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OCCURRENCE AND BIOSYNTHESIS
OF AUXINS IN CHLOROPLASTS
AND MITOCHONDRIAL FRACTIONS
FROM SUNFLOWER LEAVES

by

© Judith Anne Frégeau, B.Sc., M.Sc.

A thesis submitted to the
Faculty of Graduate Studies
and Research in partial
fulfilment of the requirements
for the degree of Doctor of
Philosophy

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ABSTRACT

The capacity of chloroplast and mitochondrial soluble enzyme fractions from sunflower leaves to synthesize the auxins, 3-indoleacetic acid (IAA) and phenylacetic acid (PAA), has been investigated. Intact chloroplasts were isolated from the primary leaves of 2-week old seedlings by centrifugation through a 40% Percoll barrier. These purified fractions showed minimal contamination, as determined by the activity of marker enzymes for other leaf cell organelles and by electron microscopy. Mitochondria were isolated by centrifugation through a Percoll step gradient. Chlorophyll contamination in the fractions was reduced to less than 0.5% of the total homogenate and peroxisomal contamination was less than 5%. Electron microscopy confirmed the low level of these contaminants.

The two auxins, along with their respective precursors, L-tryptophan and L-phenylalanine, have been found to occur endogenously in the organelles. The metabolism of unlabelled L-tryptophan to IAA and of L-phenylalanine to PAA by each organelle protein fraction has been demonstrated. Isolation and provisional identification of the two auxins was achieved by preparative-HPLC followed by GLC analysis of collected fractions. Unequivocal identification of the IAA and PAA synthesized by the chloroplast fractions has been obtained by GC-MS.

Conversion of the two ^{14}C -labelled aromatic amino acids to their respective auxin products has also been demonstrated with both organelle protein fractions. Isolation and provisional identification of the radioactive intermediates was achieved by TLC, U.V. absorption and liquid scintillation spectrometry. With each ^{14}C -amino acid, the corresponding ^{14}C -arylpyruvate and

¹⁴C-arylacetaldehyde were found in both reaction systems and their presence is consistent with the formation of IAA and PAA via the so-called arylpyruvate pathway for auxin synthesis.

The conversion of authentic 3-indolepyruvic acid to IAA and of phenylpyruvic acid to PAA was also shown to be catalyzed by the organelle enzyme fractions even though some of the auxin product appeared to arise from spontaneous degradation of the arylpyruvic acid in such in vitro systems.

Thus, the data confirm the presence of both IAA and PAA as endogenous substances in sunflower chloroplasts and mitochondria and show that the soluble protein fraction from each organelle has the enzymatic capacity to synthesize these auxins.

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Introduction

Plant hormone physiologists are constantly searching for a better understanding of the hormonal substances that control different aspects of growth and development. For this reason, the auxins, 3-indoleacetic acid, (IAA) and more recently, phenylacetic acid, (PAA), have been extensively studied in several laboratories to determine their occurrence, biochemistry and physiological activity in a range of plants.

The biosynthetic pathways leading to the formation of IAA and PAA from the aromatic amino acids, L-tryptophan and L-phenylalanine, have been studied in this laboratory for several years using various species of higher plants. Many tracer experiments have been carried out, both in vivo using excised shoots and in vitro with cell-free enzyme systems (see Literature Review). The results have shown that the primary and most universal route for the synthesis of the two auxins proceeds through the corresponding arylpyruvic acid and arylacetaldehyde intermediates, and this route is generally referred to as the 'arylpyruvate pathway' to auxin synthesis.

Knowledge of the sites of synthesis and maximum physiological action of the two auxins is fundamental to an understanding of the growth-regulating physiology of these substances. The literature contains many reports of the occurrence of auxins in whole plants, of their distribution within the plant and on the ability of certain organs to synthesize auxins from their amino acid precursor. All these studies have certainly contributed to a better understanding of the biochemistry and physiology of these phytohormones at the macro-level, namely that of the whole plant. On the other hand, few attempts have been made to obtain a comparable understanding of the intracellular level of auxin synthesis and physiological action.

Wightman and Cohen (1968) have provided some evidence that IAA synthesis will occur in cell-free cytoplasmic and mitochondrial fractions isolated from mung bean seedlings. The first enzyme in the arylpyruvate pathway, the aromatic amino acid transaminase, is now thought to be a multispecific aspartic-aromatic aminotransferase of wide or even universal occurrence in plants (Wightman and Forest, 1978) and this enzyme has been shown to be present in the chloroplasts, mitochondria, peroxisomes and cytosol from spinach leaves (Huang *et al.*, 1976). Thus, very little data is really available regarding the occurrence and biosynthesis of auxins at the subcellular level; yet on a metabolic and physiological basis, this is where they are synthesized, metabolized and exert their physiological action.

The present study was therefore undertaken in an attempt to provide information on the subcellular occurrence and biosynthesis of auxin substances in growing leaf tissue. The investigation is a logical development of the continuing study of auxin metabolism in this laboratory. Evidence was sought for a possible compartmentation of the synthesis of auxins in chloroplasts and mitochondria isolated from expanding leaves of sunflower seedlings. The main aims of the investigation were firstly, to determine whether IAA and PAA do in fact occur in chloroplasts and mitochondria and if this is the case, then secondly, to verify that the enzymic capacity for synthesizing such auxins actually resides within these organelles. In the latter phase of the work, evidence will be sought from the nature of the radioactive intermediates obtained in ^{14}C -feeding experiments, for the synthesis of auxins in chloroplasts and mitochondria primarily via the arylpyruvate pathway.

Literature Review

1. Occurrence of the auxins, IAA and PAA.

Over the past forty years, many reviews have been written on the natural occurrence and biochemistry of auxins. The most recent review (Schneider and Wightman, 1978) states that the term "natural auxin" now includes other compounds besides IAA. Some of these compounds are indoles, like 3-indolepropionic (IPA), 3-indoleacrylic (IAcA) and 4-chloro-3-indoleacetic acid (4 Cl-IAA) as well as non-indolic compounds like PAA and a group of yet unidentified auxins in citrus fruits. Although the natural occurrence of all these substances is of interest in obtaining an overall understanding of the role of auxins in the regulation of growth and development in plants, most of the substances fall outside the scope of the present work. For this reason, only the occurrence of IAA and PAA throughout the plant kingdom will be considered further.

IAA was the first naturally-occurring plant growth regulator to be isolated (Kögl et al. 1934; Haagen-Smit et al. 1946). Over the years, workers have used a variety of physicochemical procedures to demonstrate its presence in a range of plant tissues. IAA has been identified in the liverwort, Marchantia, two gymnosperms, Picea and Pinus, and at least 18 angiosperms using solvent extraction, paper and thin-layer chromatography and bioassay, (see Schneider and Wightman, 1978).

Analytical methods currently being used for IAA identification include gas chromatography (GLC), HPLC and mass spectrometry. Conclusive identification of IAA has been achieved by combined gas chromatography-mass spectrometry of extracts from roots and mesocotyls of Zea (Elliott and Greenwood, 1974; Bandurski et al., 1980), from Avena shoots (Bandurski and Schulze, 1974), from cotton ovules

(Shindy and Smith, 1975), from shoots of Douglas fir (DeVoe and Zaerr, 1976; Crozier et al., 1980), from leaves of Ricinus (Allen and Baker, 1980) and from leaves of tobacco (Wightman et al., unpublished data).

PAA has been known for some time to occur in fungi (see Wat and Towers, 1979) and its presence in the brown alga, Undaria pinnatifida, has also been demonstrated (Abe et al., 1974). Wightman (1973) and Wightman and Rauthan (1974) showed that PAA was responsible for some of the auxin activity in shoots extracts from tomato and sunflower when measured in Avena coleoptile bioassay. In a later study, using similar chromatographic and bioassay procedures along with identification and quantitation by GLC, Wightman (1977) provided further evidence for the natural occurrence of both IAA and PAA in the young shoots of tomato, tobacco, sunflower, pea, barley and corn plants. GC-MS has also been used to identify conclusively the PAA present in extracts of tobacco leaves, (Wightman and Lichty, 1981).

2. Pathways for the biosynthesis of IAA and PAA.

Several in vivo tracer studies and cell-free metabolism experiments have demonstrated that tissues from many higher plants contain enzyme systems capable of converting the aromatic amino acids, L-tryptophan and L-phenylalanine, via similar reaction pathways to the auxins, IAA and PAA, respectively (Wightman 1973; Schneider and Wightman 1978).

Three pathways of IAA biosynthesis have been distinguished in higher plants, each pathway being named after its first key intermediate, namely, the Indolepyruvate pathway, the Tryptamine pathway and the Indoleacetaldoxime pathway. The interconversions occurring in these pathways are shown in Figure 1A. Evidence will be presented in this section for the occurrence of the Indolepyruvate

Figure 1A - Summary of the three biochemical pathways demonstrated for the conversion of L-tryptophan-3- ^{14}C to 3-indoleacetic acid- ^{14}C using ^{14}C -tracer technique.

pathway, usually considered to be the major route of IAA synthesis in plants. The other two routes are considered to be less important pathways since their key intermediates are of sporadic occurrence in higher plants.

These latter pathways will not be examined further since tryptamine has not yet been found to occur in the Compositae (Smith, 1977) and indole-3-acetaldoxime seems to be a characteristic of the family Brassicaceae (Kutacek and Kefeli, 1968). However, recent gas chromatographic studies have demonstrated the presence of 3-indoleacetonitrile in a wide range of plants (Stoddard and Wightman, 1982) and these findings suggest that the Indoleacetaldoxime route for IAA synthesis may be of much wider occurrence than previously suspected.

Evidence for the biosynthetic pathways leading to PAA will be discussed together with those for IAA since, as mentioned earlier, the pathways for the synthesis of both auxins seem to be similar. The primary and most universal pathway for the conversion of tryptophan to IAA, or of phenylalanine to PAA, appears to occur via the arylpyruvate intermediate (Figure 1B). Several lines of evidence will be reviewed to support this conclusion, including occurrence of the arylpyruvates and other corresponding intermediates as native compounds, and the results from in vivo and in vitro (cell-free) tracer experiments.

A. Occurrence of intermediates as native compounds.

i) Arylpyruvic acids

IPyA has long been known to be unstable, especially under alkaline extraction conditions (Stowe, 1959). Besides 3-indolealdehyde, IAA and 3-indolecarboxylic acid, the degradation products of IPyA have never been fully determined. The question of whether IPyA is a native

Figure 1B - The arylpyruvate pathway for auxin synthesis:
precursor, intermediates and auxin product.

TPP = Tryptophan α -KG = α -Ketoglutaric acid

PHE = Phenylalanine GLU = Glutamic acid

I = aromatic amino acid

II = arylpyruvic acid

III = arylacetaldehyde

IV = arylacetic acid

V = arylethanol

Where R =

for TPP

or

for PHE

constituent of higher plants has been in doubt for many years because earlier workers, who claimed to find IPyA as an endogenous compound in certain plants, had used ammoniacal solvents for the paper chromatographic isolation of the compound and under these conditions it has been shown to decompose (Stowe, 1959). For this reason, Schneider and Wightman (1978) state that "the instability of IPyA has so far precluded its isolation as a native compound from plant tissues". An early demonstration of the presence of this keto acid in corn kernels by Stowe and Thimann (1954) was not confirmed by Srivastava (1964), although Winter (1964) claimed to have found evidence for its occurrence in this tissue.

Two groups of workers have recently shown that IPyA can be partly converted to IAA during methanol extraction and subsequent solvent fractionation (Atsumi *et al.*, 1976; Hemberg and Tilberg, 1980). Kaper and Veldstra (1958) cautioned that similar transformations could occur with phenylpyruvic acid. Loffelhardt (1977) noticed a non-enzymic degradation of PPyA into PAA under his incubation conditions.

Thus, the two aromatic pyruvic acids show some instability under certain experimental conditions and precautions must be taken to maintain their stability during in vitro experimentation. Evidence for the natural occurrence of these two key intermediates was recently obtained by Camirand (1979) who was able to identify both IPyA and PPyA in extracts of tobacco plants by first converting them to their corresponding 2,4-dinitrophenylhydrazones.

ii) Arylacetaldehydes and Arylethanols.

Like IPyA, 3-indoleacetaldehyde (IAAld) is reported to be unstable during extraction (Stowe 1959), but good evidence for its presence in vegetative tissues has been obtained. After authentic

IAAld became available as the more stable bisulfite adduct, Larsen and Aasheim (1961) were able to demonstrate the occurrence of IAAld as a native compound in etiolated epicotyls of Pisum sativum. This work was extended by Rajagopal (1967, 1968) who was able to show the presence of both IAAld and tryptophol in extracts of etiolated shoots of peas and sunflower.

IAAld was also found to be a native constituent of green and etiolated cucumber cotyledons by Purves and Brown (1978) using thin-layer chromatography of the native IAAld and of derivatives made from it. Tryptophol was identified in extracts from green shoots of cucumber seedlings by Rayle and Purves (1967) with gas chromatography and mass spectrometry. Schneider et al. (1972) demonstrated the presence of tryptophol in tomato shoots by means of paper chromatography and specific colour reactions and recently, changes in the endogenous level of tryptophol in squash hypocotyls grown under different light treatments have been studied by Segal (1980) using the gas chromatographic method of analysis.

Both IAAld and phenylacetaldehyde were shown to be present in tobacco shoots extracts when the corresponding 2,4-dinitrophenylhydrazones were prepared and isolated by TLC (Camirand 1979). Low levels of tryptophol and phenylethanol were also found in these same tobacco extracts.

B. In Vivo tracer studies.

Evidence for the biosynthesis of IAA and PAA via the Indolepyruvic or Phenylpyruvic acid pathways was obtained by in vivo metabolism experiments in which the appropriate ¹⁴C-aromatic amino acid was fed to excised plant tissues.

Wightman (1962) and Wightman (1964) demonstrated the presence of radioactive IAA, among other labelled compounds, in extracts from

cabbage shoots and tomato shoots, respectively, which had been fed ^{14}C -tryptophan. The exact pathway of synthesis was still however unclear.

Gibson, Schneider and Wightman (1972) fed ^{14}C -tryptophan to excised tomato and barley shoots which were allowed to metabolize the compound for 24 hours after which the radioactive metabolites were extracted and fractionated into neutral and acidic ether fractions. Small quantities of non-radioactive carrier substances, IPyA and IAAld, were added to the acidic and neutral ether fractions, respectively, before preparing the 2,4-dinitrophenylhydrazone derivatives for thin-layer chromatography. By this method, both IPyA and IAAld were isolated and found to be radioactive in both plants but radioactive tryptophol was produced only in tomato shoots. Radioactive IAA was also shown to be present in the two acidic ether fractions, which clearly indicated that both types of plants were able to synthesize auxin from its related amino acid.

Wightman (1973) and Wightman and Rauthan (1974) extended this work to a study of the biosynthesis of PAA. ^{14}C -Phenylalanine was fed to excised tomato shoot tissues for 24 hours after which PAA and the presumed intermediates, PPyA and PAAld, were all found to be radioactive. Whether phenylethanol was also found to be radioactive in these experiments was not mentioned in these papers. In later studies, Camirand (1979) demonstrated the formation of the corresponding radioactive arylpyruvate and arylacetaldehyde when ^{14}C -tryptophan or ^{14}C -phenylalanine was fed to excised tomato shoots. Radioactive tryptophol and phenylethanol were also found in these experiments.

Liu et al. (1978) were able to show the formation of ^{14}C -IAA and ^{14}C -tryptophol by callus tissue and petiole slices from three species

of Nicotiana which were fed ^{14}C -tryptophan. TLC and radio-chromatograms were used to identify IAA in the callus tissue whereas GLC and liquid scintillation were utilized for the petiole slices.

C. Enzyme systems.

Our present knowledge of the pathways of auxin biosynthesis rests also on the demonstration of the presence of the necessary key enzymes in cell-free systems from several plants. In vitro experiments investigating the conversion of the aromatic amino acids to the auxins will be reviewed along with more detailed studies examining the different enzymatic steps of the pathway.

i) Evidence for the formation of IAA and PAA in cell-free systems.

Cell-free enzymic preparations were first used by Gordon (1956, 1958) to describe an enzyme system from shoot tips of Phaseolus aureus seedlings which catalyzed the conversion of L-tryptophan to IAA. A supernatant fraction and several crude organelle fractions were obtained by differential centrifugation. These various preparations were incubated with L-tryptophan in phosphate buffer at pH 7.4 for two hours. Acidic ether extracts were analyzed by paper chromatography, colorimetry, and Avena curvature bioassays. IAA was produced by the high speed supernatant fraction, indicating that soluble components from plant cells were probably involved in the biosynthesis reactions. Although Gordon claimed a cytoplasmic sub-microsomal distribution for the IAA-synthesizing system, it must be pointed out that this fraction contained, besides cytoplasmic proteins, the soluble proteins from all broken organelles.

Wightman and coworkers were the next to investigate the ability of cell-free systems to metabolize L-tryptophan to IAA and fractions prepared from mungbean seedlings (Phaseolus aureus) were again used as the enzymic preparation (Wightman and Fowden 1966; Wightman and

Cohen 1968). A cytoplasmic supernatant fraction and a mitochondrial fraction obtained by high speed centrifugation followed by Sephadex G25 purification were tested as the enzymic fractions and both were incubated with L-tryptophan in presence of α -ketoglutarate, pyridoxal phosphate, thiamin pyrophosphate and oxidized NAD. The presence of a tryptophan aminotransferase in both fractions was demonstrated by measuring the amounts of glutamic acid and IPyA formed in the presence and absence of either L-tryptophan or α -ketoglutarate. Isolation of the potentially unstable indolepyruvate intermediate was achieved by converting the metabolite to its stable 2,4-dinitrophenylhydrazone derivative, when conclusive proof of its identification was obtained by infra-red spectroscopy.

In a tracer experiment, ^{14}C -L-tryptophan was incubated for 6 hours with a Sephadex-purified cytoplasmic fraction after which the radioactive metabolites were isolated either by ether extraction, or by the formation of stable 2,4-dinitrophenylhydrazones. The presence of ^{14}C -IAA and ^{14}C -tryptophol in the ether extracts was revealed by paper chromatography followed by autoradiography, using authentic ^{14}C -labelled standards for co-chromatography with the suspected radioactive metabolites. Formation of the radioactive intermediates, ^{14}C -IPyA and ^{14}C -IAAld, by the cytoplasmic enzyme system was demonstrated when the corresponding dinitrophenylhydrazone derivatives were identified (by TLC and IR spectroscopy) and both were shown to be radioactive.

In similar experiments also carried out during the late 1960's, Moore and Shaner (1967, 1968) and Moore (1969) presented evidence that ^{14}C -tryptophan could be converted to ^{14}C -IAA by cell-free extracts prepared from the shoot-tip tissues of pea seedlings. They used a dialysed supernatant fraction obtained by centrifuging a crude

homogenate at 10,000 xg as their enzyme source. The fraction was incubated with ^{14}C -tryptophan and a combination of other substances in phosphate buffer, pH 7.4, for 3 hours. Thin layer chromatography, GLC and liquid scintillation spectrometry were used to confirm the identification of ^{14}C -IAA. Maximum net ^{14}C -IAA yield was obtained in the reaction mixtures containing α -ketoglutarate, pyridoxal phosphate and thiamine pyrophosphate. The omission of any one of these substances caused a reduction in yield of ^{14}C -IAA, although no absolute dependency upon any of the cofactors could be demonstrated. The authors interpreted their results as evidence that the initial step in the conversion of tryptophan to IAA involved an aminotransferase reaction to form IPyA and glutamic acid. The stimulating effect of thiamine pyrophosphate was viewed as evidence for the occurrence of the subsequent step, namely, the decarboxylation of IPyA to IAAld. The reaction sequence was terminated with the oxidation of IAAld to IAA.

Isolation of the labelled intermediates, ^{14}C -IPyA and ^{14}C -IAAld, was attempted by Moore and Shaner (1968). Radioactive IPyA was isolated as the 2,4-dinitrophenylhydrazone derivative and found to be the main product formed in the complete reaction mixture. They encountered difficulties in their attempts to isolate IAAld and were unable to isolate free ^{14}C -IAAld or to trap it as a bisulfite adduct. Reduction of IAAld to tryptophol was tried by adding NaBH_4 to the reaction mixtures at the end of the incubation time, but this method was also unsuccessful. However, they claimed to have found evidence for the presence of ^{14}C -IAAld when a neutral toluene extract of the final reaction mixture was allowed to react with NaBH_4 . Non-radioactive tryptophol had to be added as a carrier during subsequent chromatography when low levels of ^{14}C -labelled

tryptophol were detected, indicating that ^{14}C -IAAld was probably formed in the reaction system.

Lantican and Muir (1967) reported the presence of an IAA-synthesizing system in apices of oats and peas, in tomato ovary tissues and in savoy cabbage seedlings. The protein fraction from oat apices was obtained after gel filtration on Sephadex G-25, ammonium sulfate precipitation, dialysis and gel filtration on Sephadex G-200. Thin-layer chromatography and Avena curvature bioassays were used to determine the amount of IAA-like activity produced. The highest activity was found in the system containing, besides L-tryptophan and the enzyme fraction, α -ketoglutarate and pyridoxal phosphate. A system containing only the enzyme fraction and L-tryptophan gave less than one-third of the auxin activity produced by the full reaction system. The authors concluded that the greater yield of IAA obtained with the reaction system containing α -ketoglutarate indicates that the conversion of tryptophan to IAA depends partly on the activity of an aminotransferase in the system, rather than a decarboxylase, since the latter enzyme does not require a keto acid co-substrate.

The conversion of ^{14}C -phenylalanine to ^{14}C -PAA in cell-free extracts of plant tissue was accidentally observed by Stafford and Lewis (1977). These workers were assaying the enzyme phenylammonia lyase, which catalyses the conversion of L-phenylalanine to cinnamic acid in leaf extracts, and noticed the presence of another major metabolite on their chromatograms which interfered with the measurement of cinnamic acid. They identified the interfering compound as ^{14}C -PAA. Particulate and soluble fractions from leaves of Sorghum, Spinacia and Coleus were thus found capable of converting L- and D-phenylalanine to PAA. The

particulate fraction was obtained by centrifuging the crude leaf homogenate at 37,000 xg and the remaining supernatant was subjected to ammonium sulfate precipitation and then used as the soluble fraction.

Stafford and Lewis (1977) mentioned that although the major interference product was PAA, in some cases ^{14}C -PPyA was present in significant amounts. Two dimensional paper chromatography was used for the separation. The occurrence of ^{14}C -PPyA was interpreted as being an intermediate in the pathway from phenylalanine to PAA and its formation was attributed to the activity of either an aminotransferase, or an amino acid oxidase, since only L-phenylalanine was provided as substrate. In a later study, the activity of a L-phenylalanine aminotransferase was demonstrated in Sorghum leaf extracts (Stafford and Lewis, 1979).

Löffelhardt and Kindl (1975) provided indirect evidence for the formation of PAA from phenylalanine in cell-free systems. They used a pulse-chase labelling technique in which leaf discs from Astilbe chinensis were subjected to a 1 hour-pulse of ^{14}C -phenylalanine and then incubated in water for up to 5 hours. Chloroplasts isolated from these leaf discs contained ^{14}C -labelled PAA. Considering the length of time of this "in vivo" incubation, the results cannot be regarded as direct proof of PAA biosynthesis by chloroplasts.

Gordon and Paleg (1961) claimed to have observed the formation of IAA from tryptophan by cell-free extracts of mungbean, oat and sunflower seedlings through the action of polyphenol oxidase. The enzyme is of wide occurrence in plants, particularly thylakoid-bound in chloroplasts and also in mitochondria (Mayer and Harel, 1979). The enzymic activity of plant polyphenol oxidase is, however, latent and activation can only be achieved by treating extracts or organelle

membrane fragments with detergents, mild acids or alkali, proteolytic enzymes and denaturing agents (Tolbert, 1973). Even the sonication of thylakoid preparations released the enzyme largely in a latent state (Golbeck and Cammarata, 1981), indicating that drastic conditions destroying the membranes have to be present for activation to occur. The enzyme fraction used by Gordon and Paleg (1961) was obtained from lyophilized cell-free extracts and enzyme preparations, which they claimed to be 'fresh', was made by freezing the tissue in liquid N_2 . Thus, in view of their enzyme isolation procedure and the unusual activation properties of polyphenol oxidase, the conversion of tryptophan to IAA via the route proposed by Gordon and Paleg (1961) seems very unlikely in studies involving intact organelles, or in studies with particulate and supernatant fractions.

ii) Enzymatic steps in the formation of IAA and PAA.

a) Formation of IPyA and PPyA.

The first step in the arylpyruvic acid pathway for auxin formation involves the loss of the amino group from the aromatic amino acid giving rise to the first intermediate, IPyA or PPyA. Two reactions are possible: an oxidative deamination, or a transamination.

Occurrence of appropriate enzyme.

Gamborg and Wetter (1963) and Gamborg (1965) were the first to attempt the purification of an enzyme from mungbean seedlings which they showed was able to transaminate the three aromatic amino acids. The partially purified enzyme fraction could also transaminate several other amino acids, such as aspartate, glutamate and methionine, and they found that pyruvate, rather than α -ketoglutarate, was the best amino group acceptor. They did not, however, isolate and characterize the arylpyruvic acid product in the reactions with

aromatic amino acids.

Red'kina et al. (1969) (see Wightman and Forest, 1978) demonstrated the presence of a glutamate-phenylpyruvate aminotransferase in pea seedlings which also showed 15 - 30 times greater activity as an aspartate- α -ketoglutarate aminotransferase. A reversible aromatic aminotransferase, utilizing phenylpyruvate as the amino group acceptor when aspartate or glutamate was provided, has also been demonstrated in leaves of Quercus pedunculata by Gadal et al. (1969) (see Wightman and Forest, 1978).

Forest (1971) purified aromatic aminotransferase from bushbean roots to a single protein. The enzyme was found to be a multispecific aminotransferase showing its greatest activity with aspartate as a substrate but also catalysing the transamination of the three aromatic amino acids when α -ketoglutarate was provided (Forest and Wightman 1972).

Other workers during the same period also investigated the occurrence of tryptophan or phenylalanine aminotransferase in plant cell-free extracts. Wightman and Cohen (1968) using mungbean seedling extracts and Wightman (1973) using extracts from shoot tips of tomato were able to demonstrate the occurrence of tryptophan aminotransferase activity in Sephadex-purified 35,000 xg supernatant fractions. The arylpyruvate product, IPyA, was isolated from the reaction system as the 2,4-dinitrophenylhydrazine derivative, following an incubation period of 6 hours.

Truelsen (1972) also demonstrated the conversion of tryptophan to IPyA by transamination using a 75,000 xg supernatant fraction from mungbean seedlings. IPyA was again detected in the final reaction system by forming its 2,4-dinitrophenylhydrazine derivative, or by the enol-borate complex which can be determined spectrophotometrically.

This worker was able to purify the enzyme 28 fold and showed that it could also use L-phenylalanine and L-tyrosine as substrates with α -ketoglutarate as the amino group acceptor. In a later study, Truelsen (1973) investigated the distribution of tryptophan aminotransferase activity in a range of higher plants and was able to demonstrate the presence of the enzyme in extracts from different organs of 30 plant species, including sunflower. Liu et al. (1978) studied tryptophan aminotransferase activity in extracts from seedlings of 3 species of Nicotiana and detected IPyA again as the 2,4-DNP hydrazone and as the enol-borate complex. In all the studies the pH optimum of the tryptophan aminotransferase extracted from the different plant species was found to vary between 8.0 and 8.9.

Wightman and Rauthan (1974) reported the formation of PPyA by transamination of L-phenylalanine using an enzyme preparation from tomato shoot tips. Stafford and Lewis (1979) reported the occurrence of two enzyme activities involved in the transamination of L- and D-phenylalanine by leaf extracts of Sorghum bicolor. PPyA was isolated by paper chromatography and direct spectrophotometric measurement. The anaerobic nature of these activities and the increase in synthesis of PPyA upon addition of pyridoxal phosphate and α -ketoglutarate, indicated that the reaction was due to an aminotransferase. However, since the reaction could also occur, to a certain extent, without the addition of these substances, Stafford and Lewis did not rule out the participation of an amino acid oxidase acting anaerobically with an endogenous electron acceptor.

The presence of a thylakoid-bound L-amino acid oxidase in the blue-green alga, Anacystis nidulans, responsible for the formation of PAA from phenylalanine, was described by Loffelhardt (1977). The

enzyme catalysed the formation of PPyA which was trapped and isolated as the stable oximino acid.

Very little information is available on the occurrence of an L-aromatic amino acid oxidase in higher plants; most papers dealing with this enzyme are concerned with animal enzymes.

Intracellular location of enzymes.

Cohen and Wightman (1968) reported the occurrence of an L-tryptophan aminotransferase in mitochondrial fractions prepared by differential centrifugation from etiolated mungbean seedlings. A similar tryptophan and phenylalanine aminotransferase was recovered by Forest (1971) in both mitochondrial and plastid fractions obtained by differential centrifugation of an homogenate of dark-grown bushbean roots.

Kirk and Leech (1972) were able to show the transfer of the amino group of phenylalanine and tryptophan to oxaloacetate in pure intact chloroplasts of Vicia faba. Aminotransferase activities could also be detected to a lesser degree when pyruvate was used as the amino acceptor, but α -ketoglutarate was found to be the best acceptor with tryptophan. The enzymes were restricted to the chloroplast stroma since no activity was detected in the thylakoid fraction.

As noted above, Forest and Wightman (1972) have shown that the aspartate aminotransferase of bushbean roots is multispecific, and can use all three aromatic amino acids as amino donors. The intracellular location of this enzyme clearly has some bearing on the present work. In the earlier studies with bushbean root preparations, about 90% of the aspartate aminotransferase activity was found to be in the soluble fraction with all the remaining activity occurring in the plastid (ca.6%) and mitochondrial fractions (ca.4%). Yamazaki and Tolbert (1970) reported that 17% of the total

aspartate aminotransferase activity of spinach leaves was located in the chloroplast fraction after aqueous isolation of the organelles. They found that the enzyme was easily lost from broken chloroplasts, indicating a stromal location. This chloroplast enzyme was found to be similar to the isoenzyme found in the mitochondrial fraction of spinach leaves, but distinct from the two peroxisomal forms of the enzyme (Rehfeld and Tolbert, 1972). Huang and coworkers (1976) described four distinct isoenzymes of aspartate- α -ketoglutarate aminotransferase in spinach leaf extracts. Chloroplasts, mitochondria and peroxisomes were isolated by sucrose gradient centrifugation and of the total aspartate- α -ketoglutarate aminotransferase activity, approximately 45% was represented by the chloroplast isoenzyme, 26% by the cytosol isoenzyme, 19% by the mitochondrial isoenzyme and 3-10% by the peroxisomal isoenzyme.

b) Formation of the arylacetaldehydes

The second enzyme thought to be involved in the Indolepyruvate pathway for IAA synthesis is an 'arylpyruvate decarboxylase'. Although Gibson *et al.* (1972) showed that IPyA was enzymatically decarboxylated by a high speed supernatant fraction prepared from tomato shoot tissues, the isolation and purification of the specific enzyme responsible for this activity in tomato, or any other plants, has not yet been achieved. An IPyA decarboxylase has, however, been purified from yeast and found to require thiamine pyrophosphate as a cofactor (Sukanya *et al.* 1971).

The enzyme phenylpyruvate decarboxylase has been isolated and purified about 3-fold from cell-free extracts of the bacteria, Achromobacter eurydice (Fujioka *et al.* 1968). This enzyme also requires thiamine pyrophosphate as a cofactor and was found to decarboxylate IPyA in addition to PPyA. Enzyme extracts from the

fungus Aspergillus nidulans have also been shown to decarboxylate phenylpyruvate to PAAld (Wat and Towers, 1979). The enzyme involved was not isolated, but this decarboxylation also required thiamine pyrophosphate as cofactor. Thus, the isolation and purification of enzymes capable of decarboxylating IPyA and PPyA to the corresponding arylacetaldehydes has not yet been achieved with higher plant tissue.

c) Metabolism of the arylethanols.

Wightman and Cohen (1968) partially purified an alcohol dehydrogenase from mungbean seedlings which catalysed the reversible reduction of IAAld to tryptophol in presence of NADH. The enzyme activity was detected in high speed supernatant fractions and in washed mitochondrial preparations. The same enzyme activity was also detected in cytoplasmic supernatant fractions obtained from tomato and barley shoots tissues (Gibson et al. 1972).

Brown and Purves (1980) have isolated and partially purified an indoleacetaldehyde reductase in extracts from cucumber seedlings. This enzyme catalyses the conversion of IAAld to tryptophol when the reduced pyridine nucleotide, NADH or NADPH, is provided as co-substrate. It is not certain whether the activity results from a single enzyme, which cross-reacts with both cofactors, or from two enzymes. Bower et al. (1976) reported a partial separation of these two activities in subcellular localization studies. NADPH-specific activity was associated with microsomal components in homogenates of cucumber seedlings, while NADH-specific activity appeared to be only in the cytosol. The authors stated, however, that since low activities were observed in their subcellular localization studies, these results cannot be regarded as conclusive.

Substrate specificity studies of the enzyme investigated by

Brown and Purves (1976) showed that IAAld was the preferred substrate, but both NADH- and NADPH-dependent activities also catalysed the reduction of PAAld. Liu et al. (1978) similarly reported the presence of an NADH-dependent IAAld reductase in extracts from 3 species of Nicotiana.

The reverse reaction, namely the conversion of tryptophol to IAAld, was also demonstrated by Wightman and Cohen (1968) using a pH-precipitated protein fraction isolated from mungbean seedling cytoplasmic preparations. Vickery and Purves (1972) have isolated and partially purified a tryptophol oxidase from cucumber seedlings. The enzyme catalysed the irreversible aerobic oxidation of tryptophol to IAAld, and also produced hydrogen peroxide in the reaction. The occurrence of a phenylethanol oxidase in higher plants has not yet been reported.

d) Formation of IAA or PAA from the corresponding arylacetaldehyde.

The nature of the final enzyme involved in the Indolepyruvate pathway appears to vary with the species. NAD-dependent IAAld dehydrogenase activity was detected in cytoplasmic supernatant fractions and washed mitochondrial preparations from mungbean seedlings by Wightman and Cohen (1968). Rajagopal (1971) purified an IAAld oxidase approximately 18-fold from Avena coleoptiles and showed that no cofactors were required for its activity. Bower and coworkers (1978), using a high speed supernatant fraction obtained from light-grown cucumber seedlings, demonstrated the presence of IAAld oxidase activity. No cofactors were required but the oxidase activity in the fraction was increased 2-10 fold by a mild heat treatment. Evidence was also obtained for an IAAld oxidase in high speed supernatant fractions prepared from 3 species of Nicotiana (Liu et al. 1978). Most recently, an IAAld oxidase was

purified 15-fold from extracts of etiolated pea epicotyls by Miyata et al. (1981).

Evidence for a phenylacetaldehyde dehydrogenase in plant tissues has not yet been reported in the literature. Phenylacetaldehyde dehydrogenase activity is, however, present in the bacteria Achromaobacter eurydice (Fujioka et al. 1966). The enzyme appears to be specific for PAAld, but a slow reaction was found with IAAlld. Phenylacetaldehyde oxidase activity has also not yet been reported in higher plants. However, PAAld was shown to be a good substrate for the Avena IAAlld oxidase partly purified by Rajagopal (1971). Similarly, inhibitor studies with the IAAlld oxidase found in cucumber seedlings (Bower et al. 1978) indicated that the enzyme was an oxidase capable of reacting with a limited number of other aromatic substrates, among which was PAAld.

3. Sites of auxin biosynthesis.

Probably every part of a growing plant has the capacity to synthesize auxins. However, the most important site of synthesis in the vegetative plant appears to be the young expanding leaves and cambial tissues (Schneider and Wightman, 1978). Gas chromatographic studies of auxin distribution in developing tobacco leaves show that the concentration tends to be high in the youngest leaves and to decline with age. IAA, PAA and indolepropionic acid were at their highest levels in the youngest, most rapidly expanding leaves and declined as the leaf matured and ceased to grow (Wightman, 1977). In similar studies, Allen and Baker (1980) measured the endogenous levels of IAA in the leaves of Ricinus communis by using GC-MS procedures. The highest amount of the auxin was found in the youngest expanding leaf followed by the stem apex. Low levels were recorded for the older leaves.

Another type of evidence was presented by Wightman (1973) who used in vivo and in vitro tracer studies to measure the capacity for auxin synthesis in developing tomato leaves. ^{14}C -tryptophan was fed to the shoot tips and first four leaves individually and it was found that each of the tissues was able to form radioactive IAA. The greatest amount of conversion, however, occurred in leaf 1, the youngest expanding leaf immediately below the shoot tip. Similar results were obtained with cell-free systems prepared from the shoot tips and individual leaves.

Indirect evidence for the role of the youngest tissues as the most active sites of auxins biosynthesis was also presented by Truelsen (1972). The activity of tryptophan aminotransferase, the first enzyme in the indolepyruvate pathway for IAA formation, was found to be highest in the primary leaves and shoot apex in mungbean seedlings.

4. Chloroplasts and Mitochondria

Chloroplasts are by far the most prominent organelle of leaf cells and their chief anabolic function is the photosynthetic generation of carbohydrates. However, it is increasingly appreciated that chloroplasts are able to carry out a range of other biosynthetic activities. For example, the incorporation of amino acids into proteins, of nucleotide triphosphates into nucleic acids and of UDP-galactose into galactolipids have all been shown to occur in isolated chloroplasts (Leech and Murphy, 1976). This organelle can also synthesize many kinds of small molecules in addition to monosaccharide sugars. Indeed, there is extensive evidence showing that chloroplast enzymes play a major role in the synthesis and interconversion of amino acids and lipids (Givan and Harwood, 1976). Thus, it seems reasonable to ask whether the biosynthetic pathway

leading to IAA and PAA might not also occur in this organelle.

Only the aromatic aminotransferase, the first enzyme in the arylpyruvate pathway for auxin synthesis, has yet been located in chloroplasts. As mentioned before, Kirk and Leech (1972) reported the presence of aromatic aminotransferase activity in the stroma of isolated intact chloroplasts.

Halliwell (1978), in a recent review on chloroplast enzymes, states that the pH and the supply of substrates and cofactors were two of the simplest factors that regulate the action of an enzyme in the chloroplasts. The pH of the stroma can increase by 1-2 units, reaching pH values greater than 7, in illuminated chloroplasts, conditions which could reduce or increase the activity of enzymes. Light also causes an increase in the stromal concentration of Mg^{2+} , which could act as a regulator for certain enzymes (Halliwell, 1978). The pH optimum for most plant aminotransferase has been found to be in the range of pH 8.0 - 9.0 (Forest and Wightman, 1978), and Forest (1971) demonstrated a 20% stimulation of the activity of the bushbean aromatic aminotransferase when Ca^{2+} or Mg^{2+} was added to the reaction mixture.

Chloroplasts are bounded by a double membrane; the inner membrane is the permeability barrier for the organelle and it could influence the supply of substrates or cofactors for enzymatic reactions. Kirk and Leech (1972) concluded that while transamination reactions could take place inside chloroplasts, the formation of amino acids catalysed by aminotransferase activity depended on a continuing supply of the appropriate carbon skeletons coming mainly from the cytoplasm. This implied transport of the precursors into the chloroplasts, or alternatively, import of the amino acids themselves.

Tryptophan and phenylalanine, the precursors of IAA and PAA

respectively, are the starting amino acid substrates for aromatic aminotransferase activity, along with α -ketoglutarate as the amino group acceptor. These aromatic amino acids can be synthesized in chloroplasts via the shikimic acid pathway, as demonstrated recently by Bickel and Schultz (1979). The occurrence of an NADP-specific isocitrate dehydrogenase has also been demonstrated in chloroplasts (Elias and Givan, 1977), and the presence of this enzyme suggests that at least some of the α -ketoglutarate required for transamination reactions can be generated within the organelle. Thus, chloroplasts appear to possess all the enzymic capacity necessary to synthesize the starting substrates for IAA and PAA biosynthesis, and they also appear able to catalyse the first reaction of the arylpyruvate pathway for auxin synthesis.

Although the main function of mitochondria is the synthesis of ATP by the process of oxidative phosphorylation, this is by no means the only biochemical process occurring in this organelle. Approximately 100 different types of protein have been identified in mitochondria; some of these are non-enzymatic, but the majority show catalytic functions. For example, enzymes of the TCA cycle, those of β -oxidation and enzymes active in transporting solutes across the mitochondrial membranes have all been found in this organelle (Whittaker and Danks, 1978).

The role of aminotransferase activities in mitochondria is considered mainly to allow for the complete oxidation of the carbon

✓ skeleton of amino acids via the TCA cycle, or to convert some of the TCA cycle intermediates into amino acids (Whittaker and Danks, 1978). In addition, as Wightman and coworkers have shown (see Schneider and Wightman, 1978), the transamination of tryptophan and phenylalanine by a multispecific aspartate aminotransferase results in the carbon skeletons of these aromatic amino acids being switched into the auxin biosynthesis pathways. Forest and Wightman (1972) reported the presence of the multispecific aspartate aminotransferase in mitochondria from bushbean roots. An isoenzyme of aspartate aminotransferase was located in mitochondria by Huang et al. (1976). Moreover, some enzymes of phenolic metabolism are also found in mitochondria. Appreciable shikimate dehydrogenase activity has been found associated with pea seedling mitochondria, while phenylalanine lyase (PAL) activity was detected in Ricinus mitochondria (Alibert et al. 1977).

Thus, the presence of such a range of enzyme activities in mitochondria suggests that the organelle might not be as highly specialized in its catabolic activity as previously thought and that its capacity to carry out other metabolic activities awaits to be discovered.

Material & Methods

1. Plant Material

Sunflower seeds (Helianthus annuus CM90RR) were sown in 10 inch pots in vermiculite and grown in a greenhouse for one week with natural light, plus supplementary light to give a daylength of 16 hours. The seedlings were then transferred to a growth cabinet under incandescent and fluorescent lights for 16-hour daily photoperiods, with day temperatures of 27°C and night temperatures of 21°C. The light intensity at the level of the first leaves was 1,500 ft. candles. Plants were selected for experimental work when they were 12-14 days old. At this stage, the first pair of leaves were still expanding and the second pair were just emerging from the apical bud.

2. Isolation of Organelles

A. Chloroplast Fraction

Chloroplasts were isolated from sunflower seedlings following the method of Mills & Joy (1980) with a few modifications, (see Fig. 2). Approximately 80 gm of first leaves were harvested 2 hours after the end of the dark period and placed on ice for 30 minutes. They were then cut into small pieces with scissors and placed directly into ice-cold extraction medium containing 330 mM sorbitol, 50 mM tricine-KOH (pH 8.0), 4 mM 2-mercaptoethanol, 2 mM ethylene diamine tetraacetic acid (EDTA), 1 mM magnesium chloride and 0.1% (w/v) bovine serum albumin (BSA). The chopped leaves were homogenized for 5 seconds with a Polytron PT 20 using a tissue:volume ratio of 1:5. The homogenate was squeezed through 7 layers of nylon mesh and then gravity filtered through 2 layers of Miracloth. The filtrate was centrifuged at 2,000 xg for 1 minute using a Sorvall RC-2B, refrigerated centrifuge and the pellet obtained was resuspended in extraction medium using a

Figure 2 - Procedure for the isolation of purified
chloroplasts by sedimentation through
40% Percoll medium.

HOMOGENIZATION

Grind sunflower leaves for 5 sec in Polytron in medium containing 330 mM Sorbitol, 50 mM Tricine (pH 8.0), 4 mM 2-Mercaptoethanol, 2 mM EDTA, 1 mM $MgCl_2$, 0.1% BSA

FILTRATION

Homogenate filtered through 7 layers of nylon mesh plus 2 layers of Miracloth

CENTRIFUGATION (2000 xg for 1 min)

Gently resuspended pellet in grinding medium using nylon mesh attached to a glass rod

ADDITION OF PERCOLL PAD

Place resuspended chloroplasts in centrifuge tube and underlay with a 10 ml layer of 40% Percoll (v/v) containing 330 mM Sorbitol, 50 mM Tricine (pH 8.2) and 0.1% BSA

CENTRIFUGATION (2000 xg for 1 min)

Intact chloroplasts pelleted by centrifugation in a swing-out rotor. Supernatant and Percoll layer is then removed by aspiration.

RESUSPENSION OF CHLOROPLASTS IN APPROPRIATE MEDIUM

Figure 3 - Percoll medium for the isolation of chloroplasts
before and after centrifugation.

CP = chloroplast pellet
BC = broken chloroplasts

small piece of nylon mesh attached to a glass rod.

The washed chloroplast fraction was then underlayered with 10 ml of Percoll medium containing 40% (v/v) Percoll, 330 mM sorbitol, 50 mM tricine-KOH (pH 8.2) and 0.1% (w/v) BSA. This step is illustrated on the left side of Fig. 3. The intact chloroplasts were pelleted by centrifugation at 2,000 xg for 1 minute in the Sorvall centrifuge equipped with a HB-4 swinging bucket rotor. Broken chloroplasts remained in the supernatant as shown on the right side of Fig. 3. Note that centrifugation time given is the time at the centrifugal force indicated. The supernatant and the Percoll layer were carefully removed by aspiration using a pasteur pipette and the chloroplast pellet was then gently resuspended in the appropriate medium.

B. Mitochondrial Fraction

Mitochondria were prepared from the sunflower seedlings using the method of Jackson *et al.* (1979) with some modifications, (see Fig. 4). A crude homogenate was obtained from an 80 gm sample of the first pair of leaves following the same procedure as that described for the isolation of chloroplasts, except that the grinding medium contained 330 mM mannitol, 30 mM morpholinopropanesulfonic acid (MOPS)-KOH (pH 7.2), 4 mM 2-mercaptoethanol, 1 mM EDTA, 0.2% (w/v) BSA and 0.6% (w/v) polyvinylpyrrolidone (PVP). The filtered homogenate was centrifuged at 5,000 xg for 2 minutes to sediment most of the intact chloroplasts, cell debris and broken chloroplast fragments. The supernatant was then centrifuged at 20,000 xg for 2.5 minutes to give a pellet of crude mitochondria. Both these centrifugations were performed in a Sorvall RC-2B refrigerated centrifuge using a SS-34 rotor.

Figure 4 - Procedure for the isolation of mitochondria
by sedimentation through a discontinuous
Percoll gradient.

HOMOGENIZATION

Sunflower leaves in Polytron for 5 sec. in medium containing 330 mM Mannitol, 30 mM MOPS (pH 7.2), 4 mM 2-mercaptoethanol, 1 mM EDTA, 0.2% (w/v) BSA, 0.6% (w.v) PVP

FILTRATION

CENTRIFUGATION

(5,000 xg for 2 min.)
(20,000 xg for 2.5 min.)

Gently resuspend pellet using nylon mesh attached to a glass rod.

PERCOLL STEP GRADIENT

10 ml of 60%, 5 ml of 30% and 15 ml of 21%. Percoll (v/v) containing 0.25 M sucrose, 10 mM MOPS (pH 7.2), 0.2% BSA, and 12 ml of 13.5% Percoll containing 0.239 M sucrose, 10 mM MOPS (pH 7.2), 0.2% BSA and 50 mM propane-1, 2-diol.

CENTRIFUGATION (7,500 xg for 30 min.)

Mitochondria fraction collected and diluted with resuspension medium.

WASHING (20,000 xg for 2.5 min.)

RESUSPENSION OF MITOCHONDRIA IN APPROPRIATE MEDIUM

Figure 5 - Discontinuous Percoll density gradient centrifugation of a mitochondrial fraction initially obtained by differential centrifugation.

M = mitochondria
BC = broken chloroplasts

The pellet obtained after the second centrifugation was gently resuspended in an appropriate volume of extracting medium to give a chlorophyll concentration not greater than 0.5 mg/ml. This crude mitochondrial preparation was further purified using a 4-step discontinuous Percoll density gradient. The first step of the gradient was a 12 ml layer of 13.5% (w/v) Percoll containing 239 mM propane-1,2-diol. The next three steps consisted of 15 ml of 21%, 5 ml of 30% and 10 ml of 60% (v/v) Percoll, where each layer also contained 250 mM sucrose, 10 mM MOPS-KOH (pH 7.2), 0.2% (w/v) BSA.

After layering the crude mitochondrial preparation on top of the Percoll gradient, the gradient was centrifuged for 30 minutes at 7,500 xg in a Beckman LC-50 ultracentrifuge equipped with a SW 25.2 swinging bucket rotor. Figure 5 is a picture of the gradient after centrifugation. The mitochondrial band, which sedimented in the 30% layer, was manually collected by gravity after puncturing the bottom of the cellulose nitrate tube. The mitochondrial fraction was diluted with 5 volumes of washing medium, containing 330 mM mannitol, 30 mM MOPS-KOH (pH 7.2), 1 mM EDTA and 0.2% (w/v) BSA, and the mitochondria were pelleted by centrifugation at 20,000 xg for 2.5 minutes using the Sorvall centrifuge. The pellet was then gently resuspended in the appropriate medium.

3. Characterization of Organelles

A. Markers used to identify organelle fractions

(i) Chloroplasts (intact or broken)

Chlorophyll was estimated in 96% ethanol extracts using the extinction coefficient of Wintermans and De Mots (1965) for absorption at 654 nm, where:

$$\text{conc. of chlorophyll } (\mu\text{g/ml}) = \frac{1000 A_{654}}{39.8}$$

(ii) Mitochondria

Cytochrome oxidase activity (EC 1.9.3.1) was assayed polarographically with a Clarke-type oxygen electrode using the method of Behrens and Himms-Hagens (1977), except that the reactions were run at room temperature and in air-saturated 50 mM phosphate buffer at pH 7.0. Cytochrome c was used at a concentration of 0.2 mM and 20 mM sodium ascorbate was added to keep cytochrome c in the reduced form. A 3 ml volume of air-saturated buffer containing cytochrome c and sodium ascorbate was added to the oxygen electrode vial and a background rate was recorded. A 0.2 ml aliquot of the organelle preparation was then added to the vial and the rate of O_2 consumption recorded.

(iii) Peroxisomes

a) Catalase activity (EC 1.11.1.6) was determined by a modification of the method of Van Ginkel and Brown (1978). The rate of O_2 evolution was measured with a Clarke-type oxygen electrode following the addition of 6 μl of 30% H_2O_2 to 4 ml of 50 mM phosphate buffer at

pH 7.0 containing a 10-40 μ l aliquot of the organelle fraction.

- b) Glycolate oxidase activity (EC 1.1.3.1) was also estimated with an oxygen electrode according to the method of Bergman et al. (1980). The reactions were run in air-saturated 10 mM phosphate buffer at pH 7.2 containing 10 mM glycolate and 1 mM potassium cyanide. A 0.5 ml aliquot of the organelle sample was added to 2.5 ml of the buffered solution and the rate of O_2 consumption was recorded.

B. Measurement of the intactness of organelles

(i) Chloroplasts

Chloroplast intactness was estimated by the ferricyanide test described by Lilley et al. (1975). Ferricyanide-dependent O_2 evolution was assayed in 2 ml of buffered medium containing 330 mM sorbitol, 50 mM N-2-hydroxyethylpiperazine propane sulfonic acid (EPPS)-KOH, pH 7.6, 2 mM EDTA, 1 mM $MgCl_2$, 1.5 mM potassium ferricyanide, 10 mM D,L-glyceraldehyde and 5 mM ammonium chloride. The rate of oxygen evolution by the chloroplast preparations was measured before and after an osmotic shock to give an estimation of the percentage of intact organelles.

(ii) Mitochondria

Mitochondrial membrane integrity was assessed by release of cytochrome c oxidase from Triton X-100 treated mitochondria according to the procedure of

Jackson et al. (1979). Cytochrome oxidase activity was measured polarographically as described previously, except that the reaction medium was kept isotonic with 330 mM mannitol. Mitochondria were disrupted with 0.02% (v/v) Triton X-100 (final concentration).

The oxygen electrode used for the estimation of the marker enzymes and the degree of intactness of the organelles, was calibrated against air-saturated water, the oxygen concentration of which was taken as 250 M. A blank was run in each case to correct for background variations in oxygen concentration.

C. Microscopy

The isolated organelles were fixed with 4% glutaraldehyde in 330 mM mannitol at pH 7.0 for two hours, washed with 330 mM mannitol in 50 mM phosphate buffer at pH 7.0 and postfixed in 2% osmium tetroxide for two hours. The pellets were then stepwise dehydrated in increasing acetone solutions. Propylene oxide treatment followed, for the mitochondrial pellet, before embedding in Epon, while for the chloroplast pellet, embedding in Spur was started after the final 100% acetone step. All procedures, except embedding, were done at 2-4°C. Sections were cut and finally stained with uranyl acetate and lead citrate prior to their examination in the electron microscope.

4. Procedure for the estimation of endogenous levels of free tryptophan and phenylalanine in organelle fractions.

The endogenous level of free amino acids in chloroplasts was measured on three separate occasions. Two chloroplasts samples were prepared from freshly isolated intact organelles each containing 3 mg of chlorophyll. A third chloroplast sample was prepared from six separate isolations (kept frozen) and it contained 15 mg of

chlorophyll. A mitochondrial sample was obtained by combining together six isolated fractions of this organelle (kept frozen) in order to obtain detectable quantities of most of the free amino acids.

Acetone was added to the buffered organelle suspensions to a final concentration of 80% (v/v) in order to precipitate all soluble proteins. The samples were clarified by centrifugation at 20,000 xg for 5 minutes and the volume reduced to about 2 ml using the Buchler "Evapomix". The mitochondrial sample was then ready for amino acid analysis but in the case of the chloroplast sample, the chlorophyll pigments first had to be removed. To achieve this, the aqueous chloroplast sample was adjusted to pH 4.0 with 2N phosphoric acid and extracted 3 times with diethyl ether which removed the pigments. The remaining aqueous fraction was adjusted to approximately pH 2.0 before a sample was injected into the amino acid analyzer. The mitochondrial extract was also adjusted to pH 2.0 before analysis.

The samples were analyzed in lithium buffers using a Beckman 119 BL Amino Acid Analyzer equipped with an integrator. The column (9 mm x 510 mm) was packed with W2 resin with a bed height of 280 mm. The lithium buffers (pH 2.83, 3.70 and 3.75) were run at a flow rate of 70 ml/hour and the ninhydrin flow rate was 35 ml/hour. A colorimeter analyzed the eluate for the presence of ninhydrin-reacting amino acids at 440 and 570 nm. Chromatography of known amounts of several amino acids was also performed to calibrate the analyzer for measurement of the endogenous amino acids.

5. Procedure for the extraction of the endogenous pools of IAA and PAA in organelle fractions.

Mitochondria and chloroplasts were isolated as described above and resuspended in distilled water. The membranes of the organelles were then broken by freezing (at -20°C) and thawing. In the case of mitochondria, six isolations were combined to provide a large enough sample for analysis.

After thawing the broken chloroplast preparation was filtered under suction through a layer of Celite (analytical filter aid) to remove precipitated pigments and membrane fragments. The Celite pad was washed three times with hot distilled water to ensure complete elution of all water-soluble substances. The mitochondrial preparation, containing no pigments, was not subjected to this filtration step.

The chloroplast aqueous filtrate and the broken mitochondrial preparation were then subjected to solvent fractionations. The aqueous solutions were first adjusted to pH 8.5 and neutral compounds were removed by extraction with three successive equal volumes of freshly redistilled diethyl ether. The pH of the remaining aqueous fractions was then lowered to 2.8 with 2N phosphoric acid and three further ether extractions were carried out on each fraction to yield the acidic ether extracts. These should contain all the acidic compounds that are soluble in ether, such as free indole and phenyl acids, (see Fig. 6).

The acidic ether fractions were concentrated to dryness in a test tube using a Buchler "Evapomix" evaporator. Each fraction was then analyzed for the presence of IAA and PAA initially by preparative high pressure liquid chromatography (HPLC) followed by the derivatization of collected fractions and their analysis by gas liquid

Figure 6 - Procedure for the extraction and fractionation
of endogenous auxins in chloroplasts and
mitochondria.

chromatography (GLC). Full details of these analytical procedures are given in a later section.

6. Preparation of Enzyme fraction.

The isolated organelle pellets were resuspended in 50 mM Tris (hydroxymethyl) aminomethane (TRIS)-HCl buffer at pH 8.0, containing 4 mM 2-mercaptoethanol and were placed in crushed ice (2-4°C) for 15 minutes in order to rupture the membranes by osmotic shock. The chloroplast preparation was then subjected to centrifugation at 13,000 xg for 15 minutes in a Sorvall centrifuge to pellet the membrane fraction. The remaining supernatant, containing the soluble stromal proteins, was used as the enzyme fraction in the assays for auxin biosynthesis (see Fig. 7). The resuspended mitochondrial preparation was used directly as the enzyme fraction in similar assays of auxin biosynthesis.

7. Assays for Auxin Biosynthesis.

A. Using L-tryptophan or L-phenylalanine as substrate.

(i) Non-radioactive experiments

The soluble enzyme fractions prepared from each organelle were assayed for auxin biosynthesis activity using either L-tryptophan or L-phenylalanine as the amino acid substrate. Several enzymatic steps are known to occur (Wightman 1973) in the conversion of the amino acid to the corresponding arylacetic acid product, namely, a transamination, a decarboxylation and either an oxidation or dehydrogenation reaction. The further requirements for the transamination reaction were met by providing α -ketoglutaric acid and pyridoxal-5'-phosphate in the reaction mixture. Three other substances were also added to the mixture to

Figure 7 - Procedure to isolate the chloroplast soluble enzyme fraction.

anticipate the possible cofactor requirements of the next two enzymes of the pathway; these were thiamine pyrophosphate (TPP), β -nicotinamide adenine dinucleotide (β -NAD) and β -nicotinamide adenine dinucleotide phosphate (β -NADP). A standard reaction mixture was thus made up of the following components, dissolved in 50 mM TRIS-HCl buffer at pH 8.0 containing 4 mM 2-mercaptoethanol, to a total volume of 2 ml: 25 mM amino acid, 5 mM α -ketoglutaric acid, 0.25 mM pyridoxal phosphate, 0.18 mM TPP, 0.75 mM β -NAD and 0.65 mM β -NADP plus the enzyme fraction. There were two control systems; the first contained only the amino acid substrate plus the enzyme fraction and the second contained all the components of the standard reaction mixture plus a boiled enzyme fraction. All assays were carried out at 30°C for a period of 4 hours (see Table 1). The reaction was then stopped by rapid freezing and the products formed were later isolated using the solvent extraction procedure described earlier (Fig. 6) for determining the endogenous pools of IAA and PAA in each organelle.

(ii) Radioactive experiments

The radioactive assay system was basically the same as that described for the non-radioactive experiments, except that either L-methylene ^{14}C -tryptophan (56 mCi/mmol.) or L- ^{14}C -phenylalanine (10 mCi/mmol.) was added to the 'cold' amino acid substrate at the beginning of the incubation period. Unlabelled 3-indoleacetaldehyde (IAAld) or

Table 1 - Reaction systems examined for auxin synthesis and type of components used in the assay,

Substrates	Complete System	Control 1	Boiled enzyme system
Amino Acid : L-tryptophan or (25 mM) L-phenylalanine	x	x	x
α -Ketoglutarate (5 mM)	x	-	x
Pyridoxal phosphate (0.25 mM)	x	-	x
β -NAD , β -NADP (0.75 mM, 0.65 mM)	x	-	x
TPP (0.18 mM)	x	-	x
Enzyme Fraction (1 ml)	x	x	Boiled

* Components dissolved in 50 mM Tris-HCl buffer at pH 8.0 containing 4 mM 2-mercaptoethanol. Total volume of reaction system was 2 ml and the incubation period was 4 h at 30°C. The reaction was stopped by rapid freezing.

phenylacetaldehyde (PAAld) was also included in the assay mixtures, at a concentration of 0.8 μ mole/ml, along with bovine serum albumin (BSA) (0.5 mg/ml) to facilitate recovery of the 14 C-labelled arylacetaldehyde intermediate.

3-Indoleacetaldehyde was purchased as the bisulfite adduct and was regenerated by the procedure described by Narumiya et al. (1979). The bisulfite compound was first dissolved in 1N NaOH and the liberated 3-indoleacetaldehyde was then extracted from the aqueous phase with ether. The amount of free 3-indoleacetaldehyde was calculated from the extinction coefficient devised by Brown and Purves (1976), where $\epsilon_{\text{H}_2\text{O}} = 5.4 \text{ cm. mM, } 280 \text{ nm}$

The radioactive system was incubated at 30°C for 4 hours and the reaction was stopped by rapid freezing. The system was then extracted with ether, as described previously, to give a neutral ether fraction and an acidic ether fraction. Before carrying out this fractionation, a further amount of non-radioactive IAAld or PAAld was added to the system to raise the total amount of these compounds to a level which could be detected by U.V. light after thin-layer chromatography of the neutral fraction. The acidic fraction was analyzed by HPLC and GLC, as described in section 8. Radioactivity of collected fractions was measured by liquid scintillation spectrometry, as described in section 10.

B. Using 3-Indolepyruvate or Phenylpyruvate as substrate.

In these assays, 3-indolepyruvic acid (IPyA) ($1\mu\text{mole}$) or phenylpyruvic acid (PPyA) ($1\mu\text{mole}$) were used as the substrate for auxin synthesis. In addition to the substrate, the reaction systems contained either:

- (i) enzyme only,
- (ii) enzyme plus BSA (0.5 mg/ml), or,
- (iii) enzyme, BSA and the cofactors TPP (0.18 mM), β -NAD (0.75 mM) and β -NADP (0.65 mM).

The control systems contained, instead of the active enzyme fraction, an equivalent volume of buffer or a boiled enzyme fraction. All reaction components were dissolved in 50 mM TRIS-HCl buffer at pH 8.0 containing 4 mM 2-mercaptoethanol. The incubation period was 2 hours at 30°C in a total volume of 2 ml. At the end of this time, the reaction was stopped by rapid freezing and the system was later thawed and subjected to solvent extraction as described for the previous assays. The auxin product and remaining substrate were recovered in the acidic ether fraction which was subsequently analyzed by HPLC and GLC.

8. Analysis of Acidic ether extracts of the endogenous pool of IAA and PAA and of the reaction mixtures from biosynthesis experiments.

A. High Performance Liquid Chromatography (HPLC).

HPLC was used for the preparative analysis of all acidic ether fractions. Chromatography was carried out using the reverse-phase mode on a Bondapak C_{18} column, 25 cm in length x 12 mm o.d. The solvent delivery system was an Altex Model 322 equipped with two Model 100A pumps controlled by a Model 420 microprocessor. Column eluates were monitored with an Altex

Model 152 U.V. absorbance detector set at 254 nm.

Each dried acidic ether fraction was resolubilized in 1.2 ml of 5% ethanol, sonicated for 3 minutes and then passed through a 5 μ Millipore filter. A 1 ml aliquot of the filtered extract was injected on the column and eluted with a linear gradient increasing from 5% ethanol in 0.1N formic acid to 50% ethanol in 0.1N formic acid over a period of 20 minutes. The flow rate was 5 ml min⁻¹. The elution times of authentic standards (IPyA, IAA, PPyA, PAA) were monitored at the beginning of each day and used to calibrate the column for the collection of fractions from each acidic ether extract corresponding to the four retention times. The volume of each collected fraction was usually about 10 ml and it was taken to dryness immediately at 35°C under vacuum using the Buchler Evapomix. Samples were stored in a deep freeze at -20°C until they were derivatized for GLC analysis.

Redistilled ethanol and redistilled formic acid were used in these separations. A permanganate distillation system was required to further purify the distilled water in order to minimize baseline drifts during solvent programmed runs.

B. Derivatization

The dried HPLC-purified fractions were resolubilized in acetone, transferred into 1 dram glass vials and either evaporated overnight in a desiccator under a slight vacuum or evaporated immediately under a gentle stream of nitrogen. Two different procedures were used for derivatization of the samples prior to GLC analysis. Pentafluorobenzoylation was used for the derivatization of PAA and IAA, while methyl esters were made, in some analyses, for IAA-containing samples only. The authentic

compounds used for identification by co-chromatography were derivatized in the same manner.

(i) Pentafluorobenzylation

The pentafluorobenzyl esters of PAA (PFB-PAA) and IAA (PFB-IAA) were prepared as described by Marklam et al. (1980). An excess of 1% pentafluorobenzyl bromide in acetone (0.5 ml) and 10 mg anhydrous potassium carbonate were added to the sample. The vial was closed with a teflon-coated septum cap. The mixture was heated in a Pierce Reacti-Therm heating module at 60°C for 90 minutes. The reagent was then evaporated to dryness under a stream of nitrogen. The dry residue was resolubilized in 1 ml of distilled water and this aqueous fraction was extracted three times with ethyl acetate. The ethyl acetate fractions were combined, evaporated to dryness and the derivatized residue redissolved in a known small volume of ethyl acetate for GLC analysis.

(ii) Methylation

The dried IAA-containing samples were redissolved in 10% methanol in ether (all solvents being anhydrous) and methylated according to the standard diazomethane procedure of Schlenk and Gellerman (1960). Ether saturated N₂ gas was channelled into a large test tube containing 10 ml of 10N KOH, 7 ml of dry ether and 7 ml of diethylene glycol monoethyl ether (carbitol). The diazomethane gas was generated by adding about 1 g of Diazald (N-methyl-N-Nitroso-p-toluene sulfonamide) to this mixture. The gas was then allowed to bubble through

the sample vial for a period of approximately 3 minutes to ensure that all reactive groups were methylated. The reaction mixture was dried under a stream of nitrogen and the residue redissolved in a known small volume of ethyl acetate prior to GLC analysis.

C. Gas-Liquid Chromatography

All samples were analyzed in a Pye Series 104 gas chromatograph using dual glass columns connected to heated flame ionization detectors. The columns (2 m x 2 mm I.D.) were packed with either 3% OV-17 or 3% OV-101, both coated on acid washed Chromosorb W, 80-100 mesh. Nitrogen was used as the carrier gas at a flow rate of 17 ml/min. Hydrogen and air flows to the detectors were 18 and 6 psi respectively. Attenuation varied depending on the ether extract being analyzed; the setting was usually at either 50, 100 or 200. The following isothermal conditions or temperature programs were used for good separation of the indole and phenyl compounds:

with 3% OV-17 columns

		Initial T	Program	Final T
Me-IAA		150°C	3°/min	270°C
PFB-IAA	i)	180°C	3°/min	270°C
	ii)	isothermal	at	235°C
PFB-PAA	i)	150°C	3°/min	200°C
	ii)	isothermal	at	175°C

with 3% OV-101 columns

PFB-IAA				
and		130°C	3°/min	240°C
PFB-PAA				

Measurement of the amounts of PAA and/or IAA present in each sample analyzed were made from standard curve constructed by plotting peak area against the amount of authentic standard injected. Peak areas were determined by using a Hewlett Packard No. 3385 Automation system connected to the GLC or were calculated by multiplying the peak height by the width of the peak at half its height.

9. Analysis of Neutral ether fraction.

The radioactive products extracted into the neutral ether fraction were separated by silica gel thin-layer chromatography (TLC). Commercially prepared 20 x 20 cm Silica Gel 60 plastic-backed plates (coating thickness 0.2 mm) containing F-250 fluorescent indicator were used. TLC was carried out in glass tanks (7 x 25 x 25 cm) lined with filter paper and using freshly prepared solvent system each day.

Two solvent systems were employed:

- i) Solvent 1: for separation of phenyl compounds,
Benzene/1-4 dioxane/acetic acid
- ii) Solvent 2: for separation of indole compounds,
Diethyl ether/Hexane
7:1,v/v

Chromatograms were developed one dimensionally. Appropriate authentic marker substances were always included on each chromatogram. Visual detection was achieved by using UV-light and this was made easier by the addition of small amounts of authentic compounds at the beginning of the extraction procedure. The R_f value and co-chromatography with the authentic compound were used as criteria for identification. The band corresponding to the authentic marker

compound was scraped off and the silica gel particles were collected on a glass wool plug contained in an inverted pasteur pipette connected to a vacuum line. The glass wool and silica scrapings were pushed out into a scintillation vial for the determination of radioactivity, as described in section 10.

The Rf values for the neutral compounds investigated are as follows:

Compound	TLC solvent system	
	Solvent 1	Solvent 2
	Rf	Rf
Tryptophol		0.16
3-Indoleacetaldehyde		0.32
3-Indolealdehyde		0.11
Phenylethanol	0.41	
Phenylacetaldehyde	0.61	
Phenylaldehyde	0.48	

10. Liquid scintillation spectrometry

The radioactive neutral compounds identified on TLC plates were removed by the scrapings procedure and placed in a scintillation vial containing 10 ml of TOX cocktail (Triton L-114, 250 ml; omnifluor, 4 g; xylene, 705 ml). The radioactivity in the acidic compounds was measured after their separation by HPLC (see section 8). Each collected HPLC fraction was first concentrated to dryness and then resolubilized in 1 ml of redistilled ethanol; a 0.5 ml sample was added to 10 ml of TOX cocktail while the rest of the fraction was subjected to GLC analysis.

Two scintillation spectrometers were used:

- (i) a Packard Tricarb 3003 spectrometer with an optimal

gain setting of 11%, and

- (ii) a Beckman LS-150 spectrometer with the gain set at the optimal value for the instrument.

The degree of quenching was estimated using a TOX-nitromethane quenched curve with the Packard instrument. The quench curve used with the Beckman LS-150 was generated from a series of Beckman, argon-saturated, sealed ^{14}C standards supplied with the instrument.

11. Combined Gas Chromatography - Mass Spectrometry (GC-MS)

Electron impact mass spectra of suspected and authentic samples of methylated IAA and of pentafluorobenzylated PAA were obtained with a Finnigan Model 3000 combined gas chromatograph-mass spectrometer at 70 eV. Gas chromatography was carried out on a 6 ft.-6 mm I.D. column packed with 3% Se-30 on Chromosorb W. Helium was used as the carrier gas. Spectra were recorded and analyzed with a Model 6000 Data system.

12. Protein Determination

The protein content of all reaction and control systems was determined using the Bio-Rad protein assay. Aliquots of each system, ranging from 10 μl to 30 μl were diluted to 0.1 ml with TRIS-HCl buffer, pH 8.0, and assayed using the Bio-Rad standard procedure, monitoring at 595 nm. Lyophilized bovine plasma globulin was used to prepare the standard curve.

Samples containing chlorophyll were prepared as described by Ellis (1975). Proteins were first precipitated by hot 5% trichloroacetic acid for 5 minutes and then extracted with 80% acetone to remove the pigments. The precipitated proteins were resolubilized in 1N NaOH at 90°C for 5 minutes. Aliquots of these samples were then assayed using the Bio-Rad procedure. For these samples, the standard curve was prepared using aliquots of the bovine

plasma globulin protein made up to 0.1 ml with distilled water plus a volume of 1N NaOH equivalent to the sample.

13. Source of chemicals, materials and seeds.

A. Chemical

Ascorbate, bovine serum albumin, cytochrome c, EPPS, glyceraldehyde, glycolate, 3-indoleacetic acid, 3-indoleacetaldehyde bisulfite, 3-indolepyruvic acid, 3-indoleethanol (tryptophol), α -ketoglutaric acid, MOPS, Δ -NAD, NADP, L-phenylalanine, phenylethanol, phenylpyruvic acid, pyridoxal-5'-phosphate, PVP-10, thiamine pyrophosphate, tricine, TRIS-HCl, triton X-100, L-tryptophan	Sigma
Phenylacetic acid	Aldrich
Phenylacetaldehyde	ICN-K and K
Percoll	Pharmacia
Propane-1,2-diol, sucrose	J.T. Baker Chemical
Sorbitol, EDTA	BDH Laboratories
Mannitol	Fisher Scientific
2-Mercaptoethanol	Eastman Kodak
L- U- ¹⁴ C -phenylalanine, L- methylen- ¹⁴ C-tryptophan	Amersham-Searle
Reagents for Derivatization:	
Carbitol	Fisher Scientific
Diazald	Sigma
Pentafluorobenzyl bromide	Pierce

B. Materials

μ -Bondapak C ₁₈ HPLC column packing	Waters Associates
OV-17, OV-101 GLC column packing	Chromatographic specialties
Thin-layer chromatography plates	E. Merck

C. Seeds of *Helianthus annuus* CM90RR

Seeds were obtained from Agriculture Canada Research Station, Morden, Manitoba.

Results

1. Preliminary Experiments

A. Organelles

(i) Choice of Plant Material

The aim of the cell fractionation studies attempted in this work was to determine whether chloroplasts, mitochondria and peroxisomes possess the biochemical potential for auxin synthesis. Since previous investigations in this laboratory have shown that high speed supernatant fractions isolated from various shoot tissues have the capacity to synthesize auxins (Wightman and Cohen, 1968; Gibson *et al.* 1972; Wightman 1973; Wightman and Rauthan 1974), it was clear that an important aspect of the present experimental approach must be to obtain organelle fractions showing a high level of purity and a high degree of intactness. These two conditions, however, are difficult to achieve and are usually obtained at the expense of yield. Furthermore, leaf organelles are notoriously fragile and success in their isolation usually varies considerably with the type of plant employed.

A survey of possible plant material was first undertaken to determine which plant gave the best yield of pure, intact organelles. The tissues sampled included young expanding leaves of tobacco and tomato plants and the first leaves of cucumbers, pea and sunflower seedlings. The leaves were first examined for their chloroplast fraction using the isolation procedure described by Mifflin and Beevers (1974). Between 25 and 35 grams of leaves were extracted in two volumes of a grinding medium which contained 10 mM KCl, 1 mM $MgCl_2$, 1% (w/v) Dextran T40, 1% (w/v) Ficoll, 0.1% (w/v) BSA and 30% (w/v) sucrose in 0.1 M Tricine buffer at pH 7.5. The leaves were homogenized for 5 seconds in a blender and filtered through 7 layers

of nylon mesh. The filtrate was centrifuged at 4,000 xg for 1 minute. The pellet of washed chloroplasts was further purified on a sucrose density gradient using a combination of rate and equilibrium centrifugation as described by Mifflin and Beevers (1974).

Whole chloroplasts were recovered and phase-contrast microscopy showed that the majority were intact. Chlorophyll was measured and the value expressed as a percentage of the amount present in the original filtrate. Comparisons between the chloroplast fractions obtained from the different plants were made on this basis. Pea and sunflower leaves gave similar recoveries of between 7-10% of the chlorophyll in the filtrate. The recoveries from cucumber and tomato were somewhat lower (5%) while tobacco gave the lowest recovery, only 2-3%. The main problem with tobacco leaves was found to be the large amount of starch granules present in their chloroplasts. Starch grains have a greater density than the chloroplast in which they are formed and upon centrifugation tend to migrate faster which results in many broken organelles. Different light regimes were applied to try to decrease the starch content of the leaves but no improvement in chloroplast recoveries were obtained. However, recoveries from every plant were appreciably increased by using a Polytron tissue homogenizer along with 4 volumes of isolation medium for the maceration of the leaves.

These modifications in the isolation procedure were retained during studies of the mitochondrial fraction which was isolated following the method described by Huang *et al.* (1976). Intact chloroplasts and a portion of the broken chloroplasts in the filtrate were removed by centrifugation at 2,000 xg for 10 minutes. A crude mitochondrial pellet was then obtained by centrifuging the supernatant at 10,000 xg for 30 minutes. This particulate fraction was

resuspended and further purified on a linear sucrose density gradient. Fumarase was used as the marker enzyme for mitochondria. The best recoveries were obtained with pea and sunflower leaves where 8-10% of the mitochondria present in the filtrate were recovered from the gradient, whereas with cucumber, tomato and tobacco leaves recoveries of only 3-4% were obtained.

Thus, sunflower and pea leaves were found to be the best material for the isolation of both chloroplasts and mitochondria. The final choice of sunflower as the experimental material was motivated by the better recovery of peroxisomes from this plant. Studies of the auxin biosynthesis capacity of this organelle are planned in the future and it would be desirable to use the same plant material throughout these studies.

Differential and sucrose density gradient centrifugation were used to isolate the peroxisomes by the procedure of Tolbert et al. (1969) and the marker enzyme catalase was assayed to evaluate peroxisomal recoveries. Only traces of catalase activity were detected in pea leaves whereas measurable amounts were found in sunflower leaves, amounting to 1.5% of the initial amount present in the filtrate.

Thus, sunflower leaves were selected for the present experimental work because the yields of each organelle were found to be better than those obtained from any of the other four plants examined.

(ii) Choice of Isolation Methods

Following the choice of sunflower leaves as the experimental material, the procedures used for isolating the chloroplast and mitochondrial fractions in the experimental work itself were next improved. Mannitol or sorbitol was used to generate the appropriate osmotic strength in the isolation and resuspension media. These sugar

alcohols have lower viscosities than sucrose and offer less resistance to the fragile organelles during homogenization of the leaves and subsequent differential centrifugation. The differential centrifugations were also shortened both in time and gravitational force, which resulted in much better yields of intact organelles.

Percoll replaced sucrose as the density gradient medium. Two methods employing Percoll have been published in recent years for the isolation of chloroplasts and mitochondria (Mills and Joy, 1980; Jackson et al. 1979) and these methods were adopted, with slight modifications, for the present experiments. These methods are described in detail in Material and Methods. The low viscosity of Percoll allowed more rapid sedimentation of chloroplasts and mitochondria than those observed in sucrose, which greatly reduced the time taken to carry out separation of both organelles. Such shorter purification times are better since there is much less chance for physiological degradation of the organelles to take place during centrifugation.

Thus, as will be described in later sections, recoveries of chloroplasts and mitochondria were good, both in yield and intactness, and the isolations were completed in a relatively short time.

B. Analysis of Reaction Systems

Chloroplast enzymes were assayed for IAA biosynthetic activity as described in Material and Methods. At the end of the incubation period, the reaction products were ether fractionated to give neutral and acidic fractions.

Preliminary analysis of the acidic fractions were carried out directly by gas chromatography, after ester derivatization of the acidic compounds in the fraction. Many compounds were seen to be present in these chromatograms, some in very large quantity and these

prevented the detection of the IAA formed by chloroplast enzymes. Modifications in the temperature program procedure used in such GLC analyses did not improve the separations. High performance liquid chromatography was therefore tested and adopted as a preparatory method in an effort to remove most of these major metabolites so that IAA could then be detected and measured by analytical GLC.

Preliminary analyses of the neutral ether fractions for presence of the intermediate, IAAld, were not successful. Gas chromatography of such neutral fractions was tried but no IAAld could be detected. 2,4-Dinitrophenylhydrazone derivatives of the keto- and aldehyde compounds present in the reaction systems were then made, as described by Wightman and Cohen (1968). A solution of 2,4-dinitrophenylhydrazine (2,4-DNPH) containing 3 mg/ml in 2N HCl was used as the reagent. The biosynthesis assays and the controls were stopped by adding 4 ml of this reagent to each 2 ml reaction system and hydrazone formation was allowed to proceed overnight at 8°C. Ethyl acetate was used to extract the mixed hydrazone fraction from each inactivated reaction system.

The large amount of α -ketoglutarate remaining in the complete reaction system also reacted with 2,4-DNPH and this hydrazone derivative had to be selectively removed from the ethyl acetate extract to allow for good TLC separation of the IPyA- and IAAld-dinitrophenylhydrazones. The solvent extraction method of Katsuki et al. (1961) was used to remove the α -ketoglutarate-DNPH from the mixed hydrazone fraction. TLC was then carried out on the remaining ethyl acetate extract, using pre-coated silica gel plates. Two solvent systems were used, namely benzene:ethyl acetate (90:10, v/v) for separation of neutral hydrazones and benzene:acetic acid:ethyl acetate (90:5:5, v/v) for separating the acidic hydrazones. Marker

samples of the authentic dinitrophenylhydrazones were included on each chromatogram and all the compounds were easily visible due to their yellow colour.

IPyA-DNPH was detected in large amounts only in the complete reaction system. IAAld-DNPH, on the other hand, was not found. Traces of a compound migrating to an R_f similar to the standard IAAld-DNPH were observed when all the stromal proteins (about 36 mg) from one isolation were used in a single complete reaction system. In view of these results, it was thought that the turnover in the intermediate, IAAld, was probably very fast and only very small quantities remained in solution in the reaction system at any given time. Attempts were therefore made to identify IAAld-DNPH in the hydrazone extract by gas chromatography, since this analytical method has much greater detection sensitivity. Problems were encountered, however with the derivatization of IAAld-DNPH and this method was not pursued.

Radioactive metabolism experiments were tried next, in combination with hydrazone formation. The 2,4-DNPH reagent was allowed to react for only one hour since it was noticed that an overnight reaction under such strong acidic conditions favoured the breakdown of ^{14}C -tryptophan into a compound with similar chromatographic properties to IAAld-DNPH. TLC was carried out as described earlier for the neutral hydrazone fraction. The region corresponding to the R_f of authentic IAAld-DNPH was scraped off the plate and put directly into TOX cocktail for liquid scintillation spectrometry.

Several experiments were performed but no consistent results could be obtained. In some cases, low recoveries of radioactivity (under 100 dpm) were found in the IAAld-DNPH isolated from the complete reaction system but in others, no difference was observed between the

complete reaction system and the controls. Furthermore, increasing level of radioactivity in ^{14}C -tryptophan did not always give greater amounts of radioactivity incorporated into IAAld-DNPH. The addition of non-radioactive IAAld at the end of the incubation period, to act as a carrier for the radioactive molecules during hydrazone formation and subsequent isolation did, however, favour consistent recovery of greater amounts of radioactive IAAld-DNPH from the complete reaction system.

Narumiya et al. (1979) demonstrated the presence of ^{14}C -IAAld in Pseudomonas fluorescens as an intermediate in the metabolism of ^{14}C -L-tryptophan, by adding non-radioactive IAAld to the reaction system at the beginning of the incubation period. Although this intermediate did not accumulate in significant quantity, some radioactivity was nevertheless found in IAAld by using this 'carrier method'. The same procedure was therefore tested in the chloroplast reaction systems used in the present study. A known amount of non-radioactive IAAld was included in the reaction mixture at the start of the incubation period, along with the primary substrate, ^{14}C -L-tryptophan. A further amount of non-radioactive IAAld was also added at the end of the reaction period to act as a carrier for ^{14}C -IAAld through the extraction and isolation procedures. Recovery of ^{14}C -IAAld was much improved by these two additions of non-radioactive IAAld and this procedure was therefore retained in later experiments.

Dinitrophenylhydrazone formation was, however, abandoned since no significant difference in the recovery of IAAld could be found between direct analysis of the neutral ether extract and analysis of the hydrazone derivatives. Furthermore, the yellow colour of these derivatives was found to interfere with liquid scintillation measurements.

Two other separatory methods were then investigated, besides TLC, in an attempt to minimize possible losses during chromatography. Preparative GLC on a 3% OV-17 column using temperature programmed analysis was attempted. By means of a 20:1 splitter apparatus, approximately 95% of the effluent gas from the preparative column flowed out through the collection port and could be collected and condensed in a suitable ice-cooled collection tube. This procedure allowed for the collection of any fraction of interest. The recoveries obtained by this method, however, were found to be low and the procedure was therefore abandoned. HPLC was also tried as a method for analysing the neutral ether fraction but IAald and tryptophol were found to have retention times that differed by only a few seconds from each other and this situation prevented the use of this method for adequate separation of IAald from the other neutral intermediates.

Thus, the procedure finally adopted to demonstrate the occurrence of ^{14}C -IAald in the reaction system containing radioactive L-tryptophan, included:

- (i) The addition of non-radioactive IAald to the reaction system both at the beginning and end of the incubation period,
- (ii) the use of TLC for analysis of the neutral ether extracts, and
- (iii) measurement of ^{14}C incorporation in tryptophol. These measurements were made because of the possible formation of tryptophol from IAald and the amount of ^{14}C observed in this more stable indole alcohol can therefore be used as an indirect measure of the amount of radioactivity in IAald.

The above procedures were also followed with the phenylalanine experiments and details are described in Material and Methods.

2. Growth Data

The increase in length of the blade of the first pair of leaves of sunflower seedlings is shown in Fig. 8. The leaves were harvested between 12 and 14 days after seed germination. This corresponds to the period when the first leaves are in active expansion, since by day 15 the growth rate has started to decline and by day 16, a plateau has been reached. Fig. 9 illustrates the appearance of the sunflower seedlings on day 14, showing the first pair of leaves and the emerging second pair.

3. Characterization of Organelles

A. Chloroplast

Table 2 shows the data from the estimation of the markers used to characterize the chloroplast pellet. Chlorophyll was used as the marker for chloroplasts, and possible contamination of chloroplast preparations by mitochondria and peroxisomes was investigated by assaying for the marker enzymes, cytochrome c oxidase and catalase respectively. Data from washed chloroplast preparations, obtained by pelleting the plastids at 2,000 xg, shows that the levels of these two enzymes are low, 1.4% and 2.6% of the total homogenate activity respectively, for a 21% recovery of the initial amount of chlorophyll. Sedimentation through 40% Percoll allowed an 8% recovery of the original amount of chlorophyll and reduced the mitochondria-peroxisome contamination to traces since no marker enzyme activities could be determined in the five preparations assayed.

The yield of intact chloroplasts was estimated by measuring ferricyanide-dependent O_2 evolution, as summarized in table 3. The washed chloroplasts were, on average, 52% intact, whereas the Percoll purified chloroplasts showed an average of 92% intact organelles. In the example shown in table 3, the chlorophyll yield was reduced to 47%

Figure 8 - Growth in length of the leaf blade of the first pair of leaves of sunflower seedlings. Each value is the mean of 50 leaves.

Figure 9 - Sunflower seedlings on day 14 showing first pair of leaves and emerging second pair.

Table 2 Recovery of chlorophyll and marker enzymes in purified chloroplasts. The values are expressed as percent of the total amount in the filtrate and represent the average of five preparations.

Characteristic	Washed Chloroplasts	40% Percoll Purified Chloroplasts
Chlorophyll		
% Filtrate	21 $\pm$ 3.1	8 $\pm$ 2.3
mg	6.66 $\pm$ 1.06	2.62 $\pm$ 0.56
Cytochrome c oxidase		
% Filtrate	1.4 $\pm$ 0.4	not detected
Activity (moles O ₂ consumed/min. mg chlorophyll)	64 $\pm$ 17.8	
Catalase		
% Filtrate	2.6 $\pm$ 1.5	not detected
Activity (moles O ₂ produced/min. mg chlorophyll)	1399 $\pm$ 173	

$\pm$ = standard deviation

Table 3 Ferricyanide-dependent O_2 evolution and yield of intact chloroplasts for washed and purified chloroplasts

Purification Steps	Rate (μ moles O_2 produced/min/mg chlorophyll)	Percent yield of Intact Chloroplasts*	Chlorophyll mg	Chlorophyll %	Intact Chlorophyll** mg
Washed Chloroplasts	541	1225	56 (52 $\pm$ 8)	7.39	4.14
40% Percoll Purified Chloroplasts	81	1290	94 (92 $\pm$ 4)	3.46	3.25
					79

* Figures in parentheses show the average percent obtained from five preparations.

** Chlorophyll in sample $\times$ % intactness.

$\pm$ = standard deviation

Figure 10 - Electron micrograph of the chloroplasts
obtained by pelleting a washed chloroplast
fraction through 40% Percoll. x 15,830

of the amount in the washed chloroplasts by pelleting through the 40% Percoll. However, by estimating the recovery of "intact chlorophyll" (mg chlorophyll in sample x% intactness of sample), this 47% represents 79% of the intact chlorophyll contained in these washed chloroplasts. Thus, the Percoll purified preparations were enriched in intact chloroplasts and other organelle contamination was practically eliminated.

The electron micrograph (Fig. 10) of the chloroplast pellet confirms these biochemical results. The preparation was homogeneous and the chloroplasts looked intact.

B. Mitochondria

The mitochondrial fraction was characterized using cytochrome c oxidase as the marker enzyme for this organelle. Chlorophyll was used as the marker for chloroplast material and glycolate oxidase as a marker for peroxisomes. Table 4 shows data for the estimation of these different parameters in washed mitochondria and in Percoll purified mitochondria. The washed mitochondrial fraction, obtained by differential centrifugation, contained on average 40% of the mitochondria present in the original filtrate based on the recovery of cytochrome c oxidase activity. Peroxisomes, along with broken chloroplasts, also contaminated the pellet, being present at levels 13.3% and 3.4% of those present in the original filtrate, respectively. A 10-fold reduction in chlorophyll and a 5-fold reduction in peroxisomal contamination were achieved by centrifugation through the Percoll step gradient followed by one more washing and pelleting at 20,000 xg for 2.5 minutes.

The recovery of mitochondria, however, was also reduced approximately 4-fold by these purification steps to a level of only 12% of the initial filtrate. The ratio of the average recoveries of

Table 4 Recovery of marker enzymes, chlorophyll and protein in purified mitochondria. The values are expressed as percent of the total amount in the filtrate and represent the average of five preparations.

Characteristic	Washed Mitochondria	Percoll Gradient Mitochondria
Cytochrome c oxidase		
% Filtrate	48 ± 9	12 ± 3.4
Specific activity (μ moles O_2 consumed/min. mg protein)		48.8 ± 13.3
Glycolate oxidase		
% Filtrate	13.3 ± 2.7	2.7 ± 1.3
Specific activity (μ moles O_2 consumed/min. mg protein)		34.3 ± 11.2
Chlorophyll		
% Filtrate	3.4 ± 1.1	0.3 ± 0.1
mg		0.21 ± 0.10
Protein		
mg		10.5 ± 1.9
Cyt c oxidase		
glycolate oxidase	3.6	4.4
Cyt c oxidase		
Chlorophyll	14.1	40

± = Standard deviation

Table 5. Membrane integrity of washed and gradient-purified mitochondria as measured by release of cytochrome c oxidase. Rates are expressed as μ moles O_2 consumed/min. mg protein, and are average values from 4 preparations.

Purification Step	Control	Detergent treated*	% Intact
Washed Mitochondria	34 $\pm$ 4	126 $\pm$ 13	73 $\pm$ 8
Gradient-purified Mitochondria	26 $\pm$ 3	75 $\pm$ 6	65 $\pm$ 5

*Detergent used: 0.02% (v/v) Triton X-100

$\pm$ = Standard deviation

Figure 11 - Electron micrograph of the mitochondrial fraction obtained after Percoll step gradient purification of the pelleted organelle fraction initially obtained by differential centrifugation. x 24,450

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cytochrome c oxidase to glycolate oxidase in the purified mitochondria was slightly increased compared to that of the washed mitochondrial fraction. Cytochrome c oxidase was also enriched with respect to chlorophyll after the Percoll step gradient purification. The yield in a typical preparation from 80 g leaves was about 10.5 mg protein and 0.21 mg contaminating chlorophyll giving a protein/chlorophyll ratio of approximately 50.

Cytochrome c oxidase, a marker enzyme for the inner mitochondrial membrane was used to estimate the degree of intactness of the mitochondria present in the different preparations. Measurement of the enzyme activity, before and after release of the enzyme by detergent treatment of the preparation, allowed for the calculation of the percentage of intact mitochondria. Table 5 shows that mitochondria in the washed fraction were approximately 73% intact. After purification on the Percoll gradient, a slightly lower percentage of intact mitochondria was found (65%).

The electron micrograph of the mitochondrial pellet (Fig. 11) shows mainly mitochondria with their typical cristae. Some peroxisomes are present along with debris from broken or swollen mitochondria and peroxisomes. The micrograph generally confirms the biochemical data.

4. Estimation of the endogenous levels of free tryptophan and phenylalanine in organelle fractions.

Analysis of the free amino acids extracted from isolated sunflower chloroplasts gave a pattern of distribution similar to that found in Vicia faba chloroplasts by Kirk and Leech, (1972). Glutamate and its amide, glutamine, were the two most abundant free amino acids present, and these occurred at concentrations of 131 and 117 nmoles/mg chlorophyll respectively. These were followed by alanine (19),

aspartate (8), histidine (7), serine (6), glycine (5) and threonine (3), their concentrations being shown in brackets. Valine, cystine, methionine, isoleucine, leucine, ornithine, lysine and arginine were present, but at levels below 1.5 nmoles/mg chlorophyll. Traces of proline and of the amide, asparagine, were also detected. The aromatic amino acids, tyrosine and phenylalanine, were also found at 0.86 and 1.45 nmoles/mg chlorophyll, respectively. No measurable amount of tryptophan, however, was observed. The average level of phenylalanine measured from 3 extractions gave a value of 1.25 nmoles/mg chlorophyll.

The mitochondria amino acid extract showed a pattern of distribution similar to the chloroplasts. Glutamate was again the most abundant amino acid at 2.5 nmoles/mg protein, followed by alanine (2), serine (0.7), glycine (0.6), histidine (0.3) and threonine (0.3). Valine, cystine, methionine, isoleucine, leucine, ornithine, lysine and the amide, glutamine, were present at levels below 0.1 nmoles/mg protein. Aspartate was not detected and only traces of asparagine were found. The aromatic amino acids, tyrosine and phenylalanine, were measured at 0.06 and 0.07 nmoles, respectively, whereas tryptophan occurred at 0.56 nmoles/mg protein.

Thus, as summarized in Table 6, tryptophan and phenylalanine, the precursors of IAA and PAA, were both found to occur in the isolated mitochondria whereas only phenylalanine could be detected in the purified chloroplasts.

5. Estimation of the endogenous pools of IAA and PAA in organelle fraction.

The natural occurrence of the two auxins, 3-indoleacetic acid and phenylacetic acid, in both chloroplasts and mitochondria was verified by solvent extraction followed by HPLC and GLC analysis. After the

Table 6 Free tryptophan and phenylalanine levels in purified chloroplasts and mitochondria isolated from sunflower leaves.

	Chloroplasts (nmoles/mg chlorophyll)	Mitochondria (nmoles/mg protein)
Tryptophan	not detected	0.56
Phenylalanine	$1.25 \pm 0.19^*$	0.07

*value is the average from 3 extractions

$\pm$ = Standard deviation

organelle fraction had been isolated, solvent extracted and ether partitioned as described in the Material and Methods section, the acidic ether fraction was first analysed by preparative HPLC. Collected fractions were then derivatized by pentafluorobenzoylation and further analysed by GLC. The suspected pentafluorobenzyl esters of the two auxins were identified in the appropriate HPLC fractions by co-chromatography with samples of the authentic compounds.

Figures 12 and 13 are representative scans obtained when aliquots of the derivatized HPLC-purified fraction from the chloroplasts were gas chromatographed on 3% OV-17 columns. Temperature program analysis was used to achieve good separation and identification of the growth substances since the pentafluorobenzyl esters of PAA (PFB-PAA) and IAA(PFB-IAA) have quite different boiling points and are eluted almost 50°C apart. The increase in area of the suspected peaks when co-chromatographed with authentic PFB-esters of PAA and IAA is evident in Fig. 13. PFB-PAA was eluted around 172°C whereas PFB-IAA came off the column around 223°C. Two quantitative analyses were done on the endogenous auxin pool of sunflower chloroplasts and the results are given in Table 7. These measurements were made on fractions isolated on separate occasions at different periods of the year. IAA was present at higher levels than PAA on both occasions: 634 pmoles/mg chlorophyll for IAA against 515 pmoles/mg chlorophyll for PAA in the first analysis and 474 pmoles/mg chlorophyll for IAA against 346 pmoles/mg chlorophyll for PAA in the second determination. ()

Analysis of the endogenous auxin content of mitochondria was done only once because several isolations had to be combined in order to obtain sufficient quantity of the organelles. The mitochondria were solvent extracted and the acidic ether fractions was analysed as previously described for the chloroplasts. Figures 14 and 15 are the

Figure 12 - GLC scan of pentafluorobenzylated PAA-IAA
zone trapped from PREP-HPCL analysis of
an acidic ether extract of the chloroplast
endogenous hormone pool.

Figure 13 - GLC scan of pentafluorobenzylated PAA-IAA
zone trapped from PREP-HPLC analysis of an
acidic ether extract of the chloroplast
endogenous hormone pool spiked with
authentic PFB-PAA and PFB-IAA

Figure 14 - GLC scan of pentafluorobenzylated PAA-IAA
zone trapped from PREP-HPLC analysis of an
acidic ether extract of the mitochondria
endogenous hormone pool.

TEMPERATURE PROGRAM: 150° → 240°C at
3°/min on 3% OV-101 column

Figure 15 - GLC scan of pentafluorobenzylated PAA-IAA zone trapped from PREP-HPLC analysis of an acidic ether extract of the mitochondria endogenous hormone pool spiked with authentic PFB-PAA and PFB-IAA.

TEMPERATURE PROGRAM: 150° → 240°C at
3°/min on 3% OV-101 column

Table 7 Endogenous levels of free 3-indoleacetic acid and phenylacetic acid in purified chloroplasts and mitochondria from sunflower leaves.

Auxin	Chloroplasts (pmoles/mg chlorophyll)	Mitochondria (pmoles/mg protein)
IAA	634	19.2
	474	
PAA	515	5.8
	346	

scans obtained when aliquots of the mitochondrial HPLC-purified fractions were examined by GLC. Temperature program analysis was again employed and suspected PFB-PAA and PFB-IAA were found to elute around 174°C and 220°C, respectively (Fig. 14). These suspected PFB-esters were each found to co-chromatograph with the corresponding authentic ester (Fig. 15). This finding thus provided good evidence for the presence of both IAA and PAA in sunflower mitochondria. The levels of the auxins were low, but IAA was present at about 3 times the amount of PAA: 19.2 pmoles/mg protein for IAA and 5.8 pmoles/mg protein for PAA, (Table 7).

6. Auxin Biosynthesis Assays

A. Using L-Tryptophan or L-Phenylalanine as Substrate

(i) Non-radioactive Experiments

The metabolic activity of each organelle fraction was assayed with the non-radioactive amino acids, L-tryptophan or L-phenylalanine, as described in Section 7 of Material and Methods. The complete reaction system additionally contained the cofactors probably required by the three expected enzymes of the pathway:

1. α -ketoglutarate and pyridoxal phosphate for the amino acid aminotransferase,
2. thiamine pyrophosphate for the pyruvic acid decarboxylase, and
3. oxidized NAD and NADP for the acetaldehyde oxidase or dehydrogenase.

The controls were included in each assay, one containing the amino acid substrate plus the enzyme fraction, and the other containing all the components of the complete system except that a boiled enzyme fraction was used. All reactions were stopped by rapid freezing after a 4-hour incubation period. Each reaction mixture was later thawed and sequentially extracted with ether at different pH levels to

yield neutral and acidic ether fractions, the latter containing the auxin product IAA or PAA. Qualitative and quantitative identification of the auxins was achieved by means of preparative HPLC followed by gas chromatography and mass spectrometry, as described in section 8 and 11 of Material and Methods.

In some experiments, the complete reaction mixture was divided in two equal volumes. One half was analysed as outlined above and the other half was used to determine the percent recovery of the auxin product after the reaction mixture has been through the analytical procedure. The efficiency of the method was determined by measuring the percent recovery of 5 μ g of either IAA or PAA included in the second half before starting the extraction procedure. This known amount of IAA or PAA added to the system is referred to as 'an internal spike' during final GLC analysis, as opposed to 'an external spike' which refers to the small amount (10-50 ng) of authentic PFB-IAA or PFB-PAA used for co-chromatography during the GLC analysis of the HPLC-purified fractions of the acidic ether extracts. The final percentage recovery of each auxin was calculated using the equations:

$$\text{Percent Recovery} = \frac{c}{5} \times 100\% \quad \text{and } c = b - a$$

where:

a = amount of auxin found in the non-spiked reaction system.

b = amount of auxin found in the internally-spiked system.

c = amount of auxin recovered from the original 5 μ g added at the beginning of the extraction procedure.

In these calculations, it is assumed that any losses of the auxin produced due to absorption or instability will be the same during the analysis of both halves of the system.

Thus, the results from the non-radioactive experiments will include both qualitative data on the identification of the auxin product and the likely precursor intermediates, and quantitative data indicating percentage recoveries. The results obtained with the purified chloroplast fractions will be described followed by the results obtained with the mitochondrial fractions.

(a) Chloroplasts

The qualitative data indicating the synthesis of IAA or PAA by the chloroplast fraction will first be presented. HPLC was used as the preparative analytical step to separate the auxins from other major components of the acidic ether extract which might later interfere with the final GLC analysis. 10 ml fractions of the acidic ether extract were collected during gradient elution on the reverse phase μ Bondapak C_{18} column. The fractions were selected on the basis of the retention times of authentic markers. IAA and IPyA were the markers used for the analysis of experiments using L-tryptophan. IAA should be found in the fraction eluting between 11.5 and 13.5 minutes, and IPyA should be present in the fraction eluting between 15.0 and 17.0 minutes. In the experiments supplying L-phenylalanine as the substrate, PAA was collected between 10.3 and 12.5 minutes and PPyA mainly eluted between 16.2 and 18.2 minutes with an earlier minor peak appearing between 7 and 8.4 minutes.

Figure 16 shows a typical UV scan obtained during the HPLC separation of the acidic ether extract from a complete reaction system containing L-tryptophan as substrate. Figure 17 shows the corresponding scan obtained during analysis of the acidic extract of a control system containing only the amino acid plus the enzyme fraction. The regions on the scan corresponding to the fractions collected for IAA and IPyA determinations are indicated on each

Figure 16 - PREP-HPLC scan of acidic ether extract from
chloroplast complete reaction system with
L-tryptophan as the amino acid substrate.

Figure 17 - PREP-HPLC scan of acidic ether extract
from chloroplast control No. 1 reaction
system with L-tryptophan as the amino
acid substrate.

figure. Several peaks of absorption are seen on the scan from the complete mixture, indicating the presence of many acidic compounds synthesized by the chloroplast enzymes. Comparison of the two scans shows that the enzymes were most active when all components of the complete mixture were included in the reaction system, since most of the product peaks are either absent or very small in the control scan.

The peak of the suspected IPyA intermediate was always a major component on the chromatogram of the extract from the complete reaction system. The scan in Fig. 16 illustrates this point since the fraction, eluting between 15 and 17 minutes, shows a very strong UV-absorbing peak which corresponds to the retention time of authentic IPyA. The collected fraction containing IAA appears to contain other components beside the auxin; however, the preparative HPLC procedure does eliminate many acidic substances that elute before and after IAA which would otherwise probably interfere with the final GLC determination of the auxin. The necessity for this preparative purification step will also be emphasized again during the presentation of the qualitative results from the mitochondrial reaction systems.

Preparative HPLC analysis of the acidic ether extracts prepared from final reaction mixtures in the L-phenylalanine metabolism* experiment gave similar results. Several small UV absorbing peaks were present on the HPLC scans for the complete reaction system and were mainly absent from the scans recorded from the corresponding control systems. The lower intensity of the detected peaks was mainly due to the fact that the fixed wavelength employed in the UV detector system was not optimal for phenyl compounds (Fig. 18 and 19).

Gas chromatography using flame-ionization detectors was then

Figure 18 - PREP-HPLC scan of acidic ether extract from
chloroplast complete reaction system with
L-phenylalanine as the amino acid substrate.

Figure 19 - PREP-HPLC scan of acidic ether extract from chloroplast boiled enzyme control system with L-phenylalanine as the amino acid substrate.

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performed on the HPLC-purified fractions from the different reaction systems after they had been derivatized by methylation. Figures 20 to 23 show typical GLC scans recorded from the experiment using L-tryptophan as the primary substrate. Columns packed with 3% OV-17 were used in these analyses and a temperature program running from 150°C to 300°C at the rate of 3°/minute, was employed. Figure 20 is the scan of the fraction from the complete reaction system and shows a strong peak of suspected IAA methyl ester (Me-IAA) which eluted around 202°C. The peaks of other compounds are also clearly seen later in the chromatogram but the suspected Me-IAA peak is well separated from any of these other metabolites.

An identical aliquot from this HPLC-purified fraction was then 'externally spiked' with 100 ng of authentic Me-IAA immediately prior to GLC analysis. Figure 21 is the scan obtained and clearly shows the large increase in the height of the suspected Me-IAA peak upon co-chromatography with the authentic standard. This scan provides strong evidence for the presence of Me-IAA in the derivatized HPLC fraction and therefore of IAA itself in the original acidic ether extract of the complete reaction system. Figures 22 and 23 show the scans obtained during final GLC analysis of the corresponding HPLC fractions from the ether extracts of both control systems. The only major difference between scans from the complete reaction system and those recorded from the control system which contained the active enzyme and L-tryptophan only, is clearly the presence of the large peak of Me-IAA. All other major components appear in both systems in similar amounts. Furthermore, comparison with the GLC profile recorded from the fraction obtained from the boiled enzyme control indicates that only one more component (eluting at 255°C) found on the complete reaction chromatogram beside Me-IAA,

Figure 20 - GLC scan of methylated IAA zone trapped from
PREP-HPLC analysis of acidic ether extract
of the chloroplast complete reaction system.

Figure 21 - GLC scan of methylated IAA zone trapped from
PREP-HPLC analysis of acidic ether extract
of the chloroplast complete reaction system
spiked with authentic Me-IAA.

Figure 22 - GLC scan of methylated IAA zone trapped from
PREP-HPLC analysis of acidic ether extract
of the chloroplast control No. 1 reaction
system.

Figure 23 - GLC scan of methylated IAA zone trapped from
PREP-HPLC analysis of acidic ether extract
of the chloroplast boiled enzyme control.

appears to arise from enzyme synthesis. No attempt was made to identify this compound. The peaks appearing in the scan from the boiled enzyme control fraction are probably acidic compounds which were originally present in the unpurified stromal proteins used as the enzyme fraction.

Thus, IAA was identified as a metabolic product in the complete chloroplast reaction system and did not appear to be formed in either of the control systems. These findings indicate that sunflower chloroplast proteins have the enzymic capacity to synthesize IAA from L-tryptophan. However, co-chromatography with authentic standard during GLC analysis does not provide unequivocal identification of a suspected compound. GC-MS analysis was therefore employed to achieve conclusive identification of the enzymatically synthesized IAA. The GLC traces, described earlier (Fig. 20), indicated that the methyl esters of several acidic compounds were present in the complete reaction system, but that the peak of suspected Me-IAA separated very well from these other components. Mass spectrometric analysis of such mixtures is very difficult to perform, but GC-MS analysis has the advantage of combining a separation step, in the form of gas chromatography, with the mass spectrometric examination of each compound as it elutes off the chromatographic column. Aliquots of the suspected fraction were therefore first chromatographed to get a GLC recording in order to select the proper temperature window between which a full mass spectrometric analysis was performed.

A reference mass spectrum was first obtained for authentic Me-IAA and this is shown in Fig. 24 A. This fragmentation diagram shows the mass-to-charge ratio (m/e) and the percent relative intensity (% RI) for each ion peak present in the 70 eV mass spectrum.

Figure 24 - Mass spectrum of Me-IAA: A. Authentic Me-IAA and B. Suspected Me-IAA from the chloroplast complete reaction system. RI = relative intensity; m/e = mass-to-charge ratio; electron beam energy = 70 eV.

A. AUTHENTIC Me-IAA

B. SUSPECTED Me-IAA

The behavior of Me-IAA upon ionisation by electron impact (EI) produces several major ions. The molecular ion (M^+) (formed by loss of one electron from the molecule), is found at m/e 189, which is the molecular weight of Me-IAA. The base ion (the most prominent ion fragment) has a m/e value of 130. Two structures have been proposed for this ion: the protonated methyl-3-indole cation or the quinolinium cation. Further fragmentation of this ion occurs by the loss of HCN resulting in an ion of m/e 103, and the subsequent loss of acetylene units giving rise to ions of m/e 51 (McDougall and Hillman, 1978).

Figure 24 B shows the 70 eV mass spectrum obtained when the suspected Me-IAA peak observed during analysis of the methylated HPLC fraction from the complete reaction system was re-examined by GC-MS. This type of analysis, where a full spectrum giving the detailed fragmentation pattern of the suspected compound is obtained, inevitably includes some interfering ions. Nevertheless, the mass spectrum of the suspected Me-IAA contains all the major ions typical of the authentic methyl ester and these ions occur in the same relative abundance. Thus, the suspected Me-IAA is positively identified as the methyl ester of IAA since its mass fragmentation pattern corresponds closely to the reference spectrum of the authentic compound.

The qualitative GLC results from the chloroplast metabolism experiments using L-phenylalanine as the primary substrate provide evidence that chloroplasts are also able to synthesize the auxin, PAA. Figure 25 shows the GLC trace obtained when the appropriate HPLC fraction from the acidic ether extract of the complete reaction system was chromatographed on a 3% OV-17 column, using a temperature program running from 150°C to 200°C. The HPLC-purified fraction was

Figure 25 - GLC scan of pentafluorobenzylated PAA zone trapped from PREP-HPLC analysis of acidic ether extract of the chloroplast complete reaction system.

Figure 26 - GLC scan of pentafluorobenzylated PAA zone trapped from PREP-HPLC analysis of acidic ether extract of the chloroplast complete reaction system spiked with authentic PFB-PAA.

Figure 27 - GLC scan of pentafluorobenzylated PAA zone
trapped from PREP-HPLC analysis of acidic
ether extract of the chloroplast control
No. 1 reaction system.

Figure 28 - GLC scan of pentafluorobenzylated PAA zone trapped from PREP-HPLC analysis of acidic ether extract of the chloroplast boiled enzyme control system.

first derivatized by pentafluorobenzoylation before GLC analysis. The peak eluting around 180°C is the suspected PFB-IAA, since an external spike of authentic PFB-PAA co-chromatographed with this peak when analysed along with a further aliquot of the fraction, as shown in Figure 26. Two other major peaks are present on these profiles and both again appear on the GLC traces of equivalent HPLC fractions from the two control systems. Figure 27 shows the trace from the control system containing the enzyme fraction plus amino acid only, and Figure 28 shows the comparable scan from the boiled enzyme control. These two peaks were the only peaks observed during analysis of the fractions from control systems and no evidence was found for a suspected PFB-PAA peak eluting around 180°C . The fact that the two peaks were found during analysis of the fraction from the boiled enzyme control indicates that they are due to compounds that were not synthesized by the active enzyme fraction, and are probably acidic compounds released by the unpurified stromal protein fraction used in the assay.

Thus, the results presented in Figures 25 - 28 indicate that chloroplast enzymes have the capacity to synthesize PAA, and evidence from the control system containing the active enzyme fraction further indicates that several cofactors are required for this process.

GC-MS analysis was performed on the HPLC fraction obtained from the complete reaction system to provide conclusive identification of the suspected PFB-PAA. The 70 eV mass spectrum of authentic PFB-PAA is shown in Figure 29 A. Several ions are formed during the fragmentation of PFB-PAA by electron impact. The molecular ion (M^{+}) is found at m/e 316. This ion then dissociates and the ion at m/e 181, which is the positive ion $\text{CH}_2\text{C}_6\text{F}_5^{+}$, is lost. The fragmentation pattern of the remaining ion seems to be governed by the stabilities

Figure 29 - Mass spectrum of PFB-PAA: A. Authentic PFB-PAA and B. Suspected PFB-PAA from the chloroplast complete reaction system. RI = relative intensity; m/e = mass-to-charge ratio; electron beam energy = 70 eV.

A. AUTHENTIC PFB-PAA

B. SUSPECTED PFB-PAA

of the neutral species expelled: carbon dioxide and acetylene. Thus, the ion at m/e 91 is formed by loss of carbon dioxide, which is followed by loss of acetylene to give m/e 65. Another acetylene unit is lost to give the cyclopropenium ion at m/e 39. These last 3 ions are fragments that can be typical of aromatic species, (Hill, 1972). Figure 29 B shows the mass spectrum obtained with the suspected PFB-PAA isolated from the complete reaction system. The fragmentation pattern shows the major ions described for the authentic PFB-PAA and they all occur in the same relative abundance. Based on this unequivocal evidence, it is clear that chloroplast stromal enzymes are able to synthesize the auxin, PAA.

The qualitative data just presented demonstrate the capacity of the chloroplast enzymes to synthesize the auxins, IAA and PAA. Quantitative measurements of this biochemical process, in nmoles product formed per mg of protein per 4 hours, were also carried out during each experiment along with an evaluation of the efficiency of the analytical method.

The percentage recovery of each auxin product after the different purification steps was measured according to the method described previously. Table 8 gives the results obtained when the recoveries of IAA and PAA were investigated for the chloroplast assay. Two separate measurements were carried out for each auxin. The percentage recovery of IAA varied between 63% to 74% in the two experiments, whereas 72% to 77% of the PAA produced was recovered after the purification procedures. Results are expressed as μg of auxin recovered per sample and each trial contained a different amount of protein, which explains the large variations within each group of experiments. In the first trial for the percentage recovery of IAA, 5.929 μg were measured in the unspiked complete reaction system

Table 8 Amounts of IAA or PAA recovered from chloroplast complete reaction systems and from identical systems spiked with 5 μ g IAA or PAA before commencement of the ether extraction procedure.

Auxin substance	Amounts recovered (μ g/sample)		Percentage Recovery
	Complete Reaction System	Plus Internal Spike (5 μ g)	
IAA	5.929	9.621	74
	2.272	5.420	63
PAA	3.251	6.871	72
	8.340	12.189	77

Table 9 Comparative amounts of IAA and PAA produced by the chloroplast complete reaction system in six experiments.

Amounts of:	
IAA	PAA
(nmoles formed/mg protein/4 h)	
7.71	6.10
4.29	4.41
<u>3.14</u>	<u>1.98</u>
5.05 ± 2.38	4.16 ± 2.07
Average yield:	

± = Standard deviation

whereas 9.621 μg were found in the complete reaction system that had been spiked with 5 μg of authentic IAA. The percentage recovery can then be calculated using the two equations mentioned earlier. The first equation (i.e. $c = b - a$) gives a value of 3.692 μg for the amount recovered from the original 5 μg added, and the second equation can then be written: $\frac{3.692 \mu\text{g}}{5 \mu\text{g}} \times 100\%$

which gives a final value of 73.8 as the percentage recovery for this particular experiment. Similar calculations were performed for the remaining experiments.

Although the isolation procedures were the same for both auxins, it is evident from the data in Table 8 that the recovery of IAA was slightly lower than of PAA, which suggests that IAA is probably more labile than PAA during chromatographic purification. As seen in Table 8, recovery values were not constant and the difference in some cases were significant, such as the 11% difference between the two values for IAA. Thus, quantitative measurements on the IAA or PAA formed in these biosynthesis experiments had to be done simultaneously with measurements of the percentage recovery of the auxin compound in that particular experiment. Table 9 gives the calculated amount of IAA and PAA formed by the chloroplast enzymes in the present series of experiments. The results are corrected to 100% efficiency. On average, slightly more IAA than PAA was synthesized by the chloroplast enzymes: 5.05 nmoles of IAA was formed per mg protein/4 h compared with 4.16 nmoles of PAA/mg protein/4 h. The difference is, however, not significant when the standard deviation is taken into account.

(b) Mitochondria

Mitochondrial enzymes were also assayed with L-tryptophan or L-phenylalanine to determine their capacity to synthesize IAA or PAA.

The auxin product was extracted from the final reactions following the procedure described for chloroplast systems and HPLC-purified fractions were derivatized by pentafluorobenzoylation prior to final GLC analysis.

Figures 30 and 31 show the GLC profiles obtained from the assays using L-tryptophan as the primary substrate in the complete reaction mixture and from the comparable boiled enzyme control. These profiles show the separation obtained when no preparative-HPLC purification step was performed on the acidic ether fraction before GLC analysis. Gas chromatography was carried out on a 3% OV-17 column, using a temperature program running from 180°C to 270°C at 3°C per minute. Good separation of the many compounds present in the acidic ether extracts could be achieved using these chromatographic conditions. However, GLC analysis of HPLC-purified fractions gave much cleaner profiles, as shown on Figures 32 and 33. These analyses were carried out isothermally at 235°C, again on a 3% OV-17 column. Comparison of the scan from direct analysis of the ether fraction with the one obtained after HPLC purification clearly emphasizes the need to apply this purification step to eliminate most of the acidic substances extracted into the ether fraction before GLC determination of the IAA present. This aspect of the importance of the HPLC purification step has already been described during the presentation of the chloroplast results.

The profile from direct analysis of the ether fraction from the complete reaction system (Fig. 30 A) shows several major peaks and many smaller ones, which include a distinct peak of suspected PFB-IAA eluting at the retention time of 22.84 minutes. This peak greatly increased in area when a further aliquot of the fraction was co-chromatographed with authentic PFB-IAA, (Fig. 30 B). The scan

Figure 30 - GLC scans obtained from direct analysis of pentafluorobenzylated acidic ether extract of the mitochondria complete reaction system with L-tryptophan as the amino acid substrate.

- A. Complete reaction system.
- B. Complete reaction system spiked with authentic PFB-IAA.

Spiked
with
authentic
PFB-IAA

Susp.
PFB-IAA

B.

A.

Figure 31 - GLC scans obtained from direct analysis of pentafluorobenzylated acidic ether extract of the mitochondria boiled enzyme control system with L-tryptophan as the amino acid substrate.

- A. Control system
- B. Control system spiked with authentic PFB-IAA

Elution
Temp.
of
PFB-IAA

Spiked
with
authentic
PFB-IAA

from the boiled enzyme control (Fig. 31 A), which indicates the number of acidic compounds present in the purified mitochondrial preparation used as the enzyme fraction, also displays some of the peaks of the complete reaction system; a few showing similar areas and others showing lower areas than the comparable peaks observed in Fig. 30 A. Such direct GLC analysis of the ether fraction from the complete and control reaction systems demonstrates that the mitochondrial enzymes are able to synthesize a variety of acidic compounds, in addition to IAA, from either the L-tryptophan and cofactors provided or from endogenous compounds present in the initial enzyme fraction.

Four peaks were found on the GLC profiles from the HPLC-purified fraction isolated from the complete reaction system, (Fig. 32 A).

The peak of suspected PFB-IAA was found at the retention time of 10.7 minutes and this peak greatly increased in area when a further aliquot of the fraction was co-chromatographed with 50 ng of authentic PFB-IAA (Fig. 32 B). Identification of the other peaks was not attempted. An equivalent aliquot from the HPLC-fraction from the boiled enzyme control gave a few minor peaks on GLC analysis (Fig. 33 A) and none of these small peaks co-chromatographed with a 50 ng spike of authentic PFB-IAA (Fig. 33 B). On the basis of these qualitative results, then, it is clear that mitochondrial enzymes do have the potential to synthesize IAA from tryptophan.

The qualitative data obtained from an experiment using L-phenylalanine as the primary substrate are shown in Figure 34. Direct gas chromatography of the acidic ether extracts of complete and control reaction systems was not carried out in these experiments in view of the benefits likely to be gained by first purifying each extract by preparative HPLC. The profiles shown in Fig. 34 were

Figure 32 - GLC scans of pentafluorobenzylated IAA zone trapped from PREP-HPLC analysis of acidic ether extract of the mitochondria complete reaction system.

- A. Complete reaction system
- B. Complete reaction system spiked with authentic PFB-IAA

Figure 33 - GLC scans of pentafluorobenzylated IAA zone trapped from PREP-HPLC analysis of acidic ether extract of the mitochondria boiled enzyme control system.

- A. Control system
- B. Control system spiked with authentic PFB-IAA

therefore obtained from GLC analysis of comparable HPLC fractions collected from ether extracts of the complete and control reaction systems. The HPLC fractions were derivatized by pentafluorobenzoylation and each analysis was carried out on a 3% OV-17 column by isothermal chromatography at 175°C. The complete reaction system in this experiment was divided in two equal volumes at the end of the 4 hours metabolism period and one volume was spiked with 5 μ g authentic PAA prior to ether extraction. After HPLC purification of all the ether extracts, gas chromatography of the fraction collected from the unspiked complete reaction system revealed a distinct peak of suspected PFB-PAA at the retention time of 6.82 minutes (Fig. 34 A). The same peak showed a striking increase in height and area on the profile obtained when an identical aliquot from the derivatized fraction collected from the PAA-spiked portion of the complete reaction system was gas chromatographed, (Fig. 34 B).

A very small peak at the retention time of PFB-PAA can also be seen on the profiles obtained from the two control systems examined; the first system contained the enzyme fraction plus amino acid only (Fig. 34 C) and the second contained a boiled enzyme fraction plus the substrate amino acid and all cofactors, (Fig. 34 D). The appearance of these small peaks in the two control systems indicates the presence of a low endogenous level of PAA in the mitochondrial enzyme fraction. Clear evidence that α -ketoglutarate and the other cofactors are required by mitochondrial enzymes to catalyse the conversion of L-phenylalanine to PAA is provided by the results from the control system containing the active enzyme fraction plus the amino acid only, where no indication of PAA synthesis was observed.

The qualitative results just described demonstrate that mitochondrial enzymes are able to synthesize IAA and PAA when supplied

Figure 34 - GLC scans of pentafluorobenzylated PAA zone trapped from PREP-HPLC analysis of acidic ether extract of the mitochondria reaction systems.

- A. Complete reaction system
- B. Complete reaction system spiked with authentic PFB-PAA
- C. Control No. 1 reaction system
- D. Boiled enzyme control system

Complete Mixture

Complete Mixture
with "Internal" Spiking

CONTROL No 1
(A/acid + ENZ. only)

Boiled Enzyme

Isothermal 175°C on 3% OV-17 column

with the precursor amino acids and other cofactors. Quantitative measurements were also made to determine the comparative rate of synthesis of the two auxins by such mitochondrial fractions, but the percentage recoveries obtained after the extraction and purification procedures were first calculated.

Experiments carried out to determine the recoveries of IAA included a measurement of the amount of IAA recovered when an HPLC purification step was not included in the isolation procedure. This measurement allowed for the calculation of the percentage recovery after ether extraction. Table 10 presents these results. It can be seen that about 85%-90% of the IAA synthesized was recovered from the reaction system after ether extraction, and the subsequent percentage recovery after the HPLC purification step varied from 59% in one experiment to 66% in another. The HPLC step, therefore, does appear to introduce significant losses of IAA which, in these two experiments, varied between 19-30 percent.

Similar experiments to determine the percentage recovery of PAA did not include measurements of the recoveries obtained after ether extraction. The recovery of PAA after HPLC purification of the acidic ether extract was found to be 83% in one experiment and 76% in another (Table 10). PAA thus appears to be recovered from mitochondrial reaction systems to a greater extent than IAA, which was also observed with the chloroplast reaction systems. In view of the variations observed in the recovery values, no uniform percentage recovery factor could be applied during the quantitative determination of IAA and PAA in these experiments.

The quantitative results shown in Table 11 were obtained from several experiments in which the internal spiking method for determining recovery values was applied. The results presented were corrected to

Table 10 Amounts of IAA or PAA recovered from complete mitochondrial reaction system and from identical systems spiked with 5 μ g of IAA or PAA before commencement of the ether extraction procedure.

Auxin Substrate	<u>Without HPLC purification</u>			<u>HPLC purification</u>		
	Complete Reaction System (μ g/sample)	Percentage Recovery		Complete Reaction System (μ g/sample)	Plus Internal Spike (5 μ g) (μ g/sample)	Percentage Recovery
IAA	5.490	89%		3.649	6.621	59
	2.240	85%		1.741	5.043	66
PAA	not determined			2.963	7.107	83
				2.820	6.613	76

Table 11 Comparative amounts of IAA and PAA produced by the mitochondrial complete reaction systems in six operations.

Amounts of:	
IAA	PAA
(nmol's formed/mg protein/4 h)	
20.80	19.85
18.91	16.40
<u>16.46</u>	<u>13.60</u>
Average yield: 18.72 $\pm$ 2.18	16.62 $\pm$ 3.13

$\pm$ = Standard deviation

100% recovery. The average values indicate that 18.72 nmoles of IAA were found per mg protein during the 4 hours incubation period, whereas 16.62 nmoles of PAA were synthesized per mg protein during the same period of time. As for the chloroplast results, this small difference is not significant.

(ii) Radioactive Experiments

The results from non-radioactive experiments have shown that both chloroplast and mitochondrial enzyme fractions are able to synthesize IAA and PAA from the corresponding amino acids, L-tryptophan and L-phenylalanine. This formation evidently required the presence of certain cofactors and the auxins were not the only compounds produced during the 4 hour reaction period. The major metabolite found in the acidic ether extract was the arylpyruvic acid intermediate which is produced by the activity of the aromatic aminotransferase, the first enzyme in the auxin synthesis pathway. This intermediate is believed to undergo a decarboxylation to give rise to the corresponding arylacetaldehyde, which is considered to be the intermediate precursor of the arylacetic acid, the final auxin product.

Many problems were encountered during detection of the arylacetaldehydes, as described in the section dealing with Preliminary Experiments. Evidence for the formation of 3-indoleacetaldehyde and phenylacetaldehyde was sought by looking for the incorporation of radioactivity into these substances when ^{14}C -labelled amino acids were provided as starting substrates. In such tracer metabolism experiments, the appropriate unlabelled arylacetaldehyde was added at the start of the incubation period to increase the endogenous pool of this intermediate and so endeavour to trap some of the newly synthesized radioactive molecules. The radioactivity in the corresponding arylethanol, which may be readily formed from the arylacetaldehyde

through the action of the corresponding alcohol dehydrogenase (Wightman and Cohen, 1968) or of the enzyme IAAld reductase (Brown and Purves, 1980), was also measured. The radioactivity in the arylpyruvic acid intermediates and in the main reaction products, IAA and PAA, was also determined.

These radioactive assays were carried out as described in section 7,A,ii of Material and Methods. Figure 35 outlines the procedure used for isolating the radioactive compounds present in the final reaction systems when ^{14}C -tryptophan or ^{14}C -phenylalanine was supplied as the primary substrate. The procedures used for analysis of the neutral and acidic ether extracts are described in detail in Material and Methods, sections 8 and 9, and the methods used for liquid scintillation spectrometry are given in section 10.

Two types of data are presented for each organelle: 1) the distribution of the radioactive label in the ether extracts, and 2) the radioactivity recovered in the IAA or PAA and in their biosynthetic intermediates.

(a) Chloroplast experiments

The distribution of radioactivity between neutral and acidic ether extracts when chloroplast fractions were used to provide the enzyme fractions is shown in Table 12. The values are from two experiments; one where L- methylene- ^{14}C tryptophan was supplied and the other where L- U- ^{14}C phenylalanine was provided as the primary substrate. The three types of reaction systems normally used in such assays were again examined and each was subsequently analysed to determine the nature of the radioactive products. Aliquots were taken from the final reaction mixtures and at different stages in their ether extraction; the distribution of radioactivity was determined by liquid scintillation spectrometry. Results are expressed as dpm and

Figure 35 - Procedure for isolating the radioactive products formed in organelle systems metabolizing ^{14}C -labelled L-tryptophan or L-phenylalanine.

FINAL REACTION MIXTURE

(after 4 h incubation)

Add unlabelled IAAld or PAAld

Adjust mixture to pH 8.5 and extract 3 times
with equal volumes of distilled Ether

NEUTRAL ETHER EXTRACT

(containing ^{14}C -IAAld or ^{14}C -PAAld)

TLC

Liquid scintillation
spectrometry

AQUEOUS FRACTION

Adjust to pH 2.8 and
re-extract 3 times with equal
volumes of distilled Ether

RESIDUAL
AQUEOUS
FRACTION

ACIDIC ETHER EXTRACT

(containing ^{14}C -IPyA and
 ^{14}C -IAA, or ^{14}C -PPyA and
 ^{14}C -PAA)

HPLC

GLC

Liquid
scintillation
spectrometry

also as percentage of the total dpm present in the final reaction mixture.

Examination of the results from the three assays in which ^{14}C -L-tryptophan was supplied shows that, in every case, the acidic ether extract contained more radioactivity than the neutral extract. The acidic extract of the complete reaction system gave the highest value and when either control value was substrated, 2% of the total radioactivity in the final system was recovered in this acidic extract. This is not surprising since both IPyA and IAA should be present in this extract along with other possible radioactive acidic compounds (such as 3-indole lactic acid) not identified in this study. The difference in radioactivity between the acidic extracts of the two control systems is quite small; the control containing the active enzyme fraction showed only 0.13% more radioactivity than the boiled enzyme control.

The results from the neutral ether extracts have to be examined in two steps. The extract from the complete reaction system showed 0.07% more radioactivity than that from the control system containing the active enzyme fraction. This slight increase might indicate that more neutral compounds were synthesized when the cofactors were present and since the difference in percentage amounts to 2025 dpm, this could be significant. However, the neutral extract from the boiled enzyme control contained more radioactivity than either the complete reaction system or the control with the active enzyme fraction. This finding may mean that neutral compounds can also be formed from nonenzymic breakdown of the radioactive tryptophan.

The radioactivity recovered in the neutral and acidic ether extracts from the experiment using ^{14}C -labelled phenylalanine (Table 12) were lower than the values obtained with ^{14}C -tryptophan. Again, more radioactivity was present in the acidic extracts. This extract from

Table 12 Distribution of radioactivity in the extracted ether fractions from chloroplast assays given 2.5 μ Ci of radioactive L-amino acid.*

FRACTION	COMPLETE REACTION SYSTEM		CONTROL SYSTEMS:			
	DPM	%	Enzyme plus Amino acid only	DPM	%	Boiled Enzyme
<u>L- methylene ^{14}C -tryptophan</u>						
Reaction mixture	5,447,033	100	5,647,864	100	5,516,350	100
Neutral ether extract	39,553	0.73	37,528	0.66	42,383	0.77
Acidic ether extract	163,714	3.01	65,258	1.16	56,618	1.03
<u>L- U ^{14}C -phenylalanine</u>						
Reaction mixture	5,679,843	100	5,174,903	100	5,905,182	100
Neutral ether extract	12,528	0.22	11,223	0.22	16,317	0.28
Acidic ether extract	117,564	2.07	46,375	0.90	36,454	0.62

* Total quantity of protein per reaction = 10.6 mg

the complete reaction system was found to contain 1.17% of the total radioactivity present in the final reaction system when the value from the control with the active enzyme fraction was subtracted. This same control gave 0.28% more radioactivity in the acidic ether extract than the one from the boiled enzyme control, indicating that some acidic compounds were synthesized without added cofactors. However, as mentioned in the previous experiment, the radioactivity found in the acidic extract of the boiled enzyme control again points to a possible nonenzymic breakdown of the substrate amino acid.

The difference in dpm between the neutral ether extracts from the complete reaction system and the active enzyme control amounts to 1305 dpm, but when these values are expressed as a percentage of the original radioactivity in each system, there is no difference (Table 12). This low level of radioactivity in possible neutral metabolites is also reflected in the small quantities of neutral phenyl intermediates recovered from the final reaction systems, which will be examined later. As in the ^{14}C -tryptophan experiment, the boiled enzyme control again showed more radioactivity in the neutral ether extract than did the corresponding extracts from the other two systems.

Thus, these results indicate that acidic and to a lesser extent neutral compounds are more abundant in the chloroplast assays when the active enzymes are supplied with the different cofactors along with the substrate amino acid. At the same time, the boiled enzyme control also provides evidence that some nonenzymic breakdown of the amino acid is also occurring during the 4 hours reaction period.

The neutral intermediates were separated by TLC and identified from their R_f value, as determined by their U.V. absorption. IAA and PAA, along with the corresponding arylpyruvic acids, were isolated by

preparative HPLC and identified by gas chromatography, as outlined earlier in Figure 35. The results (Table 13) are expressed as dpm/mg protein/4 hours and were obtained after values from control systems had been subtracted. Specific activities were not calculated for two reasons; first, it was impossible to identify newly synthesized ^{14}C -arylacetaldehydes without including a small amount of the authentic compound at the start of the assay to trap the radioactive molecules, and second, since unlabelled arylacetaldehydes had been included in the reaction systems at the beginning of the incubation period, it could influence the quantitative measurements of both the arylethanols and the arylacetic acids, IAA and PAA.

Three experiments were carried out using L-methylene- ^{14}C -tryptophan as the primary substrate. 2.5 μCi of the ^{14}C -labelled amino acid was supplied on one occasion whereas the other two assays received 5 μCi of the ^{14}C -substrate. The IAA recovered in each of these assays was found radioactive and, taking into account the losses occurring during ether extraction and HPLC purification, there is good correlation between the radioactivity recovered in the auxin and the initial amount of ^{14}C -label supplied. Thus, the experiment using 2.5 μCi ^{14}C -tryptophan yielded 891 dpm/mg protein/4 hours of IAA, whereas 1820 and 2065 dpm/mg protein/4 hours were found in the IAA produced in the two experiments supplying 5 μCi ^{14}C -tryptophan.

As expected, the radioactivity in IPyA was greater than that found in IAA. This result was predictable from the results obtained in the non-radioactive metabolism experiments where IPyA was found to be the major acidic compound synthesized. In the experiment using 2.5 μCi ^{14}C -tryptophan, 1155 dpm/mg protein/4 hours of IPyA were collected. Doubling the initial amount of radioactivity in the substrate amino acid, however, did not give a proportionate increase in the amount of

Table 13. Radioactivity recovered in the auxins, IAA and PAA, and in intermediates in their biosynthesis from the complete chloroplast reaction system.**

SUBSTRATE AMINO ACID		INTERMEDIATES		AUXIN PRODUCT	
Concentration Total Radioactivity		dpm/mg protein/4 h		dpm/mg protein/4 h	
¹⁴ C Tryptophan		IPyA	IAAId	Tryptophol	
L- methylene-				IAA	
25 mM	2.5 μCi	1155	10 (406)	47 (406)	891
25 mM	5.0 μCi	2929	12 (30)	122 (316)	1820
25 mM	5.0 μCi	4136	19 (78)	202 (831)	2065

¹⁴ C Phenylalanine		PPyA	PAAId	PETOH	PAA
25 mM	2.5 μCi	1634	1 (11)	9 (92)	1140
25 mM	2.5 μCi	1930	5 (19)	15 (51)	1104
25 mM	5.0 μCi	4352	11 (28)	33 (86)	3083

** Results presented are those obtained after values from control systems had been subtracted. Values in brackets show total counts recorded.

Non-radioactive indoleacetaldehyde (0.8 μ mole/ml) or phenylacetaldehyde (0.8 μ mole/ml) and BSA (0.5 mg/ml) were added to the reaction system at the start of the incubation period to facilitate recovery of the radioactive intermediates.

radioactivity recovered in IPyA. Furthermore, the two experiments using $5\mu\text{Ci } ^{14}\text{C}$ -tryptophan, showed a substantial difference between the amount of radioactivity found in IPyA; 2929 and 4136 dpm/mg protein/4 hours respectively, which is a difference of 1207 dpm/mg protein/4 hours between the two experiments.

As shown previously in Table 12, similar amounts of radioactivity were found in the neutral extracts of the complete reaction system and the control system containing active enzymes. Even greater amounts were found in the boiled enzyme control. It can be anticipated that low levels of ^{14}C -labelled neutral intermediates will be detected since the results present in Table 13 were obtained after values from control systems had been subtracted. The inclusion of non-radioactive 'carrier' IAAld in these experiments made possible the recovery of small amounts of radioactivity in this intermediate; 10, 12 and 19 dpm IAAld/mg protein/4 hours being found in the three experiments, respectively (Table 13). The values in brackets show the total dpm obtained in this compound after 4 hours of incubation.

Recovery of the ^{14}C -label was more successful with tryptophol. Radioactivity has more chance of remaining in this compound because, when synthesized, it does not appear to be metabolised to any other compound except to revert back to IAAld. The accumulation of ^{14}C -label in tryptophol was not related to the initial level of radioactivity supplied as ^{14}C -tryptophan, since 47 dpm/mg protein/4 hours were found for the $2.5\mu\text{Ci}$ experiment and 122 and 202 dpm/mg protein/4 hours for the two $5\mu\text{Ci}$ experiments.

Three experiments were similarly performed using L- $\text{U-}^{14}\text{C}$ phenylalanine as the primary substrate; two of them supplied $2.5\mu\text{Ci}$ radioactivity and one with $5\mu\text{Ci}$. As shown in Table 13, the PAA

formed was radioactive-showing 1140 and 1104 dpm/mg protein/4 hours in the two experiments using 2.5 μ Ci 14 C-amino acid, and 3083 dpm/mg protein/4 hours when 5 μ Ci were used. The values from the two experiments supplying 2.5 μ Ci initial radioactivity are virtually identical, but the result from the 5 μ Ci experiment is higher than expected if doubling the initial radioactivity in the substrate amino acid should result in a proportional increase in the radioactivity in the auxin product.

PPyA contained, as expected, more radioactivity than PAA. The results from the two assays using 2.5 μ Ci initial radioactivity were close taking into account possible losses; 1634 and 1930 dpm/mg protein/4 hours, respectively. The experiment using 5 μ Ci initial radioactivity yielded 4352 dpm in PPyA/mg protein/4 hours.

The levels of radioactivity detected in the neutral intermediates formed from 14 C-phenylalanine were very low (Table 13), even when the results are expressed simply as total dpm detected after 4 hours. PAAld contained 11, 19 and 28 total dpm/4 hours. Three times more radioactivity accumulated in phenylethanol probably for the same reason as that mentioned earlier for tryptophol. Isolation of the phenyl neutral intermediates presented an extra problem not found with the corresponding indoles, namely, their high volatility. This problem partly explains the low recoveries obtained for these compounds since they can easily be partially lost by evaporation during concentration steps in the ether extraction and chromatography procedures.

Thus, the dpm values obtained in these chloroplast 14 C-metabolism experiments show that IAA or PAA and their corresponding arylpyruvic acids contain appreciable radioactivity when 14 C-tryptophan or 14 C-phenylalanine is provided as the primary substrate. Radioactivity

was also found in the expected arylacetaldehyde and arylethanol intermediates. The levels detected were low, especially for the phenyl compounds, but evidence of radioactivity in these neutral intermediates provides further support for the synthesis of IAA and PAA by chloroplast enzymes via the arylpyruvic acid route.

(b) Mitochondria experiments

The distribution of radioactivity between different ether extracts from mitochondrial assays will be examined from two experiments where the enzyme fraction was supplied, in one instance, with 1.25 μ Ci of L- methylene- 14 C tryptophan and in the other, with 1.25 μ Ci of L- U- 14 C phenylalanine.

Distribution of the 14 C-label as indicated by the results in Table 14, shows that the acidic extracts from both complete reaction systems contained the highest amount of radioactivity. The acidic extract from the 14 C-tryptophan experiment contained 1.26% of the initial amount of radioactivity supplied to the system. This net percentage amount of 14 C must have resulted directly from the activity of the chloroplast enzymes in presence of the cofactors, since the value from the control system without cofactors had been first subtracted (i.e. $1.99 - 0.73 = 1.26\%$). The corresponding result for the acidic extract of the 14 C-phenylalanine complete reaction system showed a net recovery of 0.6% radioactivity. The difference in recovery values observed between the two complete reaction systems is related to the 14 C-amino acid supplied as the substrate, since the same amount of protein per ml was used in both experiments. Thus, the mitochondrial enzymes seemed to be twice as active in producing acidic metabolites from 14 C-tryptophan as from 14 C-phenylalanine. Differences between the acidic extracts from the two control systems in both experiments were quite small, 0.08% in the case of the

Table 14 Distribution of radioactivity in the extracted ether fractions from mitochondrial assays given 1.25 μ Ci of radioactive L-amino acid.*

FRACTION	COMPLETE REACTION SYSTEMS		CONTROL SYSTEMS:			
	DPM	%	Enzyme plus Amino acid only	DPM	%	Boiled Enzyme
<u>L- methylene ¹⁴C- tryptophan</u>						
Reaction mixture	2,722,594	100	2,770,547	100	2,720,474	100
Neutral ether extract	18,636	0.68	18,706	0.68	18,556	0.68
Acidic ether extract	54,261	1.99	20,346	0.73	17,679	0.65
<u>L- U ¹⁴C -phenylalanine</u>						
Reaction mixture	2,630,597	100	2,741,544	100	2,839,132	100
Neutral ether extract	9,628	0.37	12,218	0.45	14,528	0.51
Acidic ether extract	38,905	1.48	24,141	0.88	24,682	0.87

* Total quantity of protein per reaction = 2.3 mg

^{14}C -tryptophan experiment and 0.01% when ^{14}C -phenylalanine was used.

As found in the chloroplast experiment, the neutral ether extracts from the mitochondrial systems also gave low and/or ambiguous results. Percentage recovery data from the ^{14}C -tryptophan experiment showed identical values for the neutral extracts from each reaction system and these reflect the very low difference observed when the results are examined in terms of dpm (Table 14).

The highest amount of radioactivity in a neutral extract in the phenylalanine experiment was found in the one from the boiled enzyme control. The control containing the active enzyme fraction, but with no cofactors, gave the next highest value while the neutral extract from the complete reaction system contained the lowest amount of radioactivity. The significance of these differences in terms of the formation of neutral intermediates in PAA synthesis is difficult to understand.

Thus, the acidic extract results in Table 14 indicate that acidic compounds were synthesized in considerable amounts in both complete reaction systems, whereas the radioactivity found in the corresponding neutral extracts did not provide direct evidence for the enzymatic synthesis of neutral compounds because of the higher dpm observed in the boiled enzyme controls. However, as will be described in the next set of data, the total dpm of a neutral ether extract does not necessarily reflect the level of radioactivity in an individual compound.

Evidence for the synthesis of the neutral intermediates, IAAld and PAAld, was obtained from two sets of experiments. Two assays were performed with L-methylene- ^{14}C tryptophan, one containing 1.25 μCi and the other with 2.5 μCi . Two L-U- ^{14}C phenylalanine metabolism experiments were also carried out, both containing 1.25 μCi .

radioactivity. Table 15 presents the results from these experiments.

It can be seen that IPyA was highly radioactive in both assays and that more than twice as many dpm/mg protein/4 hours were found in this intermediate when isolated from the 2.5 μ Ci reaction system, than when isolated from the one containing 1.25 μ Ci 14 C-tryptophan. IAA was also appreciably radioactive, and as expected, lower values of radioactivity were collected in this metabolite when compared to the activity in IPyA. In the system supplying 1.25 μ Ci 14 C-tryptophan, 1450 dpm in IAA/mg protein/4 hours were found compared with 2365 dpm in IAA/mg protein/4 hours in the one supplying 2.5 μ Ci radioactivity.

The amounts of 14 C-label recovered in IAAlD were greater in these experiments than that found earlier with the chloroplast fractions. The experiments supplying 1.25 μ Ci and 2.5 μ Ci 14 C-tryptophan yielded 185 and 382 dpm in IAAlD/mg protein/4 hours, respectively. Tryptophol, however, did not seem to be synthesized by the mitochondrial enzymes since no radioactivity was detected in this compound.

The pattern of 14 C-recovery in the two assays using 14 C-phenylalanine was similar to that found with 14 C-tryptophan; 1343 and 1449 dpm/mg protein/4 hours were found in PPyA, and 1019 and 925 dpm/mg protein/4 hours were found in PAA. As expected, PPyA was more radioactive than PAA, but the difference between the two compounds was not as great as that observed between IPyA and IAA in the 14 C-tryptophan experiments. PAAld contained very low levels of radioactivity, 17 and 23 dpm/mg protein/4 hours, and no label was recovered in phenylethanol in either experiment.

Thus, the auxin substances, IAA and PAA, and their corresponding arylpyruvic acids were found to contain appreciable radioactivity as expected. Radioactivity was also found in the arylacetaldehydes but the levels detected in phenylacetaldehyde were low. No radioactivity

Table 15 Radioactivity recovered in the auxins, IAA and PAA, and in intermediates in their biosynthesis from the complete mitochondrial reaction system.**

SUBSTRATE AMINO ACID		INTERMEDIATES		AUXIN PRODUCT
Concentration Total Radioactivity		dpm/mg protein/4 h		dpm/mg protein/4 h
<u>L- methylene-¹⁴C Tryptophan</u>				
		<u>IPyA</u>	<u>IAAId</u>	<u>Tryptophol</u>
25 mM	1.25 μ Ci	3159	185 (425)	0
25 mM	2.5 μ Ci	8343	382 (880)	0
				1450
				2365
<u>L-U-¹⁴C Phenylalanine</u>				
		<u>PPyA</u>	<u>PAAId</u>	<u>PETOH</u>
25 mM	1.25 μ Ci	1343	17 (38)	0
25 mM	1.25 μ Ci	1449	23 (51)	0
				1019
				925

** Results presented are those obtained after values from control systems had been subtracted. Values in brackets show total counts recorded.

Non-radioactive indoleacetaldehyde (0.8 μ mole/ml) or phenylacetaldehyde (0.8 μ mole/ml) and BSA (0.5 mg/ml) were added to the reaction system at the start of the incubation period to facilitate recovery of the radioactive intermediates.

was recovered in the arylethanol intermediates. These results then, provide evidence for the synthesis of both IAA and PAA by mitochondrial enzymes via the arylpyruvic acid pathway.

B. Experiments using 3-indolepyruvate and phenylpyruvate as substrate.

Experiments using the arylpyruvate as starting substrate were performed with chloroplast and mitochondrial enzyme fractions to confirm their ability to metabolize these two key intermediates. The procedure for these experiments was described in section 7, b of Material and Methods. Each assay comprised two sets of reaction systems. One set involved the active enzyme fraction whereas the other consisted of control systems. In the first group, in addition to the arylpyruvate, the reaction systems contained either:

- (i) the enzyme fraction only,
 - (ii) the enzyme fraction plus bovine serum albumin (BSA) or,
 - (iii) the enzyme fraction, BSA and the cofactors TPP, NAD and NADP.
- The control group was made up of similar systems except that the active enzyme fraction was replaced by an equivalent volume of buffer or a boiled enzyme fraction.

BSA was included in some reaction systems because of its potential beneficial effect on the stability of the arylpyruvates. The presence of BSA had been shown by Stafford and Lewis (1979) to eliminate a nonenzymic reaction with phenylpyruvate in mixtures incubated without enzymes.

The IAA or PAA produced, along with the remaining substrate, were isolated following the same analytical procedures described for the experiments using the two corresponding amino acids as substrates. Reaction systems were first ether fractionated and the acidic ether extract was further purified by preparative HPLC. Collected fractions were derivatized by pentafluorobenzoylation and aliquots

were then analysed by GLC.

(i) Chloroplast assays

Table 16 presents the data obtained from reaction systems examining the activity of chloroplast fractions. The experiments were performed on two separate occasions and each enzyme fraction contained a different quantity of protein. The results for IAA and PAA are presented as the μg amount produced in 2 hours. The value from the boiled enzyme control was subtracted from each reaction system with the active enzyme fraction. These results were then expressed as nmoles produced/mg protein/2 hours for comparative purposes between IAA and PAA.

Different quantities of IAA were formed from IPyA by the active enzyme fraction depending on the composition of the reaction mixture. The active fraction with BSA and cofactors yielded $4.5 \mu\text{g}$ IAA after 2 hours. This yield of IAA was reduced to $3.2 \mu\text{g}$ after 2 hours when the cofactors were not included, but increased to $3.4 \mu\text{g}$ when BSA was also removed. In the boiled enzyme control containing BSA, $2.3 \mu\text{g}$ of IAA were formed after 2 hours and the corresponding control using buffer instead of the boiled enzyme fraction yielded only $2.0 \mu\text{g}$ IAA after 2 hours. Thus, there was a net synthesis of IAA from 3-indolepyruvate by the chloroplast enzymes and the synthesis was greater when cofactors were present. The inclusion of BSA in the complete reaction system seems to have prevented the nonenzymic formation of $0.2 \mu\text{g}$ IAA during the 2 hours incubation period. This stabilizing effect of BSA is, however, more evident in the difference between the results from the two controls using buffer instead of active enzymes. The absence of BSA in such a non-proteinaceous reaction system resulted in a major increase in the amount of IAA formed, from $2.0 \mu\text{g}/2$ hours to $7.1 \mu\text{g}/2$ hours.

Table 16 Amounts of IAA and PAA produced by chloroplast enzymes when 3-indolepyruvic acid or phenylpyruvic acid was supplied as substrate.

COMPOSITION OF REACTION SYSTEMS* (Substrates at 1 μ mole)	AUXIN/PRODUCT** (μ g/2 h)		AMOUNT OF SUBSTRATE RECOVERED (μ g)
Indolepyruvic acid (203 μ g) or Phenylpyruvic acid (164 μ g) plus:	IAA	PAA	IPyA PPyA
Enzyme fraction + BSA + cofactors	4.5 (6.28)	12.33 (5.37)	42 40
Enzyme fraction + BSA	3.2 (2.57)	9.98 (3.75)	44 51
Enzyme fraction only	3.4 (3.14)	10.06 (3.82)	50 49
Control 1 (Buffer + BSA + cofactors)	2.0	2.14	30 42
Control 2 (Buffer + cofactors)	7.1	11.64	21 23
Control 3 (Boiled enzyme fraction + BSA + cofactors)	2.3	4.48	24 35

* Components dissolved in 50 mM Tris-HCl buffer at pH 8.0 containing 4 mM 2-mercaptoethanol. BSA used at 0.5 mg/ml and the cofactors provided were NAD, NADP and cocarboxylase. Total volume of reaction system was 2 ml and the incubation period was 2 h at 30°C. The reaction was stopped by rapid freezing.

** Values in brackets are nmoles/mg protein/2 hours.

The related experiment using PPyA as the starting substrate gave similar results. More PAA was found in the complete reaction system containing BSA and the cofactors, 12.33 μg /2 hours. The corresponding controls using buffer or a boiled enzyme fraction yielded 2.14 μg PAA/2 hours and 4.48 μg PAA/2 hours, respectively. There was thus a net synthesis of PAA of about 10 μg during the 2 hour incubation period. The effect of including BSA was negligible with the systems containing the active enzyme fraction but the stabilizing effect of including this protein was again noticeable in the non-proteinaceous buffer controls where 2.14 μg of PAA after 2 hours was increased to 11.64 μg PAA/2 hours when BSA was absent.

The values in brackets show nmoles of IAA or PAA formed per mg protein/2 hours and allow a comparison to be made between the two experiments. Slightly more IAA than PAA was synthesized per mg protein but a variation of this order was also noticed in the experiment with the amino acid as the starting substrate and was found not significant.

The presence of BSA with active enzyme fractions seems to have been more effective in preventing the nonenzymic formation of auxin when a lower quantity of protein was present in the reaction system. The difference between the amounts of auxin produced by active enzymes with and without BSA was greater in the IPyA experiment, and these reaction systems contained 5 times less protein than those in the PPyA experiment.

The quantity of substrate remaining after the incubation period was also measured. The results from both experiments followed the same pattern and will be examined together. The presence of chloroplast proteins generally allowed for greater recoveries of the substrate. Proteinaceous systems in general seemed more favourable

for the stability of the arylpyruvates since recoveries from the buffer controls were low. The fact that no active enzymes were present in these controls, indicates that nonenzymic breakdown of the substrate must have occurred. A greater breakdown of substrate occurred in control No. 2 containing no BSA than in control No. 1 containing BSA. Thus, BSA can act, to a certain extent, as a stabilizer for the arylpyruvates in nonenzymic systems.

From the amounts of substrates recovered and of IAA or PAA produced in the different systems, it is evident that the arylpyruvates can either be transformed enzymatically or broken down to yield IAA or PAA. In such nonenzymic reactions, many other compounds besides the two auxins are probably also formed, but no attempt was made to identify these products.

In summary, chloroplast enzyme fractions are able to catalyse the synthesis of IAA and PAA from their corresponding arylpyruvic acids and, while there is evidence from control systems that some nonenzymic formation of IAA and PAA can occur, the formation of auxin by these reactions is probably low in proteinaceous systems.

(ii) Mitochondrial assays

The data from the assays using mitochondrial enzyme fractions and the arylpyruvate substrates are presented in Table 17. A similar description of the results can be given for both experiments in relation to the production of IAA and PAA.

The reaction systems with active enzymes, BSA and cofactors produced more IAA and PAA than the corresponding other two systems which also contained the active enzymes. Controls 1 and 3 are the two controls related to these reaction systems since the active enzyme fraction was replaced by either buffer or a boiled enzyme fraction, respectively. Although IAA and PAA were found in these

Table 17 Amounts of IAA and PAA produced by mitochondrial enzymes when 3-indolepyruvic acid or phenylpyruvic acid was supplied as substrate.

COMPOSITION OF REACTION SYSTEMS* (Substrates at 1 μ mole)	AUXIN PRODUCT** (μ g/2 h)		AMOUNT OF SUBSTRATE RECOVERED (μ g)	
	IAA	PAA	IPyA	PPyA
Indolepyruvic acid (203 μ g) or Phenylpyruvic acid (164 μ g) plus:				
Enzyme fraction + BSA + cofactors	5.4 (9.83)	3.50 (13.24)	29	21
Enzyme fraction + BSA	4.1 (5.26)	1.10 (2.43)	35	29
Enzyme fraction only	4.7 (7.37)	1.50 (4.26)	35	28
Control 1 (Buffer + BSA + cofactors)	1.4	0.15	41	33
Control 2 (Buffer + cofactors)	6.3	8.29	25	53
Control 3 (Boiled enzyme fraction + BSA + cofactors)	2.6	0.56	38	12

* Components dissolved in 50 mM Tris-HCl buffer at pH 8.0 containing 4 mM 2-mercaptoethanol. BSA used at 0.5 mg/ml and the cofactors provided were NAD, NADP and cocarboxylase. Total volume of reaction system was 2 ml and the incubation period was 2 h at 30°C. The reaction was stopped by rapid freezing.

** Values in brackets are nmoles/mg protein/2 hours.

control systems, they were formed in much lower quantities than in the reaction systems containing active enzymes. There was thus a net synthesis of both IAA and PAA by the mitochondrial fraction and the amount of PAA produced was higher than the one for IAA; 13.24 nmoles IAA per mg protein/2 hours compared to 9.83 nmoles PAA per mg protein/2 hours.

The presence of cofactors generally improved the yield. The cofactors, however, seemed to be required more in the PPyA experiment. About 5 times more PAA was formed in presence of the cofactors compared to about 2 times more IAA in the same system in the IPyA experiment.

The effect of BSA in preventing a nonenzymic breakdown of the substrate in nonproteinaceous systems was again noticeable from the difference between the auxin yields in controls 1 and 2. Clearly, there was an increase in the quantity of IAA and PAA recovered when BSA was not present in the system. The levels of auxins found in these control systems are similar to the levels found in the equivalent controls in the chloroplast assays.

In the reaction systems containing active mitochondrial enzymes, BSA was more effective in preventing the breakdown of either arylpyruvate substrate than it had been in the chloroplast assays. The protein content of the mitochondrial reaction mixtures, however, was lower than in the chloroplast systems and so it would seem that BSA is more able to stabilize a reactive substrate when soluble protein levels are not too high. Nevertheless, mitochondrial assay results tend to confirm that IAA and PAA can be found from nonenzymic breakdown of IPyA and PPyA, respectively, but the presence of proteins such as BSA does reduce the amount of either auxin that can be formed in this way.

The quantities of arylpyruvates recovered from reaction systems containing the active enzymes were lower than the amounts recovered from the corresponding controls, which indicates that mitochondrial enzymes were actively using the arylpyruvates. The controls from the IPyA experiment showed a similar pattern of results to the corresponding chloroplast controls. More breakdown of the substrate definitely occurred in control No. 2, whereas similar levels of remaining substrate were detected in the other two controls. The results from the control systems containing PPyA are more ambiguous. The boiled enzyme control (No. 3) gave the lowest recovery of PPyA and control No. 2 the highest. Control No. 2 also showed the highest nonenzymic production of PAA. This apparent contradiction suggest a possible error in the experimental results.

In summary, the mitochondrial enzymes can definitely catalyse the synthesis of IAA from IPyA, and PAA from PPyA. Some nonenzymic breakdown of the arylpyruvates to yield auxin products is possible in these complete reaction systems but seems to occur most readily in the nonproteinaceous control systems.

Discussion

Evidence for auxin synthesis by plant organelles will be discussed following basically the order in which the results were presented in the previous section. Characterization of the organelles will be dealt with first and then the data obtained from the different steps used to investigate auxin synthesis in chloroplasts and mitochondria will be discussed.

1. Plant growth and organelle characterization

As stated in the literature review, IAA and PAA can probably be synthesized in virtually all parts of the plant, although the young expanding leaves appear to be the most active sites of synthesis. The growth data presented show that the leaves used for the isolation of organelles were in the expansion phase of growth, thus it can be inferred that this material was very suitable for studies of auxin biosynthesis.

The aim of the preparative cell fractionations carried out in this work was to establish whether chloroplasts and mitochondria possess the biochemical potential for auxin synthesis. Clearly, the results of studies on such properties of a subcellular organelle are strongly influenced by the purity of the preparation. Thus, the particular organelle must be characterized for purity by enzyme markers and electron microscopical examination.

The chloroplasts isolated for use in the biosynthesis experiments were highly intact, 92% on average (Table 3), and contamination by mitochondria and peroxisomes was reduced to traces (Table 2). The criteria usually used for successful separation of chloroplasts are maximum recovery of the intact chloroplasts with minimum contamination by other organelles (Mifflin and Beevers, 1974). The degree of contamination of the chloroplasts with other organelles was remarkably

small when isolated by the Percoll method. With regard to the yield of intact chloroplasts, although only 47% of the chlorophyll from the washed chloroplasts fraction was recovered, it represented 79% of the intact chloroplasts contained in these washed preparations (Table 3). The electron micrograph (Fig. 10) showed a homogenous population of chloroplasts with intact membranes and thylakoids.

Thus, intact chloroplasts were isolated virtually free of contamination with peroxisomes and mitochondria and this preparation was therefore highly suitable for experiments on auxin biosynthesis.

Mitochondrial preparations from the leaves of higher plants are usually contaminated by peroxisomes and broken thylakoid membranes (Jackson *et al.* 1979). Thus, the contamination in washed mitochondrial preparations was not surprising (Table 4). The nature of the present study required a further procedure to separate the mitochondria from other organelles and chloroplasts. Sucrose density gradient fractionation procedures are only partially effective in removing chlorophyll contamination, in addition to peroxisomes and other material (Jackson and Moore, 1979). However, the Percoll gradient purification procedure allowed for a 10-fold reduction in chlorophyll contamination and a good decrease (5-fold) in peroxisomal contamination, with a concomitant enrichment of the fraction in mitochondria (Table 4). 65% of these mitochondria were recovered intact, which is only 8% lower than the percentage of intact mitochondria present in the washed preparations (Table 5). The electron micrograph (Fig. 11) shows the majority of the organelles present in the fraction to be mitochondria with their typical cristae, but some peroxisomes and debris from broken mitochondria and peroxisomes can also be seen.

Thus, the mitochondria were successfully isolated with much reduced contamination and this organelle fraction seemed suitable for

auxin biosynthesis studies.

2. Auxin biosynthesis

A. Endogenous levels of precursor amino acids and arylacetic acid end-products.

i) Occurrence of phenylalanine and tryptophan.

Demonstration of the occurrence of the enzymic potential for auxin biosynthesis in organelles could be considered irrelevant to the actual biosynthesis of auxins in plants unless it can be shown that the precursor amino acids and end-product, arylacetic acids, can also be isolated as endogenous substances from these same organelles.

The aromatic amino acids, phenylalanine and tryptophan, are the precursors of PAA and IAA, respectively (Schneider and Wightman, 1978). The occurrence of these two amino acids in both organelles used in this study was investigated by analysing the free amino acid pool present in each organelle. Phenylalanine was found in sunflower chloroplasts but tryptophan was not detected. There have been relatively few studies on the estimation of free amino acid levels in chloroplasts. Chapman and Leech (1979) with Zea Mays and Mills and Joy (1980) with Pisum sativum have made measurements of the concentration of individual amino acids in chloroplasts and in both cases, reported the presence of phenylalanine, but tryptophan was not determined. Kirk and Leech (1972) measured the pool sizes of the common amino acids in isolated Vicia faba chloroplasts and found both phenylalanine and tryptophan. Phenylalanine was present at 5.1 nmoles per mg chlorophyll whereas tryptophan was present in a 3-fold lower amount, at 1.7 nmoles per mg chlorophyll. Phenylalanine was found in sunflower chloroplasts at 1.25 nmoles per mg chlorophyll, which is 4-times less than the level in Vicia faba chloroplasts and about 6-times less than the level in Pisum sativum chloroplasts.

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(Mills and Joy, 1980).

The endogenous level of phenylalanine in sunflower chloroplasts was thus substantially lower than the amounts found in this organelle from two other plants. Furthermore, considering that the concentration of tryptophan in Vicia faba chloroplasts was 3-times lower than the level of phenylalanine, it may be anticipated that endogenous tryptophan is probably also at a low concentration in sunflower chloroplasts. The fact then, that tryptophan could not be detected in these organelles may be partly related to this low level and partly to the instability of tryptophan under acidic extraction conditions, since the isolation procedure called for an unavoidable acidic extraction step to remove residual pigments. Thus, free tryptophan is most likely to be present in sunflower chloroplasts, but at a concentration much lower than that of phenylalanine. Furthermore, as mentioned in the literature review, chloroplasts are probably one of the main sites of biosynthesis of aromatic compounds in plant cells (Bickel and Schultz, 1979) and therefore all three aromatic amino acids would be expected to be found in the 'free' amino acid pool.

The general pattern of concentration of the other 'free' amino acids in sunflower chloroplasts was similar to that reported for Vicia faba chloroplasts by Kirk and Leech (1972), although the levels of each amino acid were different in the two situations.

Isolated sunflower mitochondria were found to contain both phenylalanine and tryptophan. Only one measurement was made with these organelles and tryptophan was present at about 8-times the level of phenylalanine. No information could be found in the literature about the endogenous levels of free amino acids in mitochondria, probably because reliable methods for the isolation of

homogenous preparations of intact mitochondria have only been developed over the last two years.

Isolation of the free amino acid pool of sunflower mitochondria did not require any acidic extraction step, since no pigments needed to be removed. The fact that tryptophan was readily detected in these organelles makes it appear likely that the apparent absence of this amino acid in extracts from sunflower chloroplasts was due to losses during the acidic step in the isolation procedure.

Thus, both phenylalanine and tryptophan were shown to be present in the free amino acid pool in sunflower mitochondria and the comparable pool in chloroplasts contained phenylalanine. This evidence provides some support for the occurrence of a biosynthetic pathway starting with these amino acids in the organelles.

ii) Occurrence of IAA and PAA

The natural occurrence of IAA and PAA in both chloroplasts and mitochondria was also verified to provide another line of evidence for the presence of the biosynthetic pathway for these compounds in each organelle.

Free IAA and PAA were found in both the analyses carried out on sunflower chloroplasts and in the single analysis of sunflower mitochondria. Identification was provided by co-chromatography on GLC with authentic compounds, but since no GC-MS analyses were attempted, these identifications are provisional.

The GLC profiles (Fig. 12-15) obtained with aliquots of the derivatized HPLC-purified acidic fractions showed that the two auxin substances were only minor components of the endogenous pool of acidic substances present in the two organelles. This was not surprising since both organelles, as mentioned in the literature review, are involved in major metabolic activities for the formation

of many other acidic molecules. The GLC peaks of the two auxins were, however, distinctly separated from those of the other compounds present in the extract.

The results in Table 7 showed that there was a 25-30% variation between the two quantitative estimates made on the amounts of IAA and PAA in chloroplasts. These measurements, however, were carried out at two different times of the year, which may partly explain the variation. Differences between data obtained at different times of the year were also observed by Camirand (1979) with endogenous levels of auxins in tobacco shoots. On a molar basis, 1.3 times more IAA than PAA was present in both chloroplast analyses.

Mitochondria were also found to contain about 3-times more IAA than PAA. Tryptophan was found at a higher concentration in these organelles; there was 8-times more tryptophan present than phenylalanine. However, it cannot be expected that the ratio observed between the precursor amino acids will be the same for the auxin products, since the two amino acids are involved in many other reactions besides auxin biosynthesis.

While the literature contains numerous reports of the concentration of auxins in whole plants, or plant parts, no attempts have yet been made to determine the intracellular location of these growth hormones and estimate their concentrations. Thus, the results obtained in the present study cannot be compared with any others. There are, however, previous studies employing GLC that have demonstrated the association of abscisic acid (ABA) and certain gibberellins with chloroplast fractions. The ABA content of non-stressed spinach leaf chloroplasts was reported by Lovey (1977) to be around 14.4 ng per mg chlorophyll, whereas free GA_9 and GA_4 were measured at 5 ng and 1 ng per mg chlorophyll in wheat chloroplasts

* See Appendix I.

by Browning and Saunders (1977).

The amounts of IAA and PAA found in sunflower chloroplasts are in the same range as those previously reported for two other types of hormonal substances when the data is expressed in the same terms; 111 and 83 ng of IAA per mg chlorophyll and 70 and 47 ng of PAA per mg chlorophyll. Furthermore, these levels seem plausible in relation to the other hormones, since auxins are generally considered to be growth promoter substances. ABA, being a growth inhibitor, would be expected to be present at a low level in non-stressed leaves. The GAs, also considered promoter substances, were only partly characterized in wheat chloroplasts since only two of these substances, among many others, were measured.

The amounts of IAA and PAA in chloroplasts reported in this work must be considered as a low estimate of the true amounts since no account was made of possible losses from the plastids during their isolation. These data, however, are probably a good estimate of the endogenous level of free IAA and PAA in chloroplasts.

Thus, the present data show that the auxin end-products of the biosynthetic pathways investigated were found in sunflower chloroplasts and mitochondria. This evidence provides good support for the possible occurrence of the biosynthetic pathways themselves in the two organelles.

B. Biosynthesis using L-tryptophan or L-phenylalanine as substrate.

The biosynthesis data obtained when L-tryptophan and L-phenylalanine were tested as precursors of the auxins in chloroplast and mitochondria fractions will be discussed together. In this section, the emphasis will be on the different lines of evidence obtained for the occurrence of the arylpyruvate pathway in these organelles and on the analytical procedures used to gather this evidence. Section 3 of the Discussion

will deal more specifically with the quantitative data obtained with each organelle and with the implications of the localization of auxin synthesis in chloroplasts and mitochondria.

i) Analytical Methods

The occurrence of auxins in low concentrations in most higher plant tissues (Schneider et al. 1972; Wightman, 1977; Brenner, 1981), the relatively small yields obtained from in vitro biosynthesis experiments (Wightman and Cohen, 1968; Moore and Shaner, 1968) and the lability and losses of the compounds during purification (Mann and Jaworski, 1970) are all recognized problems in auxin investigations. They usually govern the kind of analytical methods that can be employed. The in vitro biosynthesis experiments carried out in the present work had a further limitation, namely, the yield of active enzymes was limited by the recovery of intact organelles. This meant that the amount of auxin synthesized might be even lower than the levels found in in vitro studies with cell-free preparations of plant tissues. Furthermore, in the present study, the small yields of auxin had to be analysed in several reaction mixtures; thus, the analytical procedure employed had to be sensitive, relatively rapid and provide reproducible results.

The modern separatory procedures of HPLC and GLC are being used with increasing frequency for analysing the many growth regulators present in plant extracts (Wightman, 1979; Brenner, 1981). Their combined use, after preliminary solvent fractionation of the organelle reaction mixtures, as described in this work for the isolation of IAA and PAA, provided good chromatographic resolution of both auxins with a speed of analysis which allowed for the handling of several extracts each day. Both methods are more sophisticated forms of liquid chromatography and are based on

different properties of biological molecules which allows for better resolving power than that obtained by the older methods of chromatography.

The HPLC profiles of the acidic ether extracts from chloroplast reaction mixtures (Fig. 16 to 19) and the GLC profiles obtained from direct analysis of the acidic ether extracts of mitochondria reaction mixtures (Fig. 30 and 31) demonstrate that the analyses were carried out on extracts of a fairly complex nature. When reverse phase preparative HPLC was used as a preliminary purification step before final GLC analysis, good results were obtained since less than five compounds including IAA or PAA were found on the GLC profiles of collected fractions from both the chloroplasts and mitochondria reaction mixtures.

The peak resolution obtained with GLC was excellent; the auxin peak consistently separated well from the other substances in the fraction (Fig. 20, 25, 32 and 34). The flame-ionization detector (FID's) used with the GLC showed high sensitivity. The FID is the most widely used detector system because it has sensitivity limits approaching 1 ng for almost all organic compounds (Wightman, 1979). However, the FID's are nonselective since they will detect any compound that ionizes upon combustion in a hydrogen flame. Further proof of identification of the auxin substances biosynthesized in these experiments was therefore needed beyond that obtained by co-chromatography with the authentic standards. This was obtained from combined GC-MS analysis of the purified HPLC fractions isolated from chloroplast complete reaction mixtures. These analyses confirmed the presence of IAA methyl ester or of PAA pentafluorobenzyl ester in the derivatized fractions (Fig. 24 and 29). These findings provided unequivocal proof that IAA and PAA were synthesized by the chloroplast

enzymes.

These GC-MS analyses were carried out by an outside laboratory (CBRI, Agriculture Canada) and unfortunately, similar analyses of the purified HPLC fractions from the mitochondrial reaction mixtures could not be obtained. However, since the same procedures were followed for analysing the final complete reaction mixtures from both organelles, the co-chromatographic GLC identification of the two auxins synthesized by the mitochondrial systems is most probably correct.

Isolation of the synthesized auxins required several purification steps before each auxin could be quantified. The percentage recovery of each auxin was therefore measured to evaluate the losses encountered during the purification procedure. Brenner (1981) has stated that the net recovery of an internal standard added right at the start of tissue extraction is now the most accepted procedure for estimating recovery efficiency. In the present work, the internal standard used in each experiment was a known amount of the appropriate authentic auxin added to the final reaction mixture at the beginning of the extraction procedure. The concentration of the internal standard was similar to the levels of synthesized auxin found in the reaction systems. It was felt that using concentrations of this order would give a more accurate account of the losses.

The percentage recoveries obtained at the end of the analytical procedure varied from 59 to 74% for IAA and from 72 to 83% for PAA (Tables 8 and 10). Such differences between the recoveries of the two auxins were expected since the two substances differ in their chemical structure and most likely in their stability to acidic extraction and chromatographic conditions. Brenner (1981) also recently mentioned that recovery efficiencies for different plant

growth regulators are quite variable. IAA, in particular, is known to show some instability during solvent fractionation and its oxidative degradation during the purification of a plant sample was considered by Mann and Jaworski (1970) as a possible major source of loss of the compound. Such oxidative losses could be partly responsible for the differences observed between the recoveries of the two auxins obtained in the present work.

The recovery of IAA after ether fractionation, derivatization and GLC was measured with the complete reaction mixtures from mitochondria only, since fewer compounds were found to be present in the acidic ether extracts and direct GLC quantification could be performed. 85-89% of the IAA was recovered after this preliminary fractionation procedure in which the HPLC purification step was omitted (Table 10), which clearly indicates that subsequent purification of the ether extracts by HPLC was achieved at the expense of recovery of auxin. However, inclusion of the HPLC step is justified in view of the great reduction in interfering compounds that is achieved by this purification procedure.

Brenner (1981) attributes losses of plant growth regulators during extraction and analysis to several factors; incomplete partitioning between solvents, entrapment in other components of the system such as proteins and chemical reaction or adsorption onto glassware or onto solid chromatographic supports. Entrapment in proteins when partitioning against ether could be a significant factor in the losses of IAA and PAA observed in the present work since half of the reaction mixture was made up of soluble protein from the organelle under investigation.

Mann and Jaworski (1970) reported erratic recoveries of IAA ranging from 25% to 70% with a more lengthy procedure than the one

used here and their most important finding was that a substantial portion of their losses could be attributed to sublimation of IAA in vacuo, even when the temperature was kept below 40°C. DeYoe and Zaerr (1976) also attributed significant losses of IAA (up to 60%) to this factor during the in vacuo concentration of ether extracts from shoots of Douglas fir. Since concentration under vacuum was used here to reduce the volume of ether extracts and to dry the fractions collected from HPLC, it must be considered as a factor that could have contributed to the losses observed for IAA. PAA has not yet been studied as extensively as IAA in terms of stability in acidic ether solution and efficiency of recovery, but it could be sensitive to the same factors mentioned above for IAA. It can, however, also be partly lost during the evaporation of solvent extracts because of its relatively low boiling point and therefore higher volatility (J. Phipps, personal communication).

Thus, the present purification procedure had the advantage of being relatively rapid, of giving good chromatographic resolution of the two synthesized auxins and the recoveries obtained were reasonably consistent and of a satisfactory level. Since this 3-step purification procedure has not been followed before by other workers using similar plant material or cell-free preparations, a comparison of the percentage recoveries previously reported cannot be made. However, we consider that the present procedures do give efficient recoveries in view of the many purification steps involved.

Attempts to reduce losses through adsorption were made by the use of silanised glassware whenever possible and by deactivating sites on the GLC chromatographic column prior to injection of samples. Concentration by flash evaporation of the ether extracts and of the HPLC collected fractions were, however, unavoidable in this procedure.

Thus, measurement of the percentage recovery of internal standard is an essential requirement for quantitative determination made following this procedure.

The radioactive experiments were conducted mainly to find qualitative evidence for the arylpyruvate pathway of auxin biosynthesis after attempts to detect nonradioactive intermediates of this pathway had failed (see Results, Preliminary experiments). Radioactive L-tryptophan and L-phenylalanine were metabolised into neutral and acidic compounds by the enzymic fractions isolated from both organelles. The percentage of radioactivity incorporated into neutral and acidic intermediates was measured after ether fractionation and was found to be low (Tables 12 and 14). These levels, however, were similar to the one observed by Cohen (1968) using a reaction system containing soluble mitochondrial enzymes prepared from etiolated mung bean seedlings. Cohen prepared the 2,4-dinitrophenylhydrazone derivatives of the aldehyde and keto compounds present and found that only 1.41% of the radioactivity originally supplied to the reaction was recovered in the combined acidic and neutral ethyl acetate extracts.

The majority of the ^{14}C -label was found in the final aqueous fraction and was probably present as the unmetabolized aromatic amino acid. The acidic ether extracts from both the chloroplast and mitochondria radioactive reaction mixtures were analyzed in the same manner as described earlier for the determination of IAA and PAA in nonradioactive experiments. Losses during these earlier experiments probably again occurred during the purification and chromatography of the radioactive acidic extracts. Chromatography of the neutral extracts was carried out by TLC on silica gel plates. Moore and Shaner (1968) mentioned that the possible instability of IAAld, due to oxidation, could be responsible for the lack of recovery of IAAld

during TLC separation. Losses when applying the extracts to the TLC plates were probably important for the phenyl intermediates. Losses due to adsorption could also be a factor when TLC is employed.

No internal standards were used during the radioactive experiments and consequently, the losses encountered at different stages during purification of the extracts could not be assessed. Specific activities were not calculated partly for this reason but mainly because nonradioactive arylacetaldehyde intermediate was added as a carrier substance both at the beginning and end of the reaction period. This procedure prevented any measurement of the amounts of the corresponding synthesized intermediate and auxin product. Thus, it must be emphasized that the radioactivity values cited in Tables 13 and 15 for the different intermediates and the auxin end-products have to be considered as qualitative evidence for the synthesis of these compounds.

ii) Qualitative evidences for the arylpyruvic acid pathway

The protein fraction isolated from both sunflower chloroplasts and mitochondria appeared to have the potential for auxin biosynthesis. Identification of IAA and PAA as acidic products formed during the metabolism of L-tryptophan and L-phenylalanine is based on qualitative evidence obtained from both nonradioactive and radioactive experiments; firstly, from co-chromatography of the suspected and authentic derivatized auxins on GLC, (Fig. 20-21, 25-26, 32 and 34), secondly, from the incorporation into IAA and PAA of ^{14}C -label from the radioactive aromatic amino acid substrates (Tables 13 and 15), and thirdly, from GC-MS identification of Me-IAA and PFB-PAA in the derivatized purified fractions obtained from chloroplast final reaction systems (Fig. 24 and 29).

Three types of reaction mixtures were assayed. The reaction

mixtures containing the boiled enzyme fractions represented the true controls and neither IAA or PAA was produced in these systems as determined by GLC analysis (Fig. 23, 28, 33, 34). The other two reaction mixtures contained the active enzyme fraction and the amino acid substrate along with, or without, α -ketoglutarate and various cofactors. Only the complete reaction mixtures for the two amino acids provided GLC evidence for the synthesis of IAA or PAA. Thus, the addition of α -ketoglutarate, pyridoxal phosphate and the other cofactors to the active chloroplast and mitochondrial fractions stimulated the conversion of L-tryptophan to IAA and of L-phenylalanine to PAA. The requirement for α -ketoglutarate indicates that transamination of the aromatic amino acid to form the corresponding arylpyruvic acid was probably the first reaction occurring under these experimental conditions. This conclusion suggests then, that the main pathway by which L-tryptophan or L-phenylalanine were converted to IAA or PAA respectively, was very probably via the corresponding arylpyruvic acid and arylacetaldehyde intermediates.

This conclusion is similar to the findings reported by previous workers using cell-free plant systems. Moore and Shaner (1967) showed that the addition of α -ketoglutarate, pyridoxal phosphate and thiamine pyrophosphate to reaction mixtures containing L-tryptophan and a cell-free protein extract from pea seedlings greatly enhanced the formation of IAA. Wightman and Cohen (1968) also reported evidence for greater IAA synthesis by mitochondrial protein fractions from mung bean seedlings when α -ketoglutarate and the same cofactors mentioned above were supplied along with L-tryptophan in the complete reaction mixture.

The assay systems containing no α -ketoglutarate and cofactor additives served to examine whether the pathway suggested by

Loffelhardt (1977) for blue-green algae was occurring in the present organelle reaction systems. Loffelhardt proposed that an L-amino acid oxidase was the enzyme responsible for the formation of the arylpyruvic acid intermediate in his blue-green algae system. The production of this key intermediate only occurred under aerobic conditions and did not require the presence of α -ketoglutarate or other cofactors in his system. Since aerobic conditions were maintained throughout the present experiments, oxygen was not a limiting factor during the incubation period, yet no IAA or PAA could be detected in the reaction mixtures where only the L-aromatic amino acid was provided (Fig. 22, 27, 34). These findings would seem to eliminate the possibility that an L-amino acid oxidase could be the enzyme responsible for the formation of the arylpyruvic acid intermediate observed in the complete reaction systems.

Two approaches have been taken in the past to seek more direct evidence for the occurrence of a specific pathway in plants for the synthesis of an auxin: a) investigation of the simultaneous occurrence of the expected intermediates in the pathway along with the newly synthesized auxin in the final in vitro reaction mixture or fed plant, and b) the isolation and measurement of the activity of certain of the enzymes in the pathway under study. As reported in the Literature Review, only the aromatic aminotransferase has been completely isolated and its properties examined; most of the other enzymes have only been partially purified and their stability and properties were found to vary from species to species. In the investigations reported here, the amount of enzyme protein available for each experiments was limited due to the fact that only small quantities of intact organelles could be isolated from each sample of sunflower leaves. Since these experiments represent the first detailed study of the capacity of leaf

cell organelles to synthesize auxins, the first approach was chosen to try to confirm more directly the formation of auxins via the arylpyruvate pathway.

The occurrence of the appropriate arylpyruvic acid and arylacetaldehyde was therefore investigated. The nonradioactive experiments first provided good evidence for the formation of the two arylpyruvic acids, depending on which aromatic amino acid was provided as the primary substrate. Either IPyA or PPyA was found to be present in the complete reaction mixture from both organelles and could be easily detected in ether extracts even on the profiles obtained during preparative HPLC (Fig. 16 and 18). The radioactive experiments fully confirmed the formation of these intermediates since substantial amounts of radioactivity were recovered in these substances (Tables 13 and 15). Wightman and Cohen (1968) were able to detect significant amounts of ^{14}C -IPyA, isolated as the 2,4-dinitrophenylhydrazone, in their in vitro experiments in which DL-tryptophan-2- ^{14}C was supplied to both cell-free enzymes and mitochondrial enzymes from mung bean seedlings. Moore and Shaner (1968) also reported recovering radioactivity in IPyA, isolated again as the 2,4-dinitrophenylhydrazone, using a cell-free preparation from pea seedlings.

It was much more difficult to detect the arylacetaldehyde intermediate, as was described earlier in the Preliminary Experiments section. Similar problems were encountered by Moore and Shaner (1968) in their detection of enzyme synthesized IAAld. The procedure used here to isolate the radioactive intermediates was based on the method of Narumiya et al. (1979), who also experienced difficulties in their investigation of IAAld as an intermediate in a biochemical pathway occurring in Pseudomonas fluorescens. Wightman and Cohen (1968) found

evidence for the presence of radioactive IAAld in their reaction system by isolating it as the 2,4-dinitrophenylhydrazone. They stated, however, that the amount of ^{14}C -IAAld present was much less than that of ^{14}C -IPyA, but since IAAld was known to be readily metabolized by plant enzyme preparations, its presence was likely to be very transitory.

Other reasons have been put forward to explain the problem encountered with the detection of arylacetaldehydes. Clarke and Mann (1957) reported lower recoveries of IAAld than was expected in their reaction mixtures and they partly attributed this to the polymerization of IAAld. They also mentioned that the same phenomenon had been noticed with phenylacetaldehyde in aqueous solutions. Larsen *et al.* (1962) also noticed an instability of IAAld in reaction mixtures. In addition, there is also the possibility that there was a rapid conversion of the arylacetaldehyde to the corresponding arylacetic acid or that the enzyme preparations contained factors catalysing other reactions involving arylacetaldehydes.

Radioactive IAAld was found to be present in both organelle reaction systems, although lower levels were detected in the chloroplast systems (Tables 13 and 15). Similar results were found with PAAld but the levels here were even lower, which was probably due to poor efficiency in recovery because of the volatility of this intermediate.

The presence of radioactivity in the arylethanol intermediate, which can arise from a side reaction involving reduction of the arylacetaldehyde, was also determined in the complete reaction systems. Differences were encountered between the two organelles reaction systems with respect to the arylethanol intermediates. Radioactivity was detected in tryptophol and phenylethanol only in the chloroplast

systems. Mitochondrial enzymes did not seem to possess the capacity for this synthesis. Cohen (1968) reported that mung bean soluble mitochondrial enzymes exhibited tryptophol formation activity, but only when NADH_2 was provided in the reaction mixtures. As mentioned in the Literature Review, formation of the arylethanol from the corresponding arylacetaldehyde has been studied with cell-free enzyme preparations and the enzyme responsible has been partially purified by some workers (Wightman and Cohen, 1968; Brown and Purves, 1980).

In all these earlier investigations, whether the enzyme studied was an alcohol dehydrogenase or an aldehyde reductase able to use both IAAld and PAAld, activity was dependent on the presence of either NADH_2 or NADPH_2 . In the experiments reported here, the reaction mixtures contained only oxidized NAD and NADP, which were provided as cofactors for the enzyme catalysing the formation of IAA and PAA from their corresponding arylacetaldehyde. The absence of tryptophol and pheylethanol formation can be interpreted either as an indication of the inability of the soluble mitochondrial enzyme systems to generate reduced cofactors from the NAD or NADP provided, or as an indication of the absence of the enzyme itself. The soluble stromal enzymes from sunflower chloroplasts seemed to have the capacity for this synthesis and the presence of this activity may be partially responsible for the low radioactivity detected in the arylacetaldehyde intermediates.

This point brings up another aspect of the present experimental method which should be borne in mind since it could partly explain some of the discrepancies observed between the quantitative data per se which further prevents our taking these values as true quantitative results. The choice of the approach used to investigate the occurrence of the arylpyruvate pathway also meant that the organelle

protein preparations used contained, in addition to the enzymes for the formation of auxins, many other active enzymes. Certain of these enzymes can probably utilize one or both of the intermediates formed in the auxin pathway as a substrate for the formation of other products that are not necessarily potential precursors of the auxins. The synthesis of other compounds in the complete reaction mixtures was observed from the fact that many extra peaks were present on the preparative HPLC profiles from chloroplast reaction systems (Fig. 16 to 19). Many reaction products were also observed on the GLC profiles obtained during direct analysis of the mitochondrial reaction mixtures when L-tryptophan was used as substrate (Fig. 30 and 31).

Moreover, IAA and PAA are not themselves end-products. The possibility of other reactions occurring with these compounds certainly exists (see Schneider and Wightman, 1978) but no attempt was made in the present study to examine for auxin degradation or conjugation reactions occurring in the sunflower organelle reaction mixtures.

Thus, because of the reasons referred to in the previous section and in view of the two possible additional complications mentioned above, a quantitative assessment of the arylpyruvate pathway using the approach employed here is very difficult.

Nevertheless, the qualitative and quantitative data reported in this thesis are consistent with the operation of the arylpyruvate pathway for auxin biosynthesis in the soluble protein fractions prepared from sunflower chloroplasts and mitochondria. These findings therefore agree with the results reported by Wightman and Cohen (1968) which demonstrate the presence of an auxin-synthesizing enzyme system in mitochondria from mung bean seedling.

C. Biosynthesis using IPyA or PPyA.

The arylpyruvic acids were used as substrates to confirm that enzymatic steps were involved in the conversion of this intermediate to the corresponding arylacetic acid end-product. These experiments were tried in view of the results reported by Loffelhardt (1977) in his study of phenylalanine metabolism by Anacystis nidulans. This worker used PPyA as the starting substrate in some of his assays to investigate further the pathway leading to PAA. Even though Loffelhardt could not completely rule out enzyme activity for the formation of PAA from PPyA, his results indicated that PPyA underwent a significant non-enzymatic transformation into PAA under the incubation conditions of the experiments. The comparison was made against control systems where the enzyme fraction had been replaced by either buffer only, or a boiled enzyme fraction.

Breakdown during the incubation period could be responsible for the formation of the arylacetates in the experiments described here, as was observed by Loffelhardt (1977) for some of the PAA in his study. The results presented in Tables 16 and 17 indicate that the enzymes from both sunflower chloroplasts and mitochondria were partly responsible for the synthesis of the arylacetic acids from the arylpyruvic acids supplied under the present experimental conditions. The addition of cofactors increased this level of synthesis but their presence was not essential. As was observed by Loffelhardt (1977), non-enzymatic formation of arylacetic acid was substantial in the control system containing buffer only. However, the addition of BSA in the other controls had a stabilizing effect, in that it appreciably prevented the non-enzymatic formation of the arylacetates.

These experiments, therefore, did demonstrate that enzyme synthesis is involved in the formation of IAA and PAA from the corresponding

arylpyruvic acid, but under the in vitro conditions used, some non-enzymic breakdown could not be ruled out. It must be remembered, however, that the arylpyruvates would normally be formed by enzyme synthesis in the organelle protein fractions used in the earlier experiments of this investigation. This implies that these intermediate compounds would be produced and maintained in a proteinaceous environment which, in the light of results obtained when BSA was included in reaction mixtures, seems to prevent the non-enzymatic formation of IAA or PAA. Furthermore, it could seem unlikely that the normal in vivo synthesis of important hormones, like IAA or PAA, would rely, to a large extent, on the instability of one of the intermediates.

Thus, enzyme activity is most likely the factor responsible for the synthesis of IAA and PAA in intact tissues and also, to a large extent, in the chloroplast and mitochondrial in vitro systems used in the present study when the precursor aromatic amino acid was provided as the primary substrate.

Measurement of the possible conversion of the authentic arylpyruvic acids to the arylacetic acids during the purification procedure was not done as such. Previous workers have shown that IPyA may undergo spontaneous oxidative decarboxylation during solvent extraction and chromatographic purification, mostly when these steps were done under alkaline conditions (Bentley et al. 1956; Atsumi et al. 1976). In the method used here to extract and purify the enzyme-synthesized IAA and PAA, acidic solvent conditions, which are advantageous to the maintenance of the more stable enol form of IPyA (Cohen, 1968), were maintained both for the ether extraction and the HPLC separation.

Hemberg and Tillberg (1980) reported less than 1% conversion of

authentic IPyA to IAA upon dissolving the compound in methanol and analysing immediately the IAA content with the indolo- α -pyrone method. The percentage in vitro conversion raise to 2-3% when the methanol solution was kept for 24 hours at 4°C, solvent extracted through several steps and then the IAA content measured again with the indolo- α -pyrone method. Initial alcohol extraction of the reaction products was not required in the procedure used here and the ether fractionation steps were finished within 10-15 minutes. The acidic ether fractions were evaporated to dryness in a very short time since the volumes involved were never more than 10 mls. The residues were then stored overnight in the dark at -20°C and resolubilization for HPLC purification was only done just before chromatography. During this chromatographic step, the arylacetic acids were separated within 15 minutes from the arylpyruvic acids, which eluted later.

Thus, in view of the results of Hemberg and Tillberg (1980) and of the acidic conditions and short length of time of the purification procedure used here, breakdown of the arylpyruvic acids during the isolation of the auxin product was probably low.

3. Organelles

The enzymes from both sunflower chloroplasts and mitochondria showed auxin-synthesizing capacities under the experimental conditions used. The two organelles, however, cannot be compared in terms of amounts of auxin produced since no common factor, to which the level of synthesis could be related, was available. The criteria used for quantitative assessment of the synthesis is "the nmoles amount of IAA or PAA produced per mg protein", which included every soluble protein present in the fraction since the organelle enzyme fractions were used without any purification step. The soluble protein fraction from chloroplasts was probably made up of a large quantity of the

enzyme ribulose biphosphate carboxylase, since this protein is known to account for up to 50% of the soluble protein fraction in the leaf of a C_3 plant (Halliwell, 1978). Thus, even though chloroplast fractions seemed to synthesize less auxin than mitochondrial fractions, on a per mg protein basis, the activities could be similar on a per organelle basis.

The quantitative data for each organelle (Tables 9 and 11) does, however, indicate that the IAA and PAA synthesizing capacity of the enzymes was similar, on a per mg protein basis, under the conditions of the experiments. There was a slight difference between the average values, but this was shown not to be important when considering the standard error.

Some considerations must be taken into account when interpreting the quantitative data. Firstly, as mentioned earlier, no attempt was made to study any possible concomitant degradation or transformation of the auxin produced in each complete reaction system. Moore (1969) observed in his in vitro experiments with cell-free systems from pea shoots that enzyme oxidation of IAA was also taking place along with synthesis of the compound. Auxins are not metabolic end-products and in vivo feeding experiments have shown that both IAA and PAA can be subjected to a variety of catabolic reactions (see review by Schneider and Wightman, 1978). It is possible that some of these reactions could have taken place during the incubation period of the present experiments, along with the synthetic reactions themselves.

Another important point to remember when considering the present results is that it is not known to what extent the auxin-synthesizing capacity of each organelle protein fraction reflects the actual in vivo capacity of the intact organelles. Except for the aromatic aminotransferase, none of the other enzymes of the pathway have yet

been purified; the activity of these enzymes has mainly been determined in either soluble enzyme fractions from plant homogenates or in partially purified enzyme preparations. Thus it is difficult to ascertain whether the conversion of intermediates observed with the organelle protein fractions is due to the activity of certain enzymes working specifically on the different aromatic intermediates in the pathway, or the result of the activity of certain enzymes showing a broad spectrum of substrate specificity.

In this connection, the aromatic aminotransferase, the first enzyme of the pathway, has been shown to be a multispecific enzyme which can effect the transamination of aspartic and glutamic acids in addition to the three aromatic amino acids (Forest and Wightman, 1972). These workers found that the purified enzyme could catalyse the transamination of aspartic and glutamic acids at a much higher rate than that obtained with tryptophan, phenylalanine and tyrosine. They also found that the aromatic aminotransferase activity of the enzyme was almost completely inhibited when L-aspartate was included in the reaction medium at a 1:1 mole ratio with an aromatic amino acid. Since glutamate was found as the most abundant substance in the free amino acid pool of each type of organelle used in this study and that at least 5 times more aspartate than phenylalanine was detected in the amino acid pool in chloroplasts, it seems likely that such high mole ratios would influence the rate of auxin synthesis in organelles in the in vivo situation. However, just as the plant subcellular structure is complex, there most likely also exists different microenvironments within each organelle, but at present there is no way of knowing what range of concentration of substrates, or of intermediary metabolites, is likely to be found in the microenvironment of the auxin-synthesizing enzymes in vivo.

Wardrop and Polya (1980), studying a soluble auxin-binding protein from bean and pea leaves showed that the binding activity could be accounted for by the enzyme RuBPCase. This protein was found to have a high capacity and a high affinity for both IAA and PAA and other naturally-occurring auxins. The binding was rapid, reversible, non-degradative and occurred optimally at pH 8.0, which also happens to be the optimum pH for aromatic aminotransferase activity. As mentioned before, RuBPCase is a major chloroplast protein and Rubery (1981) stated that "an auxin-binding protein located within chloroplasts at a concentration greatly in excess of its K_D for IAA (or PAA) would be an important component in the synthesis-destruction-transport-sequestration network determining free auxin levels, particularly if its auxin-binding capacity could be modulated". Furthermore, Heber and Heldt (1981) suggested that the IAA molecule (and probably PAA as well) was most probably subjected to a coupling with the proton gradient across the chloroplast envelope, similar to the abscisic acid gradient already observed by Heilmann *et al.* (1980). The latter authors found that ABA is distributed according to the pH gradient between the cytoplasm and the chloroplast stroma. The ABA molecule is normally trapped as an anion in the alkaline stroma whereas, upon acidification of the medium, the protonated ABA is able to penetrate the chloroplast membranes and be released into the cytoplasm. These findings add strength to the present observations indicating the occurrence of auxin synthesis in sunflower chloroplasts, for such a location of auxin synthesis would facilitate the coordination of some of the factors that determine free auxin levels in the cytoplasm of leaf cells.

In conclusion, although some reservations should rightly be

expressed about the reliability of the present data with respect to the actual auxin synthesis in intact chloroplasts and mitochondria, nevertheless, on the basis of the experimental methods used in this study, it is clear that the enzymatic potential for the synthesis of IAA and PAA does reside in the soluble protein matrix of both chloroplasts and mitochondria of sunflower leaves.

CONCLUSIONS

1. The endogenous occurrence of the auxins, IAA and PAA, and of their respective amino acid precursors, L-tryptophan and L-phenylalanine, was investigated in sunflower chloroplasts and mitochondria. Phenylalanine was present in both organelles whereas tryptophan was detected only in mitochondria. The presence of tryptophan in the free amino acid pool of chloroplasts can, however, be inferred from evidence in the literature.
2. The soluble enzyme fraction isolated from sunflower chloroplasts and mitochondria was able to synthesize IAA or PAA when supplied with L-tryptophan or L-phenylalanine, respectively. The auxin products from the chloroplast enzyme system were unequivocally identified by GC-MS.
3. Radioactive metabolism experiments provided qualitative evidence for auxin synthesis occurring via the arylpyruvate pathway, since radioactivity from the ^{14}C -labelled aromatic amino acid was found in the arylpyruvate and arylacetaldehyde intermediates of this pathway. The corresponding arylethanol intermediate was also shown to be radioactive in the chloroplast enzyme assays.
4. Because of the known though possibly different instabilities of the arylpyruvate intermediates, experiments were carried out to confirm the role of the enzyme fractions in catalysing the further metabolism of indole- and phenyl-pyruvate to the corresponding auxin product. Enzyme activity was found to be mainly responsible for the formation of IAA and PAA when the corresponding authentic arylpyruvate was provided as substrate, but some non-enzymic formation of each auxin was also observed. The presence of bovine serum albumin and/or a large amount of soluble organelle protein in a reaction system, seemed, however,

to have a considerable stabilizing effect in these intermediate compounds. This suggests that in the intact organelle and even in the present cell-free systems, surrounding the enzymatically-formed arylpyruvate with a proteinaceous environment is most likely stabilizing this intermediate. In such situations, then, the amount of IAA or PAA formed by non-enzymic reactions would be greatly reduced.

5. The modern analytical procedures used for the chromatographic separation of IAA and PAA from other reaction products provided for an accurate determination of each auxin substance, since their presence in the appropriate fractions was confirmed by GC-MS. To ensure reliable estimates of the amount of auxin present, the recovery of internal standards was measured concomitantly, since losses can occur during the purification procedures and recoveries can be erratic.

Limitations

The experimental approach used in this study to investigate auxin biosynthesis at the subcellular level does not, unfortunately, resolve the issue completely, namely, of the in vivo occurrence of auxin synthesis in leaf chloroplasts and mitochondria. Some experimental factors put limitations on the interpretation of results. The fact that a disrupted organelle enzyme system was used in seeking evidence for the reactions meant that any internal organization or microenvironment for this enzyme system was lost and thus some of the modification controls known to regulate the activity of enzymes (Miflin, 1977) were also lost. Furthermore, partial purification of the soluble protein fraction was not attempted, which meant that many unspecific enzymes, including possible auxin degradative enzymes, could have been present in the assay systems.

The results, however, provide a useful first step in the study of intracellular auxin metabolism, since they indicate that the enzymic potential for auxin biosynthesis is present in leaf chloroplasts and mitochondria. As pointed out by Leech (1977):

"Demonstration of the mere presence of an enzyme in association with a particular organelle is, of course, insufficient evidence to establish its contribution to the physiology of the tissue from which it is derived. A quantitative assessment relating one aspect of the physiology of a tissue to the biochemical attributes of its organelles involves a major research effort over many years."

Suggestions for future work.

Since intact chloroplasts have been found to be permeable to both L-tryptophan and L-phenylalanine (Bickel and Schultz, 1979), feeding these auxin precursors directly to highly purified, intact organelle fractions should next be attempted. Similar experiments have already been carried out by Hartung *et al.* (1981) for investigating ABA synthesis in plant cells. These authors fed ¹⁴C-labelled mevalonic acid, the precursor of ABA, to various spinach preparations, among which were intact protoplasts and intact chloroplasts in order to elucidate the problem of the compartmentation of ABA synthesis. There is thus good reason to feel that this method of approach might also prove successful in future studies of the compartmentation of auxin synthesis in leaf cells.

As for the purely enzymatic aspect of such future investigations, since only the multispecific aspartate aminotransferase has been isolated and purified from various plant organelles, further separation and characterization of other enzymes in the system should be attempted. However, the purification of these enzymes from different organelles might have to wait until each enzyme has

been purified and characterized from cell-free homogenates.

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Appendix I

Although comparison of the levels of auxins in chloroplasts cannot be made with other chloroplast data in the literature, these concentrations can however be examined in relation to the levels found in individual leaves. A quantitative estimation of the IAA and PAA present in developing tobacco leaves was reported by Wightman (1977). In this study, the leaf which was found to be elongating most actively was the leaf in position 3 down the stem from the apex. This young, expanding tobacco leaf was at a growth stage comparable to the sunflower leaves used to isolate the intact chloroplasts in the present experiments. The various growth parameters of tobacco leaf No. 3 reported by Wightman (1977) are as follows:

Fresh Weight (F.Wt.): 0.75 gram

Chlorophyll content: 1.68 mg (F. Wightman pers. comm.)

Auxin content: IAA = 305 ng/g. F. Wt.

PAA = 1405 ng/g. F. Wt.

These levels of IAA and PAA can be expressed in relation to the chlorophyll content of the leaf which will then allow for a comparison with the sunflower chloroplast auxin levels. The following table provides a summary of the data from both plants for comparative purposes:

	Sunflower Chloroplasts	Tobacco Leaf No. 3
	(pmoles/mg chlorophyll)	
IAA	554 ± 113	1383
PAA	431 ± 120	8199

± = standard deviation

The levels of IAA in sunflower chloroplasts represent about 40% of the IAA found in the tobacco leaf, whereas for PAA, the distribution percentage is only 5%. This difference in distribution for the two auxins might indicate difference either in their relative rates of synthesis, or in the sequestration capacity of the chloroplasts for each auxin. However, either way, chloroplasts are clearly involved in determining part of the free auxin levels in the leaf.

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