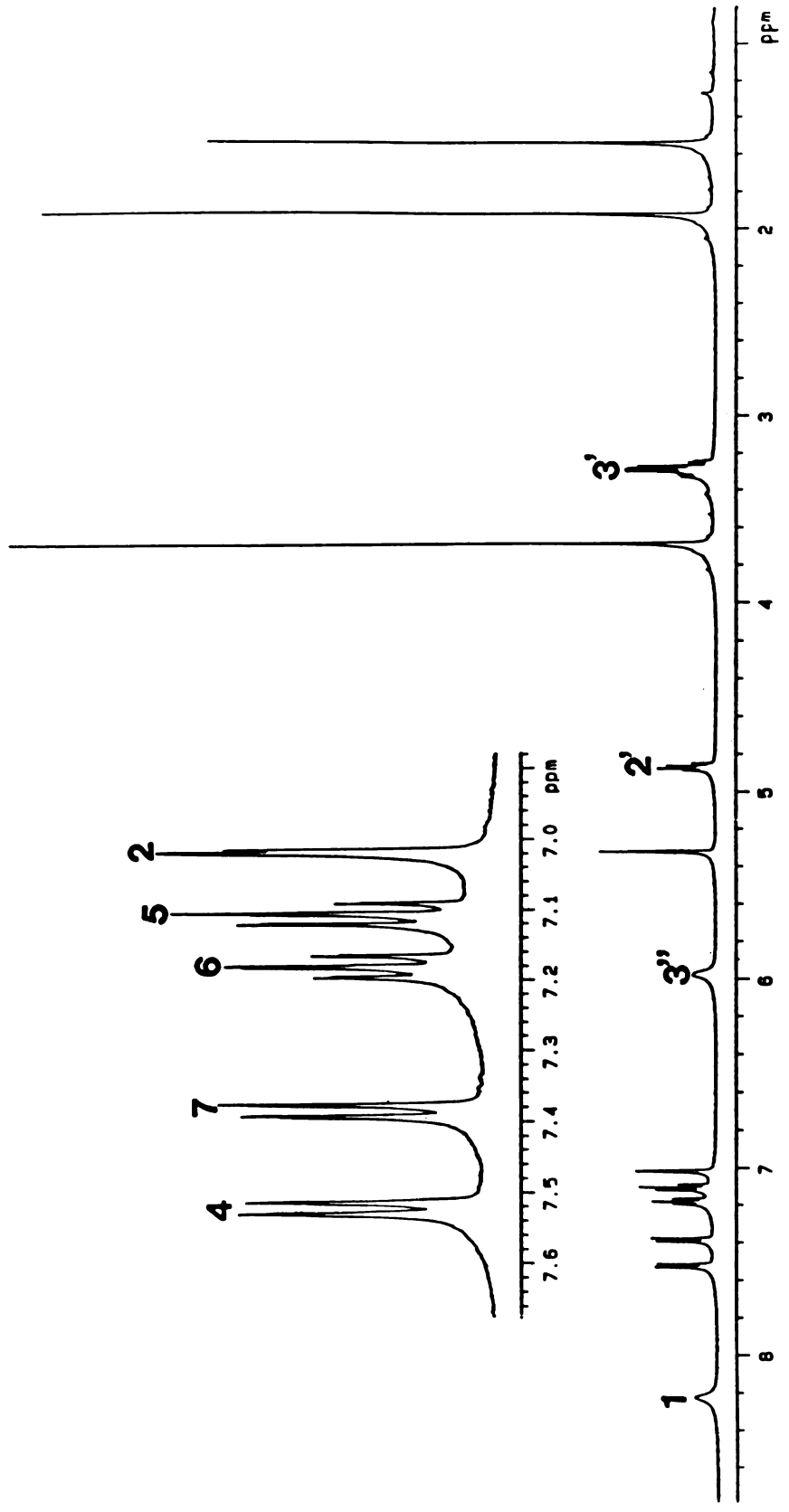
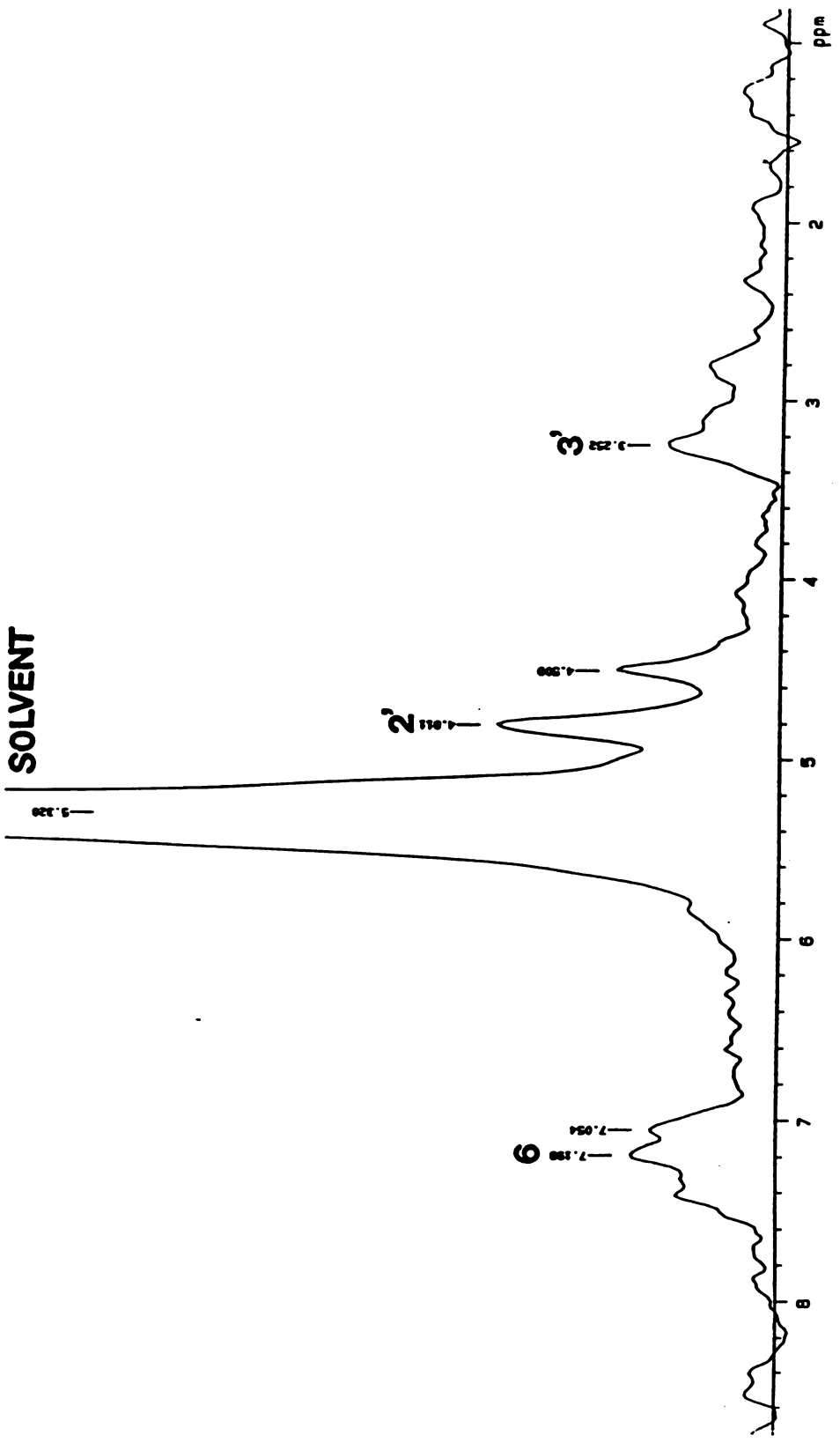

1
2
3
4
5
6
7
2'
3'
3''
ppm
7.6 7.5 7.4 7.3 7.2 7.1 7.0 ppm

Figure 4B. ^{2}H-NMR spectrum of methylated and acetylated tryptophan isolated from *Zea mays* seedlings grown in 30% D_2O for 7 days in the dark.


SOLVENT
5.320
4.014
2
4.000
3.252
3
7.390
7.054
6
ppm
2
3
4
5
6
7
8

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EXPERIMENTAL II

METABOLISM OF INDOL-3-YLACETATE IN MATURING KERNELS OF *ZEA MAYS*

ABSTRACT

Mature kernels of Zea mays sweet corn contain one per cent of total indole-3-acetic acid (IAA) as free IAA and the remainder as esters with a small percentage as amide linked conjugates (Plant Physiol. 1977. 60:211-213). The origin of IAA in the kernel has never been determined. The ultimate source is the leaves, but it is not known whether IAA, an IAA precursor, or an IAA conjugate is transported from the plant leaf into the developing endosperm of the kernel. In an attempt to distinguish between the above alternatives, GC-MS assays of free IAA and ester IAA were made at zero to 45 days after pollination. Free IAA remains relatively constant throughout the entire period at about 0.2 ξmol kernel^{-1} and there is essentially no ester IAA until day 10. Ester IAA increases from day 10 to day 45 reaching a final value of 500 ξmol kernel^{-1}. Additional experiments have lead us to conclude *de novo* synthesis of IAA is occurring in the endosperm. Since free IAA never accumulates we conclude that IAA biosynthesis in the kernel or transport of IAA precursors, and not esterification, is the rate limiting step in the accumulation of IAA in the developing kernel. Thus we established the rate at which IAA accumulated in the kernel and this enabled us later to determine whether that rate of synthesis occurred in the kernel.

INTRODUCTION

Conjugation and storage of plant hormones in developing plant seeds is a general phenomenon (Avery *et al.*, 1942, Hatcher, 1943, Miller *et al.*, 1987, Bialek and Cohen, 1989). The stored hormones are available as sources of hormone for the developing seedling plant (Epstein *et al.*, 1980), are protected against oxidation (Cohen and Bandurski, 1978) may serve as transport forms (Nowacki and Bandurski, 1980), and ultimately serve in mechanisms for hormonal homeostasis (Bandurski *et al.* 1988). Seeds, as rich sources of plant hormones, have been used in agriculture in antiquity to promote rooting of cuttings. Definitive chemical studies began with Cholodny (1935) who recognized that the endosperm of cereals was a rich source of growth hormone after mild treatment that would hydrolyze bound forms. Pohl, Laibach and Meyer, Hatcher and Gregory, Avery *et al.*, [for reviews see Thimann and Went (1937), Cohen and Bandurski (1982)] showed that the seed hormone precursor was readily converted to the same hormone found in the tip of grass coleoptiles. Finally, Heyn (1935) and Skoog (1937) demonstrated that removal of the endosperm made the seedling more responsive to applied IAA thus developing the concept of a seed auxin precursor.

In this paper we describe a time course for the biosynthesis of free and ester IAA in the endosperm of *Zea mays*. These data demonstrate that free IAA does not build up in the kernel but that the IAA is esterified as rapidly as it

reaches, or is synthesized in, the kernel. In much earlier studies, and using less exacting analytical methods, Corcuera (1967), Avery *et al.* (1942), and Teas and Newton (1951) for maize, and Hatcher, for rye (1943), had described time courses. In an earlier paper, we studied changes in free and ester IAA during maize kernel germination (Ueda and Bandurski, 1969) and observed a similar, but reverse, sequence of events. Additional data have led us to conclude IAA is produced *de novo* in the seed (see experimental III).

MATERIALS AND METHODS

Plant Material

Experiments were conducted on sweet corn plants (*Zea mays* L., cv Silver Queen) grown under field conditions. Ear shoots were bagged prior to silk emergence and then sib pollinated once the silks had emerged. In an attempt to compensate for variability due to weather conditions, two groups of ears were pollinated on different dates. For the IAA determinations two ears from each pollination group were harvested every 5 days from 0 days until 45 days after pollination (DAP). Additional ears were harvested at 58 DAP and allowed to dry down at room temperature. Free and total IAA determinations were conducted on fresh tissue. Each sample consisted of kernels from one ear for a total of four samples per age.

IAA purification

For the determination of free and total IAA, 15 to 90 fresh kernels were extracted with acetone (final conc.= 70%) after being frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. An internal standard of [$^{13}C_6$]-IAA (100 μg/sample) and approximately 80,000 cpm of [^{3}H]-IAA for peak detection were added to the samples. The samples were allowed to extract for 18 hours at 4 ^{o}C then centrifuged at top speed for five minutes in a benchtop centrifuge (1300 G, Internationl Equip. Co.) and the supernatant liquid divided for free and total IAA analysis.

Free and total IAA were purified using a modification of a method developed by Chen *et al.* (1988). Quantification was accomplished through the use of stable isotope labelled IAA as an internal standard (Magnus, 1980, Cohen *et al.*, 1986). For the determination of free IAA, 6 ml of the extract was diluted with 5 ml of double distilled H_2O and applied to a DEAE-Sephadex A-25 column (acetate form, 1.5 ml bed vol., Pharmacia) packed in a 3 ml syringe fitted with a polypropylene frit. Using a vacuum manifold, the column was sequentially washed with 2 ml of 50% ethanol, methanol, ethyl acetate, hexane, ethyl acetate, methanol, 50% ethanol and 50% acetonitrile containing 0.75% acetic acid. Though the packing underwent drastic changes in volume with the use of 100% organic solvents the IAA was still retained. IAA was eluted from the column with 3 ml of 50% acetonitrile

containing 2% acetic acid and dried under vacuum at 50 ^{o}C. The sample was taken up in solvent and chromatographed on an HPLC (LDC/Milton Roy model CM 4000 pump with a model 3000 spectro Monitor variable UV detector set at 280 nm) equipped with a C18 column (5 micron ODS, 25 X 4.6 cm, Val-U-Pak, Regis). The mobile phase was 20% acetonitrile/water containing 1% acetic acid. The 3 ml fraction at the retention time of IAA was checked for radioactivity, and dried *in vacuo*. The sample was then taken up in 200 μl of methanol and methylated with 200 μl of diazomethane at room temperature for 5 minutes. After methylation the sample was dried at 45^{o}C under a stream of nitrogen, taken up in solvent and rechromatographed on the C18 HPLC column using 45% acetonitrile/water as the solvent. The IAA fraction was dried *in vacuo* and taken up in acetonitrile for GC-MS analysis.

For the determination of total IAA, 2 ml of the extract was dried to aqueous phase *in vacuo* then an equal volume of 2N NaOH was added to give a final concentration of 1N NaOH. The sample was purged with nitrogen, incubated for one hour at room temperature, acidified to pH 2.5 with 1N HCl, diluted with 2 ml of double distilled water and applied to a conditioned (washed sequentially with 5 ml of hexane, methanol, water, and 1% acetic acid prior to sample addition) C18 solid phase extraction cartridge (J.T. Baker Co.). The cartridge was washed with 10 ml of double distilled water and the IAA eluted with 2 ml of acetonitrile. The sam-

ple was dried *in vacuo*, taken up in solvent and HPLC purification was as above for the free IAA. Final recoveries ranged from 19% to 50% with an averaged of 34%.

GC-MS-SIM analysis

GC-MS-SIM analysis of the purified methylated IAA was conducted on a Hewlett-Packard 5890 GC with a capillary direct interface to a model 5970 MSD. The GC column was a 15.0 m DB-17 fused silica capillary column (0.25 mm id., 0.25 μm film thickness, J & W Scientific). The GC temperature program consisted of an initial 1 minute hold at 100 ^{o}C followed by a ramp to 230 ^{o}C at 30 ^{o}C/minute. The ions at m/z 130, 136, 189 and 195 were monitored and the peak areas measured to determine the $^{13}C/^{12}C$ ratio.

RESULTS

Developing kernel of *Zea mays* contain levels of IAA several orders of magnitude greater than that reported in vegetative tissue. For the first 10 days after pollination IAA levels increase only slowly. Beginning 10 DAP (days after pollination) there is over a thousand fold increase in the amount of total IAA reaching a maximum of 90 μg kernel^{-1} at 45 DAP then decreasing by approximately half for dry seed (Figure 1a). The change in IAA levels parallels the increase in fresh weight but slightly precedes the onset of dry weight accumulation (Figure 1b).

Plotting free and total IAA on a per gram dry weight basis clearly shows IAA levels begin to rise before the onset of dry weight accumulation and at a rate independent of the change in dry weight (Figure 2a). When the levels of free and total IAA are plotted on a per gram fresh weight basis it is obvious that as rapid kernel growth begins, the concentration of IAA remains relatively constant (Figure 2b).

Compared to the amount of total IAA, the free IAA levels remain relatively constant at about 1 to 2% of the total. Considered alone the free IAA undergoes a large increase between 5 to 20 DAP from 28 ng/kernel to approximately 1000 ng/kernel and then remains at relatively constant level for the rest of the growth period (Fig 3). During the period of rapid increase in total IAA the ratio of ester to free IAA is maintained at a constant percentage (Figure 4) when computed on a fresh weight, dry weight, or per kernel basis. Free IAA constitutes approximately 1-2 percent of the total the remaining IAA exists as ester conjugates, there was no detectable amide linked IAA (see appendix B).

Figure 1. Comparison of changes in the levels of free and total IAA (A) and the accumulation of fresh and dry weight (B) during the growth and maturation of maize kernels. Vertical bars indicate the standard error from the mean of four replicate samples.

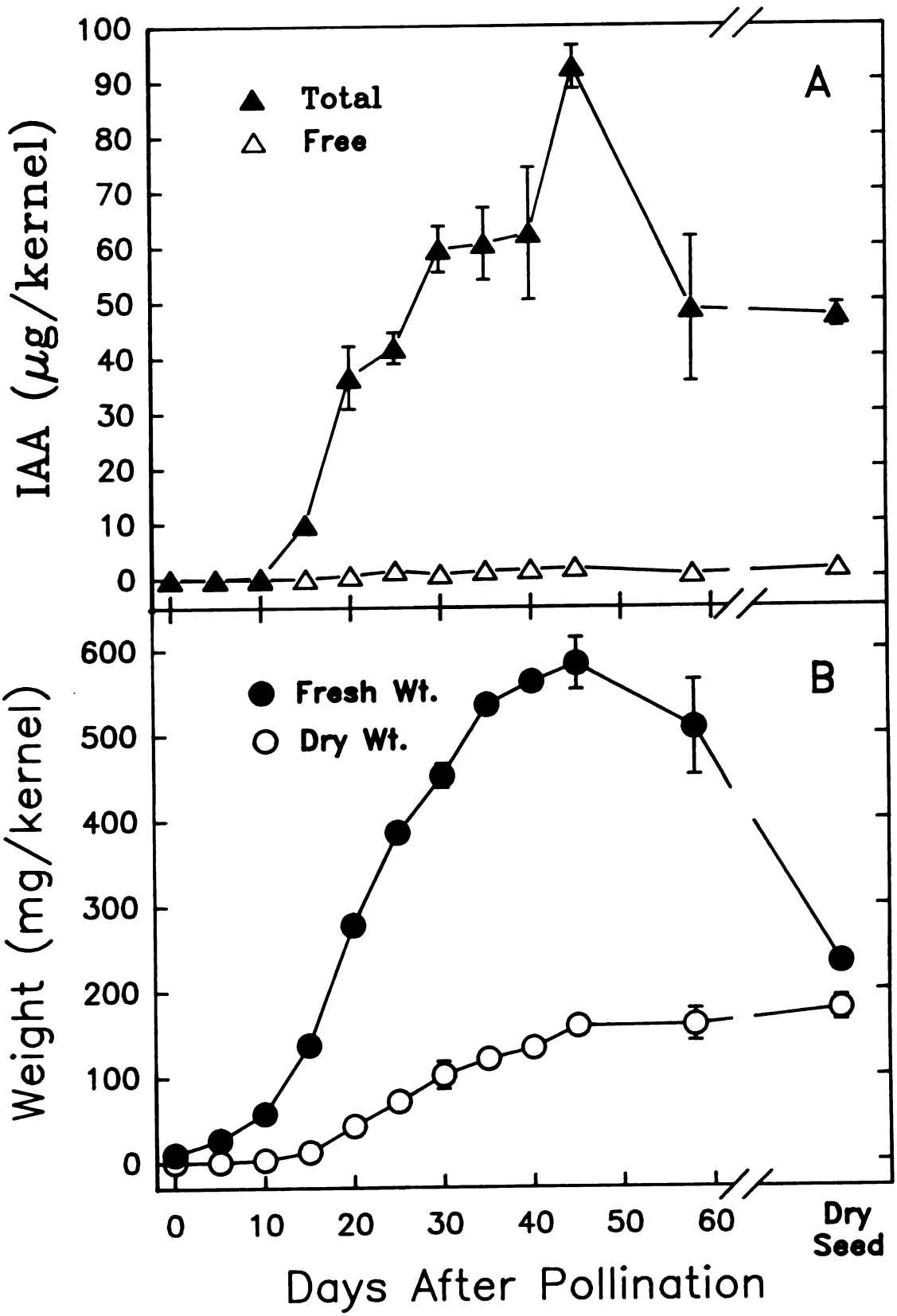
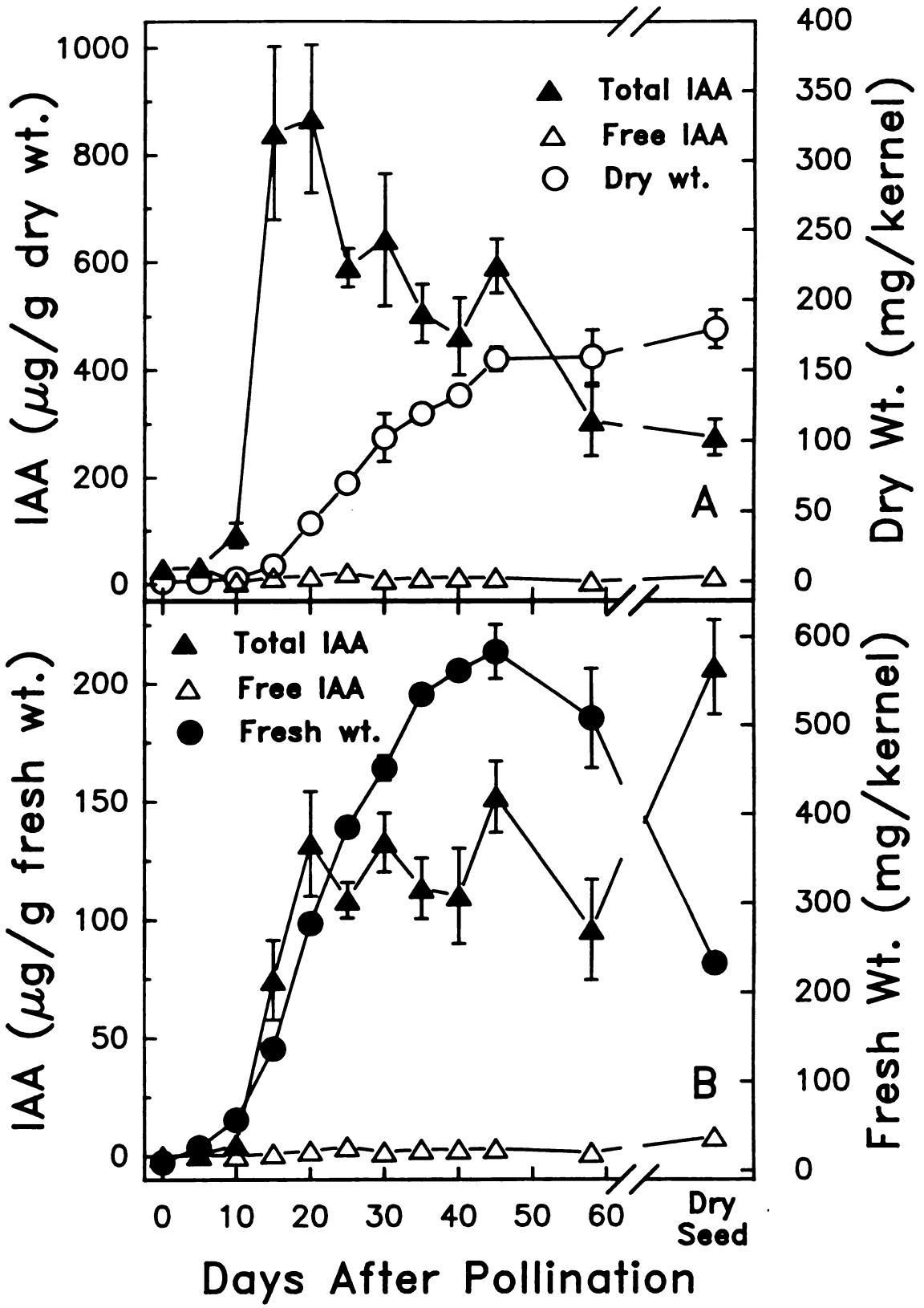

IAA (μg/kernel)
100
90
80
70
60
50
40
30
20
10
0
Total
Free
A
Weight (mg/kernel)
600
500
400
300
200
100
0
Fresh Wt.
Dry Wt.
B
0
10
20
30
40
50
60
Dry Seed
Days After Pollination

Figure 2. Comparison of changes in the levels of free and total IAA when calculated on a per gram dry weight basis (A) and a per gram fresh weight (B) basis during the growth and maturation of maize kernels. Vertical bars indicate the standard error from the mean of four replicate samples.


IAA (μg/g dry wt.)
Dry Wt. (mg/kernel)
Total IAA
Free IAA
Dry wt.
A
IAA (μg/g fresh wt.)
Fresh Wt. (mg/kernel)
Total IAA
Free IAA
Fresh wt.
B
0 10 20 30 40 50 60 Dry Seed
Days After Pollination

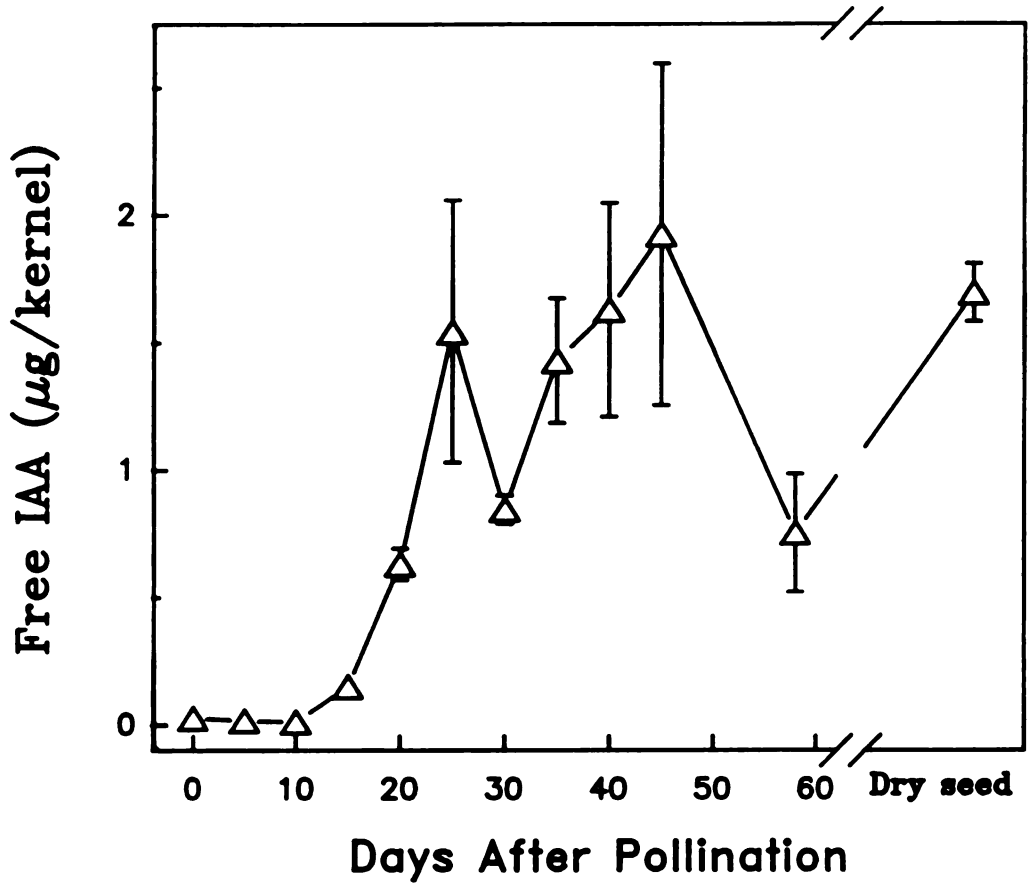


Figure 3. Changes in the levels of free IAA in the kernel during development

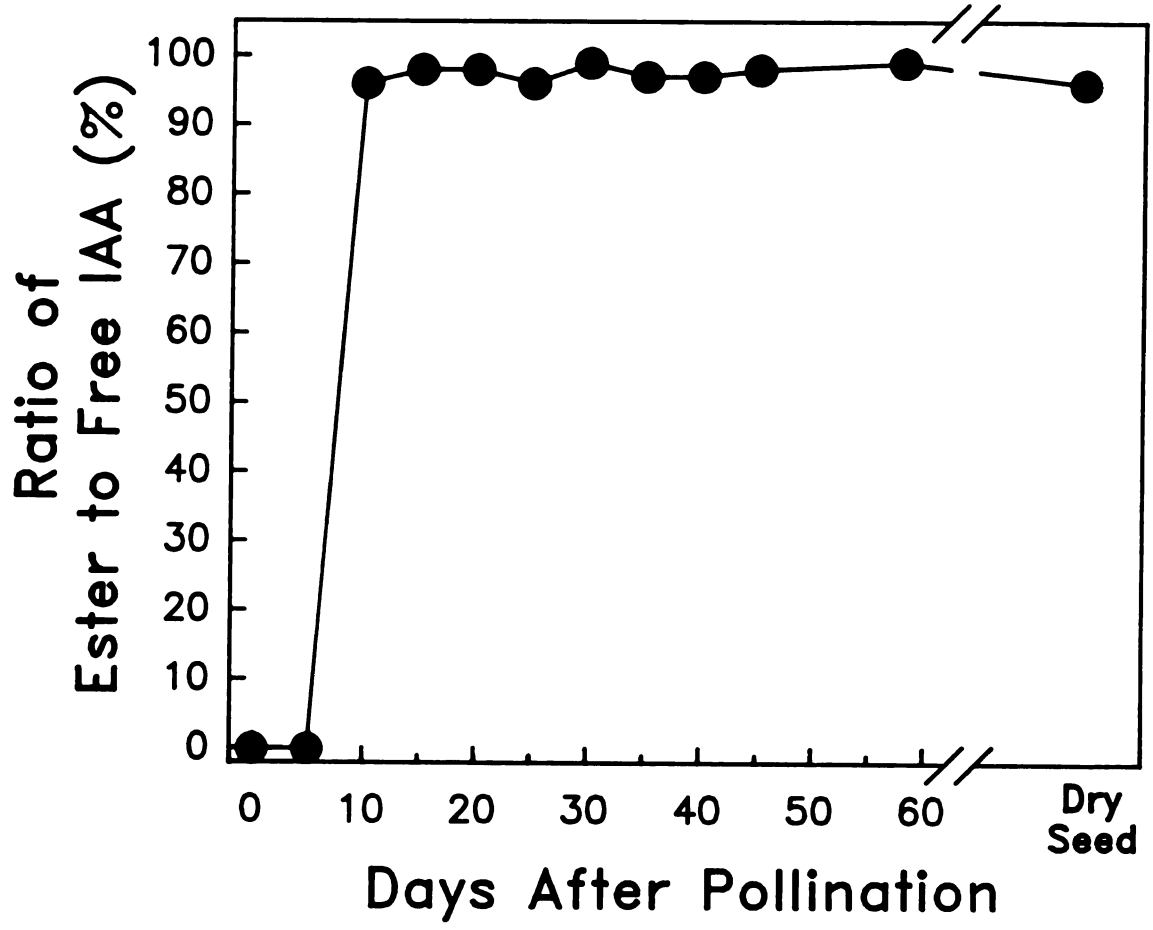


Figure 4. Percent of total IAA in the ester form during kernel development. The remaining IAA is in the free acid form.

DISCUSSION

The accumulation of IAA in the developing kernel can be divided into three phases. These phases are characterized by the periods of: (1) vascular development of the cob, (2) kernel filling, and (3) dormancy induction. During the first 10 DAP, IAA is present in the kernel in quantities typical of vegetative tissue. Only after the cob has reached a particular stage of development around 10 DAP does IAA become available for storage along with other typical storage compounds. This delay may simply be due to a lack of vascular development preventing the transport of IAA or its precursors into the kernel. Preliminary experiments (see appendix A) have been conducted involving the removal of blocks of kernels from ears 5 DAP resulting in a curvature of the cob towards the dekerneled area. When 1% IAA in lanolin is applied to the dekerneled area there is no subsequent curvature of the cob. This suggests the kernels and IAA play a role in the development of the cob similar to what Nitsch found with the removal of seeds from the achenes of strawberries (1950). Based on this, we believe IAA has some physiological role in the development of the cob. As a consequence of development, IAA is catabolized. At this early stage, it may be the nucellus which provides IAA to the growing cob. After cob growth is nearly complete and

the nucellus has disintegrated, it is likely that the endosperm is then responsible for the *de novo* biosynthesis of IAA for storage. This has been proposed earlier by Hatcher (1943) who, working with rye, conclude the grain was the site of auxin production based on the fact that prematurely harvested grains continued to accumulate auxin. Additional studies show that on hormone free media, *in vitro* grown *Zea mays* kernels produce almost normal levels of IAA and endosperm suspension cultures also produce IAA *de novo* (see experimental III).

Beginning with the period of rapid growth, large amounts of ester IAA appear in the kernels. The level of total IAA in the developing kernel clearly increases as the kernel matures. Earlier investigators working with *Zea mays*, also observed a large increase in IAA during development (Avery *et al.*, 1942, Teas and Newton, 1951). After reaching maximum levels the total IAA decreased 80% or greater depending on the variety or inbred used (Avery *et al.* 1942). Throughout development, sugary endosperms contained more IAA than starchy ones (Avery *et al.*, 1941, Teas *et al.*, 1952). The developmental stage at which maximum IAA levels were reached also varied with the variety or inbred. The levels of IAA were expressed on a per gram dry weight basis in their studies. The choice of units greatly affects the appearance of the data whether it be per kernel (Figure 1.), per gram fresh (Figure 2b.) or dry (Figure 2a.) weight as has been

suggested by Bialeck and Cohen (1989). In these earlier studies they failed to sample to the maximums in fresh weight and likely missed the true IAA maximum. Again this may be the result of how they calculated their IAA levels. When calculated on a per gram dry weight the IAA maximum occurs early during development (figure 2a). Differences between the previous work and the data presented here may be due to the use of primitive analytical techniques, the variety of maize, and in the case of Hatcher, the grain studied.

In seedlings of *Zea mays* around 88% of the total IAA exists as ester forms, the remainder as free (Bandurski and Schulze, 1977). In contrast to this, while the kernel is rapidly growing and accumulating IAA, the ester forms account for 97 to 99% of the total. This is indicative of the fact that as free IAA appears in the kernel it is immediately conjugated. This consistency can be explained by examining the thermodynamics of the first two steps of conjugation ending in the formation of IAA *myo*-inositol (IAInos).

	Δ G (calories)
IAA + UDPG ⟶ 1-O-IAGlu + UDP	+1400
1-O-IAGlu + myo-inositol ⟶ IAInos + Glu	-4200
IAA + UDPG + myo-inositol ⟶ IAInos + UDP + Glu	-2800

For every 1400 calories there is a one order of magnitude shift in the equilibrium of the reaction. A Δ G of -2800 calories for the sum of these reactions approximates a 99:1

ratio of ester to free IAA. This is identical to what we observed in the developing kernel. Therefore the observed rise in free IAA during grain filling is most likely only a result of the kinetics of the conjugation reactions. The difference between the ratio of ester to free IAA between seedlings and the developing kernel is noteworthy. This suggests a different or more simplified control mechanism operates in the kernel. It seems likely that production of IAA is regulated by some as of yet unknown means and it is the availability of free IAA that regulates the levels of IAGlu synthase and other enzymes involved in conjugation.

From the present study we can conclude that a control mechanism is operating that prevents accumulation of free IAA in the endosperm of the developing kernel. That such rigid control should be operative during both ripening and germination is indicative of the importance of controlled levels of the hormone both in seed and vegetative growth.

Unlike corn, it has been reported by Bialek and Cohen (1989) that during bean seed development the level of total IAA on a per seed basis remains fairly constant while the percentages of free, ester and amide change. A possible explanation for the lack of change in the amount of total IAA could be that IAA in bean seeds is produced elsewhere in the plant and then transported to the seed. This is exactly opposite to what we have observed in corn. In a study on the location of indole alkaloid biosynthesis in *Ipomea* it was

concluded that alkaloids were produced in the leaves and then transported to the seed (Mockaitis *et al.*, 1973). This again is opposite what we have observed in corn for IAA, but may represent an intrinsic difference between monocots and dicots. More recently, Bialek and Cohen have shown that *de novo* synthesis provides the developing bean seedling with IAA. In this system, IAA conjugates in the seed have little or no role in this regard (1991). This is contrary to what we have found with maize seedlings where there is no *de novo* biosynthesis and the seedling is totally reliant on IAA from conjugates stored in the seed (see experimental I).

No role for IAA in the regulation of embryo growth is known at this time. The changes in total IAA levels run parallel to the change in total protein. A time course of total protein levels in corn during kernel development shows a plateau between 25 and 35 DAP (Ingle *et al.*, 1965). We observed an identical plateau at this age in the levels of total IAA. These two may in fact be related developmental processes as was suggested by Bialek and Cohen for beans (1989), but no direct evidence for this relationship has been demonstrated.

Several researchers have been using *in vitro* culture of *Zea mays* kernels to examine the biochemical and physiological aspects of kernel growth and development (Cully *et al.*, 1984, Singletary and Below, 1989, 1990, Singletary *et al.* 1990). These researchers report that growth of kernels in culture

is roughly equivalent to growth of kernels in the field. We propose the use of *in vitro* kernel culture as a powerful tool for studying the biosynthesis of IAA and it's regulation. We believe this system is ideal for elucidating the complete biosynthetic pathway for IAA. The high levels of IAA in *Zea mays* kernels indicate this pathway is extremely active in this tissue, making the system easier to work with. Preliminary studies with endosperm suspension cultures also indicates their ability to synthesize IAA *de novo*. We are currently conducting further experiments to determine the feasibility of these systems for the study of IAA metabolism.

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EXPERIMENTAL III

METABOLISM OF INDOLE-3-ACETIC ACID IN ENDOSPERM SUSPENSION CULTURES OF *ZEA MAYS*

ABSTRACT

The kernel of *Zea mays* accumulates large amounts of IAA during development. The origin of this IAA has not yet been determined. A clue to this question is the knowledge that *Zea mays* endosperm can grow *in vitro* on media containing no growth hormones. This suggests the endosperm can make its own IAA, and is therefore the site of synthesis in the developing kernel. We have conducted a time course study using endosperm cells in suspension culture to determine if indeed they are synthesizing IAA. Levels of free IAA reached a maximum of 65 ng·g fwt^{-1} on the fourth day after subculture. The total IAA reached a maximum of 620 ng·g fwt^{-1} on the eight day after subculture, 2 days after the maximum in fresh weight. No amide IAA was found. Clear evidence that these cells are indeed synthesizing IAA is demonstrated by calculating the IAA levels on a per flask basis. Between the second and eighth days after subculture there was an increase of nearly 10 μg of total IAA in the flask. During this period the increase in fresh weight was only 10 gms. The high levels of IAA being produced by these cells make them an ideal system for studying the biosynthesis of IAA and its regulation.

INTRODUCTION

The existence of a growth regulating compound in plants was first proposed by Ciesielski in 1871 (Ciesielski, 1872). It was then more than fifty years before the structure of IAA was determined (Kogl *et al.*, 1934), and shown to be an endogenous component of plants in 1942 (Haagen-Smit). A definitive pathway for IAA biosynthesis in plants has yet to be demonstrated. This shortcoming has slowed progress in the other aspects of auxin physiology such as regulation and mode of action. A biosynthetic pathway of IAA involving tryptophan has been well established in several different bacterial species (Kosuge, *et al.*, 1966, Sequira and Williams, 1964, Thomashaw *et al.*, 1984). In plants, tryptophan has also been suggested to be a precursor of IAA. Several lines of evidence including lack of growth induction by tryptophan (Winter, 1966, Thimann and Grochowska, 1968) and questions about microbial contamination (Libbert *et al.*, 1966) contradict this hypothesis. More conclusive evidence that disproves the role of tryptophan as a precursor in *Zea mays* seedlings has been presented recently (Wright *et al.*, 1991). It has been proposed that two pathways for the biosynthesis of IAA may exist, a major one involving a pathway excluding tryptophan, and one from tryptophan which may be associated with stress (Bandurski *et al.*, 1992).

The literature on the various theoretical precursors and biosynthetic pathways is extensive yet a definitive

pathway has not been demonstrated in plants. One of the problems responsible for the slow progress in this field is the lack of a system that is unequivocally producing IAA *de novo* and does so at a readily quantifiable rate. Only recently working with *Lemna* (Baldi *et al.* 1991) and with whole mutant seedlings of *Zea mays* (Wright *et al.* 1991) has the production of IAA from labelled precursors been definitively demonstrated.

The endosperm of *Zea mays* kernels contain large amount of IAA ester conjugates which are used by the seedling during germination (Ueda and Bandurski, 1969). Working with rye in the 1940's Hatcher proposed that the increase in IAA during seed development was not the result of transport from the mother plant but that IAA was synthesized within the seed. *Zea mays* endosperm suspension cultures also have been initiated and grown for many years without the need for hormone supplementation (Shannon 1982). Given the likelihood that IAA is indeed produced *de novo* by developing *Zea mays* kernels we undertook a study to determine if endosperm cells of *Zea mays* produce IAA *de novo* in culture. In this report we will show that IAA is produced *de novo* and at levels greater than in normal vegetative tissue.

MATERIALS AND METHODS

Plant Material

Suspension cultures of endosperm cells derived from the *Zea mays* inbred A636 were kindly provided by Dr. Jack Shannon of the Pennsylvania State University. The cells were grown on liquid medium containing Murashige and Skoog salts, 3% sucrose, 2 g/L of asparagine and 0.4 mg/L of thiamine HCl. The pH was adjusted to 5.6 prior to autoclaving. Cultures were grown in the dark at 29 ^{0}C on a rotary shaker at 120 RPM. Two one liter stock cultures grown for seven days after transfer served as a source of innoculum for the experimental flasks. Each stock culture was subcultured into one set of six 250 ml flasks, each containing 80 ml of media. Every two days after subculture one flask from each set was harvested, fresh weight determined and the tissue lyopholized for later analysis.

Zea mays kernels (hybrid B73 X LH51) grown *in vitro* were provided by Dr. Fred Below of the University of Illinois. The kernels were grown on solid medium containing Murashige and Skoog salts, 15% sucrose, 2 g/L of asparagine and 0.4 mg/L of thiamine HCl. No hormones were added to the media. Kernels were harvested at 20 and 32 days after pollination.

IAA Purification

For the determination of free IAA 5% of each sample, based on dry weight, was extracted with 70% acetone under vacuum for 2 hrs. at room temperature. [$^{13}C_6$]-IAA (100

μg/sample) as an internal standard and approximately 80,000 dpm of [^{3}H]-IAA for peak detection were added to the sample. After two hrs the sample was centrifuged at top speed for five minutes in a benchtop centrifuge (1300 G, International Equip. Co.) and the supernatant decanted off. The supernatant was then reduced in volume to aqueous phase and then diluted with two ml of water. The sample was then loaded using a vacuum manifold onto a preconditioned 10 mm X 1 cm semi-prep guard column (Upchurch Scientific) loaded with C18 pellicular packing (Whatman). The preconditioning, loading and washing procedures are identical to those of Chen *et al.* (1988) for a C18 SPE column. The column was conditioned by washing sequentially with 5 ml of hexane, methanol, water, and 1% acetic acid. After washing with 10 ml of water, the precolumn was inserted in place of the injector loop on the HPLC (LDC/Milton Roy model CM 4000 pump with a model 3000 spectro Monitor variable UV detector set at 280 nm) and the sample was eluted off the precolumn onto the analytical C18 column (5 micron ODS, 25 X 4.6 cm, VAL-U-PAK, Regis) with the HPLC solvent (20:80 Acetonitrile:1% Acetic acid). The peak containing the IAA was dried down then taken up in 200 μl of methanol and methylated with 200 μl of diazomethane at room temperature for 5 minutes. After methylation the sample was dried at 45 ^{o}C under a stream of nitrogen, taken up in acetonitrile for GC-MS analysis.

For the determination of total IAA an additional 5% of the sample, plus the internal standard, was extracted with 1N NaOH under vacuum for two hrs at room temperature. The sample was then acidified to pH 2.5 with 1N HCl and applied to the precolumn and purified as above for the free IAA. Final recoveries for both free and total IAA ranged from 20% to 50% with an averaged recovery of 36%.

GC-MS-SIM analysis

GC-MS-SIM analysis of the purified methylated IAA was conducted on a Hewlett-Packard 5890 GC with a capillary direct interface to a model 5970 MSD. The GC column was a 15.0 m DB-17 fused silica capillary column (J & W Scientific; 0.25 mm id., 0.25 μm film thickness). The GC temperature program consisted of an initial 1 minute hold at 100 ^{0}C followed by a ramp to 230 ^{0}C at 30 ^{0}C/minute. The ions at m/z 130, 136, 189 and 195 were monitored and the peak areas measured to determine the $^{13}C/^{12}C$ ratio.

RESULTS AND DISCUSSION

The growth curves (fresh and dry weights) of the *Zea mays* endosperm suspension cells during the course of the culture cycle are shown in Figure 1. The curves are typical for cells in culture inoculated at a high density. IAA levels determined at two day intervals are shown in Figure 2. IAA biosynthesis by these tissues supports the hypothesis that

the endosperm is a site of IAA production in the developing kernel.

In proposing a new model for studying IAA biosynthesis it is essential to show that the system is producing IAA *de novo* under normal conditions. The levels of free IAA during the growth cycle of the endosperm suspension cells are shown in Figure 2a. The maximum in free IAA occurs at four days after subculture. In other cultures where the levels of IAA have been determined the majority also have their free IAA maximum early during the exponential growth phase (Bekker *et al.*, 1976, Moloney and Elliot, 1982, Mousdale, 1982). It has been proposed that this is important in initiation of the exponential growth phase in autonomous and habituated cultures (Beiderbeck, 1977, Mousdale *et al.*, 1985). The high levels of free IAA we observed may explain the autonomous nature of our cultures. Normal cultures require the addition of an auxin to maintain growth. Rather than acting as an auxin itself, 2,4-D has been shown to promote IAA biosynthesis in cell cultures (Moloney *et al.*, 1983, Michalczuk *et al.*, 1992). In normal cultures the cells do not produce enough IAA for autonomous growth, such cultures require addition of an external auxin for growth to occur.

The levels of total IAA increase up to a maximum of 12.6 μg/flask eight days after subculture and then decreases to 6 μg/flask. The maximum in total IAA occurs 2 days after the maximum in fresh weight (Figure 2b). The continued in-

crease in total IAA after the maximum in fresh weight has been reached is noteworthy. At this time the significance of this is unclear. Presenting the data on a μg/flask basis most clearly shows that *Zea mays* endosperm suspension cells are indeed producing large amounts of IAA. 9.5 μg of IAA was produced per flask in six days therefore the biosynthetic rate is approximately 66 ng/hr/flask. Since only inorganic salts, sucrose, thiamine and asparagine are present in the media, the increase in the levels of IAA is direct proof that *de novo* synthesis has occurred. Preliminary experiments indicate a large amount (up to 20 μg/flask) of IAA is excreted into the media. It is likely therefore that the biosynthetic rate may be up to twice that mentioned above.

The curve for IAA levels in the endosperm suspension culture is very similar to that in developing kernels (Jensen and Bandurski 1991). The cultured cells appear to retain the characteristics of the intact kernel with respect to IAA dynamics. The apparent IAA biosynthetic capacity is only 10% that of the intact kernel on a per gram dry weight basis. Another major difference between the intact kernels and the cultured cells is the percent of the IAA in the ester form. In the developing kernels the ester IAA is maintained at around 98% of the total for most of the developmental period (see experimental II). In the suspension cells the ester IAA reaches a maximum of only 92% of the total, which is similar to observed levels in vegetative tis-

sue. This suggests that while in the kernel IAA is produced for storage, in the suspension cells the IAA produced is actively being utilized by the cells.

Moloney and Elliot have reported on IAA levels in normal *Acer* cell cultures (1982). In their system, free IAA increases to a maximum of approximately 275 ng/g dry weight as compared to our system where the maximum level of free IAA is approximately 1000 ng/g dry weight. Unfortunately they did not determine the level of IAA conjugates present in their samples as is true for almost all similar studies in other cultures. the levels of free and total IAA have been measured in carrot suspension cultures (Michalczuk *et al.*, 1992) and are similar to what we have reported. In their embryogenic line however, the levels of IAA drop when 2,4-D is removed from the medium. In the non-embryogenic line only a small decrease was observed when grown on 2,4-D free media.

The amount of free and total IAA found in *in vitro* cultured kernels is shown in Table 1. There has clearly been an increase in the amount of IAA present. The kernels were cultured on hormone free media so the observed increase in IAA is clear evidence that the kernel contains all of the enzymes required to biosynthesize IAA. Therefore, the large amounts of IAA appearing in the kernel during development are synthesized in the kernel, likely in the endosperm.

We have shown that *Zea mays* endosperm cells in suspension culture and *in vitro* grown kernels can synthesize IAA. Additionally, we know the rate of production which will allow us to determine the significance of any conversion of applied precursors to IAA. In doing so we can evaluate the importance of a variety of purported precursors of IAA to determine which pathway(s) is operating in these cells.

The high capacity of these cells to produce IAA is another attractive feature of this system. Any conversion of precursors should be readily observable and not require any rigorous or time consuming analytical procedures. This system should therefore yield results which are significantly above background with a high proportion of precursors being converted to IAA.

Endosperm cells in suspension culture are an ideal system for studying IAA biosynthesis. In the future we hope to further characterize the IAA metabolism of this system, including examining the apparent excretion of IAA into the media. Once this has been completed we can begin testing various compounds for their involvement in the production of IAA.

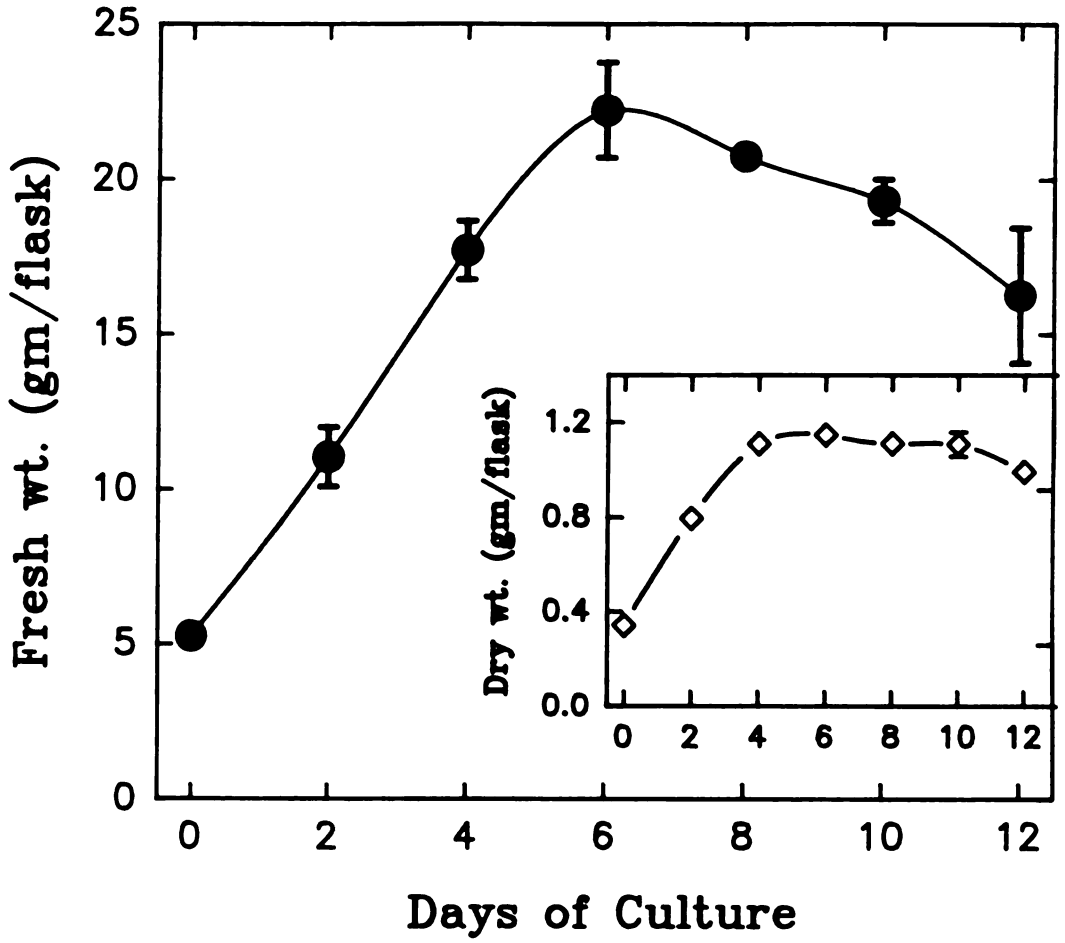

Figure 1. Fresh and dry weight of *Zea mays* endosperm cultures during the culture cycle. Culture were inoculated at a high density to obtain a rapid growth rate.

Figure 2. Comparison of changes in the levels of free and total IAA (A) and the accumulation of fresh and dry weight (B) during the life cycle of *Zea mays* endosperm suspension cells. Vertical bars indicate the standard error from the mean.

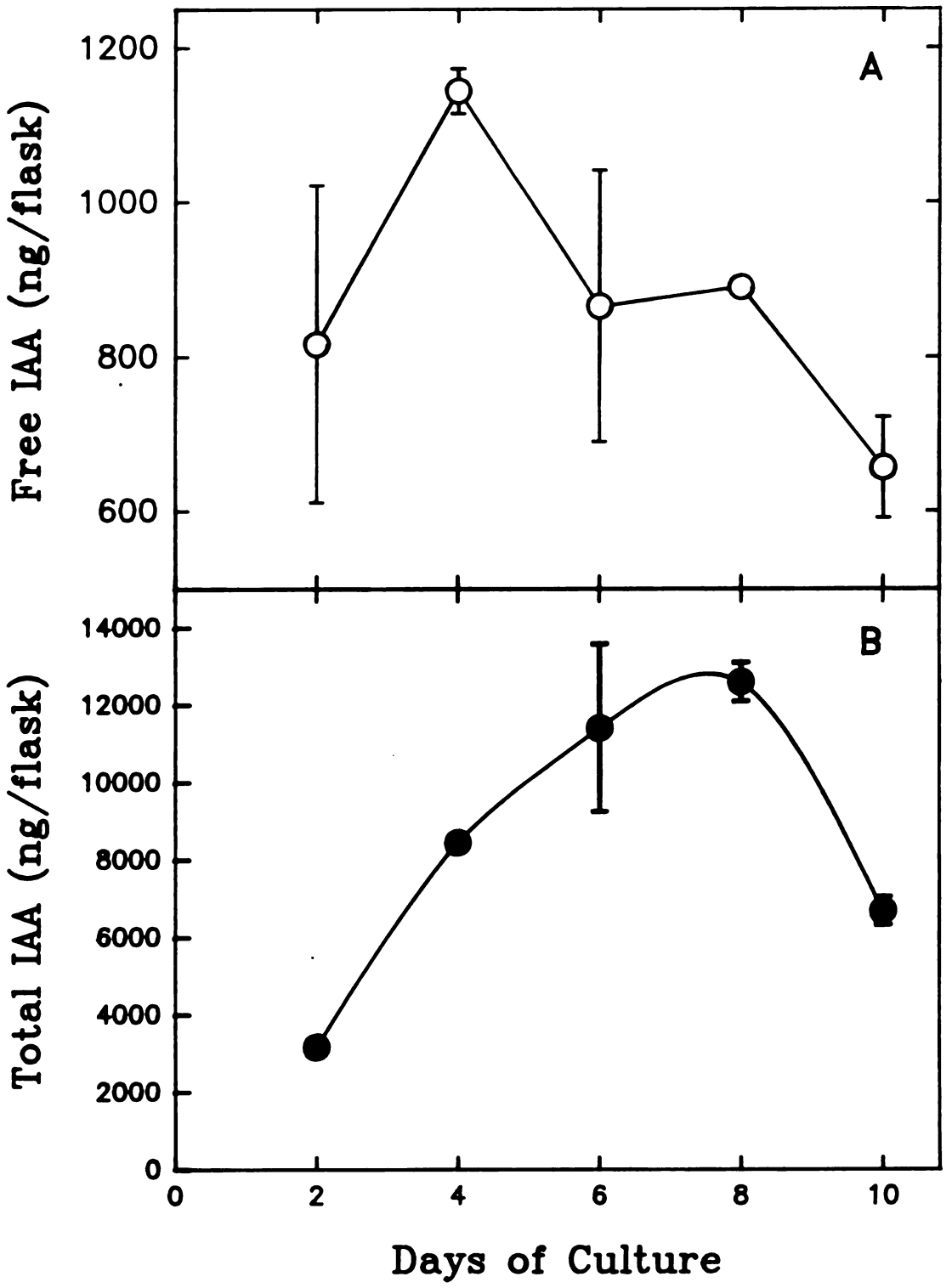
A
B
Free IAA (ng/flask)
Total IAA (ng/flask)
1200
1000
800
600
14000
12000
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
Days of Culture

Table 2. Synthesis of IAA and IAA esters by *in vitro* cultured kernels of *Zea mays*.

	IAA ($\mu g \cdot kernel^{-1}$)	
	Day 20	Day 32
Free	3.5	3.5
Total	16	33

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EXPERIMENTAL IV

ISOLATED IMMATURE *ZEA MAYS* ENDOSPERM AS A MODEL SYSTEM FOR STUDYING INDOLE-3-ACETIC ACID METABOLISM

ABSTRACT

The endosperm of *Zea mays* sweet corn contains as much as 0.5 μmol of esterified indole-3-acetic acid (IAA) in a single kernel. It was not known where, in the plant, IAA synthesis occurred: in the leaves, and then transported to the kernel as IAA or an IAA precursor, or in the kernel from sugars biosynthesized in the leaves. To that end, this laboratory has demonstrated that developing kernels cultured *in vitro* on hormone and tryptophan free media continued to accumulate large amounts of IAA. Further, endosperm tissue in suspension culture grow freely on hormone free media and retain almost 2% of the IAA biosynthetic rate of the attached kernel. Therefore it is certain that the endosperm of *Zea mays* contains all of the enzymes required for the *de novo* biosynthesis IAA. With the knowledge of the large capacity for IAA synthesis of the maize endosperm this system would be ideal for examining putative IAA precursors for their ability to sustain IAA synthesis. To determine the feasibility of this system we have examined the conversion of ^{14}C-anthranilic acid to IAA in isolated endosperm. Anthranilic acid was used because it is common to all proposed pathways yet occurs early enough so as not to presuppose one pathway over another. Data relating to the rate of IAA synthesis from anthranilic acid and proof as to the identity of the IAA will be presented.

INTRODUCTION

It was has long been assumed that Tryptophan (TRP) is a precursor of Indole-3-acetic acid (IAA). Thimann (1935) was the first to suggest TRP as a precursor of IAA based on his work with the fungus *Rhizopus suinus*. Wildman *et al.* (1947) provided the first demonstration of the conversion of TRP to IAA in plants. Numerous studies have been conducted since then which corroborate these findings.

Recently several reports have been published which present evidence that tryptophan is not a precursor of IAA in some plants (Baldi *et al.* 1991, Jensen and Bandurski 1990, Wright *et al.* 1991). The most dramatic of these used a tryptophan auxotroph mutant of maize (Wright *et al.* 1991). Embryos were germinated on media containing (^{15}N-indole)-tryptophan and after 6 or 10 days when the IAA was isolated there was no incorporation of the ^{15}N into the IAA molecules, even though the mutant produced 10 times more IAA than the non-mutant.

These experiments demonstrate the need to carefully reexamine the question of the biosynthetic pathway of IAA. Those experiments conducted in the past have failed to unequivocally demonstrate one pathway or the other in plants. To avoid this problem in the future a number of steps need to be taken in order to provide more conclusive results.

It must be demonstrated that the system normally makes IAA. Many studies on IAA biosynthesis have been conducted

using cereal coleoptiles. There is evidence however that the coleoptile of some cereals do not make IAA and are totally reliant on seed stores of the compound (Pohl 1935, Sheldrake 1973, Jensen and Bandurski 1990). Conversion of precursors to IAA using such systems may be the result of a pathway(s) not normally operative in the intact plant.

The pool sizes of the potential precursors and products should be determined so the relative importance of a compound as a precursor can be determined. There is a vast amount of data demonstrating the conversion of TRP to IAA (for reviews see Schneider and Wightman, 1974, Marumo, 1986) but the relative importance of this conversion has not been demonstrated. In a recent report Baldi *et al.* (1991) grew *Lemna* on media containing ^{15}N-L-Trp and was able to demonstrate the appearance of ^{15}N in IAA only when the level of Trp applied was 400-1600 times that of the normal pool size. When ^{15}N-D-Trp was fed, no conversion was observed at any feeding level. On the basis of pool size and the amount of labelled IAA produced they concluded Trp was not a significant precursor of IAA in *Lemna*.

There must be conclusive proof of the identity of any labelled compound produced as a result of metabolism. Identification of reaction products has often been made on the basis of a compound's Rf on paper chromatography. The radioactivity at the Rf's of interest is then determined and conclusions made based on this information. This however

does not constitute conclusive proof. When using radioactive precursors the product(s) first need to be purified to a constant specific activity to insure the radioactivity is associated with a particular peak. The compound must then be positively identified by mass spectral or some other qualitative analysis.

When possible a system that produces large amount of IAA should be used. The low levels of IAA, especially when compared to that of many proposed precursors, increases the difficulty in differentiating between biological and artifactual conversion. One potential source of error that has been demonstrated is the conversion of radioactive Trp to IAA with a 30% yield by simply drying in a flash evaporator (Epstein *et al.*, 1980). Using a system with a high capacity for IAA biosynthesis would help guarantee any conversion observed was of a biological nature.

The endosperm of maize contains large amounts of IAA (Haagen-Smit *et al.*, 1942, Avery *et al.*, 1944, Ueda and Bandurski, 1969, Piskornik, 1975), stored mostly as ester conjugates (Epstein *et al.*, 1980). During kernel development, IAA is produced in the seed and then rapidly conjugated for storage (see experimental II). The endosperm should therefore contain all of the enzymes required for IAA biosynthesis and conjugation. We have found that isolated endosperm system retains its ability to synthesize IAA. We tested the capacity of this system to biosynthesize IAA by using ^{14}C-

anthranilic acid as a precursor. Anthranilic acid was used because all IAA biosynthetic pathways proposed to date have this compound in common yet anthranilic acid occurs early enough so as not to presuppose one pathway over another. At this stage we do not wish to make conclusions as to the biosynthetic pathway of IAA. Rather, we concentrated on the suitability of using a maize endosperm system for further IAA biosynthesis research.

MATERIALS and METHODS:

Ears of *Zea mays* cv. Silver Queen were harvested between 22 and 30 days after pollination and used immediately. The ears were surface sterilized for 10 minutes in 10% bleach and then rinsed 4 times with sterile water. Several endosperms were removed from the kernels, placed in pairs in sterile microfuge tubes and gently homogenized with a glass rod. Additional endosperms were frozen at -80^{o}C for later analysis to determine the freezing stability of the system. Alternatively many endosperms were place in a sterilized mortar, homogenized, and then transferred to microfuge tubes in amounts approximately equal to two endosperms. To each tube approximately 250,000 dpm (8.6 nM) of anthranilic acid was added. Control samples were boiled for five minutes and cooled prior to the addition of the radiolabel. The samples were allowed to incubate for 0, 4, 12, and 24 hours, after

which the samples were analyzed immediately or frozen with liquid nitrogen and stored at -80^{o}C for later analysis.

IAA purification

Total IAA was determined using a modification of the method developed by Chen *et al.* (1988). To each sample an equal volume of 2N NaOH was added to hydrolyze the conjugates and any remaining enzymes. Approximately 80,000 cpm of [^{3}H]-IAA was added to the samples for determining recoveries and to aid in peak detection . After 1 hour at room temperature the samples were then acidified to pH 2.5 with 1N HCl, and centrifuged for five minutes in a microfuge. The supernatant was applied to a conditioned (column was washed sequentially with 5 ml of hexane, methanol, water, and 1% acetic acid prior to sample addition) C18 solid phase extraction column (J.T. Baker Co.). The column was washed with 10 ml of double distilled water and the IAA eluted with 2 ml of acetonitrile. The sample was dried under vacuum and taken up in solvent for HPLC purification. The sample was further purified on an HPLC (LDC/Milton Roy model CM 4000 pump with a model 3000 spectro Monitor variable UV detector set at 280 nm) equipped with a C18 column (5 micron ODS, 25 X 4.6 cm, Val-U-Pak, Regis). The mobile phase was 20% acetonitrile/water containing 1% acetic acid. The 3 ml fraction at the retention time of IAA was then checked for ra-

dioactivity (Beckman LS 1701). Final recovery values ranged from 32% to 70% with an average of 56%.

Some samples were additionally purified as follows. The fraction containing the putative IAA was dried under vacuum then taken up in 200 μl of methanol and methylated with 200 μl of an ethereal solution of diazomethane at room temperature for 5 minutes. After methylation the sample was dried at 45 ^{o}C under a stream of nitrogen, taken up in solvent and rechromatographed on the C18 HPLC column using 45% acetonitrile/water as the solvent. The radioactivity in the 3 ml fraction at the retention time of IAA was then determined. The sample was then dried under vacuum and taken up in acetonitrile for GC-MS analysis.

GC-MS analysis

GC-MS-SIM analysis of the purified methylated IAA was conducted on a Hewlett-Packard 5890 GC with a capillary direct interface to a model 5970 MSD. The GC column was a 15.0 m DB-17 fused silica capillary column (J & W Scientific; 0.25 mm id., 0.25 μm film thickness). The GC temperature program consisted of an initial 1 minute hold at 100 ^{o}C followed by a ramp to 230 ^{o}C at 30 ^{o}C/minute. The spectrometer was set to scan from m/z 50 to m/z 350.

RESULTS

The amount of radioactivity associated with the IAA HPLC peak after ^{14}C-anthranilic acid feeding for various in-

cubation times is shown in Table 1. As much as 42,500 DPM was found to be associated with the IAA peak after 24 hrs. of incubation. Two to eight percent of the total radioactivity applied was recovered in the IAA fraction. Based on the original specific activity, approximately 1.2 μg of anthranilic acid was added per experiment. Using an average conversion percentage of 6%, then roughly 72 ng of labelled IAA has been produced in 24 hours at a rate of 17 pmol/hr.

The number of DPM associated with IAA continued to increase as incubation times were increased (Fig 1.) leveling off between 12 to 24 hours

^{3}H-IAA was added at the beginning of purification to aid in peak identification and for determination of recoveries. The ratios of ^{14}C to ^{3}H in the samples at various points of the purification process are shown in Table 2. As expected the ratio decreases as other radioactive metabolites are removed from the sample. The ratios after both HPLC steps however remained unchanged, indicating no additional metabolites were removed following methylation and a second HPLC step. A GC-MS analysis of the IAA fraction resulted in a single peak (Figure 2a) with a spectrum identical to that of an IAA standard (Figure 2 b,c).

Table 1. DPM recovered in IAA fraction after HPLC purification step. IAA was isolated from *Zea mays* endosperm after incubation with ^{14}C-anthranilic acid.

Results of 3 separate feeding experiments.

sample	incubation times (hrs) 12	24	24
1	17,850 (3.5)*	42,539 (7.9)	29,891 (4.9)
2	11,200 (2.0)	40,997 (7.6)	42,292 (7.6)
3	20,050 (3.5)		28,053 (6.4)

* value in parenthesis is percent of applied ^{14}C converted to IAA

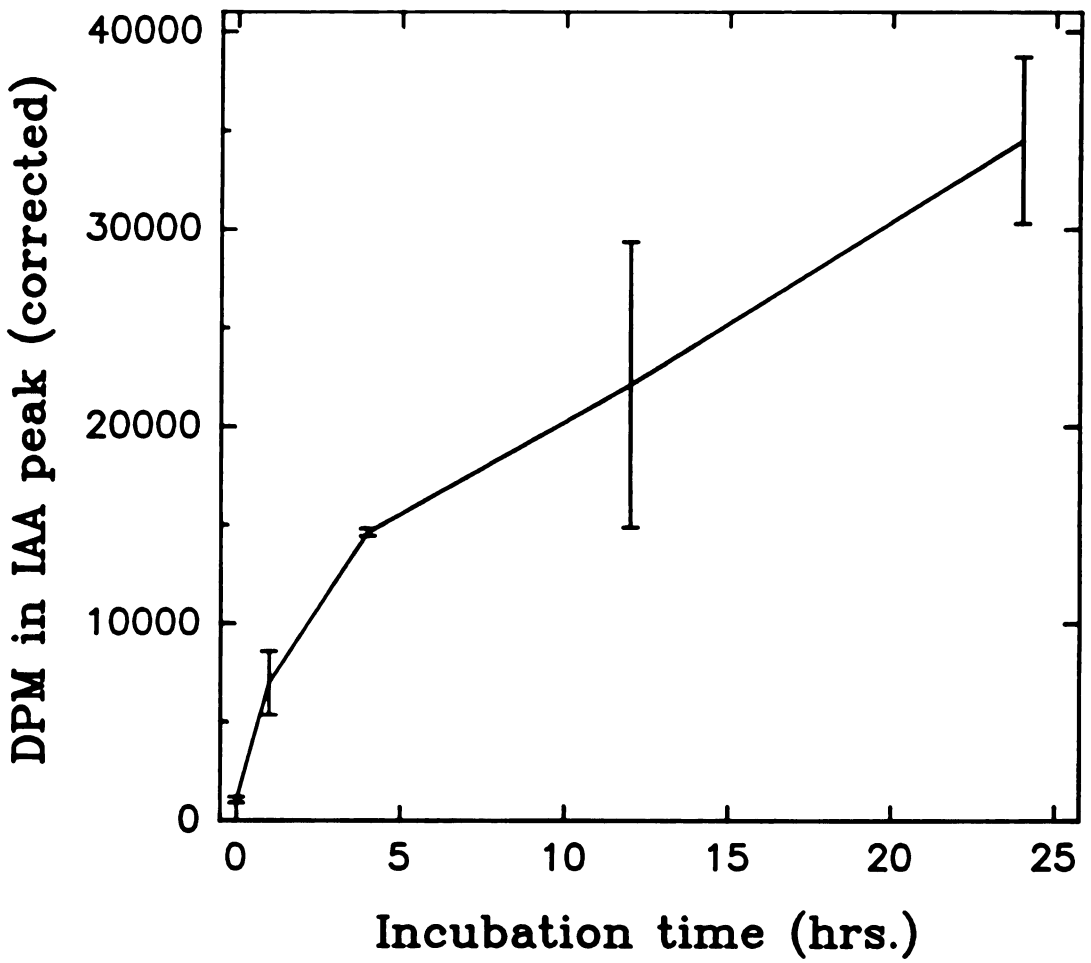


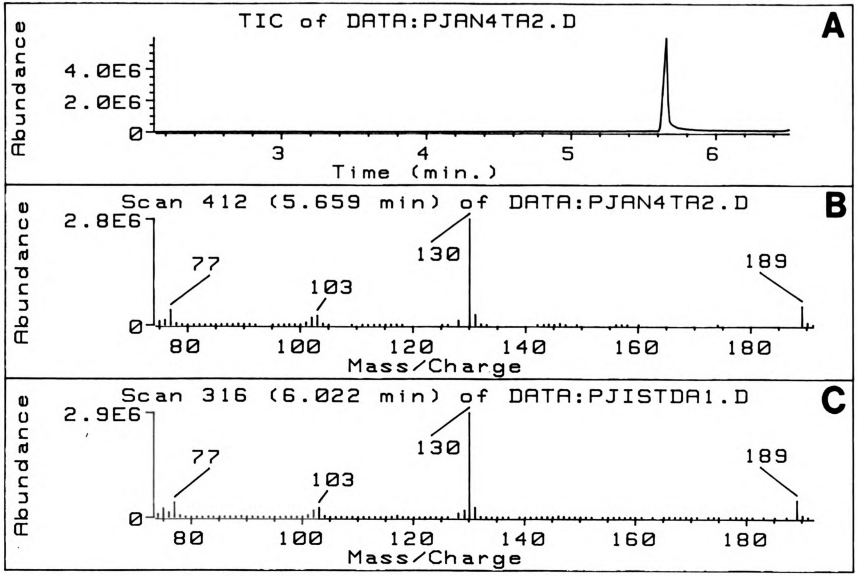
Figure 1. Time course showing DPM associated with IAA fraction from isolated endosperm incubated with ^{14}C-anthranilic acid.

Table 2. Ratio of ^{14}C to ^{3}H in IAA fraction at various stages of purification.

sample*	pre-SPE	post-SPE	post 1st HPLC	post 2nd HPLC
1	2.318	0.648	0.089	0.089
2	2.015	0.890	0.100	0.104
3	2.661	0.690	0.119	0.120

* all sample were incubated for 4 hrs. with ^{14}C-anthranilic acid. ^{3}H IAA was added at the beginning of purification.

Figure 2. (A) GC-MS chromatogram of HPLC fraction containing methylated IAA purified from isolated endosperm incubated with ^{14}C-anthranilic acid. (B) Mass spectrum of single peak observed in GC-MS chromatogram. (C) Mass spectrum of a methylated IAA standard.


TIC of DATA:PJAN4TA2.D
A
Abundance
4.0E6
2.0E6
0
3
4
5
6
Time (min.)
Scan 412 (5.659 min) of DATA:PJAN4TA2.D
B
Abundance
2.8E6
77
103
130
189
0
80
100
120
140
160
180
Mass/Charge
Scan 316 (6.022 min) of DATA:PJISTDA1.D
C
Abundance
2.9E6
77
103
130
189
0
80
100
120
140
160
180
Mass/Charge

DISCUSSION

The kernel of *Zea mays* contains more IAA than any plant tissue examined thus far (Bandurski and Schulze, 1977) The IAA, stored as esters can approach a concentration of 0.5 μmol/kernel (Jensen and Bandurski, 1991). We have previously demonstrated that the IAA is synthesized in the kernel from simple sugars and an amino donor, and is not synthesized in the leaves and transported to the kernel as IAA or an IAA precursor (Jensen and Bandurski 1991). The seed as a site of auxin production has been known for many years (Nutman, 1939, Muir, 1942), but has largely been ignored as a tissue to use for IAA biosynthesis experiments. Based on this we believe the endosperm of maize contains all of the enzymes required to synthesize IAA. Additionally the large amount of IAA found in the kernel suggests the endosperm has a high capacity for IAA production.

In most experimental systems studied thus far, less than 0.05 percent of the applied radioactive precursors have been converted to IAA. In our system we have observed conversion rates of up to 8 percent. The counts we obtained were significantly greater than the background and that obtained with boiled controls (data not shown). Conversions of this magnitude raise little doubt that we are indeed observing IAA biosynthesis in our system. Reported values for IAA turnover range from 1-10 pmol/hr in the shoot and up to 70 pmol/hr in germinating kernels of *Zea mays* (for review see

Bandurski, 1989). We have calculated the rate of IAA biosynthesis in our system to be 17 pmol/hr. This value, which is already greater than most observed values is based purely on the amount of radiolabelled anthranilic acid fed, and does not take into account the endogenous pool of anthranilic acid. As a result this value is an underrepresentation of the actual rate. In the future we will measure the pool size of anthranilic acid and other precursors so we can obtain a accurate rate of IAA biosynthesis.

Many of the early studies on IAA biosynthesis did not use rigorous methods to positively identify the metabolite(s) of interest. As a result, conclusive proof that the radioactivity associated with that metabolite is actually due to labelled metabolite has often not been presented. By adding ^{3}H-IAA to our samples we have been able to provide strong evidence that the radioactivity we observe is in fact due to labelled IAA produced through the metabolism of anthranilic acid. We have purified our samples to a constant ratio of ^{14}C to ^{3}H and therefore conclude the radioactivity observed was as IAA. It is highly unlikely that another metabolite would co-elute with both the free acid and methylated forms of IAA from an HPLC column. Contamination is indicated by changes in the ^{14}C to ^{3}H ratio as purification proceeds. This ratio will continue to change until on all contaminating metabolites have been removed. At this point the rates will remain constant throughout any further pu-

rification steps. Definitive proof that the compound we isolated is in fact IAA has been demonstrated through GC-MS analysis.

We know that maize kernels biosynthesize IAA *in situ*, and have shown that isolated endosperm tissue retains this capacity. Large amounts of IAA can be produced by this system and at a rate at least comparable to observed IAA turnover rates. In all likelihood the rate is probably several times greater than reported here. With such high activity the precursor pool sizes will be more readily determined. Preliminary experiments indicate this enzyme system is stable to freezing (data not shown). This will insure a plentiful year-round supply of experimental material. We believe this system will prove to be very useful in our further studies on IAA metabolism.

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APPENDEXES

APPENDIX A

Amide IAA Content in Kernels of *Zea mays* var. Silver Queen.

Amide linked IAA conjugates are abundant in beans (Bialek and Cohen, 1989), in contrast to that observed in corn (Epstein *et al.*, 1980). 1N NaOH is sufficient to hydrolyze ester-linked IAA conjugates. Hydrolysis of amide-linked IAA conjugates requires incubation in 7N NaOH at 100°C for one hour. Mature seeds from the same planting previously used for the survey of IAA levels in developing kernel were assayed for amide-linked IAA. These assays were conducted to check the previous findings and determine if amide-linked IAA has a function in the seeds of *Zea mays*.

Materials and methods:

Twenty-five dry seeds were frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. Two aliquots each equivalent to one kernel were weighed out. Samples were hydrolyzed in either 1N or 7N NaOH containing 1 μg of [$^{13}C_6$]-IAA as an internal standard and approximately 50,000 cpm of [^{3}H]-IAA as a radiotracer. The sample in 1N NaOH was hydrolyzed for one hour at room temperature. The sample in 7N NaOH was hydrolyzed for 1 hour at 100°C. Hydrolysis was carried out in Teflon PFA vials (Tuf-tainer, Pierce Chemical Co.). The caps were fitted with an inlet and outlet for nitrogen purging gas. The nitrogen gas was

passed through an oxygen scrubber and saturated with water prior to reaching the samples. After hydrolysis, the sample were acidified to pH 2.5 with HCl and centrifuged at 1300 G for 5 minutes. The acidified hydrolysates was purified for GC-MS analysis using SPE mini columns and two HPLC steps as described in experimental II.

Results and Conclusions

both hydrolysis procedures gave results that were nearly identical. The 1N NaOH hydrolyzed sample resulted in a value of 57,000 ng/kernel of total IAA. The 7N NaOH hydrolyzed sample resulted in a value of 56,700 ng/kernel of total IAA. Based on the lack of a difference in IAA amounts between the two hydrolysis procedures, there is no amide-linked IAA in the kernel of *Zea mays* var. Silver Queen.

APPENDIX B

Affect of the Kernel on Cob Development in *Zea mays*.

Studies by Nitsch (1950, 1954) have shown that removal of the seed greatly affects the development of the receptacles of strawberries. This effect is believed to be the result of a loss of growth promoters, presumably IAA, produced in the seed. Asymmetric growth could be obtained by removing the seeds from portions of the receptacle. The cob of an ear of corn is very similar in anatomy to the receptacle of strawberries. An experiment was conducted to determine the role of the kernel, and any IAA produced therein, in cob development. Large amount of IAA appear in the kernels of *Zea mays* during development (see experimental II). Cob growth is presumably regulated to some degree by IAA. Removal of kernels prior to the period of rapid cob growth might potentially cause a dramatic change in growth. The kernel may be the source of IAA, which in turn controls growth. The kernel may also somehow regulate the levels of IAA appearing in the cob, or may have no affect at all.

Materials and Methods

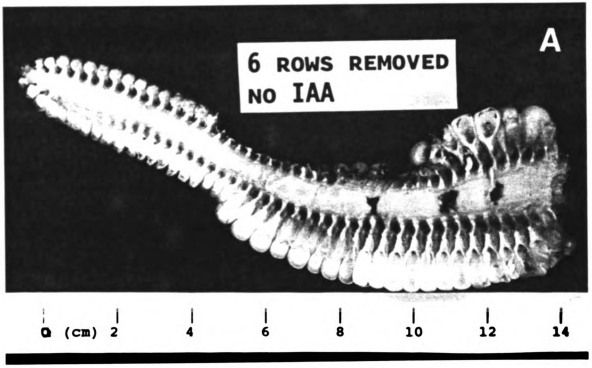
At 5 DAP (days after pollination) a block of 72 kernels (6 rows X 12 kernels) was removed from the mid portions of several ears of sweet corn (Illini Xtra Sweet). To accomplish this, the husks were sliced with a razor blade

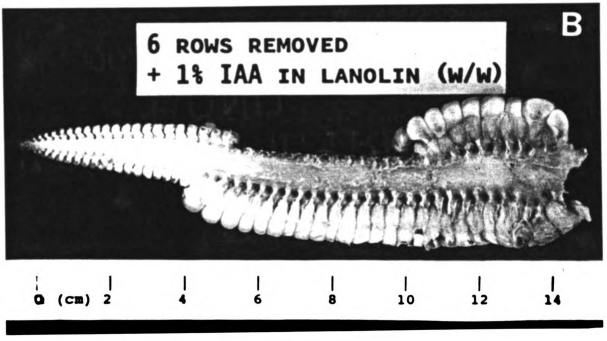
and pulled back to expose the kernels. The kernels were then removed by gently prying with a small spatula. The wounded area was then covered with lanolin, or lanolin containing 1% IAA (w/w). At 25 DAP the ears were harvested and the resultant growth observed.

Results and Conclusions

Ears to which lanolin alone had been applied to the dekerneled area generally exhibited curvature towards the dekerneled area (Figure 1A). This may be the result of a decrease in IAA on the dekerneled side resulting in Asymmetric growth. Those ears which were treated with lanolin containing 1% IAA generally maintained a straight growth pattern (Figure 1B). This conceivably is the result of the applied IAA supplementing what was lost upon kernel removal. While by no means conclusive, this data suggests *in situ* biosynthesis is the source of IAA which appears in the kernel during development.

Figure 1. (A). Cob development after removal of a 6 X 12 block of kernels from the ears five days after pollination and (B) subsequent application of 1% IAA in lanolin (w/w).


6 ROWS REMOVED
NO IAA
A
0 (cm) 2
4
6
8
10
12
14


6 ROWS REMOVED
+ 1% IAA IN LANOLIN (W/W)
B
0 (cm) 2
4
6
8
10
12
14

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