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COMPARTMENTATION OF INDOLE-3-ACETIC
ACID IN PISUM SATIVUM L.

By

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Thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy

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This thesis is dedicated to my wife
and daughter, in gratitude for their
patience and understanding through-
out my studies.

SUMMARY

Studies were initiated on the compartmentation of indole-3-acetic acid (IAA) in Pisum sativum cv. Meteor. Particular attention has been given to the biosynthesis and catabolism of IAA in isolated pea chloroplast preparations. By the use of high performance liquid chromatography (HPLC), gas chromatography (GC) and combined gas chromatography-mass spectrometry (GC-MS), data were obtained which provided convincing evidence for the presence of IAA and indole-3-ethanol (I_{EtOH}) in light-grown pea seedlings. HPLC, GC and GC-MS analyses also confirmed IAA as endogenous constituent in pea chloroplast fractions while HPLC and GC provided strong evidence for the presence of I_{EtOH} in chloroplast preparations. IAA breakdown during extraction and purification procedures was also investigated.

The catabolism of IAA by pea chloroplasts was investigated using both cell-free systems derived from chloroplast fractions and isolated chloroplast preparations. Experiments with both experimental systems have indicated that the major pathway of enzymic catabolism of IAA in pea chloroplasts is by decarboxylative oxidation. Incubations of isolated chloroplast fractions with [1-¹⁴C]IAA resulted in the loss of the carbon-1 as ¹⁴CO₂. A distinct light-effect on the decarboxylation of IAA was observed. Results of control experiments suggested that a negligible proportion of this catabolism was directly attributable to photo-oxidation. HPLC analyses of extracts from [2-¹⁴C]IAA-

fed incubations suggested that the major detectable catabolite of IAA decarboxylation by pea chloroplast fractions was indole-3-methanol (IMeOH). The identification of this reaction product was confirmed by GC-MS analysis of a purified extract derived from a crude enzyme preparation incubated with IAA.

Preliminary investigations into the possible biosynthesis of IAA by chloroplasts of Pisum sativum were also carried out. Isolated chloroplast suspensions appeared to possess the ability to synthesis [^3H]IAA when incubated with [^3H]tryptophan. No apparent difference was observed in the amounts of putative IAA formed by chloroplasts incubated in the light and darkness. While an enzymic conversion of 0.17% [^3H]tryptophan to putative [^3H]IAA by chloroplast fractions was observed, boiled chloroplast and buffer control incubations resulted in 0.05% non-enzymic synthesis of [^3H]IAA.

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ABBREVIATIONS

| | |
|---------------|---|
| ATP | Adenosine triphosphate |
| BSFTA | Bis-(trimethylsilyl)-trifluoroacetamide |
| chl | Chlorophyll |
| chlps | Chloroplasts |
| cpm | Counts per minute |
| DMCS | Dimethyl-dichlorosilane |
| dpm | Disintegrations per minute |
| EDTA | Ethylenediamine tetra-acetic acid |
| E.P. | Enzyme preparation |
| <u>et al.</u> | <u>et alia</u> |
| EtOAc | Ethyl acetate |
| EtOH | Ethanol |
| fsd | Full scale deflection |
| f.wt. | Fresh weight |
| GC-MS | Combined gas chromatography-mass spectrometry |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| HPLC | High performance liquid chromatography |
| IAA | Indole-3-acetic acid |
| IAA-Me | IAA methyl ester |
| I.D. | Internal diameter |
| IEtOH | Indole-3-ethanol |
| MeOH | Methanol |
| MES | 2[N-morpholino]ethanesulfonic acid |
| m/z | Mass to charge ratio |

| | |
|------|---|
| ODS | Octadecylsilane |
| PVP | Polyvinylpyrrolidone |
| RC | Radioisotope counting |
| Rt | Retention time |
| TLC | Thin-layer chromatography |
| TMS | Trimethylsilyl derivative |
| Tris | 2-amino-2-(hydroxymethyl)propane-1,3-diol |

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INTRODUCTION

1.1 BRIEF HISTORY OF IAA RESEARCH

The auxins consist of a group of physiologically-active plant growth substances, of which indole-3-acetic acid (IAA) is now regarded as the most significant (Bearder, 1980). Other major groups of phytohormones include gibberellins, cytokinins, abscisic acid and ethylene. Research into the occurrence and effects of auxins has been said to have originated with Charles Darwin, who concluded that the phototropic response of a grass coleoptile was due to "some influence being transmitted from the upper to the lower point, causing the latter to bend" (see Audus, 1953; Bearder, 1980). The intriguing story of the discovery of alleged auxins, A and B, by K \ddot{u} gl in 1933, and the eventual isolation of IAA from human urine in 1934, is well documented (Went and Thimann, 1937; Audus, 1953; Jacobs, 1979). The first isolation of free IAA from plants was reported by Haagen-Smit et al. (1946), who extracted the compound from immature Zea mays kernels, utilizing acid and base destruction tests and melting-point properties for final identification. Subsequent characterization of the endogenous hormone made use of paper chromatography for separation, followed by detection using bioassays or semi-specific chromogenic sprays (Bennet-Clark et al., 1952). Studies on auxins using paper chromatography in the 1950's, and thin-layer chromatography (TLC) in the early 1960's, resulted in numerous reports claiming the detection of IAA in various plant species (e.g. Iwahori, 1967;

Wheeler, 1968; Alden, 1971). However, due to the inherent poor resolution of these chromatographic techniques, together with the lack of specificity of the colour-forming reagents and the lability of IAA and related indoles, these findings are now regarded with some suspicion (see Schneider and Wightman, 1978; Yokota et al., 1980; Reeve and Crozier, 1980).

In the last 15 years, several more sophisticated physico-chemical methods have been employed for the detection, separation and identification of IAA from plants, e.g. gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC) and combined gas chromatography-mass spectrometry (GC-MS). Details of these procedures have been summarized by McDougall and Hillman (1978) and Yokota et al., (1980). In 1970, Jamieson and Hutzinger published mass spectra of IAA and 19 other naturally occurring indoles which they either synthesized or obtained from commercial sources. However, the following year, GC-MS was employed to identify IAA from the fruit of Citrus unshui (Igoshi et al., 1971). This work is generally accepted as being the first positive identification of endogenous IAA from plants. Since then, unequivocal identification of IAA by GC-MS has been attained from surprisingly few plant sources (see Bearder, 1980).

1.2 ROLE OF IAA IN HIGHER PLANTS

1.2.1. TROPIC RESPONSES

Almost every growth phenomenon that has been observed in plants has, at some time in the past, been attributed to IAA. As previously described, phytohormone research originated with Darwin's observations on the phototropism of coleoptiles and many reports have been presented correlating phototropic response with the presence of IAA (e.g. Went and Thimann, 1937; Gardner et al., 1974). Gravitropism is another phenomenon which has received much attention with regards to IAA, both in the early days of phytohormone research (see Went and Thimann, 1937) and more recently (Wilkins, 1984). According to the Cholodny-Went hypothesis, growth curvatures are due to the unequal distribution of auxin between the two sides of a curving organ. It was also postulated that, in the tropisms induced by light and gravity, this unequal distribution is brought about by a transverse polarisation of the cells, resulting in the lateral transport of auxin (see Went and Thimann, 1937). However, some questions have been raised as to the importance of IAA in tropic responses. Firn and Digby (1980) believe that the lateral movement of IAA across a coleoptile may be insufficient to elicit the observed curvature. More recently, Mertens and Weiler (1983), using immunoassay for quantification, failed to detect any significant lateral asymmetry of IAA, or any other growth regulator, in gravitropically reacting root tips of several

plant species.

1.2.2. CELL ELONGATION

IAA has been long known for its effects on cell elongation, leading to plant growth (Went and Thimann, 1937). This phenomenon is apparently due to an increase in the plasticity of cell walls. Early studies demonstrated that the cellulosic microfibrils in the cell wall were virtually at right angles to the long axis of the plant prior to cell enlargement. However, after expansion, the microfibrils became almost parallel to the long axis of the plant. This observation would therefore account for the fact that an increase in the turgor pressure of the protoplast resulted in an elongation of the cell. Many hypotheses have been put forward on the mechanisms involved in increasing cell wall elasticity but none has explained it satisfactorily (Zeroni and Hall, 1980). One of the most common explanations is that of auxin-induced proton efflux, and at least six mechanisms have been proposed for this process (Cleland, 1982). In the presence of exogenous IAA, an outward pumping of H^+ ions from the cytoplasm to the cell wall has been observed after a lag of 8-10min. This reduction in the pH resulted in a "loosening" of the cell wall, allowing extension. However, the process of cell extension induced by weak acids has been known since the early days of auxin research, so this acid growth effect is not exclusive to phytohormones. Auxin-induced cell extension has

also been explained by gene activation, causing altered RNA and protein synthesis. Key (1969) reported that IAA has been shown to stimulate the incorporation of radio-labelled nucleotides into RNA, and convincing evidence has been presented for the de novo synthesis of cellulase mRNA in auxin-treated pea epicotyls (see Stoddart and Venis, 1980). Theologis and Ray (1982) have also reported on changes in mRNA following application of IAA. A few specific mRNA sequences were observed to increase in amount or translation activity within 15-20min of applying IAA to pea hypocotyl segments. It has been suggested that auxin-induced cell elongation may be mediated by the combined effects of both proton efflux and nucleic acid synthesis (Stoddart and Venis, 1980). Although extensive research has been carried out on this process of cell elongation, no universally-established theory has been accepted. As increasing numbers of researchers concentrate on IAA-induced growth phenomena at the molecular level, the more complex and intricate the overall picture becomes.

1.2.3. OTHER RESPONSES

Other growth phenomena attributed to IAA include apical dominance (see Hillman, 1984), delaying of leaf senescence and inhibition of leaf abscission (see Sexton and Woolhouse, 1984). IAA and other auxins are also well known for their ability to promote rooting in certain plant species, and to

increase fruit setting. Indeed, both these properties of IAA analogues have been exploited commercially (Avery and Johnson, 1947). At the cellular level, IAA has been shown to stimulate cambial cell division, xylem and phloem differentiation and also regulate callus tissue morphogenesis.

1.2.4. IAA AND PHOTOSYNTHESIS

Another IAA-mediated phenomenon, which may be of relevance to this present study, is its effect on certain photosynthetic processes. When IAA was sprayed onto Phaseolus vulgaris leaves, it increased the rate of CO₂ fixation and the rate of transport of photosynthates from the leaf (Bidwell et al., 1967). The plant hormone also caused a substantial increase in the photorespiration of recent products of photosynthesis. More recently, it has been reported that exogenous IAA has a significant effect on the photosynthetic apparatus of Sinapis alba (Zerbe and Wild, 1980a). IAA treatment appeared to induce a significantly lower rate of CO₂ fixation and a depressed nitrite reductase activity. It also caused a decrease in dry weight of chlorophylls, carotenoids and cytochrome f. Interestingly, in similar experiments, Zerbe and Wild (1980b) observed the effect of kinetin on the photosynthetic apparatus. Kinetin treatment apparently induced higher rates of CO₂ fixation and also higher activity of nitrite reductase was found. The effect of exogenous IAA on chlorophyll P-700 of Raphanus seedlings has also

been described (Buschmann and Lichtenthaler, 1974). After treatment with IAA, the Photosystem I chlorophyll concentration increased markedly, as did the rates of Hill-activity. These results suggested an increase in the number of photosynthetic reaction centres.

Changes in photosynthetic reactions induced by IAA treatment have been observed in isolated chloroplasts from pea and oat leaves. The rate of CO_2 fixation by chloroplasts treated with 10^{-7} M IAA increased markedly, after 4min of exposure to light (Tamas et al., 1972, 1973). Correlation was also shown between the responses of photophosphorylation control to IAA-treatment. In addition, oat chloroplasts incubated with 10^{-7} M IAA showed an increase in the value of the ATP/e_2^- ratio (Tamas et al., 1974). Suggestions have been made of the existence of a key regulatory site in the photochemical apparatus which is responsive to IAA, and that IAA may have a direct role in the export of photosynthetic products towards the growing regions of the plant. However, since at the moment, there is no direct evidence of endogenous IAA stimulating these photosynthetic reactions, this suggested role is purely speculative.

1.2.5. SUMMARY

Most of the research purported to directly involve IAA in growth phenomena and intracellular functions has been carried

out by external application of the plant growth substance. It is therefore quite possible that in many cases, the response to IAA treatment was merely de-toxification of abnormally high doses. Regardless of the external concentration of the applied hormone, the internal concentration of that same substance would be different (Zeroni and Hall, 1980). For example, the internal concentration of IAA would be dependant on the initial endogenous IAA level, the rate of penetration of exogenous IAA through the cuticle into the tissues and cells, and the rate of metabolism of the applied substance. Metabolic rates would depend on the distribution of both endogenous and exogenous IAA, and the accumulation of IAA at an intracellular site which did not normally contain such levels could well elicit atypical physiological responses. Bearing this in mind, it is now evident that the mechanisms by which IAA affects plant growth and development are not as firmly established as was previously inferred.

1.3 OCCURRENCE OF IAA IN HIGHER PLANTS

1.3.1. WHOLE PLANTS

Analysis by GC-MS is now generally considered to be essential for the assured identification of IAA (Schneider and Wightman, 1978), and indeed for any plant hormone (Horgan, 1981). By this criterion, there are very few reports in the literature where IAA has been positively identified in higher

plants. Bandurski and Schultze (1974) investigated the concentrations of free IAA and IAA esters in etiolated seedlings of Zea mays and Avena sativa by GC-MS. Free IAA has also been identified in Pinus sylvestris seedlings (Sandberg et al., 1981), in 3 week-old seedlings of Douglas fir (Caruso et al., 1978) and in dark-grown Pisum sativum epicotyls (Allen et al., 1982).

1.3.2. TISSUES AND ORGANS

More often, the occurrence of IAA has been investigated in specific plant tissues or organs. Greenwood et al. (1972) employed the use of GC-MS to detect IAA in Zea mays coleoptile tips. The presence of IAA was also confirmed by GC-MS in the steles, cortices and tips of Zea primary roots (Bridges et al., 1973), sterile roots of Zea (Elliott and Greenwood, 1974), and the root cap and apex of the same plant (Rivier and Pilet, 1974). More recently, IAA has been positively identified in the cambial region of Picea sitchensis (Little et al., 1978); Douglas fir shoot tips (Caruso et al., 1978) and Phaseolus lateral buds (Hillman et al., 1977).

1.3.3. SUB-CELLULAR LOCALISATION OF IAA

It has been suggested that there is a great need in plant physiology to investigate the intercellular and intracellular occurrence of plant growth substances (Horgan, 1981). However, a

survey of the literature reveals very little reference to the presence of IAA in specific intracellular organelles. In fact, surprisingly few studies have been carried out on the sub-cellular compartmentation of plant hormones in general. Positive identifications (e.g. by mass spectrometry) of plant growth substances from clearly-defined cellular fractions are even fewer. Stoddart (1968) has reported on the presence of a significant gibberellin-like activity in crude chloroplast fractions from Brassica. Detection was carried out using bioassay procedures. Gibberellin-like substances have also been found in undefined chloroplast preparations from Pisum sativum seedlings (Railton and Reid, 1974a; Railton and Rechav, 1979). The procedure for the detection of the substances consisted of loading the crude extracts onto a silica gel partition column and the collection of fractions from the eluate, followed by bioassay.

Several reports have demonstrated the localisation of ABA in chloroplasts from spinach (Loveys, 1977; Heilmann et al. 1980). In both cases, GLC was carried after methylation of the semi-purified extracts. A more convincing identification of ABA from chloroplasts has been carried out by Railton et al. (1974). Combined GC-MS with selected ion monitoring (SIM) was performed on a purified extract derived from an undefined pea chloroplast fraction. Although from the data presented there can be little doubt as to the presence of ABA in the sample, lack of efficient

chloroplast isolation procedures prevents valid interpretation of the results. Browning and Saunders (1977) used the detergent, Triton X-100, to disperse membranes in chloroplast preparations obtained from Triticum aestivum first leaves. Both GA_q and GA₄ were convincingly identified by gas chromatography-single ion current monitoring (GC-SICM). Approximately equal amounts of gibberellin were found in the membrane and supernatant pellets when extracted with methanol, whereas chloroplast preparations extracted with Triton X-100 were found to contain excessive amounts of gib^berellins, which were detectable only in the membrane pellet fractions. However, this work has not been repeatable (Saunders, unpublished, cited by Sembdner et al., 1980).

More recent reports on intracellular localisation of plant hormones consist mainly of studies into gibberellin and ABA metabolism in chloroplasts (e.g. Hartung et al., 1981,1982) and in vacuoles (see Keith et al., 1982). However, a small number of reports have quite recently been presented on the compartmentation of IAA. Heilmann et al. (1981) have reported on the localisation of IAA in mesophyll cells of spinach. Apparently, 45% of the total amount of free IAA was found within the chloroplasts, although these plastids occupy only 7% of the tissue volume. It has been suggested that this is due to the dissociation properties of IAA, since the chloroplast envelope of spinach has been demonstrated to be highly permeable to IAA

(Gimmler et al., 1981). However, as is the case with the majority of these studies, inadequate analytical procedures were employed. Chloroplast and cytoplasm extracts were subjected to thin-layer chromatography (TLC) and the suspected endogenous IAA was detected by the 2-methylindolo- α -pyrone fluorescence assay, as described by Mousdale et al. (1978). This method has since been found by Sandberg and Dunberg (1982) and others to be imprecise and prone to inaccuracies when inadequate sample purification steps have been carried out (see Horgan, 1981). The occurrence of IAA has been demonstrated in purified intact chloroplast and mitochondrial fractions from sunflower leaves (Wightman and Fregeau, 1982). Combined GC-MS analyses were carried out and the identification of IAA-Me in the two samples was claimed. The endogenous level of IAA in both organelles was estimated, although the method employed for the quantification is not described. From the data presented, it has been inferred that sunflower leaf chloroplasts contain many times more IAA than leaf mitochondria. However, since different units of concentration were adopted for each measurement, no direct comparison of levels is pertinent.

1.3.4. SUMMARY

As previously stated, few reports are available on the positive identification of IAA in plant tissues. The fact that IAA was the first plant hormone ever to be investigated may have

contributed to the reason why many workers in the field still adopt inefficient and imprecise analytical techniques. As a consequence, auxin research has lagged behind that of the other groups of phytohormones (see Sandberg and Crozier, 1984). It is quite evident from the literature cited in the previous section, and from the comments of Horgan (1981), that convincing data on the sub-cellular localisation of IAA is sadly lacking. The use of HPLC together with recently-developed monitors which possess both high sensitivity and high selectivity could provide the means by which intracellular levels of IAA may be determined. For example, Crozier et al. (1980) have reported on the use of a spectrophotofluorimeter for the analysis of picogram quantities of IAA extracted from plant tissue. An alternative may be the use of immunological techniques. A solid-phase enzyme immunoassay has been developed by Weiler et al. (1981) which is capable of detecting as little as 3-4pg of IAA. Although problems of cross-reactivity may be encountered with crude extracts, development and refinement of this technique could provide a powerful tool for use in compartmentation studies.

It therefore seems apparent that the problems of detecting very low amounts of plant hormones in organelles etc. are not insurmountable. However, these problems must be solved before any studies on the intracellular localisation of plant hormones can be considered relevant to plant physiology as a whole.

1.4. IAA CATABOLISM

1.4.1. INTRODUCTION

There are many reports in the literature on the degradation of IAA by both in vitro and in vivo experimental systems, and numerous comprehensive reviews in recent years have concentrated on this aspect of IAA metabolism (see Schneider and Wightman, 1974, 1978; Moore, 1979; Sembdner et al., 1980; Bandurski, 1982, 1984; Bandurski and Nonhebel, 1984). Many compounds have been proposed as being products of IAA catabolism although in some cases identification of intermediates may not have been sufficiently rigorous (Sandberg, 1984; Bandurski and Nonhebel, 1984).

The destruction of IAA is said to occur by two basic oxidative processes, namely that of enzymatic reaction and by photo-oxidation. Since photo-oxidation is currently considered to have little physiological importance (Moore, 1979), few recent reports have been presented regarding this degradative process (however, see Koch et al., 1982). Investigations into the enzymic catabolism of IAA have produced reports postulating both decarboxylative and non-decarboxylative oxidation pathways.

1.4.2. DECARBOXYLATIVE DEGRADATION OF IAA

The decarboxylative oxidation of IAA results in the loss of carbon-1 of the side chain as CO₂. For example, Wilkins et al. (1972) reported on the evolution of substantial amounts of ¹⁴CO₂

after [1-¹⁴C]IAA was applied to Zea root segments. When Epstein and Lavee (1975) incubated old, non-growing apple callus tissue with [1-¹⁴C]IAA they observed a loss of 90% of the IAA by decarboxylation. It was suggested that decarboxylation took place at cut surfaces (see also Waldrum and Davies, 1981). This decarboxylation of IAA is catalysed by enzymes known as IAA oxidases, which are now considered to be widespread in higher plants (Schneider and Wightman, 1974). The consensus of opinion is that the enzymes are peroxidases acting as oxidases and are at least partially independent of exogenous H₂O₂, since they carry a peroxide-producing system with them (Moore, 1979). However, it has not yet been established whether IAA oxidase and peroxidase activity are present on the same molecule (Sembdner et al., 1980). Horseradish peroxidase has been widely used for in vitro studies into the catabolic fate of IAA (e.g. Gelinas, 1973; Haard, 1978) and isozyme analysis has been carried out on commercially available preparations of this enzyme (Gove and Hoyle, 1975). It was found by isoelectric polyacrylamide gel electrophoresis that the preparations contained 20 peroxidase isozymes, each of which also possessed IAA oxidase activity.

The cellular location of IAA oxidase/peroxidase activity has been discussed by Schneider and Wightman (1974), although the information available on its compartmentation seems somewhat vague. Peroxidase activity is allegedly partly soluble in the

cytoplasm but other reports have suggested that it may be associated with the cell wall, plasmalemma and tonoplast. More recently, it has been reported that IAA oxidase activity in pea epicotyl segments is most closely associated with the Golgi apparatus and also to a lesser extent with lysosomes and endoplasmic reticulum (Waldrum and Davies, 1981).

The major products of IAA decarboxylation are reputed to be 3-methyleneoxindole and indole-3-aldehyde. However, these findings are almost exclusively the result of in vitro studies using cell-free systems or crude homogenates and various IAA oxidase preparations or commercially-obtained horseradish peroxidase (e.g. Hinman and Lang, 1965; BeMiller and Colilla, 1972; Ricard and Job, 1974). Also, the products of the reactions were normally separated by TLC and detected by UV absorption spectroscopy, methods which are now considered inadequate for such identifications (Reeve and Crozier, 1980; Horgan, 1981, Sandberg, 1984). Whether 3-methyleneoxindole or indole-3-aldehyde is formed during IAA decarboxylation in these systems is reported to be dependant on the substrate concentration and on pH. Incubation at pH6.0 and a substrate concentration of less than 2×10^{-4} M resulted in the formation of 3-methylenoxindole from its precursor 3-hydroxymethyloxindole (Hinman and Lang, 1965), whereas at higher IAA concentrations and at pH4.0, indolealdehyde was produced. These products of IAA decarboxylation have also been observed after in vivo incubations (e.g.

Magnus et al., 1971; Epstein and Lavee, 1975) but, as with the majority of the in vitro studies, tentative identifications of products were made by TLC and imprecise detection methods. Magnus et al. (1971) also reported that indole-3-aldehyde formed by pea stem sections was reversibly reduced to indole-3-methanol. More recently, Magnus et al. (1982) have demonstrated the production of indole-3-methanol and indole-3-carboxylic acid as catabolic products when IAA was incubated with sections of plant material from Orobanche spp.

The co-factor requirements of the IAA oxidase/peroxidase system has also been widely studied using horseradish peroxidase (see Schneider and Wightman, 1974; Sembdner et al., 1980). Most IAA oxidases reported are said to require manganese as a co-factor, although Hoyle and Routley (1974) have commented that Mn^{2+} can inhibit, have no effect or promote IAA decarboxylation. Monophenols, such as 2,4-dichlorophenol (DCP) or p-coumaric acid are also reported as having stimulatory effects on IAA oxidase activity, whereas ortho-diphenols or polyphenols, such as catechol or caffeic acid inhibit the enzymic decarboxylation of IAA (see Sembdner et al., 1980). Hoyle and Routley (1974) have also commented that the most commonly used co-factor in IAA-oxidase studies, i.e. DCP, can give spurious results. Quite recently, over 60 mono- and diphenols were tested for their effect on IAA decarboxylation in the presence and absence of DCP (Lee et al., 1982). Although the data obtained was complex, an

attempt was made to elucidate a structure-activity relationship between phenolic compounds and horseradish peroxidase. The relationship between phenolic compounds, H_2O_2 and peroxidase has also been recently investigated. Grambow and Langenbech-Schwich (1983) demonstrated that the fate of IAA was dependent on the occurrence and concentration of H_2O_2 and phenols in the reaction medium. Presence of phenolic compounds resulted in the formation of indole-3-methanol as the major catabolite, whereas without phenols, or at low concentrations, 3-hydroxymethylenoxindole was the predominant product. Grambow and Langenbeck-Schwich also reported that indole-3-methanol preceded indole-3-aldehyde in the oxidation pathway of IAA, an observation which contrasts with former presumptions regarding the decarboxylative catabolism of IAA (see Schneider and Wightman, 1974). Unfortunately, as with earlier in vitro studies, IAA catabolites were separated using TLC and detected by UV absorption spectroscopy. Also, since reactions were catalysed by commercially-obtained horseradish peroxidase and not even crude plant homogenates, the relevance of this data with respect to in vivo catabolism of IAA is uncertain.

The prevailing weakness of IAA decarboxylation studies is the lack of satisfactory identifications of catabolites isolated from in vivo test systems (Sandberg and Crozier, 1984). UV and NMR spectroscopy techniques were used to identify indole-3-carboxylic acid as a catabolite from in vivo metabolism of exo-

ogenous IAA (Magnus et al., 1982), but other than this, there appears to be no unambiguous identifications of decarboxylative oxidation products. The relevance of decarboxylative degradation of IAA as a method of controlling IAA levels in higher plants has been questioned by a number of workers (e.g. Waldrum and Davies, 1982). This criticism is compounded by the fact that very few substances reported as being products of IAA decarboxylation have been satisfactorily identified in plants, either as endogenous constituents, or as catabolites of IAA (Sandberg et al., 1984). Recently, however, indole-3-methanol (Sundberg et al., 1984) has been positively identified in etiolated Pinus sylvestris seedlings, and indole-3-carboxylic acid (Sandberg et al., 1984) has been established as a natural constituent of Pinus needles. Figure 1 illustrates the schematic pathway of IAA decarboxylative catabolism, as summarized by Sembdner et al. (1980). It must be stressed, however, that since few of the intermediates have been identified on the basis of strict physicochemical evidence, this scheme is in part speculative.

Very little information is available on the intracellular localisation of IAA catabolism although quite recently Sandberg et al. (1983) have reported on the catabolism of [2-¹⁴C]IAA by protoplast, chloroplast and cytoplasmic preparations from barley. It was found that light enhanced the rate of catabolism by protoplasts and to a lesser extent, the chloro-

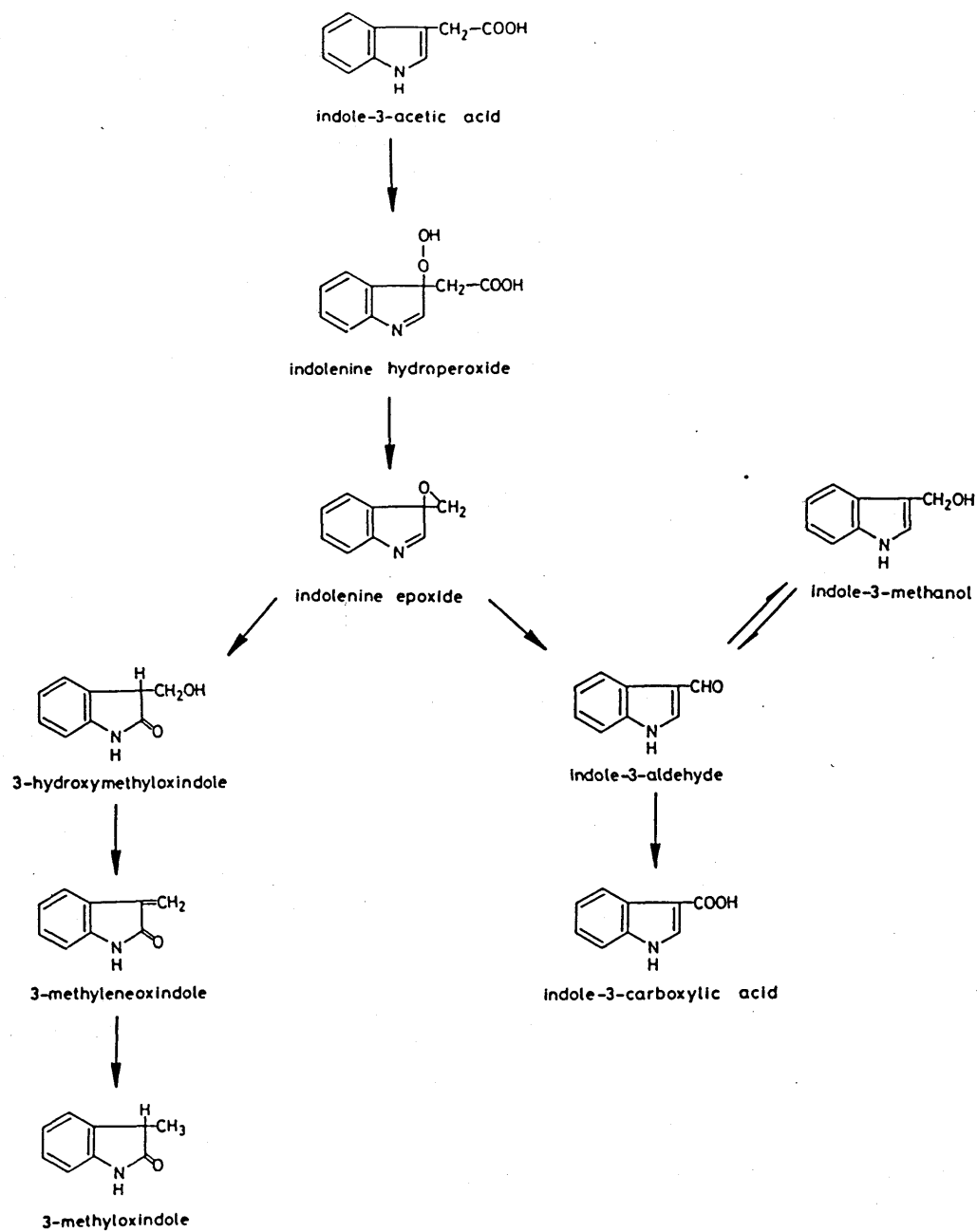


FIGURE 1

Schematic pathway for the decarboxylative catabolism of IAA.

plast-rich fraction although no light-enhancement was observed in the crude, cytoplasmic fraction. Unfortunately, no attempt was made to identify the products of this enzymic reaction.

1.4.3. NON-DECARBOXYLATIVE DEGRADATION OF IAA

In recent years, evidence has been produced for the non-decarboxylative degradation of IAA, that is, IAA catabolism with the retention of the carboxyl carbon. When Epstein et al. (1980) applied $[1-^{14}\text{C}]$ IAA to Zea mays endosperm, they found that although IAA was metabolised rapidly, very little $^{14}\text{CO}_2$ was produced. This indicated that IAA degradation was occurring by a mechanism not involving the loss of the carboxyl carbon (Bandurski, 1982, 1984). Reinecke and Bandurski (1981) have identified oxindole-3-acetic acid as the major product of IAA catabolism of Zea mays kernels and more recently this compound has been identified by mass spectroscopy as an endogenous constituent of Zea endosperm (Reinecke and Bandurski, 1983). Work carried out in another laboratory has resulted in a report indicating that oxindole-3-acetic acid may also be the major catabolite of IAA oxidation in Zea roots (Nonhebel et al., 1983). Reverse phase HPLC analysis of a methanolic extract from the incubation medium revealed a number of products, the most prominent of which co-chromatographed with authentic oxindole-3-acetic acid.

At first glance it appears that the scheme for non-

decarboxylative IAA degradation has been derived exclusively from studies with Zea mays. However, the methyl ester of oxindole-3-acetic acid and the esters of several structurally-related compounds, e.g. dioxindole-3-acetic acid, 5-hydroxyindole-3-acetic acid and 5-hydroxydioxindole-3-acetic acid, have been isolated from rice bran (Kinashi et al., 1976). Since analyses were carried out by UV, IR and mass spectrometry, there can be little doubt as to the presence of these methyl esters in rice bran. Kinashi et al. also proposed a schematic pathway of non-decarboxylative IAA degradation leading to β -acid, although much of this route is speculative. Dioxindole-3-acetic acid derivatives have also been reported in Vicia roots, both as natural constituents and as catabolites of exogenous IAA (Tsurumi and Wada, 1980). However, in this case, identification must be regarded as tentative since TLC, acid and base hydrolysis and UV spectrometry techniques were employed.

Very recently, it has been demonstrated that oxindole-3-acetic acid is metabolised by roots, shoots and caryopses of dark grown Zea mays seedlings to 7-hydroxyoxindole-3-acetic acid-glucoside (Nonhebel and Bandurski, 1984). This was found to be the major product when [5-³H]-2-oxindole-3-acetic acid was incubated with intact plants or root and coleoptile sections (Nonhebel et al., 1984) and the compound was also confirmed as a product of radiolabelled IAA degradation by root segments. Mass spectrometric identification has also verified 7-hydroxy-

oxindole-3-acetic acid-glucoside as an endogenous constituent of Zea mays and on the basis of these findings, the principal catabolic pathway for IAA in Zea seedlings has been proposed (Nonhebel and Bandurski, 1984). Figure 2 illustrates the non-decarboxylative catabolic pathway for IAA, as summarized by Sembdner et al. (1980), but including the propositions of Kinashi et al. (1976) and the recent findings of Nonhebel and Bandurski (1984). Again, it must be noted that major parts of this scheme are conjectural.

1.4.4. IAA CONJUGATION

IAA conjugation is the process by which free IAA is covalently attached to a substituent molecule via an ester or amide linkage (Cohen and Bandurski, 1982; Bandurski and Nonhebel, 1984). In the strict sense, conjugation does not constitute catabolism since breakdown of the IAA molecule to a simpler form does not occur. However, the formation of conjugates does represent a significant part of the metabolism of IAA in certain plant systems (Sembdner et al., 1980) and since conjugation includes reversibility (via hydrolysis), it is quite possible that it may be involved in regulating the level of free IAA. For example, Epstein et al. (1980) have concluded that seedlings of Zea mays utilize IAA conjugate esters for the IAA requirements of the germinating plant. The occurrence of IAA conjugates and their formation have been discussed in great detail in

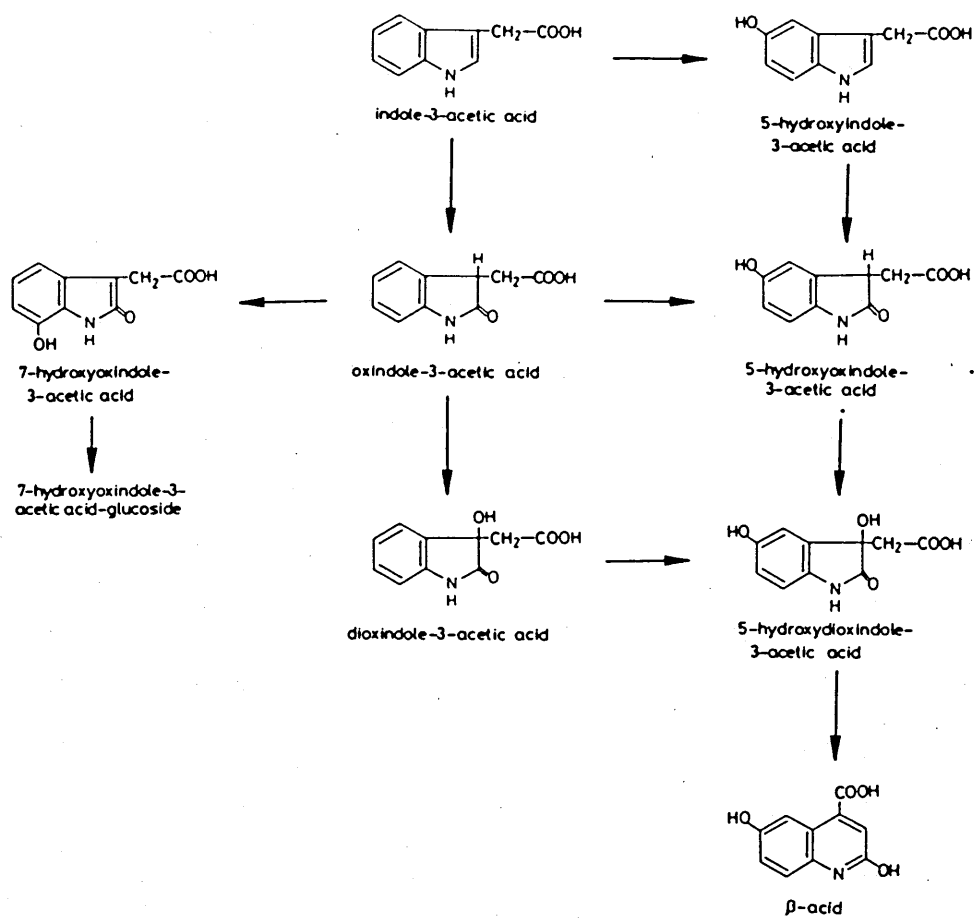


FIGURE 2

Schematic pathway for the non-decarboxylative catabolism of IAA.

recent years (e.g. Sembdner et al., 1980; Cohen and Bandurski, 1982; Bandurski, 1982, 1984; Cohen and Bialek, 1984). While most elegant studies have been described on both the production and hydrolysis of IAA conjugates in Zea mays kernels, little is yet known on the importance of this aspect of IAA metabolism in other experimental systems.

1.5. IAA BIOSYNTHESIS

1.5.1. PATHWAYS

The biosynthesis of IAA has been comprehensively discussed in several reviews in the last decade (see Schneider and Wightman, 1974, 1978; Sembdner et al., 1980) and very recently, an updated account has been given (Cohen and Bialek, 1984). Several reports have also been presented on more specific aspects of IAA biosynthesis in higher plants (see Cohen and Bandurski, 1982; Sandberg and Crozier, 1984).

The pathway of IAA biosynthesis should really be considered from the de novo synthesis of the aromatic indole ring although at present there is very little known about aromatic biosynthesis in plants (Bandurski, 1982). The protein amino acid, L-tryptophan is generally considered to be the precursor for IAA biosynthesis because of its close chemical similarity to IAA and its ubiquitous occurrence in higher plants (Schneider and Wightman, 1978; Sembdner et al., 1980), and it is from this intermediate that the majority of the numerous studies have been

initiated. There have been 4 major pathways proposed for the conversion of tryptophan to IAA and these have been named according to their key intermediates, indole-3-pyruvic acid, tryptamine, indole-3-acetoldoxime and indole-3-ethanol (tryptophol)(Schneider and Wightman, 1978). In some plant systems, one or other of these pathways may occur to the exclusion of the others, whereas in other systems, more than one biosynthesis route may be taken (Moore, 1979). Unfortunately, the vast majority of reports detailing IAA biosynthetic pathways has been produced by indirect evidence, for example, the biological activity of suspected intermediates, the presence of intermediates as endogenous constituents and in vivo conversions of exogenous precursors (Schneider and Wightman, 1978). This criticism is compounded by the fact that few of these presumed intermediates have been positively identified on the basis of strict physicochemical evidence (Sandberg, 1984). Consequently, the exact pathways of IAA biosynthesis which function in vivo have not been elucidated. The routes diagrammatically illustrated in Figure 3 represent current ideas on the biosynthesis of IAA from tryptophan in higher plants (see also Cohen and Bialek, 1984).

Indole-3-pyruvic acid is purported to be intermediary in the formation of indole-3-^{acyl}aldehyde from tryptophan. However, due to the high instability of this compound, it has never been satisfactorily isolated and identified from plants (Schneider and Wightman, 1978; Sembdner et al., 1980). In an attempt to

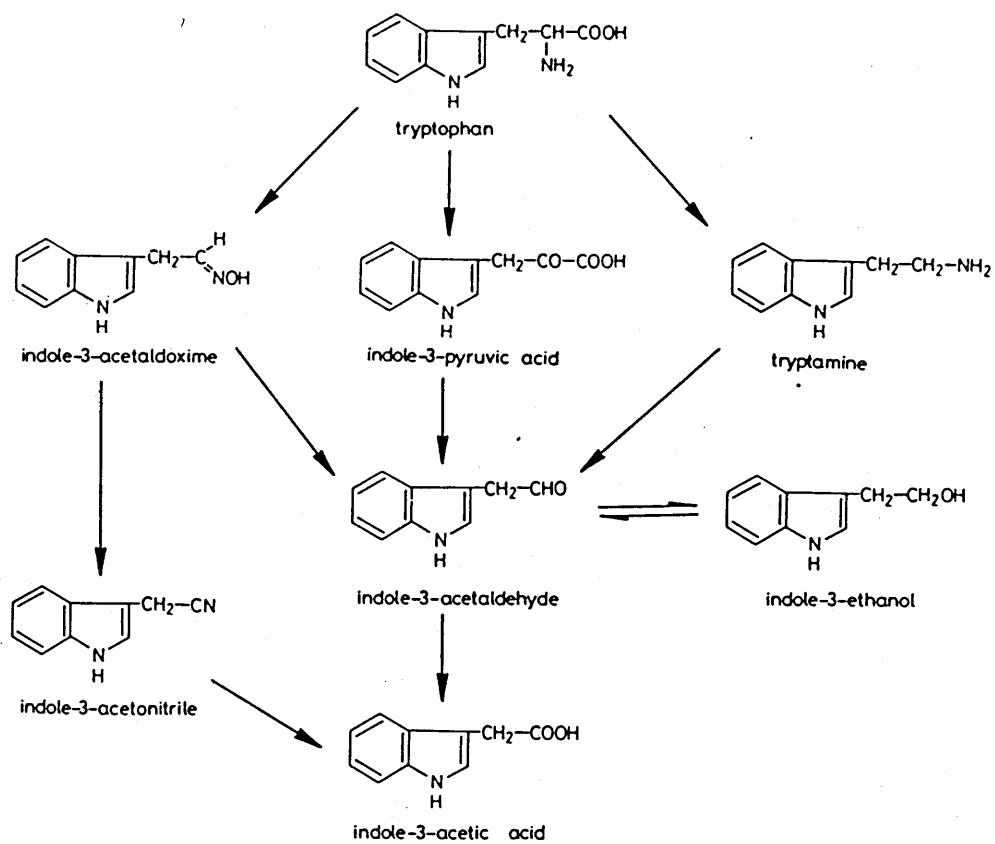


FIGURE 3

Schematic pathways for the biosynthesis of IAA.

overcome this problem Gibson et al. (1972) derivatized extracts from radiolabelled tryptophan feeds prior to separation and identification by TLC and colour-forming reagents. By feeding ¹⁴C-labelled tryptophan to excised barley and tomato shoots, they demonstrated conversions to indole-3-pyruvic acid, tryptamine and indole-3-acetaldehyde. The application of [2-¹⁴C]tryptamine also resulted in the formation of radiolabelled indole-3-acetaldehyde. The biosynthesis of IAA via tryptamine has also been demonstrated using cell-free systems from tobacco (Phelps and Sequiera, 1967). Identification of [¹⁴C]tryptamine was based on paper chromatography, colour reaction and radioactivity scanning techniques. Indole-3-acetaldehyde and indole-3-ethanol have been reported as naturally occurring compounds in etiolated shoots of Pisum sativum (Rajagopal, 1968). However, the qualitative and quantitative evidence presented was based on TLC and the Avena curvature test respectively. The enzymic reduction of indole-3-acetaldehyde to indole-3-ethanol has been reported by Brown and Purves (1980). Reaction rates of in vitro incubations were determined by recording the decrease in absorbance at 340nm and identification of the product was based solely on the absorbance of the reaction mixture in the presence of a chromogenic reagent. TLC and colour reaction methods have also been used to identify indole-3-acetaldehyde and indole-3-ethanol in etiolated shoots of pea and sunflower seedlings (Rajagopal, 1967). Positive identification of indole-3-ethanol in cucumber shoots

has been achieved using TLC and GC separation techniques, followed by UV, IR and mass spectrometric detections (Rayle and Purves, 1967a). It has been suggested that the reduction of indole-3-acetaldehyde to indole-3-ethanol is of great importance with respect to indole metabolism in higher plants and that indole-3-ethanol may be a storage product involved in the regulation of IAA biosynthesis (Sembdner et al., 1980). While Rajagopal (1967) found indole-3-ethanol inactive in the Avena bioassay, it has been found to promote growth when applied to cucumber hypocotyl segments and intact seedlings (Rayle and Purves, 1967b). However, this auxin-like activity has been attributed to the conversion of indole-3-ethanol into IAA (Rayle and Purves, 1967a, 1967b). Very recently, indole-3-ethanol has been confirmed by mass spectrometry as an endogenous constituent of Pinus sylvestris needles (Sandberg, 1984). Excised Pinus needles were also incubated with [3-¹⁴C]-tryptophan or [2-¹⁴C]tryptamine and in both cases, HPLC-RC analysis revealed a ¹⁴C-labelled peak with identical chromatographic properties to [2-¹⁴C]indole-3-ethanol. When [2-¹⁴C]-ethanol was used as substrate, a radiolabelled compound eluted from an HPLC column at the retention time of a [2-¹⁴C]IAA standard.

1.5.2. INTRACELLULAR STUDIES

Several recent reports have described investigations into

IAA biosynthesis at the intracellular level. Heilmann et al. (1982) used protoplasts isolated from leaf mesophyll cells of Spinacia oleracea. By applying [^{14}C]tryptophan as a precursor, they found that chloroplasts as well as extraplastidic compartments were able to convert tryptophan to IAA. It was also found that light inhibited IAA synthesis in all fractions. Since analyses of products were carried out by chromatographing crude, methanolic extracts onto TLC-plates, followed by detection using chromogenic reagents, then these results must be considered inconclusive. The serious limitations of TLC for analysing metabolic products are exemplified by Epstein et al. (1980) who found that simply chromatographing radiolabelled tryptophan onto TLC-plates and drying in vacuo resulted in a 30% conversion to IAA.

The IAA synthesising capacity of purified chloroplast and mitochondrial fractions from sunflower leaves has been investigated (Wightman and Fregeau, 1982). Soluble enzyme preparations derived from both fractions were incubated with both unlabelled and ^{14}C -labelled tryptophan, together with certain co-factors. The mitochondrial reaction system appeared to synthesise more IAA than the chloroplast reaction system. Confirmation of IAA in the chloroplast enzyme incubate was achieved by GC-MS analysis although no such analysis was carried out on the extract derived from the mitochondrial enzyme incubation. Since no dialyses of enzyme preparations were carried out, and only data from

unlabelled tryptophan feeds were presented, then these findings must be regarded with some caution.

Sandberg et al. (1982) have reported on the biosynthesis of IAA in protoplasts, chloroplasts and a cytoplasmic fraction from barley leaves. A total of 6 HPLC-RC systems were utilized to show that protoplasts enzymatically converted [5-³H]-tryptophan to [³H]IAA, and chloroplasts and a cytoplasmic fraction, isolated from protoplasts which had previously been fed [5-³H]tryptophan, contained measurable amounts of [³H]IAA. Radiolabelled IAA extracted from chloroplasts and cytoplasmic preparations which had been fed [5-³H]tryptophan after isolation from protoplasts, was found to be appreciably less than that detected in in vivo feeds.

1.5.3. SUMMARY

In comparison with catabolism studies, the pathways of IAA biosynthesis seem to be better established, since a greater proportion of in vivo investigations have been made. However, in common with the majority of data presented on IAA degradation, identifications of the intermediates from tryptophan to IAA have mainly been based on outmoded analytical techniques (see Reeve and Crozier, 1980). While the use of inadequate analytical procedures is a serious limitation when investigating metabolic processes, there are several other problems, specific to IAA biosynthesis, which are more difficult to resolve. For

example, tryptophan is considered to be universally distributed through out higher plants and is presumed present in all cells (Schneider and Wightman, 1978). The concentration of tryptophan in Zea kernals has been shown to be several orders of magnitude greater than that of IAA (Epstein et al., 1980) and since it is an amino acid, it is quite reasonable to presume that tryptophan is the precursor for many different plant substances. For these reasons it is very difficult to carry out in vivo feeding experiments and obtain sufficient product for positive identification. This problem is compounded by the fact that many of the proposed intermediates in the IAA biosynthetic pathway are very unstable and hence even detection may prove extremely difficult.

1.6. PLANT CELL FRACTIONATION

1.6.1. INTRODUCTION

Plant cell fractionation involves the isolation of subcellular particles from the rest of the cell components. Ideally, these particles should be recovered morphologically and physiologically intact, free from contamination and recovery should be quantitative (Price, 1983). However, in practice it is very rare for all these criteria to be met and a compromise is usually made. The isolation of intracellular organelles involves firstly the disruption of the tissues and cells, followed by separation of subcellular components. Difficulties may arise

when isolating larger membrane-bound organelles, such as nuclei or chloroplasts, since the method employed to disintegrate the plant cell walls is often sufficient to destroy the organelle. Various methods have been employed for the subsequent separation of intracellular organelles but centrifugation techniques are still considered the most common (see Hall and Moore, 1983).

1.6.2. CHLOROPLAST ISOLATION

A literature survey of the past 20 years reveals numerous methods which have been used for the isolation of chloroplasts. It has been said that there are almost as many types of chloroplast preparations as there are workers in the field (Leech, 1966) and indeed, most knowledge of the biochemistry of chloroplasts has been derived mainly from studies of isolated organelles. Procedures have differed according to the intended experimental purpose. For example, for many studies on the mechanism of photosynthesis, it is sufficient to prepare chloroplast-rich suspensions (Quail, 1979), whereas for the analysis of endogenous constituents, the purity of the chloroplast fraction should be clearly defined (see Reeves and Hall, 1980).

The initial disruption of the plant cells has been carried out using a pestle and mortar, with and without the addition of an abrasive (e.g. sand, carborundum powder) although it is now more common to employ the use of motor-driven homogenisers such as a Waring blender or polytron mixer. A Waring blender is

generally thought to be too destructive although it has the advantage over manual techniques in that results are more reproducible (Walker, 1966). Cellular debris is subsequently removed by straining the homogenate through cotton wool and muslin. It has been reported that the use of fibrous cloth in preference to material such as nylon, facilitates greater retention of chloroplast fragments and hence improves intactness (Leegood and Walker, 1983). Since chloroplasts are usually isolated in order to investigate photosynthetic reactions, it has been desirable to produce preparations containing intact chloroplasts. Walker (1964) and Jensen and Bassham (1966) employed the use of 0.33M sorbitol as an osmoticum during isolation and assay procedures, since it was found that intact chloroplasts produced by these methods resulted in enhanced rates of CO_2 fixation. The simplest method of obtaining chloroplast preparations is by differential centrifugation, where particles are resolved solely on the basis of their rates of sedimentation. This method has the disadvantage of possessing low resolution. For example, it can be used to separate intact chloroplasts from mitochondria but it cannot resolve mitochondria from glyoxisomes (Price, 1983). However, the advantages of employing this method are that of simplicity, speed and high sample capacity, each of which is beneficial when investigating the presence and metabolism of plant growth substances. Density gradient centrifugation has also been extensively used for the

separation of intracellular components (e.g. Tolbert, 1974; Wagner and Siegelman, 1975), although cross-contamination can occur since mitochondria are sandwiched between intact and ruptured chloroplasts (Quail, 1979). Leech (1966) has reported on the use of discontinuous sucrose density centrifugations to obtain chloroplast preparations substantially free from contamination. However, these gradients have been found to be unsatisfactory since sharply changing sucrose concentrations lead to increased breakage and lower recovery (Mifflin and Beevers, 1974). It has also been suggested that discontinuous gradients substantially reduce the resolving power of density gradient centrifugation while simultaneously creating the illusion of clear-cut separation (DeDüve, 1971). Gel filtration has been used in attempts to purify crude plastid pellets. The method was developed using Avena etioplast pellets which were passed through Sephadex G-50 (Wellburn and Wellburn, 1971) and has also been employed for the purification/^{of} intact chloroplasts from the alga, Codium fragile (Cobb, 1972). However, the procedure has since been found to be inadequate for the separation of intact plastids from mitochondria (Quail, 1979; Hilton and Smith, 1980), since about 15% of the original homogenate mitochondrial marker is found in the fraction before and after passage through the column (Quail, 1977). Indeed, the elution profiles of all the marker enzymes tested have been found to be identical, due to all particulate material above the steric exclusion limit of

the gel eluting in the void volume (Hilton and Smith, 1980). Another method for the purification of chloroplast preparations is that of partition in an aqueous polymer two-phase system (see Larsson, 1983). By this technique, membrane particles are separated according to differences in their surface properties in a phase system usually consisting of the polymers, Dextran T-500 and polyethylene glycol (PEG) (Kanai and Edwards, 1973). Although good separations can be achieved by this method, the temperature during mixing and settling of the two-phase system is very critical (Larsson, 1983) and this, together with the high cost of the polymers has probably prevented the method from becoming more popular. It has been reported that the most impressive chloroplast isolations have resulted from the use of protoplasts as starting material (Quail, 1979). Chloroplasts were first isolated from protoplasts by Wagner and Siegelman (1975) who separated vacuoles and chloroplasts by osmotic shock. Nishimura et al. (1976) succeeded in isolating chloroplasts from spinach leaves by passing enzymically-prepared protoplasts through a syringe, over which a piece of fine nylon mesh had been placed, followed by sucrose density gradient centrifugation. A clear separation of a single band of chloroplasts, practically free from stripped chloroplasts, was achieved. By the use of enzyme marker assays, it was found that the chloroplast fraction was virtually free from contamination.

1.6.3. ASSESSMENT OF PURITY

Before any valid conclusion can be made with regards to the intracellular function of any plant substance, the purity of the sub-cellular compartment in which it is being studied must be adequately determined. As mentioned in section 1.6.1., a compromise must usually be made between several factors when choosing an isolation procedure. Each successive purification step normally results in a considerable decrease in the amount of plant organelle material to be investigated. In the case of plant hormone analysis, rigorous purification procedures could result in insufficient sample after extraction for even tentative identification.

Two basic methods are employed for the assessment of organelle purity, namely morphological and biochemical marker techniques (Quail, 1979). The advantage of quantitative morphometry with regards to chloroplast isolations is that by producing electron micrographs a visual assessment of the structural integrity of the chloroplasts and also the level of contamination can be made. However, there are several major disadvantages with this technique for assessing organelle purity. It has been commented that the amount of material represented by 10-100 electron micrographs is of the order of 10^3 less than that used for a biochemical assay of a sub-cellular fraction (Siebert, unpublished, cited by Quail, 1979). Also, in addition to the time involved in preparing electron micrographs, much

care is needed in processing samples, to ensure that unbiased, statistically valid electron micrographs are obtained (Quail, 1979). Ideally, centrifuged pellets should be sectioned vertically so that the entire depth of the pellet can be analysed. The use of biochemical markers usually involves assaying the activity of enzymes which have been established as occurring only in a specific sub-cellular component. For example, if an aliquot from a preparation is found to catalyse a particular reaction which is known only to occur in one cellular compartment, then it is reasonable to conclude that the preparation is contaminated to some degree by that sub-cellular component. The practical use of biochemical markers has been extensively discussed by Quail (1979). Mitochondrial enzyme markers include cytochrome c oxidase (Smith, 1955), fumarase (Racker, 1950; Tolbert, 1974), succinic dehydrogenase (Slater and Bonner, 1952) and cytochrome c reductase (Douce et al., 1972). The most common marker used for the detection of microbodies is catalase (Beevers, 1979) although isocitrate lyase has been used as a specific glyoxisome marker (Dixon and Kornberg, 1959). As a general cytoplasmic marker, acid phosphatase has been employed, using p-nitrophenyl phosphate as substrate (Lindhart and Walter, 1963; Cobb, 1977). The most convenient marker used for chloroplasts is chlorophyll which is assayed spectrophotometrically. Although most enzyme marker assays afford high sensitivity, this method of detecting intracellular contamin-

ation is not without disadvantages. Failure to detect activity of any marker enzyme does not necessarily mean that the corresponding organelle or component is absent from the preparation, since it is quite possible that inhibitors of the enzymic reaction may be present in the fraction, thus preventing the reaction occurring. Also, the assumption that a particular enzyme is naturally found in only one specific sub-cellular compartment may not be fully warranted.

1.7. CHOICE OF PLANT TISSUE

The pea plant has a long history of use in experimental systems (Sutcliffe, 1977), and there are many obvious reasons why it has gained wide popularity for research in plant physiology. Briefly, peas have high viability and are readily available throughout the year. Since they do not possess a hard seed coat, germination is rapid and large food reserves stored in the cotyledons permit growth for several weeks at low light intensity, without mineral supplement. Until relatively recently, pea and spinach leaves were the only plant materials suitable for the isolation of structurally-intact chloroplasts (Leegood and Walker, 1983). This success in isolating functional organelles was mainly due to the fact that both plant species possess soft leaves which contain little thick-walled tissue and therefore allow relatively easy disruption of cells. Also, high metabolic activities have been achieved in vivo since both pea

and spinach leaves contain very low levels of phenolic inhibitors. Although spinach is considered an obvious choice for the isolation of chloroplasts by mechanical means (Leegood and Walker, 1983), peas have the advantage of healthy growth throughout the year and provide high yields of fresh leaf tissue. For example, in approximately 14 days, 200g dry wt. of pea seeds will produce ca. 200g f.wt. of suitable leaf and shoot material (Walker, 1966). For this reason, the availability of plant material does not inflict a severe limitation on intracellular plant hormone analysis.

1.8. OBJECTIVES

As previously stated (see section 1.3.3.), very little information is available regarding the occurrence and metabolism of IAA at the sub-cellular level. It is the opinion of some investigators (e.g. Horgan, 1981) that accurate studies on the intercellular and intracellular analysis of plant hormones are necessary if the precise role of these substances is to be fully understood. This present study deals with the compartmentation of IAA in peas, with particular emphasis on metabolism by isolated chloroplast preparations.

The primary objectives of this present study were:

- (1) To detect the presence of IAA in chloroplast preparations from Pisum sativum and to extract for positive identification. There is little convincing data available on the intracellular

localisation of IAA in any plant species. Detection of endogenous IAA in chloroplast preparations would provide the first steps in rectifying this situation.

(2) To investigate the catabolism of IAA by chloroplast fractions. It has been suggested that catabolism may represent an important aspect of the mechanism by which IAA promotes growth and development (Bandurski, 1982). Exogenous IAA has been shown to have some effect on certain photosynthetic processes (Tamas et al., 1973, 1974) and therefore, if active metabolism of IAA by chloroplasts can be demonstrated, then this may lead to further investigations to determine whether IAA has any regulatory role in photosynthesis.

(3) To determine whether pea chloroplasts possess the ability to synthesis IAA. Although an attempt has quite recently been made to establish the intracellular site of IAA biosynthesis in spinach (e.g. Heilmann et al., 1982), many more studies are obviously required before any sort of general picture can emerge.

MATERIALS AND METHODS

2.1. PLANT MATERIAL

Seeds of Pisum sativum L. cv Meteor (Sinclair McGill, Boston, Lincolnshire) were soaked in running tap water overnight and then sown in moist vermiculite or perlite. The seedlings were grown in a controlled growth room at a temperature of 20°C under a bank of "Atlas" warm white (80W) fluorescent lights giving an intensity of ca. 80W m^{-2} at plant level. The illumination was set for a 12h photoperiod and the seedlings were grown for 12-14 days.

2.2. CHLOROPLAST ISOLATION

The pea seedlings were harvested within 1h of the onset of the 12h light period in order to minimise the production of starch grains by the chloroplasts. This is advisable since during differential centrifugation, any starch grains which have formed in the chloroplasts would possibly sediment more rapidly than the chloroplasts themselves. Consequently, a substantial number of chloroplasts would have ruptured envelopes (Walker, 1965). The pea leaf tissue (ca. 100g) was cut finely with scissors and then homogenised in 200ml ice-cold grinding medium in a Waring blender at high speed for 3sec. It was found that, while a Polytron homogeniser increased the overall yield of intact chloroplasts, less leaf tissue could be accommodated at one time. In practice, this meant that there was no significant difference between the amounts of intact chloroplasts which

could be produced by both methods. The grinding medium consisted of 0.33M sorbitol; 50mM Na_2HPO_4 ; 50mM KH_2PO_4 ; 5mM MgCl_2 ; 0.1% NaCl and 0.2% sodium isoascorbate (Walker, 1967). This was adjusted to pH6.5 with HCl and stored at -20°C until needed. Prior to homogenisation, $200\mu\text{g ml}^{-1}$ of freshly-prepared penicillin-G and streptomycin sulphate were added to the grinding medium. The crude homogenate was filtered through 2+8 layers of muslin, (with non-absorbant cotton wool spliced between the 8 layers), by squeezing through the 2 uppermost layers but allowing the homogenate to drip through the remaining layers of muslin and cotton wool. The filtrate was then centrifuged in clean, scratch-free 100ml-centrifuge tubes at $4000 \times g$ for 50sec in a swing-out head. The supernatants were poured off and retained for enzyme marker assays. The pellets were then resuspended in 2-3ml wash medium using a soft paintbrush in order to cause as little mechanical damage to the chloroplasts as possible. The medium consisted of 0.33M sorbitol, adjusted to pH7.6 with Tris base (Nakatani and Barber, 1975). After a second centrifugation at $4000 \times g$ for 35sec the tubes were gently shaken to resuspend the top layer of the pellets only. This supernatant contained a larger proportion of broken chloroplasts and mitochondria and was carefully pipetted off. The pellets were subsequently resuspended in a known volume of assay medium using the soft paintbrush. The assay medium consisted of 0.33M sorbitol; 2mM EDTA; 1mM MgCl_2 ; 1mM $\text{MnCl}_2 \cdot \text{H}_2\text{O}$ and 50mM HEPES.

This was prepared double-strength, adjusted to pH7.6 with KOH and stored at -20°C until needed. It was diluted by half before use. The combined chloroplast suspension was covered with foil and stored on ice until needed. Using the procedure as described, 100g of pea leaf tissue yielded a chloroplast fraction corresponding to 12-15mg chlorophyll. Chlorophyll was determined by the method of Arnon (1949).

2.3. PURITY OF FRACTION

In order to assess the degree of contamination by other organelles in the chloroplast preparation, enzyme marker assays were routinely carried out. The chloroplast isolations were carried out as described earlier. After the first centrifugation the supernatants were combined and stored in darkness at 4°C. The pellets were subsequently resuspended in wash medium and re-centrifuged, after which the supernatants were again combined and retained. The pellets were then resuspended and the volume of each fraction was determined. Two aliquots from each fraction were removed and ultrasonicated in ice for 5min in order to disintegrate all intracellular constituents. The chloroplast content of all 3 fractions was determined by measuring chlorophyll in 80% acetone in a spectrophotometer at 652nm (Arnon, 1949).

(a) Cytochrome c oxidase.

To determine the mitochondrial presence in each fraction,

cytochrome c oxidase activity was assessed using the method of Smith (1955). This method determined the rate of oxidation of reduced cytochrome c by measuring spectrophotometrically the decrease in optical density (O.D.) at 550nm as the cytochrome c was oxidised by the enzyme in the fraction. The reaction was carried out in a 3ml cuvette and readings were taken every 10s for 3min. Saturated $K_3Fe(CN)_6$ solution was added to determine the O.D. of completely oxidised cytochrome c. The possibility of quenching by chlorophyll in each fraction was compensated for by the addition of an equal amount of the fraction to the blank cuvette.

(b) Catalase.

Contamination by microbodies (peroxisomes, glyoxisomes) was assessed by measuring catalase activity in each fraction. To an aliquot of fraction was added a known amount of hydrogen peroxide (H_2O_2) in buffer. The assay involved measuring the time taken for the O.D. at 240nm to decrease from 0.450 to 0.400. This corresponds to the decomposition of $3.45\mu moles H_2O_2$ in 3ml solution. From the values obtained, the rate of the reaction was determined.

(c) Acid phosphatase.

Acid phosphatase activity was determined in each fraction as a marker for general cytoplasmic contamination (Cobb, 1977). The assay was carried out using the method from Sigma Technical Bulletin No.104 (Sigma Chemical Co. Ltd., 1981), and involved

the hydrolysis of p-nitrophenol phosphate by phosphatase under acidic conditions. This reaction produces p-nitrophenyl and inorganic phosphate as products. After incubation at 37°C for 30min, alkali was added to the reaction mixture and the solution turned yellow. The intensity of colour was directly proportional to the enzyme concentration. The O.D. at 410nm was measured and the enzyme activity was calculated from a calibration curve.

All assays were carried out on the same day within 4h of the chloroplast isolation. Assays were carried out in triplicate on each of the 2 aliquots taken from each fraction. After calculating the total measurable activity in the preparations, the mean activity in each fraction was determined.

2.4. STRUCTURAL AND FUNCTIONAL INTEGRITY OF ISOLATED CHLOROPLASTS

The structural intactness of the chloroplasts was determined by an assay which utilizes ferricyanide as a Hill oxidant. Since the chloroplast envelope is impermeable to ferricyanide then its reduction can only be carried out by "broken" chloroplasts. The reaction was measured in an oxygen electrode in a mixture consisting of 1ml assay medium; 0.77ml H_2O ; 0.1ml chloroplast suspension; 3mM $K_3Fe(CN)_6$. In order to prevent CO_2 -dependent O_2 evolution, 10mM D,L-glyceraldehyde was added (see Lilley et al, 1975). After 3min of illumination 5mM NH_4Cl was added which uncouples the chloroplasts. From the difference in

oxygen evolution rates between the chloroplast preparation and an osmotically shocked suspension, the percentage intactness could be determined. The isolation procedure described earlier, yielded chloroplast preparations which were typically 78-84% intact, as determined by this method. The metabolic activity of the chloroplasts was assessed by measuring CO_2 -dependent oxygen evolution in an O_2 -electrode (see Walker, 1976). Using a reaction mixture containing assay medium, chloroplast suspension and 10mM NaHCO_3^- , rates of ca. $85\mu\text{moles O}_2\cdot\text{h}^{-1}\cdot\text{mg}^{-1}\text{ chl}$ were obtained.

2.5. CHLOROPLAST INCUBATIONS

The chloroplast suspension was normally diluted with assay medium to a concentration corresponding to $0.30\text{mg chl. ml}^{-1}$. Generally, for experiments investigating IAA catabolism by chloroplast suspensions, the incubation medium consisted of 1mM ATP ; 10mM HCO_3^- ; chloroplast suspension (final concentration: $0.15\text{mg chl ml}^{-1}$); $3 \times 10^6\text{ dpm } [2\text{-}^{14}\text{C}]\text{IAA}$ (final concentration: $7.0 \times 10^{-7}\text{M}$) in isotonic assay medium. After incubation at 20°C , the reactions were stopped by the addition of ice-cold methanol containing 20mM sodium diethyldithiocarbamate as an anti-oxidant. The suspensions were subsequently ultrasonicated using an Ultrasonics A180G probe ultrasonicator at full power for 5min at 0°C . The preparations were covered in foil and stored at -20°C for not more than 1h prior to extraction. When

investigating the possible biosynthesis of IAA by chloroplast fractions, the reaction mixture contained 5.0×10^6 dpm [^3H]tryptophan. At the end of the incubation period unlabelled IAA was added to each preparation as carrier, in addition to ice-cold methanol and anti-oxidant.

2.6. EXTRACTION PROCEDURES

(a) Solvent Extraction.

After ultrasonication, the methanolic extract was vacuum-filtered. The residue was then washed with several volumes of methanol until no more colour could be removed. The combined filtrates were reduced to aqueous phase in vacuo and 0.5M pH8.0 phosphate buffer was added. This was subsequently partitioned against half-volumes of toluene until no more colour could be removed from the aqueous phase. Occasionally, the extract was filtered through cellulose powder which removed emulsions. The toluene was discarded and the aqueous phase was slurried with polyvinylpyrrolidone (PVP) (Glenn et al., 1972) and after 10min was filtered and the filtrate adjusted to pH2.5 with 50% sulphuric acid. The extract was subsequently partitioned five times with 2/5 volumes of ethyl acetate. When extracting samples to determine the presence of endogenous IAA, the aqueous phase was adjusted to pH3.0 with sulphuric acid and partitioned against 2/5 volumes of freshly-distilled diethyl ether. The acidic, ethyl acetate-soluble or diethyl ether-

soluble fraction was then dried with anhydrous sodium sulphate and reduced to dryness in vacuo. After transferring to a 500 μ l vial with methanol and ethyl acetate (1:1), the sample was reduced to dryness with oxygen-free nitrogen and stored in darkness at -20°C. Prior to HPLC analysis, each sample was dissolved in a known volume of methanol and buffer.

(b) Buffer Extraction.

0.1M pH8.0 phosphate buffer containing 0.02M anti-oxidant was added to the chloroplast preparation, resulting in a final volume of 3-4 times that of the initial chloroplast suspensions. The preparation was then ultrasonicated in ice for 15min and subsequently filtered. The residue was washed with buffer, filtered and both filtrates were combined. Ammonium sulphate was then added to saturation level in order to precipitate proteins which might include degradative enzymes. After centrifugation at 10000 x g for 40min, the supernatants were decanted and re-adjusted to pH8.0. All filtration and centrifugation procedures were carried out at 4°C. The buffer extract was then eluted onto a 5 x 300mm XAD-7/^{ion-exchange} column (0.3g) which had been pre-eluted with 0.1M pH8.0 phosphate buffer. At pH8.0, acidic indoles pass through the column whereas neutral and basic indoles are adsorbed to the XAD-7 (Andersson and Andersson, 1982). The eluent was retained, adjusted to pH2.7 and run through a second XAD-7 column which had previously been

equilibrated to pH2.7. Acidic indoles such as IAA and indole carboxylic acid are adsorbed to the XAD-7 at pH2.7. After washing the first column with ca. 10ml buffer solution, it was eluted with 10ml 70% ethyl acetate in hexane which removed the indoles from the column. The aqueous phase of the eluent was pipetted off and the organic phase was reduced to dryness under a stream of nitrogen. To remove acidic indoles, the second XAD-7 column was eluted with 10ml 40% ethyl acetate in hexane. Both pH8.0 and pH2.7 fractions were stored in darkness at -20°C until HPLC analysis. The buffer extraction procedure is illustrated in Figure 4.

2.7. PROTOPLAST ISOLATION

Approximately 10g f.wt. pea leaves were harvested from 12-14 day-old pea seedlings. The leaf tissue was then cut manually into segments 0.5-1.0mm in width using a new, sterile razor blade. Intact protoplasts were then prepared by a modified method of Sandberg and Hållgren (1984) (see Results).

2.8. PREPARATION OF CELL-FREE SYSTEM

Phosphate buffer (0.1M, pH8.0) containing $200\mu\text{g ml}^{-1}$ each of penicillin-G and streptomycin sulphate was added to the chloroplast suspension, to a total volume of 25ml. The preparation was ultrasonicated for 5min at 0°C and subsequently centrifuged at $100000 \times g$ for 90min in a Mistral High Speed 25

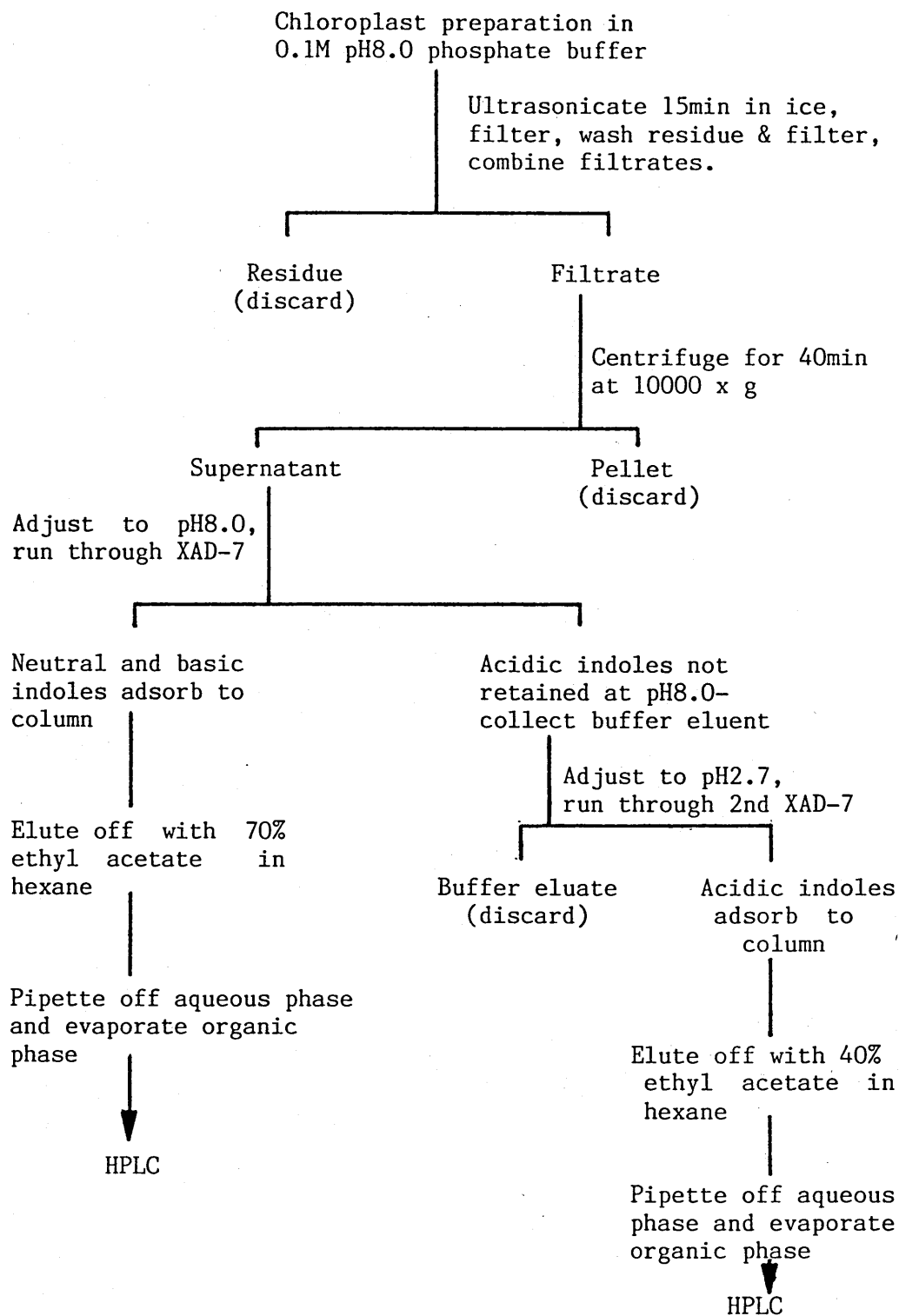


FIGURE 4

Flow diagram illustrating buffer extraction procedure.

centrifuge with swing-out head. After centrifugation, the pellets were resuspended, and the supernatant and pellet fractions were dialysed, separately, against 0.1M pH8.0 phosphate buffer for 36h at 4°C. The buffer was changed at least twice during the course of the dialysis. Subsequently, both fractions were freeze-dried and then stored in darkness at -20°C until needed. The supernatant fraction was subsequently used as the soluble enzyme preparation and the pellet fraction was referred to as the insoluble or membrane-bound enzyme preparation.

2.9. DETERMINATION OF ENZYME ACTIVITY

Each crude enzyme preparation was tested at regular intervals after storage, in order to determine its ability to decarboxylate [1-¹⁴C]IAA. Equal dry weights of the preparation were dissolved in 0.1M pH6.5 phosphate buffer, together with co-factors where appropriate, and [1-¹⁴C]IAA. The reaction mixtures were incubated in plastic scintillation vials, and shaken gently, in darkness for 2h at 20°C. The set-up is illustrated in Figure 5. On completion of the incubation, the inserts containing 0.25M KOH were removed and scintillant was added. After washing the outer surface of each vial to remove adhering solution, the relative amounts of ¹⁴CO₂ absorbed by the KOH was determined by liquid scintillation counting.

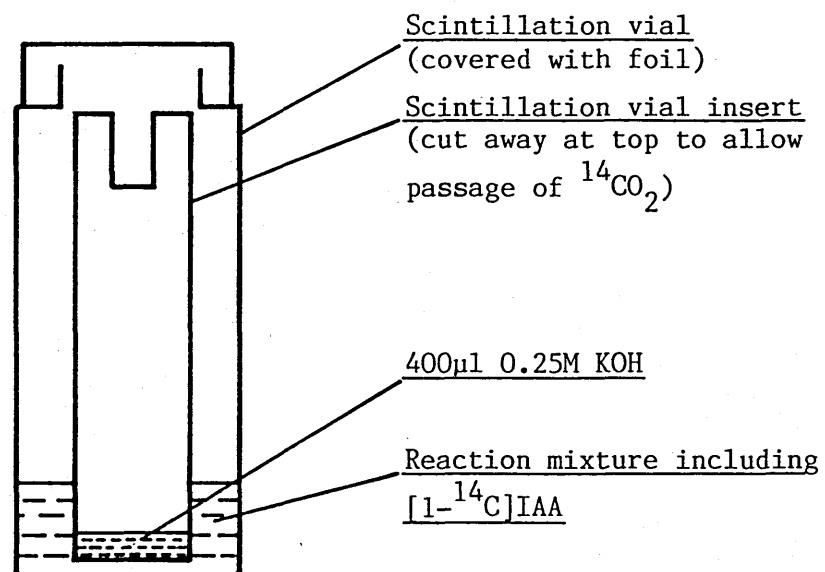


FIGURE 5

Scintillation vial with modified insert used for [1- ^{14}C]IAA
decarboxylation assay.

2.10. LIQUID SCINTILLATION COUNTING

Samples containing radio-labelled substances were routinely assayed by counting aliquots in either 6ml or 20ml plastic vials. The scintillation cocktail comprised of 5g 2,5-diphenyloxazole (PPO); 0.3g 1,4 bis-2(4-methyl-5-phenyloxazole)benzene (dimethyl POPOP); 200ml Triton X-100 and 800ml distilled toluene. Aliquots were counted in an LKB 1211 Minibeta liquid scintillation counter. Samples were corrected for colour quenching by counting known amounts of [^{14}C]IAA in scintillation cocktail and then repeatedly counting the same samples after the addition of increasing amounts of chlorophyll. A calibration curve of the sample channels ratio (SCR) against relative efficiency was plotted and this was used to determine the radioactivity in counts per minute (cpm) of all [^{14}C]-labelled samples. Scintillation counting of samples containing [^3H]-labelled compounds were corrected for colour quenching and counting efficiency. In this case, a standard curve was obtained using [^3H]hexadecane quenched with increasing amounts of chlorophyll in acetone.

2.11. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Solvents were delivered from a Spectra-Physics 8700 liquid chromatograph. Aliquots of extract were injected off-column via a Valco valve injector fitted with a 200 μl sample loop. Column eluates were monitored with a Perkin-Elmer LS-3 spectro-

photofluorimeter fitted with a 16 μ l flow-cell. The detector was set to an excitation at 280nm and an emission at 350nm for optimum sensitivity for IAA. Analyses of samples from radioactively-labelled feeds utilized a Reeve Analytical Radioactivity Monitor connected in series with the fluorescence detector. For the detection of ^{14}C , the monitor was used in heterogenous mode for normal- and reverse phase HPLC, giving *static* counting efficiencies of ca. 80% and ca. 20% respectively with a 200 μ l flow-cell packed with cerium-activated lithium glass scintillant. When analysing [^3H]-labelled compounds, the radioactivity monitor was used in homogenous mode for reverse phase HPLC, giving a counting efficiency of ca. 15% with a 200 μ l coiled glass flow-cell. Liquid scintillant was delivered at a constant rate from a Reeve Analytical Reagent Delivery pump. The scintillant was mixed with the column eluate (3:1 v/v) before entering the monitor. Normal phase chromatography was carried out using a 5 x 240mm 5 μ m Nucleosil CN column eluted at a flow rate of 1.0 ml min $^{-1}$. The mobile phase consisted of ethyl acetate in hexane (25:75 v/v) and 1% acetic acid was added to the solvents when acidic indoles were analysed. The liquid scintillant used for ^3H detection comprised of 10g 2,5-diphenyl-oxazole (PPO); 330ml triton X-100; 670ml xylene and 150ml methanol. Reverse phase chromatography employed the use of a 5 x 250mm ODS Hypersil column eluted with methanol in 20mM ammonium acetate buffer. Both isocratic and gradient elution were carried

out at a constant flow rate of 1ml min^{-1} . When analysing acidic indoles, the ammonium acetate was prepared to pH3.5. This was adjusted to pH6.5 when analysing samples containing indoles of neutral pH. Homogenous mode radioactivity detection utilized a scintillant comprised of 12g PPO, 150g naphthalene, 50ml Triton X-100 and 1litre distilled toluene. All solvents used for chromatography were HPLC-grade and the aqueous buffer was purified by elution through a $10 \times 100\text{mm}$ glass column containing $10\mu\text{m}$ ODS silica gel.

2.12. CHEMICALS

The specific activities of the radio-labelled substances used were as follows:-

| | | |
|--|------------------------------|----------------------------|
| $[2-^{14}\text{C}]$ indole-3-acetic acid | $2.04 \text{ GBq mmol}^{-1}$ | (55mCi mmol^{-1}) |
| $[1-^{14}\text{C}]$ indole-3-acetic acid | $1.78 \text{ GBq mmol}^{-1}$ | (48mCi mmol^{-1}) |
| L- $[5-^3\text{H}]$ tryptophan | $962 \text{ GBq mmol}^{-1}$ | (26Ci mmol^{-1}) |

Standards of $[^2\text{H}_5]$ IAA were kindly supplied by Dr. Göran Sandberg, Swedish University of Agricultural Sciences, Umeå, Sweden. Dilutions of radiochemicals were routinely analysed by HPLC for homogeneity. All bulk chemicals were of standard laboratory grade. Organic solvents were glass-distilled prior to use.

2.13. PREPARATION OF DERIVATIVES

(a) Silylation.

In order to conduct gas chromatography (GC) analyses, samples from endogenous extracts were silylated. 30µl of acetonitrile and bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (1:1 v/v) was added to each sample which was incubated for 10min at 70°C.

(b) Methylation.

Samples were methylated with a solution of diazomethane in ether using a modification of the method of Schlenk and Gellerman (1960). Each extract was dissolved in 100µl of methanol before 100µl of diazomethane was added. After incubation for 10min, the reaction was neutralised by the addition of a few drops of 1% acetic acid and the solvents were subsequently evaporated. The efficiency of the methylation was tested by the addition of [2-¹⁴C]-IAA to a duplicate sample. After methylation, the sample was analysed by HPLC-RC.

2.14. GAS CHROMATOGRAPHY

GC was carried out on several methylated and silylated extracts. The equipment comprised of a Hewlett- Packard 5880 gas chromatograph equipped with a flameless NP detector. The column was a 15m x 0.25mm I.D. fused silica column and the crosslinked stationary phase was DB-s, with a film thickness of 0.25µm. The

splitless injection technique was used, with the injector temperature at 250°C. The oven temperature was held at 170°C and the detector temperature was 200°C.

2.15. COMBINED GAS CHROMATOGRAPHY-MASS SPECTROMETRY

The analysis of endogenous extracts by combined GC-MS was performed by Dr. Einar Jensen, University of Tromsø, Norway. The instrument used consisted of a Hewlett-Packard 5710A gas chromatograph linked via a jet separator to a VG micromass 7070H mass spectrometer equipped with a DEC PDP8A computer system. Silylated and methylated aliquots from extracts were introduced via an injector at 240°C onto a glass column (2m x 2mm I.D.) packed with 100-200 mesh Supelcoport with a 1% SP2100 stationary phase. The column temperature was held at 120°C for 4min after injection and then programmed from 120-200°C at 16°C min⁻¹. The helium carrier gas flow rate was 30ml min⁻¹. The interface temperature was 240°C. Positive ion electron impact spectra were recorded at 70eV with a 1.0s decade⁻¹ scan rate and a 0.5s interscan delay.

The GC-MS analyses from [¹H]-, [²H]- and [2-¹⁴C]IAA feeds were carried out by Dr. Laurent Rivier, University of Lausanne, Switzerland. The instrument used comprised of a Hewlett Packard 5985A combined GC-MS under computer control. Samples were derivatized with BSFTA and 1% dimethyl-dichlorosilane (DMCS) in pyridine (1:1) at 60°C for 20min. Aliquots of sample were intro-

duced onto a fused-silica capillary column (25m x 0.31mm I.D.) coated with 0.3 μ m cross-linked SE-54 stationary phase, which was connected to the ion source without an interface. The column temperature was programmed to 100°C for 1min after injection then increased at a rate of 25°C min⁻¹ to 240°C. The helium carrier gas was at a pressure of 0.5kg cm⁻², giving a flow rate of approximately 1.5ml min⁻¹ at 200°C. Positive ion electron impact spectra were recorded at 70eV with an emission current of 300 μ A. The temperature of the ion source was 200°C and the resolution of the quadrupole filter was 2.5 times the mass when measured at half peak height. For repetitive scanning, the acquisition of data commenced at 4min for a duration of 5min. For IMeOH, scanning was carried out from m/z 50 to 350 at a scan rate of 1 scan s⁻¹.

3.1. PREPARATORY TECHNIQUES

3.1.1. PURITY OF CHLOROPLAST PREPARATIONS

Enzyme marker assays were carried out in order to determine the efficiency of differential centrifugation in separating intact chloroplasts from other organelles and intracellular debris. The assays were regularly carried out on the supernatant and pellet fractions obtained by centrifuging the pea leaf homogenate. The enzyme reactions were assayed spectrophotometrically in 3ml glass cuvettes, as described earlier (see section 2.3).

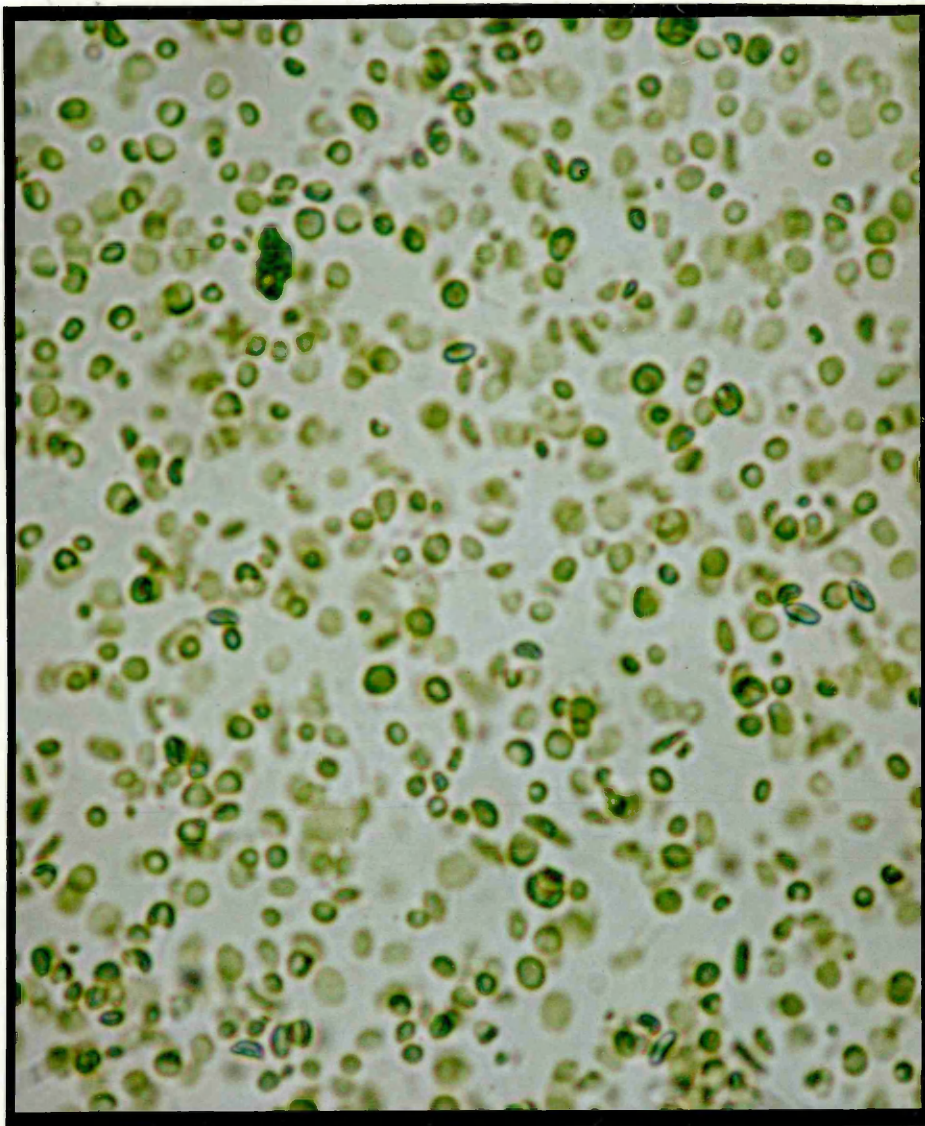
It was found that any increase in yield of isolated chloroplasts (as determined by chlorophyll concentration), resulted in a proportional increase in the activities measured of all 3 marker enzymes in the chloroplast pellet. This therefore suggests that the efficiency of the isolation procedure was consistent with regards to purity. Table 1 summarizes typical results obtained.

TABLE 1

Balance sheet of components measured in 1st supernatant, supernatant after washing crude pellet and final chloroplast pellet. Data is expressed as a percentage of total crude filtered homogenate.

| Fraction | % component in fraction | | | |
|-------------------------|-------------------------|----------|----------|------------------|
| | chlorophyll | cyt.c ox | catalase | acid phosphatase |
| 1st Sup. | 54.9 | 87.6 | 99.2 | 97.1 |
| 2nd Sup. | 20.4 | 8.1 | 0.6 | 1.7 |
| chl _p pellet | 24.7 | 4.3 | 0.2 | 1.2 |

These results show that, although detectable, the degree of contamination by microbodies and cytoplasm was low. Mitochondrial contamination was predominant and this was to be expected since mitochondria have a density which approximates that of broken chloroplasts (see Quail, 1979). Therefore, during differential centrifugation, the mitochondria would sediment with the broken chloroplasts. Figure 6 gives a pictorial representation of the chloroplast suspensions obtained by this method.



x 1000

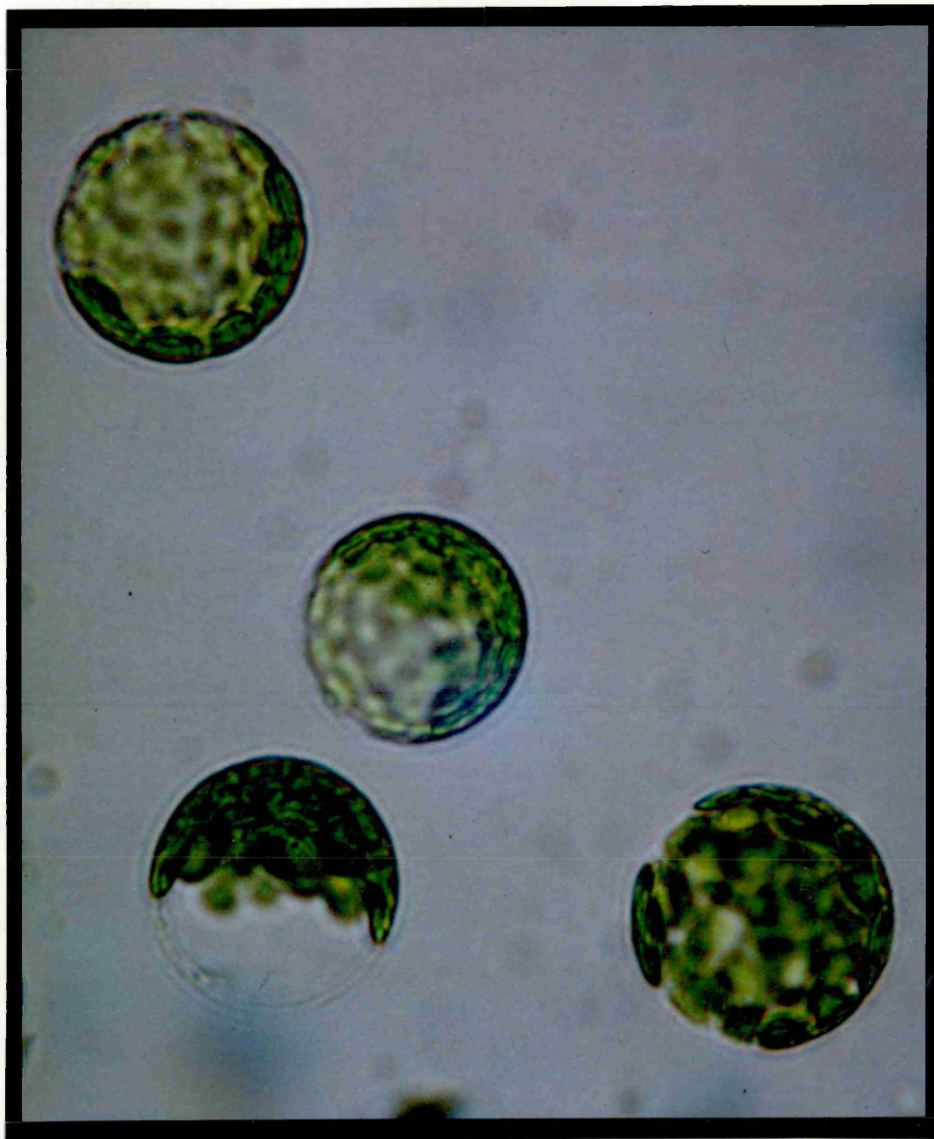
FIGURE 6

Photomicrograph of isolated chloroplast suspension.

3.1.2. PREPARATION OF PROTOPLASTS

It was decided to investigate the possibility of preparing isolated protoplasts from pea leaf tissue since it has been reported that chloroplasts approaching 100% purity and intactness can be obtained by using protoplasts as starting material (Quail, 1979). Approximately 5-10g f.wt. pea leaves were harvested and then cut into segments 0.5-1.0mm wide in a wash medium in ice. The wash medium consisted of 0.5M sorbitol; 5mM HEPES; 1mM CaCl₂; 0.05% bovine serum albumen (BSA); 5% polyethylene glycol (PEG) at pH 7.0. The segments were soaked for 1h at 4°C in the medium and then filtered and washed twice. The tissue was then incubated in digestion medium in a 50ml conical flask. The digestion medium consisted of 1.5% pectinase ("Macerzyme"); 0.3% cellulase ("Cellulysin"); 0.5% sorbitol; 1mM CaCl₂ 0.1% BSA; 5mM Mes at pH 5.0. The incubation was carried out in the light at room temperature on a gentle shaker and the preparation was kept under a slight vacuum for the 1st 1h of the 3h incubation period. At the end of the incubation the tissue was filtered off and gently washed with 2 x 50ml volumes of the wash medium. Both the initial filtrate and the wash filtrates were centrifuged at 500 x g for 5min at 4°C and the pellets were resuspended in solution of 0.2M sucrose and 0.3M sorbitol. After a second centrifugation at 500 x g for 5min, a portion of the pellet was resuspended for examination under the light microscope. A representative photomicrograph of the protoplasts in

the preparation is shown in Figure 7. The remainder of the protoplast pellet was resuspended in chloroplast assay medium and chloroplasts were isolated by pressing the suspension 3 times through a syringe covered with a 20 μ m nylon mesh. The broken suspension was subsequently centrifuged at 1000 x g for 90s and the pellet was discarded. After centrifugation of the supernatant at 9000 x g for 90s the chloroplast pellet was carefully resuspended and stored at 4°C. On examination by light microscopy (x 1000 magnification, oil-immersion), the chloroplast suspension appeared to consist uniformly of intact chloroplasts with no observable contamination by other organelles. No enzyme marker assays were carried out. The chlorophyll concentration of the isolated chloroplasts was determined spectrophotometrically at 652nm in 80% acetone. The amount of chlorophyll per gram f.wt. leaf tissue compared favourably with that obtained by the chloroplast isolation procedure described earlier. Due to the very limited amount of plant material that could be accommodated by the enzyme medium at one time, no further use was made of protoplasts as starting material for the isolation of chloroplasts.



x 1700

FIGURE 7

Photomicrograph of purified protoplasts from Pisum sativum cv.
Meteor.

3.1.3. IAA BREAKDOWN

It was anticipated that qualitative studies of IAA in chloroplasts would involve the detection of very low levels of indoles, due to the limited amount of isolated intracellular material. For this reason, steps were taken to reduce, wherever possible, loss of compounds caused by breakdown and inefficient recovery during purification.

(a) Preliminary Incubations with Chloroplast Fractions.

Preliminary experiments were carried out to determine whether isolated pea chloroplasts possess the ability to metabolize [2-¹⁴C]IAA. A chloroplast fraction was prepared by the method described in section 2.2. The preparation was incubated with 3.0×10^6 dpm [2-¹⁴C]IAA in the light at 20°C. The incubation medium also contained 1mM ATP and 10mM NaHCO₃⁻ in assay medium. After 15min the incubation was stopped by the addition of ice-cold methanol and the preparation was extracted through a solvent series. The acidic, ethyl acetate-soluble fraction was subsequently analysed by reverse phase HPLC with an on-line radioactivity monitor. The data obtained are shown in Figure 8. It would appear that the chloroplast preparation rapidly metabolized [2-¹⁴C]IAA to at least 8 other radio-labelled compounds. However, analysis of the boiled chloroplast control revealed that much of this conversion was apparently non-enzymic. Extensive control experiments were

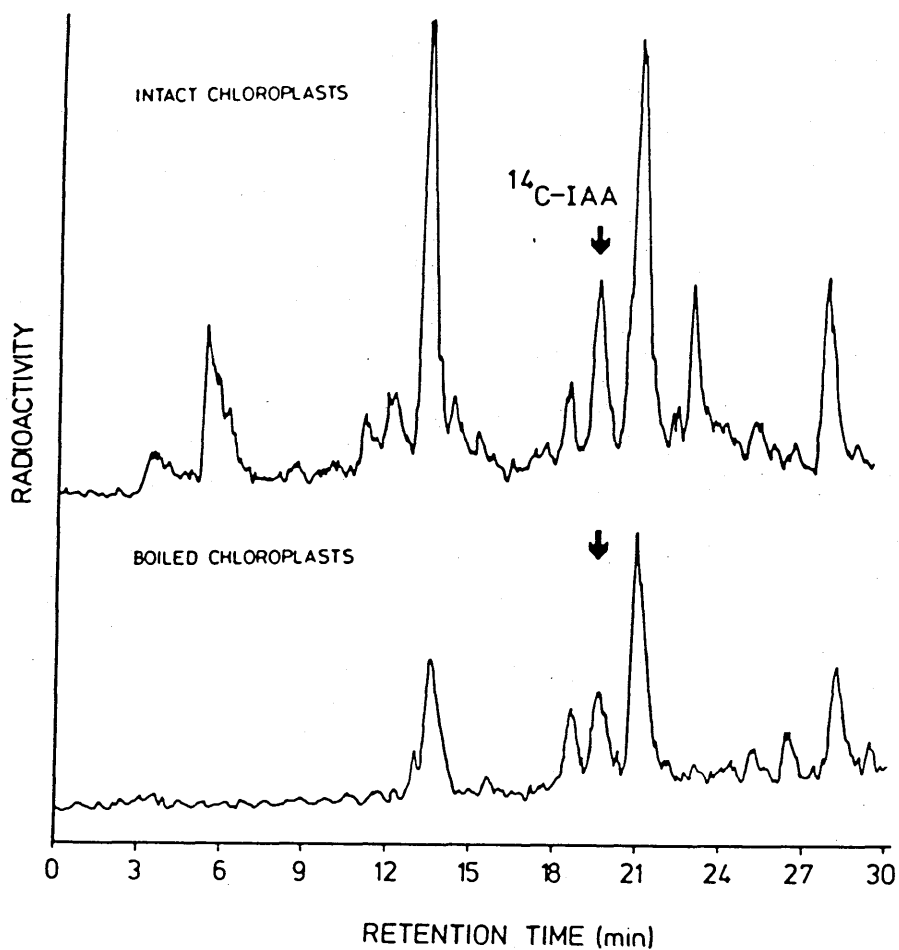


FIGURE 8

Reverse phase HPLC of chloroplast extracts. Column: 250 x 5mm (I.D.) 5 μ m ODS Hypersil; Solvent: 25min gradient of 20-70% MeOH in 20mM, pH3.5 ammonium acetate; Solvent flow rate: 1ml min⁻¹; Sample: (A) 1/30 aliquot extract from intact chloroplasts incubated for 15min with 3×10^6 dpm [2-¹⁴C]IAA, (B) 1/30 aliquot extract from chloroplasts boiled for 15min before incubation with radioactive IAA. Detector: radioactivity monitor, 32cps full scale deflection (fsd)

therefore devised to determine whether this breakdown was occurring during the incubation itself or during the subsequent extraction.

(b) Effect of adding anti-oxidant during solvent extraction.

Freshly-prepared dilutions of [2-¹⁴C]IAA were added to 50ml volumes of distilled methanol and then extracted through a solvent series (see section 2.6 (a)). Duplicate [¹⁴C]IAA samples were extracted with solvents and buffer in which 20mM diethyldithiocarbamate had been added as an anti-oxidant. After extraction, HPLC analysis revealed that extensive breakdown of [¹⁴C]IAA had taken place in the samples without anti-oxidant. Breakdown was greatly reduced in the preparations which had been extracted with solvents containing anti-oxidant.

(c) Effect of adding cold carrier during IAA extraction.

An experiment was also carried out to determine the effect of adding excess unlabelled IAA to samples in the absence of anti-oxidant. IAA extraction through a solvent series was carried out as in Experiment (b), but with 30µg non-radioactively-labelled IAA added to the methanol prior to commencing the extraction and partitioning procedure. Again, radioactivity detection of HPLC eluates indicated that the addition of unlabelled IAA carrier considerably reduced [¹⁴C]IAA breakdown during sample extraction.

By adding both anti-oxidant and unlabelled IAA carrier to samples, [2-¹⁴C]IAA breakdown during extraction and partitioning was virtually eliminated (Figure 9).

(d) Occurrence of IAA breakdown during Solvent Extraction.

An attempt was made to pin-point the steps in the extraction procedure where [¹⁴C]IAA breakdown was occurring. The solvent extraction procedure was carried out on several identical samples of [2-¹⁴C]IAA and at various steps in the procedure, unlabelled IAA and anti-oxidant were added. The extractions were then completed and the samples were analysed by HPLC with on-line radioactivity detection. It was found that, although breakdown was detectable at all steps, the most extensive breakdown occurred during partitioning with ethyl acetate at pH2.5. Also, if [¹⁴C]IAA samples which were dissolved in methanol, ethyl acetate or diethyl ether were reduced to dryness under a stream of nitrogen, then extensive breakdown occurred. However, the use of oxygen-free nitrogen greatly reduced any breakdown and in the presence of anti-oxidant, no breakdown was detectable at this step.

(e) Light-induced degradation of IAA.

The extent of IAA breakdown by light was also investigated. [2-¹⁴C]IAA in assay medium without chloroplasts was incubated in the light for 3h at 20°C. After the addition of ice-cold

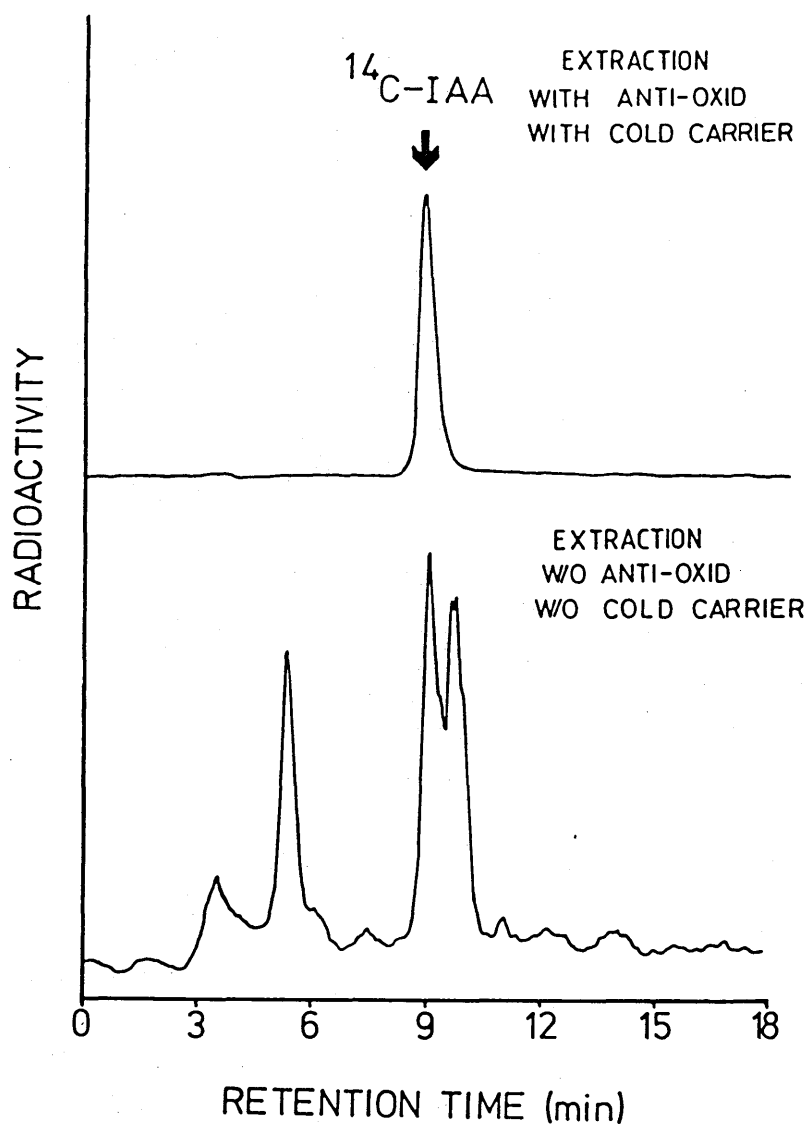


FIGURE 9

Reverse phase HPLC of [^{14}C]IAA standard solutions. Column: 250 x 5mm (I.D.) 5 μm ODS Hypersil; Solvent: 40% MeOH in pH3.5 ammonium acetate, isocratic elution; Sample: (A) anti-oxidant and unlabelled IAA added to [^{14}C]IAA solution prior to extraction; (B) as (A) but without anti-oxidant and without unlabelled IAA added; Detector: radioactivity monitor, homogenous mode, 16cps fsd.

methanol containing anti-oxidant and cold carrier, the incubation medium was extracted and partitioned in solvents in which 20mM anti-oxidant had been added. The HPLC analysis of the extract produced a single peak that co-chromatographed with authentic [^{14}C]IAA. This indicated that light-induced breakdown of IAA was not occurring during incubation of the chloroplast preparation.

(f) Buffer extraction.

The results obtained from these control experiments suggested that extraction and purification by solvent partitioning was not optimally suited for the identification of IAA in pea chloroplast preparations. It was therefore decided to employ the use of buffer extraction techniques for subsequent experiments investigating endogenous indoles. Buffer extraction had the advantage of using minimal quantities of organic solvents and it was also less time-consuming. This meant that each extract was subjected to room temperatures for shorter periods, hence reducing the risk of breakdown of unstable compounds. The purification of endogenous IAA from chloroplast fractions, by both solvent partitioning and buffer extraction techniques, is described in section 3.2.

3.1.4. MINIMISING LOSSES OF IAA DURING EXTRACTION

Extraction and purification procedures can affect recoveries of endogenous IAA by a number of ways. It has been found that IAA can be non-enzymically broken down to a number of chromatographically-distinct compounds (see section 3.1.3). However, losses can also be incurred due to the inefficiency of the purification procedures in recovering the IAA from the system.

(a) Effect of Anti-oxidant on Sample Solubility.

When solutions of [^{14}C]IAA containing 20mM diethyldithiocarbamate were reduced to dryness, difficulties arose when transferring the sample from one vessel to another. A known amount of [$2\text{-}^{14}\text{C}$]IAA was added to a volume of methanol containing 20mM anti-oxidant. This was then reduced to dryness in vacuo and ca. 5ml diethyl ether was added to the flask. The flask was then gently agitated to dissolve the sample and the solvent pipetted into a scintillation vial. After evaporating off the diethyl ether, the radioactivity in the sample was determined by liquid scintillation counting. It was found that the sample contained about 35% of the radioactivity present in the original [^{14}C]IAA solution. Repeated washing of the flask removed a further 10-15% of the original radioactivity. Similar results were obtained when transferring samples in ethyl acetate. It was found that by using methanol or ethanol to

transfer [^{14}C]IAA samples, recoveries of 97-99% were obtained.

(b) Recovery of IAA from XAD-7 Column.

It was decided to test the recovery of [^{14}C]IAA from the XAD-7 column under basic pH conditions. A solution of [2- ^{14}C]IAA in 50ml 0.1M pH8.0 phosphate buffer was loaded onto an XAD-7 column. When the buffer solution had passed through, the column was eluted with 10ml 70% ethyl acetate in hexane (v/v). The organic phase was removed, reduced to dryness and the radioactivity present in the sample was determined by liquid scintillation counting. This procedure was repeated adding 20mM anti-oxidant to the [^{14}C]IAA solution prior to loading onto a freshly-prepared column. The effect of washing the column with 10ml buffer before eluting with organic solvent was also investigated. The results obtained are summarized in Table 2.

TABLE 2

Recovery of [^{14}C]IAA from XAD-7 column. Radioactivity is expressed in cpm $\times 10^{-3}$

| Treatment | Initial radioactivity | Radioactivity from EtOAc:hexane elution | amount adsorbed |
|-------------------------------------|-----------------------|---|-----------------|
| 1. [^{14}C]IAA in buffer | 252.3 | 44.9 | 17.8% |
| 2. as 1. with anti-oxidant | 252.2 | 23.4 | 9.3% |
| 3. as 2. but column washed | 252.3 | 10.8 | 4.3% |

3.2. ENDOGENOUS INDOLES

3.2.1. PEA SEEDLINGS

An attempt was made to identify several indoles from whole pea seedlings. About 70g f.wt. of 14 day-old pea shoot tissue was harvested and added to 150ml 0.1M pH8.0 phosphate buffer containing anti-oxidant. After homogenisation in ice, the preparation was buffer extracted as described in section 2.6. Both pH8.0 and pH2.7 fractions were stored in darkness at -20°C prior to HPLC analysis.

(a) pH8.0 fraction.

The pH8.0 fraction was first subjected to normal phase HPLC. Aliquots of sample were eluted isocratically in 25% ethyl acetate in hexane (v/v). A substantial fluorescent peak corresponding to the retention time (Rt) of indole ethanol (IEtOH) was observed. This peak co-chromatographed with authentic IEtOH. A minor peak with retention characteristics of indole methanol (IMeOH) was also present. This IMeOH-like peak co-chromatographed with an authentic IMeOH standard. The remainder of the sample was loaded onto the column and the IEtOH and IMeOH zones were collected and reduced to dryness. Gradient elution from a reverse phase HPLC column was then carried out on both fractions. The IEtOH-like fluorescent peak again showed identical retention properties to authentic IEtOH and on co-

chromatography, peak areas were additive. However, with this system, the IMeOH-like fraction produced a single fluorescent peak with a R_t of 14.1min, whereas authentic IMeOH gave a single peak at 12.2min. Therefore, no further analysis was carried out on this fraction. The remainder of the putative IEtOH sample was then eluted onto the reverse phase column and the IEtOH zone, 15.6–17.3min, was collected, reduced to dryness and stored under nitrogen in methanol until further analysis. The sample was subsequently divided equally in two aliquots, one of which was derivatized to produce di-trimethylsilyl-IEtOH (di-TMS-IEtOH). Both underivatized and silylated samples produced GC profiles which closely correlated with their authentic standards (Figure 10). Combined GC-MS of both these samples confirmed the presence of IEtOH in pea seedlings (Figures 11 & 12). The chromatographic data are summarized in Table 3.

(b) pH2.7 fraction.

The acidic fraction from the XAD-7 column underwent similar analytical procedures to that of the pH8.0 fraction. Aliquots of sample were eluted isocratically from a normal phase column in ethyl acetate:hexane:acetic acid (24.5:74.5:1). The zone corresponding to the R_t of IAA was collected and reduced to dryness. A sample of indole carboxylic acid (ICA) standard eluted from the column with a similar R_t to IAA indicating that the putative IAA peak collected may not be homogenous. Gradient elution from a reverse phase column was then carried out on the

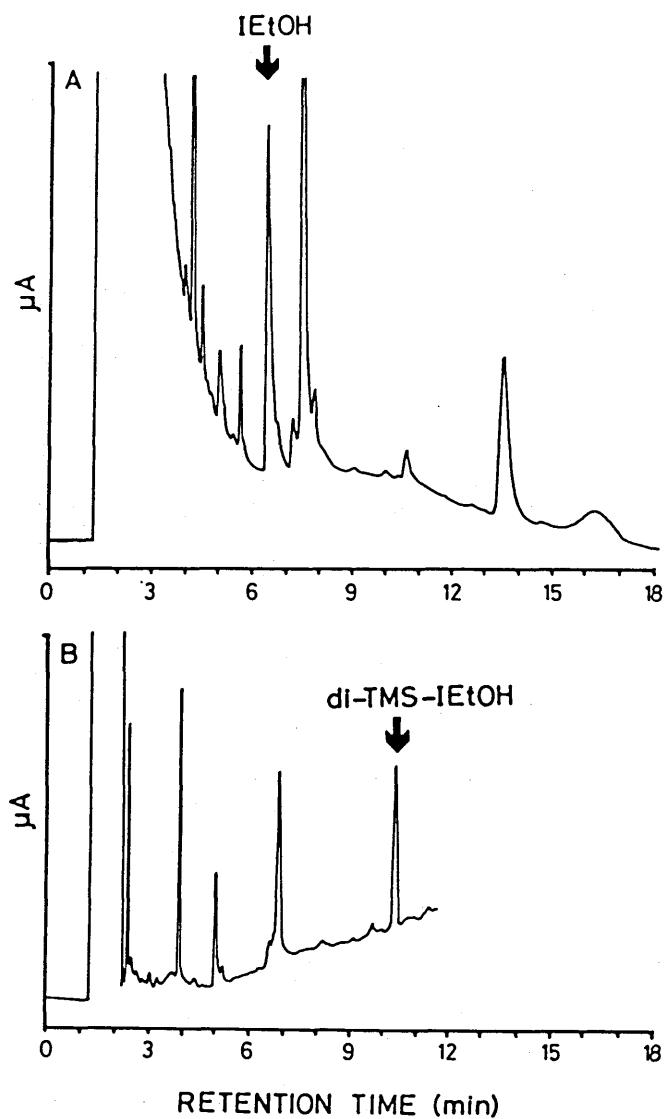


FIGURE 10

GC-NP of semi-purified extract from pea seedlings. Column: 15m x 0.25mm (I.D.) fused silica with a crosslinked DB-s stationary phase; Splitless injection; injector temperature: 250°C; oven temperature: 170°C, isothermal; helium carrier gas flow rate: 30ml min⁻¹; Sample: (A) IEtOH zone collected from reverse phase HPLC; (B) extract after silylation. Detector: flameless N-P.

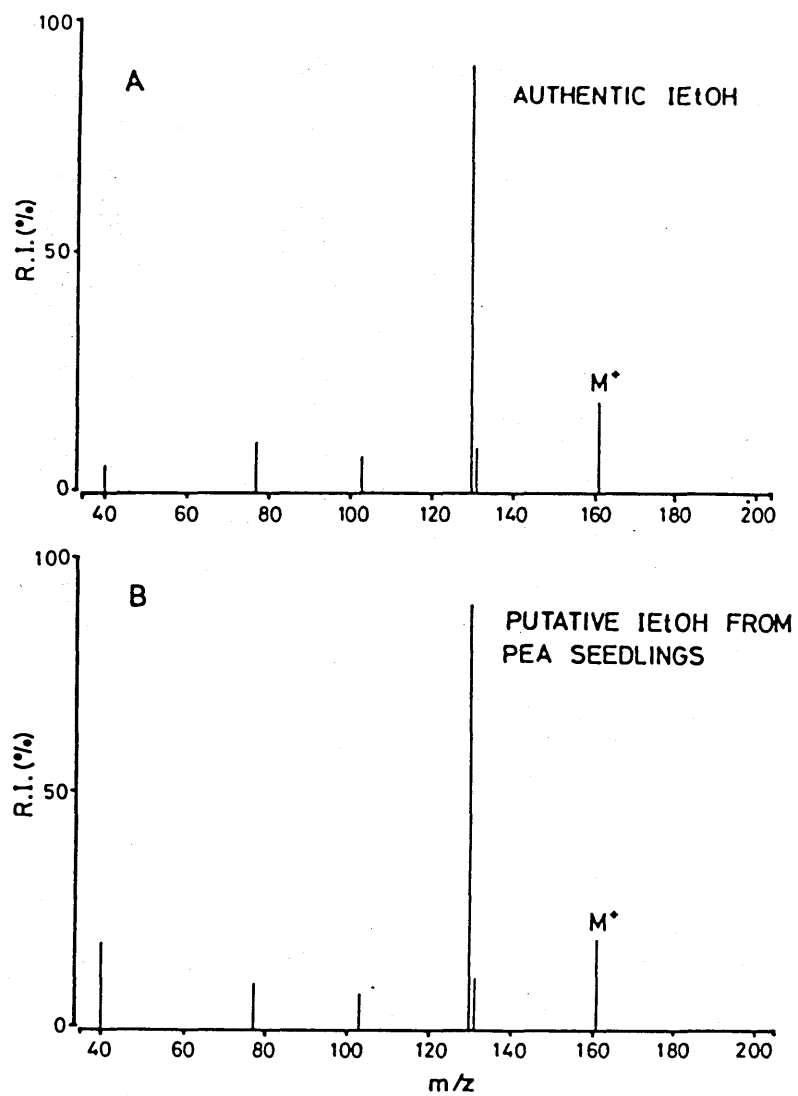


FIGURE 11

Electron impact mass spectra of IEtOH. (A) authentic IEtOH; (B) putative IEtOH from pea seedlings.

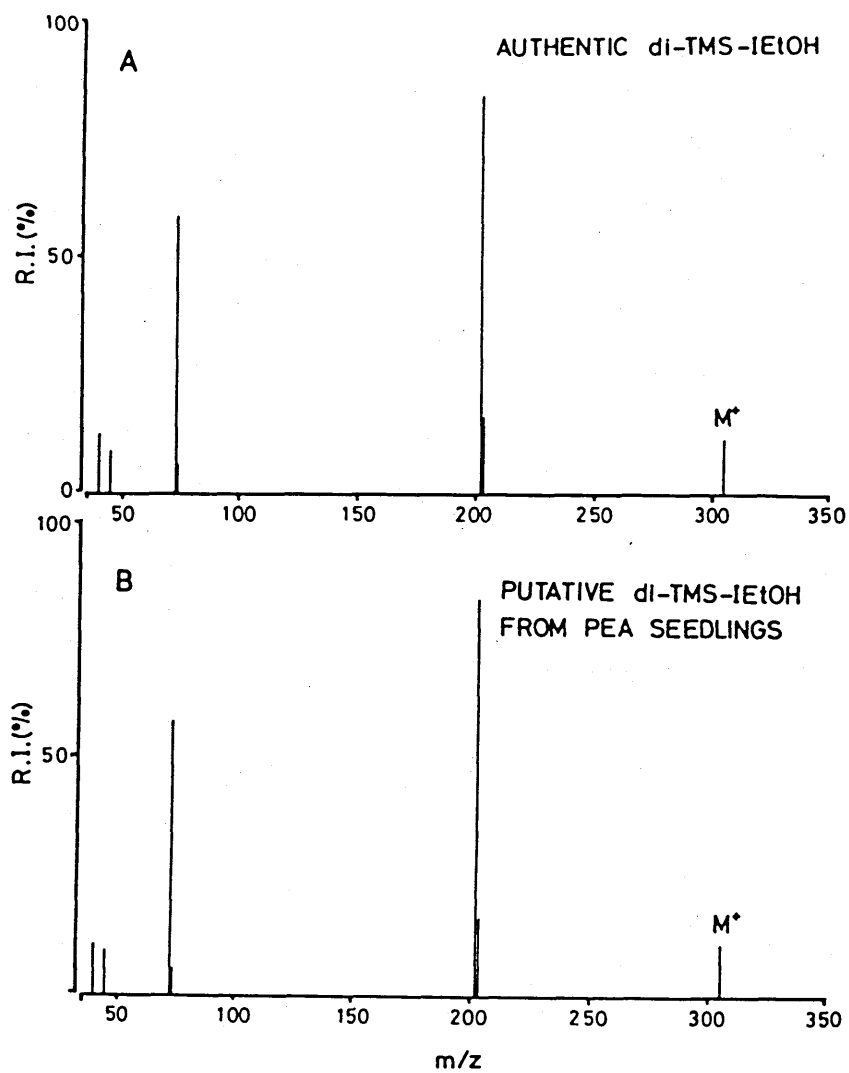


FIGURE 12

Electron impact mass spectra of di-TMS-IEtOH. (A) Authentic di-TMS-IEtOH; (B) TMS-derivative of putative IEtOH from pea seedlings.

TABLE 3

Chromatographic properties of endogenous IEtOH-like compound from pea seedlings.

| Chromatographic System | Retention time | | | |
|---|-----------------|----------------|------------------------|-----------------------|
| | Authentic IEtOH | Putative IEtOH | Authentic di-TMS-IEtOH | Putative di-TMS-IEtOH |
| Normal phase HPLC, isocratic elution in EtOAc:hexane (25:75) | 18.7min | 18.7min | - | - |
| | | | | |
| Reverse phase HPLC, 25min gradient of 25-75% MeOH in pH6.5 ammonium acetate | 16.4min | 16.4min | - | - |
| | | | | |
| GC - isothermal | 6.46min | 6.44min | 10.29min | 10.26min |

fraction and it produced a single fluorescent peak which co-chromatographed with authentic IAA. However, this system could not completely resolve IAA and ICA standards when co-injected. The remaining volume of sample was injected and the zone corresponding to the R_t of IAA was collected, reduced to dryness and subsequently methylated with diazomethane. Prior to GC analysis, part of the extract was further derivatized to produce the TMS-methyl ester of the compound in the sample. GC of the putative IAA-Me sample resulted in a trace containing a peak which had the same R_t as authentic IAA-Me. The silylated methyl ester sample produced a trace containing a clearly resolved peak at the R_t of TMS-IAA-Me standard (Figure 13). Combined GC-MS of the methylated and silylated samples confirmed the presence of IAA in pea seedlings but provided no evidence for the presence of ICA. The mass spectra obtained are illustrated in Figures 14 and 15 together with that of authentic IAA-Me and TMS-IAA-Me.

3.2.2. BUFFER EXTRACTION OF CHLOROPLAST PREPARATIONS

Pellets from 6 separate chloroplast isolations were combined to give a suspension containing a total of 115mg chlorophyll from ca. 560g f.wt. pea leaf tissue. After the addition of 140ml 0.1M pH8.0 phosphate buffer containing anti-oxidant, the preparation was ultrasonicated for 20min in ice and then buffer extracted as described in section 2.6. A pH8.0 fraction and a pH2.7 fraction were obtained from the

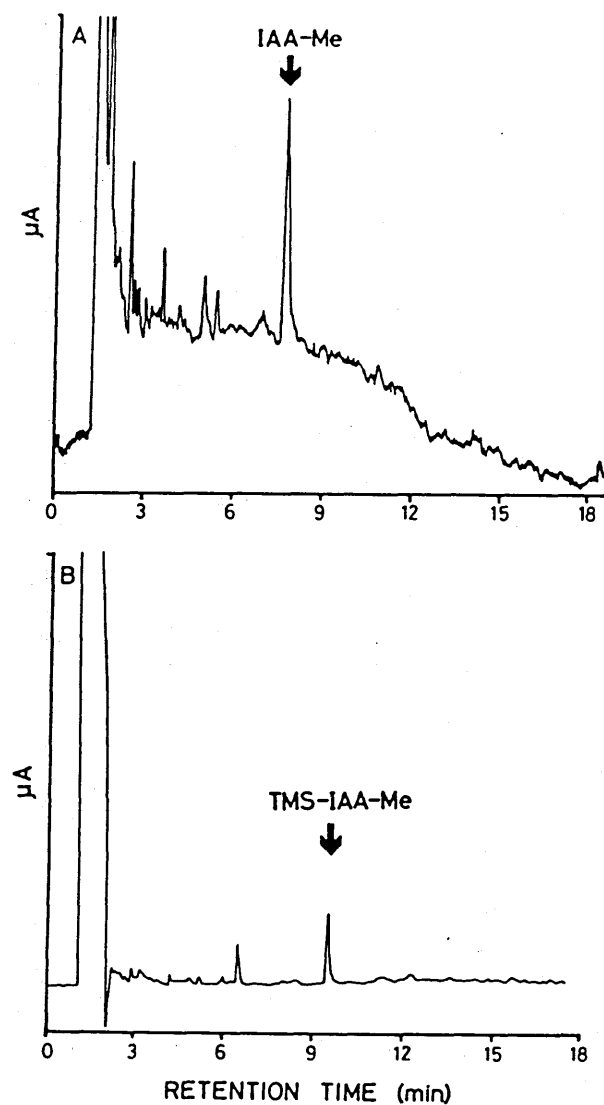


FIGURE 13

GC-NP of semi-purified extract from pea seedlings. Column: 15m x 0.25mm (I.D.) fused silica with a crosslinked DB-s stationary phase; Splitless injection; injector temperature: 250°C; oven temperature: 170°C, isothermal; helium carrier gas flow rate: 30ml min⁻¹; Sample: (A) IAA zone collected from HPLC and methylated; (B) IAA zone from HPLC after methylation and silylation. Detector: flameless N-P.

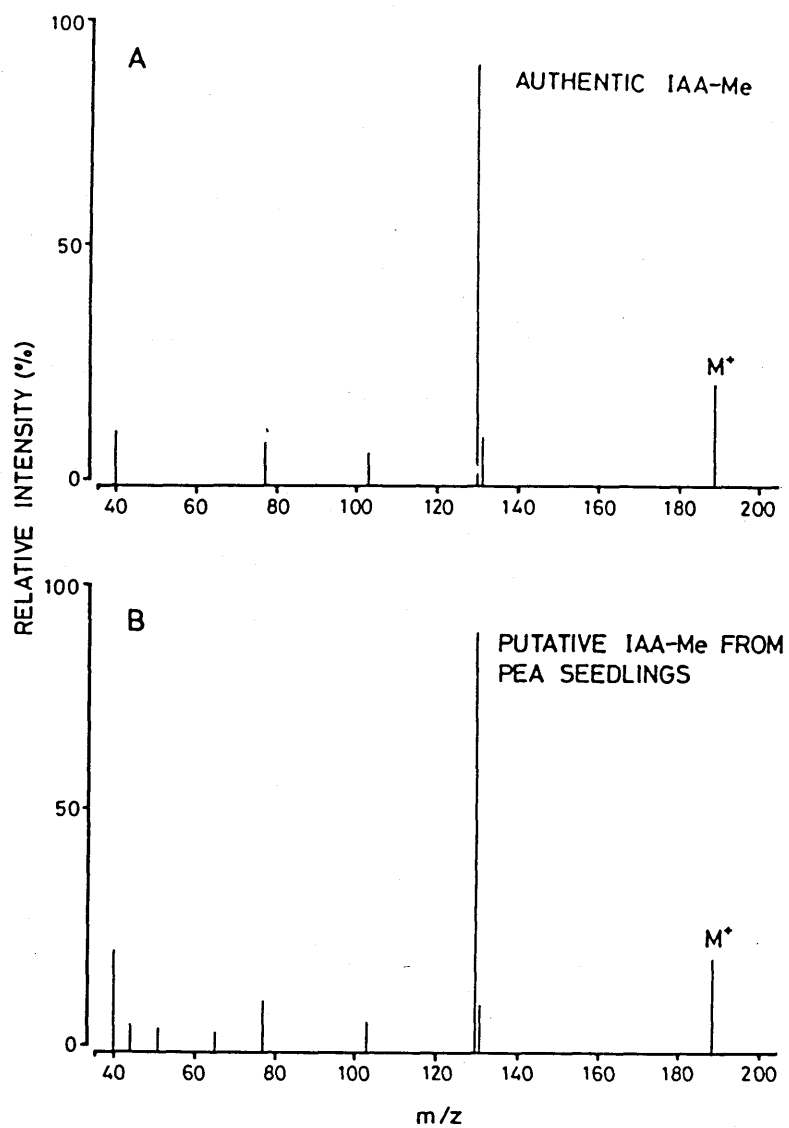


FIGURE 14

Electron impact mass spectra of IAA. (A) Authentic IAA-Me; (B) putative IAA from pea seedlings after methylation.

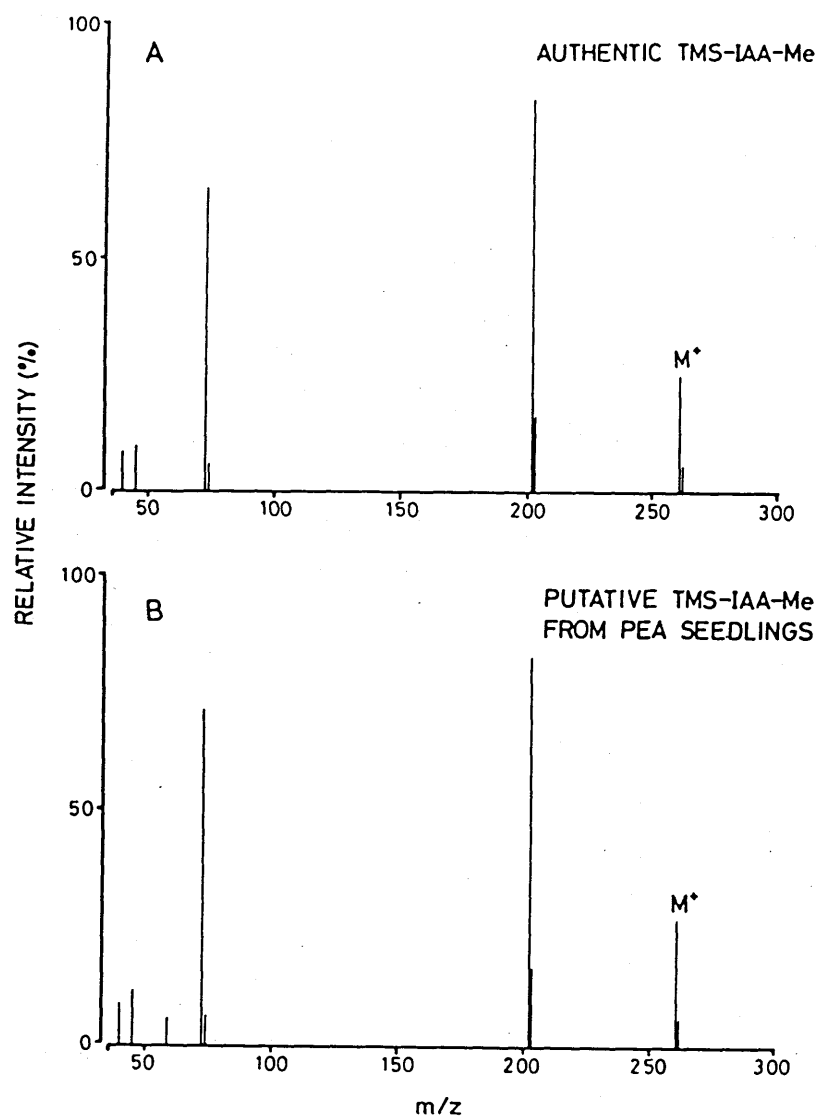


FIGURE 15

Electron impact mass spectra of TMS-IAA-Me. (A) authentic TMS-IAA-Me; (B) purified extract from pea seedlings after methylation and silylation.

extraction.

(a) pH8.0 fraction.

This sample was analysed in a similar manner to the pH8.0 fraction from pea seedlings (see section 3.2.1). Normal phase HPLC was carried out and the IEtOH and IMeOH zones were collected and reduced to dryness. When analysed by reverse phase chromatography, the IMeOH-like sample did not co-chromatograph with authentic IMeOH and therefore this sample was not analysed further. On gradient elution, the IEtOH-like fraction produced a substantial peak at Rt 16.4min (Figure 16), which co-chromatographed with authentic IEtOH. By reference to a known quantity of IEtOH standard under identical conditions, the total amount of IEtOH-like substance in the sample was calculated to be approximately 7ng. This estimate did not account for losses incurred during extraction or purification procedures. GC analysis of the silylated sample produced data which closely correlated with authentic di-TMS-IEtOH. Due to lack of sample remaining, GC-MS analysis could not be carried out. The chromatographic properties of the sample are summarized in Table 4.

(b) pH2.7 fraction.

Normal phase HPLC, reverse phase HPLC, GC and combined GC-MS analyses were carried out on the pH2.7 fraction from the chloroplast preparation. At each chromatographic step a clearly-resolved IAA-like peak was present which co-chromatographed

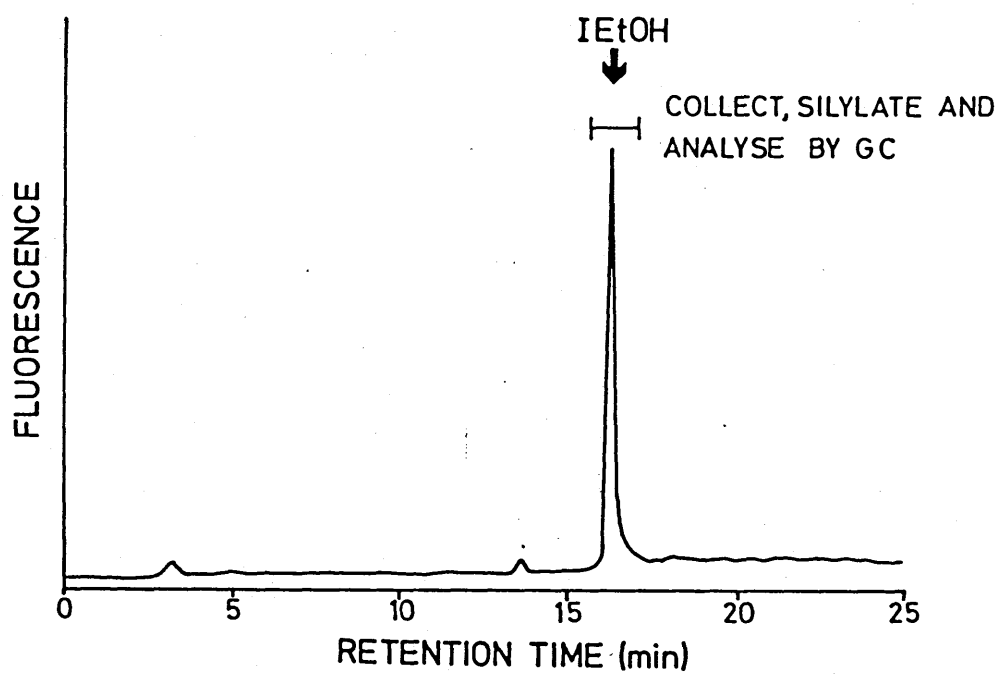


FIGURE 16

Reverse phase HPLC of semi-purified extract from pea chloroplast fractions. Analytical conditions: 25min gradient of 25-75% MeOH in 20mM pH6.5 ammonium acetate; Sample: 2/5 aliquot of IEtOH zone collected from normal phase HPLC; Detector: fluorimeter, excitation : 280nm, emission: 350nm.

TABLE 4

Chromatographic properties of endogenous IEtOH-like compound from pea chloroplast fractions.

| Chromatographic System | Retention time | | | |
|---|--------------------|-------------------|---------------------------|--------------------------|
| | Authentic IEtOH | Putative IEtOH | Authentic di-TMS-IEtOH | Putative di-TMS-IEtOH |
| Normal phase HPLC, isocratic elution in EtOAc:hexane (25:75) | 18.7min | 18.7min | - | - |
| Reverse phase HPLC, 25min gradient of 25-75% MeOH in pH6.5 ammonium acetate | 16.4min | 16.4min | - | - |
| GC - isothermal | 6.41min | 6.42min | 11.30min* | 11.29min* |

(* - lower gas flow rate than in Table 3, therefore increased Rt.)

with authentic IAA and the peak heights were additive. The methylated sample also produced a peak which co-eluted exactly with a methylated IAA standard. The chromatographic data are presented in Table 5.

The presence of IAA as an endogenous constituent of pea chloroplast fractions was confirmed by GC-MS of the methylated sample and its TMS-derivative. A positive ion electron impact mass spectrum of the suspected IAA-Me component from the chloroplast fraction is illustrated in Figure 17. A mass spectrum of the TMS-derivative of putative IAA-Me from the chloroplast preparation is shown in Figure 18.

3.2.3. SOLVENT EXTRACTION OF CHLOROPLAST PREPARATIONS

Extraction of 6 combined chloroplast preparations from ca. 500g f.wt. pea leaf tissue was carried out by the solvent extraction method described in section 2.6. The acidic, diethyl ether-soluble fraction was reduced to dryness, re-dissolved in 20% MeOH in 20mM pH3.5 ammonium acetate, and subjected to gradient elution from a reverse phase HPLC column. Fluorescence detection of the column eluent produced a trace with a substantial peak at the Rt of IAA, and this peak co-chromatographed exactly with authentic IAA. From the peak heights of putative and authentic IAA aliquots, it was estimated that the total sample contained ca. 400ng IAA. The IAA zone was collected, reduced to dryness, and dissolved in 40% MeOH in

TABLE 5

Chromatographic properties of IAA-like compound from pea chloroplast fractions.

| Chromatographic System | Retention time | | | | | | | |
|---|------------------|-----------------|---------------------|--------------------|-------------------------|------------------------|-------------------------|------------------------|
| | Authentic IAA | Putative IAA | Authentic IAA-Me | Putative IAA-Me | Authentic TMS-IAA-Me | Putative TMS-IAA-Me | Authentic TMS-IAA-Me | Putative TMS-IAA-Me |
| Normal phase HPLC, isocratic elution in EtOAc:hexane:HAc | 7.6min | 7.7min | - | - | - | - | - | - |
| Reverse phase HPLC, gradient of 25-75% MeOH in 20mM, pH3.5 ammonium acetate | 15.0min | 15.0min | - | - | - | - | - | - |
| Reverse phase HPLC, isocratic elution with 35% MeOH in pH3.5 ammonium acetate | 14.9min | 15.0min | 19.4min | 19.5min | - | - | - | - |
| GC - isothermal | - | - | 7.77min | 7.77min | 9.58min | 9.56min | - | - |

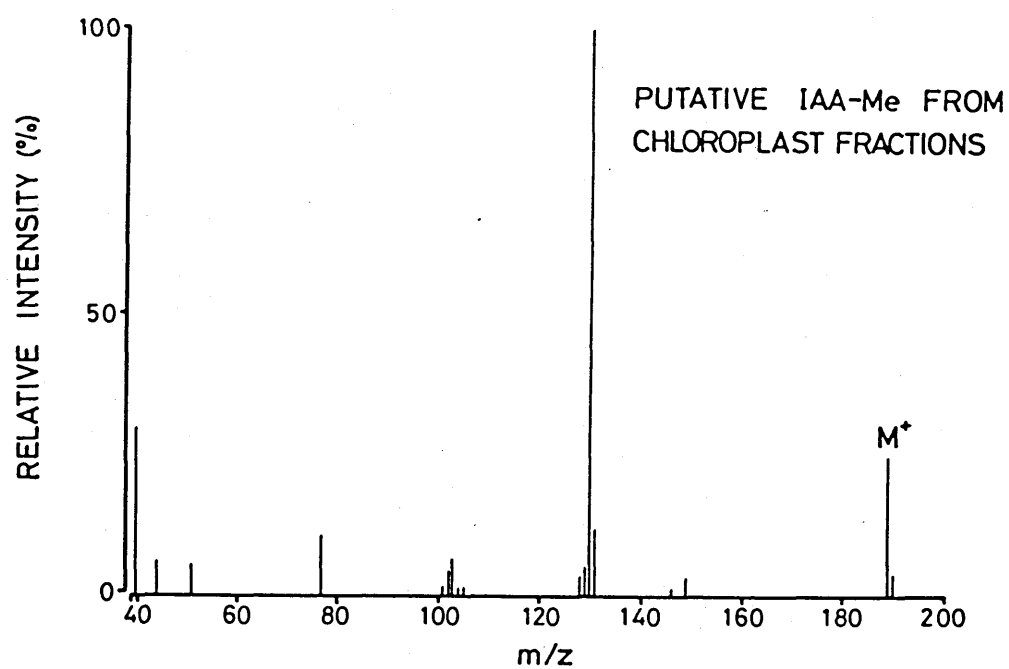


FIGURE 17

Electron impact mass spectrum of suspected endogenous IAA from
pea chloroplast fraction. Analysed as purified, methylated
derivative.

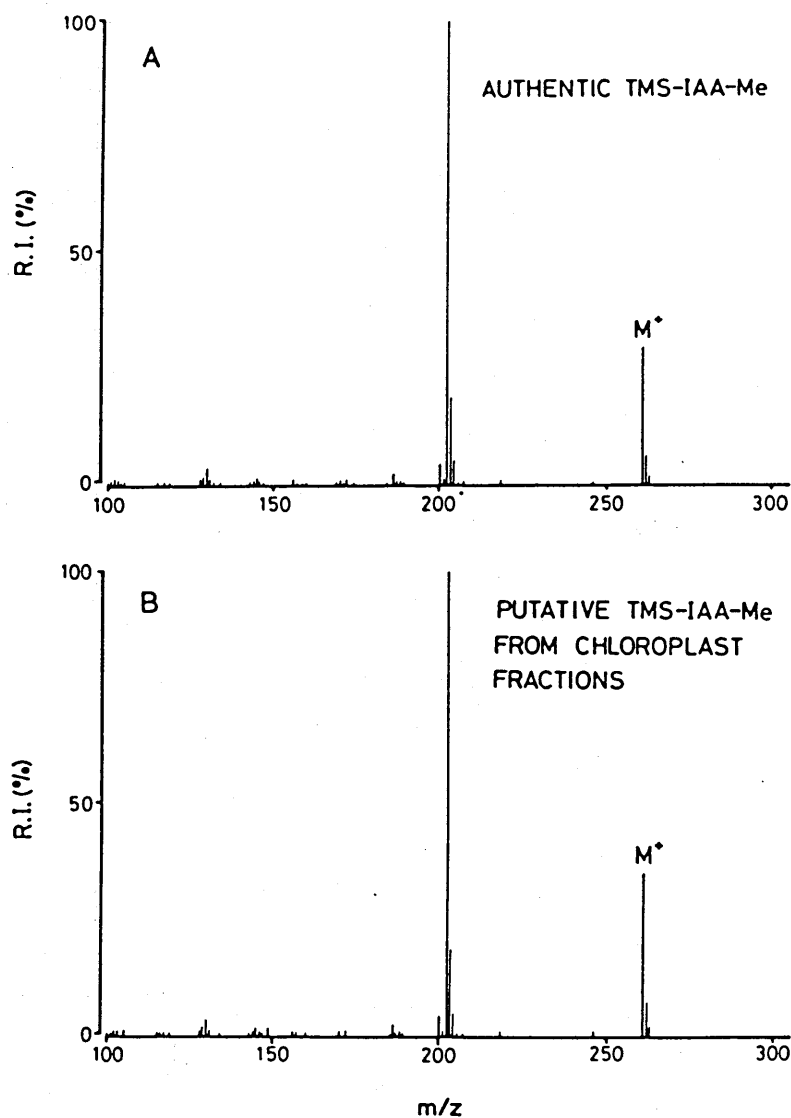


FIGURE 18

Electron impact mass spectra of TMS-IAA-Me. (A) Authentic TMS-IAA-Me; (B) purified, methylated and silylated extract from pea chloroplast fractions.

ammonium acetate. When re-chromatographed, the sample produced an IAA-like peak which also co-chromatographed perfectly with an IAA standard (Figure 19). The IAA zone was again collected, reduced to dryness and methylated with diazomethane. Combined GC-MS analysis of this methylated sample provided a mass spectrum which was virtually identical to that obtained with authentic IAA-Me.

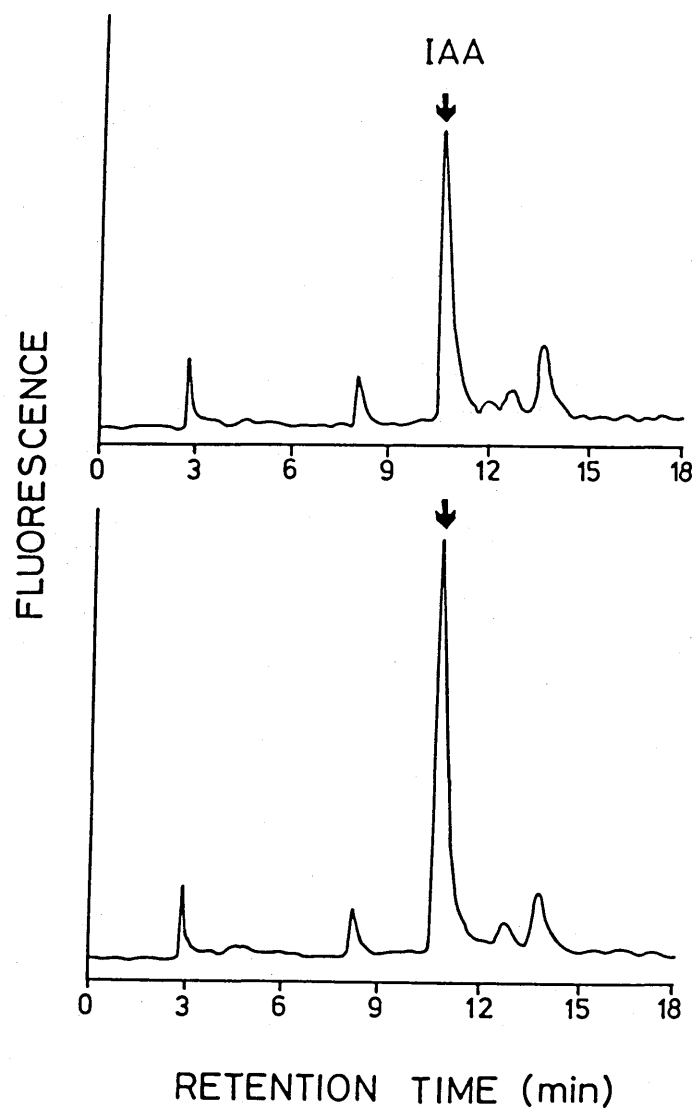


FIGURE 19

Reverse phase HPLC of semi-purified extract from pea chloroplast fractions. Sample extracted by solvent partitioning; Analytical conditions: 250 x 5mm (I.D.) 5 μ m ODS Hypersil column; Solvent: 40% MeOH in pH3.5 ammonium acetate; isocratic elution; Sample: (A) 1/40 aliquot of chloroplast extract; (B) 1/40 aliquot of chloroplast extract co-injected with 300pg IAA; Detector: fluorimeter; Excitation: 280nm, emission: 350nm.

3.3. CATABOLISM OF IAA

3.3.1. INTRODUCTION

The possible catabolism of IAA by pea chloroplast fractions was investigated using two experimental systems:- (a) a crude enzyme preparation (cell-free system) derived from chloroplast preparations and (b) metabolically-active isolated chloroplast fractions. Experiments were carried out to determine whether IAA catabolism occurred by decarboxylative or non-decarboxylative degradation. HPLC and combined GC-MS were employed in order to identify catabolic products.

3.3.2. CELL-FREE SYSTEM

The crude enzyme preparations were prepared from chloroplast fractions as described in section 2.8. Initially, the preparations were stored in solution, in 0.1M pH6.5 phosphate buffer at 4°C. It was found that freeze-drying the preparations after dialysis, had little or no detrimental effect on their ability to decarboxylate [1-¹⁴C]IAA.

This method for obtaining crude enzyme preparations resulted in a substantial amount of chlorophyll being present in the insoluble membrane fraction, and to a much lesser extent, in the soluble enzyme fraction. After freeze-drying, the soluble and insoluble fractions were weighed and chlorophyll content was determined by the method of Arnon (1949). Typical results

obtained are presented in Table 6.

TABLE 6.

Chlorophyll content of crude enzyme preparations.

| Enzyme Preparation | Dry weight (mg) | Amount of Chlorophyll ($\mu\text{g chl mg}^{-1}$ dry wt.) |
|-----------------------|-----------------|---|
| 1. soluble | 267 | 1.4 |
| insoluble | 177 | 90.0 |
| 2. soluble | 450 | 1.2 |
| insoluble | 210 | 76.0 |
| 3. soluble | 430 | 1.1 |
| insoluble | 180 | 43.0 |

Crude enzyme preparations were subsequently stored in powder form, in darkness at -20°C . As described in section 2.9., each preparation was routinely tested to determine its ability to decarboxylate $[1-^{14}\text{C}]\text{IAA}$. The results obtained from these assays gave a qualitative assessment of the cell-free system. Several experiments were carried out to determine the effect of different factors on the decarboxylative capacity of the enzyme

preparations.

(a) Relative Activity of Soluble and Insoluble Fractions.

This experiment was carried out to determine whether IAA-decarboxylation activity could be clearly assigned to the soluble enzyme fraction or the membrane-bound fraction. To 4ml 0.1M pH6.5 phosphate buffer was added a known weight of crude enzyme preparation. The weights of insoluble and soluble powders added, corresponded to the ratio of each derived from the chloroplast fraction. When co-factors were added, their concentrations were as shown in Table 7.

TABLE 7

Concentrations of co-factors used in cell-free system. All co-factors were dissolved in 0.1M phosphate buffer at the appropriate pH.

| Co-factor | Concentration |
|--|----------------------------|
| hydrogen peroxide (H_2O_2) | $1 \times 10^{-4}\text{M}$ |
| 2,4-dichlorophenol (DCP) | $5 \times 10^{-5}\text{M}$ |
| Mn^{2+} (as MnCl) | $1 \times 10^{-4}\text{M}$ |

In order to reduce the risk of spurious results from bacterial contamination, $200\mu\text{g ml}^{-1}$ each of penicillin-G and

streptomycin sulphate were added to each vial. All co-factors and antibiotics were dissolved in 0.1M pH6.5 phosphate buffer. 20ul [$1-^{14}\text{C}$]IAA in buffer (1.1×10^5 cpm) was added to each vial and the incubation was carried out in darkness at room temperature for 3h. The vial inserts containing KOH were subsequently removed and the $^{14}\text{CO}_2$ absorbed by the KOH was determined by liquid scintillation counting. The results obtained are given in Table 8.

TABLE 8

Decarboxylative capacity of soluble and insoluble enzyme fractions. Incubations with co-factors contained DCP, H_2O_2 and Mn^{2+} (see text for concentrations).

| Preparation | $^{14}\text{CO}_2$ absorbed by KOH ($\times 10^3$ cpm) | |
|--------------------|---|-----------------|
| | without co-factors | with co-factors |
| Soluble E.P. | 2.3 | 34.9 |
| Insol. E.P. | 4.9 | 44.9 |
| Sol. E.P.-boiled | 1.0 | 2.0 |
| Insol. E.P.-boiled | 1.4 | 1.8 |
| Buffer only | 1.3 | 1.7 |

From the results in Table 8 it appears that both the membrane-bound fraction and the soluble enzyme fraction have

the same capacity to decarboxylate [$1-^{14}\text{C}$]IAA. The addition of co-factors markedly increased this degradation of [^{14}C]IAA. This experiment was carried out on each enzyme preparation after freeze-drying, and routinely during its storage at -20°C . In each case, similar results were obtained.

(b) Enhanced Decarboxylation by Combining Fractions.

It was decided to repeat the experiment again, but combining soluble and insoluble fractions in order to determine if the extent of decarboxylation was increased (Table 9). All other factors remained identical to those in Expt. (a).

TABLE 9

Effect of combining soluble and insoluble fractions. Incubations with co-factors contained DCP, H_2O_2 and Mn^{2+} .

| Preparation | $^{14}\text{CO}_2$ absorbed by KOH ($\times 10^3 \text{cpm}$) | |
|------------------------|---|-----------------|
| | without co-factors | with co-factors |
| Soluble E.P. | 6.9 | 32.0 |
| Insol. E.P. | 8.2 | 50.0 |
| sol. + insol.(0.5:0.5) | 10.2 | 36.8 |
| Sol. E.P.-boiled | - | 2.7 |
| Insol. E.P.-boiled | - | 3.9 |
| Buffer only | 0.4 | 0.6 |

There appeared to be no enhancement effect when both fractions were combined. This experiment was repeated 3 times using enzyme preparations derived from separate chloroplast fractions. In each case, results were in close agreement with those given in Table 9.

(c) Effect of Co-factors on Decarboxylation.

It has been shown in Tables 8 and 9 that the addition of H_2O_2 , DCP and Mn^{2+} together, to the reaction medium, greatly increased the extent of decarboxylation by the cell-free system. However, it has not been established which of these co-factors has any effect singly, or in the presence of only one other. An experiment was therefore devised to test all combinations of the 3 co-factors used, on their ability to enhance decarboxylation of $[1-^{14}C]IAA$ by the soluble enzyme preparation.

10mg dry wt. soluble enzyme preparation was added to 4ml 0.1M pH6.5 phosphate buffer containing $200\mu g\ ml^{-1}$ penicillin-G and streptomycin sulfate. Each co-factor was added in 250 μ l buffer, resulting in concentrations as given in Expt. (a). To maintain constant volumes, 250 μ l buffer only, was added to each vial when no co-factor was being added. 3×10^5 dpm $[1-^{14}C]IAA$ was added to each vial in 50 μ l buffer and the vials were incubated in darkness at 25°C and shaken gently for 3h. The results obtained showed that in the presence of DCP and H_2O_2 , extensive decarboxylation took place, with 94% of the radio-

TABLE 10

Effect of co-factors on the decarboxylation of IAA by enzyme preparation. The amount of $^{14}\text{CO}_2$ absorbed by KOH is expressed as cpm $\times 10^{-3}$. Relative decarboxylation is expressed as a percentage of the total radioactivity added.

| Incubation Medium | Buffer Control | | Soluble E.P. | |
|--|--------------------|-----|--------------------|------|
| | $^{14}\text{CO}_2$ | % | $^{14}\text{CO}_2$ | % |
| No co-factors | 3.3 | 1.1 | 14.7 | 4.9 |
| DCP | 6.1 | 2.0 | 29.9 | 9.9 |
| H_2O_2 | 9.6 | 3.2 | 34.2 | 11.3 |
| Mn^{2+} | 6.2 | 2.0 | 13.2 | 4.3 |
| DCP, H_2O_2 | 9.5 | 3.2 | 282.9 | 94.3 |
| DCP, Mn^{2+} | 10.9 | 3.6 | 125.2 | 41.7 |
| H_2O_2 , Mn^{2+} | 7.3 | 2.4 | 12.3 | 4.1 |
| DCP, H_2O_2 , Mn^{2+} | 7.9 | 2.6 | 121.4 | 40.4 |

activity fed being absorbed as ^{14}CO in the KOH solution (Table 10). The addition of Mn^{2+} to the reaction mixture had little enhancing effect and in some cases Mn^{2+} appeared to inhibit the effect of the other co-factors. Similar experiments were carried out 4 times and in each case the effect of adding DCP and H_2O_2 was consistent with that shown in Table 10. However, inconsistent results were obtained from the incubations containing Mn^{2+} where both enhancing and inhibiting effects were observed. For this reason, Mn^{2+} was omitted from all subsequent incubations.

(d) Catabolism of $[2-^{14}\text{C}]\text{IAA}$ by the Cell-free System.

In order to investigate the products of IAA degradation, experiments were carried out involving the incubation of $[2-^{14}\text{C}]\text{IAA}$ in the cell-free system. 10ml volumes of 0.1M pH 6.5 phosphate buffer, each containing 25mg dry wt. soluble enzyme preparation, DCP, H_2O_2 and antibiotics, were incubated in darkness at 25°C with either $[2-^{14}\text{C}]\text{IAA}$ or $[1-^{14}\text{C}]\text{IAA}$. A third preparation was boiled for 10min and allowed to cool to 25°C before the addition of $[2-^{14}\text{C}]\text{IAA}$. After 3h the incubations were stopped by the addition of anti-oxidant and immediately adjusted to pH 8.0, partitioned 5 times with 2/5 volumes of EtOAc and the organic phase reduced to dryness. The samples were dissolved in 400 μl 25% MeOH in 20mM pH 6.5 ammonium acetate prior to HPLC analysis.

On gradient elution from a reverse phase column, it was found that the sample from the [2-¹⁴C]IAA incubation contained a substantial radioactive and fluorescent peak at the Rt of IMeOH. A smaller, less polar peak was also observed (Figure 20). Both peaks were collected, reduced to dryness and silylated with BSFTA prior to GC-MS analysis. Due to suspected breakdown during storage, insufficient sample remained after preliminary purification steps to allow further analysis. Hence, no positive GC-MS data were obtained from these collected fractions. The sample from the [1-¹⁴C]IAA incubation produced an HPLC trace with a substantial fluorescent peak which had identical retention properties to IMeOH. However, in this case, no accompanying radioactive peak was observed. HPLC analysis of the boiled control produced a trace with no significant catabolite peaks. In each sample, a radioactive peak was observed which eluted from the column 4.4min after injection of the sample. This corresponded to [¹⁴C]IAA which partitioned slightly into EtOAc at pH8.0.

(e) Catabolism of unlabelled IAA.

2ml volumes of buffer containing 15mg soluble enzyme preparation, co-factors (DCP, H₂O₂) and antibiotics were incubated in darkness at 25°C with 35µg unlabelled IAA. One preparation was boiled for 10min prior to incubation. After 1.5h the reaction was stopped by adding anti-oxidant and precipi-

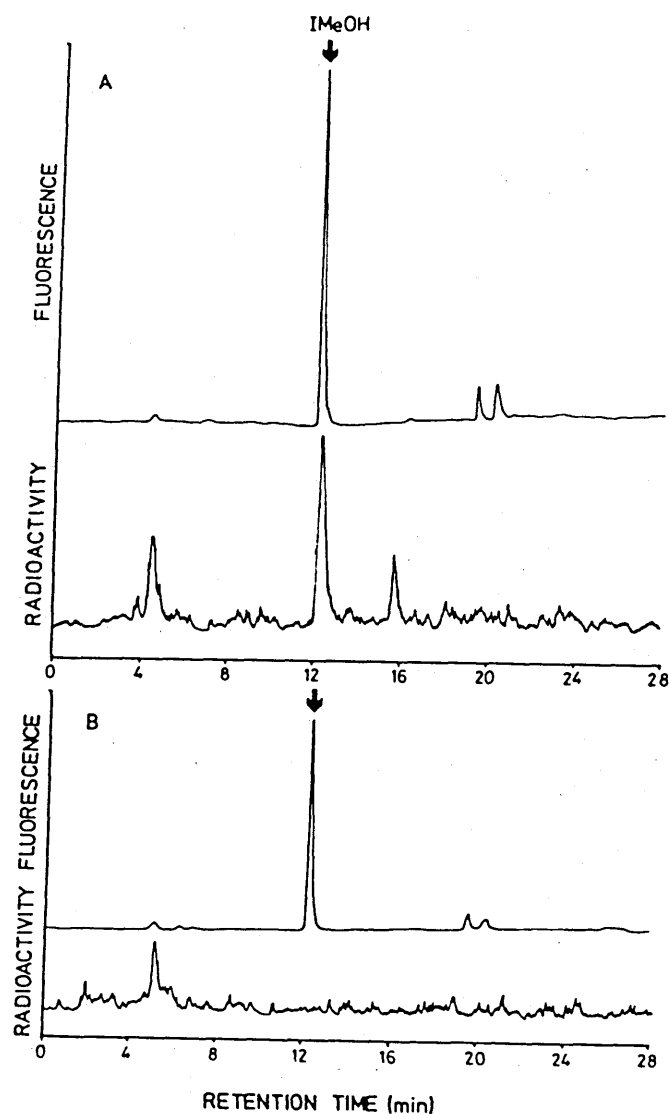


FIGURE 20

Reverse phase HPLC of extracts from cell-free system incubated with [2-¹⁴C]- and [1-¹⁴C]IAA. Analytical conditions: 25min gradient of 25-75% MeOH in 20mM pH6.5 ammonium acetate; Samples: (A) 1/40 aliquot pH8.0, EtOAc-soluble fraction from cell-free system incubated with [2-¹⁴C]IAA; (B) 1/40 aliquot pH8.0, EtOAc-soluble fraction from cell-free system incubated with [1-¹⁴C]IAA; Detectors: Fluorimeter, excitation: 280nm, emission: 350nm; Radioactivity monitor, 10cps fsd.

tating dissolved proteins with 2ml acetone. After centrifugation at 2000 x g for 4min, the supernatants were pipetted off, adjusted to pH8.0 and partitioned with EtOAc. The samples were subsequently reduced to dryness, redissolved in 25% MeOH in 20mM pH6.5 ammonium acetate and analysed by reverse phase HPLC. Gradient elution from the column revealed one peak corresponding to the Rt of IMeOH, in addition to the IAA peak. From the peak height, it was estimated that the sample contained a total of ca. 5µg of an IMeOH-like compound. At a fluorescence range giving full scale deflection (fsd) with the IMeOH-like peak, no other catabolites were detected. The putative IMeOH peak was collected, reduced to dryness and stored under nitrogen in methanol containing anti-oxidant prior to GC-MS analysis. After silylation with BSFTA, the sample was analysed by both GC and GC-MS. Selected ion monitoring was carried out, focussing on the ions characteristic of TMS-IMeOH, at m/z 202, 290 and 291. In each case a trace of intensity of the selected ion against GC retention time produced a peak at the Rt of a TMS-IMeOH standard. The relative abundance of the ions in the putative sample closely correlated with authentic TMS-IMeOH (Table 11). An averaged, electron impact positive ion mass spectrum of the suspected TMS-IMeOH is shown in Figure 21, together with authentic TMS-IMeOH. HPLC of the boiled control produced a trace with no detectable catabolite peaks.

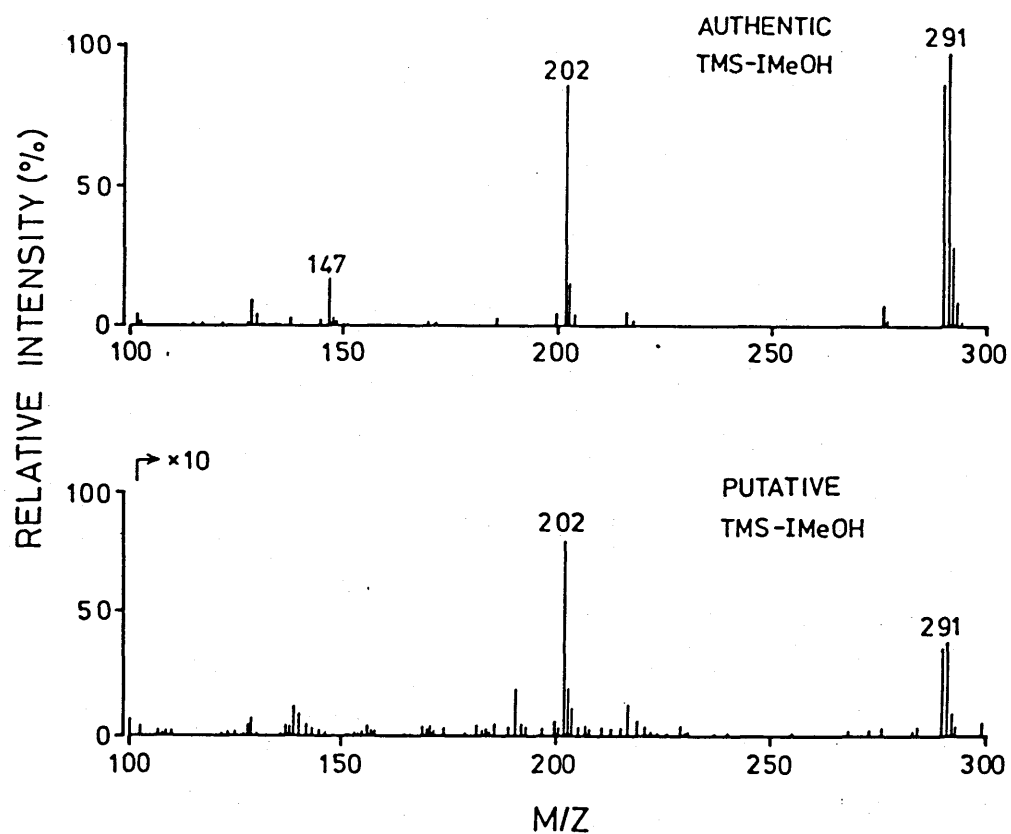


FIGURE 21

Electron impact mass spectra of TMS-IMeOH. (A) Authentic TMS-IMeOH; (B) averaged spectrum (total= 88 scans) of HPLC-purified, pH8.0, EtOAc-soluble fraction from cell-free system fed with IAA; sample analysed as TMS-derivative.

TABLE 11

Details of putative IMeOH analysed by mass spectromery.

| m/z | Relative Abundance (%) | |
|-----|------------------------|----------------|
| | Authentic IMeOH | IAA-metabolite |
| 202 | 100 | 100 |
| 290 | 55.6 | 53.2 |
| 291 | 56.8 | 57.4 |

(f) Extraction of Soluble Enzyme Preparation.

Since Expt. (e) involved an incubation which resulted in an unlabelled IMeOH-like catabolite, then it was possible that this compound originated from the crude enzyme preparation. To 5ml 0.1M pH6.5 phosphate buffer was added 50mg soluble enzyme preparation, which was then stirred continuously for 5min. After adjusting to pH8.0, the buffer solution was partitioned 5 times with 2/5 volumes EtOAc. The organic phase was subsequently reduced to dryness and redissolved in 200 μ l 25% MeOH. A 100 μ l aliquot of the sample was injected onto a reverse phase column and the column eluate was monitored by fluorescence detection. With the fluorimeter set to a range 50 times higher than that used in Expt. (e), no IMeOH-like peak was detected.

(g) Catabolism of [$^2\text{H}_5$]IAA by the Cell-free System.

In order to verify that IMeOH was not an endogenous constituent of the enzyme preparation, Experiment (e) was repeated with deuterium-labelled IAA. 30 μg [$^2\text{H}_5$]IAA containing excess diethyldithiocarbamate was dissolved in 40 μl phosphate buffer and incubated in the cell-free system for 1.5h. HPLC analysis of the pH8.0, ethyl acetate-soluble fraction revealed no catabolic products. An HPLC trace of a sample derived from a [^1H]IAA-fed incubation under identical conditions, contained one large catabolite peak at the R_t of IMeOH. The incubation with [$^2\text{H}_5$]-labelled IAA was repeated and again no catabolites were observed.

The [$^2\text{H}_5$]IAA standard was dissolved in phosphate buffer, adjusted to pH3.0 and partitioned 5 times with 2/5 volumes diethyl ether. The organic phase was then reduced to dryness prior to purification by preparatory HPLC. The IAA zone eluting from a reverse phase column was collected, reduced to dryness and stored at -20°C without anti-oxidant. The experiment was subsequently repeated and HPLC analysis of the [$^2\text{H}_5$]IAA-fed sample revealed a substantial fluorescent peak which co-chromatographed perfectly with authentic IMeOH. No other catabolite peaks were observed (Figure 22). The IMeOH zone was collected, reduced to dryness and stored under methanol at -20°C prior to GC-MS analysis. After silylation and purification by GC, selected ion monitoring was carried out on the sample, focussing

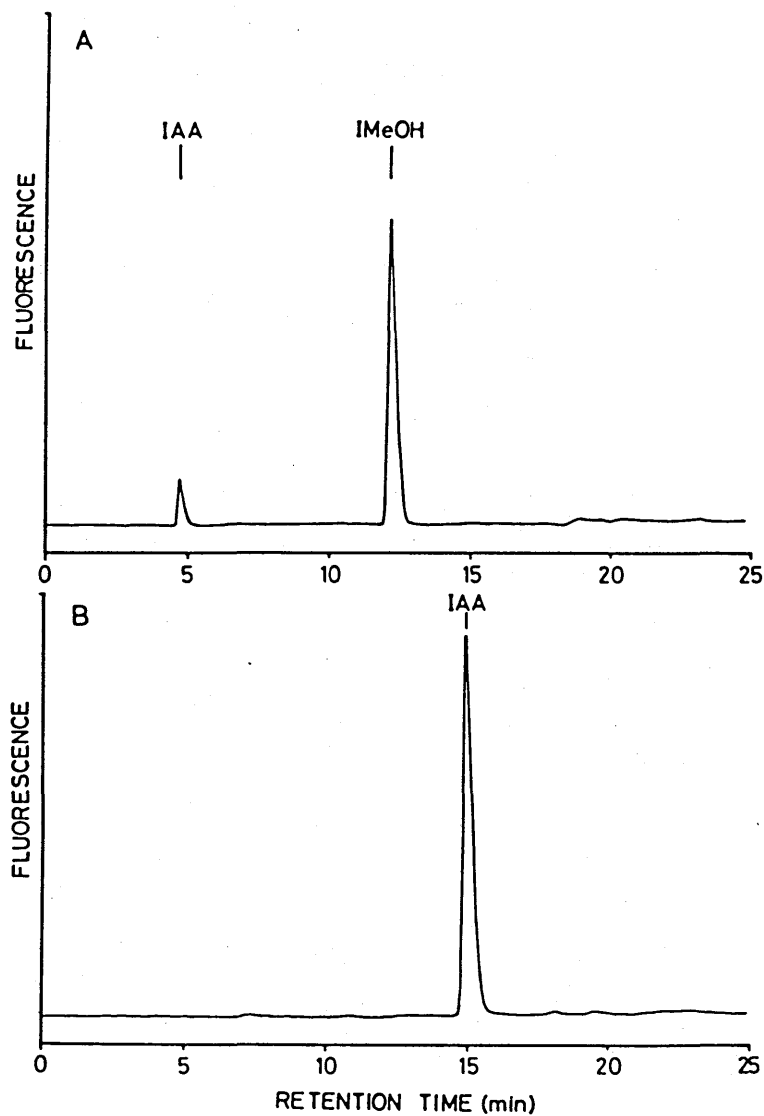


FIGURE 22

Reverse phase HPLC of extract from chloroplast cell-free system incubated with [$^2\text{H}_5$]IAA. Analytical conditions: (A) 25min gradient of 25–75% MeOH in 20mM pH6.5 ammonium acetate; (B) 25min gradient of 25–75% MeOH in pH3.5 ammonium acetate; Sample: (A) 1/3 aliquot pH8.0, EtOAc-soluble fraction derived from incubation; (B) 1/4 pH2.7, EtOAc-soluble fraction derived from same incubation; Detector: fluorimeter, excitation: 280nm, emission: 350nm.

on the ions of m/z 207, 295 and 296. These ions are each 5 mass units greater than those characteristic of TMS-ImeOH, and are expected to be detected in [$^2\text{H}_5$]-labelled compounds. When monitoring m/z 207, a trace of intensity of the ion against GC retention time produced a detectable peak at the R_t of TMS-ImeOH. However, no peaks were detected when monitoring ions m/z 295 or 296. Insufficient sample remained for further purification and analytical procedures to be carried out.

The aqueous fraction from the [$^2\text{H}_5$]-fed sample was adjusted to pH 2.7 and again partitioned against EtOAc. The organic phase was reduced to dryness, re-dissolved in 400 μl 25% MeOH and analysed by reverse phase HPLC. Gradient elution produced a trace with no significant fluorescent peaks other than IAA, indicating that no acidic, EtOAc-soluble catabolites of IAA were produced by the crude enzyme system (Figure 22).

(h) Effect of Co-factors on IAA Breakdown Pattern.

It has been suggested that the diphenol:peroxide ratio in horseradish peroxidase systems affects the products obtained from IAA degradation (Grambow and Langenbeck-Schwich, 1983). It was therefore decided to investigate whether different catabolites were produced in this cell-free system when incubated with DCP, H_2O_2 or both co-factors together. To 2ml volumes of phosphate buffer containing 15mg dry wt. soluble enzyme preparation was added 2.5×10^5 dpm [$2\text{-}^{14}\text{C}$]IAA, together with

5×10^{-5} M DCP and 10^{-4} M H_2O_2 as indicated in Table 12.

TABLE 12

Combinations of co-factors added in Experiment (h). To each preparation was added 2.5×10^5 dpm $[2-^{14}C]IAA$.

| Preparation | Co-factors added |
|-------------------------|------------------|
| 1. Soluble enzyme prep. | DCP |
| 2. Soluble enzyme prep. | H_2O_2 |
| 3. Soluble enzyme prep. | DCP, H_2O_2 |
| 4. Boiled enzyme prep. | DCP, H_2O_2 |
| 5. Buffer only | DCP, H_2O_2 |

The preparations were incubated for 2h at 25°C and the reactions were stopped by acetone precipitation and the addition of anti-oxidant. Both pH8.0 and pH2.7, EtOAc-soluble fractions derived from each incubation medium were reduced to dryness and re-dissolved in 400µl 25% MeOH. HPLC of pH8.0 fractions from samples 1 and 2 indicated little catabolism whereas sample 3 revealed a radioactive IMeOH-like peak and a smaller, less polar radioactive peak (Figure 23). The putative IMeOH peak co-chromatographed perfectly with a non-radioactively-labelled IMeOH standard. The IAA degradation pattern from sample 3 was very similar to that obtained in Experiment (d)(see Figure 20). No catabolic products were detected in the samples from the boiled

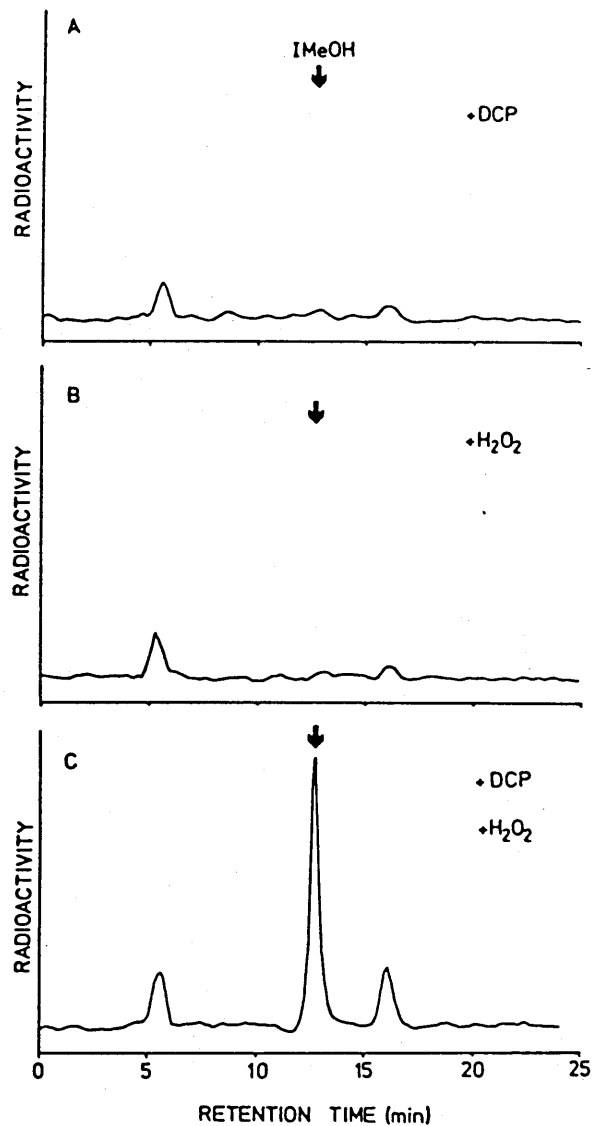


FIGURE 23

Reverse phase HPLC of extracts from cell-free system incubated with [2-¹⁴C]IAA. Analytical conditions: 25min gradient of 25-75% MeOH in 20mM pH6.3 ammonium acetate; Sample: (A) 1/4 aliquot pH8.0, EtOAc-soluble fraction from incubation medium containing only DCP; (B) as (A) but containing only H₂O₂; (C) as (A) but containing DCP and H₂O₂; Detector: radioactivity monitor, 32cps fsd.

enzyme and buffer control incubations and, except for a radioactive IAA peak, no peaks were observed in the pH2.7, EtOAc-soluble fractions from any of the samples.

3.3.3. ISOLATED CHLOROPLAST FRACTIONS

Similar experiments to those described in section 3.3.2 were carried out using freshly-isolated pea chloroplast fractions which were subsequently incubated with [1-¹⁴C]- or [2-¹⁴C]-labelled IAA. The chloroplast preparations were routinely assayed to determine their ability to undergo ¹⁴CO₂-dependent oxygen evolution. Incubations were initiated within 1h of the chloroplast isolation.

(a) Decarboxylation of [1-¹⁴C]IAA by Chloroplast Fractions.

90g f.wt. pea leaf tissue was harvested and an isolated chloroplast fraction was prepared by the method described in section 2.2 to yield a pellet corresponding to a total of 12mg chlorophyll. The pellet was resuspended in 10ml pH6.0 assay medium and stored in darkness at 4°C prior to use. Aliquots of chloroplast suspension were added to 4ml-volumes of isotonic assay medium, resulting in a chloroplast incubation medium corresponding to 0.14mg chl ml⁻¹. 1×10^5 dpm [1-¹⁴C]IAA was added to each vial together with 5×10^{-5} M DCP and 10^{-4} M H₂O₂, where appropriate, and the vials were incubated in darkness for

2h at room temperature while being shaken gently. Duplicate vials containing assay medium and co-factors but without chloroplasts, were also incubated. The assay system used was identical to that described in section 2.9 (see Figure 5). On completion of the incubation period, the vial inserts containing 400ul 0.25M KOH were removed and their outer surfaces were washed in MeOH. The amount of $^{14}\text{CO}_2$ absorbed by the KOH was determined by liquid scintillation counting. Incubations were carried out in duplicate and the mean values obtained are presented in Table 13.

TABLE 13

Decarboxylation of $[1-^{14}\text{C}]\text{IAA}$ by a chloroplast fraction. To each preparation was added 10^5 dpm $[1-^{14}\text{C}]\text{IAA}$.

| Preparation | $^{14}\text{CO}_2$ absorbed by KOH ($\times 10^3$ cpm) | |
|------------------------|---|-------------------------------|
| | + DCP, H_2O_2 | - DCP, H_2O_2 |
| Chloroplast suspension | 12.9 | 12.4 |
| Buffer control | 0.4 | 0.4 |

The results obtained from this experiment indicated that chloroplast fractions possessed the ability to decarboxylate $[1-^{14}\text{C}]\text{IAA}$ when incubated in darkness. The assay was carried out 4 times using different chloroplast preparations with similar

results.

(b) Light-mediated Decarboxylation by Chloroplast Fractions.

An experiment was carried out to determine whether light had any effect on the ability of the chloroplast fractions to decarboxylate $[1-^{14}\text{C}]\text{IAA}$. Aliquots of chloroplast suspension were added to 4ml pH7.6 or pH6.0 assay medium containing H_2O_2 and DCP, resulting in an incubation mixture corresponding to $0.12\text{mg chl ml}^{-1}$. After the addition of 1.5×10^5 dpm $[1-^{14}\text{C}]\text{IAA}$, the vials were incubated in the light for 3h at 25°C , on a gentle shaker. Controls were carried out by incubating vials containing chloroplast suspension in the dark, and vials without chloroplasts in the dark and light. On completion of the incubation period, the radioactivity absorbed as $^{14}\text{CO}_2$ in the KOH solution was determined by liquid scintillation counting. The results obtained are shown in Table 14.

It is evident from the results presented in Table 14 that light had a stimulatory effect on the ability of the chloroplast suspension to decarboxylate $[1-^{14}\text{C}]\text{IAA}$. Also, decarboxylation at pH6.0 was greater than at pH7.6, but the amount of $^{14}\text{CO}_2$ absorbed by KOH in the buffer control was also greater at pH6.0. This experiment was carried out several times with different chloroplast fractions and in each case, incubation of chloroplast suspensions in the light resulted in a significant increase in $[1-^{14}\text{C}]\text{IAA}$ decarboxylation compared with identical

incubations in the dark.

TABLE 14

Effect of light on the decarboxylation of $[1-^{14}\text{C}]\text{IAA}$ by a chloroplast fraction. To each preparation was added DCP, H_2O_2 and 10^5 dpm $[1-^{14}\text{C}]\text{IAA}$. Relative decarboxylation is expressed as a percentage of the total radioactivity added.

| Preparation | $^{14}\text{CO}_2$ absorbed by KOH ($\times 10^3$ cpm) | | | |
|-------------------------|---|------|------|-----|
| | LIGHT | % | DARK | % |
| chloroplast prep. pH7.6 | 23.9 | 15.9 | 1.0 | 0.7 |
| assay medium pH7.6 | 0.4 | 0.3 | 0.4 | 0.3 |
| chloroplast prep. pH6.0 | 66.4 | 44.3 | 8.3 | 5.6 |
| assay medium pH6.0 | 1.9 | 1.3 | 0.5 | 0.3 |

(c) Chlorophyll-sensitized Decarboxylation of $[1-^{14}\text{C}]\text{IAA}$.

It has been reported that photooxidation can occur when IAA is exposed to light in a solution containing chlorophyll (Koch et al., 1982). It is possible that the results obtained in Experiment (b) might be explained by this chlorophyll-sensitized degradation of IAA. For this reason, an experiment was carried out in which an aliquot of chlorophyll stock solution was incubated in assay medium in the light. 50g f.wt. 12 day-old pea leaf tissue was harvested and added to 150ml 80% acetone. After

homogenisation in a Waring blender at high speed for 10sec, the brei was vacuum-filtered to remove cellular debris and precipitated proteins. The filtrate was stored at -20°C in darkness for 12h and subsequently vacuum-filtered to remove water. After reducing the acetone extract to dryness in vacuo, it was redissolved in approximately 12ml ethanol, resulting in a concentration of 0.6mg chl ml^{-1} , and subsequently stored in darkness at -20°C until needed. Prior to use, the chlorophyll solution was concentrated to 1ml.

To 4ml volumes of pH6.0 assay medium containing DCP and H_2O_2 , was added 65 μl chlorophyll solution, resulting in an incubation medium containing $0.11\text{mg chl ml}^{-1}$. 65 μl ethanol was added to the controls vials without chlorophyll. After the addition of 1.4×10^5 dpm [$1\text{-}^{14}\text{C}$]IAA, the vials were incubated at 25°C for while being shaken gently. On completion of the incubation period, the vial inserts were removed and absorbed $^{14}\text{CO}_2$ was determined by liquid scintillation counting. Incubations were carried out in duplicate and the mean values obtained are presented in Table 15.

From these results, there appeared to be negligible chlorophyll-sensitized decarboxylation of [$1\text{-}^{14}\text{C}$]IAA. In all samples, less than 1.2% of the total radioactivity added was present in the KOH. Interestingly, both light- and dark-incubated vials in which co-factors had been added, contained less radioactivity

than the preparations without co-factors. This experiment was repeated using a concentration of $0.01\text{mg chl ml}^{-1}$ which was similar to the chlorophyll concentration in the cell-free system experiments (see section 3.3.2 (e)). Again, the results obtained indicated that negligible $[1-^{14}\text{C}]\text{IAA}$ decarboxylation had taken place.

TABLE 15

Chlorophyll-sensitized photo-degradation of $[1-^{14}\text{C}]\text{IAA}$. Incubations were carried out in duplicate and the mean values are given. To each preparation was added 1.4×10^5 dpm $[1-^{14}\text{C}]\text{IAA}$. Relative decarboxylation is expressed as a percentage of the total radioactivity added.

| Preparation | $^{14}\text{CO}_2$ absorbed by KOH ($\times 10^3$ cpm) | | | |
|--------------------------------------|---|-----|------|-----|
| | LIGHT | % | DARK | % |
| chl only | 1.6 | 1.1 | 0.7 | 0.5 |
| chl + DCP, H_2O_2 | 0.3 | 0.2 | 0.2 | 0.1 |
| Buffer + DCP, H_2O_2 | 0.2 | 0.1 | 0.1 | - |

(d) Preliminary Incubations with [2-¹⁴C]IAA.

Initial studies investigating the degradation of [2-¹⁴C]IAA by chloroplast fractions were carried out by analysing acidic, EtOAc-soluble fractions derived from the incubations (see section 3.1.3 (a)). A chloroplast suspension in pH7.6 assay medium containing 1mM ATP and 10mM HCO₃⁻ was incubated with 2.5 x 10⁶ dpm [2-¹⁴C]IAA for 45min in the light at 25°C. On completion of the incubation, ice-cold MeOH containing 20mM diethyldithiocarbamate and cold carrier was added and the preparation was subsequently ultrasonicated for 5min. The incubation medium was then subjected to extraction through a solvent series, as described in section 2.6 (a), and the acidic, EtOAc-soluble fraction was analysed by reverse phase HPLC with an on-line radioactivity monitor. On isocratic elution with 40% MeOH in 20mM pH3.5 ammonium acetate, an aliquot of sample revealed at least two substantial radioactive peaks in addition to [¹⁴C]IAA (Figure 24). Both peaks eluted after the [¹⁴C]IAA peak, indicating that they comprised of less polar compounds. Negligible breakdown of [2-¹⁴C]IAA occurred in the boiled chloroplast control, suggesting that catabolism was enzymic. Since no partitioning of the aqueous phase was carried out at pH8.0, it was not established whether any neutral or basic indole catabolites had been formed from [¹⁴C]IAA degradation. Consequently, all subsequent incubations with [2-¹⁴C]IAA were analysed by HPLC after the extraction of pH8.0 and pH2.7

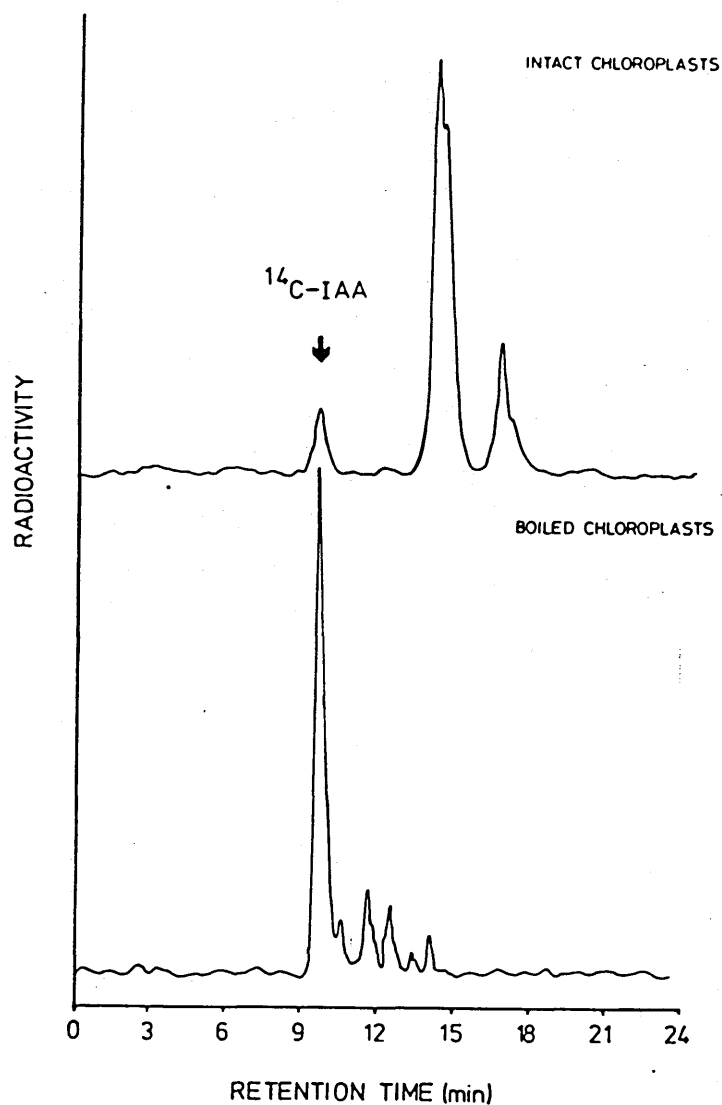


FIGURE 24

Reverse phase HPLC of extracts from chloroplast suspensions incubated with $[2-^{14}\text{C}]$ IAA. Analytical conditions: 40% MeOH in pH3.5 ammonium acetate, isocratic elution; Sample: (A) acidic, EtOAc-soluble extract from intact chloroplasts incubated $[2-^{14}\text{C}]$ IAA in the light for 45min; (B) as (A) but preparation boiled for 20min prior to incubation; Detector: radioactivity monitor, 16cps fsd.

fractions.

(e) Stability of IMeOH under acidic conditions.

A stock solution of IMeOH was prepared in HPLC-grade methanol and stored at -20°C in darkness. An aliquot of the standard was diluted with 40% MeOH in pH6.5 ammonium acetate and eluted isocratically onto a reverse phase column. Elution from the column in 40% MeOH in pH6.5 ammonium acetate produced a single fluorescent peak, indicating homogeneity of sample. A second aliquot of standard was diluted with 40% MeOH in pH3.5 buffer and immediately injected onto a reverse phase column which had been previously equilibrated with MeOH and pH3.5 ammonium acetate (40:60). Detection of the eluate resulted in a double peak at Rt 15.0min. A second aliquot from the same sample was injected onto the column within 20min of the previous injection. Fluorimetric detection revealed a double peak after 15.0min and a smaller, poorly-resolved peak at Rt 16.6min. The IMeOH standard had apparently broken down when transferred to acidic conditions.

(f) $[2-^{14}\text{C}]$ IAA Catabolites from Chloroplast Incubations.

To 10 ml volumes of pH7.6 assay medium containing DCP, H_2O_2 , ATP and HCO_3^- were added aliquots of chloroplast suspension, resulting in a final concentration of 0.4mg chl ml^{-1} . After the addition on 6.5×10^5 dpm $[2-^{14}\text{C}]$ IAA, the preparations were

incubated in the light at 25°C for 2h. Duplicate incubations were carried out in the dark. In order to determine whether non-decarboxylative degradation occurred in chloroplast fractions, an incubation was carried out feeding [1-¹⁴C]IAA for 2h in the light at 25°C. A summary of the experiment is given in Table 16.

TABLE 16.

Details of chloroplast incubation with [¹⁴C]IAA carried out in Experiment (f). See text for concentrations of co-factors.

| Preparation | Constituents of incubation medium |
|-------------|--|
| A | chlps + [2- ¹⁴ C]IAA + H ₂ O ₂ , DCP, ATP, HCO ₃ ⁻ LIGHT |
| B | chlps + [2- ¹⁴ C]IAA + ATP, HCO ₃ ⁻ LIGHT |
| C | chlps + [2- ¹⁴ C]IAA + DCP, H ₂ O ₂ LIGHT |
| D | chlps + [2- ¹⁴ C]IAA + H ₂ O ₂ , DCP, ATP, HCO ₃ ⁻ DARK |
| E | chlps + [1- ¹⁴ C]IAA + H ₂ O ₂ , DCP, ATP, HCO ₃ ⁻ LIGHT |
| F | buffer control + [2- ¹⁴ C]IAA + H ₂ O ₂ , DCP, ATP, HCO ₃ ⁻ LIGHT |

At the end of the incubation period, anti-oxidant was dissolved in each preparation before the addition of 10ml 80% acetone. The preparations were ultrasonicated for 3min and subsequently centrifuged at 2000 x g for 4min. The supernatants were retained and the pellets were resuspended in 2ml acetone in 0.1M pH8.0 phosphate buffer (1:1), agitated thoroughly for 3min and

re-centrifuged. The supernatants from both centrifugations were then combined and phosphate buffer was added to the preparations, resulting in a final volume of 40ml.

It was found from preliminary experiments that the addition of 10ml 80% acetone to the incubation medium (giving a final concentration of 40% acetone), resulted in acceptable recoveries of radioactivity. After ultrasonication and centrifugation procedures, the combined supernatants contained ca. 70% of the original radioactivity added. However, the advantage of this method was that the acetone concentration was low enough to allow a large proportion of the chlorophyll to sediment with the insoluble membraneous debris, resulting in a semi-purified supernatant fraction.

The preparations were adjusted to pH8.0, partitioned 5 times with 2/5 volumes EtOAc and the organic phase reduced to dryness. The aqueous phase was adjusted to pH2.7 with acidified phosphate and subsequently re-partitioned with EtOAc. Both pH8.0 and pH2.7 fractions were transferred to 500 μ l V-vials and dissolved in 25% MeOH in pH6.5 and pH3.5 ammonium acetate respectively. Prior to HPLC analysis, the vials were centrifuged at 2000 x g for 5min which sedimented the remaining precipitated chlorophyll in each sample.

The pH8.0, EtOAc-soluble fraction from each incubation was analysed by reverse phase HPLC. The solvent system used was MeOH in 20mM pH6.5 ammonium acetate, and gradient elution of a 1/4

aliquot of sample A revealed 4 predominant radioactive peaks (Figure 25). The compound eluting at Rt 4.4min corresponded to [2-¹⁴C]IAA which was known to partition slightly into the EtOAc at pH8.0. When collected and eluted isocratically in 35% MeOH in pH3.5 ammonium acetate, this peak co-chromatographed with a freshly-prepared [2-¹⁴C]IAA standard. The radioactive peak eluting at Rt 11.9min co-chromatographed with a non-radioactively-labelled IMeOH standard when co-injected. The IMeOH zone was collected, reduced to dryness and re-chromatographed in 40% MeOH in pH6.5 ammonium acetate. With fluorescence detection, this radioactive fraction again co-chromatographed with authentic IMeOH and peak heights were additive. A third peak which eluted from the column after 15.3min was approximately equal in size to the suspected [¹⁴C]IMeOH peak.

Both samples B and C produced HPLC traces which were very similar to sample A. When identical aliquots of sample were injected, there were no significant differences in the sizes of the radioactive peaks between samples (Figure 26). This suggested that no enhancement effect was produced when all 4 co-factors were present in the incubation medium. Sample D, which was derived from a chloroplast suspension incubated in the dark, produced an HPLC trace with a similar profile to the previous samples described. However, in this case, the sizes of the radioactive catabolite peaks were proportionately smaller (Figure 26). HPLC analysis of sample E produced a trace in which

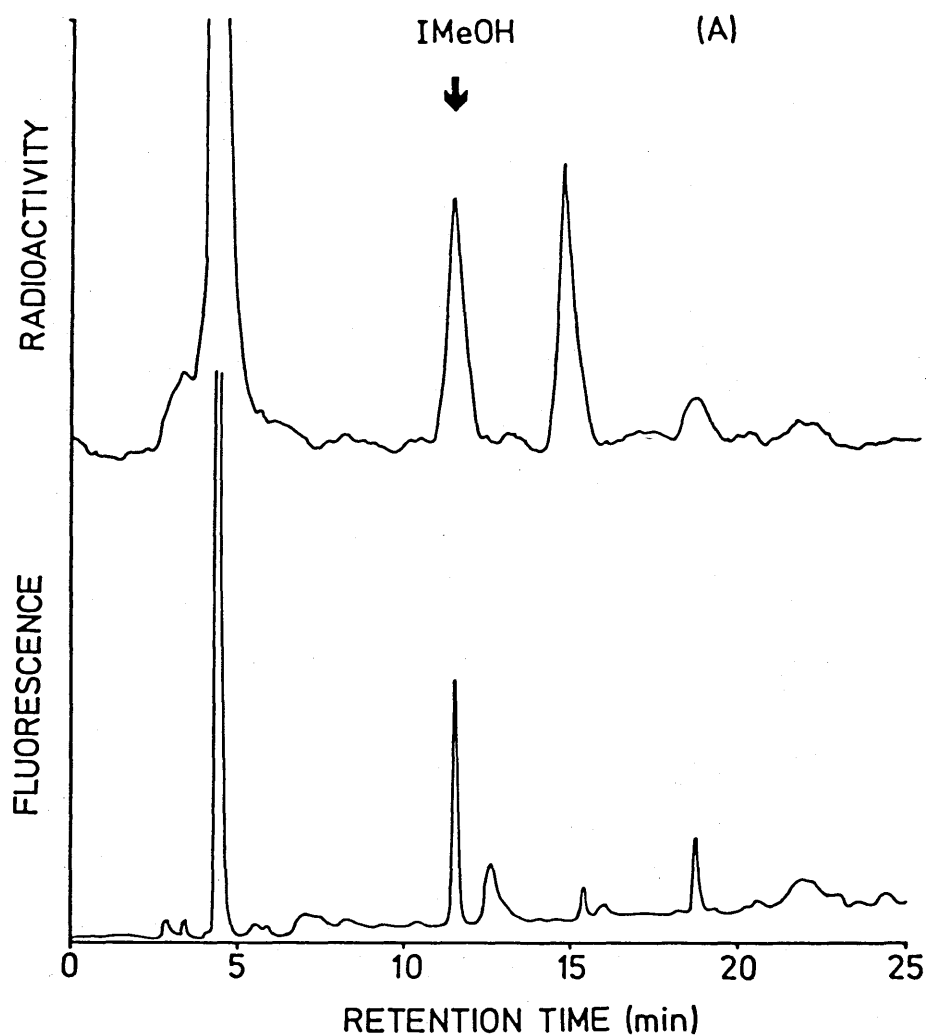


FIGURE 25

Reverse phase HPLC of extracts from chloroplast fractions incubated with [14 C]IAA. Analytical conditions: 25min gradient of 25-75% MeOH in 20mM pH6.5 ammonium acetate; Sample: 25% pH8.0, EtOAc-soluble extract derived from sample A in Experiment (f) (see Table 16); Detectors: radioactivity monitor, 16cps fsd; fluorimeter, excitation: 280nm, emission: 350nm.

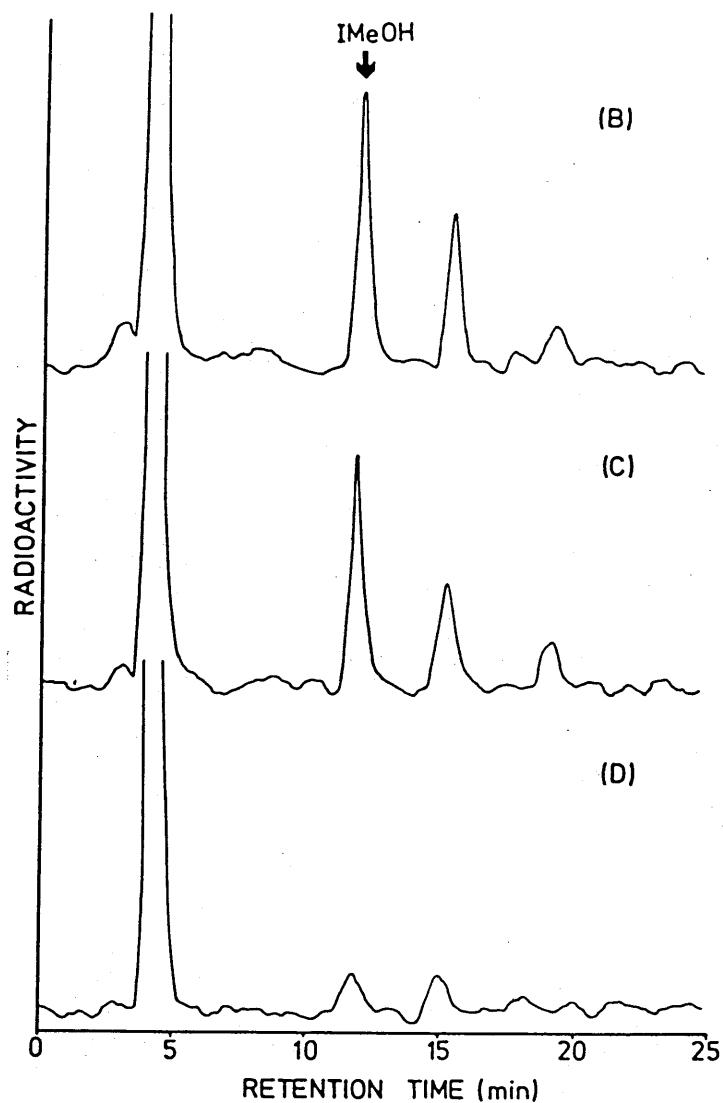


FIGURE 26

HPLC analysis of extracts from samples B, C and D from Experiment (f); Analytical conditions: 25min gradient of 25-75% MeOH in 20mM pH6.5 ammonium acetate; 25% pH8.0, EtOAc-soluble extract derived from each sample; Detector: radioactivity monitor, 16cps fsd.

no significant radioactive catabolite peaks were detected (Figure 27). However, an IMeOH-like fluorescent peak was present whose size would discount it from representing an endogenous component of the chloroplast suspension (see section 3.2). Gradient elution of the control incubation (sample F) revealed no apparent breakdown of [2-¹⁴C]IAA (Figure 27).

The pH2.7, EtOAc-soluble fraction derived from each incubation was analysed by reverse phase HPLC, using a solvent system of MeOH in pH3.5 ammonium acetate. Gradient elution of sample A revealed a single radioactive peak at Rt 15.0min (Figure 28). This peak co-chromatographed with authentic [2-¹⁴C]IAA, indicating that no acidic, EtOAc-soluble catabolites were formed by the chloroplast suspension. Analysis of the [1-¹⁴C]IAA-fed sample also revealed a single IAA-like radioactive peak. No catabolism was observed in the pH2.7, EtOAc-soluble fractions from any of the other samples.

(g) Catabolism of [2-¹⁴C]IAA by Broken Chloroplast Fractions.

Several incubations were carried out in an attempt to determine whether chloroplast preparations with broken envelopes metabolised IAA at a different rate from intact chloroplast fractions. The chloroplast suspensions were prepared by the usual method and stored for not more than 1h at 4°C prior to use. Two different methods were employed in order to rupture the chloroplast envelope. Samples were osmotically shocked by

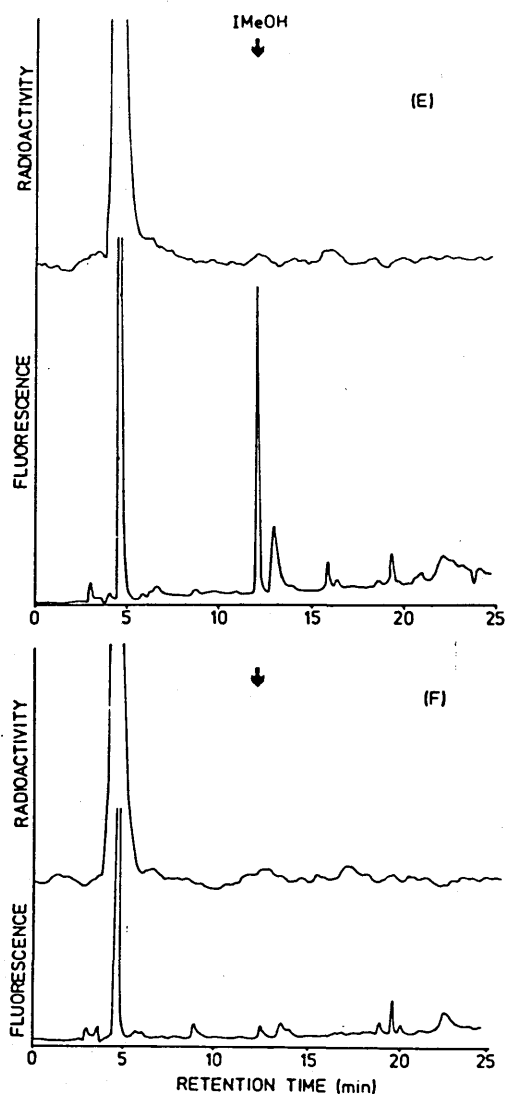


FIGURE 27

HPLC analysis of extracts from samples E and F from Experiment (f); Analytical conditions: 25min gradient of 25-75% MeOH in 20mM pH6.5 ammonium acetate; Sample E: pH8.0, EtOAc-soluble extract from chloroplast fraction incubated with $[1-^{14}\text{C}]$ IAA; Sample F: extract from buffer control incubation with $[2-^{14}\text{C}]$ IAA; 25% of each sample injected; Detectors: radio-activity monitor, 16cps fsd; fluorimeter, excitation: 280nm, emission: 350nm.

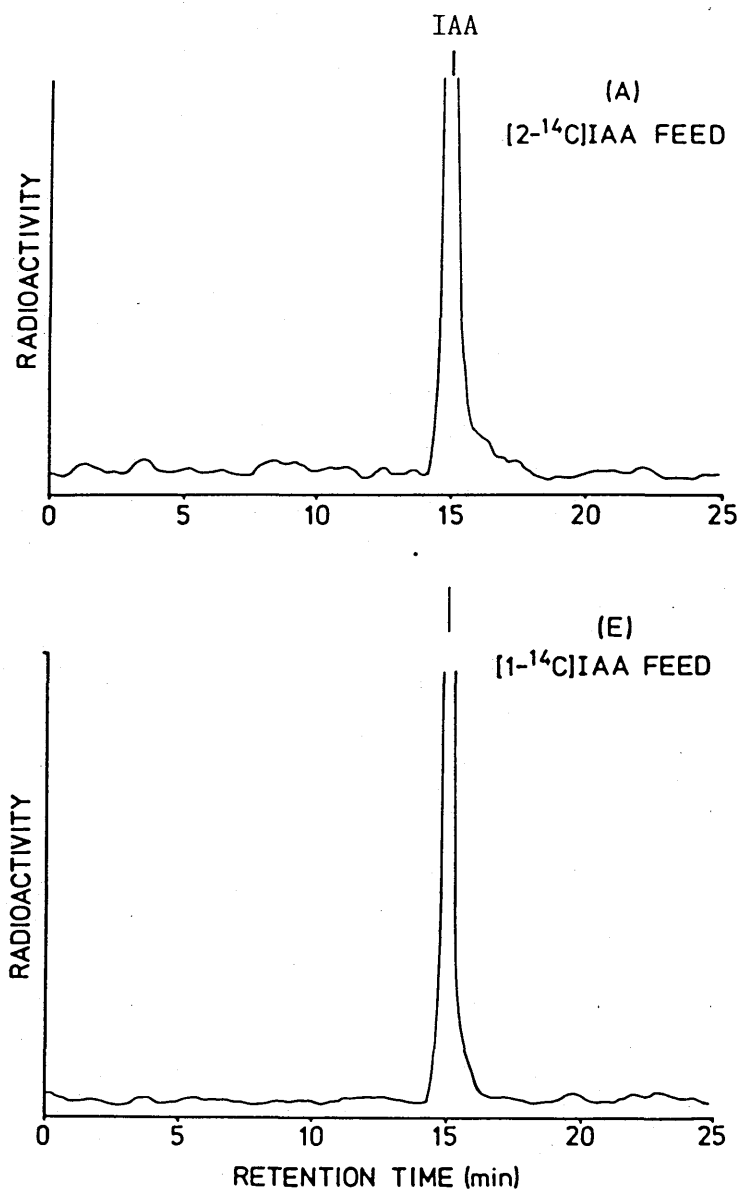


FIGURE 28

HPLC analysis of acidic extracts derived from samples A and E.
 Analytical conditions: 25min gradient of 25-75% MeOH in 20mM pH3.5 ammonium acetate; (i) 25% acidic, EtOAc extract from chloroplast suspension incubated with $[2-^{14}\text{C}]$ IAA; (ii) 25% acidic, EtOAc extract from chloroplast suspension incubated with $[1-^{14}\text{C}]$ IAA; Detector: radioactivity monitor, 16cps; radioactive IAA peaks approx. 2.5 times fsd.

diluting aliquots of chloroplast suspension in hypotonic assay medium (0.16M) prior to incubation. Alternatively, chloroplast suspensions were gently sonicated by placing the incubation flasks in an ultrasonic bath containing ice-cold water for 5min. Incubations were subsequently carried out as described in Experiment (f), and after extraction and purification, the extracts from each incubation were analysed by reverse phase HPLC.

In each case, similar results were obtained to those described in Experiment (e). The pH8.0, EtOAc fraction revealed an IMeOH-like radioactive peak but the presence of additional catabolite peaks was inconsistent. No significant catabolites were detected in the pH2.7, EtOAc fractions. There appeared to be no difference between intact and broken chloroplast suspensions with regards to breakdown pattern or levels of conversion.

(h) Time-course of [2-¹⁴C]IAA Degradation.

Experiment (e) was repeated several times using independently-isolated chloroplast fractions. In each case, a radioactive IMeOH-like peak was most evident in the light-incubated sample and to a lesser extent, in the dark-incubated sample. However, the occurrence of [¹⁴C]-labelled compounds eluting after IMeOH was inconsistent and in some cases, no other catabolites were detected at all. Consequently, a time-course

experiment was carried out in order to determine the sequential fate of [2-¹⁴C]IAA catabolism. Equal quantities of chloroplast suspension were incubated for 15, 30, 45 and 90min in the light with 1.5 x 10⁴ dpm [2-¹⁴C]IAA. HPLC analysis of the pH8.0, EtOAc extracts from each incubation revealed similar profiles to that observed in Experiment (f)(see Figure 25). In the extracts from 15, 45 and 90min incubations, two smaller radioactive peaks were observed eluting after the putative [¹⁴C]IMeOH compound although their peak heights could not be correlated with incubation time.

3.4. BIOSYNTHESIS OF IAA

3.4.1. INTRODUCTION

Experiments were initiated to investigate the possibility of IAA biosynthesis by pea chloroplast fractions. Both cell-free and isolated chloroplast systems employed in section 3.3., were utilized in this study. Incubations were carried out feeding compounds reputed to be precursors of IAA biosynthesis. Results were assessed by HPLC analysis of acidic, diethyl ether-soluble extracts derived from the incubations.

3.4.2. CELL-FREE SYSTEM

The freeze-dried, soluble and insoluble crude enzyme preparations used for IAA catabolism studies were also used for biosynthesis experiments. Several incubations also utilized α -ketoglutaric acid, pyridoxal phosphate (Moore and Shaner, 1967), ATP and bicarbonate as co-factors. The effects of adding these co-factors independently from each other were not investigated.

(a) Dialysis of Soluble Enzyme Preparation.

An experiment was carried out in order to determine whether significant levels of endogenous IAA were present in the soluble enzyme preparation after dialysis. A preparation was derived from an isolated chloroplast fraction, as described in

section 2.8. To the total volume of 6.7ml soluble protein solution was added 2×10^5 dpm [$2-^{14}\text{C}$]IAA. The preparation was then inserted into pre-washed dialysis tubing and dialysed against 2 litres 0.1M pH6.5 phosphate buffer. Two changes of buffer were made during the 36h dialysis period. At 12h intervals, 50 μ l-aliquots of solution were removed from the tubing and radioactivity was measured by liquid scintillation counting. The results obtained are presented in Table 17.

TABLE 17

Diffusion of [^{14}C]IAA from dialysis tubing. Radioactivity remaining is calculated as a percentage of the total [^{14}C]IAA added. Counts are corrected for chlorophyll quenching.

| Dialysis Time | Radioactivity remaining | |
|------------------|-------------------------|-------|
| | $\times 10^3$ cpm | % |
| 0h | 200.0 | 100.0 |
| 12h | 3.3 | 1.7 |
| 24h | 0.8 | 0.4 |
| 36h | 0.2 | 0.1 |

These results therefore suggest that negligible amounts of endogenous IAA remained in the soluble enzyme preparation after dialysis.

(b) Extraction of Soluble Enzyme Preparation.

In order to verify the results in Experiment (a), an extraction of the acidic indoles from the soluble enzyme preparation was carried out. To 10ml 0.1M pH6.5 phosphate buffer was added 50mg dry wt. soluble enzyme preparation. The solution was stirred continuously for 15min to ensure that all the preparation had dissolved. After adjusting to pH3.0, the buffer solution was partitioned with 2/5 volumes of freshly-distilled diethyl ether. The organic phase was subsequently reduced to dryness and re-dissolved in 400 μ l 35% MeOH in pH3.5 ammonium acetate. An aliquot of sample was injected onto a reverse phase HPLC column and the column eluate monitored by fluorescence detection. No IAA-like peak was observed when the fluorimeter was set to the range normally used for the detection of metabolites.

(c) Incubation of Cell-free System with L-tryptophan.

To a 2ml-volume of 0.1M pH6.5 phosphate buffer containing 200 μ g ml⁻¹ each of penicillin-G and streptomycin sulfate, was added 15mg dry wt. soluble enzyme preparation. After the addition of 50 μ g L-tryptophan in 50 μ l buffer, the preparation was incubated in the dark for 1.5h at 27°C. Incubations containing 0.1mM pyridoxal phosphate and 5mM α -ketoglutaric acid, and a boiled enzyme control containing co-factors were also carried out. The reactions were stopped by acetone

precipitation and the addition of anti-oxidant. After centrifugation, the supernatant fractions were adjusted to pH3.0 by adding acidified 0.1M phosphate and then partitioned 5 times with 2/5 volumes of diethyl ether. The acidic, diethyl ether-soluble fractions were subsequently reduced to dryness, re-dissolved in 25% MeOH in pH3.5 ammonium acetate and analysed by reverse phase HPLC. Elution from the column with a 25min gradient of 25-75% MeOH in pH3.5 ammonium acetate revealed no detectable IAA-like fluorescent peaks in any of the samples.

This experiment was repeated several times with various additives, e.g. NADH and ATP, and also feeding L-[³H]tryptophan as substrate. Incubations with insoluble enzyme preparations were also carried out. In each case, no IAA-like peak was detected during HPLC analysis of the acidic, diethyl ether-soluble fraction from the incubation mixture.

(d) Incubation of Cell-free System with IEtOH.

Similar studies to those described in Experiment (e) were carried out incubating IEtOH with the soluble enzyme preparation. 15mg dry wt. crude enzyme preparation was added to 2ml 0.1M pH7.6 phosphate buffer containing penicillin-G and streptomycin sulfate. The incubation mixture also included 0.1mM pyridoxal phosphate, 5mM α -ketoglutaric acid and 1mM ATP. After the addition of 30 μ g IEtOH, the preparation was incubated in the dark for 3h at 25°C. The appropriate control experiments were

also carried out. HPLC analysis of the acidic, diethyl ether-soluble extract produced a trace in which no IAA-like peak could be seen. This experiment was carried out twice, and also with insoluble enzyme fractions. In each case, no apparent IAA biosynthesis occurred.

(e) Incubation of Cell-free System with tryptamine.

To 1ml 0.1M pH7.6 phosphate buffer containing penicillin-G and streptomycin sulfate, was added 20mg dry wt. soluble enzyme preparation. The incubation medium also contained ATP, α -keto-glutaric acid and pyridoxal phosphate. After the addition of 30 μ g tryptamine in 50 μ l buffer, the mixture was incubated in the dark for 3h at 27°C. Test-tubes containing insoluble enzyme preparations and boiled enzyme controls were also incubated. On completion of the incubation period, the reaction was stopped by acetone precipitation and the addition of anti-oxidant. After centrifugation, the supernatant fraction was adjusted to pH3.0, partitioned with diethyl ether and the organic phase reduced to dryness under a stream of nitrogen. The acidic, diethyl ether-soluble extract was re-dissolved in 35% MeOH and eluted isocratically with 35% MeOH in pH3.5 ammonium acetate onto a reverse phase HPLC column. The data obtained are illustrated in Figure 29.

Incubation of the soluble E.P. with tryptamine resulted in a small peak at Rt 13.5min when the extract was analysed by

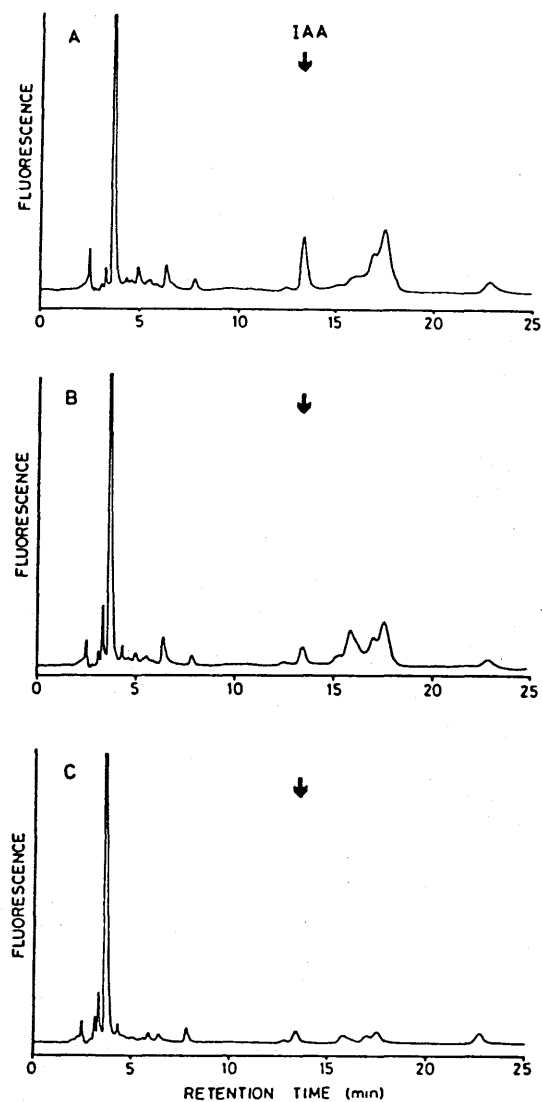


FIGURE 29

Reverse phase HPLC of acidic extracts from cell-free system incubated with tryptamine. Analytical conditions: 35% MeOH in 20mM pH3.5 ammonium acetate, isocratic elution; Samples: (A) 25% pH3.0, diethyl ether-soluble extract from soluble enzyme preparation; (B) 25% extract from insoluble enzyme preparation ; (C) 25% extract from soluble enzyme preparation boiled for 10min prior to incubation with tryptamine; Detector: fluorimeter, excitation: 280nm, emission: 350nm.

HPLC. A 1/4 aliquot from this sample co-chromatographed with 250pg authentic IAA. From the height of the IAA-like peak produced by co-injection, it was estimated that less than 0.02% conversion of tryptamine to IAA occurred. However, since HPLC analyses of the insoluble E.P. and boiled control samples also revealed detectable IAA-like peaks, the significance of this conversion was unclear.

3.4.3. ISOLATED CHLOROPLAST FRACTIONS

Experiments were carried out incubating freshly-isolated chloroplast suspensions with L-[³H]tryptophan. Due to the possibility of endogenous components confusing results, no experiments were carried out with non-radioactively-labelled substrates.

(a) Incubation of Chloroplast Fractions with L-[³H]tryptophan

A chloroplast suspension was prepared by the method described in section 2.2., and subsequently covered with foil and stored on ice prior to use. Aliquots of the suspension were added to 1ml-volumes of 0.33M pH7.6 assay medium containing ATP, HCO₃⁻, pyridoxal phosphate and α-ketoglutaric acid. After the addition of 3×10^6 dpm L-[³H]tryptophan, the preparations were incubated either in the dark or in the light for 3h at 27°C. A preparation without chloroplasts was also incubated in the light for 3h. On completion of the incubation, 30µg non-radioactively-

labelled IAA was added to each incubate, together with anti-oxidant. 1ml 80% acetone was added to preparation which was then gently ultrasonicated for 3min at 0°C. After centrifugation at 2500 x g for 4min, the pellets were washed, re-centrifuged and the supernatant fractions combined. Each sample was adjusted to pH3.0 with acidified 0.1M phosphate, partitioned 5 times with 2/5 volumes freshly-distilled diethyl ether and the organic phase subsequently reduced to dryness. After re-dissolving in 35% MeOH in pH3.5 ammonium acetate, the acidic, diethyl ether-soluble fractions were analysed by HPLC-RC. On isocratic elution from a reverse phase column, an aliquot from the light-incubated sample produced a trace with a radioactive peak at Rt 15.0min (Figure 30). On co-injection, this peak co-chromatographed perfectly with a [2-¹⁴C]IAA standard. The [³H]IAA-like peak was collected and an aliquot measured by liquid scintillation counting. The compound was estimated to contain a total of 5100 dpm radioactivity, suggesting a conversion of L-[³H]-tryptophan to [³H]IAA of 0.17% ^{applied substrate}. The remaining [³H]IAA-like fraction was methylated with diazomethane and re-chromatographed. A radioactive peak was eluted from the column after 19.5min, which corresponded to the Rt of an authentic [¹⁴C]IAA-Me standard (Figure 31). The acidic, diethyl ether-soluble fraction derived from the dark-incubated preparation also produced a radioactive peak at the Rt of [¹⁴C]IAA, although it was considerably smaller in height (Figure 30.(b)). The extract derived from the assay

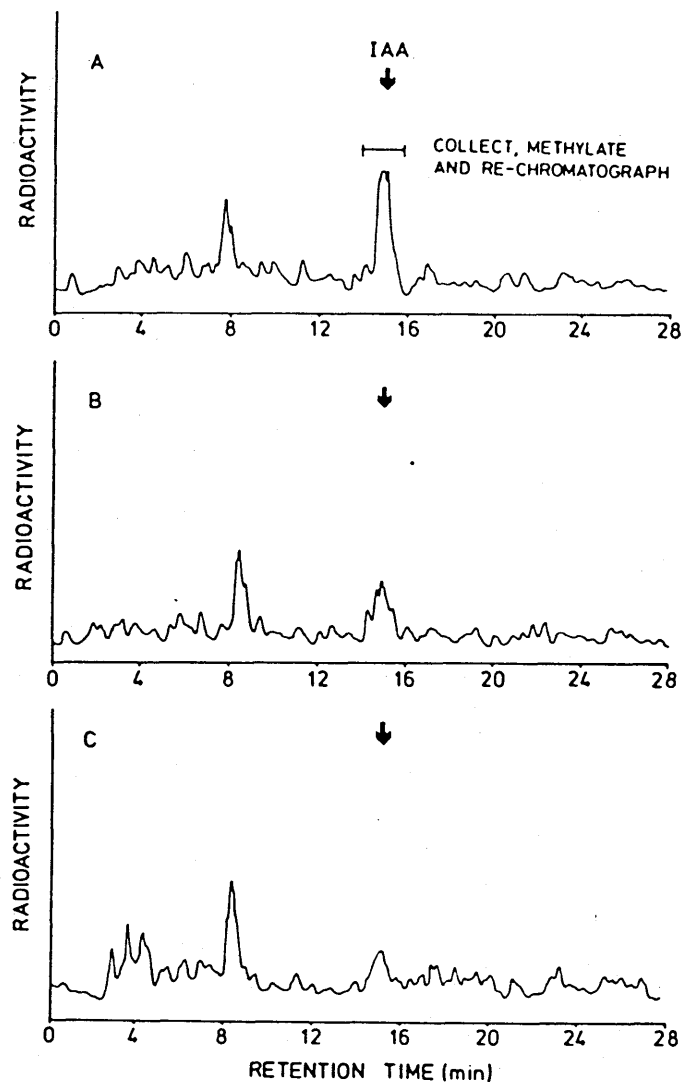


FIGURE 30

Reverse phase HPLC of extracts from chloroplast fractions incubated with [3 H]tryptophan. Analytical conditions: 35% MeOH in 20mM pH3.5 ammonium acetate, isocratic elution; Samples: (A) 1/8 aliquot acidic, diethyl ether-soluble extract from chloroplast fraction incubated in light with [3 H]tryptophan; (B) 1/8 aliquot acidic fraction from chloroplast fraction incubated in dark with radioactive tryptophan; (C) 1/8 aliquot extract from assay medium control incubated in light with radioactive tryptophan; Detector: radioactivity monitor, 16cps fsd.

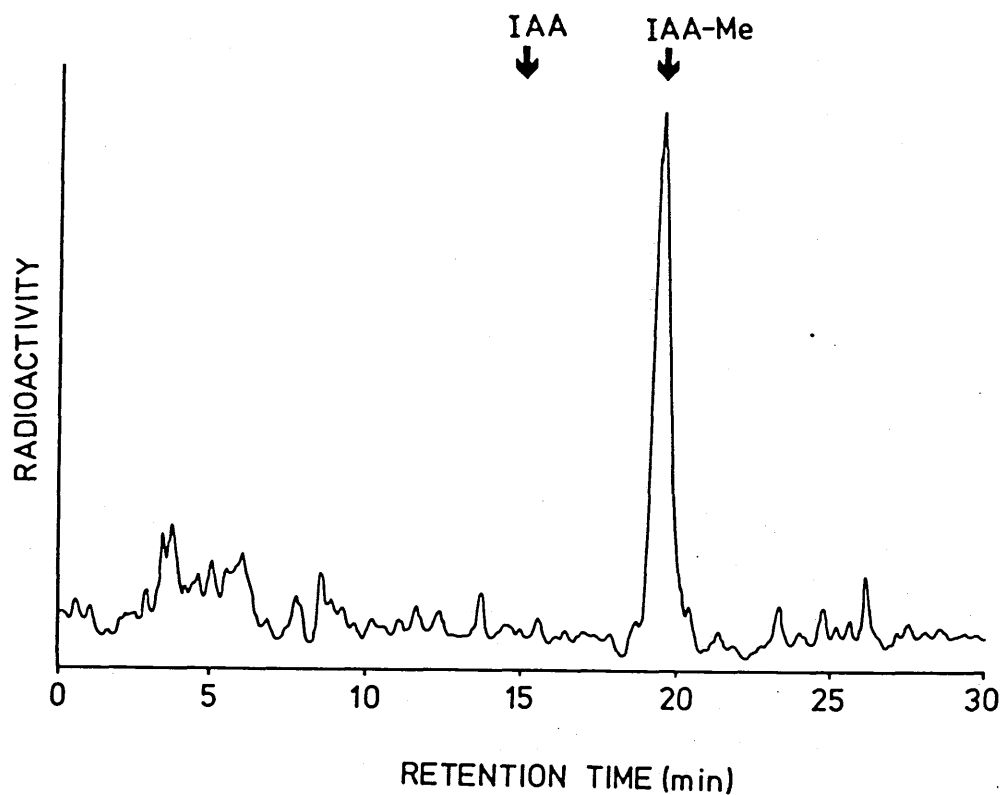


FIGURE 31

HPLC of methylated metabolite from chloroplast incubation with
[³H]tryptophan. Analytical conditions: 35% MeOH in 20mM pH3.5
ammonium acetate; IAA zone from sample in Figure 30 (A),
collected, methylated and re-chromatographed; Detector:
radioactivity monitor, 16cps fsd.

medium control revealed a small, but distinguishable radioactive peak at the R_t of IAA, indicating a conversion of ca. 0.05% and therefore suggesting the possibility of non-enzymic conversion of radioactive tryptophan to a [^3H]IAA-like compound (Figure 30.(c)).

(b) Additional Experimentation

Experiment (a) was repeated several times with independently isolated chloroplast fractions. In each case, there was no significant difference in metabolism between incubates containing co-factors and those without additives. On several occasions, boiled chloroplast control incubations resulted in an HPLC trace with a small, but detectable [^3H]IAA-like peak. In order to discount the possibility of this peak resulting from carry-over in the HPLC injection valve, a control experiment was carried out. 3×10^6 dpm L-[^3H]tryptophan was added to a boiled chloroplast preparation which was then incubated for 3h. After acetone precipitation and centrifugation steps, the supernatant fraction was adjusted to pH3.0 and subsequently partitioned 5 times with 2/5 volumes freshly-distilled diethyl ether. The organic phase was reduced to dryness, re-dissolved in 35% MeOH and subjected to reverse phase HPLC analysis. Prior to injection of the sample, the column and injection valve were washed through with 100% MeOH for 30min and after re-equilibration, a blank injection of 35% MeOH was made. With the radioactivity

detector set to 8cps, no radioactive peaks were observed. 50% of the control extract was subsequently injected and a small radioactive peak was observed at the Rt of authentic IAA. From the height of the peak, it was estimated that the total extract contained ca. 1000 dpm radioactivity, indicating a non-enzymic conversion of ca. 0.03%. No co-injection with a radioactive IAA standard was carried out.

DISCUSSION

4.1. INTRODUCTION

Compared with other areas of analytical phytochemistry, the analysis of plant growth substances is a technically complex subject. By far the major problem encountered when investigating endogenous hormones is their presence in trace quantities in complex multicomponent samples. Many procedures have therefore been developed in attempts to extract, purify and identify these compounds as accurately as possible. However, each class of plant growth regulator requires special attention since no universal extraction and purification procedures have yet been developed which afford equal efficiency and ease of use to all substances. The limitation of low endogenous levels becomes even more severe when investigating the presence or metabolism of phytohormones at the sub-cellular level. This, together with the fact that IAA and some of its precursors and catabolites are notoriously unstable, makes any investigation into the intracellular compartmentation of IAA a most formidable task.

4.2. EXTRACTION TECHNIQUES

The results presented in section 3.1. support previous reports that IAA is unstable under certain conditions. It was found that varying degrees of breakdown occurred at every step during sample purification by solvent partitioning, and although these observations were based on control experiments with IAA standards, it is reasonable to assume that breakdown occurs at

all steps during solvent extraction of crude plant extracts. The addition of anti-oxidant and cold carrier (when investigating radiolabelled feeds) virtually eliminated IAA degradation during extraction and partitioning. The effective use of anti-oxidants during the extraction of IAA has been described by Mann and Jaworski (1970), who also found that rotary film evaporation caused substantial losses of IAA by sublimation. However, their report dealt only with effecting good recoveries of endogenous IAA, whereas in most cases, losses of extractable IAA can be compensated for by the addition of an internal standard (Reeve and Crozier, 1980). When investigating IAA metabolism, where the products are usually not known, it is imperative to identify all possible steps of the extraction procedure where IAA can be non-enzymically degraded since failure to do so would allow erroneous conclusions to be made regarding the products of in vivo metabolism. For example, during extraction, IAA can be degraded into indole-3-carboxylic acid (Sandberg et al., 1984) or indole-3-methanol (Sundberg et al., 1984), both of which are reputed to be catabolic products of endogenous IAA. Although in both cases the percentage of non-enzymic conversions were less than 1% of the applied substrate, these results should not be ignored. While photo-oxidation has long been known as a process by which IAA can be degraded (see Moore, 1979, Yokota et al., 1980), no such light-mediated breakdown was observed in this present study, either when IAA was incubated

alone in solution, or in the presence of chlorophyll. It is therefore questionable whether light induces the destruction of IAA per se rather than through some additional factor present in the solution.

Results have shown that the widely-used solvent extraction and partitioning procedure is not ideally suited for the purification of indolic compounds. Buffer extraction followed by sample concentration using XAD-7 proved to be a more satisfactory alternative, and with adequate precautions to prevent enzymatic conversions, this method was preferable for qualitative analysis of endogenous indoles from pea seedlings. Although the majority of subsequent experimentation utilized buffer extraction techniques, a simpler, more rapid technique was later adopted for metabolic studies (see section 3.3.3(f)). The procedure involved protein precipitation in 40% acetone followed by centrifugation and subsequent EtOAc partitioning steps. The complete extraction, from cessation of incubation to storage of sample prior to HPLC, could be accomplished in less than 20min.

An enzyme immunoassay (Weiler et al., 1981) was employed to assess the efficiencies of each extraction method for the recovery of endogenous IAA from chloroplast fractions (data not presented). This, together with semi-quantitative HPLC data on the relative recoveries of metabolites, indicated that no appreciable differences in extraction efficiencies could be detected between each of the three methods employed. It would

appear that buffer extraction is a most useful technique for the isolation of IAA from large, crude extracts where the high load capacity of the XAD-7 column (Andersson and Andersson, 1982) provides a suitable method for concentrating samples. However, when dealing with small samples, such as 10-20ml chloroplast preparations, the speed and simplicity of acetone precipitation seems to be quite adequate. It must be noted, however, that the use of anti-oxidant in all buffers and solvents is essential irrespective of the method employed for the isolation of indoles.

4.3. ENDOGENOUS INDOLES

From the data presented in section 3.2.1, it can be concluded, with a high degree of certainty, that both indole-3-acetic acid and indole-3-ethanol are present as endogenous constituents of light-grown Pisum sativum seedlings. IAA has previously been identified and quantified by GC-MS in dark-grown pea epicotyls (Allen et al., 1982) where levels of $5-15\text{ng g}^{-1}$ tissue were determined. It is interesting to note that Hattori and Marumo (1972) failed to detect either IAA or its methyl ester in immature pea seeds, although in this case the limits of detection imposed by their analytical techniques were considerably higher. Despite the mass of literature relating to IAA and the pea plant (see Sutcliffe and Pate, 1977), all other evidence for the presence of endogenous IAA in peas rests on bioassays

and paper and thin-layer chromatography (McComb, 1977). It therefore appears that the report presented in this thesis is the first to positively establish IAA as an endogenous constituent of light-grown pea seedlings.

The data presented here also confirm the presence of IEtOH in seedlings of Pisum sativum. Endogenous IEtOH has previously been positively identified in cucumber seedlings (Rayle and Purves, 1967a) and more recently in Pinus sylvestris needles (Sandberg, 1984). IEtOH is reported to be a precursor of IAA biosynthesis (see Figure 3) and is described as a transitory side-reaction product (Schneider and Wightman, 1978), although its importance in the biosynthetic pathway of IAA has not been fully established. However, Sandberg (1984) has recently demonstrated that Pinus sylvestris needles were capable of converting both radiolabelled tryptophan and tryptamine to IEtOH, and IEtOH itself was metabolised to IAA. In view of these findings, subsequent studies may show that IEtOH has more significance in the in vivo biosynthesis of IAA than is presently implied.

On the basis of data obtained from HPLC, GC and GC-MS analyses of purified extracts, it can be concluded that chloroplast preparations from Pisum sativum contain detectable amounts of IAA. Although GC-MS analysis could not be carried out on the suspected IEtOH fraction, there is strong chromatographic evidence for the presence of IEtOH in chloroplast fractions. This tentative identification exemplifies the problems encoun-

tered when investigating the intracellular localisation of plant hormones, namely that the lack of sufficient organelle material will often prevent spectroscopic identification. While HPLC and GC analyses give encouraging support to the possibility of IEtOH in chloroplast fractions (see Table 12), data obtained by these analytical techniques should always be interpreted with caution. For example, it was found that only after GC-MS analysis could ICA be discounted from being a constituent of the IAA-like fraction from pea seedlings. Also, pH8.0 fractions from both chloroplast and pea seedling preparations appeared to contain an IMeOH component when analysed by normal phase HPLC. When subjected to reverse phase chromatography, the IMeOH-like samples did not co-chromatograph with authentic IMeOH.

It is quite possible that these limitations of certain chromatographic systems may prevail when analysing IEtOH-like fractions. Since as already stated, many of the presumed intermediates of IAA biosynthesis and catabolism are very unstable under certain conditions, failure to detect them does not preclude their natural occurrence. In addition, since no studies were carried out on the occurrence of IAA in preparations of other intracellular compartments, there is little reason to assume any physiological relevance for its detection in chloroplast fractions, other than it provides justification for studies on metabolic processes. It is interesting to note that Heilman et al. (1981) found that about

45% of the total free IAA in spinach leaves was localised within chloroplasts. They concluded that since the chloroplast envelope was easily permeable to IAA (Gimmler et al., 1981), the plant hormone accumulated within the organelle as a result of a pH gradient between the cytoplasm and the chloroplast.

As previously described (see section 1.3.3), the report by Wightman and Fregeau (1982) appears to be the only other investigation carried out where IAA has been detected in chloroplast fractions. However, it is most unfortunate that no analytical data were presented, since only by careful interpretation of both chromatographic and spectroscopic data can valid conclusions be drawn.

4.4. IAA CATABOLISM

Studies with cell-free systems have indicated that the major pathway of IAA catabolism in pea chloroplast fractions is by decarboxylative oxidation. Both soluble and insoluble or membrane-bound, crude enzyme fractions possessed the ability to decarboxylate [$1-^{14}\text{C}$]IAA. However, it is quite possible that the activity observed in the membrane fraction resulted from soluble enzymes which were present in the membrane pellet, due to insufficient separation. The use of co-factors in studies investigating IAA degradation has been well documented (see Semdner et al., 1980) and the most common used in horseradish peroxidase systems have been DCP, H_2O_2 and Mn^{2+} (e.g. Grambow

and Langenbeck-Schwich, 1983).

The results presented here (see Table 10) support the observation by Hoyle and Routley (1974) that the addition of Mn^{2+} in cell-free systems can give rise to inconsistent results. In contrast, the presence of both DCP and H_2O_2 resulted in a very marked increase in the decarboxylative capacity of the soluble enzyme preparation. Similar experiments with isolated chloroplast suspensions also resulted in the evolution of $^{14}CO_2$ from incubates fed with $[1-^{14}C]IAA$, although in this case, no enhancement effect was observed when co-factors were added. One suggestion for this is that co-factors were already present as endogenous components in the chloroplast fractions. Although IAA decarboxylation occurred in darkness, a significant increase was observed when chloroplast suspensions were incubated in the light. Sandberg et al. (1983) have reported on the effect of light on the rate of catabolism of $[2-^{14}C]IAA$ in protoplasts, a chloroplast-rich fraction and a crude cytoplasmic fraction from barley leaves. While the rate of degradation was slower in chloroplasts than in protoplasts, both preparations catabolised $[2-^{14}C]IAA$ more rapidly in the light than in darkness. Since IAA has been reported as being susceptible to photo-oxidation, results on light-enhanced catabolism should be interpreted with caution. Koch et al. (1982) demonstrated chlorophyll-sensitized photooxidation of IAA, using micellar preparations containing chlorophyll extracted from pea leaves. However, from

the data presented in Table 15, it is evident that a negligible proportion of IAA catabolism by pea chloroplast preparations was directly attributable to photo-oxidation.

The major products of the decarboxylative degradation of IAA are purported to be 3-methylenoxindole and indole-3-aldehyde (see Figure 1), although, as previously described (section 1.4.2), the majority of the investigations demonstrating the production of these catabolites have involved in vitro studies using cell-free systems or commercially-obtained horseradish peroxidase. In this present study, it has been demonstrated, by both cell-free systems and metabolically-active chloroplast suspensions, that IMeOH is the major detectable catabolite of IAA degradation in pea chloroplast fractions. It has been reported that indole-3-aldehyde is readily and reversibly reduced to IMeOH (see Sembdner et al., 1980). Quite recently, Grambow and Langenbeck-Schwich (1983) have concluded that IMeOH is the first detectable product of in vitro degradation of IAA in the horseradish peroxidase system, and that IMeOH is subsequently oxidised to indole-3-aldehyde.

Results presented here suggest that this is also the case in chloroplast fractions from Pisum sativum. It is possible that the smaller catabolite peak observed by HPLC analysis of several incubates was indole-3-aldehyde (see Figures 20, 25 and 26). However, this peak did not co-chromatograph with an indole-3-aldehyde standard, but since authentic indole-3-

aldehyde itself was very unstable and degraded readily when analysed by HPLC in both pH3.5 and pH6.5 systems, the possibility of this catabolite being indole-3-aldehyde cannot be dismissed with absolute confidence. IMeOH may therefore be the predominant catabolite which is subsequently converted to indole-3-aldehyde under unfavourable analytical conditions, although it is equally possible that indole-3-aldehyde is formed first, as a transitory intermediate which is then rapidly converted to IMeOH. The findings reported here therefore cast doubt on the validity of previous studies involving the detection of indole-3-aldehyde by less sophisticated methods.

The soluble crude enzyme contained no detectable IMeOH and therefore from HPLC and GC-MS analyses of extracts from incubates, it can be concluded that IAA was enzymically degraded to IMeOH. When [$^2\text{H}_5$]IAA containing excess sodium diethyldithiocarbamate was incubated in the cell-free system, no catabolites were formed (section 3.3.2 (g)). Since sodium diethyldithiocarbamate is a strong reducing agent, then this observation can be explained by the anti-oxidant depleting the system of O_2 , thus preventing the decarboxylative oxidation of IAA (see Moore, 1979). Grambow and Langenbeck-Schwich (1983) have also reported that the presence of phenolic compounds in horseradish peroxidase systems results in the formation of IMeOH, while in their absence, 3-hydroxymethyloxindole is produced. However, no evidence of this could be found in the

cell-free system from pea chloroplast fractions (Figure 23).

It is always a possibility that certain catabolites may not be detected in experimental systems due to inappropriate extraction or chromatographic techniques. Until recently, many of the polar metabolites of non-decarboxylative IAA catabolism in Zea experimental systems were not detected, due to their inability to partition into ether from acid solution (Bandurski, 1984). In this present study, since up to 94% decarboxylation was recorded (Table 10), it is very unlikely that any non-decarboxylated catabolites of IAA were produced in chloroplast systems. Also, no catabolite peaks were observed when acidic, ethyl acetate-soluble fractions from incubates were analysed by reverse phase HPLC. Since IMeOH is unstable under acidic conditions (3.3.3(e); also Sundberg et al., 1984) it is reasonable to assume that the catabolites found during preliminary incubations of chloroplast suspensions with [2-¹⁴C]-IAA (Figure 24) were IMeOH and a spontaneously-formed breakdown product.

Since IMeOH was not confirmed as an endogenous constituent of pea chloroplast fractions, the physiological relevance of the findings presented in this present study cannot be fully appreciated. However, it can be reasonably concluded that exogenous IAA is enzymically catabolised by isolated chloroplast preparations from pea, and that degradation is enhanced by light. The mechanism of this catabolism is by decarboxylation

and the predominant detectable catabolite is IMeOH.

4.5. IAA BIOSYNTHESIS

Several recent reports have demonstrated that chloroplasts from certain species possess the ability to synthesis IAA from tryptophan (see section 1.5.2). Soluble crude enzyme preparations from purified sunflower chloroplasts and mitochondria have also been used to demonstrate the biosynthesis of IAA (Wightman and Fregeau, 1982). Studies described in this thess were initiated to investigate the possible biosynthesis of IAA in chloroplast preparations from Pisum sativum . However, HPLC analysis of extracts derived from [³H]tryptophan-fed crude enzyme incubates revealed no detectable [³H]IAA. Incubations of the cell-free system with a high dose of unlabelled tryptophan, together with the co-factors, pyridoxal phosphate and α -keto-glutaric acid (Moore and Shaner, 1967; Wightman and Fregeau, 1982) also failed to produce any detectable IAA.

Since the cell-free system had previously been used for the investigation of IAA catabolism, the reaction system was not optimised for IAA biosynthesis studies, and therefore several factors may have been antagonistic to the formation of IAA. While incubations of the soluble crude enzyme preparation with IEtOH also failed to produce IAA, a small, but detectable IAA-like peak was observed when an extract derived from a tryptamine-fed incubate was analysed by reverse phase HPLC. A

conversion of less than 0.02% unlabelled tryptamine to putative IAA was observed. Since HPLC analysis of an extract from a dialysed enzyme preparation revealed no endogenous IAA-like peak (section 3.4.2(b)), it would seem that the putative IAA was a product of the applied tryptamine. However, since HPLC analysis of a boiled control sample also revealed a detectable IAA-like peak, no valid conclusion regarding IAA biosynthesis can be arrived at from these cell-free system investigations.

Isolated chloroplast suspensions appeared to possess the ability to synthesise [^3H]IAA when incubated in the light with [^3H]tryptophan. Radioactivity associated with putative IAA indicated a conversion of ca. 0.17% which compares favourably with the findings of Sandberg et al. (1982), who demonstrated a 0.15% conversion of tryptophan to IAA by isolated chloroplasts from barley. There appeared to be no significant differences in the amounts of putative IAA synthesised by chloroplasts incubated in the light and darkness (see Figure 30), which is consistent with studies on barley protoplasts and chloroplasts. However, in contrast with the report of Sandberg et al. (1982), a small but distinguishable radioactive IAA-like peak was observed during HPLC analysis of boiled chloroplast and buffer controls. As previously described, Epstein et al. (1980) found that drying [^3H]tryptophan in vacuo could result in a 30% conversion to a radiolabelled product with IAA-like TLC properties. In this present study, incubations with chloroplast fractions

showed only a 3-fold increase in putative [^3H]IAA biosynthesis over boiled chloroplast and buffer control incubations. Due to these non-enzymic conversions observed in control incubations, it would perhaps be premature for conclusions to be made concerning the biosynthesis of IAA by chloroplasts of Pisum sativum.

4.6. ADDITIONAL CONSIDERATIONS

While the natural occurrence of IAA in chloroplast fractions from Pisum sativum has been demonstrated in this thesis, no data on the accurate quantification of endogenous levels have been presented. Wightman and Fregeau (1982) have claimed levels of 88ng IAA mg^{-1} chl. in chloroplasts of sunflower, although details of the quantification method were not fully described. By reference to HPLC-fluorescence peak heights of authentic IAA standards, results presented in this current study indicate average recoveries of 4ng IAA mg^{-1} chl. in pea chloroplast fractions. However, since no internal standards were added, these estimates do not account for losses incurred during extraction and purification procedures. Consecutive estimations of several extracts indicated similar yields of IAA suggesting that the efficiency of the extraction procedure was consistent. Both [^3H]- and [^{14}C]IAA have received widespread use as internal standards for the quantitative determination of IAA levels in plant tissues. For several reasons, these were considered unsatisfactory for use in this present study. A commercially-

obtained standard of [^3H]IAA was found to be very unstable when stored undiluted and degradation appeared to vary depending on the volume of solvent used for working solutions. Since loss through breakdown of both endogenous IAA and [^3H]IAA could occur at different rates, recovery of radioactivity associated with IAA at the final stage of the analysis would not result in an accurate determination of endogenous IAA levels. [^{14}C]IAA was considered to be of too low specific activity for use as an internal standard in the analysis of endogenous IAA in chloroplast preparations. It was envisaged that the mass associated with detectable quantities of radioactivity would mask the low nanogram levels of endogenous IAA previously detected in extracts from chloroplast fractions.

In an attempt to overcome the problems encountered with the internal standard method for quantification, an enzyme immunoassay (see Weiler et al., 1981) was carried out on several semi-purified extracts from pea chloroplast fractions. However, results indicated levels of two orders of magnitude greater than previous estimations by HPLC-fluorescence. It is suspected that, due to inadequate sample purification, extracts may have contained interfering substances, resulting in erroneous results. A recent report has demonstrated certain limitations of immunoassays for the quantification of endogenous IAA in crude plant extracts (Sandberg et al., 1985). It was found that extracts from Pinus sylvestris needles contained contaminants that

adversely affected both the precision and accuracy of a radio-immunoassay for the quantitative estimation of IAA.

It is clearly evident that the methods employed for estimating recoveries of IAA after purification need to be thoroughly tested prior to carrying out quantitative analysis. Failure to do so may lead to claims of IAA levels in plant tissues which bear little relation to the actual endogenous levels present. Despite numerous reports on the quantification of plant hormones from various tissues, no data have yet been produced on the absolute levels of any phytohormone. The reason for this is that no extraction method can be guaranteed to extract all the endogenous hormone from the plant material. The efficiencies of different extraction methods are also extremely difficult to accurately assess, since the extraction of more hormone by one method over another may be merely the result of increased hydrolysis of bound or conjugated forms.

It has been suggested that the magnitude of the errors resulting from unknown extraction efficiencies may completely outweigh post-extraction errors (Horgan, 1981). As well as hydrolysis of bound hormones, the reverse situation can also occur where the extraction method results in the depletion of the free form of the phytohormone by conjugation etc. For example, it has been demonstrated that when plant material from Pisum or Zea is extracted by methanol or ethanol, esterification of IAA can occur during sample preparation

(Allen et al., 1982). Using methanol as a solvent, up to 10% of an IAA standard was converted into IAA-Me, while a similar conversion was observed to take place in methanolic plant extracts. This report by Allen et al. exemplifies the possibility of incurring erroneous results through the use of even well-established methods for the quantitative analysis of plant hormones, if prior qualification for their use has not been satisfactorily achieved.

The presence of contaminating sub-cellular material in an isolated organelle fraction must always be taken into consideration when formulating conclusions from compartmentation studies. Results have indicated that the isolated chloroplast fractions employed throughout this present study contained consistent levels of such contamination, as assayed biochemically with enzyme markers (section 3.1.1). It is not inconceivable for an organelle present as a minor contaminant to contain a 1000-fold increase in the levels of a phytohormone over that naturally occurring in the organelle being analysed. In fact, the concept of plant hormone compartmentation would support this possibility. While it has been suggested that the provision of complete balance sheets should be mandatory for publication of subcellular fractionation studies (Quail, 1979), the literature contains many reports of studies in which no account of organelle purity has been given.

Although the successful isolation of pea protoplasts was

not fully exploited in this present study (see section 3.1.2), it is considered that their use in future studies provides the most viable means of studying the compartmentation of plant growth substances. Since it has been demonstrated that isolated chloroplasts approaching 100% intactness and purity can be attained from the use of protoplasts (Nishimura et al., 1976), future studies on the intracellular localisation of IAA would benefit from such procedures. However, as described previously, lack of sufficient purified organelle material would have prevented quantitative analysis of endogenous IAA in this present study. Future experimentation could rectify this by carrying out quantitative analysis of IAA in pooled extracts from many chloroplast suspensions isolated from protoplasts. This procedure may also be practicable for quantitative studies on the biosynthesis and catabolism of IAA in pea chloroplasts, either by feeding substrates to protoplasts prior to isolation of chloroplasts or feeding after chloroplast isolation (see Sandberg et al., 1982, 1983). With careful considerations of factors affecting protoplast incubations e.g. pH, digestion medium and osmotic environment (Sandberg and Crozier, 1984), and adequate extraction methods, conclusive evidence based on accurate results should be obtained.

4.7. CONCLUDING REMARKS

For the reasons given above, it cannot be concluded that chloroplasts of Pisum sativum contain IAA per se, only that IAA is present and metabolised in chloroplast fractions of pre-defined purity. Studies carried out with such chloroplast fractions have demonstrated a distinct light-effect on the decarboxylation of IAA, whereas the possibility of this degradation resulting from chlorophyll-sensitised photo-oxidation has been discounted. While there can be little doubt as to the presence of both IAA and IEtOH in light-grown seedlings of Pisum sativum lack of GC-MS analysis prevents similar conclusions regarding endogenous IEtOH in chloroplast fractions from being made. Since the work presented here has mainly concerned IAA metabolism in isolated chloroplasts, then it should perhaps be regarded as a preliminary step in determining the compartmentation of IAA in Pisum sativum. However, it is envisaged that the results presented in this thesis provide justification for subsequent investigation into the possible physiological role of IAA in chloroplasts.

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