AN INVESTIGATION OF THE FATE OF EXOGENOUS AUXIN IN THE PEA, WITH SPECIAL REFERENCE TO THE FORMATION AND ROLE OF AUXIN CONJUGATES

A Thesis submitted for the degree of

Doctor of Philosophy

in the

University of Southampton

by

G.A. Rider

Department of Biology

University of Southampton

JUNE 1981

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

BIOLOGY

Doctor of Philosophy

AN INVESTIGATION OF THE FATE OF EXOGENOUS AUXIN IN THE PEA, WITH SPECIAL REFERENCE TO THE FORMATION AND ROLE OF AUXIN CONJUGATES

by GILL A. RIDER

The physiological significance of indole-acetyl aspartic acid (IAAsp) in auxin metabolism was examined with special reference to its role as part of a homeostatic mechanism to control hormone levels in pea seedlings (Pisum sativum L.).

A technique is described for the chemical synthesis of IAAsp. This was successfully used to obtain samples of the authentic compound. Techniques for the isolation, purification and concentration of IAAsp from plant tissues were examined in detail and a technique giving improved recoveries of the compound is described. Several techniques were used in an attempt to obtain more reliable identification of the presumed IAAsp in plant extracts. These included paper and thin-layer chromatography in several solvent systems; mass spectroscopy (MS); gas chromatography/mass spectroscopy (GC-MS) and acid and alkali hydrolysis of the isolated compound. Although the presumed IAAsp consistently co-chromatographed with samples of the authentic compound, technical problems during isolation and derivatization prevented its successful identification by MS and GC-MS.

When the presumed ^{14}C-labelled IAAsp was reapplied to intact pea plants, ^{14}C was found with indoleacetic acid (IAA) indicating that the conjugate could be hydrolysed at the presumed peptide bond to release the free hormone. The formation of IAAsp by plants may therefore represent a means of storing excess IAA in a protected and inactive form from which it can be released if tissue concentrations fall.

The time course of uptake, conjugation and export of ^{14}C-IAA following its application to the apices of intact pea seedlings was examined in relation to photomorphogenesis and dwarfism.

ACKNOWLEDGEMENTS

I should like to thank the following:-

Southampton University - Biology Department

Dr. David A. Morris - for his invaluable supervision and guidance during all stages of this thesis.

Mrs. Rosemary P. Bell - for excellent technical advice and continual encouragement.

Mrs. Anne Brixton - for expertly typing the thesis.

- and all those who provided so many friendships during my years at the department.

- Chemistry Department

Dr. David Evans - for his interest, discussions, the use of the chemistry department's facilities and the invaluable help of his postgraduate students especially Bill Speed.

Glasgow University

Dr. John R. Hillman - for willing involvement with the attempted GC-MS of IAAsp.

Additionally

Science Research Council - for providing the grant and also the CRAC summer school.

Mr. G.R. Elliott - for help during the preparation of this text.

THESIS INDEX

Section I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

AUXINS, THE REGULATION OF PLANT GROWTH AND DEVELOPMENT

Auxins are the group of natural and synthetic compounds defined by their ability to regulate plant growth, in particular cell differentiation, enlargement and division (Thimann, 1969). The stimulatory effects of auxin on plant growth vary considerably between tissues, and high concentrations of auxin generally bring about growth inhibition rather than stimulation. Roots are particularly sensitive to inhibition by auxins. In addition to the effects on plant growth, a large number of developmental processes in plants are regulated by auxins, including the initiation of adventitious roots, the differentiation of secondary vascular tissue and wound regeneration. Auxins play a major role in 'correlation' phenomona, that is in the overall organisation of plant processes, for example, apical dominance. Tropic responses partially result from the regulation of differential growth rates by auxins.

Indole acetic acid

Indole-3-acetic acid (IAA) was the first auxin to be identified (Kögl *et al.*, 1934) and is still regarded as the principal member of this group of plant hormones. IAA remains the only naturally-occurring auxin whose existence is definitely established. It has now been identified by colorimetric, chromatographic and chemical methods in a great many higher plants and is widely distributed throughout the plant kingdom.

It is known that the concentration of auxin available to the tissues can have a determining effect on growth and differentiation.

This behaviour is employed to bioassay auxins. It has also been shown that the number of xylem elements which formed in decapitated Coleus shoots was directly proportional to the amount of IAA applied to the shoots (Jacobs and Morrow, 1957). Investigations of fruit drop revealed a positive correlation between the concentration of auxin present in fruit extracts and the periods of fruit retention on the tree (Luckwill, 1959).

The regulation of hormone concentrations

An essential requirement of any hormone-mediated growth regulatory system is a mechanism for controlling the concentration of the active hormone within the plant. In addition to regulation of the levels of synthesis the plant has four known options for the removal or disposal of IAA. It can be 1) transported, 2) compartmentalized, 3) enzymatically degraded, as by the IAA-oxidase system, or 4) metabolically converted into inactive derivatives.

The major pathways of metabolism of both exogenous and endogenous IAA are still incompletely understood. The free IAA concentrations found in plant tissue may represent a balance of the processes outlined in Figure 1.

Assessment of the evidence for the control of active IAA concentration by compartmentalization has been made difficult by the loose terminology employed by authors. The confusion has arisen with the use of "binding". The concept of compartmentalization should refer to molecules held in different components of the cell and thus kept within separate systems. This may involve binding with protein. However, 'auxin-binding' has wider usage than in relation to compartmentalization, as binding may be associated with other

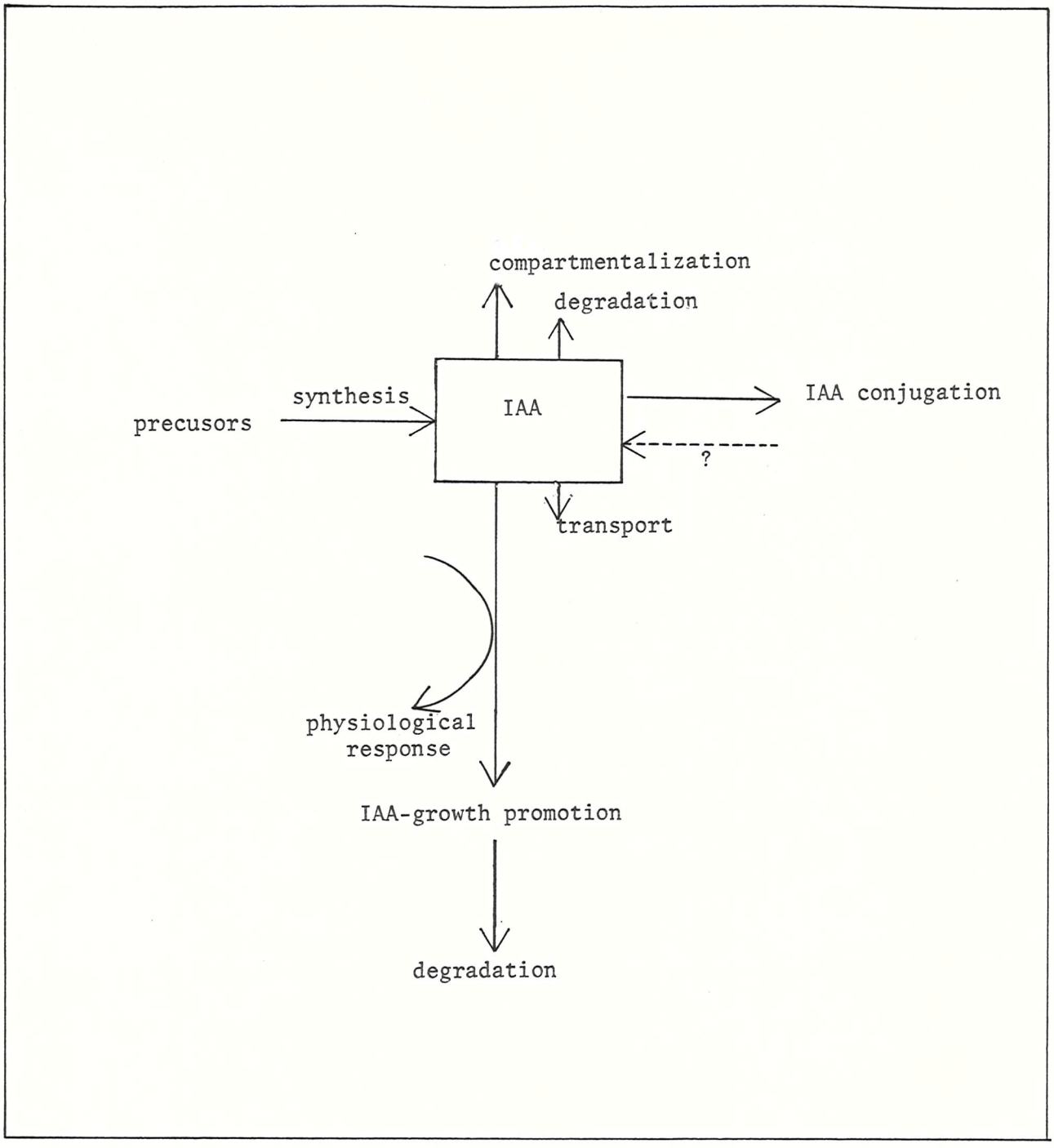


Figure 1 Scheme illustrating the possible mechanisms involved in regulating concentrations of free auxin in plant tissues (based on Cohen and Bandurski, 1978).

processes such as transport or action sites for auxin. There are many reports of the binding of IAA to proteins and confusion arises from a limited understanding of the processes in which these are involved. A non-transportable bound auxin was recorded by Winter and Thimann (1966) in Avena coleoptiles. Some auxin remained in the tissue even after a two-hour export period, at the end of which further diffusion of IAA from the tissue had largely ceased. From the extraction properties of this component they deduced an association of IAA with protein. Further characterization of auxin-protein complexes, described as "binding sites" for auxin and localized on membranes of the endoplasmic reticulum, have been published recently (Kasamo and Yamaki, 1966; Batt et al., 1976; Ray, 1977; Venis, 1977). It is now suggested that the binding of auxin to protein rather than representing compartmentalization, and thus removing and regulating free auxin concentration, may have a direct role in cell wall synthesis (for example, Davis, 1976). Ray, Dohrmann and Hertel (1977) concluded that auxin binding sites may be receptors for auxin action. Compartmentalized auxin may be represented by IAA stored, for example, in vacuoles, and the actual influence within the plant of such 'bound' auxin remains unknown.

Another possibility for the control of IAA concentrations indicated in Figure 1 is degradation. The regulation of plant growth and development by the oxidative degradation of IAA has been explored (Ockerse and Mumford, 1973). Jasdanwala, Singh and Chinoy (1977) analysed growth parameters of developing cotton hairs (Gossypium hirsutum) and divided fibre development into four distinct phases. Changes in oxidase and peroxidase activity showed that IAA catabolism while low during one phase, the elongation, increased four-fold during

a phase of secondary thickening. They suggested that the level of free IAA, thus regulated,might determine the termination of primary wall extension and the initiation of cellulose deposition in cotton fibre. A relationship has also been reported between IAA-oxidase activity and the growth periods of Lupinus hypocotyls (Sabater et al., 1978). A similar parallel between IAA catabolism and the rate of growth has been recorded in tobacco (Nicotiana spp.) callus tissue (Lee, 1971). Again the full significance of such regulation of concentration in terms of auxin action remains to be elucidated.

AUXIN CONJUGATES

A third pathway offering potential control of free IAA concentrations exists in the scheme outlined in Figure 1. The conversion of IAA into inactive metabolic derivatives affords an effective means for the rapid removal of auxin from the growth regulatory system and conjugation, unlike catabolism, does not destroy the IAA molecule. Such metabolites in which the IAA molecule is covalently linked to another compound, have been found in most higher plants examined.

Bandurski and Schulze (1977) assayed a wide range of plants including the seeds of oats (Avena sativa L.), coconut (Cocos nucifera L.), soyabean (Glycine max L.), rice (Oryza sativa L.), millet (Panicum milliaceum L.), kidney bean (Phaseolus vulgaris L.), buckwheat (Fagopyrum esculentum Moench.), wheat (Triticum aestivum L.) and maize (Zea mays L.), as well as the vegetative tissue of oats, pea (Pisum sativum L.) and maize. They concluded that all the plant tissues examined contained most of their IAA in the form of ester

or peptide derivatives. The predominant forms of IAA found in leguminous plants were amide conjugates, while esterified IAA was detected in most monocotyledons.

IAA-esters

The esters identified vary in structure, ranging from IAA esterified to myo-inositol, myo-inositol glycosides, arabinose and glucan polysaccharides found in Z. mays (Labaracca et al., 1965; Piskornik and Bandurski, 1972; Bandurski et al., 1977) to IAA-glucose detected in cereals and P.sativum by Zenk, 1961.

IAA-peptides

The conjugation of IAA with peptides has also been demonstrated to be a general reaction for many plant tissues. Reported examples of amide IAA include conjugation with aspartic and glutamic acids, glycine, alanine and valine in Parthenocissus tricuspidata Planch. crown gall callus tissue (Feung et al., 1976). Hutzinger and Kosuge (1968) also isolated the L-lysine conjugate of IAA from the plant pathogen Pseudomonas savastanoi Planch.

Additionally the conjugation of externally applied plant growth regulators, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and benzoic acid have been observed. Andreae and Good (1957) concluded that condensations with aspartic acid were not limited to IAA, but also occurred when P.sativum epicotyls were incubated with indolepropionic, indolebutyric, benzoic and 2,4-D acids. Aspartic acid conjugates of 2,4-D, benzoic and napthalenacetic acid (NAA) have also been reported often (Beyer and Morgan, 1970; Veen, 1972; Venis, 1972; Goren and Bukovac, 1973). Feung et al. (1971, 1972,

1973) also detected 2,4-D conjugates of seven amino acids, aspartic and glutamic acid, alanine, valine, leucine, phenylalanine and tryptophan in soyabean callus. An aspartic acid conjugate of the chlorinated natural auxins has also been identified (Hattori and Marumo, 1972). Hofinger and Lis (1977) reported that indolacrylic acid (IAcA), which they considered a true auxin in Lens culinaris Med., also formed a conjugate with aspartic acid.

Many plant tissues have been reported to accumulate primarily the aspartic acid conjugate. The major occuring peptidyl conjugate is indoleacetyl aspartic acid (IAAsp). IAAsp was first detected by Andreae and Good (1955) after the incubation of pea tissue in concentrations of IAA admittedly higher than physiological levels.

INDOLEACETYL ASPARTIC ACID

After Andreae and Good had identified IAAsp as a major metabolite of exogenous IAA in P.sativum tissue, they further demonstrated its formation in etiolated coleoptiles of oats, maize and barley (Hordeum vulgare L.); etiolated epicotyls of pea; etiolated hypocotyls of sunflower (Helianthus annus L.), cucumber (Cucumis sativus L.), and buckwheat; etiolated potato (Solanum tuberosum L.) sprouts, and the stems and petioles of pea, tomato (Lycopersicum esculentum Mill.) and cabbage (Brassica oleraceae L.) (Good et al., 1956). Examination of the conjugation processes in roots, where much lower concentrations of IAA have growth inhibitory effects, revealed that the tips of intact roots accumulated IAAsp at a rate considerably greater than epicotyl segments (Andreae and Van Ysselstein, 1960).

Natural occurrence of IAAsp

IAAsp was subsequently reported in the above and many other plant species, both as a naturally-occurring compound and as a metabolite of exogenously applied IAA. Row, Sanford and Hitchcock (1961) demonstrated the natural occurrence of IAAsp in tomato seedlings. IAAsp has also been identified in peach, Prunus persica Stokes (Weaver and Jackson, 1963), a lilaceae, Veratrum tenuipetalum L. (Olney, 1968) and P.vulgaris seedlings (Tilberg, 1974).

IAAsp, a metabolite of exogenous IAA

The formation of IAAsp from exogenous IAA has been reported in a wide range of plants, (see literature cited by Mollan et al., 1972; also Venis, 1972; Goren, Bukovac and Flare, 1974; Lau, Murr and Yang, 1974; Feung, Hamilton and Mumma, 1976). Many of these identifications rely heavily on the previously published Rf values of the sample. Often only one chromatography solvent system was employed and the co-chromatography of authentic IAAsp was not always recorded. Colour tests on the chromatograms and, more occassionally, biological activity have also been employed. Minchin and Harmey (1975) have identified IAAsp as the major form of IAA in barley endosperm using spectroscopic analysis. Andreae and Good (1955) demonstrated that alkaline hydrolysis of their plant metabolite released equimolar quantities of IAA and aspartic acid. Generally 'identifications' must be considered tentative unless supported by chemical analysis or at least co-chromatography with authentic sample in several solvent systems.

As well as the identification of IAAsp as a plant metabolite in whole plants it has also been found in various callus tissue cultures

and cell suspensions (Feung et al., 1976; Epstein et al., 1977; Maillard and Zryd, 1977; Rekoslavskaya and Gamburg, 1977; Bogers et al., 1978). The synthesis of IAAsp by a cell free system prepared from peas was reported by Lantican and Muir (1969). Venis (1972) was unable to repeat these observations. Higgins and Barnett (1976) demonstrated the conjugation of [1-^{14}C]IAA with aspartic acid in a tissue homogenate of etiolated pea seedlings. The conjugation of applied IAA with aspartic acid has been found in many tissues, although the mechanism for the formation of conjugates and their biological function remains unknown.

The formation of IAAsp

The presence of ATP and CoA has been described as essential for the formation of IAAsp and other auxin conjugates. Zenk (1960) demonstrated a model system using octanoate thiokinase, an enzyme isolated from liver mitochondria, that catalysed the formation of conjugates of IAA with amino acids, in the presence of IAA, the amino acid, CoA and oxygen. Two intermediate products in the formation of indoleacetylglycine were, a) indoleacetyladenosine monophosphate, from IAA and ATP, which in turn combined with CoA to yield b) indoleacetyl-CoA. Zenk showed that several amino acids could be acylated with both the indoleacetyl-CoA and indoleacetyl-AMP. IAA-ester formation is stimulated by ATP and CoA (Kopcewicz, Ehmann and Bandurski,1974). These authors also suggested that the reaction proceeds by the formation of adenyl-IAA, followed by the formation of CoA-IAA and then acylation of cycliotol or glucose. Feung et al. (1973) had previously described a similar pathway for the formation of 2,4-D conjugates in soyabean callus tissue. 2,4-dinitrophenol

(DNP), which uncouples oxidative phosphorylation thus leading to a depletion of ATP in the tissue, suppressed the formation of IAAsp in experiments performed by Lau, Murr and Yang (1974). Higgins and Barnett (1976) described the enhancement of IAA and aspartic acid conjugation by ATP, as indicated by increased levels of IAAsp with the addition of phosphoenol pyruvic acid and pyruvate kinase to the incubation medium.

It has also been reported that the rate of formation of IAAsp can be increased by pre-incubation with IAA (Venis, 1964; Südi, 1964; Hofinger and Lis, 1977; Rekoslavskaya and Gamburg, 1977). This indicated the existence of an L-aspartic acid acylase whose adaptive formation can be induced by pretreatment, and explained reported lag periods between the application of exogenous IAA and the formation of IAAsp. Veen (1967) stated that he could not find evidence for induction, as conjugates were formed even at the lowest concentrations of external NAA (0.1 μM). However, cycloheximide, puromycin and actinomycin-D, inhibit the conjugation of IAAsp, providing evidence for the involvement of m-RNA and protein synthesis in the process (Venis, 1964; Kang et al., 1971; Rekoslavskaya and Gamburg, 1977). Südi (1966) and Dahlhelm (1969) postulated an allosteric interaction of auxin and the protein regulating the synthesis of the enzyme(s) responsible for the production of the conjugate, to explain the auxin-induced formation of IAAsp. There exists no further characterization of the systems involved.

THE PHYSIOLOGICAL FUNCTION OF IAAsp

A review has been presented which demonstrates that much of the

IAA present in plant tissues is incorporated in conjugates. The role of such conjugates in the hormonal relationships of the plant is not understood, but the information that is available will be examined.

Auxin mobility

Hoad, Hillman and Wareing (1971) produced data suggesting that in intact plants IAAsp functioned as the major form of transported auxin. Their conclusions were based on the involvement of the phloem in auxin transport. Bonnemain (1971), Bourbouloux and Bonnemain (1973, 1974), Little et al. (1978) and Morris and Thomas (1978) considered it more likely that a cambial pathway for auxin (IAA) movement exists, and that this is the major transport pathway in the intact plant. Field and Peel (1971, 1972) and Field (1973) have assumed the mobility of IAAsp both laterally and in sieve tubes based on observations of willow (Salix viminalis L.) stems. These results may be accounted for by the IAA-mobile component being conjugated as it moved through the tissue. Their results were also in contradiction to those of Veen (1967), Eschrich (1968), Morris, Briant and Thomson (1969), and Lepp and Peel (1971), who found IAAsp to be immobile in pea, broad bean (Vicia faba L.), willow and Coleus (Coleus rhenaltianus L.). Bourbouloux and Bonnemain (1973) studied the transport of auxin from young pods of broad bean and found IAA was the main or only mobile auxin. The collection of IAAsp in agar receiving blocks from stem segments of willow and bean suggested the basipetal movement of this metabolite (Hoad et al., 1971; Patrick and Woolley, 1973). This may reflect the diffusion of conjugate from the cut surface of the segment where it was formed, rather than its transport. However, Morris, Briant and Thomas (1969) only found

IAA and indole-3-aldehyde in diffusates into agar from pea stems even though large amounts of IAAsp formed in the stem itself. The question of auxin-conjugate mobility should be more closely examined. Krul and Colclasure (1977) recommended further exploration of the mobility of IAA-glucoside, another conjugate for which contradictions exist. Hertel and Flory (1968) offered evidence that one auxin, NAA does not move in a complexed form. When corn coleoptiles were fed with NAA-glucose only the free acid (NAA) was detected at the protein binding sites which are believed to be the receptors associated with auxin transport. In spite of the inconsistency, it appears unlikely that auxin conjugates are a transported form of IAA.

IAAsp and ethylene interaction

Several authors explored the role of aspartic-conjugated IAA in relation to the inhibition of auxin transport by ethylene. The treatment of tissue with ethylene was shown to significantly stimulate IAAsp formation by Row, Sanford and Hitchcock (1961), Beyer and Morgan (1970) and Valdovinos, Ernest and Jensen (1970). However, they did find that the 'immobilization' of free IAA due to the increase in conjugate formation was not alone sufficient to account for the disruption of auxin transport by ethylene, and was more likely the result rather than the cause of the phenomena.

Kang et al. (1971) found that IAA-induced ethylene production and growth in excised segments of etiolated pea shoots paralleled the free IAA concentration of the tissue, which in turn depended upon the rate of IAA conjugation and decarboxylation. The control of free IAA concentration by such processes as conjugation and decarboxylation could therefore play an important role in the regulation of plant

growth, in this case by the alteration of ethylene production. It was suggested that IAAsp might be involved in the interaction of other plant hormones with IAA (Kang et al., 1971). Potato tubers treated with ethylene showed an increased rate of IAA conjugation and decarboxylation (Minato and Okazawa, 1978). It is possible that these systems provide a homeostatic mechanism balancing free IAA levels and ethylene production. Imaseki and Sakai (1973) reported the isolation of a proteinaceous inhibitor of ethylene production from mung bean (Phaseolus aureus Roxb.), which reversibly inhibited IAAsp formation. Possibly the enzymological mechanisms of ethylene production and IAAsp formation are interrelated and regulated at one stage by the same mechanism, affected by mung bean protein.

Kinetin was shown to have a synergistic effect on auxin-stimulated ethylene production in mung bean hypocotyls (Lau and Yang, 1973; Lau, Yang, Yung, 1973; Imaseki, Kondo and Watanabe, 1975). In contrast with the other references discussed in this section, an increase in ethylene production in kinetin treated tissue was associated with the suppression of IAAsp formation. At first these authors believed the regulation of IAA concentrations by IAAsp formation explained the interaction between kinetin and IAA-induced ethylene production. However, the case for such a regulatory role of IAAsp formation dwindled as they felt that the decrease in IAAsp conjugation and the corresponding increase in free IAA were not sufficient to account for the doubling of the ethylene production rate. Goren et al. (1974), and Lieberman and Knegt (1977) offered further data to demonstrate that conjugation was not the mechanism by which ethylene effects auxin behaviour or vice versa.

The biological activity of IAAsp

A further role of IAAsp which can be considered concerns its function as an active auxin. There is great variety in the reports of the biological activity of this conjugate. IAAsp had little activity in split pea curvature or pea stem elongation bioassays although it was a major IAA-metabolite in the tissue (Andreae and Good, 1955). IAAsp greatly exceeded IAA in the stimulation of tobacco pith cell suspension cultures, although it was inactive in the induction of cell division in the first 10-24 hours (Robinson et al., 1968). IAAsp also had a weak activity on potato and soyabean suspension cultures. Feung, Hamilton and Mumma (1977) demonstrated that the aspartate conjugate was highly active in the Avena coleoptile and soyabean callus bioassay. Thurman and Street (1962) observed that IAA and IAAsp possessed similar biological activity in the inhibition of growth of excised tomato roots and Tillberg (1974) found the promotion of Avena coleoptile elongation by IAAsp.

Generally, IAAsp appeared less active than IAA in tissues where it was a major metabolite. IAAsp may have direct biological function, or its activity may depend on the ability of the tissue to release free IAA from the conjugate. Feung et al. (1974) discussed this with reference to the activity of 2,4-D conjugates. They found higher activity of 2,4-D conjugates compared to 2,4-D, which may suggest that the conjugate is itself the required, or alternative, active form. However, the higher activity of the conjugate could be related to secondary factors such as altered uptake or metabolism. In dealing with exogenous hormones it becomes likely that the response may reflect differences in the uptake of the tested compounds rather than differences in activity. The lower activity of IAAsp in tissues

in which it is known to occur suggests that the conjugate may function as a storage form of IAA, releasing IAA only when the equilibrium favours free IAA and growth.

The regulation of IAA concentration: Two examples

The role of IAAsp in the regulation of 'critical' auxin levels has been explored in two recent references. Epstein, Kochba and Neuman (1977) reported the far greater formation of IAAsp in embryogenic callus than non-embryogenic ovular callus lines of "Shamouti" orange (Citrus sinesis). They found the non-embryogenic ovular callus tissue formed very little IAAsp and then only after much longer incubation periods. They concluded that 'by this mode of IAA removal' (IAAsp formation) the auxin level in embryogenic callus was reduced to a level inducive to embryogenesis. More recently, Liu, Gruenert and Knight (1978) discussed the involvement of IAA conjugates in tumorigenesis of Nicotiana species. They noted that genetic tumors arose most often in Nicotiana hybrids apparently in response to a signal aimed at potentially meristematic cells. The signal for tumorigenesis appeared to be a reduction in endogenous levels of IAA. The response is the activation of the IAAsp-synthetase system. The authors suggested that the release of IAA from a 'bound form' (IAAsp) played an important role in the activation mechanism causing tumorigenesis in the genetic hybrid plants. Thus both cases suggest the function of the IAAsp system is to provide regulation of the free IAA concentrations, the alteration of which results in a major developmental change within the tissue.

A biosynthetic intermediate

An additional function of IAAsp which may explain its presence, is that it represents a biosynthetic intermediate for the production of IAA. No confirmation of this exists, although thorough investigation has been made into the biosynthesis of IAA (Schneider, Gibson and Wightman, 1972 for example) without indication of such metabolic involvement of IAAsp. It has also been suggested that the role of auxin conjugates may be one of detoxification, to maintain the endogenous concentration of IAA at the desired level, thus controlling or balancing the physiological behaviour of the plant organs and tissues (Zenk, 1966). Various functions of IAA-conjugates have been discussed in this section, and from the examples cited here the most likely function of IAAsp is that of homeostatic control of auxin concentrations, the effects of such a system being seen in the examples of tumoregenesis and embryogenesis. The formation of IAAsp could therefore be seen as a storage of IAA, from which IAA can be released under conditions where higher free IAA concentration is required.

AN ANALYSIS OF THE EVIDENCE FOR HOMEOSTASIS

If a system exists for controlling auxin levels by the maintainance of an equilibrium between IAA and IAAsp, two categories of evidence might be expected. Firstly, the formation of IAAsp has been established; now the fate of IAAsp *in vivo* should be examined. Evidence concerning the release of IAA from IAAsp will be discussed. Secondly, having postulated such a system for the regulation of auxin levels, variation in the equilibrium must occur with environmental stimulation known to result in physiological response. The literature

concerning these two categories is now reviewed.

The fate of IAAsp

There is little information about the fate of IAAsp in plant tissue. Thurman and Street (1962) bioassayed chromatograms from exised tomato roots treated with IAAsp and showed two zones slightly stimulatory to coleoptile straight growth. The zone of strongest proportion corresponded to the Rf value of IAA and the second stimulatory zone occured near the Rf value of IAAsp. It was considered unlikely endogenous IAA could account for the stimulatory effect and so this may be indirect evidence for the release of IAA from exogenous IAAsp. Sakai and Imaseki (1973) reported a personal communication from Igari demonstrating the formation of free IAA from IAAsp in isolated pea epicotyl segments. However, incubations of wheat coleoptile segments with IAAsp failed to produce growth and hydrolysis of free IAA from IAAsp was not detected even at high concentrations of IAAsp (Klämbt, 1961). There is no other examination of IAAsp hydrolysis *in vivo*, nor have the enzyme(s) which might cleave IAAsp to release free IAA been characterized.

Two reports exist which describe the hydrolysis of other auxin conjugates. Feung *et al.* (1973) observed the hydrolysis of 2,4-D[1-^{14}C]-glutamate. When 2-4,D-glutamate was metabolized for 12 days by soyabean callus tissue it was converted to other amino acid conjugates such as 2,4-D-aspartate (approximately 50 per cent). 2,4-D (approx. 25%), unmetabolized 2,4-D-glutamate (approx. 20%) and some hydroxylated metabolites accounted for the rest of the activity. Studies by Bandurski and Schulze (1974) supported the occurence of the enzymatic hydrolysis of IAA-esters by *Z.mays*.

The lack of evidence for the in vivo hydrolysis of IAAsp may reflect the lack of examination of plant tissue in which the supposed equilibrium between IAAsp and IAA favours the former.

The sensitivity of the homeostatic mechanism to physiological conditions

One facet of the postulated system for the control of auxin levels, the existence of an equilibrium between IAAsp and IAA, is that it would be expected to relate to changes in the physiological status of the plant. Reports have demonstrated a stimulation of conjugation in conditions adverse to growth, and increased levels of free IAA in conditions favouring growth. It is believed that this system may account for differences between tall and dwarf peas. Lantican and Muir (1969) concluded that greater activity of the IAAsp-conjugation system in the dwarf pea than the tall pea, resulted in less free IAA for growth and thus a 'dwarfed' form. Experiments using pea internodes and Coleus explants produced results indicating that conjugation increased in tissues which had aged and were no longer growing (SÜdi, 1966; Veen and Jacobs, 1969). Davidonis, Hamilton and Mumma (1978) discussed the significance of their observation that older soyabean callus tissue regulated the level of free 2,4-D within the tissue at, what they described as, a "saturation" level by converting any excess 2,4-D to glutamic and aspartic acid conjugates. This would indeed suggest a homeostatic function of auxin conjugates, especially as the conjugation of 2,4-D occured to a far lower extent in younger tissues. Differentiated roots were found to maintain a "saturation level" in a similar manner to older callus tissue. The authors speculated that the tissues in which auxin induction of aspartate conjugation has been observed would show the

regulation of auxin levels by the mechanism of conjugation.

Levels of IAAsp have also been compared in plants grown in different light environments. Lantican and Muir (1969) and Muir (1970) reported observations that light-grown peas contained IAAsp, while dark-grown peas contained none. Morris (1970) concluded that light stimulated the synthesis of IAAsp after the application of IAA to pea seedlings, and Hofinger and Lis (1977) described the necessity of light for the biosynthesis of IAAsp by lentil epicotyls. By contrast, Tillberg (1974) found concentrations of endogenous IAAsp were similar in light- and dark-grown bean seedlings making it possible that the differences observed by Lantician, Muir, Morris and Hofinger reflect differences in the behaviour of plants to exogenous IAA in varying light environments. In fact IAAsp formation was shown not to vary with different light treatments in rice coleoptiles, a variation in absorption of IAA being noted. A light flash, which was sufficient to cause inhibition of growth of Z.mays seedlings, has been shown to cause a decline in the concentration of free IAA (-10 μg. kg^{-1}) and an increase in ester IAA (+ 9 μg. kg^{-1}) by Bandurski, Schulze and Cohen (1977). This does provide a clear demonstration of changes in hormone concentration, involving the formation of a covalently-bound hormone conjugate in response to environmental stimulation. This aspect of the conjugation mechanism has been investigated during the course of this project (Section V).

Interrelations between control mechanisms

The postulated regulation of free IAA concentration by conjugation may show some links to other known mechanisms of control. Cohen and Bandurski (1978) produced results indicating that conjugation

protected IAA from degradation *in vivo*. This would further implicate IAAsp in the storage of the IAA moiety. They found that horseradish peroxidase, and peroxidase activity from *Z.mays* and *P.sativum* degraded IAA but not the IAA-conjugate native to the plant from which the peroxidase was isolated. IAAsp has also been shown to be protected from IAA-oxidase in tobacco cell cultures (Rekoslavskaya and Gamburg, 1976). Such a system adds another dimension to the IAA-conjugate system, which can be seen as providing the means for rapid detoxification of excess IAA, and also a source of rapidly available IAA within the plant. It is possible there may be a fine balance between the different pathways for the regulation of IAA concentrations illustrated in Figure 1.

CONCLUSION

Metabolites which incorporate the intact IAA moiety have been identified in many plant species, often accounting for a high proportion of the IAA present in the tissue. Little is known about the significance of these compounds to the hormonal relations of the plant. Various suggestions concerning the role of IAAsp have been described above. The formation of the amide-conjugate in *P.sativum* provides a suitable system to investigate the physiological function of conjugation. It is proposed that IAAsp represents a storage product involved in the regulation of free IAA concentrations and that an equilibrium may exist between IAA and conjugated IAA, regulated by environmental and physiological conditions.

In the present investigation an attempt has been made to study

the involvement of IAAsp in auxin metabolism. Initially observations were made of the fate of IAAsp *in vivo*, in relation to the release of free IAA from the conjugate. To obtain information leading to a more comprehensive understanding of the function of conjugation in the regulation of plant development the role of IAAsp formation in photomorphogenesis in pea was examined. Attention has also been paid to the role of uptake and transport in the fate of exogenous IAA in intact plants. Other major objectives of the work described in this thesis were the development of an improved isolation technique for ^{14}C-IAAsp and the positive identification of this conjugate as a metabolite of IAA in the pea.

Section II

THE ISOLATION AND RE-APPLICATION OF IAAsp

THE ISOLATION AND RE-APPLICATION OF IAAsp

INTRODUCTION

The widespread occurrence of conjugated forms of IAA in plant tissues (see General Introduction) suggests that these compounds may play an important role in the regulation of free auxin levels in the plant. In a wide range of species IAAsp is both a major naturally-occurring conjugate of IAA and one of the main metabolites of applied IAA. In the case of exogenously applied IAA the conjugate is formed after uptake, as was demonstrated by Andreae and Van Ysselstein (1956) and this together with the observation that the IAAsp "synthetase" enzyme system may be induced by exposure of tissues to high external auxin concentrations (Andreae and Good, 1955; Row, Sanford and Hitchcock, 1961) supports the view that conjugation is a mechanism for regulating internal concentrations of IAA rather than part of the uptake mechanism itself.

Little is known about the fate of the conjugates once they have been synthesised. However, changes with time in the levels of ^{14}C-labelled IAAsp formed in intact pea plants following the application of [1-^{14}C]IAA to their apical buds suggested that the compound was re-metabolised to release IAA (Morris, Briant and Thomson, 1969). This led them to suggest that the formation of IAAsp was a mechanism for storing excess IAA in a protected form from which it can be released later if free auxin levels become sub-optimal.

If auxin conjugates form part of a homeostatic mechanism for controlling endogenous free auxin concentrations the means must

exist for their re-hydrolysis to release the active hormone molecule (see Fig. 1). A major objective of the present project was to investigate the fate in plant tissues of IAAsp, and to determine whether this compound could be hydrolysed by the enzyme systems of plant tissues to release free IAA.

The plant material chosen for investigation was the pea (Pisum sativum). Previous studies using the dwarf variety, 'Meteor', have indicated that following the application of [1-^{14}C]IAA to the apical bud a considerable proportion of the applied IAA was rapidly converted by the apical tissue to IAAsp (Morris, Briant and Thomson, 1969; Morris, 1970). An attempt was therefore made to isolate and purify [^{14}C]IAAsp from this variety and to study the metabolic fate of this compound following its re-application to unlabelled pea plants.

MATERIALS AND METHODS

Plant Material

Seeds of dwarf pea (P.sativum cv. 'Meteor') were surface sterilized in calcium hypochlorite solution (5%) for 15 minutes, washed thoroughly and allowed to soak overnight in running tap water. The seeds were planted singly in 60 mm pots containing vermiculite in a growth chamber at a temperature of 21°C in a 16-hour photoperiod. The illumination was provided by Philips 'Warm White' fluorescent tubes (intensity 7.7 k lux). The plants were watered regularly with half strength Hoaglands' mineral nutrient solution. Plants were selected for uniformity and experiments were carried out when the plants were 12 to 14 days

old. Generally leaf 4 was fully expanded at this stage of growth.

Application of ^{14}C-labelled IAA

[1-^{14}C]IAA (specific activity 57 mCi $mMol^{-1}$) was obtained either as the solid ammonium salt or in benzene/acetone solution from the Radiochemical Centre, Amersham, Bucks., U.K. When in solution a gentle stream of nitrogen was blown over the sample to evaporate the solvent and completely dry the sample. Prior to use, the [1-^{14}C]IAA was re-dissolved in a known volume of distilled water containing 0.1% polyoxyethylene sorbitan monolaurate ('Tween 20') to act as a wetting agent to aid uptake by the plant tissues. As a precaution the radiochemical purity of the compound was checked before use by paper chromatography.

The apical bud was exposed using a pair of fine forceps to open the stipules (usually of leaves 5 and 6) and the [1-^{14}C]IAA was applied to the bud with a previously calibrated 'Agla' micrometer syringe. A 5 µl droplet containing a known concentration of IAA was applied to each plant.

Extraction of the plant tissue

After the required translocation period the plants were dissected into individual organs, cut into small pieces and placed in cold 70% aqueous ethanol (Burroughs, U.K.) in Erlenmeyer flasks. The flasks were kept in darkness at 3°C ± 1°C during the extraction period. The extraction fluid was changed after 72 hours and again after 36 hours, after which the extracts were bulked and made up to a known volume. 3 x 1 ml aliquots were taken from each sample for liquid scintillation counting. 10 ml of a

1,4-dioxan-based fluid was used, containing 100g napthalene (BDH Chemicals Ltd., England), 0.3g 1,4-di-[2-(4-methyl-5-phenyloxazolyl)]-benzene (Dimethyl-POPOP) and 7.0g 2,5-diphenyloxazole (PPO) (both Koch-Light Laboratories Ltd., England) per litre. The samples were counted for 20 minutes in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3375). The counts recorded were corrected for background and quenching, measured by the external standard ratio method.

Additional ethanol extractions were sometimes employed. The tissue was homogenized in 70% ethanol using a Silverson blender, washed with more ethanol, and filtered on No. 1 Whatman filter paper with a Büchner funnel. The residue was further washed, the filtrate made up to volume and 3 x 1ml aliquots again taken for liquid scintillation counting. Finally NaOH extraction was also used to remove ethanol-insoluble ^{14}C. The residual tissue was placed in beakers and oven dried at 80°C to remove any remaining ethanol. 10ml of 10% NaOH was added to the dried residue and left for 12 hours at room temperature. 10ml of distilled water was used to rinse the beakers and the samples centrifuged at 2000g (3500 rpm) for 3 minutes. The supernatent was removed and aliquots counted.

Chromatography of the extract

Initially the chromatography system traditionally employed for auxin investigations was employed (for its development see Bennet-Clark, Tambiah, Kefford, 1952; Stowe and Thimann, 1953; Kefford, 1955). The extracts were reduced to a small volume by rotary evaporation under reduced pressure at 25°C. Approximately 20 µl of ethanol was added and aliquots spotted on Whatman No. 1

chromatography paper (40mm x 480mm) and dried in a cool airstream. The chromatograms were developed in the ascending direction in isopropanol: ammonia (0.88):H_2O (10:1:1 v/v) over a distance of 150 or 200mm. The chromatograms were air-dried and scanned using a Packard radiochromatogram scanner (Model 7201). Authentic samples of IAA (Koch-Light Laboratories Ltd., U.K.) and IAAsp (initially Calbiochem, U.S.A.) were always co-chromatogrammed as standards. UV radiation and Ehrlich's spray reagent (B.D.H., Dorset, U.K.) were used to locate the authentic compounds on developed chromatograms.

Elution of the radioactive material

The Rf value of authentic IAAsp in isopropanol:NH_3:H_2O (10:1:1 v/v) was 0.04. A radioactive spot was present on the chromatograms of the ethanol extracts from plants labelled with [1-^{14}C]IAA corresponding to IAAsp. To elute the compound this region of the chromatogram was cut out and the paper strip placed in 70% ethanol in the dark for 2-3 days under refrigeration.

Purification

The elutant was filtered through a fine scinter to remove paper fibres and concentrated under reduced pressure at 25°C. The purity of an aliquot was determined by paper chromatography and if necessary the sample was rechromatogrammed, eluted and reduced. The sample was dried and the residue taken up in a known volume of 0.1%'Tween 20'. The radioactive sample was then applied to the apical bud in a manner identical to that described for [1-^{14}C]IAA. The initial activity of the sample was determined by counting. 5 µl droplets were applied to the apical buds. The

activity applied per plant ranged from 2,500 dpm to 3,500 dpm in different experiments. The translocation time was 8 hours in continuous light at 20° ± 1°C. The plants were then extracted, and the samples chromatogrammed as before.

RESULTS

A poor recovery of [^{14}C]IAAsp was achieved from plants labelled with [1-^{14}C]IAA, high losses occuring during the purification procedure (Table 1). As low percentage returns of IAAsp were obtained it was necessary to use large quantities of [1-^{14}C]IAA. The radiochemical purity of [^{14}C]IAAsp so isolated is shown in Figure 2.

An examination was made of the uptake of [^{14}C]IAAsp by the apices to which it had been applied. Prior to extraction the apical surface was rinsed in 0.1% 'Tween 20' and distilled water to remove any compounds remaining on the surface. 55% of the activity applied to the plant was removed by this treatment, compared to only 7% removed when [1-^{14}C]IAA was applied under the same conditions (Section V, Fig. 32). The ^{14}C-material in the washings chromatogrammed as a single spot which corresponded to the Rf value of the applied compound (Fig. 3b).

Although pure radioactive compounds corresponding to IAAsp, eluted and purified in the same manner were applied to the apical buds, considerable variability was observed in the patterns of metabolism and transport. The application of [^{14}C]IAAsp was repeated ten times and typically activity was only detected in the apex, occasionally the internode below it (Internode 6) and the expanding leaf (Leaf 6) as illustrated in Table 3. In this

Table 1 ^{14}C-IAAsp recovered from light-grown, 14-day old dwarf peas 8 hours following the application of [1-^{14}C]IAA to the apical buds. The 70% ethanol apical extract was reduced, and the sample purified twice by paper chromatography (isopropanol:NH_3:H_2O, 10:1:1 v/v).

	dpm. plant^{-1}	% recovery
Applied Activity	403,225	100
Apical Extract	322,580	80
After Rotary Evaporation	15,548	3~~8~~
"^{14}C-IAAsp", activity eluted from Rf 0.04	15,121	3~~8~~

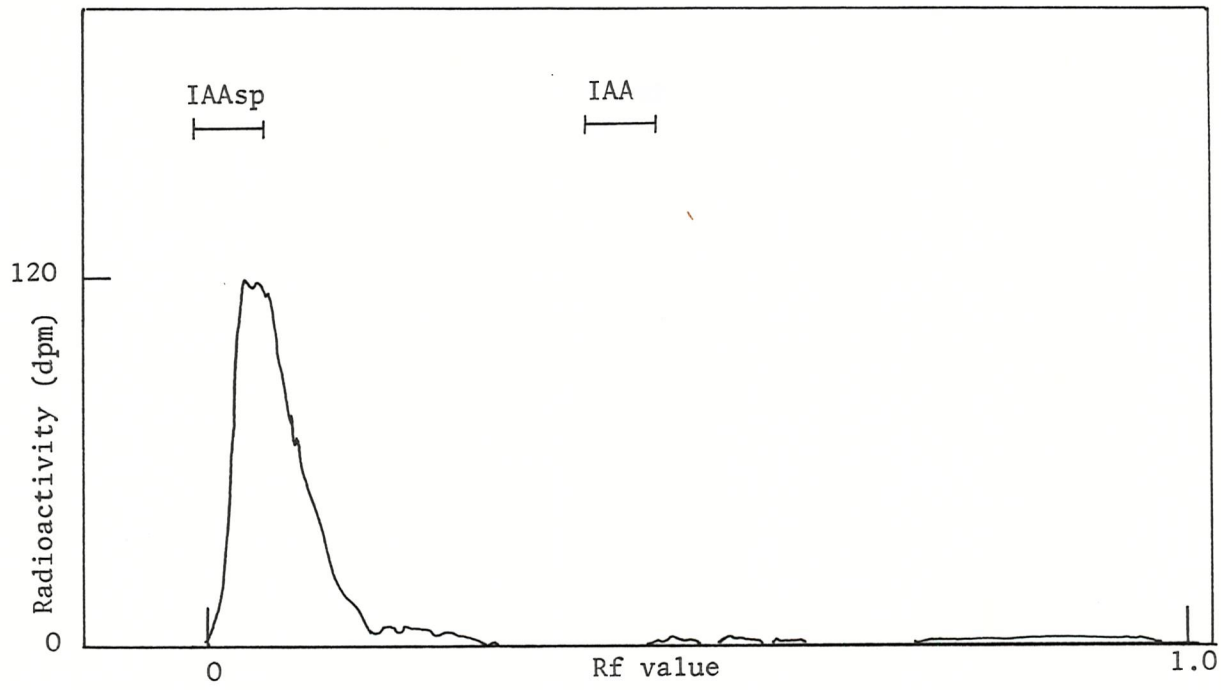
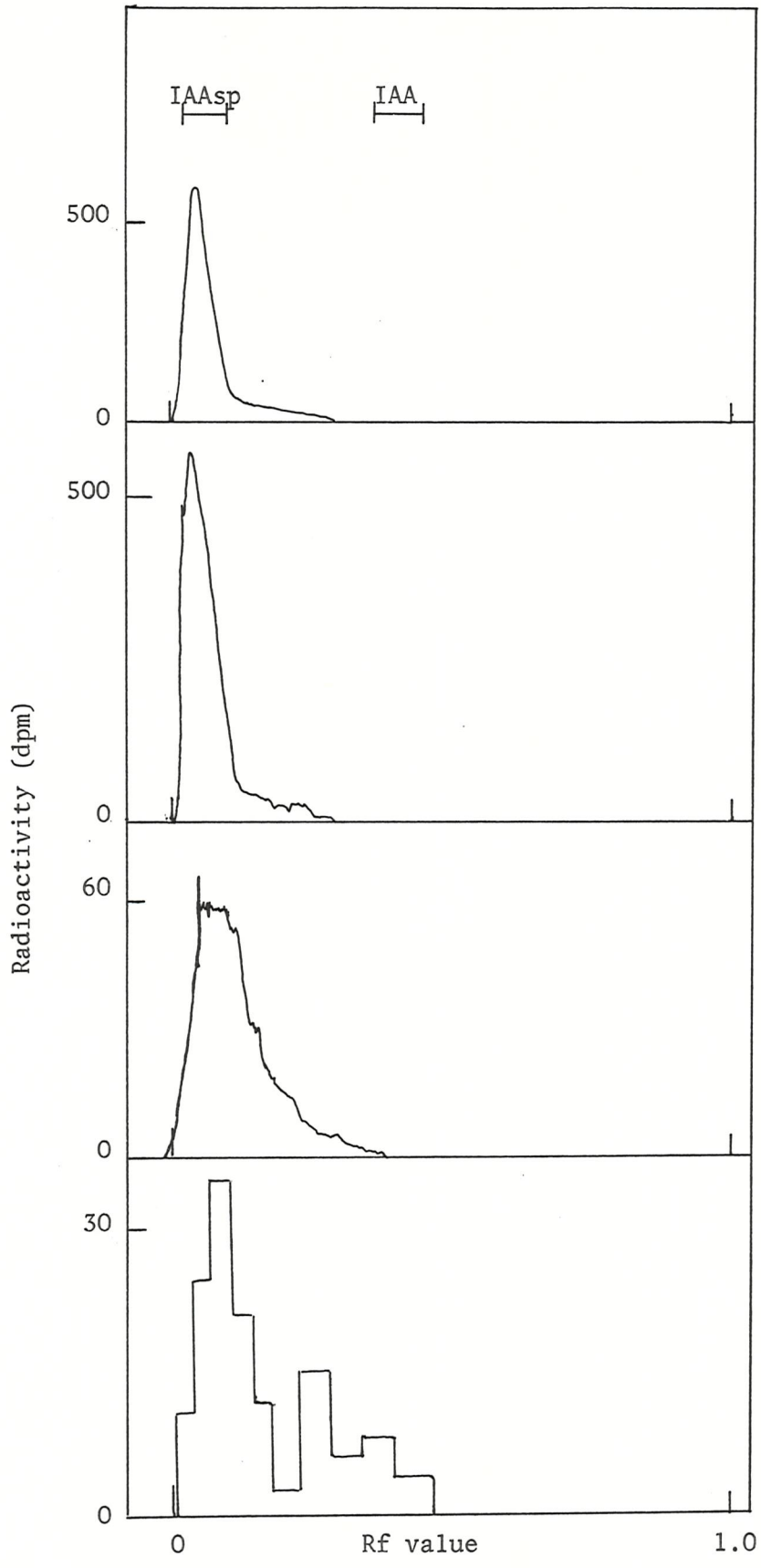


Figure 2 Radiochromatogram scan of ^{14}C-activity extracted, purified and concentrated 8 hours after the application of [1-^{14}C]IAA to the apical buds of light-grown 12-day old pea seedlings. The horizontal bars represent the position of authentic IAA and IAAsp. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v).



a) ^{14}C-activity applied to apical buds (scan).

b) ^{14}C-activity washed from the surface of apical buds (scan).

c) ^{14}C-activity extracted from apical buds (scan).

d) ^{14}C-activity extracted from Internode 3 (counted).

Figure 3 Radiochromatograms of the radioactivity applied, the apical wash and the 70% ethanol extractions 8 hours after the application of [^{14}C]IAAsp to the apical buds of light-grown 12-day old pea seedlings. The horizontal bars represent the position of authentic IAA and IAAsp. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v).

Table 2 ^{14}C-activity recovered by apical washing and 70% ethanol extraction of 12 day old, light-grown 'Meteor' peas 8 hours after the application of [^{14}C]IAAsp to the apical buds. The apical buds were surface washed prior to ethanol extraction. Applied activity = 7800 dpm. plant^{-1}

All values are an average of 3 replicates ± sd (dpm. plant^{-1})

	Mean ± sd
Apical wash	4302 ± 287
Apical extract	2701 ± 164
Leaf 3	0
Internode 3	20 ± 3.5
Remainder of Stem	1.9 ± 3.4
Cotyledons	0
Roots	0
Total activity recovered	7025
% recovery	90

Table 3 ^{14}C-activity recovered by 70% ethanol extraction of 14-day old, light-grown 'Meteor' peas after the application of [^{14}C]IAAsp to the apical buds. The apices were not washed prior to extraction.
Applied activity = 3550 dpm. plant^{-1}
All values dpm. plant^{-1}

	Harvest time (h)		
	2	5	8
Apical Extract	2562	2301	2310
Leaf 6	0	104	340
Internode 6	52	33	75
Rest of plant	0	0	0
Total activity recovered	2614	2438	2725
% recovery	74	69	77

experiment 15% of the activity recovered from the plant had been exported after eight hours. Unfortunately the amount of transported activity was often too low to identify the labelled compounds. Where a measure of uptake was recorded it was found that less than 1% of the [^{14}C]IAAsp that entered the apex was exported (Table 2). Only on one occasion was appreciable (29%) ^{14}C-activity exported from the apex (Table 4).

There would appear to be a clear link between the amount of transport and the metabolism of the [^{14}C]IAAsp applied. There was no evidence in any of the experiments for the metabolism of [^{14}C]IAAsp within the apex (Fig. 3c, Fig. 4, Fig. 5). The ^{14}C-material extracted from the apex chromatogrammed in isopropanol:NH_3:H_2O (10:1:1 v/v) (IAW) at Rf 0.04, identical to authentic IAAsp. Typically the major proportion of the radioactivity recovered from the stem co-chromatogrammed with IAAsp (Fig. 3d). A small peak corresponded with the Rf value of authentic IAA and an unidentified peak occured at Rf 0.28 in IAW. On the one occasion when there was significant transport of the ^{14}C-material (Table 4), conversion of IAAsp to IAA was clearly demonstrated (Fig. 5) in all parts of the plant except the apex. [^{14}C]IAA represented the majority of the radioactivity extracted from the older foliage leaves and lower stem in this experiment.

DISCUSSION

The results described above reveal considerable variability between experiments in the ability of the plants to metabolise the applied IAAsp to other compounds. Similarly, differences occurred

Table 4 ^{14}C-activity recovered by 70% ethanol extraction of 14-day old, light-grown 'Meteor' peas 8h following the application of ^{14}C-IAAsp to the apical buds. The apical buds were not washed prior to extraction. Applied activity = 2500 dpm. plant^{-1}

	Dpm. plant^{-1}
Apex	1684.0
Stipules Leaf 6	39.9
Rest of Leaf 6	140.0
Internode 6	245.9
Leaf 5	48.2
Leaf 4	27.2
Leaf 3	18.8
Stem (Internodes 1-5)	118.3
Roots	64.0
Total activity extracted	2386.3
% recovery	95

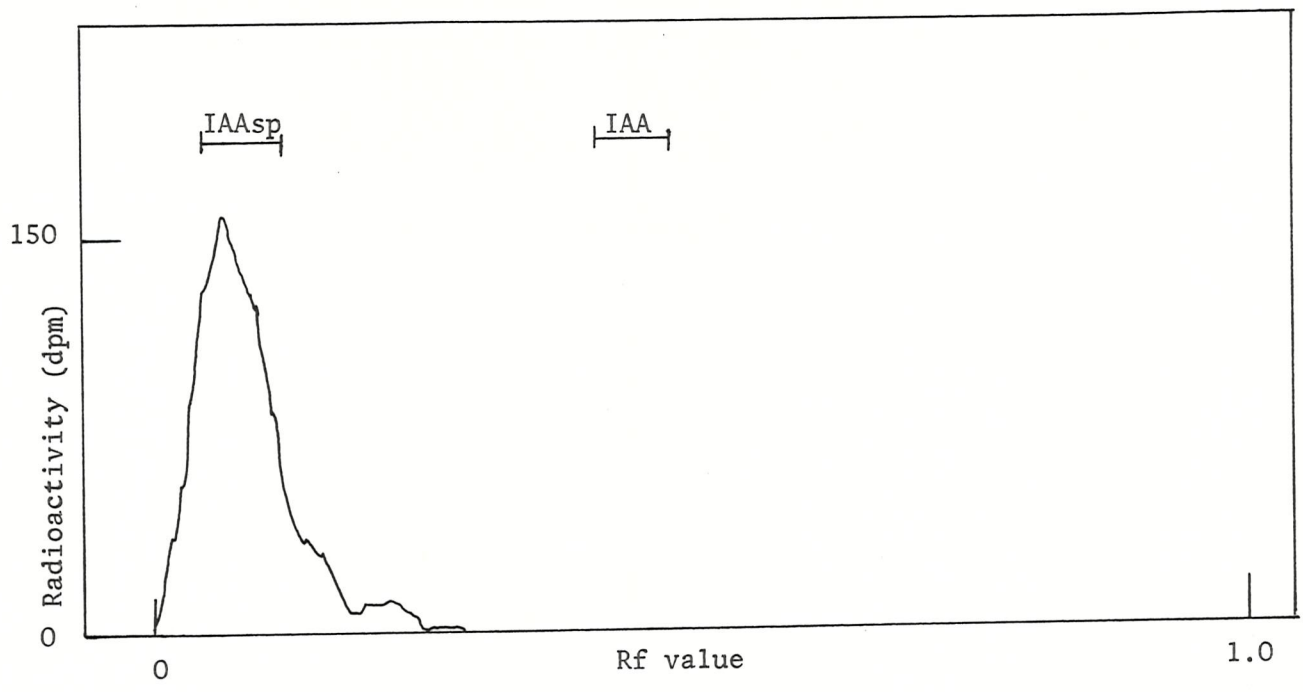


Figure 4 Radiochromatogram scan of the ^{14}C-activity extracted by 70% ethanol from the apex of 14-day old light-grown 'Meteor' peas, 5 hours after the application of [^{14}C]IAAsp to the apical buds. The chromatogram was developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars represent the positions of authentic IAA and IAAsp.

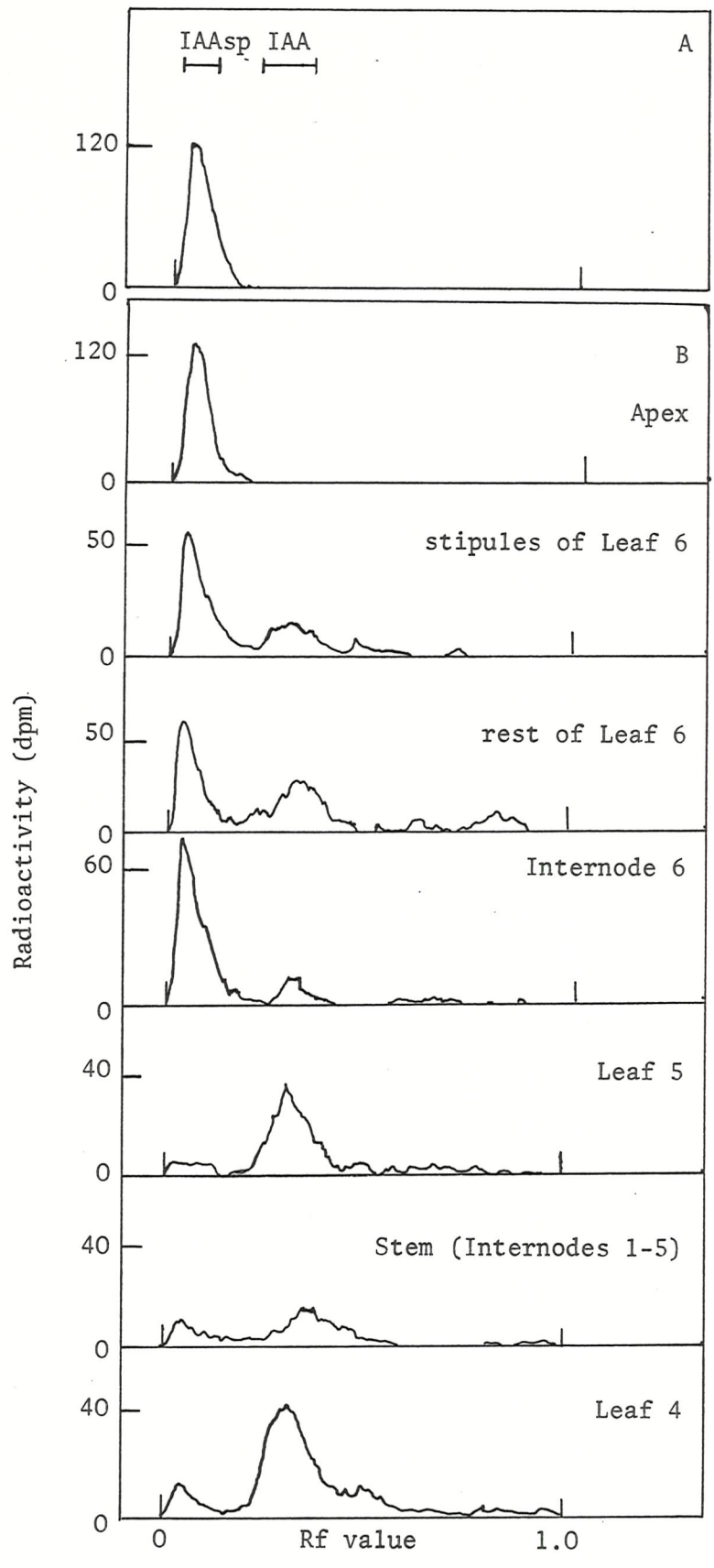


Figure 5

Radiochromatogram scans of the ^{14}C-activity applied (A), and the 70% ethanol extractions (B) of dissected plant parts 8 hours following the application of [^{14}C]IAAsp to the apical buds of light-grown, 14-day old dwarf peas. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars represent position of authentic IAA and IAAsp.

in the extent to which ^{14}C-material was exported from the labelled apex. The reason for this variability is unknown. Uniform plants, grown under identical environmental conditions, were chosen for each experiment. The daily watering regime and the labelling techniques, including the time of application, were also identical. No variation occurred in the harvesting, extraction or purification procedures. Variation in the ^{14}C-compound applied is also unlikely as identical techniques were employed in its isolation. One possibility is that there may have been subtle changes in plant vigor. This is unlikely as identical conditions were maintained throughout the experiments. Another explanation is that there were differences between the batches of seed obtained from the suppliers (Gillet and Son Ltd., Wisbech, England). The selection procedure for the plants makes this unlikely.

Later experiments (Section V) suggest that differences in the uptake of [1-^{14}C]IAA occurred more often than variation in its conjugation. The metabolic differences when IAAsp was applied to peas may be due to differences in uptake. The one examination of the uptake of [^{14}C]IAAsp revealed that under 50% of the [^{14}C]IAAsp entered the apex. The different pattern of metabolism when there was a high transport rate (Table 4, Fig. 5) may have been due to increased uptake. No explanation can be offered to account for this.

In this experiment the isolated auxin conjugate, IAAsp, was broken down in vivo to release free IAA (Fig. 5). It is believed this is the first evidence for the conversion of IAAsp to IAA by plant tissue. Having demonstrated the formation of IAA from IAAsp, the site of conversion remains unknown. There is no possibility of

distinguishing between the following two alternatives. Firstly IAAsp may be immobile, the conversion of IAAsp occuring in the apical tissue, the site of application. There was no detectable IAA in the apex. If this was the site of conversion all the IAA formed was rapidly exported to the stem. IAA was found in the stem when a large proportion of ^{14}C-material was exported. IAAsp was also found in stem and leaf tissue. The second alternative is that the conversion of IAAsp to IAA occurs throughout the plant and IAAsp itself is mobile. It is possible, although unlikely, that some of the IAAsp entered the phloem and was distributed around the plant.

The site of conversion of IAAsp to IAA would most easily be identified by a better understanding of the mobility of the two compounds, although, the evidence on the mobility of IAAsp is conflicting (Section I). Observations from aphid-feeding experiments have demonstrated transport of IAAsp in the phloem of willow (Hoad, Hillman and Wareing, 1971). In contrast, Eschrich (1968) using the same technique demonstrated the absence of IAAsp in the phloem of V.faba although large amounts of IAA were detected. Both the mobility and immobility of IAAsp in various plant tissues has been recorded. Further evidence is essential before any conclusion can be reached as to the mobility of IAAsp or the location of the enzyme system responsible for the conversion of IAAsp to IAA. However some indication may be given by the predominance of IAA in the lower stem, (Fig. 5) suggesting that IAA is the transported form and that IAAsp formation occurs during transport. The link between the amount of transport and metabolism of IAAsp has already been mentioned. In the experiment that

demonstrated the greatest conversion of IAAsp to IAA, transport occurred throughout the plant. Transport may, therefore, depend on the prior metabolism of IAAsp to IAA.

Problems in obtaining adequate amounts of [^{14}C]IAAsp prevented further investigation of the relationship between uptake and metabolism of IAAsp, or indeed of factors controlling both. It was hoped that characterization of the enzyme system would be achieved, including the cellular location, and that further evaluation of the physiological significance of IAAsp could be made. As a result of problems in the isolation of IAAsp it was considered necessary to explore the techniques used and assess any improvements which could be made (Section III).

These results have shown that metabolic release of IAA from IAAsp. The plant therefore possesses the enzyme system for this conversion. This being so it appears more likely that IAAsp may represent a storage form of IAA from which it can be released when required, and that the postulated homeostatic system for the control of free IAA levels does exist.

Section III

THE DEVELOPMENT OF IMPROVED ISOLATION TECHNIQUES

THE DEVELOPMENT OF IMPROVED ISOLATION TECHNIQUES

INTRODUCTION

In view of the difficulties experienced in obtaining pure radioactive IAAsp (Section II) an examination was made of the techniques available for the production of IAAsp. These techniques could be divided into two categories, firstly the extraction from IAA-labelled plant tissue known to have synthesised the conjugate, and secondly, the chemical synthesis of the compound. The latter technique was investigated as circumstances made essential the synthesis of authentic IAAsp for use as a standard.

The extraction of IAAsp from intact pea plants labelled with [1-^{14}C]IAA

No entirely satisfactory method for the extraction of auxins and their metabolites from plant material has yet been published. The efficiency of any isolation procedure employed here is obviously of considerable importance and every effort must be made to maximize the recovery and concentration of IAAsp. Many papers have reported low and erratic percentage returns from IAA applied to plant tissues. Recoveries of radioactive IAA from corn shoots, using the same extraction, concentration and chromatography methods, varied from 0 to 28%, with an average of 14% according to Hamilton et al. (1961). Udea and Bandurski (1974) achieved recoveries of 65-79%. However this was for a high concentration of IAA added to ground maize just before extraction, and not for metabolized IAA. Using the isolation procedure described by Kefford (1955),

Mann and Jaworski (1970) reported erratic recoveries ranging from 25-70%. Greenwood et. al. (1972) noted a 60% loss of [1-^{14}C]IAA and metabolites during the later stages of isolation, partitioning and chromatography. In most cases reported in the literature recovery data for only one or two steps of the extraction procedure were given. Large losses of IAA during extraction procedures have commonly been observed by others (Scott and Jacobs, 1964; DeYoe and Zaerr, 1976). Little et al. (1978) also found inconsistencies, recording a range of recoveries for [^{14}C]IAA between 0.5 and 27.1%. Even in those experiments in which several extracts of IAA were purified simultaneously 5-fold differences in recovery were commonly observed. The basic procedure employed has already been outlined in Section II and a more detailed consideration will now be given to the individual methods employed.

a) Extraction

The three most commonly employed solvents for the extraction of auxins from plant material are ethanol, ethanol-water mixtures and diethyl ether. Reports exist describing the continuing action of enzymes during extraction with diethyl ether allowing for the formation of additional IAA (Wildman and Muir, 1949; Tambiah, 1951). Extraction with ethanol was demonstrated to dehydrate enzymes preventing any further metabolism (Thimann and Skoog, 1940; Kefford, 1955). Davies (1973) suggested that the lower yields of IAA and its metabolites resulting from diethyl ether extraction are probably due to the immiscibility of ether with the aqueous cell contents. He found 95% aqueous ethanol to be an efficient extraction solvent for most of the metabolites of exogenous IAA in

pea and bean tissue (Davies, 1972). Hemberg and Tillberg (1978) considered the effects of time and temperature on the extraction of IAA from maize kernels by methanol. Yields increased with extraction time and temperature. A 24 hour extraction at 4°C gave about 3 times more IAA than a 3 hour extraction, and a 24 hour extraction at 30°C gave about 1.5 times more than extraction for the same time at 4°C. Hillman (1978), however, found no difference between methanol extractions at 20° to 25°C and 4°C in their efficiency at removing IAA and its metabolites into solution from the plant material.

b) Rotary evaporation

Concentration is the next major step in the separation of plant hormones. Mann and Jaworski (1971) reported "unacceptable" losses of between 50 and 85% occuring when diethyl ether solutions containing IAA, with or without soyabean leaf tissue respectively, were taken to dryness under reduced pressure in a rotary evaporator. They also quoted recoveries of 34-42% for [^{14}C]IAA dissolved in ether and reduced by rotary evaporation. The explanation appeared to be the sublimation of IAA *in vacuo* (Mace, 1965). Such losses of auxins during extraction are often erratic and responsible for continued difficulties in the isolation of auxins by plant physiologists.

c) Chromatography

Methods for the chromatographic separation on paper of indole compounds for bioassay techniques were developed by Bennet-Clark and his co-workers in the early 1950s. Since then this technique

has been successfully used for the separation of IAA and its metabolites, but has also been found responsible for some of the losses occuring during the isolation procedure. Authors have investigated ways of increasing the efficiency of the technique. Kefford (1955) found no advantage in developing the chromatograms in a nitrogen atmosphere - his major loss occurred during storage leading to a recommendation for the immediate use of chromatograms both before and after development. If it was necessary to store chromatograms, he suggested an atmosphere of nitrogen, in darkness at -10°C. Mann and Jaworski (1971) further advised the use of an anti-oxidant, Santoquin, if the chromatograms were stored undeveloped. A 44% loss of IAA caused by overnight storage of such chromatograms could be reduced to 19% by pretreatment with Santoquin.

Two other methods of chromatography were investigated in this section, thin-layer chromatography (TLC) and the use of a Sephadex column. TLC initially appeared to be superior to paper chromatography for the separation of small quantities of indole compounds (Stahl and Kaldewey, 1961, 1964). Moore and Shaner (1967) however reported reproducible recoveries of only 45 to 50% of [^{14}C]IAA after the development of the chromatograms. The use of TLC is further discussed in Section IV. Sephadex gels have been used by various authors for the separation of IAA and other indoles (Anderson, 1968; Eliasson, 1969; DeYoe and Zaerr, 1976). Bandurski and Schulze (1974) concentrated IAA on an LH-20 column after purification of the extract using a DEAE-cellulose column. In order to distinguish between free and bound forms of IAA, Liu, Gruenert and Knight (1978) applied extracts from Nicotiana species to a Sephadex G-25 column. The material was eluted with a NaCl

linear gradient. There is one report of the successful use of Sephadex LH-20 for the separation of endogenous IAAsp from bean tissue, and this technique was followed here (Tillberg, 1974).

The isolation of IAAsp from stem segments incubated in [1-^{14}C]IAA

In addition to an investigation of the isolation of IAAsp from intact plants, the isolation of IAAsp from stem segments incubated with IAA was also explored. Much of the published information concerning the characteristics of auxins in plant tissue, especially of auxin transport, has been gained from experiments involving isolated plant parts, including coleoptiles, stem, leaf and root segments. It was considered that the incubation of [1-^{14}C]IAA with these different tissues might provide an ideal system from which to extract its main metabolite, presumed to be IAAsp.

The chemical synthesis of IAAsp

Production by Calbiochem Ltd. of synthetic IAAsp was terminated and the lack of an alternative source meant that the chemical synthesis of the compound had to be undertaken. As both IAA and aspartic acid can be obtained radioactively labelled such a technique might prove invaluable in the production of the radioactive metabolite for further experimentation. Good (1956) used the method of Boissonas (1951) to synthesise unlabelled IAAsp, recording a yield of 57% of the indoleacetic acid used. This method is in some respects unsuitable for IAAsp formation, due to the problems of zwitterion formation and problems associated with the formation of a peptide-linkage directly with a free amino-acid. From a choice of the other recorded synthesis methods, that of Mollan

et al. (1971) was chosen as being simpler and better. This involved the coupling of the p-nitrophenol ester of IAA to the tetramethyl-guanidine salt of L-aspartic acid.

MATERIALS AND METHODS

The extraction of IAAsp from intact pea plants labelled with [1-^{14}C]IAA

a) Plant material

The plants were grown as previously described in Section II. 0.10 μCi [1-^{14}C]IAA was applied to the apex of 12- or 14-day old light-grown dwarf pea plants as a 5.0 μl droplet in "Tween 20" (0.1%) (0.34 μg IAA plant^{-1}, 1.92 x 10^{-6} μmol plant^{-1}). After 4 hours translocation, the activity was extracted from either the dissected plant organs or the isolated apical buds.

b) Extraction

Three investigations were made of extraction procedures. Firstly, the extraction efficiency of diethyl ether, acetone and ethanol (absolute and aqueous) on apical tissue were examined. Secondly, the extraction procedure described in Section II was more fully investigated. The 70% ethanol extraction of dissected whole plants was extended to include an additional ethanol extraction. After homogenization of the tissue and prior to NaOH extraction, the residual tissue was washed off from the filter paper with 70% ethanol and stored in Erlenmeyer flasks under refrigeration.

After 48 hours the tissue and aqueous ethanol solution were separated by centrifugation (2000g, 3500 rpm for 5 minutes). Aliquots from the known volume of supernatent were counted, and the supernatent reduced for chromatography. The residual tissue was placed in 50 ml beakers for NaOH extraction. Thirdly, the possibility of aqueous extraction was explored. Labelled apices were harvested, macerated in saturated sodium bicarbonate solution and left stirring for at least one hour. The debris was removed by filtration with a Büchner funnel and rinsed with distilled water. The filtrate was made up to a known volume and aliquots taken for liquid scintillation counting. The filtrate was then adjusted to pH 9.0 with 0.1N NaOH if necessary and partitioned against petroleum ether (b.pt. 80°-100°C) as described below. Plant extracts were stored, when necessary, either by refrigeration in the dark at 3°C ± 1°C for short term storage, or in the deep freeze at -10°C when longer term storage was required.

Concentration and purification

c) Fractionation

A method of partitioning was devised. Radio-activity from apical buds was extracted using 70% aqueous ethanol as previously described and the extracts reduced to the aqueous phase by rotary evaporation. The activity in a known volume of the sample was then determined. The pH of the aqueous sample was found to be between 8.0 and 9.0. The extract was then partitioned against redistilled petroleum ether (boiling point 80°-100°C) 3 times using half the volume of the sample. The amount of radioactivity in known volumes of the petroleum ether fractions and water fraction was determined.

Diethyl ether, ethyl-acetate and chloroform were rejected as these removed a greater proportion of the [1-^{14}C]IAA metabolites from the sample. The pH of the aqueous fraction was reduced to between 2.0 and 2.5 with 0.1 N HCl. The sample was partitioned with petroleum ether, as before, and the radioactivity in both fractions determined. A final partitioning of the aqueous fraction against butan-1-ol was employed 3 times using half the volume of the aqueous sample. The ^{14}C content of the organic and aqueous phases was determined. The two ether fractions, the butan-1-ol and the aqueous fractions were concentrated at reduced pressure and chromatogrammed. The large proportion of lipids in the petroleum ether layers caused streaking of the samples on the chromatograms. The petroleum ether fractions were therefore completely air-dried and distilled water added to the residue. The sample was then chromatogrammed. Determination was made of the fate of [1-^{14}C]IAA and its metabolites in plant extracts, and of cold synthetic IAAsp and IAA. The latter two were traced, non-quantitatively, by Ehrlich's reagent.

d) Chromatography

Paper

Paper chromatography was employed as described in Section II and the areas under the peaks on radiochromatogram scans were measured with a planimeter to determine the percentage distribution of radioactivity between metabolites.

Analytical TLC

Ascending TLC using silica gel (BDH, Dorset, England; silica

gel incorporating 13% $CaSO_4$, thickness 0.25 mm) was used as an alternative to paper chromatography. The solvent was allowed to move 100-120 mm from the line of application. The plates were divided into 10 mm fractions, the silica gel removed and placed in liquid scintillation vials for counting. Ehrlich's reagent was used to localize compounds.

Gel Filtration

The method used by Tillberg (1974) to separate endogenous IAAsp from bean extracts was attempted. Sephadex LH-20 (Pharmacia Fine Chemicals, U.K.) was swollen in 30% phosphate buffer (pH 6.0, final concentration 1 mM) 70% ethanol and packed in a 300 mm x 15 mm Pharmacia column. Samples were dissolved in and eluted from the column using the phosphate buffer ethanol mixture at a temperature of 20° - 30°C.

e) Elution

Paper strips or TLC silica gel portions were placed in Erlenmeyer flasks containing either absolute ethanol, 70%, 80% or 90% aqueous ethanol or water, and left stirring overnight in darkness at room temperature. The paper fibres and silica were removed by either centrifugation or filtration.

f) The estimation of ^{14}C loss

The determination of radioactivity in plant extracts and other solutions by liquid scintillation counting has already been described in Section II.

[1-^{14}C]IAA was also employed in controlled studies of the

individual steps in the concentration and purification procedure, for example rotary evaporation. A known amount of [1-^{14}C]IAA in 70% aqueous ethanol solution was subjected to the same procedure as a plant extract and the loss of ^{14}C determined at each stage, either by taking aliquots from known volumes of solutions or, in the case of paper chromatography, by directly counting the paper in liquid scintillation fluid.

The isolation of IAAsp from stem segments incubated in [1-^{14}C]IAA

P.sativum cv. Meteor and Alderman were grown in continuous light or in darkness. Freshly cut 3 mm stem segments were transferred after weighing to 25 ml Erlenmeyer flasks containing 10 ml of the incubation medium (0.005 M malate buffer, pH 5.6 [1-^{14}C]IAA (2 µg ml^{-1}) and incubated for 4 hours at 25°C in a shaking water bath in diffuse daylight. In another experiment, segments were pretreated for 8 hours in incubation medium containing unlabelled IAA (2 µg ml^{-1}), prior to transfer to a medium containing ^{14}C-IAA. Between treatments the segments were washed with distilled water, blotted and weighed. This was also carried out at the end of all experiments. After incubation 1 ml aliquots from the combined incubation medium and washings were counted. The tissue was placed in 10 ml 70% aqueous ethanol and left refrigerated overnight. The segments were homogenized and stirred at room temperature for 1 hour, centrifuged and aliquots of the supernatant counted. The supernatant and the combined experimental solution and washes were reduced and paper chromatogrammed (IAW).

Controls in which [1-^{14}C]IAA was incubated in 10 ml of the medium in the absence of stem segments, revealed negligible change

in ^{14}C content during the experimental period.

The chemical synthesis of IAAsp

The method of Mollan, Donnelly and Harmey (1971) was followed. The two stages of the reaction were, firstly, the synthesis of the p-nitrophenol ester of IAA (5.13 g) from IAA (5.8 g) and p-nitrophenol (4.8 g) (mole equivalents). Secondly, the synthesis of indole-3-acetyl-L-aspartic acid (2.54 g) from IAA p-nitrophenol ester (5.5 g) and L-aspartic acid (2.5 g) (mole equivalents). A full report, including the slight modification made, is included in the appendix.

RESULTS

The isolation of IAAsp from intact pea plants labelled with [1-^{14}C]IAA

a) The distribution and metabolism of [1-^{14}C]IAA

It was shown that after a 4 hour translocation period 95% of the activity applied was extracted from the apex (Tables 5, 6). Radioactivity was detected throughout the whole plant, including a small proportion in the older foliage leaves. A very small percentage had been transported to the roots during this period. The bulk of the labelled-material found in the apex was present in a compound which had an Rf value of 0.04 in IAW (Table 7). Assuming this to be IAAsp, a high percentage of the ^{14}C applied as [1-^{14}C]IAA theoretically can be recovered as IAAsp by extraction of the apex under these conditions.

Various unidentified metabolites were noted, especially from tissue some distance from the shoot apex (Table 7).

Table 5 The recovery of ^{14}C from light-grown 14-day old dwarf pea seedlings following the application of [1-^{14}C]IAA to the apical buds and 4 h translocation period. The dissected plants were extracted with 70% ethanol, then homogenized and re-extracted. The residual plant material was finally extracted with 10% NaOH.

Average activity applied = 750000 dpm plant^{-1}.

All values dpm plant^{-1}.

10 plants per treatment, 2 replicates per treatment.

	Ethanol extract	Ethanol wash (homogenized)	Ethanol extract and wash. TOTAL	Further ethanol extraction	NaOH extraction	Total
Apical extract	567510	40500	608010	677	162	608850
Leaf 6 and Internode 7	2580	1980	4560	46	0	4606
Internode 6	1989	53	2042	0	0	2042
Leaf 5	6197	258	6455	0	0	6455
Internode 5	4661	82	4743	0	0	4743
Leaf 4	2278	2465	4744	179	37	4961
Internode 4	2186	50	2236	0	0	2236
Leaf 3	563	111	674	0	0	674
Remainder of shoot	635	3346	3981	261	45	4287
Root	1009	154	1163	0	0	1163

Table 6 The percentage distribution of ^{14}C recovered from light-grown 14-day old dwarf pea seedlings following the application of [1-^{14}C]IAA to the apical buds and translocation for 4 h. The dissected plants were extracted with 70% ethanol, homogenized and re-extracted. The residual plant material was finally extracted with 10% NaOH.

Average activity applied = 795000 dpm plant^{-1}.

All values dpm plant^{-1}.

10 plants per treatment, 2 replicates per treatment.

	Ethanol extract	Ethanol wash (homogenized)	Ethanol extract and wash. TOTAL	Further ethanol extraction	NaOH extraction	Total
Apical extract	88.60	6.30	95.00	0.001	0.003	95.1
Leaf 6 and Internode 7	0.04	0.31	0.71	0.01	0	0.72
Internode 6	0.31	0.01	0.32	0	0	0.32
Leaf 5	0.97	0.04	1.01	0	0	1.01
Internode 5	0.73	0.01	0.74	0	0	0.74
Leaf 4	0.36	0.39	0.74	0.03	0.01	0.78
Internode 4	0.34	0.01	0.35	0	0	0.35
Leaf 3	0.01	0.01	0.02	0	0	0.11
Remainder of shoot	0.01	0.52	0.62	0.04	0.01	0.67
Root	0.16	0.02	0.18	0	0	0.18

Table 7 The proportions of the major ^{14}C-metabolites recovered by 70% ethanol extraction, and homogenization followed by extraction of dissected light-grown 14-day old dwarf pea seedlings 4 h following the application of [1-^{14}C]IAA to the apical buds. The extracts were separated by chromatography in isopropanol:NH_3:H_2O (10:1:1 v/v).

All values ^{14}C as a percentage of total chromatogram - ^{14}C.

* results approximate due to low level of ^{14}C.

	Ethanol extraction					Homogenization and ethanol wash				
	IAAsp Rf 0.04	Rf 0.23	IAA Rf 0.33	Rf 0.52	Rf 0.7	IAAsp Rf 0.04	Rf 0.19	Rf 0.23	IAA Rf 0.33	Rf 0.56
Apical extract	90		10			100				
Leaf 6 and Internode 7	78		22			100				
Internode 6	61		39							
Leaf 5	69		31			43		22	35	
Internode 5	61		39							
Leaf 4	40		60			57		6	37	
Internode 4	47		53							
Leaf 3	52		19		29	42	20		14	24
Remainder of shoot *	48	24	12		(Rf 0.4) 12	91				9
Root *	43	11	14	32						

b) Extraction

Of the organic solvents assessed redistilled diethyl ether presented problems with the formation of emulsions and poor yields. The recovery of ^{14}C-material achieved with acetone (absolute or aqueous) was much lower than with ethanol. The recovery of radioactivity and presumably therefore, the recovery of the metabolites of interest, by $NaHCO_3$ extraction did not compare favourably with 70% aqueous ethanol extraction (Table 8). Further $NaHCO_3$ extraction did not significantly increase the ^{14}C recovered. The advantages of not extracting chlorophyll and other hydrophobic compounds were outweighed by problems of increased viscocity of the samples. Paper and TLC (IAW) indicated that a greater reduction occurred in the amount of IAAsp extracted than IAA (Fig. 6, 7).

The highest recovery was achieved by extraction with 70% aqueous ethanol, a range from 70 to 90% being recorded, with an average of 88%. Preliminary experiments suggested little difference between 20°C and 4°C ethanol extraction, provided the extraction time was over 12 hours. Table 9 shows the efficiency of each step of the ethanol extraction procedure. A final NaOH extraction of the tissue debris released negligible ^{14}C. Of the ^{14}C-applied 20% was unaccounted for in this case and may have been released as $^{14}CO_2$.

c) Storage

Table 10 illustrates the negligible effects of storage on 70% aqueous ethanol solutions containing [1-^{14}C]IAA. This is in agreement with the results obtained from the storage of plant extracts. The ^{14}C ethanol apical extracts had been reduced to the aqueous phase and filtered to remove any solid debris (volume 2 to 5 ml).

Table 8 Comparison of ^{14}C recovered from the apices of 14-day old light-grown dwarf pea seedlings 4 h following the application of [1-^{14}C]IAA to the apical buds. Extraction by either saturated aqueous $NaHCO_3$ or 70% ethanol. Each extraction the average of 2 replicates.

	$NaHCO_3$ extract	Ethanol extract
Activity Applied (dpm plant^{-1})	496855	460515
^{14}C extracted from apices (dpm plant^{-1})	265140	433061
% recovery	54	94

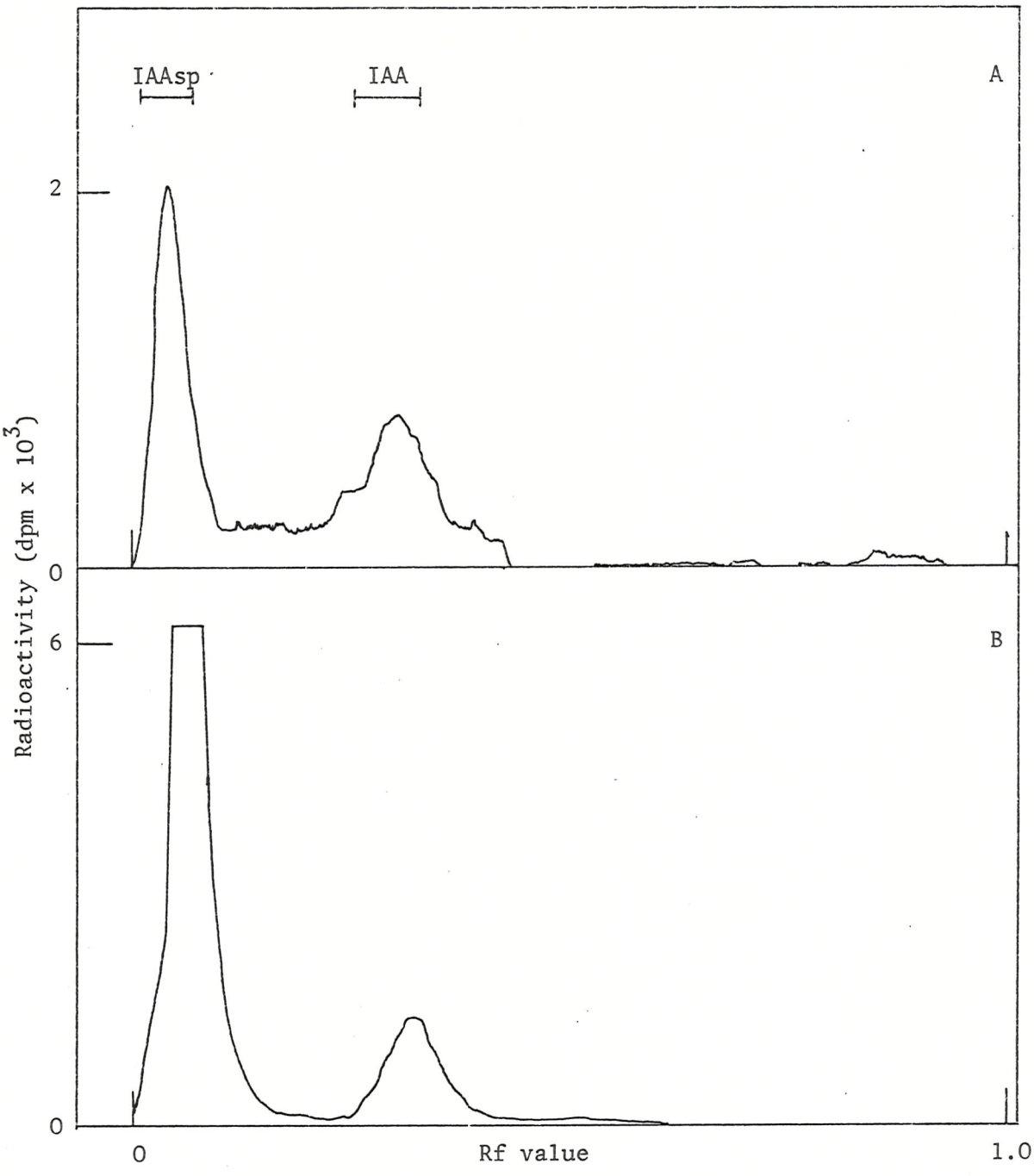


Figure 6 Radiochromatogram scans from apices labelled with [1-^{14}C]IAA and extracted after 4 hours by either, A: $NaHCO_3$ extraction or B: 70% ethanol extraction. Chromatograms developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars represent positions of authentic IAA and IAAsp.

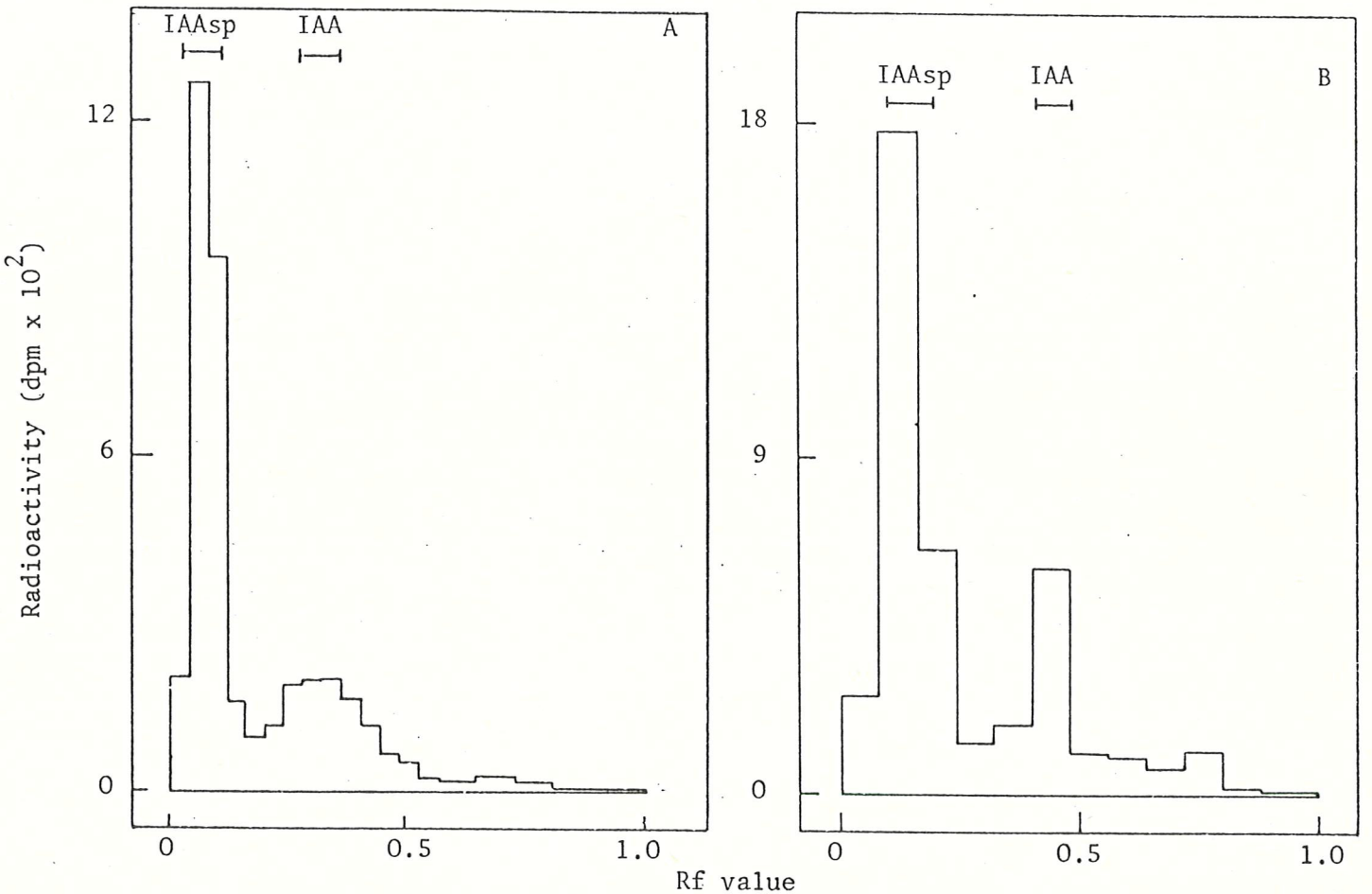


Figure 7

The separation by TLC of radioactivity present in apical extracts from plants labelled with [1-^{14}C]IAA 4 hours prior to extraction by A: $NaHCO_3$ or B: 70% ethanol. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars represent the position of authentic IAA and IAAsp.

Table 9 The efficiency of 70% ethanol extraction of ^{14}C from 14-day old light-grown dwarf pea seedlings 4 h following the application of [1-^{14}C]IAA to the apical buds.

Applied activity = 750000 dpm plant^{-1}.

All values dpm plant^{-1}.

	Ethanol Extraction	Homogenization and ethanol wash	Further ethanol extraction	NaOH extraction	Total
Apices	567,510	40,500	677	163	608,850
Rest of Plant	22,100	8,500	487	88	31,177
Total	589,610	49,000	1164	251	640,027
% recovery	74.6	6.2	0.14	0.03	81.0

Table 10 The effect of storage on ^{14}C extracted from 12-day old light-grown dwarf pea apices 4 h following the application of [1-^{14}C]IAA to the apices. The samples were

a) refrigerated for 14 days, dark at 3°C ± 1°C

or b) stored under nitrogen in the deep freeze, -14°C.

The plant extracts and [1-^{14}C]IAA were stored in 70% ethanol solutions.

	Activity before storage (dpm)	Activity after storage (dpm)
[1-^{14}C]IAA	269,200 ± 3712	273,700 ± 1893
a) Plant Extract 1.	24,653	23,800
b) Plant Extract 2.	33,463	33,515

Estimates of the radioactivity before and after storage do not differ.

Purification and Concentration

d) Rotary evaporation

A 12% loss was recorded during controlled studies of rotary evaporation using a known concentration of [1-^{14}C]IAA in 70% aqueous ethanol (Table 11). Care was taken not to dry the sample completely, an advantage of using aqueous ethanol rather than diethyl ether. When a 70% aqueous ethanol extract of apical tissue previously labelled with [1-^{14}C]IAA was reduced, a large increase in the percentage of ^{14}C lost was recorded (12% compared to 48%, Tables 11, 12). This demonstrates the danger in extrapolation from experiments based on pure [1-^{14}C]IAA to losses from plant extracts containing its radioactive metabolites.

e) Fractionation

The partitioning of the aqueous radioactive plant extract against petroleum ether cleared the sample considerably, but resulted in an approximately 20% loss of ^{14}C from the aqueous phase. This loss was unaccounted for, a very small proportion entering the ether layer. It was found that the butanol:H_2O partitioning must preferably be left fifteen minutes after shaking to allow adequate separation to occur. 97% of the activity was transferred from the aqueous to the butanol fraction which was easily concentrated to a small volume (Table 13).

Partitioning with petroleum ether had little effect on the radioactive composition of the sample (Fig. 8). It was difficult to determine the metabolites present after the second partitioning as

Table 11 The loss of [1-^{14}C]IAA from a 70% ethanol solution during rotary evaporation.

All values averaged from a minimum of 5 replicates ± standard deviation.

	Initial activity in the solution	Reduced to 1 ml	Additional rinse from flask	Total activity after rotary evaporation
dpm	278,180 ± 7960	244,180 ± 18730	8,590 ± 1044	252,770
%	100	85	3	88

Table 12 The effect of rotary evaporation on 70% ethanol extracts of apical tissue harvested from 12-day old light-grown dwarf pea seedlings 4 h following the application of [1-^{14}C]IAA to the apical buds.

	Before rotary evaporation (dpm)	After rotary evaporation (dpm)	% loss
Extract 1	649,600	308,070	53
Extract 2	567,510	306,350	46
Extract 3 (Homogenized)	139,850	75,470	46
Extract 4 (Homogenized)	40,500	21,430	47
Average loss (%)			48

Table 13 The recovery during partitioning of 70% ethanol-soluble ^{14}C-compounds from apices labelled with [1-^{14}C]IAA, reduced to the aqueous phase and partitioned against, firstly A: petroleum ether (pH 8.0 to 9.0), secondly B: petroleum ether (pH 2.5 to 2.0) and finally C: butanol (pH 2.5 to 2.0).

All values represent an average of 4 replicates.

A: petroleum ether (pH 8.0-9.0)

		dpm	% recovery
Activity before partitioning		306250	100
Activity after partitioning	petroleum ether	2142	0.7
	H_2O	242564	79
	Total	244706	79.7

B: petroleum ether (pH 2.5-2.0)

		dpm	% recovery
Activity before partitioning		219157	100
Activity after partitioning	petroleum ether	1058	0.5
	H_2O	179982	82
	Total	181040	82.5

C: Butan-1-ol (pH 2.5-2.0)

		dpm	% recovery
Activity before partitioning		131632	100
Activity after partitioning	H_2O	4025	3
	Butanol	127305	97
	Total	131330	100

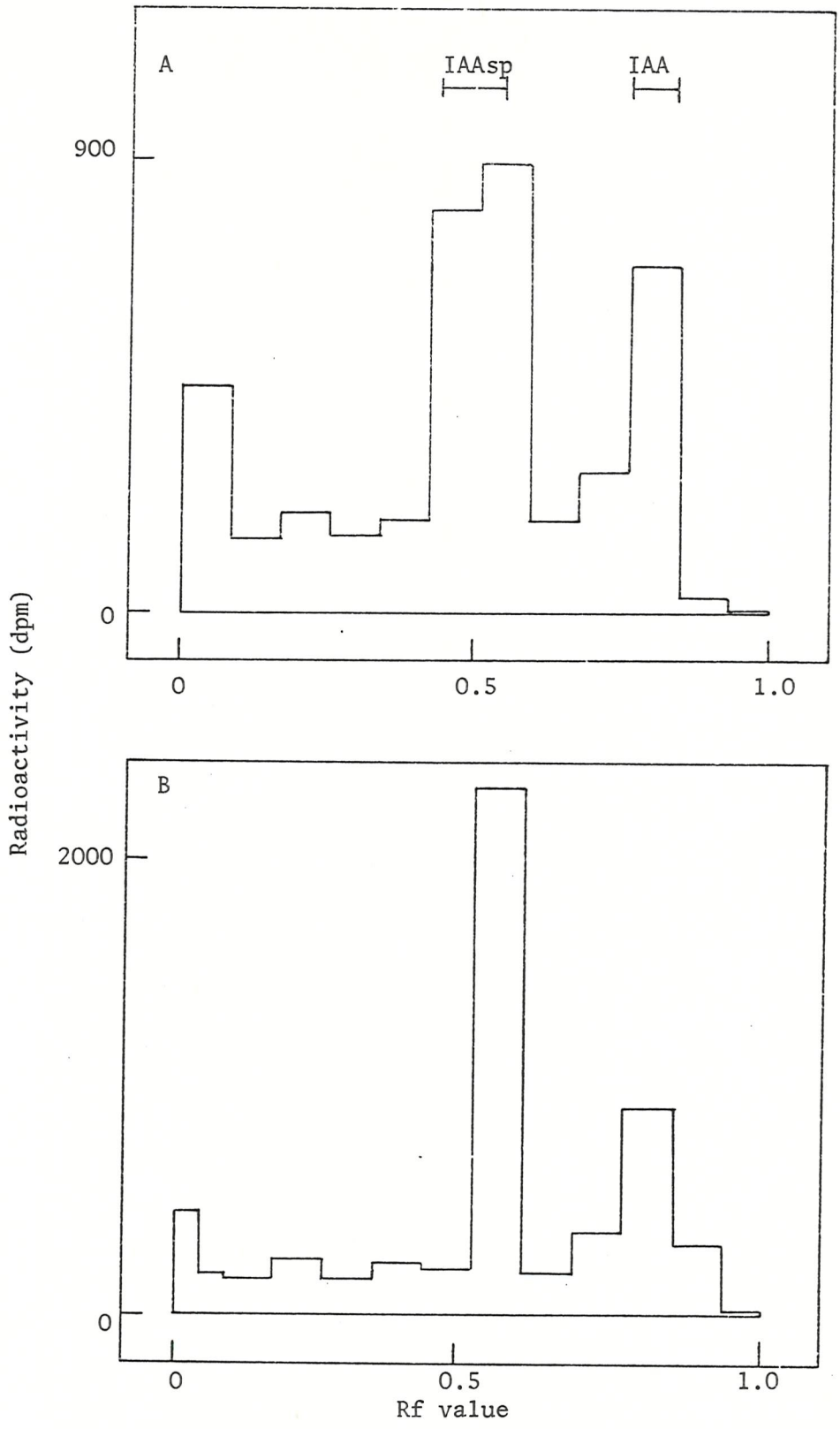


Figure 8

The separation by TLC of ^{14}C-compounds present during the purification of 70% ethanol extract from apices labelled with [1-^{14}C]IAA. A: 70% ethanol extract reduced to aqueous phase, B: the aqueous phase (pH 8.0) after partitioning with petroleum ether. The chromotograms were developed in chloroform:ethyl acetate:formic acid (35:55:10 v/v). Horizontal bars represent the position of authentic IAA and IAAsp.

the presence of HCl in the aqueous phase effected the compounds when reduced and thereby concentrated. This can clearly be seen in Fig. 9B which is essentially identical to Fig. 9A except for the adjustment of pH to 2.5 before rotary evaporation. Additional experiments were carried out with synthetic IAA, IAAsp and HCl at pH 2.5. Within 10 hours at room temperature additional compounds were detectable in the solution containing IAAsp and HCl. An Ehrlich's positive, ninhydrin negative spot occurred at Rf 0.09, and a less prominent Ehrlich's positive spot at Rf 0.1 - 0.15 (TLC, chloroform:ethyl acetate:formic acid, 35:55:10 v/v). As the butanol fraction (Fig. 9C) contained the same ^{14}C metabolites as the first aqueous sample it can be concluded that the second ether extraction (pH 2.5) does not effect the composition of the sample. Fig. 10 shows the chromatography of the ^{14}C-metabolites in the aqueous extract of the dried petroleum ether fractions. Only a small percentage of activity was transferred to the petroleum ether layer and it can not be accounted for by a single metabolite. Four spots are discernable, two of which co-chromatogram with authentic samples of IAA and IAAsp. The unidentified compounds occur at Rf 0-0.7 and 0.2-0.25 in this solvent system.

The main peaks of radioactivity extracted from the plants, co-chromatogrammed with authentic samples of IAAsp and IAA, mainly the former (Fig. 8, 9C). There was a higher percentage of IAA in these samples than usually noted (IAAsp:IAA = 64:36 compared with 88:12 recorded above). A four hour translocation period was employed as before, although approximately 40% less [1-^{14}C]IAA was applied to the apex (0.75 µg apex^{-1} compared to 1.20 µg apex^{-1} in the experiment described earlier). All other conditions were

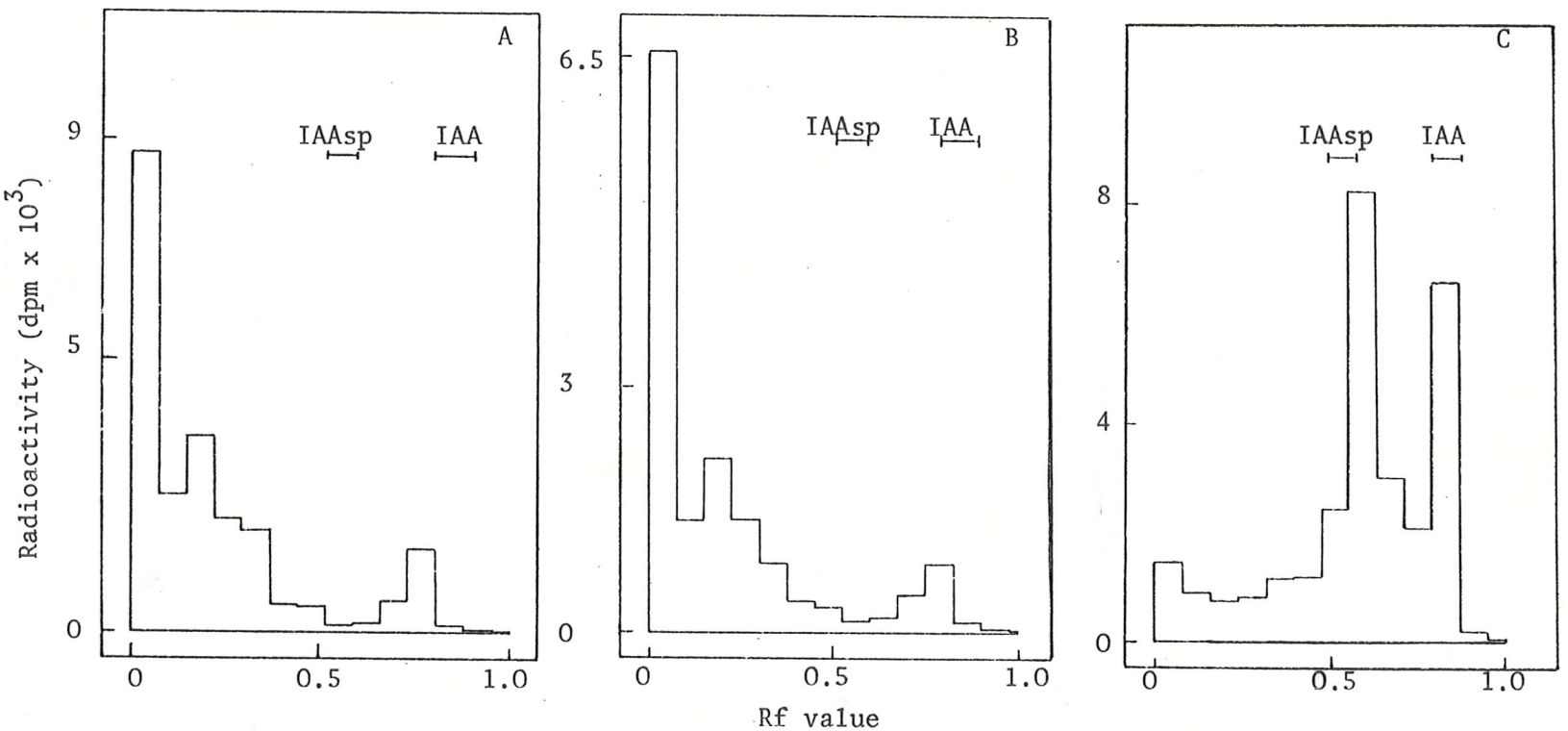


Figure 9 The separation by TLC of the 70% ethanol-soluble ^{14}C-compounds extracted from apices labelled with [1-^{14}C]IAA, reduced to the aqueous phase and partitioned against petroleum ether (pH 8.0), A: following the reduction of pH to 2.5 using 0.1 N HCl, B: after the second partitioning with petroleum ether (pH 2.5) and C: the butanol fraction following partitioning of the aqueous phase (pH 2.5) against butanol. The thin-layer plates were developed in chloroform:ethyl-acetate:formic acid (35:55:10 v/v). Horizontal bars represent the position of authentic IAA and IAAsp.

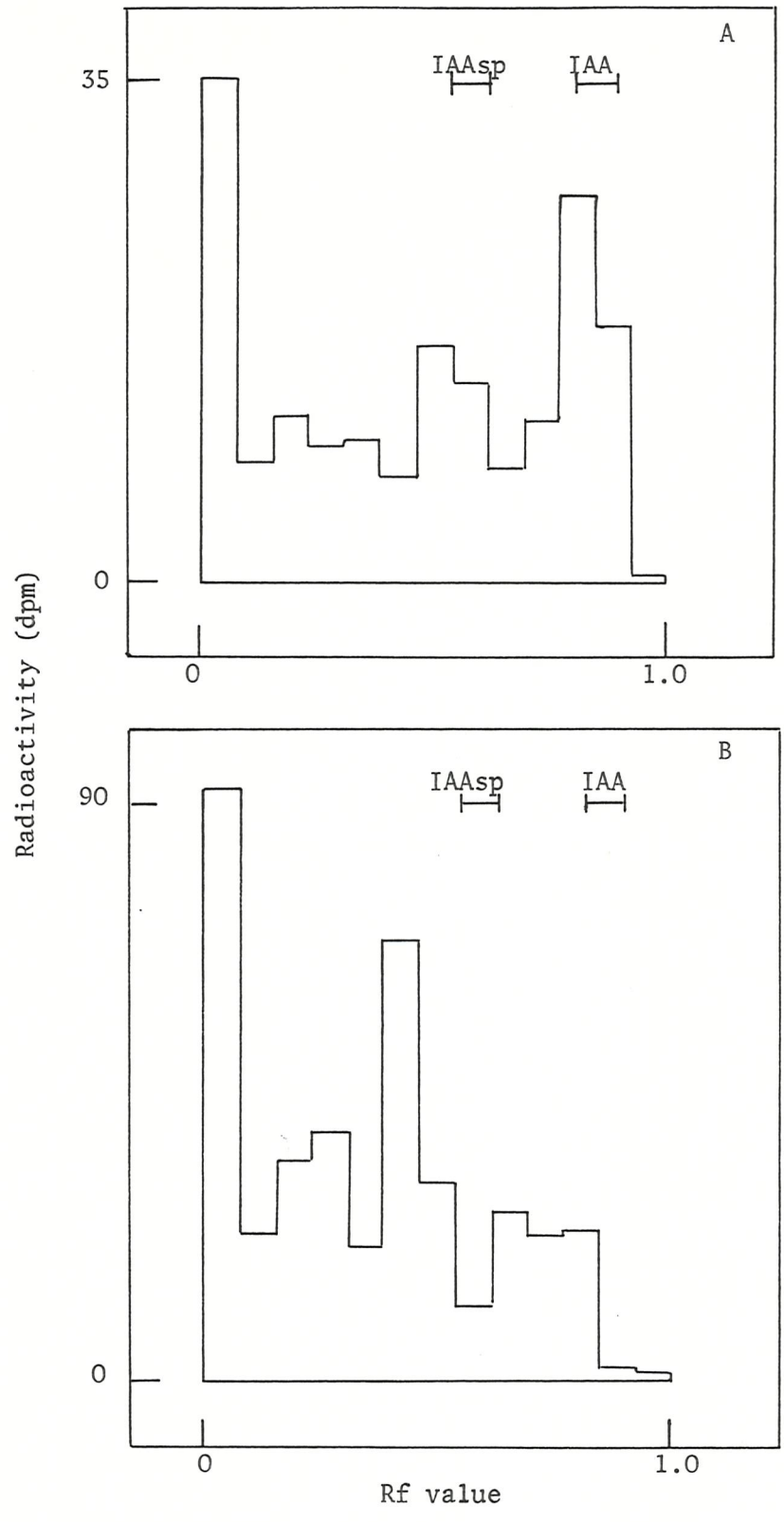


Figure 10 The separation by TLC of ^{14}C-compounds in the petroleum ether fractions after partitioning against the aqueous plant extract from apices labelled with [1-^{14}C]IAA, A: at pH 8.0 and B: at pH 2.5. The chromatograms were developed in chloroform:ethyl-acetate:formic acid (35:55:10 v/v). The horizontal bars represent the position of authentic IAA and IAAsp.

Table 14 The loss of [1-^{14}C]IAA during the preparation of paper chromatograms. The [1-^{14}C]IAA was dissolved in 70% ethanol.

All values averaged from a minimum of 5 replicates ± standard deviations.

Average activity of 5µl aliquot counted directly	Average activity of 5µl aliquot spotted, dried and counted on paper
269,200 ± 18430	278,730 ± 28940

Table 15 The recovery of [1-^{14}C]IAA during the development of paper chromatograms. The [1-^{14}C]IAA was applied to the paper in a 5 µl droplet of 70% ethanol. All values averaged from a minimum of 5 replicates ± standard deviations.

	Average activity applied to the paper	Chromatograms stored		Chromatograms developed in isopropanol:NH_3:H_2O
		light	dark	
dpm	268,200 ± 18200	219,050 ± 18257	213,940 ± 4608	193,430 ± 18686
% recovery	100	82	80	72

identical.

f) Chromatography

Paper chromatography

Table 14 demonstrates that no loss of ^{14}C occurred during the preparation of the chromatograms. The major loss of ^{14}C, 18-20% during storage, can be avoided by the immediate development of the chromatograms once prepared, and elution, counting or other usage, immediately upon drying. The loss during development can not be avoided (Table 15).

In general the figures produced by experiments using [1-^{14}C]IAA agree well with results from plant extracts.

TLC

Thin-layer plates gave a ten-fold increase, at least, in the minimum concentration of compounds detectable by colour reagents compared to paper chromatography.

Gel Filtration

It was found impossible to repeat the method of Tillberg (1974) as standards of synthetic IAA and IAAsp had identical elution volumes. Alternative elution solvents (70% aqueous ethanol, 30% aqueous ethanol) were used without success.

g) Elution

Absolute ethanol eluted only a small proportion (approximately 10%) of the radioactive plant metabolite from the paper chromatograns on which it had been separated. 70% aqueous ethanol was found the

Table 16 The recovery of ^{14}C during the elution of paper chromatograms. [1-^{14}C]IAA was applied to the paper in a 5 µl droplet of 70% ethanol. The chromatograms were eluted in 70% ethanol.

All values averaged from a minimum of 5 replicates ± standard deviations.

	Average activity applied to paper	Average activity in elutant	Activity remaining on paper	Total activity recovered
dpm	203, 681 ± 18374	215,450 ± 7867	2,720 ± 349	218,170
% recovery		98.7	1.3	100

Table 17 Estimated recoveries during the isolation of [^{14}C]IAAsp from 70% ethanol extraction of intact light-grown dwarf pea plants labelled with [1-^{14}C]IAA to the apical buds. Results are expressed as % ^{14}C applied to the plants and are cummulative after each experimental stage.

Procedure		Alternative method	
Application	100		
Activity extracted from apical tissue	81		
Rotary evaporation	42		
Paper chromatography	30	Partitioning (petroleum ether pH 8.0-9.0)	42
Proportion as IAAsp	27	Partitioning (petroleum ether pH 2.5)	27
Elution	26	Partitioning (butan-1-ol pH 2.5)	26
Rotary evaporation	14	Rotary evaporation	14
To purify further			
Paper chromatography	13		
Elution	12		
Rotary evaporation	6		

Table 18 The incubation of [1-^{14}C]IAA with stem segments from 12-day old dwarf and tall pea seedlings grown under different light conditions. The segments were incubated for 4 h with [1-^{14}C]IAA (2 µg ml^{-1}). The pretreated dark-grown tall pea segments were previously incubated for 8 h with unlabelled IAA (2 µg ml^{-1}).

The results are expressed as a percentage of the total ^{14}C added as [1-^{14}C]IAA to the experimental solutions.

Segments incubated	Percentage ^{14}C in the experimental solutions and washes after incubation	Percentage ^{14}C extracted from the segments (70% ethanol)	Percentage ^{14}C unaccounted for
Light-grown Dwarf peas	87.0	2.5	10.5
Dark-grown Dwarf peas	77.0	2.0	21.0
Light-grown Tall peas	57.0	2.0	41.0
Dark-grown Tall peas	62.0	2.5	35.5
Pretreated Dark-grown Tall peas	88.0	3.0	9.0

most efficient solvent leaving only 2% of the activity on the paper. Distilled H_2O, 80% and 90% aqueous ethanol all had the ability to remove about 90% of the ^{14}C-material (presumably IAAsp) from the chromatography paper. These results also agree with control experiments using [1-^{14}C]IAA, the efficiency of 70% ethanol is seen in Table 16.

Balance sheet for the recovery of [^{14}C]IAAsp

Table 17 provides a summary of the cummulative loss of ^{14}C-material during the extraction and purification procedure based on the results presented above. The final predicted recovery agrees accurately with recoveries achieved from plant extraction where between 3% and 7% of the applied [1-^{14}C]IAA was isolated as [^{14}C]IAAsp. Comparisons of recoveries from experiments using [1-^{14}C]IAA as a standard and those using [^{14}C] plant extracts appeared equivalent except in the case of rotary evaporation. Table 17 also illustrates that the substitution of the first preparatory paper chromatography with partitioning has little effect on the overall recovery of [^{14}C]IAAsp.

The isolation of IAAsp from stem segments incubated in [1-^{14}C]IAA

The percentage recoveries recorded for ^{14}C were extremely variable. Pretreatment appeared to reduce this loss (Table 18). However, these results were produced by preliminary experiments and were not well replicated. The percentage activity extracted from the tissue was small, although a proportion of that found in the incubation medium had been metabolised (Rf 0.07 in IAW). This may have resulted from combining the washes from the segments with

the incubation medium. The metabolite may have been washed from the surface of the segments but this is unlikely as other workers have found metabolites in experimental solutions which have not been combined with washes. There may be surface sites for the conversion of IAA to IAAsp, either on the epidermal or cut surfaces. Alternatively leakage of enzymes from the segments may occur, or the IAAsp formed may leak out into the external solution.

Generally the proportion of IAAsp to IAA was the same in the tissue, as in the incubation solution and wash. The percentage conversion of [1-^{14}C]IAA to IAAsp by segment incubation was considerably lower than that achieved by the application of exogenous IAA to the apical buds of intact plants (results above). A range of 3% to 40% conversion to [^{14}C]IAAsp was recorded, with an average of 15%. This may relate to differing metabolic activity of stem and apical tissue.

The chemical synthesis of IAAsp

The chemical synthesis of unlabelled IAAsp gave a percentage return of 25% of IAA used. A full report including the identification of IAAsp is submitted in the Appendix. Although this method might provide the basis for the synthesis of [^{14}C]IAAsp there are a number of serious technical difficulties that would need investigation before this method could be used routinely. The major problem would be in obtaining an acceptable yield of [^{14}C]IAAsp from the [1-^{14}C]IAA employed. Firstly a TLC (preferably using cellulose plates) system must be devised which would separate all the components involved from the end product, IAAsp. The use

of TLC should aim to remove the necessity for partitioning and recrystallization. Secondly the reactions must be scaled down to µg quantities for a reasonable specific activity to be obtained and this would increase problems of recovery. [1-^{14}C]IAA should be added to excess amounts of the constituents, and the end products and intermediates separated by chromatography. It is not known what percentage recovery could be achieved in the above manner.

DISCUSSION

The extraction of IAAsp from intact pea plants labelled with [1-^{14}C]IAA

A thorough investigation has been made into the efficiency of techniques used for the isolation of IAAsp from plant tissues. These results have shown that the recorded recovery of 3-7% [^{14}C]IAAsp can be explained by inherent losses in the procedures used for its isolation, concentration and purification.

Consistently the majority of the [1-^{14}C]IAA applied to the pea apex was metabolised to IAAsp which remained almost wholly within the apical tissue. The problem(s) found in obtaining pure [^{14}C]IAAsp samples for further work are therefore due to losses during isolation and concentration, rather than the low metabolic formation of the conjugate.

The efficiency of 70% ethanol extraction was described and a relatively small percentage of radioactivity remains unaccounted for at this stage. A surprisingly low amount of ^{14}C was released by NaOH extraction which indicated little protein-bound IAA. This

was perhaps due to the short translocation period. Alternatively, homogenization may have released the protein-bound IAA complexes prior to NaOH extraction. An alternative extraction system, $NaHCO_3$, that was explored proved unsuccessful in the extraction of [^{14}C]IAAsp. Davies (1972) had reported that H_2O, as an extraction solvent, removed material which was probably present in the free space, or which diffused out of the plant cells. This included a high proportion of IAA. Davies also suggested that the larger percentage of IAA present in alkaline extracts (IN NaOH or KOH) may indicate the hydrolysis of bound IAA (Davies and Galston, 1971). Such observations may explain the proportionally higher recovery of IAA compared to IAAsp by $NaHCO_3$ extraction. Further investigations were not made as this extraction system was less efficient than 70% ethanol.

The potential losses of the steps comprising the isolation and purification procedure were then investigated. Large differences in the ^{14}C losses from ethanol solutions containing [1-^{14}C]IAA and ethanol plant extracts containing metabolised [1-^{14}C]IAA were noted during rotary evaporation. Other indole compounds may behave differently and substance(s) within the plant extract may increase the ^{14}C-losses.

Mann and Jaworski (1970) claimed that most of the losses of IAA during chromatography consisted of oxidation during the application of solutions to the origin of chromatograms. Data presented here suggests that this is not the case, the major loss of [^{14}C]IAA and its metabolites occuring during storage of chromatograms. The following precautionary measures were adopted to minimize the loss of IAAsp during purification.

1) Rotary evaporation was responsible for the major loss. By not reducing the sample to dryness and having a maximum temperature of 35°C this loss could be held to a minimum and fairly constant level.
2) The chromatograms were loaded rapidly and development and elution following immediately. The elimination of the losses occuring during storage has gone some way to improve the percentage returns.
3) Where the storage of partially purified extracts could not be avoided losses were minimized by storage in ethanol, under refrigeration in darkness.
4) The isolation procedure was completed as quickly as possible.

A major problem with the use of paper chromatography was the interference from solid material extracted and concentrated with the 70% ethanol extract. Consequently the amount of extract that could be loaded onto the paper was small. Partitioning against petroleum ether and butanol successfully overcame these problems. This clean-up procedure was shown to have little effect on the content (radioactive metabolites) of the plant extract. Hoad, Hillman and Wareing (1971) also demonstrated that partitioning, in this case of honeydew samples containing ^{14}C-IAA and ^{14}C-IAAsp, with ethyl-acetate at pH 2.6 did not alter the radioactive compounds present in the sample compared to 80% ethanol extraction. It was also shown that the losses inherent in partitioning were no more than those involved in the initial preparatory paper chromatography and this technique therefore provides an useful alternative method for cleaning the sample. It is especially

useful for large plant extracts (30 or more plants).

The final procedure adopted to isolate and purify IAAsp from pea plants is shown in Figure 11. This technique was followed to produce the [^{14}C]IAAsp which was used experimentally. No difference was noted between the behaviour of this [^{14}C]IAAsp when applied to the apical buds of intact peas, and that purified by preparatory paper chromatography as described in Section II. This procedure failed to yield [^{14}C]IAAsp in sufficient quantity to make it economically viable. However, this technique was successfully employed when [^{14}C]IAAsp was produced during other experimentation, and also in the isolation of IAAsp from unlabelled plants for identification by mass spectroscopy.

The isolation of IAAsp from stem segments incubated in [1-^{14}C]IAA

The recoveries of ^{14}C from this system were extremely erratic. Andreae and Good (1957) had related the loss of activity during incubation to the loss of $^{14}CO_2$. In a later paper they described large variable losses from the experimental solution (70-90%). Zenk and Müller (1964) found the loss of activity as $^{14}CO_2$ could exceed 50%, and that the cut surface of the segment increased the rate of destruction of IAA. Others have also discussed the decarboxylation of IAA at the cut surface, especially with reference to donor and receiver blocks (Kirk and Jacobs, 1968; Epstein et al., 1975; Beyer and Morgan, 1970; Krul and Colclasure, 1977). Such losses do not appear uncommon. Zenk and Müller (1964) also found the epidermis to be active in oxidising IAA. It is possible that the loss of IAA represented bacterial activity. Libbert and Rische(1969) noted the existence

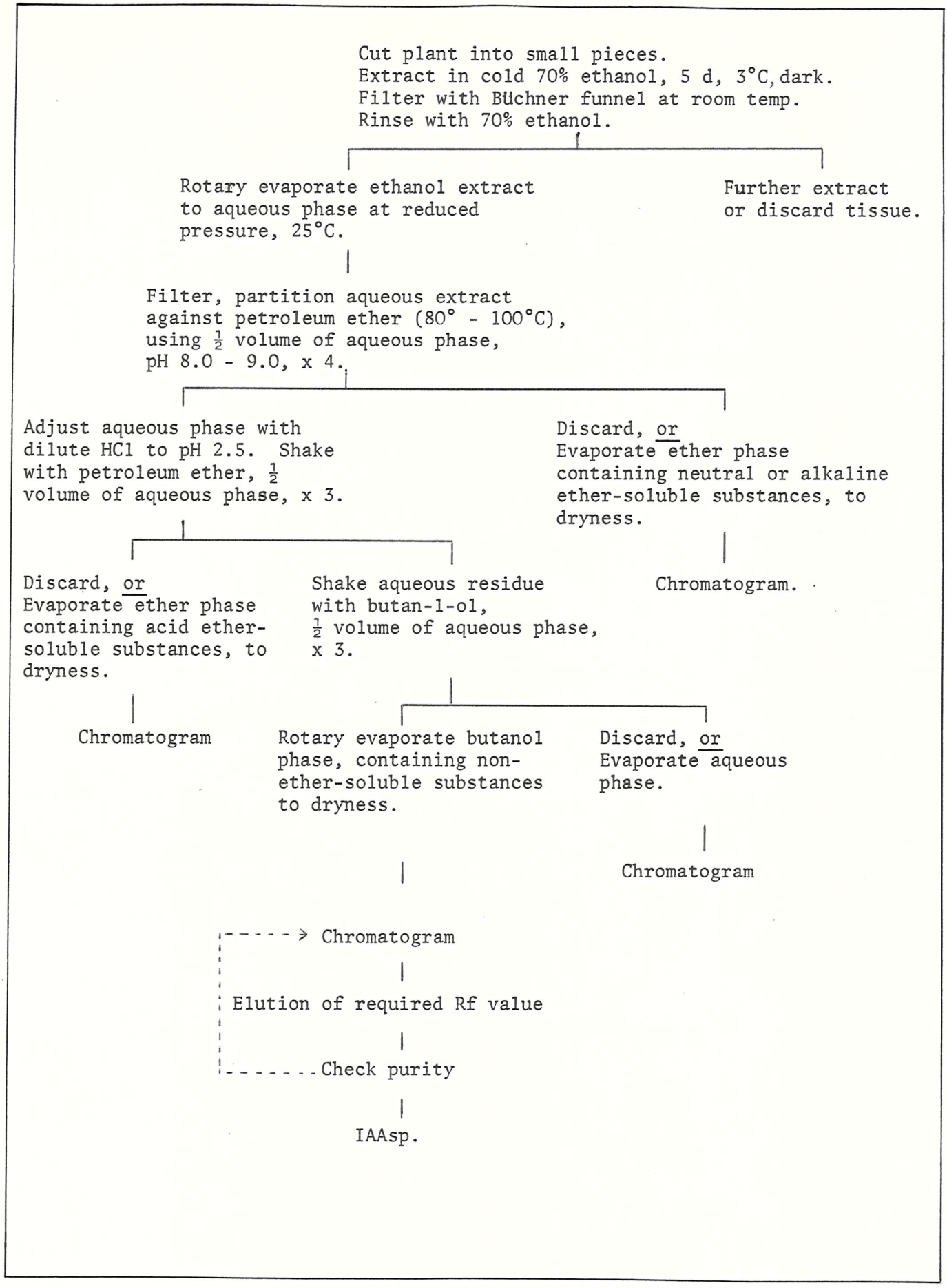


Figure 11 Flow diagram showing the general procedure for the extraction and purification of ^{14}C-metabolites from P.sativum seedlings labelled with [1-^{14}C]IAA.

of epiphytic IAA-degrading bacteria, although they were far less numerous than IAA-synthesis bacteria, and expressed the opinion that not only were degrading bacteria of minor importance, but would preferentially use carbon sources other than IAA. Libbert et al. (1970) also found that in spite of the presence of IAA-synthesising bacteria there was no difference in the conversion of tryptophan to IAA by sterile or non-sterile systems.

Various authors have also observed the presence of metabolites in the incubation fluid as was recorded here. Andreae et al. (1961) suggested such metabolites were formed in the segments after the uptake of IAA and diffused out. Zenk and Müller (1964) were also in favour of leakage into the solution while Mino (1968) and Magnus et al. (1971) believed the metabolites in the experimental solution were due to the liberation of enzymes from the damaged cells of the cut surface. Kenny et al. (1969) also described changes in the external solution that were not due to microorganisms but related to the metabolic changes in the tissue and leakage into the solution. Such leakage would appear to be the explanation for the results recorded here, and is substantiated by the similarity in the proportions of metabolites found in the experimental solutions and the tissue itself.

The poor percentage conversion from IAA to IAAsp may reflect the uptake rate of the tissue, or alternatively lower capacity for the conversion in stem tissue compared with apical tissue. However, this system has no advantage over the extraction of the radioactive metabolite from apically-labelled intact plants, and has the disadvantage that a large, variable and unaccountable percentage of the ^{14}C may be lost during incubation. This is in

addition to the losses of ^{14}C which occurred during the extraction, purification and concentration procedure described earlier in this Section. This technique was therefore rejected as a method for the synthesis of [^{14}C]IAAsp.

The chemical synthesis of [^{14}C]IAAsp

The modifications necessary for the use of this method to synthesis radioactive IAAsp have already been described. The low recoveries coupled with the low specific activity of commercial available [1-^{14}C]IAA and the high cost excluded the use of this method.

CONCLUSION

This thesis questioned initially the fate and function of IAAsp formed from exogenous IAA in pea. Under exploration in this Section was the isolation of radioactive IAAsp, the object being to generate pure [^{14}C]IAAsp for utilization in experiments investigating the proposed hypothesis, the control of auxin levels by conjugation. An improved isolation technique allowing the purification and concentration of the auxin conjugate, IAAsp, from plant tissue has been described. Two alternative systems were considered and preliminary experiments completed on one. Although the percentage yields of the method selected were low and did not allow the preparation of sufficient [^{14}C]IAAsp for experimental work, the technique provides a replicable and fairly simple method for the isolation of IAAsp and was subsequently used to prepare samples of the unlabelled compounds for attempted identification procedures (Section IV).

Section IV

THE IDENTIFICATION OF IAAsp

THE IDENTIFICATION OF IAAsp

INTRODUCTION

It was assumed at the start of the project that the immobile conjugate of IAA reported to occur in pea was IAAsp (Andreae and Good, 1955; Morris et al., 1969). However, a closer examination of the literature revealed that in many instances, in the pea and other species, identification had been based upon inadequate criteria and was therefore unreliable.

Many identifications of IAAsp have been based on paper chromatography in one solvent system, isopropanol:NH_3:H_2O (10:1:1 v/v)(IAW) (Südi, 1966; Aasheim and Iversen, 1971; Kendall et al., 1971; Goren et al., 1974; Lau et al., 1974; Epstein, Kochba and Neuman, 1977). In practice, if the metabolite co-chromatogrammed with authentic IAAsp this was taken as positive identification (Kang et al., 1971). This type of 'identification' can only be described as tentative as no attempt was made to further characterize the metabolite or to eliminate the possibility of other labelled compounds co-chromatogramming with IAAsp. Several authors have discussed this problem. Davies (1972) reported the occurrence of several labelled metabolites of IAA with common Rf values in certain solvent systems, and Schneider et al. (1972) reported that malonyl tryptophan co-chromatogrammed with IAAsp in isopropanol:NH_3:H_2O. Characterization of the IAA-metabolite as IAAsp has been attempted by authors using a combination of three (Weaver and Jackson, 1963; Olney, 1968; Davies, 1972; Patrick and Woolley, 1973) or four (Minchin and Harmey, 1975; Feung et al., 1976; Epstein et al., 1977) different solvent systems. A firm identification was not

claimed in every case.

Tillberg (1974) offered identification of endogenous IAAsp by correlation of the behaviour of authentic IAAsp and extracted endogenous metabolite using two-dimensional TLC, gel filtration, electrophoresis, alkaline hydrolysis and bioassay. However, she found the bulk of the metabolite from the plant extract to be soluble in an acid-ether fraction on partitioning. This is in contrast with the results presented in Section III, and the partitioning of synthetic IAAsp. Minchin and Harmey (1975) using UV spectroscopy claimed the identification of IAAsp by comparison with the UV spectra published by Mollan et al., (1971). As few experimental details were given it is difficult to judge the reliability of this report.

As a positive identification of the metabolite as IAAsp can not be provided solely using the above methods, the possibility of characterization by other techniques was investigated.

MASS SPECTROSCOPY

Biologists are becoming increasingly aware of the advantages of mass spectroscopy (MS) in the identification of compounds. In recent years the successful characterization of IAA and other plant indoles by MS has been reported including Jamieson and Hutzinger (1970), Greenwood et al., (1972) and Allen et al., (1979). The MS fragmentation pattern of a chloro-IAAsp (mono-methyl-4-chloroindolyl-3-acetyl-L-aspartate) isolated from extracts of immature seeds of P.sativum has been obtained (Hattori and Marumo, 1972).

The procedure described in Section III provides an ideal system for the purification of IAAsp from plant extracts and was used here to obtain material for a MS analysis. To ensure an

adequate quantity of purified IAAsp, cold IAA was applied to the pea apices and extraction started after an eight hour metabolism period. The sensitivity of MS is such that 1µg of pure chemical is adequate for a positive identification.

Method

For each attempt at identification a minimum of ninety (later between two and three hundred) plants were grown. Cold IAA (0.5µg $plant^{-1}$) was applied to the apices of 12-day old light-grown 'Meteor' peas. Ten to 15 additional plants were labelled with [1-^{14}C]IAA using the same procedure in order to monitor the behaviour of IAA during the preparation. The IAA was applied in a 5µl droplet of 'Tween-20'. After an eight hour translocation period the apices were removed and extracted in cold 70% ethanol for three days at 3°C in darkness. The samples were then reduced by rotary evaporation (25°C) to the aqueous phase, filtered under suction to remove solid matter and partitioned against petroleum ether (pH 8.0) as described in Section III. The aqueous phase was further reduced and chromatogrammed using pre-washed TLC plates (silica gel, incorporating 13% $CaSo_4$; BDH, Dorset, England; 0.35 mm thickness). Between 15 and 20 samples were spotted across a 100 mm x 100 mm plate and the plates were then developed in the ascending direction in chloroform: ethyl acetate:formic acid (35:55:10 v/v). One developed sample was sprayed with Ehrlich's reagent while the other samples on the plate were adequately protected.

During the initial experiments silica gel from a wide band of the plate, corresponding to the Rf of IAAsp, was removed and compounds eluted by stirring overnight in 70% ethanol (Fig. 12). In later

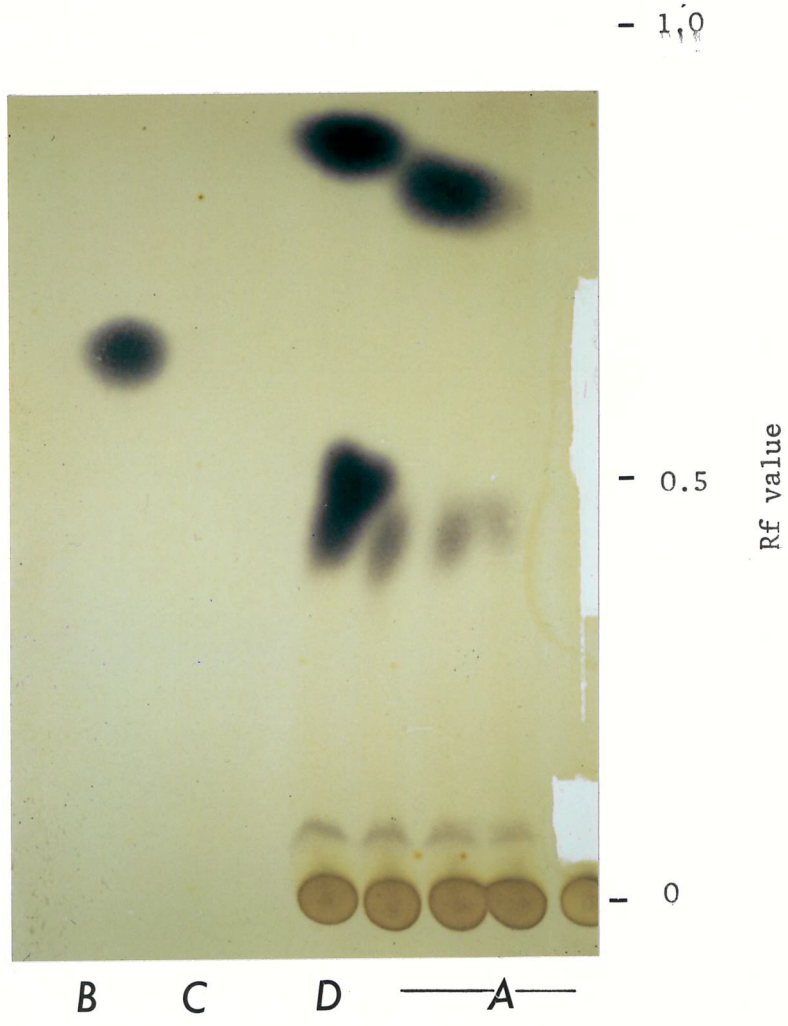


Figure 12 TLC separation of A: unlabelled plant extract prepared for mass spectroscopy, B: authentic IAAsp, C: authentic IAA (note veer to right), and D: plant extract run with IAA and IAAsp standards. The eluted regions can be seen. The chromatogram was developed in chloroform:ethyl-acetate:formic acid (35:55:10 v/v) and developed in Ehrlich's reagent.

experiments narrower zones were removed corresponding to Rf values of 0.6, IAAsp, and 0.08. On spraying the chromatogrammed sample of plant extract (non-radioactive) with Ehrlich's reagent a blue spot developed at Rf 0.08. The elutant was filtered through a scinter under suction, reduced and the purity of the sample checked by micro TLC techniques. On many occassions it was necessary to further purify the sample by TLC.

After elution and concentration both samples displayed a yellow colour. The mass spectra were obtained by direct probe analysis employing an AEl MS 12 model mass spectrometer.

Due to the required purity of the sample care was taken to avoid the use of any plastics or grease during the course of the experiment. All water was doubly distilled in glass only.

Results

The methyleneindole ion, characteristic of all indole compounds (Fig. 13), was not detected in any of the spectra produced. Several attempts were required before 2 good spectra were obtained for the material running at Rf 0.08 and Rf 0.60 (Fig. 14). Neither were indole compounds. The 8-peak mass spectra catalogue was consulted but it was found impossible to identify the compounds isolated. An NMR was attempted but there was an insufficient quantity of the compound available. A MS of authentic IAAsp is included in the appendix.

Discussion

The spectra obtained were not from indole compounds. It is possible that two compounds, unrelated to IAA-metabolism,

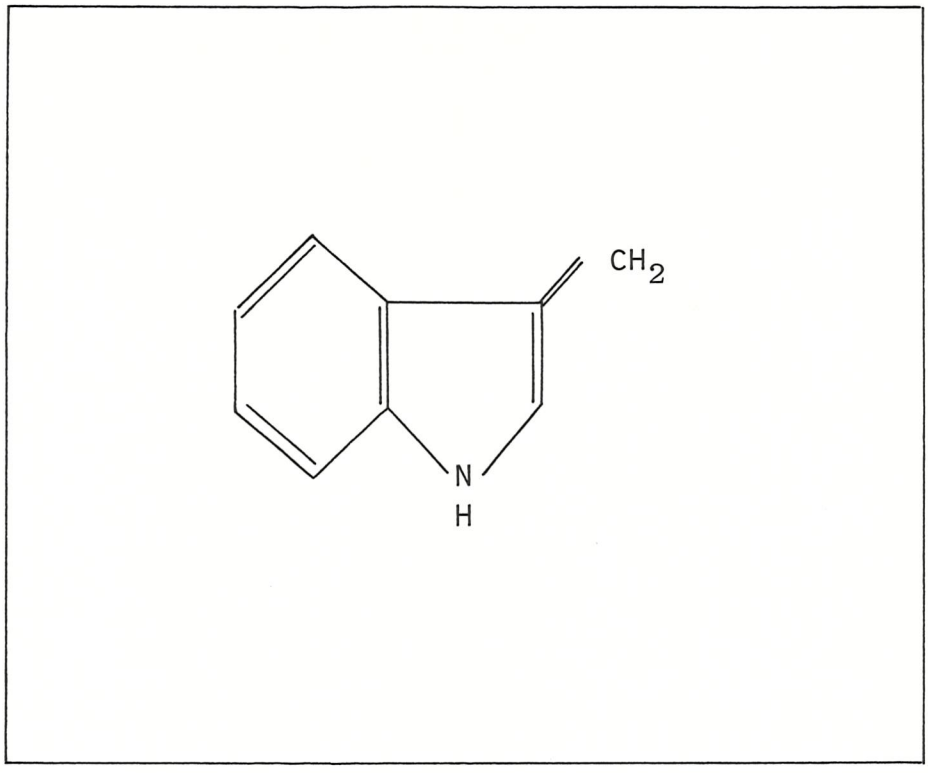


Figure 13 The methyleneindole ion.

MASS	INT %B
236.20	0.45
168.16	0.55
158.16	0.55
154.16	0.29
152.16	1.56
140.11	0.29
140.06	0.39
139.11	0.35
136.19	0.32
129.12	0.45
128.13	0.52
127.16	0.45
126.16	1.62
125.18	0.71
124.18	0.61
123.17	1.26
122.13	0.35
121.11	0.45
113.17	0.65
112.14	1.40
111.10	1.36
110.06	1.04
109.10	0.84
108.15	0.52
107.16	0.29
106.18	0.29
102.13	0.32
101.09	7.03
100.04	4.55
99.07	35.23
98.12	4.20
97.15	2.53
96.20	1.59
95.18	0.74
94.19	0.52
93.16	0.32
87.15	0.45
86.21	1.23
85.19	4.10
84.18	8.66
83.15	-
82.12	3.67
73.19	3.77
72.15	3.38
71.10	78.67
58.22	4.62
57.26	2.70
56.28	22.56
55.30	43.30
54.29	6.80
46.34	17.06
45.37	4.39
43.38	6.74
29.97	2.83
28.07	5.01

Figure 14

Mass spectrogram of the compound isolated from the 70% ethanol extract of dwarf pea apices 8 hours following the application of IAA. The compound chromatogrammed with authentic IAAsp at Rf 0.60 in chloroform:ethyl-acetate: formic acid (35:55:10 v/v).

chromatogrammed at the Rf values of IAAsp and the unknown compound. Radioactive metabolites from plants labelled with [1-^{14}C]IAA chromatogrammed at the Rf value (0.6-0.65) of the compound thought to be IAAsp, and also between Rf 0-0.1, which correlates with the other compound. Both Rf values from which the samples were eluted also showed a reaction to Ehrlich's reagent, although this is not absolutely specific for indole derivatives and tryptophan (Zweig and Sherma, 1972).

The compound detected at Rf 0.60 may be a side chain metabolite of IAA. Davies (1972) noted an inconsistancy during studies using [1-^{14}C],[2-^{14}C] and [^{3}H]IAA. He failed to detect IAAsp in two out of three chromatography solvent systems when [^{3}H]IAA was used. The metabolite detected when [1-^{14}C] and [2-^{14}C]IAA were used may represent a side chain metabolite separated from the indole ring. Aasheim and Iversen (1971) recorded similar inconsistancies in results after labelling with [2-^{14}C] and [^{3}H]IAA. They suggested this was due to tritium exchanged when using [^{3}H]IAA. Bender and Neumann (1978) incubated carrot tissue cultures with [1-^{14}C]IAA. A large recovery, 75% of the ^{14}C-activity within the tissue, was found in the 'organic acid fraction'. Their separation technique also gave acid, basic and neutral indoles, amino acid and carbohydrate fractions. They felt this may indicate that IAA taken up by the tissue was readily broken down by the side chain splitting. However, Kulescha (1971) used ring-labelled ^{14}C-IAA in tissue culture experiments and found a compound co-chromatogramming with IAAsp formed as a metabolite. A side chain metabolite would have been non-radioactive and undetected.

Another interesting point relates to the quantity of tissue used.

During these experiments between 50 and 80 g (fresh weight) of tissue was extracted, from plants to which exogenous IAA had previously been applied and metabolised. IAAsp could not be identified. However, Row et al. (1961) had detected the natural occurrence of IAAsp in 24 g (fresh weight) of tomato tissue using relatively unsophisticated methods, chromatography and colour reagents. Tillberg (1974) reported the extraction of endogenous IAAsp from between 10 and 20 g of bean tissue. Scheider et al. (1972) having carried out a detailed study of the metabolism of IAA in tomato and barley plants felt that Row et al. were more likely to be detecting malonyl tryptophan than IAAsp. Schneider et al. (1972) found malonyl tryptophan at levels of 400µg/kg of plant tissue, more in keeping with the detection range of Row et al. (1961).

Either the technique used here was inadequate for the purification of IAAsp (perhaps due to inherent losses or masking by other, more predominant compounds) or the existence of IAAsp must be questioned. As identification can only result from the accuracy and competence of the technique, it was decided more success might be achieved by combined gas-chromatography (GC-MS). This system was unavailable in Southampton at the time of the investigation and co-operation was sought, and gratefully received, from the Department of Botany at Glasgow University.

GAS CHROMATOGRAPHY - MASS SPECTROSCOPY (GC-MS)

At Glasgow, McDougall and Hillman (1978) had successfully developed a system (GC-MS) for the analysis of IAA from biological samples. This technique produced a reliable identification of IAA,

and it was hoped could be extended with slight modification to the resolution of IAAsp.

Method and Results

Initially a sample of synthesised IAAsp was sent to Glasgow for preliminary investigation.

Derivatization and Purification

A small portion (6 mg) of the synthetic IAAsp was esterified with excess of etherial diazomethane. The ether soluble fraction was further purified by TLC on a silica plate using ether, or ether-n-Hexane as the solvent system. The compound separated into 2 bands (Rf 0.55 and Rf 0.85 using ether as solvent). The less polar fraction was recovered and examined. It indicated that dimethyl-IAAsp (IAAsp.Me) had been formed and was fairly pure. The mass spectral analysis showed the molecular ion at m/e 318 and peaks at m/e 287, 286, 259, 174 and 130 (100%).

GC-MS analysis

Three IAAsp derivatives were analysed by GC-MS, IAAsp.Me, trimethylsilyl IAAsp (IAAsp.TMS) and dimethyl trimethylsilyl IAAsp (IAAsp.Me.TMS). A variety of columns were used including OV-101, SE-30, QF.1 and Dexil 300 1-3%, from 1.5 ft to 9 ft length and at temperatures 240°C-280°C. The results indicated that these compounds did not possess good gas chromatographic properties. When selected ion monitoring was examined in conjunction with the GC traces obtained, it indicated that detrimethylsilylation occurred on the column in an unpredictable manner. Additionally it was realized

that unless the hydrogens on the ring nitrogen and the amide group were properly blocked, the derivatives were unsuitable for quantitative GC-MS analysis.

Attempts to block these sites by the preparation of trifluoroacetyl or acetyl derivatives of IAAsp.Me were unsuccessful. The Glasgow team concluded that until these or similar stable and more volatile derivatives of IAAsp.Me could be prepared the best available means of identification was paper chromatography followed by probe analysis. This technique and the lack of satisfactory results have already been described.

Analysis of a biological sample

A sample of plant extract believed to contain IAAsp was sent to Glasgow. The sample was prepared as described for Mass Spectroscopy except for the use of 80% methanol as the extraction solvent (Hillman, personal recommendation). It proved a far quicker extraction solvent than ethanol and was equally efficient (12 hour extraction period at 20°C in darkness). The samples were chromatogrammed initially on paper in isopropanol:NH_3:H_2O (10:1:1 v/v), eluted and re-chromatogrammed on paper in n-butanol:glacial acetic acid:water (4:1:1 v/v). This technique was adopted to avoid problems which had arisen through the use of silica gel (see later discussion on the use of TLC). The partially purified sample failed to provide positive results either after examination by GC-MS or by direct probe analysis at Glasgow.

GC-MS did not prove to be a successful technique for either the identification of IAAsp or the recovery and purification of IAAsp from a biological sample. The later could have been extremely

useful in providing clean biological samples for further experiments.

CHROMATOGRAPHIC PROPERTIES OF THE PLANT METABOLITE

To provide additional information for the identification of the auxin conjugate, its chromatographic properties were investigated. Using radiochemical methods these details can easily be found for submicrogram quantities of the metabolite. The Rf values of the radioactive plant extract and authentic IAAsp were compared by TLC and paper chromatography in several solvent systems.

Methods

The methods used for the preparation of radioactive material, extraction, fractionation and chromatography have been previously described in detail (Section III). Analytical TLC plates (silica gel) were prepared to 0.25 mm thickness. The solvent systems are listed below. The authentic indole compounds were located with Ehrlich's reagent (BDH, England).

1. Paper Chromatography:

Solvent

A Isopropanol:NH_3:H_2O (I.A.W.). 10:1:1 v/v

B Isopropanol:H_2O 5:95 v/v

C 8% NaCl in 1% aqueous solution of glacial acetic acid (w/v)

D Butan-1-ol:acetic acid:H_2O (B.A.W.). 4:1:1 v/v

E Petroleum ether:diethyl ether:methanol:acetic acid:H_2O
60:60:15:2:1 v/v

2. TLC

Solvents

A Isopropanol:NH_3:H_2O (I.A.W.). 10:1:1 v/v

F Chloroform:methanol:acetic acid 75:20:10 v/v

G Chloroform:ethyl acetate:formic acid 35:55:10: v/v

Results and Discussion

Table 19 demonstrates the correlation between the Rf values of the major radioactive metabolite found in plant extracts, and authentic IAAsp.

IAA was also detected in the majority of the chromatograms, often as only a small proportion of the total radioactivity. Table 20 lists the more consistantly found additional metabolites.

An unidentified ^{14}C-metabolite which did not correspond to the Rf value of either IAA or IAAsp was occassionally detected in the plant extracts separated by solvents F and G. The proportion of radioactivity found at this Rf value (0.35 - 0.45) varied considerably between plant extracts and represented a large proportion of the ^{14}C recovered from one extract (Fig. 15). In other extracts it was not present. An additional ^{14}C-compound was found at the origin on separation of some extracts in solvents F and G. Four peaks are therefore found after separation of the various extracts from [1-^{14}C]IAA labelled apices in these solvents, Rf 0, 0.35 - 0.45, 0.6 (IAAsp), 0.8 (IAA).

Zenk (1962) described the separation of malonyl-D-tryptophan (MTPP) from IAAsp in solvent G. Schneider et al. (1972) concluded that a metabolite found in tomatoes was more likely to be MTPP than IAAsp on the basis of chromatography in the same solvent.

Table 19 The Rf values of the major radioactive metabolite extracted by 70% ethanol from the apices of 12-day old light-grown dwarf peas 8 h following the application of [1-^{14}C]IAA to the apices. The Rf values of authentic IAA and IAAsp are also shown.

* the major ^{14}C peak.

· separation inadequate for purification method unless used in conjunction with other systems.

Solvent system	Paper chromatography					T.L.C.		
Solvent	A	B·	C·	D·	E	A	F	G
Plant Extract*	0.04	0.8	0.8	0.79	0.07	0.07	0.69	0.59
IAAsp	0.04	0.84	0.80	0.79	0.08	0.10	0.69	0.60
IAA	0.33	0.79	0.60	0.89	0.70	0.50	0.86	0.83

Table 20 Minor ^{14}C-metabolites detected following the chromatography of 70% ethanol extracts from the apices of 12-day old light-grown dwarf peas 8 h after the application of [1-^{14}C]IAA to the apices.

* detectable when [2-^{14}C]IAA was applied.

	Solvent	Rf values
A	isopropanol:NH_3:H_2O - paper	0.23, 0.42, 0.58, 0.74, 0.84*, 0.93
B	isopropanol:H_2O - paper	0.44, 0.58*, 0.70
G	chloroform:ethyl acetate:formic acid - TLC	0.07, 0.37
F	chloroform:methanol:acetic acid - TLC	0.08, 0.20, 0.35

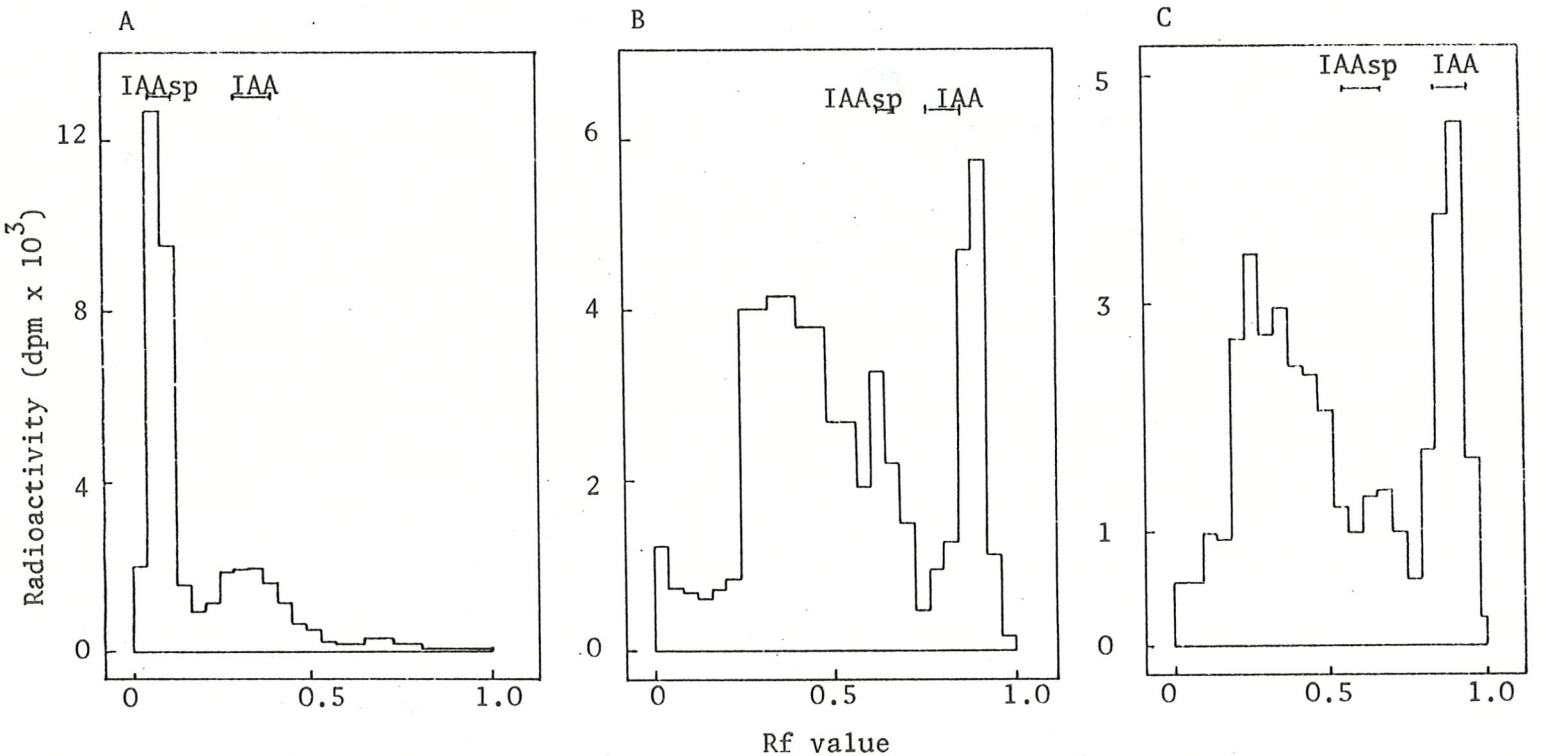


Figure 15 The separation by TLC of a 70% ethanol plant extract from apices labelled with [1-^{14}C]IAA in A: isopropanol:NH_3:H_2O (10:1:1 v/v), B: chloroform:ethyl-acetate: formic acid (35:55:10 v/v), and C: chloroform:methanol:acetic acid (75:20:10 v/v).

Additionally Good and Andreae (1957) and Elliott (1971), reported that IAAsp and MTPP co-chromatogram in many solvents. The cited Rf value of MTPP in solvent G is Rf 0.37 and this may correspond to the ^{14}C-compound found at Rf 0.35 to 0.45 in these experiments (Fig. 15). MTPP runs slightly higher than IAAsp in IAW (Rf 0.23) and may explain the small additional peak occasionally detectable in this solvent system. The chemical similarity between IAAsp and MTPP can be seen in Fig. 16. Zenk (1964) suggested that MTPP formation is another mechanism for the immobilization and detoxification of IAA, possibly the formation of MTPP occurring from an indole precursor of IAA. No correlation could be found between any factors, for example partitioning, and the variation in the occurrence of the ^{14}C-metabolite at Rf 0.35 to 0.45 in solvents F and G. It is not certain if a pathway exists which would allow for the conversion of [1-^{14}C]IAA into [^{14}C]MTPP.

There are many alternatives for the identities of the various compounds detected. For example indolepyruvic acid also has a low recorded running position in IAW, Rf 0.20 (Stowe and Thimann, 1953). Two additional metabolites were revealed by separation of plant extracts with solvent B, at Rf 0.70 and 0.58. The latter may possibly be indoleacetic aldehyde (IAAId) as it only appeared when [2-^{14}C]IAA was used. IAAId has an Rf value of 0.84 in IAW and is acid-ether soluble. The removal of an indole metabolite at Rf 0.23 by acid-ether partitioning is illustrated in Figure 17, by comparison of plant extracts D and C.

Schneider et al. (1972) employed chloroform:ethyl acetate: formic acid in different proportions to those used here (50:40:10, IAA-Rf 0.9) and recorded Rf values of 0.05 for 5-hydroxytryptamine

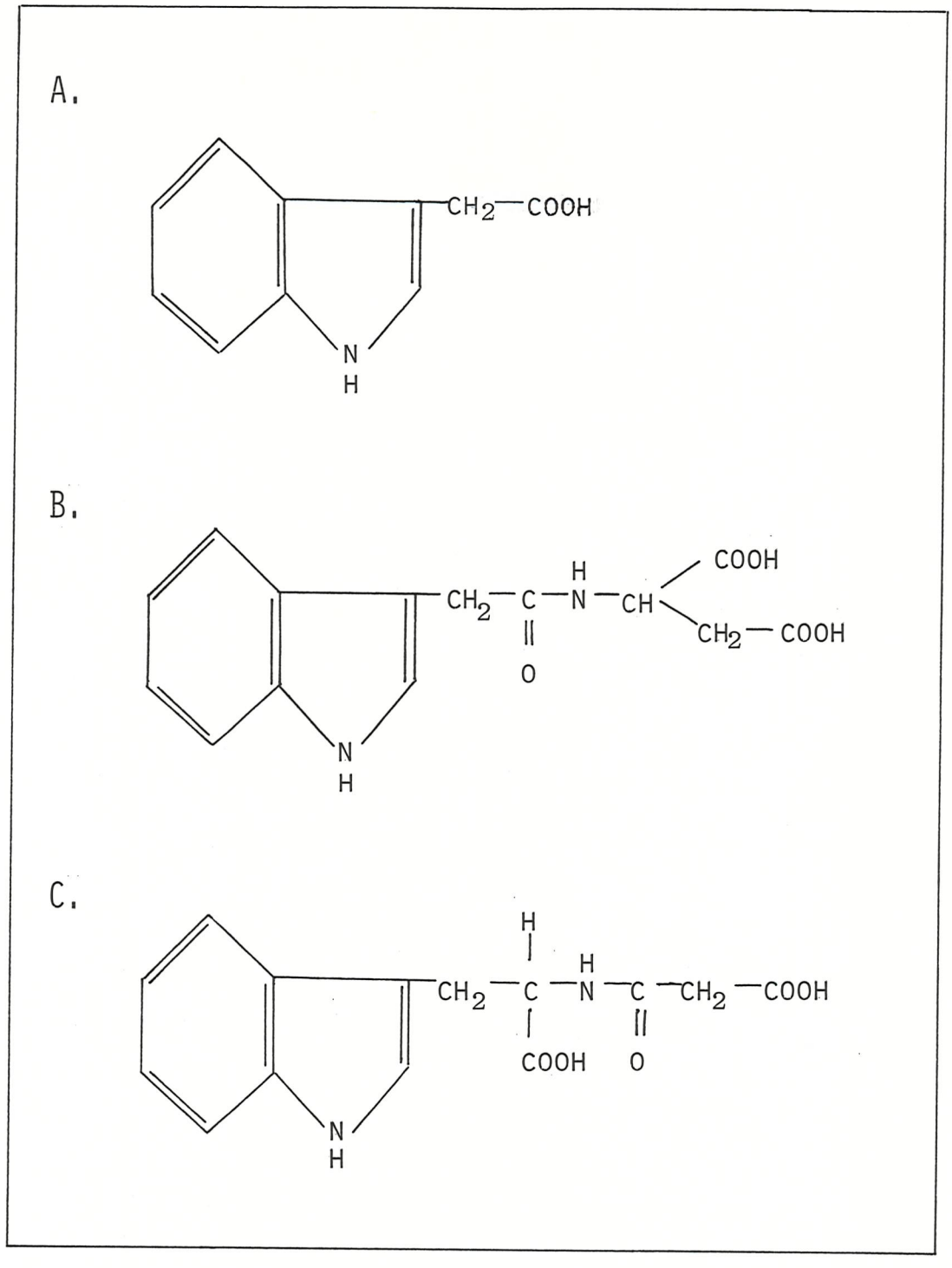


Figure 16 The molecular formulae of A: IAA, B: IAAsp, and C: malonyl tryptophan (MTPP).

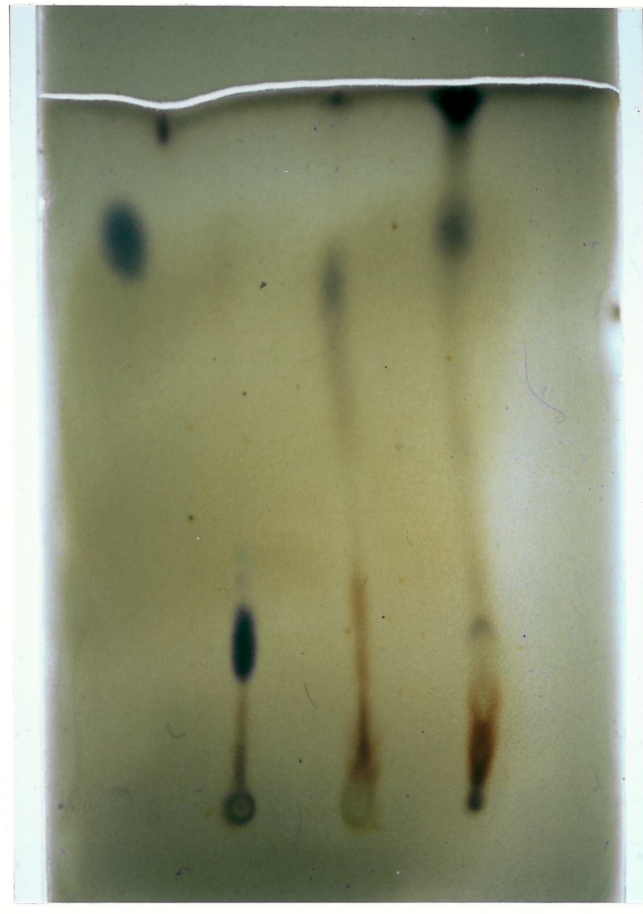


Figure 17 TLC separation of A: the butanol fraction of a plant extract from [1-^{14}C]IAA labelled apices after partitioning (petroleum ether and butanol), and B: authentic IAAsp. C and D are plant extracts of [2-^{14}C]IAA labelled apices, before (D) and after (C) partitioning. The chromatogram was developed in chloroform:methanol: acetic acid (75:20:10 v/v) and sprayed with Ehrlich's reagent.

and tryptophan. Both these compounds correspond to the radioactivity remaining at the origin in Solvent G, and would be radioactive providing metabolism did not cleave the side chain carboxyl from the indole nucleus. 5-hydroxytryptamine has a recorded Rf value of 0.71 in IAW, and tryptophan and Rf of 0.43 in the same solvent (Weaver and Jackson, 1963). Metabolites were detected at both these Rf values. However, positive identification was impossible.

Several authors, including Good et al. (1956) have shown indoleacetamide to be a fast moving compound in IAW, and this may account for one of the metabolites recorded here. Another compound mobile in IAW is indoleacetonitrile (Rf 0.8, Kefford, 1955) but there was no evidence for its presence in these extracts. 3-ethyl indoleacetate was detected at Rf 0.93 in the same solvent by Weaver and Jackson (1963). The list of IAA-metabolites which might be present is large and it is impossible to identify the metabolites occuring here with the information available. Frequently authors have relied on Rf values for the identification of plant metabolites. This method is far from satisfactory.

The use of TLC

The major advantage of silica-gel TLC was the improved resolution of the individual compounds. Consequently the detection thresholds were lowered, for example with Ehrlich's reagent. Separation was also possible over a shorter distance and time of development. There were, however, problems associated with this TLC technique.

One problem that occurred during the use of acid solvents on silica gel was the oxidation of IAA. This was occassionally

observed during development, indicated by a pink spot discernible in daylight at the position of IAA. This faded after development. A pinker colour reaction than normal with Ehrlich's reagent was also observed. Moore and Shaner (1967) experienced this problem and Tsurmi and Ohwaki (1976) referred to the partial destruction of IAA by silica gel. As this was believed to occur only at Rf values above IAAsp it was not considered a major problem in these experiments, but should certainly be avoided in other experiments.

An additional disadvantage in the use of silica gel plates was the increased loss of ^{14}C-material and presumably of IAAsp and other metabolites. The loss of ^{14}C from the silica-gel plate was about 50% of the activity applied, a larger loss occurring in solvents F and G than in IAW. Moore and Shaner (1967) recorded similar percentage losses in their experiments using [1-^{14}C]IAA and attributed these to the oxidation of IAA.

The variable appearance of unknown metabolites during the separation of plant extracts has already been discussed (Fig. 15). A more frequent inconsistency occurred in the samples where indoles were detected with Ehrlich's reagent. A plant metabolite corresponding to the Rf value of IAAsp in IAW (Figure 18, extract A) and various other solvents, appeared not to be IAAsp when run in chloroform: methanol:acetic acid (75:20:10 v/v). This metabolite had a varying Rf value in the same solvent system under seemingly identical conditions, Rf 0.23 and 0.88 (Fig. 17 and 19). However on other occasions a compound was detected at the Rf value of IAAsp in chloroform:methanol:acetic acid (75:20:10 v/v). No explanation can be offered for such variation, except that

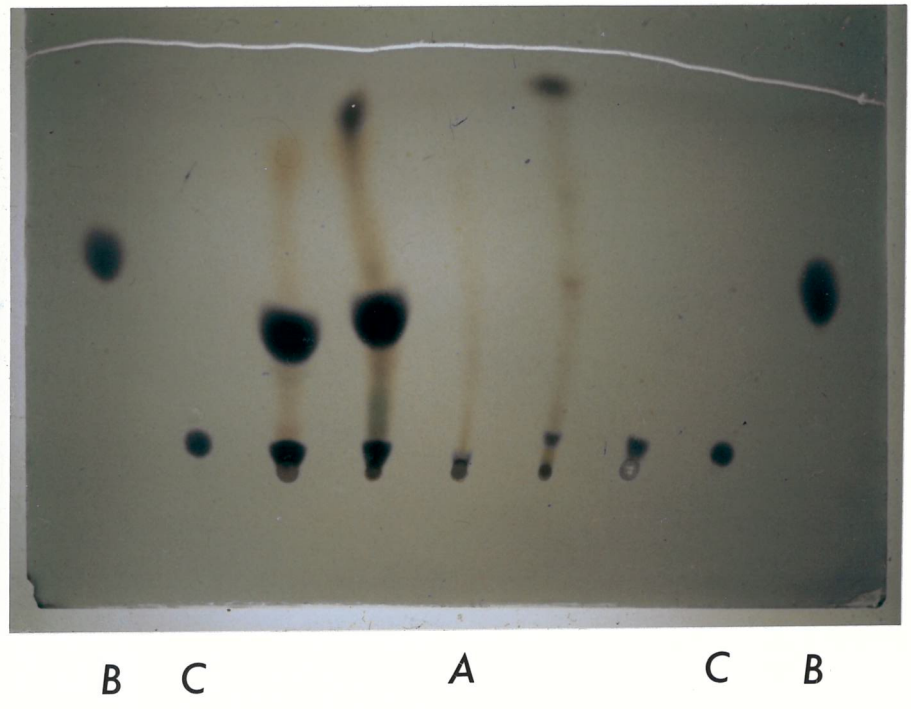


Figure 18 TLC separation of A: the butanol fraction of a plant extract from [1-^{14}C]IAA labelled apices after partitioning (petroleum ether and butanol), B: authentic IAA and C: authentic IAAsp. The other samples represent plant extracts before and after partitioning, and with and without authentic IAA and IAAsp. The chromatogram was developed in isopropanol:NH_3:H_2O (10:1:1 v/v) and sprayed with Ehrlich's reagent.

Figure 19 TLC separation of A: the butanol fraction of a plant extract from [1-^{14}C]IAA labelled apices after partitioning (petroleum ether and butanol), and B: authentic IAAsp. The chromatograms were developed in chloroform:methanol: acetic acid (75:20:10 v/v) and sprayed with Ehrlich's reagent.

discrepancies arose only on silica gel plates in the acid solvents F and G. Such difficulties were associated with only a few of the plant extracts prepared, but should be borne in mind by future investigators and demonstrate the necessity for replication.

In conclusion a positive correlation exists between the radioactive plant metabolite and authentic IAAsp in the eight solvent systems examined. The significance of this in relation to results from alternative methods for the identification of plant metabolites will be discussed at the end of this Section.

HYDROLYSIS OF THE PLANT CONJUGATE

An attempt was made to demonstrate by a series of hydrolysis experiments the liberation of IAA and aspartic acid from the conjugate believed to be IAAsp. Andreae and Good (1955) demonstrated that alkaline hydrolysis of the plant metabolite produced equimolar quantities of IAA and aspartic acid. Hydrolysis of a compound believed to be IAAsp was also shown by Thurman and Street (1962). Liu *et al.* (1978) and Bandurski and Schulze (1977) defined peptidyl-IAA as that liberating free IAA on hydrolysis with NaOH, but offered no further identification of the conjugates they had examined. Earlier workers Bennet-Clark and Wheeler (1959) had liberated aspartic acid from a metabolite by hydrolysis with concentrated acid and had also shown the release of free IAA and aspartic acid from the metabolite treated with 7N NaOH. Similar results would provide additional evidence for the identification of the plant conjugate.

Peptide Hydrolysis

Method

A radioactive plant extract was obtained as previously described. After fractionation with petroleum ether at pH 8.0, and filtration, the aqueous sample was reduced to dryness and taken up in phosphate buffer (pH 7.1). The plant extract (0.5 ml) was incubated with an equal volume of peptidase enzyme at 37°C (0.5 mg ml^{-1}. Sigma, U.S.A. peptidase preparation from hog intestine). Standards of IAAsp were similarly prepared and incubated for 0.5, 1.0, 1.5 and 2.0 h. Later the experiment was repeated with changes in concentration, pH and the length of incubation time. The samples were reduced and chromatogrammed. Three solvent systems were used, chloroform:ethyl acetate:formic acid (35:55:10 v/v), isopropanol:NH_3:H_2O (10:1:1 v/v) (IAW) and butanol:acetic acid:H_2O (4:1:1 v/v)(BAW). BAW was useful in the identification of aspartic acid (Rf 0-0.1) as the radioactive metabolite and standard IAA and IAAsp have high Rf values (0.75 - 0.85) in this solvent.

Results

The pepidase had no effect on the authentic IAAsp or the plant extract. Three possible explanations exist. Firstly, the peptidase was not active. This was not the case as the peptidase was active on bovine serum albumin. Secondly, the incubation system may have been incorrect. Extending the time period, and changes in concentrations and pH did not hydrolyse the IAAsp. The third, possible explanation is that this peptidase was not suitable for action on the peptide bond of IAAsp. However, as Sigma describe this enzyme as having general proteolytic and aminopeptidase activity this also

appears unlikely.

Alkaline Hydrolysis

An attempt was made to hydrolyse the peptide bond with strong alkali following the method of Liu et al. (1978).

Method

To an equal volume of partially purified plant extract or IAAsp standard, 1ON NaOH was added. This was incubated for 4 hours at 37°C (water bath). The pH was then adjusted to 3.0 with HCl and the samples chromatogrammed without reduction. Experimental data is based on 4 replicates.

Results

Separation by paper chromatography (IAW) showed that alkaline hydrolysis of synthetic IAAsp led to the formation of a compound chromatogramming at Rf 0.15 and not to the release of free IAA (Fig. 20). There was also no evidence for the release of aspartic acid. The appearance of a compound at Rf 0.15 was also noted following the hydrolysis of a plant extract (Fig. 21). This compound was positive with Ehrlich's reagent and ninhydrin. Although all the synthetic IAAsp was converted by hydrolysis to the unknown compound at Rf 0.15, only a small proportion of the ^{14}C chromatogramming with IAAsp in the plant extract was so converted. This may suggest that more than one metabolite is associated with the Rf of IAAsp in IAW. Alternatively substances present in the partially purified plant extract may interfere with the action of NaOH.

Aliquots of the same ^{14}C-plant extract prior to hydrolysis

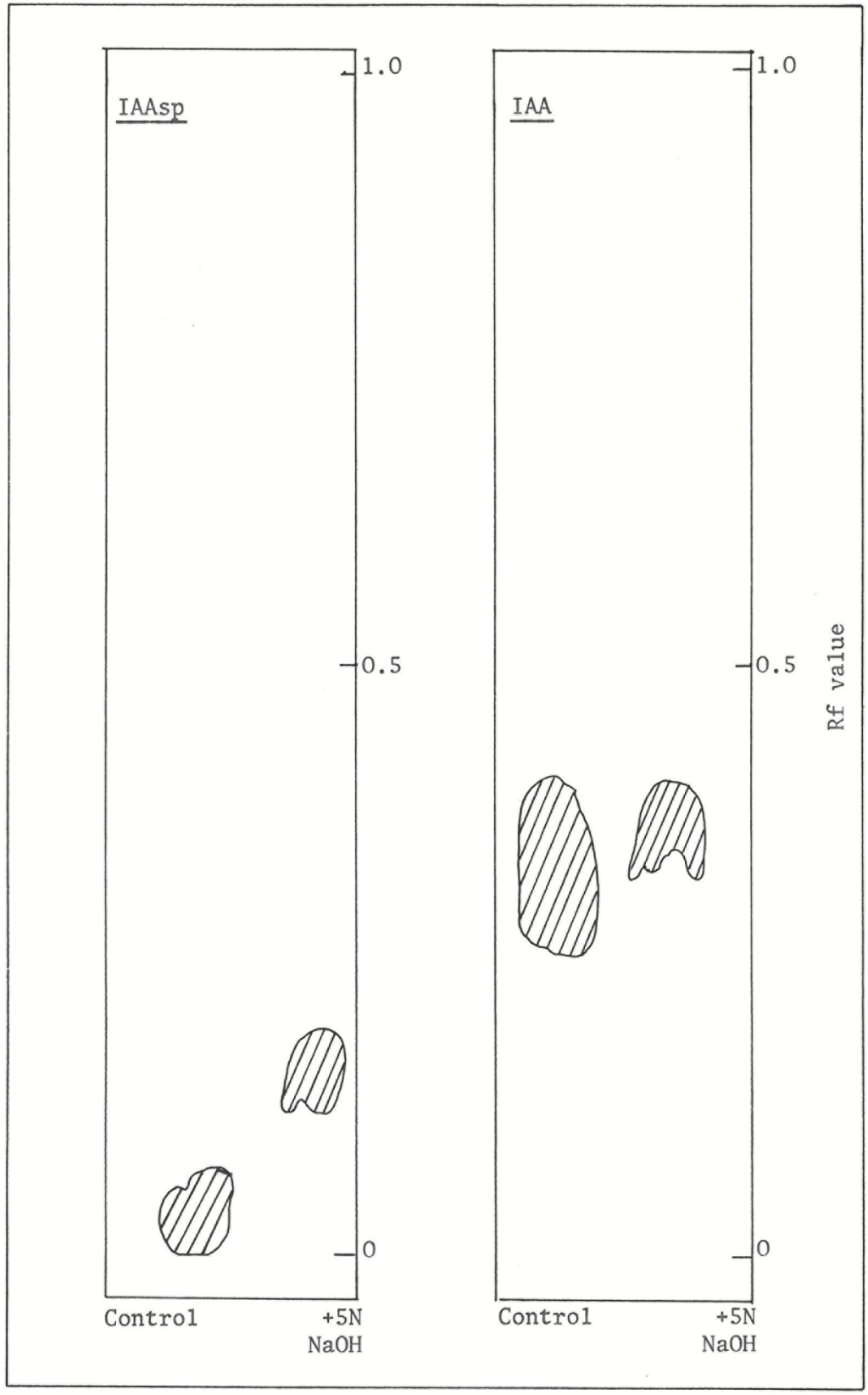


Figure 20 The alkaline hydrolysis of authentic IAA and IAAsp. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v) and sprayed with Ehrlich's reagent.

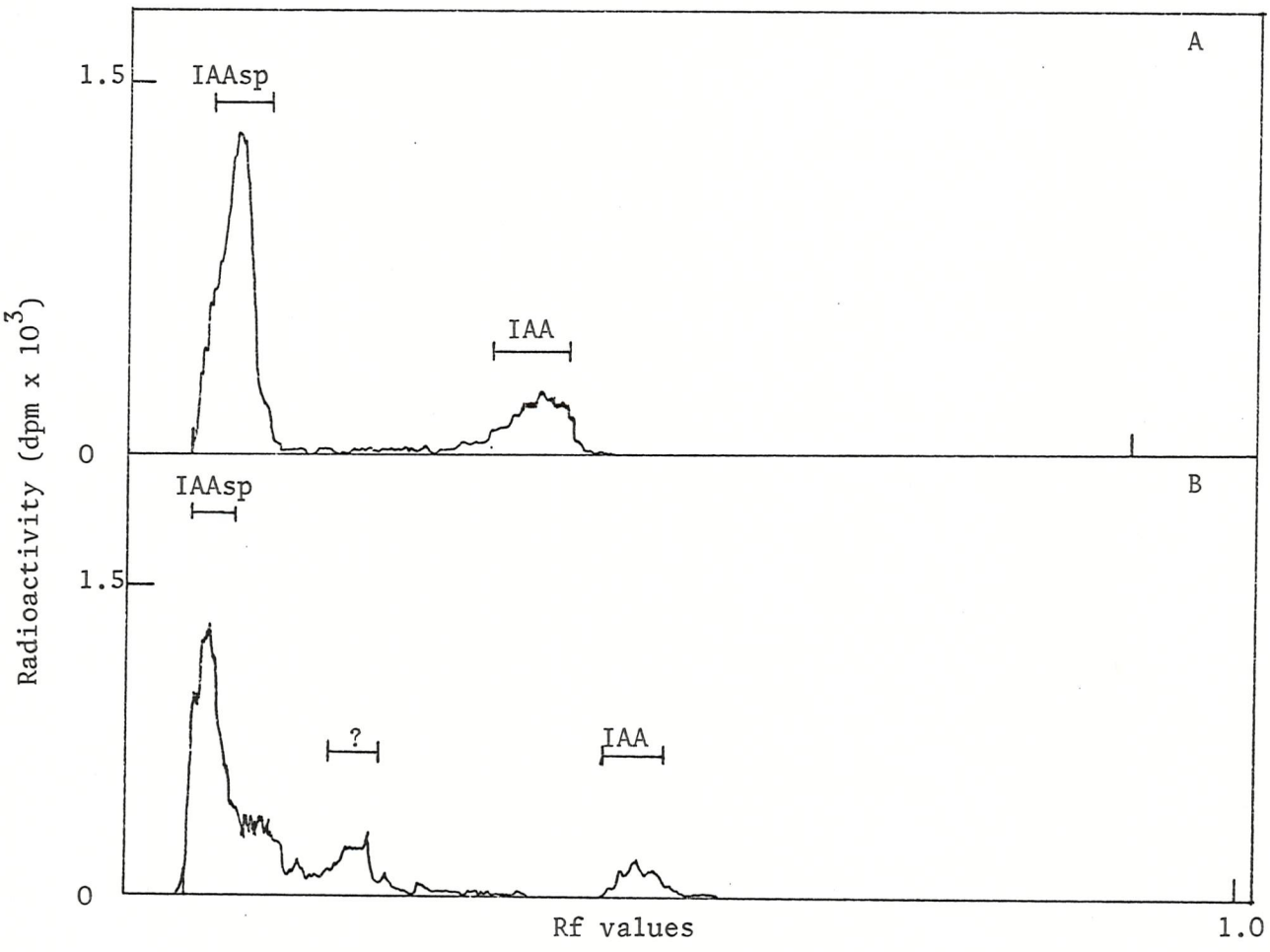


Figure 21 Radiochromatogram scans of partially purified ^{14}C-plant extracts. A: incubation control and B: incubation with 5N NaOH. The chromatograms were developed in isopropanol: NH_3:H_2O (10:1:1 v/v). Horizontal bars represent the position of authentic IAA and IAAsp.

separated into four ^{14}C-zones in chloroform:ethyl-acetate:formic acid (35:55:10 v/v)(Table 21). After hydrolysis a far greater proportion of the ^{14}C-material was immobile in the same solvent system and the Rf values 0.6 to 0.7 (IAAsp) and 0.4 to 0.5 were correspondingly reduced. The smaller amount of ^{14}C-compounds at higher Rf values may account for the clearer separation between IAA and IAAsp, revealing an additional small peak. The possibility that the activity at Rf 0.4 to 0.5 may be malonyltryptophan was discussed above. The hydrolysis of MTPP would be expected to yield tryptophan which runs at Rf 0.05 in this solvent system.

The variation between the separation of ^{14}C-metabolites by these two chromatography systems (IAW and chloroform:ethyl-acetate: formic acid) extended to the chromatography of standard IAAsp after incubation. Authentic IAAsp, following NaOH hydrolysis was chromatogrammed in chloroform:ethyl-acetate:formic acid (35:55:10 v/v) and appeared to have been totally hydrolysed to a compound corresponding to the Rf value of authentic IAA (Table 21, Fig. 22). A ninhydrin positive spot was detected at Rf 0.03 after TLC coinciding with authentic aspartic acid. In one replicate only 20% conversion occurred, however these experiments show the successful hydrolysis of authentic IAAsp. The inconsistency of chromatography in isopropanol:NH_3:H_2O could not be explained.

The behaviour of the plant extract differed from that of standard IAAsp. One explanation might be the conversion of ^{14}C-IAA once released by hydrolysis to the compound at Rf 0 to 0.1 as the result of a chemical reaction between the plant extract and NaOH. The production of a pure radioactive metabolite of [1-^{14}C]IAA would overcome this problem. The difficulties in obtaining pure ^{14}C-IAAsp

Table 21 The effect of alkaline hydrolysis by 5N NaOH on standard IAA and IAAsp and the ^{14}C metabolites present in a partially purified plant extract. Each result is the average of 4 replicates. The chromatograms were developed in chloroform:ethyl-acetate: formic acid (35:55:10 v/v). The ^{14}C at each zone of the chromatogram is expressed as a percentage of the total ^{14}C on the TLC plate.

	Rf value	0-0.1	0.4-0.5	0.6-0.7	0.78-0.85	0.74
IAA	A. Before hydrolysis				+	
	B. After hydrolysis				+	
IAAsp	A. Before hydrolysis			+		
	B. After hydrolysis				+	
Plant Extract (^{14}C-%)	A. Before hydrolysis	18.5	24.0	41.6	15.7	-
	B. After hydrolysis	64.5	-	16.1	14.5	4.8

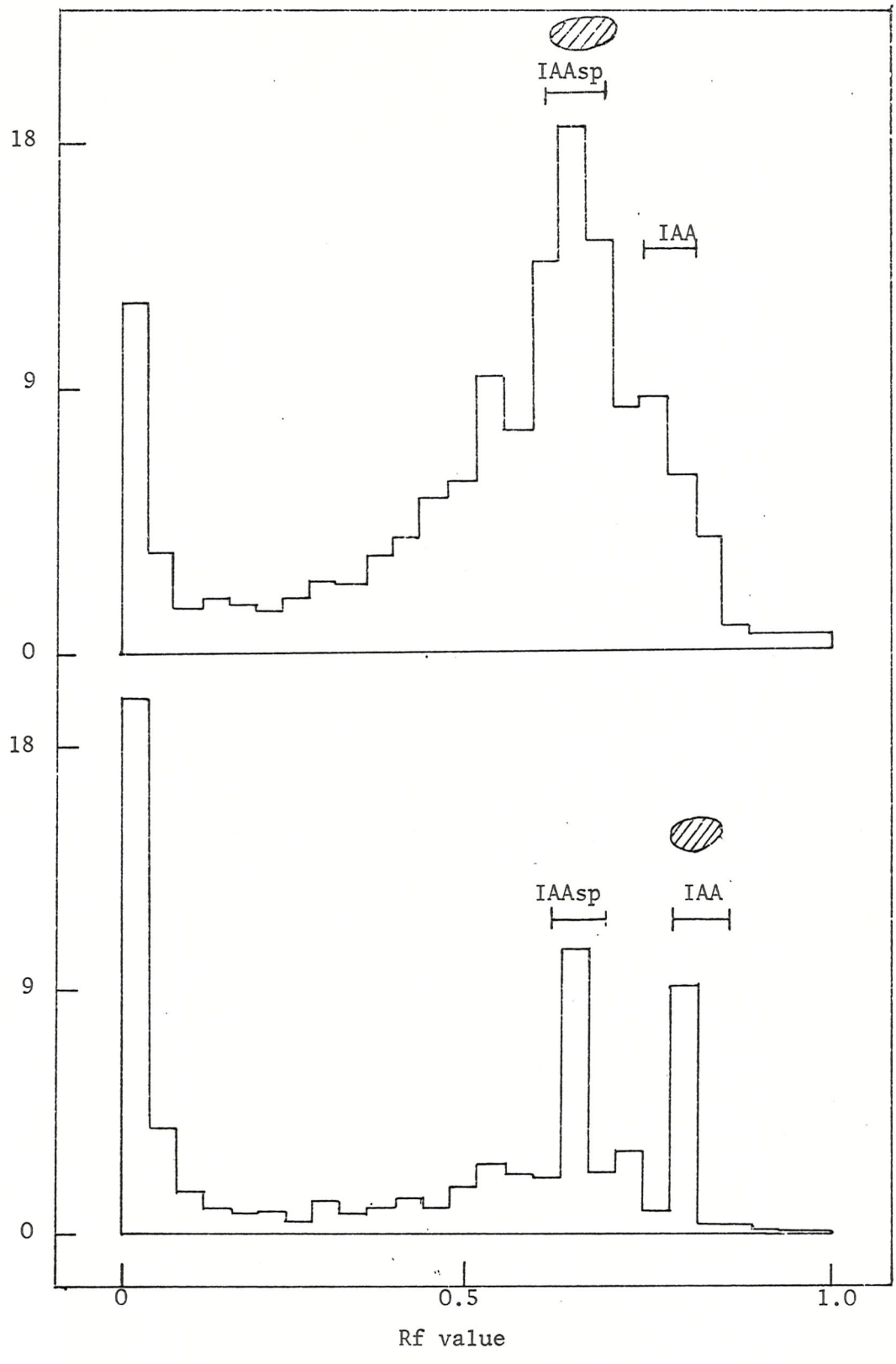


Figure 22 The separation by TLC of a partially purified ^{14}C-plant extract following incubation during alkaline hydrolysis, A: control and B: with 5N NaOH. The chromatograms were developed in chloroform:ethyl-acetate: formic acid (35:55:10 v/v). Horizontal bars represent the position of authentic IAA and IAAsp. The shaded areas show the effect of incubation with 5N NaOH on authentic IAAsp.

from plant extracts were examined in Section III.

The contradictory results produced by chromatography in different solvents were not resolved. Such inconsistency was unsatisfactory but could not be overcome.

Acid Hydrolysis

Hydrolysis of the peptide bond by concentrated HCl was also investigated. This had been used as an identification method for naphthylacetylaspartic acid (NAAsp) by Riov et al. (1979). Their partially purified metabolite co-chromatogrammed with authentic NAAsp in 3 solvent systems. Hydrolysis in 6N HCl yielded two substances, one chromatogrammed with authentic NAA and the other, which was ninhydrin positive, co-chromatogrammed with authentic L-aspartic acid.

The acid hydrolysis of the plant metabolite believed to be IAAsp was performed indirectly. Figure 9 A and B (Section III) demonstrate the loss of the radioactive metabolite that co-chromatogrammed with IAAsp, from a sample when the pH was adjusted to 2.5 with HCl prior to rotary evaporation. During reduction and the concentration of the acid, a large proportion of the ^{14}C-activity previously at the Rf of IAAsp became immobile when separated by chloroform:ethyl-acetate:formic acid (35:55:10 v/v). There was no increase in the percentage of free IAA.

A preliminary acid hydrolysis experiment followed the method of Maan (1978). A partially purified plant extract, standard IAA and IAAsp were incubated in 6N HCl for 7 hours at 80°C. This resulted in the loss of all IAA and an 80% loss of ^{14}C-activity. There was no detectable release of aspartic acid. An increase in

the proportion of immobile ^{14}C-compound(s) was noted on chromatograms developed in chloroform:ethyl-acetate:formic acid (35:55:10 v/v). Bennet-Clark and Wheeler (1959) recorded the destruction of IAA by strong acid. Such high losses of IAA and ^{14}C reduce the suitability of this technique as an 'identification' method for IAAsp.

Discussion

Of the three methods examined the alkaline hydrolysis proved the most promising for the cleavage of the peptide bond, offering the possibility of monitoring free IAA and aspartic acid, if released, by TLC (chloroform:ethyl-acetate:formic acid 35:55:10 v/v). However, the successful hydrolysis of authentic IAAsp was not reflected in the behaviour of the compound isolated from the plant extract. The changes in the composition of the [^{14}C]IAA metabolites were presented. This investigation has not helped establish the identity of the plant conjugate believed to be IAAsp. Inconsistencies within techniques were reported and the importance of developing and utilizing more sophisticated isolation, purification and identification techniques must be stressed.

DUAL-LABELLING

Another approach to the identification of the auxin metabolite was considered. The application of [^{3}H] aspartic acid and [^{14}C]IAA simultaneously to the plant apices may result in the formation of a dual-labelled compound. This would depend on the metabolism of IAA to IAAsp incorporating at least a proportion of the [^{3}H] aspartic

acid applied. This technique has been successfully used by other workers. Field and Peel (1971) investigated the radial movement of growth regulators and herbicides in willow stems, first determining the extent of the metabolism of the relevant compounds. A large proportion of the [^{14}C]IAA was metabolized to one compound which co-chromatogrammed with IAAsp in two solvent systems (Rf 0.05, isopropanol:NH_3:H_2O and Rf 0.81, butanol:acetic acid:H_2O). The application of [1-^{14}C]IAA and [^{3}H] aspartic acid to bark strips of willow, extraction and chromatography showed both tracers to be present in the metabolite. Chromatographic analysis of the hydrolysate following acid hydrolysis showed two labelled compounds whose Rf values corresponded to IAA and aspartic acid. Field and Peel (1972) also used this technique to examine the mobility of IAA and IAAsp.

An attempt was therefore made to use the dual labelling technique to establish the identity of the [^{14}C]IAA-metabolite.

Methods

Twelve-day old, light-grown 'Meteor' peas were prepared as previously described and labelled with [1-^{14}C]IAA and L-[2,3-^{3}H] aspartic acid alone or in combination. The labelled compounds were applied in 5 µl droplets of 0.1% "Tween 20" at the following concentrations: [1-^{14}C]IAA, 0.125 µ Ci, 3.85 x 10^{-1} µg, 2.2 x 10^{-3} µM per plant and L-[2,3-^{3}H] aspartic acid, 5 µ Ci, 5.6 x 10^{-2} µg, 4.2 x 10^{-4} µM per plant. All radioactive compounds were obtained from the Radiochemical Centre, Amersham, U.K. The specific activities were [1-^{14}C]IAA 325 µ Ci mg^{-1}, L-[2,3-H^{3}] aspartic acid 90 mCi mg^{-1}. The pH of the three solutions were checked using standard chemicals of

the same concentrations (aspartic acid only pH 3.75, IAA only pH 3.6, IAA and aspartic acid pH 3.6).

After an eight-hour translocation period the apices were surface washed in dilute "Tween 20" and distilled water. This was achieved by cutting the stem and smearing the cut surface with vaseline to prevent leakage. The apical surface was held under water, with gentle stirring, for 5 minutes. The apices were cut into small pieces and placed in 70% cold ethanol for extraction. The remaining plant was separated into root and shoot material and extracted.

The dual-labelled material was counted on the overlap channels of the scintillation counter and the ^{3}H value corrected for overlap resulting from the presence of ^{14}C. Scintillation procedures and all other methods were otherwise as previously described. Reduced samples were chromatogrammed in isopropanol:NH_3:H_2O (10:1:1 v/v. IAW) and butanol:acetic acid:H_2O (4:1:1 v/v. BAW), and 10 mm divisions of the paper chromatograms counted. The results shown are means of at least two experiments.

The chromatogram zone in IAW corresponding to both aspartic acid and IAAsp (Rf 0 - 0.1) was eluted and re-chromatogrammed in BAW. This was difficult to achieve due to the low levels of radioactivity present and the destructive nature of scintillation counting of chromatograms. It was only completed once.

Results and Discussion

In all three treatments the only compounds detected in the apical washes were the compounds applied.

Of the ^{3}H-aspartic acid applied to the apical surface less

than 20% was taken up by the plant (Table 22) and below 0.1% of the ^{3}H-activity taken up left the apical tissue. The ^{3}H translocated was metabolised to unknown compounds (Fig. 23). On the basis of these results these metabolites cannot be identified, however it is known that a major pathway for the metabolism of aspartic acid in plants is the formation of asparagine (Beevers, 1976). Other amino acids are often derived from aspartic acid, for example methionine, isoleucine and lysine (Miflin and Lea, 1977). The low percentage recovery of ^{3}H may reflect the conversion of aspartic acid to proteins (which were not examined).

The fate of [1-^{14}C]IAA (Fig. 24) was as previously described in Section III. There was less uptake of ^{14}C by the dual-labelled plants (Table 22) than the [1-^{14}C]IAA labelled plants. Of the ^{14}C recovered 32% was washed from the surface of dual-labelled plants compared to 19% from single-labelled plants. The differences in uptake were reflected in the amount of ^{14}C extracted from the apical tissue although the ^{14}C translocated, expressed as a percentage of the total ^{14}C recovered, was very similar (3.9% compared with 3.4%).

Dual-labelling appeared to affect the metabolism of [^{14}C]IAA (Fig. 25). The ^{14}C-material extracted from IAA-only labelled plants was found to chromatogram mainly with IAAsp. In the dual-labelled plants a large proportion of the ^{14}C-material in apical, stem and root extracts coincides with the Rf value of IAA (Fig. 25).

Therefore dual-labelling had two major effects, firstly on [1-^{14}C]IAA uptake by the plant and, secondly, on the metabolism of the [1-^{14}C]IAA within the plant.

Table 22 Radioactivity (^{14}C and ^{3}H) recovered by 70% ethanol extraction from 12-day old light-grown dwarf pea seedlings labelled with [^{3}H] aspartic acid, [^{14}C]IAA and dual-labelled with [^{3}H] aspartic acid and [^{14}C]IAA.

Radioactivity measured as dpm plant^{-1}.

	^{3}H Asp alone	^{3}H Dual labelled	^{14}C IAA alone	^{14}C Dual labelled
Apical Wash	2 109 157.0	2 419 773.0	13 914.0	23 807.5
Apical Extract	471 531.0	598 139.0	56 673.5	48 984.0
Stem Extract	3 124.0	642.0	1 498.0	1 201.0
Root Extract	276.5	88.0	1 382.0	1 352.0
Activity transported	3 400.5	730	2 880.5	2 553.0
Total Activity Recovered	2 684 088.5	3 018 642.0	73 478.0	75 344.5
Percentage recovery, from total applied	49.7	55.9	60.7	62.2

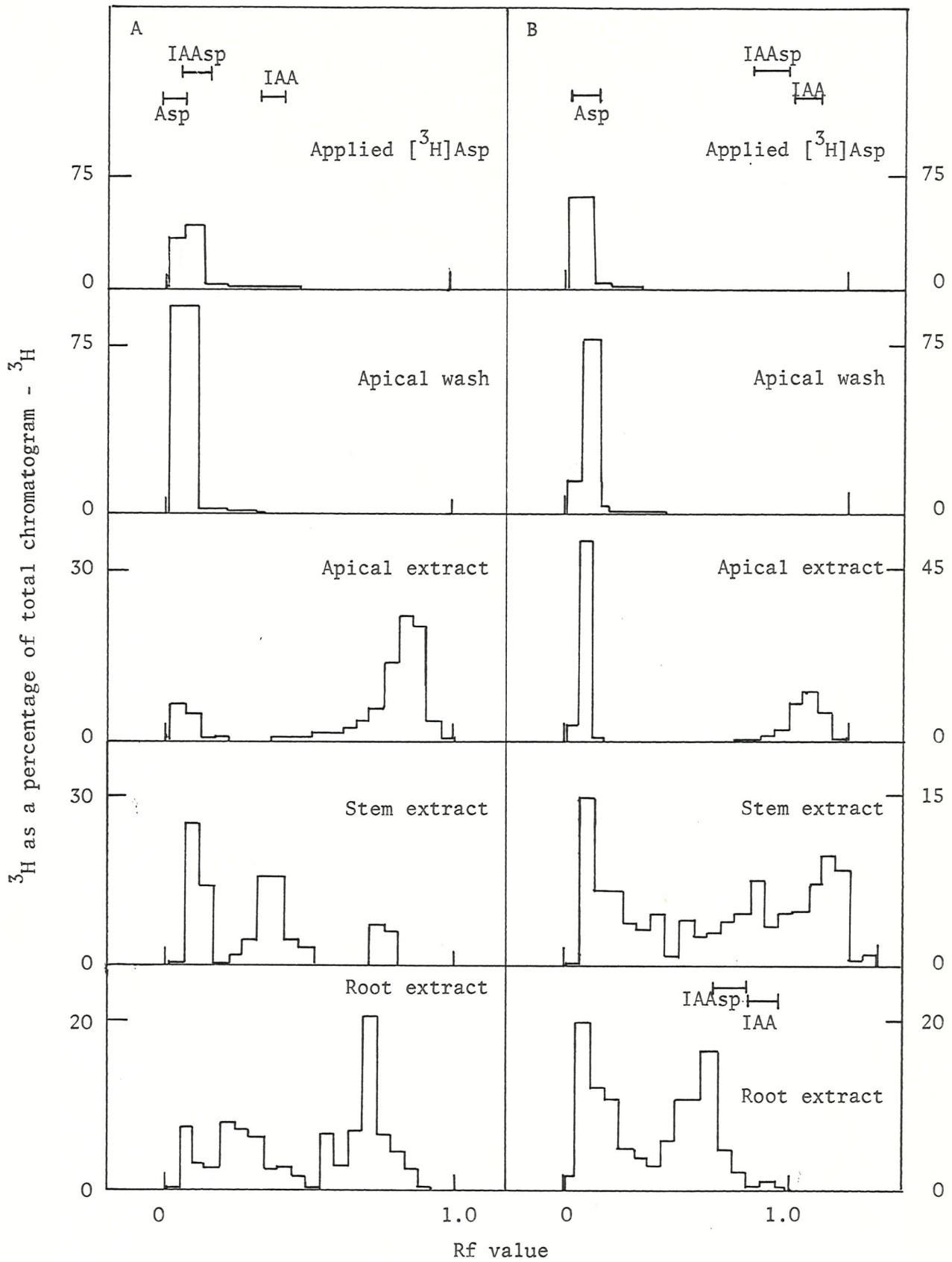


Figure 23 Radiochromatograms of the ^{3}H activity applied, the apical washes and 70% ethanol extracts from light-grown 12-day old dwarf peas 8 hours following the application of [2,3 - ^{3}H] aspartic acid to the apices. The chromatograms were developed in A: isopropanol:NH_3:H_2O (10:1:1 v/v) and B: butanol:acetic acid:H_2O (4:1:1 v/v) and then counted as 1 cm portions in scintillation fluid. Horizontal bars represent the position of authentic IAA, IAAsp and aspartic acid.

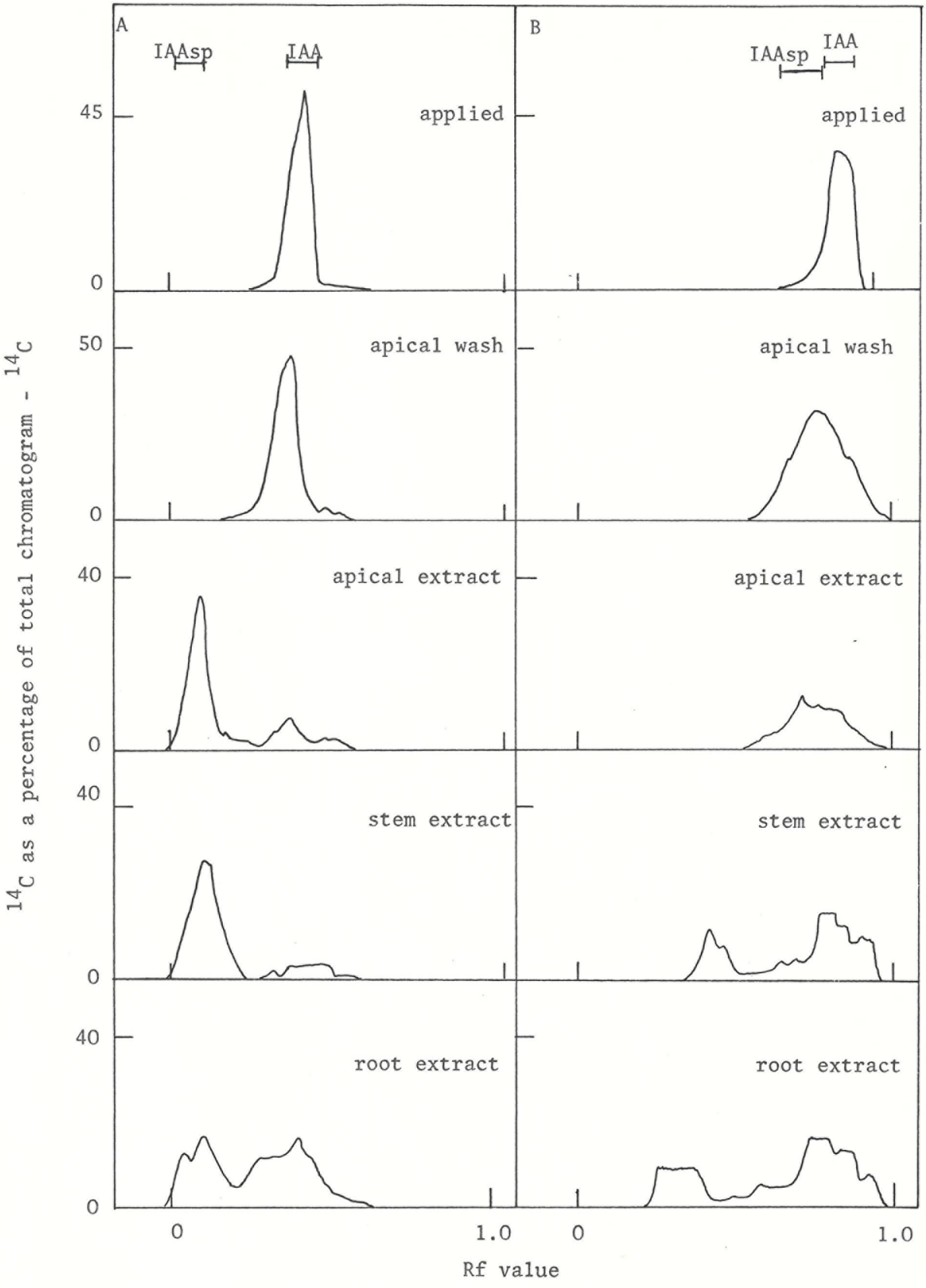


Figure 24 Radiochromatogram scans of the ^{14}C-applied, the apical wash, and the 70% ethanol extracts from 12-day old light-grown dwarf peas 8 hours following the application of [1-^{14}C]IAA to the apices. The chromatograms were developed in A: isopropanol:NH_3:H_2O (10:1:1 v/v) and B: butanol:acetic acid:H_2O (4:1:1 v/v). The horizontal bars represent the position of authentic IAA and IAAsp.

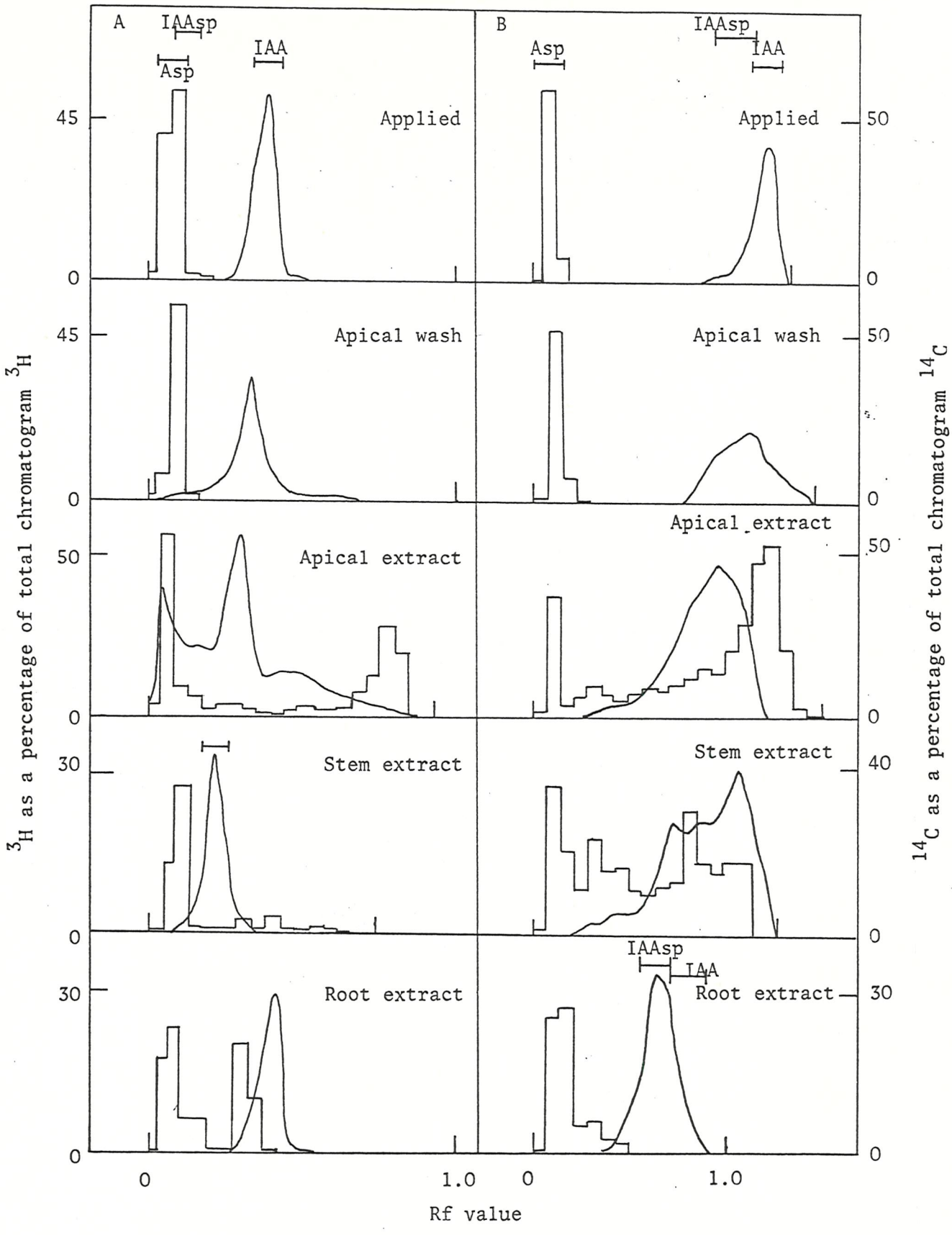


Figure 25 Chromatograms of the radioactivity applied, apical washes, and 70% ethanol extractions from 12-day old light-grown dwarf peas 8 hours following the application of [1-^{14}C]IAA and (2,3-^{3}H]aspartic acid to the apices. The chromatograms were developed in A:isopropanol:NH_3:H_2O (10:1:1 v/v) and B:butanol:acetic acid:H_2O (4:1:1 v/v). The chromatograms were scanned for ^{14}C-material and counted in 1 cm portions to detect ^{3}H-compounds (histogram). Horizontal bars represent the position of authentic IAA, IAAsp and aspartic acid

The object of this experiment was not realized. There was little correlation between the ^{3}H and ^{14}C material in the two chromatography systems. The results achieved by Field and Peel (1971) were far clearer. When elution and re-chromatography were accomplished (Fig. 26) the bulk of the ^{14}C-material separated from the ^{3}H compound(s) in BAW. This investigation did not detect the formation of a dual-labelled metabolite.

It is possible that the aspartic acid utilized during conjugation may come from a pool into which the exogenous [^{3}H] aspartic acid did not enter. Field and Peel (1971) extracted 16 hours after dual-labelling. This time period (double that employed here) may have allowed the greater incorporation of [^{3}H] aspartic acid during IAA-conjugation in the willow. However, lengthening the translocation period would be unlikely to have an effect on IAA-metabolism in peas as conjugation occurs in the early hours after labelling (Section V). The possibility of pretreatment with [^{3}H] aspartic acid was also considered. Increased amounts of unknown ^{3}H-metabolites would probably complicate chromatography. The danger of tritium-exchange would also increase with time. Dual-labelling did not provide aid in the identification of IAAsp in pea seedlings.

Additional Information

In an attempt to understand the differences recorded in the uptake and metabolism of [^{14}C]IAA during the dual-labelling experiment [1-^{14}C]IAA with and without cold aspartic acid was applied to pea apices using the method described above. The same concentrations of IAA and aspartic acid were applied.

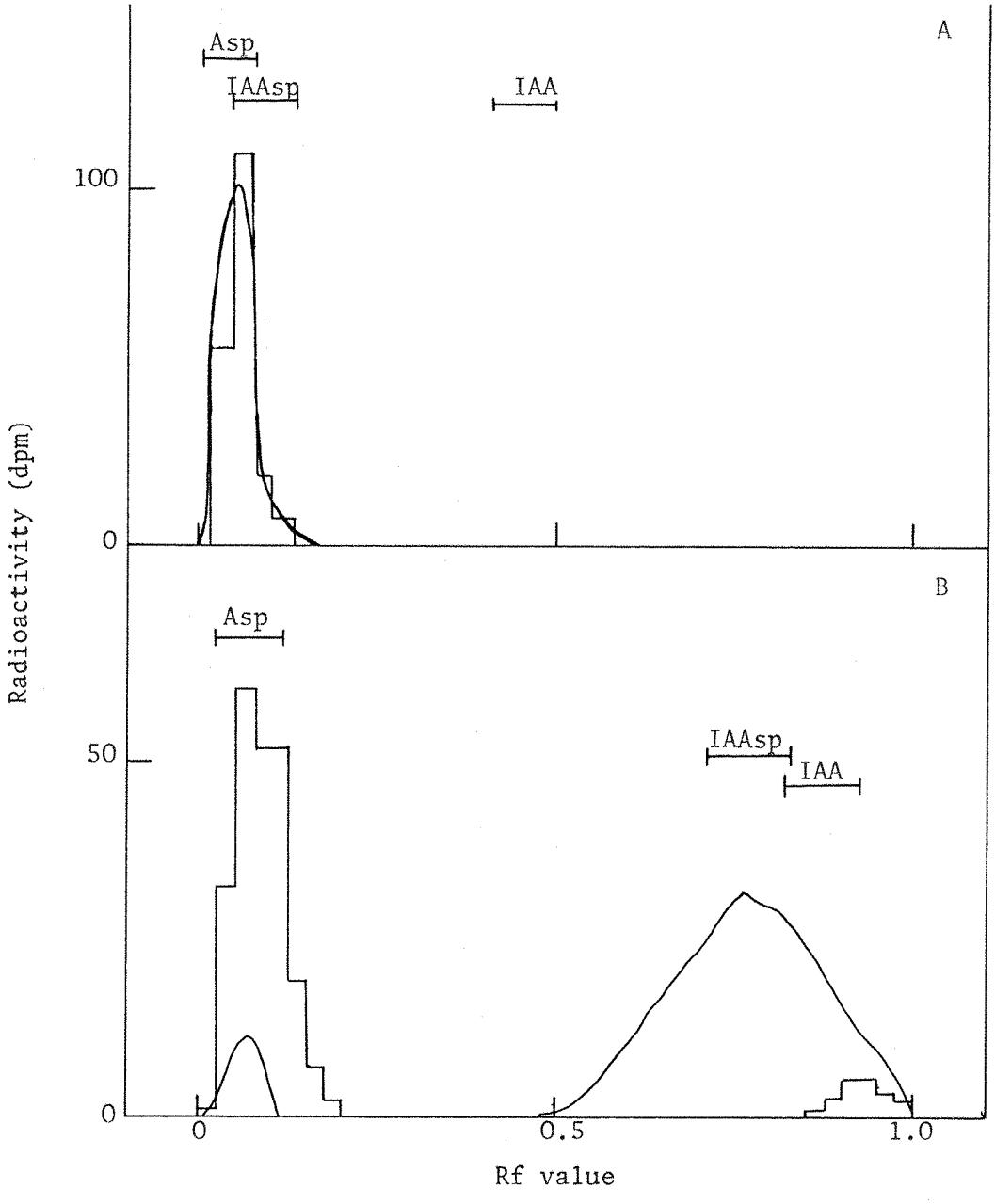


Figure 26

Chromatograms of the eluted and concentrated Rf zone 0 - 0.1 taken from 70% ethanol extracts of dual labelled apices chromatogrammed in isopropanol:NH_3:H_2O (10:1:1 v/v). The chromatograms were developed in A: isopropanol:NH_3:H_2O (10:1:1 v/v) and B: butanol:acetic acid:H_2O (4:1:1 v/v). The chromatograms were scanned for ^{14}C-material and counted in 1 cm portions to detect ^{3}H-compounds (histogram). Horizontal bars represent the position of authentic IAA, IAAsp and aspartic acid.

Results

Uptake was found to be reduced in the presence of exogenous aspartic acid. In this experiment 21% of the [^{14}C]IAA applied alone was removed by surface washing, while 48% of that applied with aspartic acid had remained on the apical surface (Table 23). Apical metabolism did not appear to differ between the two treatments (Fig. 27).

Discussion

The presence of aspartic acid with exogenously applied auxin inhibited the uptake of that IAA. In these experiments a concentration difference existed between the IAA applied alone and that applied with aspartic acid (both treatments were applied as 5 µl droplets). Concentration is known to have an effect on the uptake of [1-^{14}C]IAA by pea seedlings (Eliezer, 1978). However, this effect would be expected to be negligible after an eight hour uptake period. The lower uptake in dual-labelled plants may be an effect of aspartic acid competing with IAA for uptake sites. It is unlikely to be an effect of pH as the applied solutions were within 0.15 of a pH unit. The reason for reduced uptake of IAA in the presence of aspartic acid remains unknown.

SUMMARY

The identification of the [^{14}C]IAA-metabolite was investigated in five different ways:

MASS SPECTROSCOPY - Spectra were not achieved of any indole compounds.

Table 23 The recovery of ^{14}C from 12-day old dwarf pea seedlings following the application of [1-^{14}C]IAA with and without aspartic acid to the apices. Translocation was for 8 hours and the plants extracted, after apical washing in 70% ethanol. 171, 197 dpm [1-^{14}C]IAA applied per plant. Radioactivity measured as dpm. plant^{-1}.

	IAA alone	IAA and aspartic acid
Apical Washings	20 118	46 684
Apical extract	72 108	49 834
Stem extract	837	611
Root extract	848	631
Total ^{14}C recovered	93 911	97 760
% recovered, from total applied	55	57

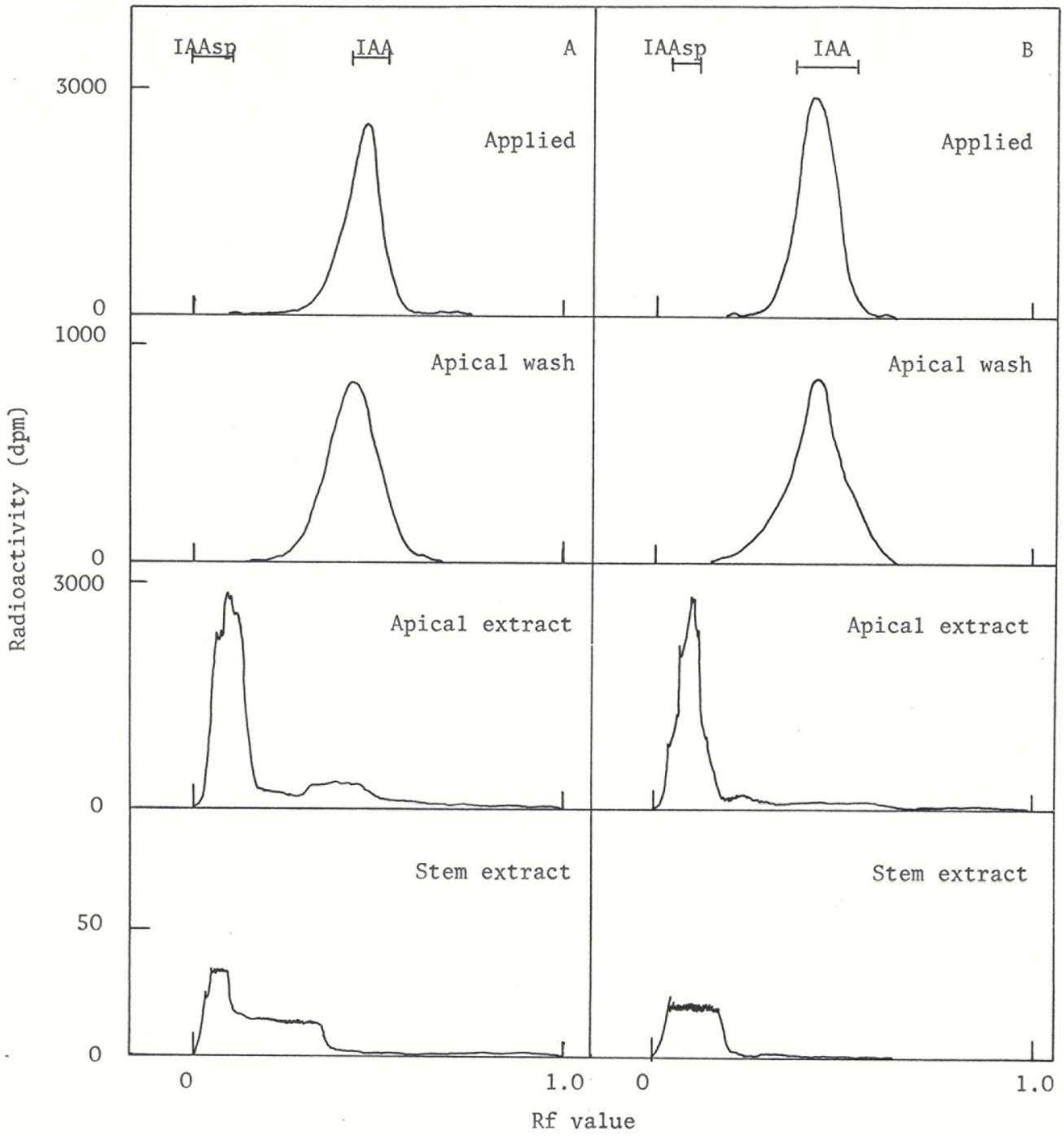


Figure 27

Radiochromatogram scans of the ^{14}C-activity applied, the apical wash and 70% ethanol extracts from 12-day old light-grown dwarf peas 8 hours following the application of A: [1-^{14}C]IAA and B: [1-^{14}C]IAA and unlabelled aspartic acid to the apices. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). The horizontal bars represent the positions of authentic IAA and IAAsp.

GC-MS	-	IAAsp derivatives proved unsuitable for GC.
CHROMATOGRAPHY (paper and TLC)	-	Positive correlations were found between the ^{14}C-metabolite extracted from pea seedlings and IAAsp in eight chromatography systems.
HYDROLYSIS	-	Alkaline hydrolysis of authentic IAAsp was successful but could not be reproduced in a partially purified plant extract containing the ^{14}C-metabolite.
DUAL-LABELLING	-	A dual-labelled metabolite was not detected.

A strong correlation was found between authentic IAAsp and the [^{14}C]IAA-metabolite using paper and thin-layer chromatography. This suggests that the compound is IAAsp. Additional evidence for the identification of the metabolite was not achieved and consequently a more detailed examination of the identity of this plant conjugate is still required. Technical problems were encountered which could not be overcome. A pure sample of the metabolite is essential for positive identification by MS. Further work developing the GC technique could provide an ideal source of pure metabolite if successful. Increasingly botanists are aware of the need to use the chemical methods available today for the identification of

organic compounds. The development of integrated projects between botanists and chemists must be encouraged if botanical research is not to be based on 'tentative assumptions'.

Section V

STUDIES ON THE PHYSIOLOGICAL SIGNIFICANCE OF AUXIN CONJUGATION IN PEA SEEDLINGS

STUDIES ON THE PHYSIOLOGICAL SIGNIFICANCE OF AUXIN CONJUGATION IN PEA SEEDLINGS

INTRODUCTION

Although the roles played by auxin conjugates in the regulation of plant growth and development are still far from clear, it has been suggested by several authors that reversible conjugation may act to regulate the levels of free (physiologically-active) auxin in plant tissues (Morris et al., 1969; Feung et al., 1976; Bandurski and Schulze, 1977). It has been found that morphological differences between light- and dark-grown pea plants can be correlated with the quantities of "diffusible" auxin obtained from such plants (Scott and Briggs, 1963; Moore and Shaner, 1967), and it has been suggested that differences in the extent of auxin conjugate formation in light and darkness may control the "diffusible" auxin levels (Lantican and Muir, 1969; Bandurski, Schulze and Cohen, 1977). In support of this, the rates of conversion of [^{14}C]IAA to an immobile compound co-chromatogramming with IAAsp were found to be considerably higher in light-grown pea plants than etiolated plants (Morris, 1970). It has also been suggested by Lantican and Muir (1969) that differences in the formation of IAAsp from IAA in tall and dwarf varieties might be the basis for dwarfism in this species.

The work reported below was designed to obtain further information on the possibility that conjugation might act to control free IAA levels. The time-course of uptake, export and metabolism of [1-^{14}C]IAA applied to the apical buds of intact light- and dark-grown tall and dwarf pea varieties was investigated. The experiments fell into three categories: firstly, studies of auxin uptake, metabolism

and transport in dwarf pea seedlings grown and transported in light; secondly, uptake, metabolism and transport in light- and dark-grown dwarf pea seedlings transported in light and darkness; thirdly, studies on tall and dwarf pea varieties.

MATERIALS AND METHODS

Time-course experiments

All experiments were carried out on light-grown dwarf pea seedlings (cv. Meteor) grown as previously described (Section II). Transport experiments were commenced when the plants were 12-days old except in the longer time-course experiments (1-10 d) when they were labelled at 11-days. Three separate time-course experiments were completed, 1 to 12 h, 5 to 48 h and 1 to 10 d. The harvests were taken at 1, 2, 4, 8 and 12 h; 5, 8, 12, 18, 24, 30 and 48 h; 1 to 10 d (harvests every 24 h). All labelling, extraction and chromatography techniques were as described in Section III. [1-^{14}C]IAA was applied as a 5µl droplet in 'Tween 20', 146,800 dpm plant^{-1}, (0.22µg plant^{-1}).

The influence of light on auxin uptake, metabolism and transport in dwarf peas

Twelve-day old Meteor peas were grown either in light (16 h light period) or in total darkness. The dark-grown plants were maintained in a ventilated, light-proofed compartment in the same growth room as the light-grown peas. All manipulations of the dark-grown seedlings (including watering) were carried out under weak illumination from a green safelight (Ilford "Spectrum Green" filter,

transmission 490-530 nm), which had previously been shown to be phototropically inactive. To allow for any difference in IAA metabolism reflecting the light conditions of the plants during translocation, half the light-grown and dark-grown plants were transferred to the opposite light conditions 12 h prior to labelling. After the transfer the light treatments were maintained in continuous light for the duration of the experiment. Plants from the four treatments (light-grown, light-transported; light-grown, dark-transported; dark-grown, dark-transported; and dark-grown, light-transported) were labelled as previously described with [1-^{14}C]IAA (158,000 dpm, 0.23µg, plant^{-1}) and harvested 5, 8, 12, 18, 24, 30 and 48 h later.

Comparison of auxin uptake, metabolism and transport in tall and dwarf varieties

Tall peas cv. Alderman were grown under light and dark conditions as described above. These were also divided and half the plants transferred to the opposite light regime as described for the Meteor peas. The two varieties were labelled when 12 d old with [1-^{14}C]IAA (180,000 dpm, 0.27µg, plant^{-1}) and harvested at five and eight hours.

A stem segment incubation experiment was also performed using etiolated and light-grown plants. The preparation of the plant material has been described previously (see Section III).

The estimation of uptake of [1-^{14}C]IAA by apical bud tissue

The total uptake of ^{14}C by the apical bud from the applied aqueous droplet was determined by washing the buds in water after

specified time periods and counting the ^{14}C in the wash solutions. The apical buds were excised at the end of the allowed uptake period and their cut surfaces were smeared with vaseline to prevent any ^{14}C leakage from the tissues. The "apical bud" was taken to be all tissues above the internode which subtended the apical bud at the time of application of IAA. This distinction was particularly important in the case of long-term studies in which considerable growth of the "apical" tissues occurred between the time of application of [^{14}C]IAA and the time of harvest. For example, in the 1-10 d experiment leaf 3 was included as apical material in the first harvest, and further growth meant that leaves 3 to 6 were included as "apical" material by the final harvest.

The ^{14}C-labelled material remaining on the surface of the bud was washed off by totally immersing the buds in known volumes of 0.1% 'Tween 20' solution with gentle agitation for 10 min and rinsed several times in distilled H_2O. Triplicate 1 ml aliquots were mixed with 1 ml absolute ethanol in polyethylene vials and ^{14}C determined by liquid scintillation counting after the addition of 10 ml of scintillation fluid. The addition of 1 ml of ethanol to the washes prior to adding the scintillation fluid improved the miscibility of the sample with the scintillation fluid.

Estimation of radioactivity in the plant material

At the end of each uptake period the apices were carefully removed (with or without washing, depending on the experiment), cut into small pieces and placed in cold 70% ethanol. The remainder of the plant was subdivided as required (see Results) and the individual parts extracted in 70% ethanol as previously described

(Section III).

The metabolic fate of the applied [1-^{14}C]IAA was studied by paper chromatography of the ^{14}C-labelled compounds in the extracts and washings. Samples were reduced by rotary evaporation at 25°C under reduced pressure and aliquots were spotted on Whatman No. 1 chromatography paper strips. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v. IAW), dried and scanned to locate radioactive spots. In some experiments chromatograms were also developed in butan-1-ol:acetic acid:H_2O (4:1:1 v/v. BAW) and in some cases extracts were analysed by TLC in chloroform:ethyl-acetate: formic acid (35:55:10 v/v). Samples of authentic unlabelled IAA and IAAsp were also chromatogrammed and located using UV light (366 nm) and/or Ehrlich's reagent.

In one experiment autoradiographs of developed chromatograms were prepared by exposing them to Kodachrome X-ray film for 21 d. These provided no additional information to radiochromatogram scanning.

EXPERIMENTAL STUDIES

Time-course studies of the uptake, metabolism and export of [1-^{14}C]IAA in light-grown dwarf pea seedlings

Results

Changes with time in the amount of ^{14}C recoverable by washing the apical bud following the application of [1-^{14}C]IAA are shown in Figures 28 and 29, together with the time-course of uptake by the plant. Uptake was estimated in two ways: as the difference

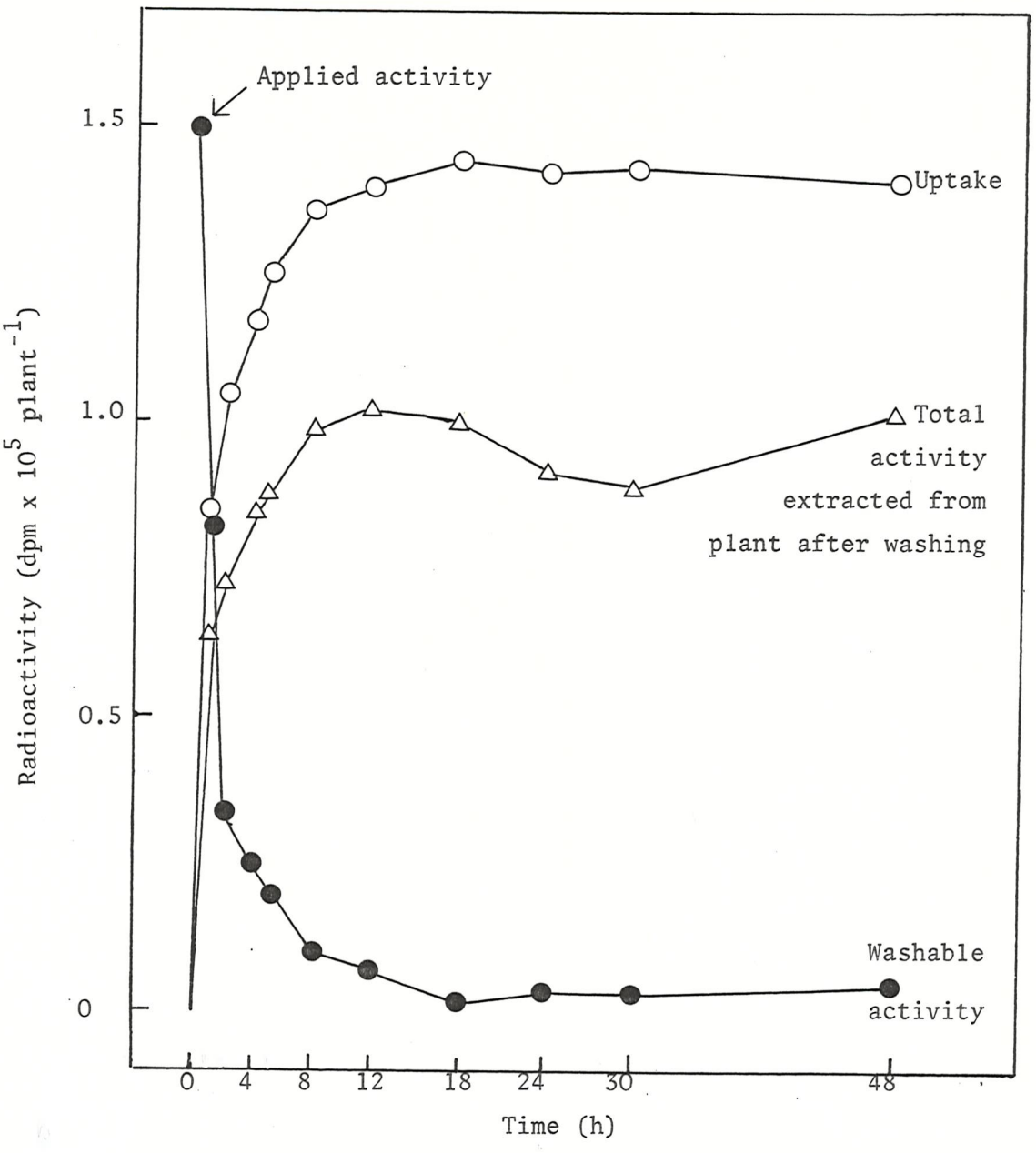


Figure 28 Time course changes in washable activity and uptake following the application of [1-^{14}C]IAA to the apical buds of intact, light-grown 12-day old dwarf pea seedlings. Uptake estimated as: applied activity-activity removed by washing. Applied activity = 146,800 dpm $plant^{-1}$.

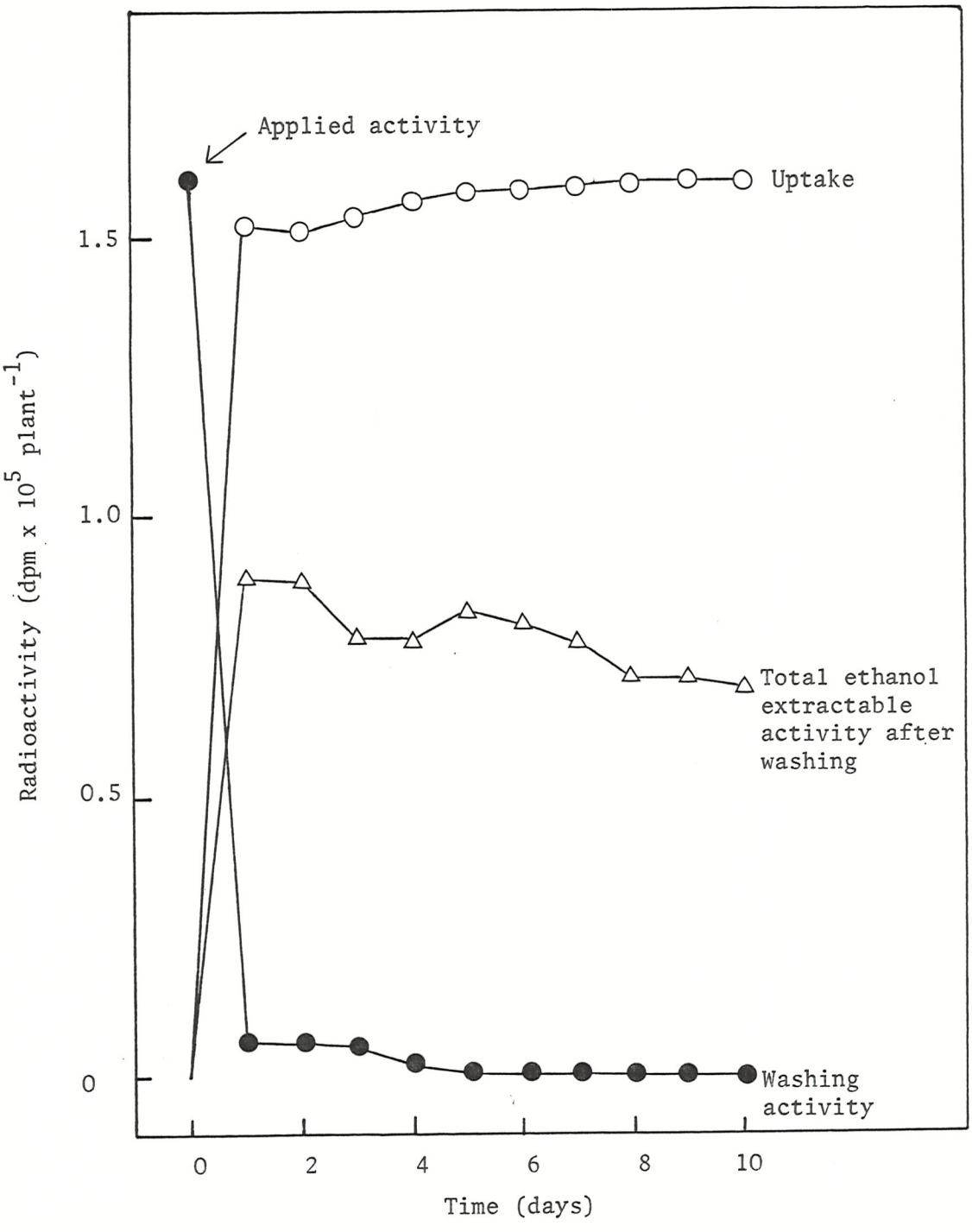


Figure 29 Time course changes in washable activity and uptake following the application of [1-^{14}C]IAA to the apical buds of intact, light grown 11-day old dwarf pea seedlings. Uptake estimated as: applied activity - activity removed by washing. Applied activity = 158,900 dpm $plant^{-1}$.

between applied activity and the activity removed by washing, and as the total ethanol-soluble ^{14}C extracted from the plant. The difference between these two methods must result from a combination of incomplete extraction of ^{14}C from the plant (probably due to metabolism to non-ethanol soluble components) and the likely decarboxylation of IAA. The increase in the difference between these two values with time supports this view.

Initial uptake by the apical bud tissue occurred very rapidly, 85% of the [1-^{14}C]IAA being taken up from the applied solution within 5 hours of application. Subsequently the rate of uptake declined, no ^{14}C being detected in the surface washings by day 7. The washable component represents not only the IAA remaining in the surface droplet, but also any which has diffused into the free space (that is any IAA not taken up by the plant).

Chromatography of the radioactive material washed from the surface of the apical tissue revealed one compound, indistinguishable from the applied [1-^{14}C]IAA by the methods used. The IAA, on entering the plant was rapidly metabolised to a compound with the same chromatographic properties as IAAsp. The ethanol-soluble extracts contained an appreciable amount of this compound one hour after the application of [1-^{14}C]IAA, indicating the very rapid conversion of the IAA taken up (Fig. 30). This fraction increased, while the ^{14}C-IAA declined, reaching a maximum eight hours after labelling (Fig. 30, 34). The ^{14}C chromatogramming with IAAsp remained the major metabolite present in the apical tissue for ten days (Fig. 31 to 34). There was no detectable decline in the amount of ^{14}C-IAAsp within the apex.

An unknown compound was present at the origin of chromatograms

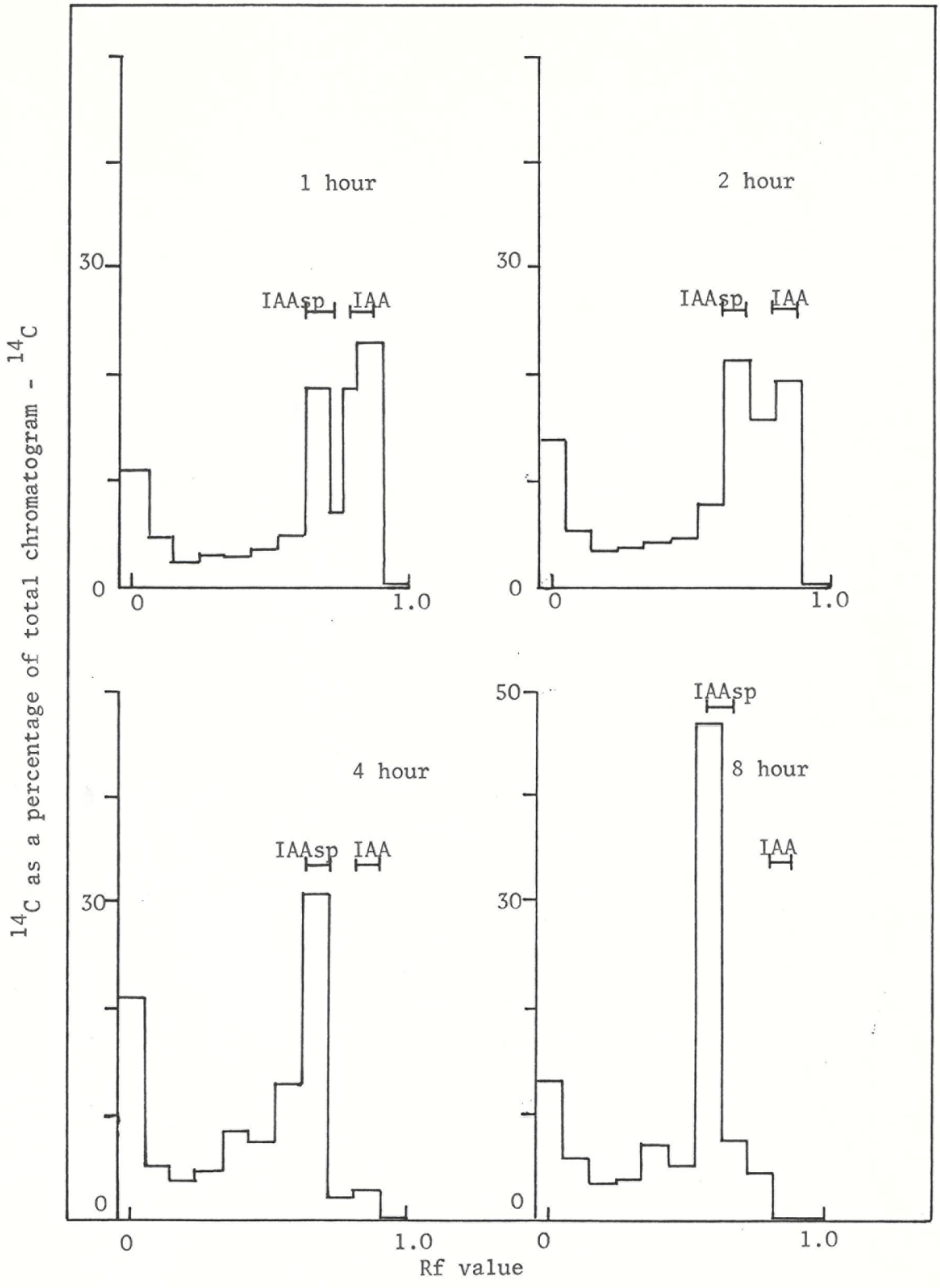


Figure 30

Chromatograms of 70% ethanol-soluble ^{14}C extracted from washed apical bud tissue after increasing time periods following the application of [1-^{14}C]IAA to 14-day old light-grown dwarf pea seedlings. The thin layer chromatograms were developed in chloroform:ethyl-acetate:formic acid (35:55:10 v/v) and counted as 1 cm portions in scintillation fluid. The horizontal bars represent the positions of authentic IAA and IAAsp.

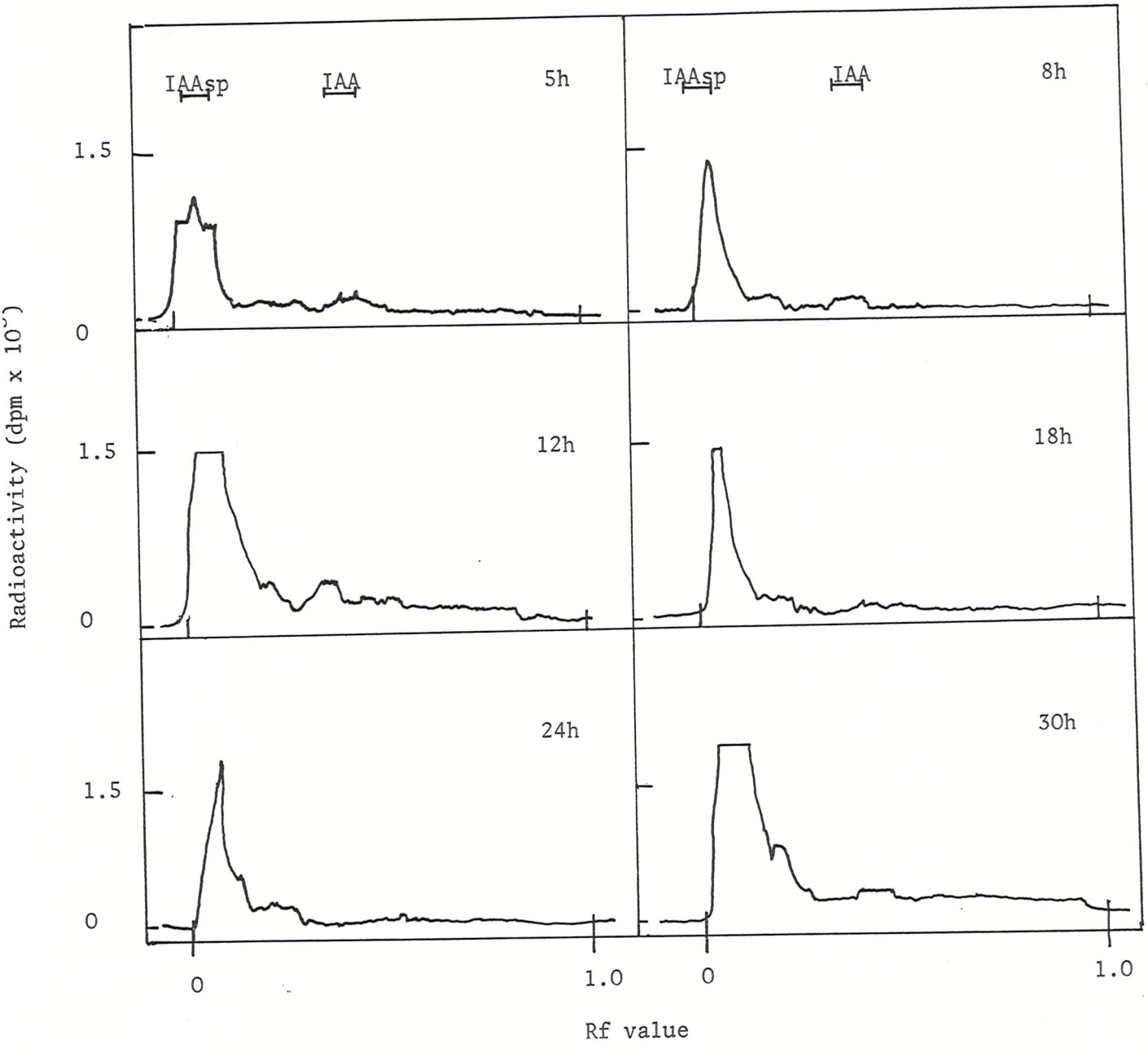


Figure 31 Radiochromatogram scans of ethanol-soluble ^{14}C extracts of apices at different time intervals following the application of [1-^{14}C]IAA to the apical buds of intact 12-day old dwarf peas. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars indicate the position of authentic IAA and IAAsp.

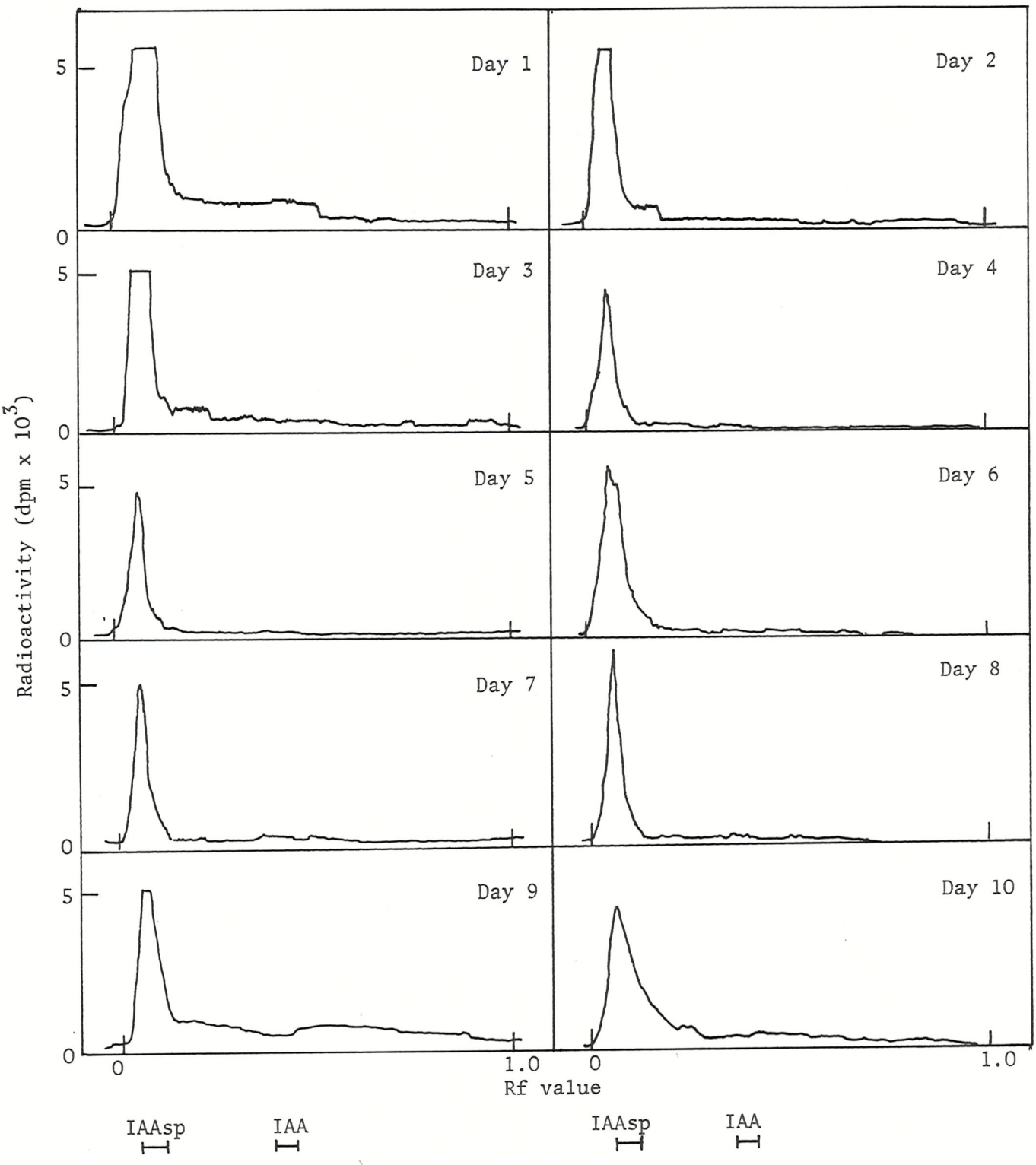


Figure 32 Radiochromatogram scans of ethanol-soluble ^{14}C extracts of apices at daily intervals following the application of [1-^{14}C]IAA to the apical buds of intact 11-day old light-grown dwarf peas. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars indicate the position of authentic IAA and IAAsp.

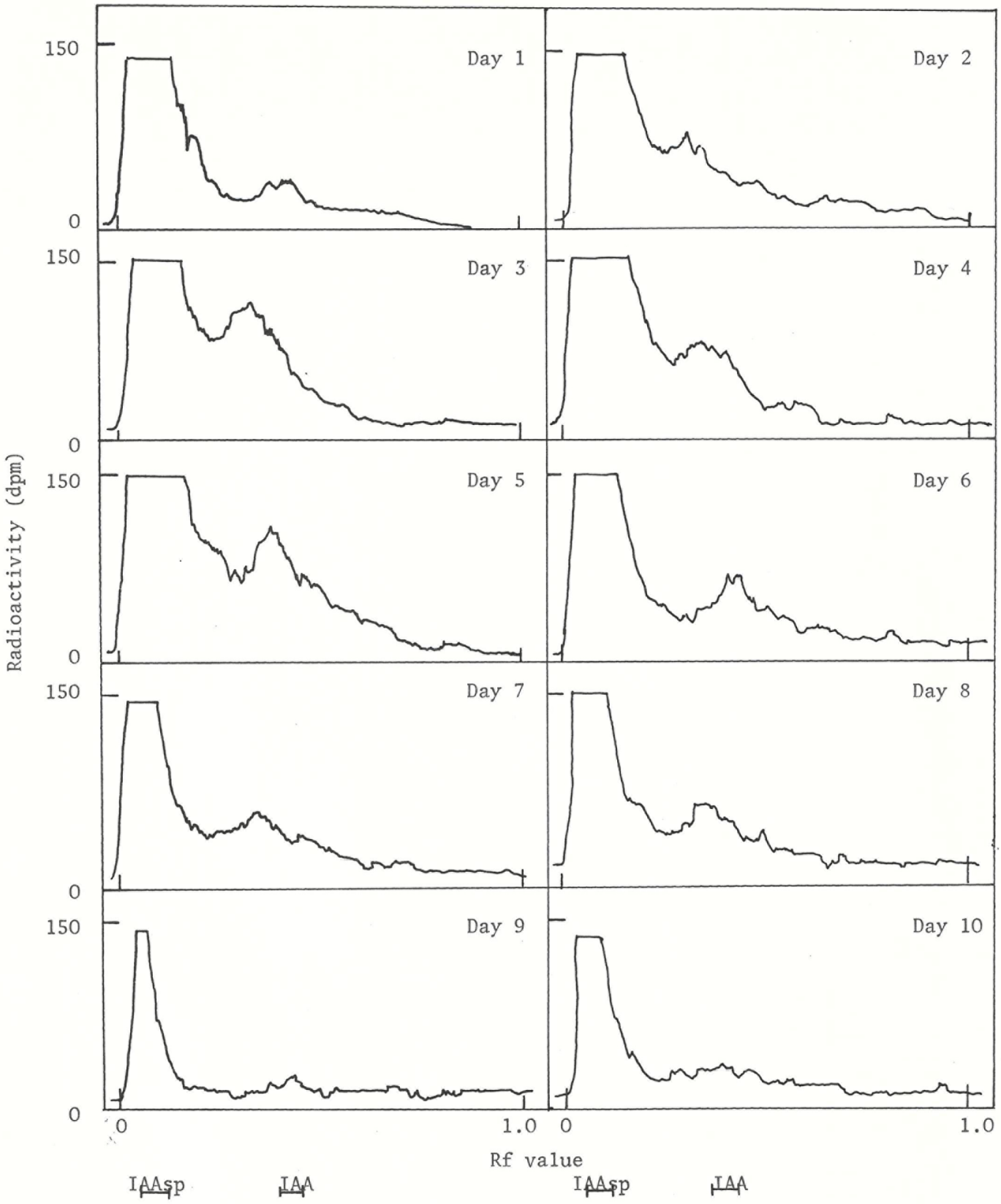


Figure 33 Radiochromatogram scans of ethanol-soluble ^{14}C extracts of apices at daily intervals following the application of [1-^{14}C]IAA to the apical buds of intact 11-day old light-grown dwarf peas. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars indicate the position of authentic IAA and IAAsp. Note the expanded scale compared to Figure 32, [^{14}C]IAAsp represents 99% of the ^{14}C present on each chromatogram.

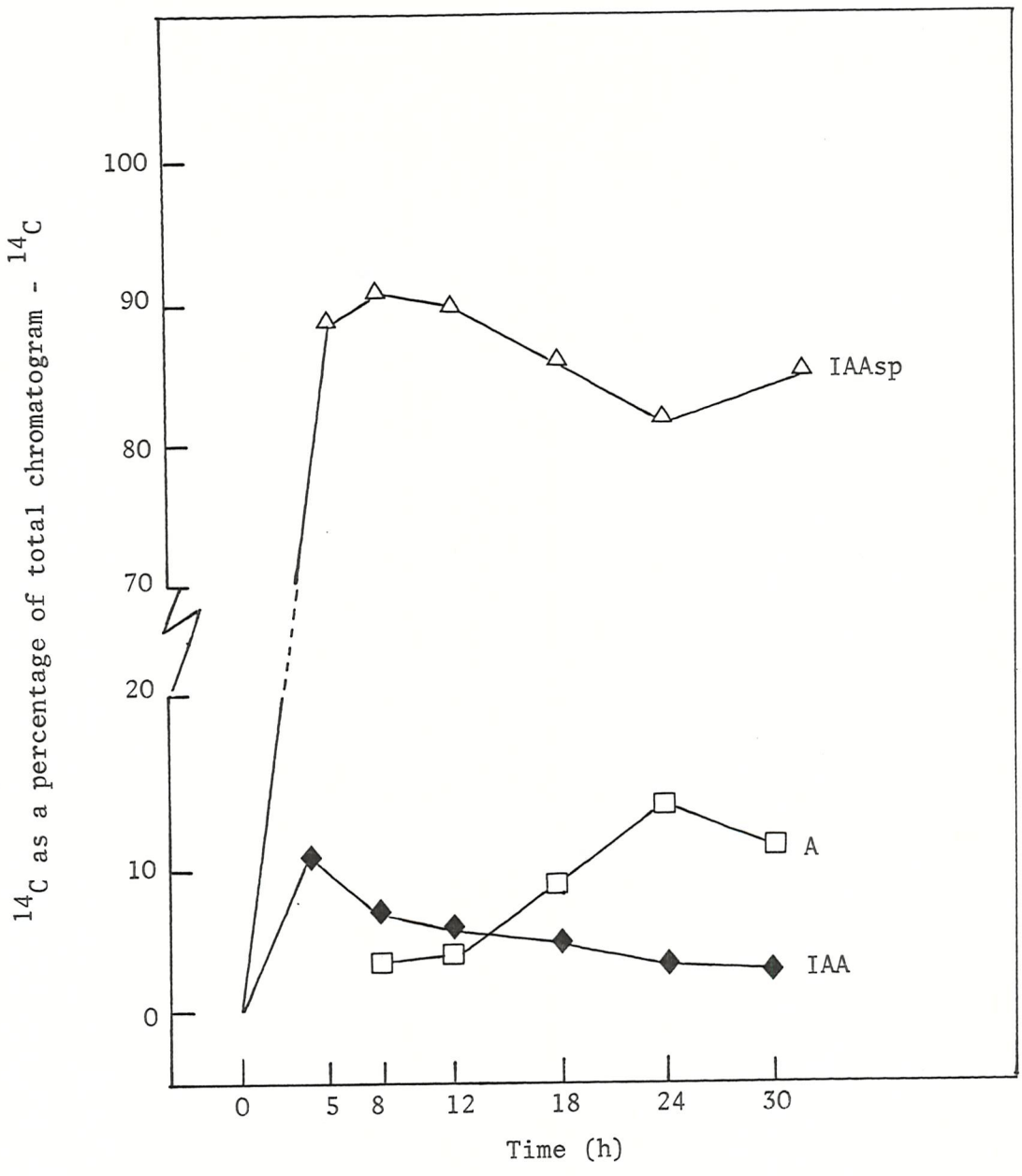


Figure 34 Changes with time in the proportions of major ^{14}C-metabolites recovered by 70% ethanol extraction of washed apical buds of intact 12-day old light-grown, light-transported dwarf peas following the application of [^{14}C]IAA to apical buds.

developed in chloroform:ethyl-acetate:formic acid (35:55:10 v/v) within one hour of the application of [1-^{14}C]IAA. This compound increased to a peak at four hours, and had declined as a percentage of total chromatogram ^{14}C by eight hours (Fig. 30). This may be the same compound as that noted at Rf 0.19 (designated 'A') in isopropanol:NH_3:H_2O (10:1:1 v/v. IAW) after 8 hours (Fig. 31). 'A' increased to a maximum of 15% of the total ethanol-soluble ^{14}C recovered, a reciprocal loss of IAAsp being recorded (Fig. 34). 'A' declines at 30 h and went undetected in the IAW developed chromatograms of the two to ten day harvests (Fig. 33). A small peak can be clearly seen at Rf 0.16 on chromatograms developed in butanol:acetic acid:H_2O (4:1:1 v/v) (Fig. 35). It was not apparent six days after the application of [1-^{14}C]IAA, and no information exists concerning its fate.

The time course of ^{14}C export from the apical tissue was estimated by measuring the total ethanol-soluble radioactivity in the remainder of the plant (i.e. stem, leaves and root system) at each harvest time. ^{14}C was transported from the apex at a high and almost constant rate for the first 8 h; thereafter export continued at a slower, steady rate for the remainder of the 48 h transport period (Fig. 36). The longer time-course study of export (Fig. 37) showed that the level of ^{14}C in the shoot reached a maximum 4 d after labelling and then declined very gradually over the next six days. The ^{14}C-extracted from the root showed no indication of reaching a maximum level with time. Total export from the apical tissue ceased after eight days, the increase in root ^{14}C being translocated from the stem.

Eliezer (1978) found that export was directly proportional to

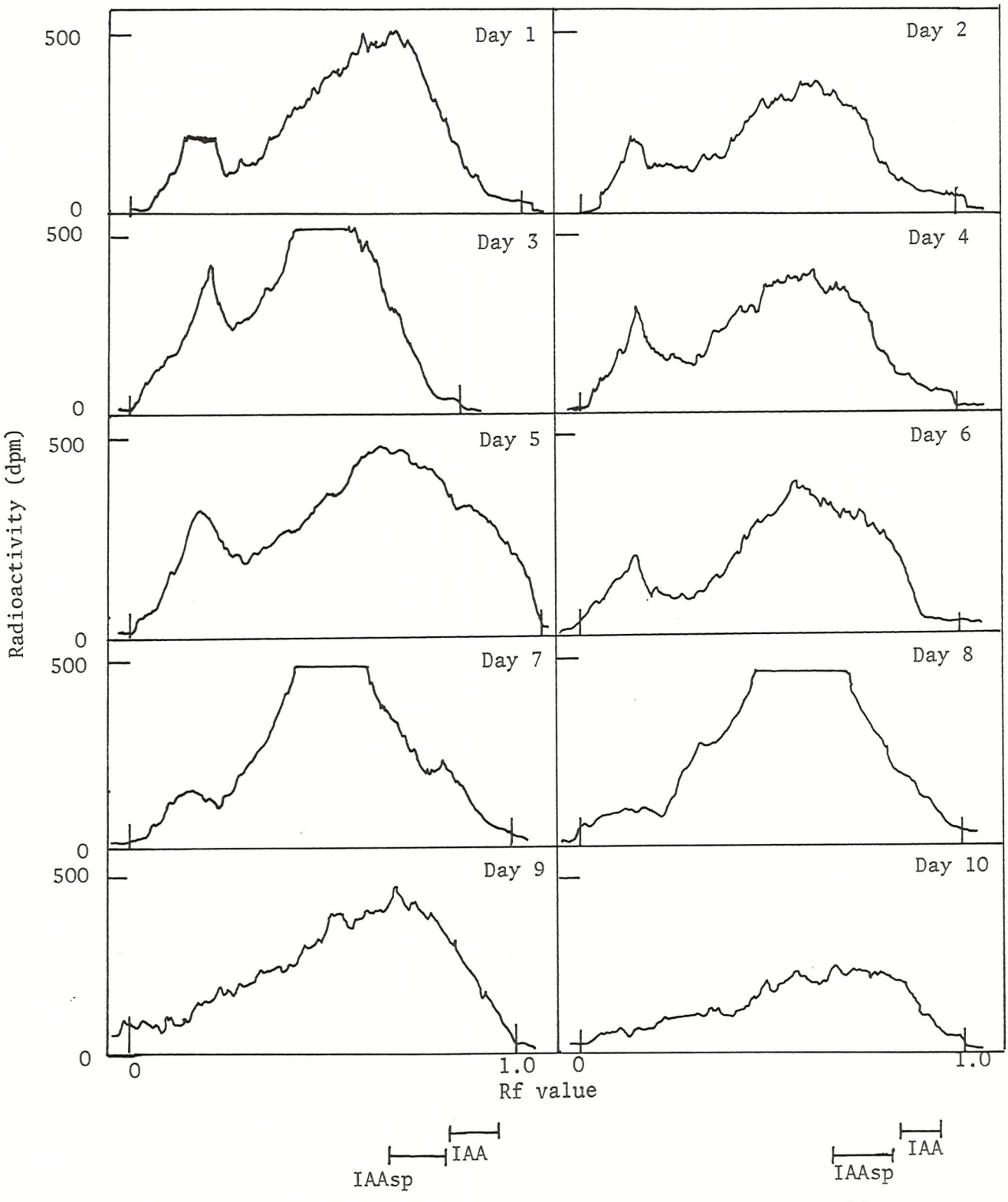


Figure 35 Radiochromatogram scans of 70% ethanol-soluble ^{14}C extracted from apices at daily intervals following the application of [1-^{14}C]IAA to the apical buds of intact 11-day old light-grown dwarf peas. The chromatograms were developed in butanol:acetic acid:H_2O (4:1:1 v/v). Horizontal bars represent the position of authentic IAA and IAAsp.

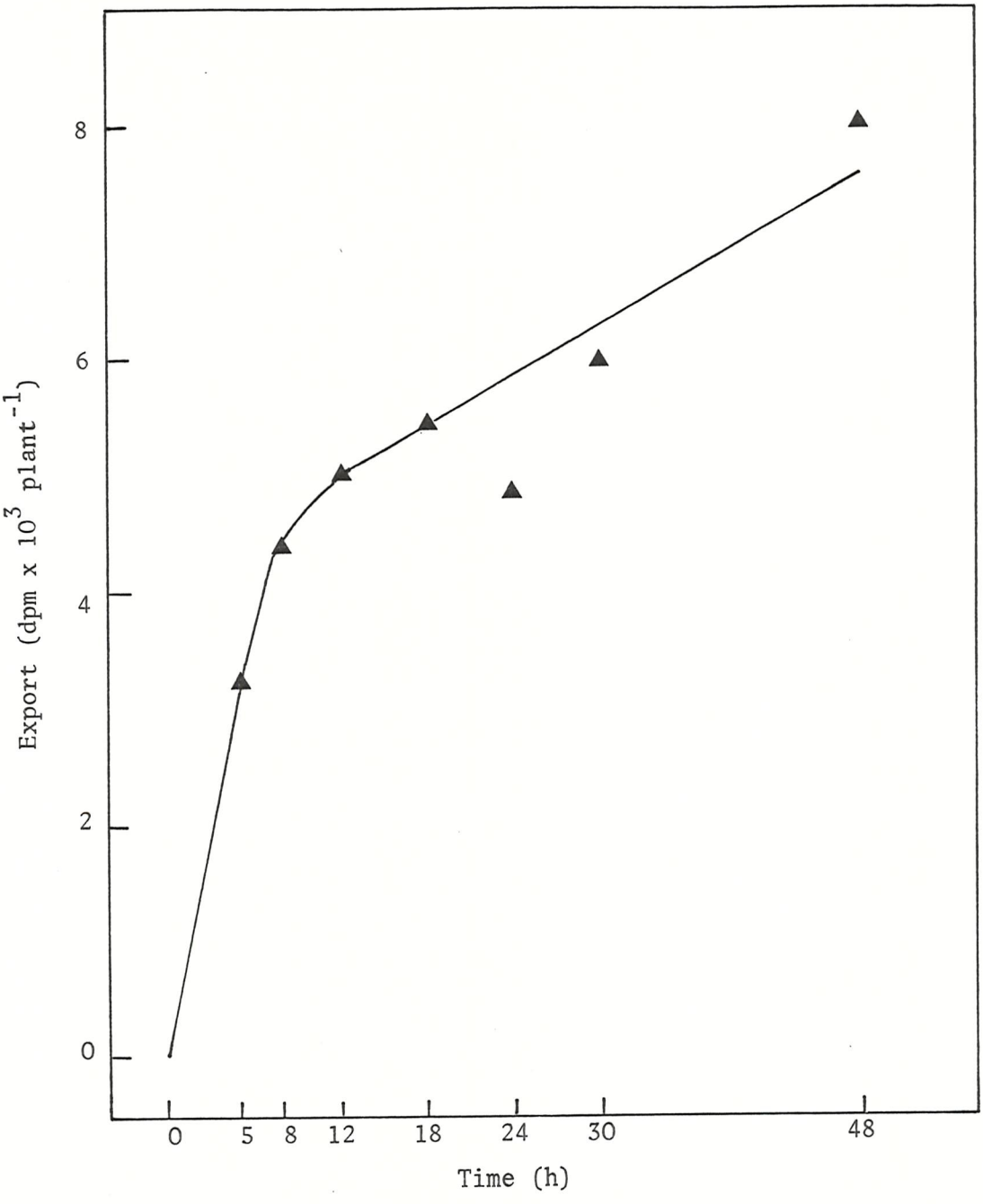


Figure 36 Time-course of export of ^{14}C out of the apices following the application of [1-^{14}C]IAA to the apical buds of intact 12-day old light-grown dwarf pea seedlings. Applied activity = 146,800 dpm plant^{-1}.

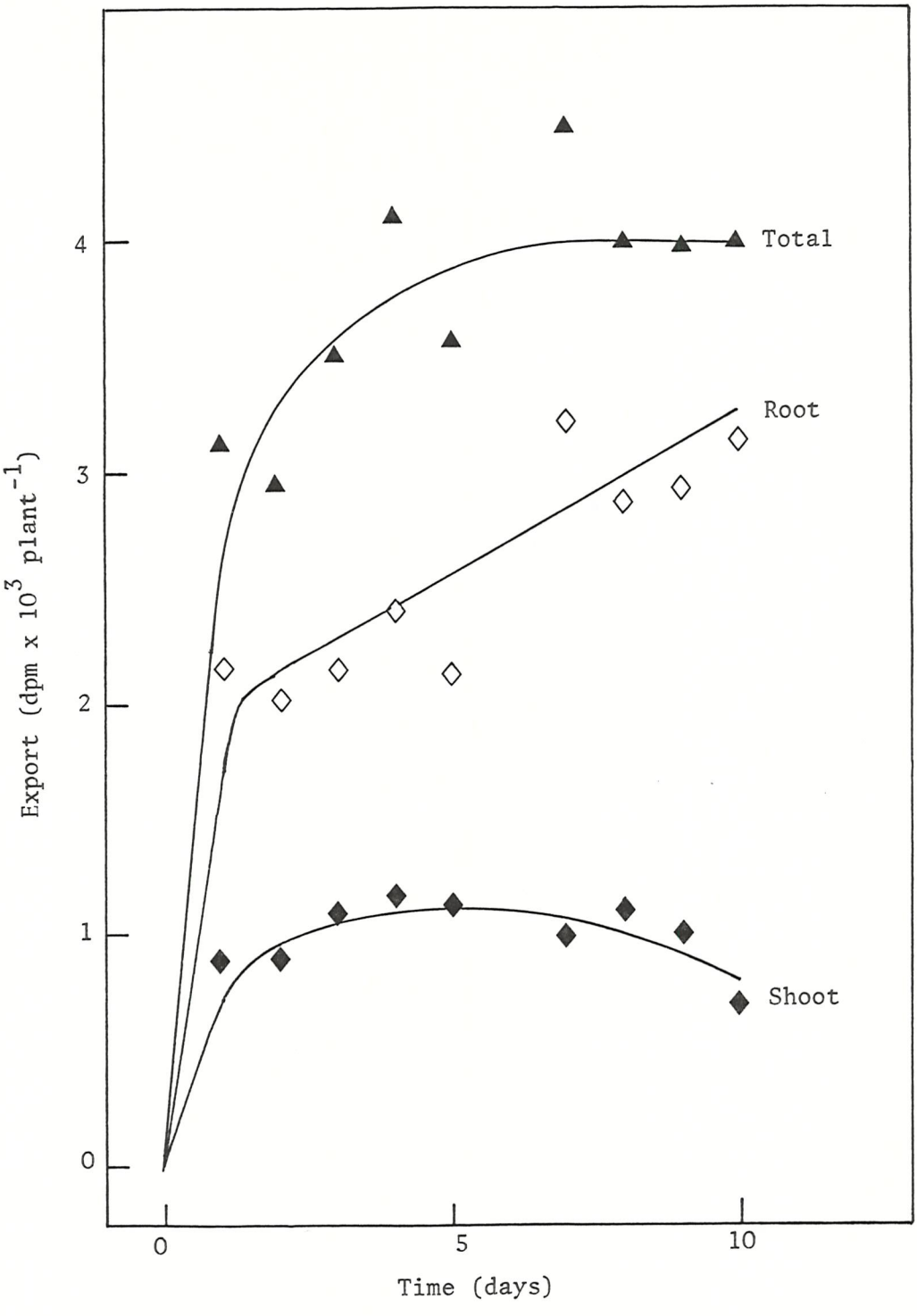


Figure 37 Time-course of export of ^{14}C from the apices to the root and shoot systems following the application of [1-^{14}C]IAA to the apical buds of intact 11-day old light-grown dwarf pea seedlings. Applied activity = 158,900 dpm plant^{-1}.

uptake for about the first two hours after labelling dwarf peas. A constant proportion of about 2.5% of the ^{14}C taken up during the first few hours was exported to the stem, thereafter export continued at a declining rate, less rapid than the decline in uptake rate. Eliezer (1978) concluded that this indicated a pool of transportable auxin in the apical bud that continued to drain faster than it was replenished by further uptake. Similar characteristics are found here. For the first five hours 2.6% of the ^{14}C taken up was exported. A rapid decline in the export rate followed. The first 5 h accounted for 40% of the total activity exported during the 48 h period of this experiment. As uptake ceased eight days after labelling, so also did export of ^{14}C from the apical tissue.

The major ^{14}C-metabolite extracted from both shoot and root systems co-chromatogrammed with IAAsp (Fig. 38, 39, 40). Other authors have also recorded the rapid formation of IAAsp in dwarf peas and *V.faba* stems transporting IAA (Morris *et al.*, 1969; Bonnemain, 1971). A small peak co-chromatogramming with IAA was detected in the 8, 12 and 18 h and 6, 9 and 10 d stem extracts. Consideration was given in Section II to the problem of interpreting ^{14}C-transport. Two alternatives exist: IAA is transported and then metabolised to IAAsp, or IAAsp itself is mobile. Data accumulated here indicated that the amount of ^{14}C-IAA within the apical tissue was sufficient to account for the ^{14}C exported. A small amount of IAA remained detectable within the apex after export had ceased at eight days. The amount of ^{14}C-IAA within the stem was not sufficient to account for the export of ^{14}C to the root which continued after day 8 (Table 24). The proportion of IAA within the

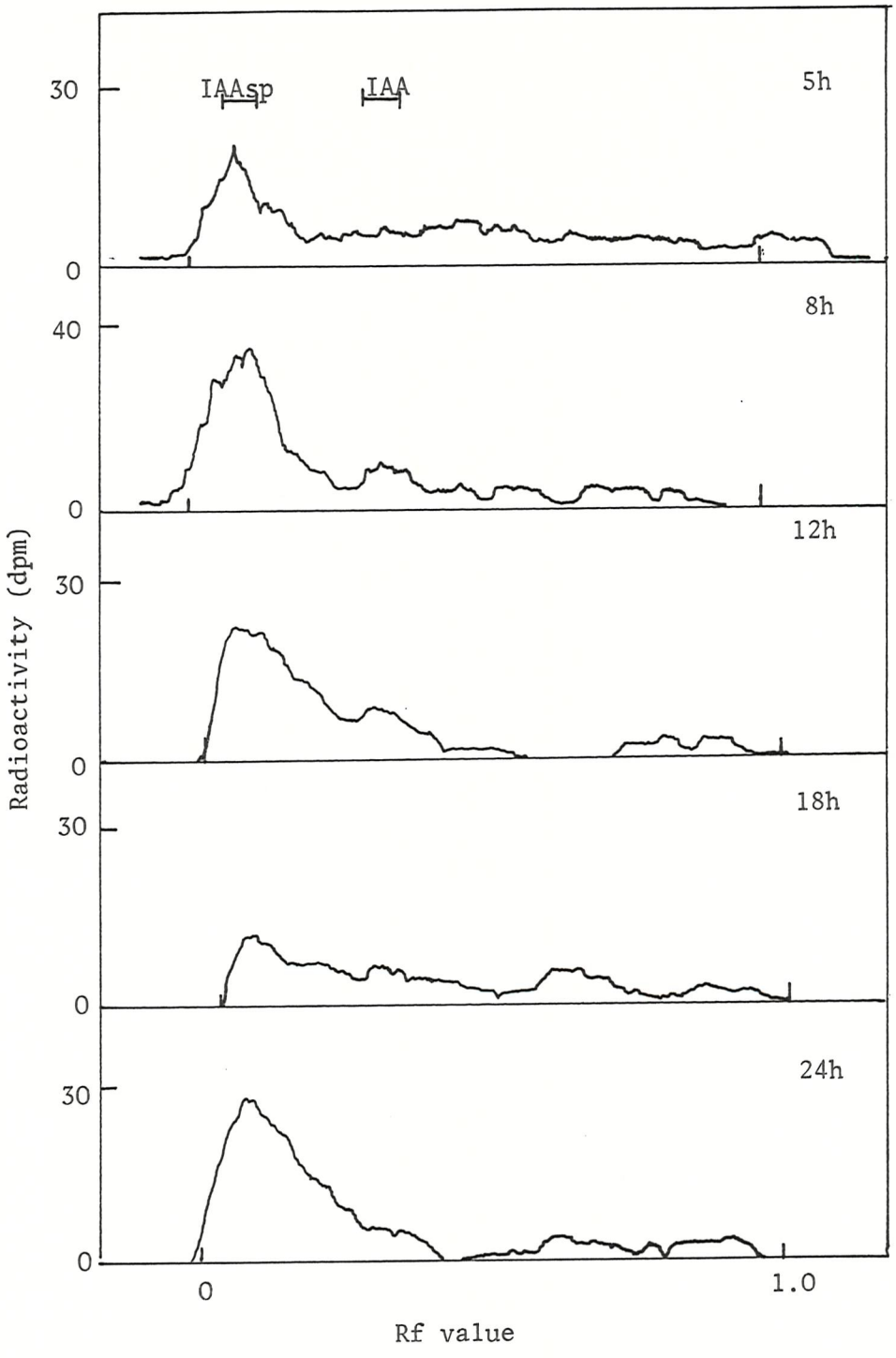


Figure 38 Radiochromatogram scans of ethanol-soluble ^{14}C extracts of stems following the application of [1-^{14}C]IAA to the apical buds of intact 12-day old dwarf peas. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars indicate the position of authentic IAA and IAAsp.

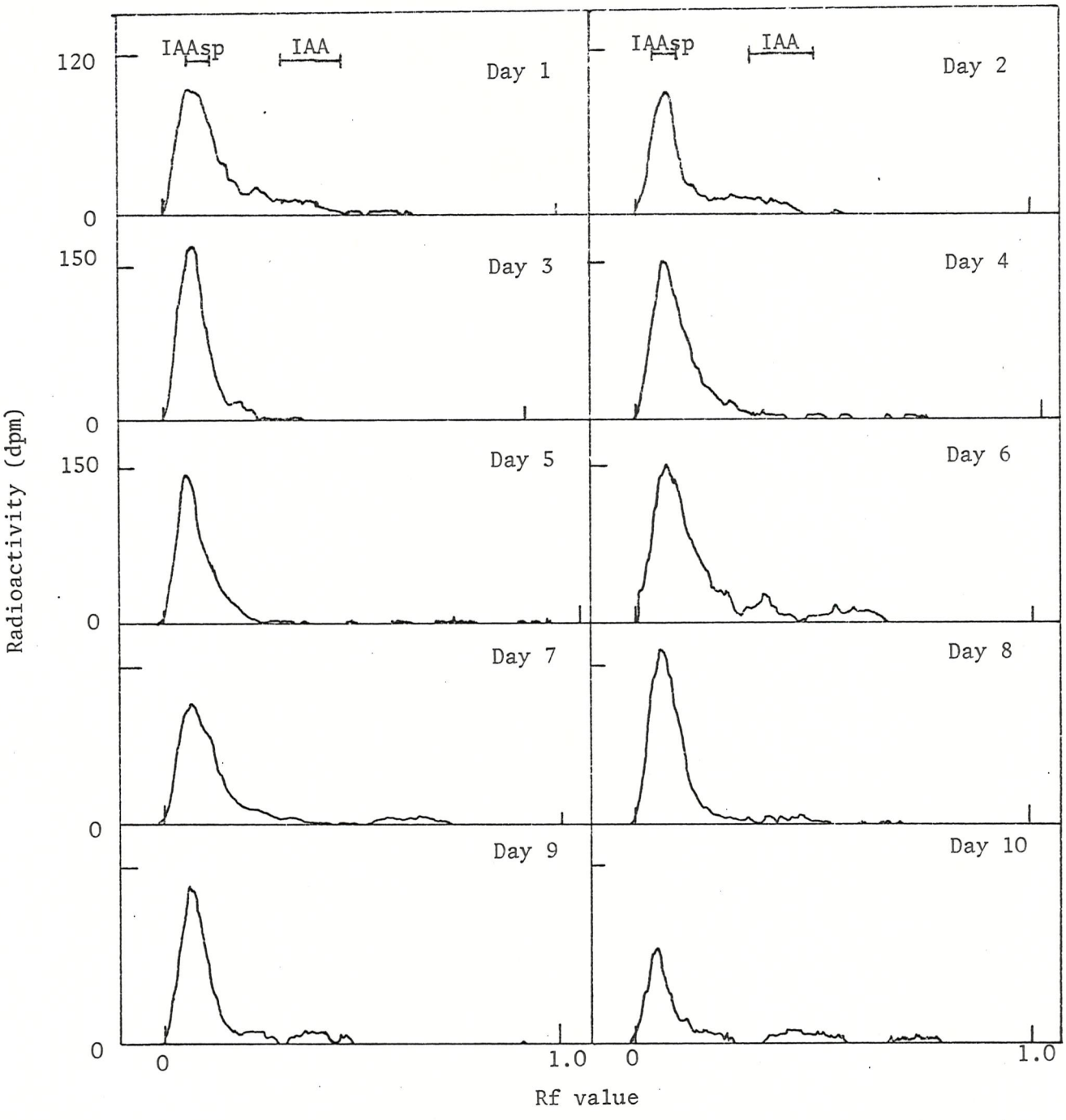


Figure 39

Radiochromatogram scans of ethanol-soluble ^{14}C extracts of stems following the application of [1-^{14}C]IAA to the apical buds of intact 11-day old light-grown dwarf pea seedlings. Harvest were taken every 24 hours. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars indicate the position of authentic IAA and IAAsp.

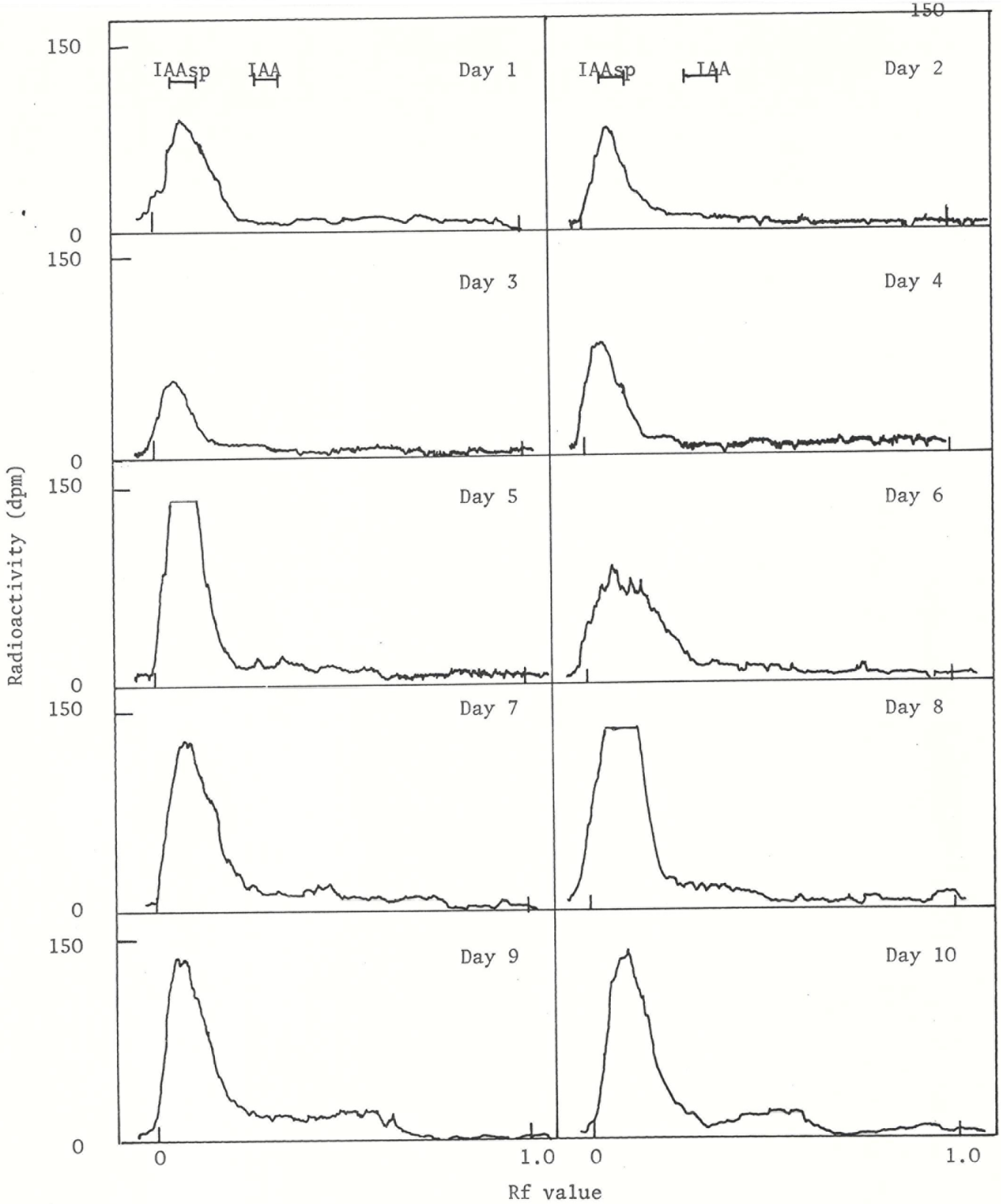


Figure 40 Radiochromatogram scans of ethanol-soluble ^{14}C extracts of roots taken at daily intervals following the application of [1-^{14}C]IAA to the apical buds of intact 10-day old light-grown dwarf pea seedlings. Horizontal bars indicate the position of authentic IAA and IAAsp. Chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v).

Table 24 The ^{14}C extracted by 70% ethanol from the stem and root 8, 9 and 10 days after the application of [1-^{14}C]IAA to the apical buds of 11-day old dwarf pea seedlings. The amount of ^{14}C-IAA in the stem was estimated from the proportions of ^{14}C-compounds separated by chromatography of the extracts.

Activity applied = 158,000 dpm. plant^{-1}.

Apical activity = 67,500 dpm. plant^{-1}.

All values dpm. plant^{-1}.

	Harvest time (d)		
	8	9	10
Total ^{14}C-ethanol soluble activity extracted from the stem	1199	1042	658
^{14}C-IAA extracted from the stem (estimated)	80	106	132
Total ^{14}C-ethanol soluble activity extracted from the root	2797	2875	3327

stem increased from day 8 to day 10 (Fig. 39) and this may have been due to slow release from IAAsp.

Discussion

From these experiments an account of the events following the application of exogenous IAA to the apical buds of intact light-grown dwarf pea seedlings has been developed. The major role played by conjugation of ^{14}C-IAA with aspartic acid was clearly confirmed. Within one hour of the application of exogenous IAA conjugation had commenced, and five hours after labelling 90% of the ^{14}C-auxin taken up was in the form of a compound presumed to be IAAsp. Conjugation continued while additional [1-^{14}C]IAA was taken up by the apex. A large percentage of the IAA molecules taken up by the plant remained 'compartmentalized' in the form of this conjugate for at least ten days. The total-ethanol soluble ^{14}C (mainly IAAsp) only declined slowly over the 10-day period (Fig. 39) and this suggests that IAAsp undergoes little further metabolism. Conjugation thus has a major influence in reducing the amount of free ^{14}C-IAA within the plant.

An indication was obtained that IAAsp was mobilized, or was re-metabolized to release free IAA in the stem. Following the end of export from the apex eight days after labelling, transport continued to the root system. Assuming that IAAsp is not transported (Section II), the release of IAA from the conjugate must have occurred in the stem to account for the amount of transport to the root between day 9 and 10.

The opinion expressed by other workers that IAAsp formation is a method for reducing potentially toxic levels of IAA within the plant

has been discussed in Section I. The large percentage of ^{14}C-IAA conjugated, the rapidity with which conjugation occurred, and the fact that the conjugate remained unchanged for many days, can be interpreted as supporting this view. Conjugation may give the plant the ability to rapidly reduce the amount of incoming IAA to a concentration closer to the endogenous level. Some evidence however has also been presented which can be used to support the hypothesis that IAAsp formation represents a method of storing IAA.

Uptake occurred rapidly at first, then slowed to a steady rate for 18 h. This process probably initially involved the diffusion of IAA molecules within free space to a position of contact with the plasma membrane. It has been suggested that the rate limiting step in IAA uptake occurs at the plasmalemma (Sherwin and Furuya, 1973) prior to uptake to the intra-cellular compartments. From observations of the uptake of IAA from external solutions by alga cells and tissue segments it seems likely that this process will involve some carrier mediated uptake together with the diffusive entry of IAA into cells (Davies and Rubery, 1978). Metabolism of IAA was recorded one hour after labelling indicating the entry of IAA to the cells within that time.

Other workers have described a biphasic uptake process although these investigations were based mainly on the behaviour of stem segments of Avena sativa and Pisum sativum (Johnson and Bonner, 1956; Kenny et al., 1969; Davies, 1973; Davies and Rubery, 1978). The initial rapid uptake defined by these authors was considered to be largely due to the rapid entry of IAA through the cut surface of the segments, which cannot be the case with application to the intact apex. Quantitative differences also exist between observations of

stem segments and those completed here and by Eliezer (1978) on the intact pea. For example, Davies and Rubery (1978) record an initial phase, with a $\frac{1}{2}$-time of about ten minutes, which is completed in 30 min. Johnson and Bonner (1956) concluded that the slower continuing rate of uptake leading to accumulation was under metabolic control, a fact confirmed by subsequent workers. Davies and Rubery (1978) felt that this continuing uptake represented the compounded result of three processes, transport to cells further down the tissue, internal compartmentalization in areas of the cell such as the vacuole and metabolic conversions, although Davies (1973) found this to be negligible in the first hour. It appears that the pattern of uptake of exogenous IAA by apical tissues of intact plants differs from that of cut segments.

The IAA molecules entering the apical tissues were rapidly removed by conjugation, and also by the steady process of export. Eliezer (1978) reported that the transport rate of IAA correlated with the availability of IAA molecules in a labile pool within the apical tissue. Both uptake and conjugation rates might thus be expected to influence transport. Uptake ceased eight days after labelling and so did export from the apical bud, although IAA was still detectable in the apex after 10 days. This suggests that some [^{14}C]IAA entered the labile, transportable pool of auxin in the apex directly on uptake and was not replenished from any other [^{14}C]IAA 'source'. As the root continued to import ^{14}C throughout the course of this experiment (1 to 10d), it was realized that remobilization of ^{14}C from the stem must have occurred. It seems likely that a relationship exists between transport and conjugation, through the proposed equilibrium of

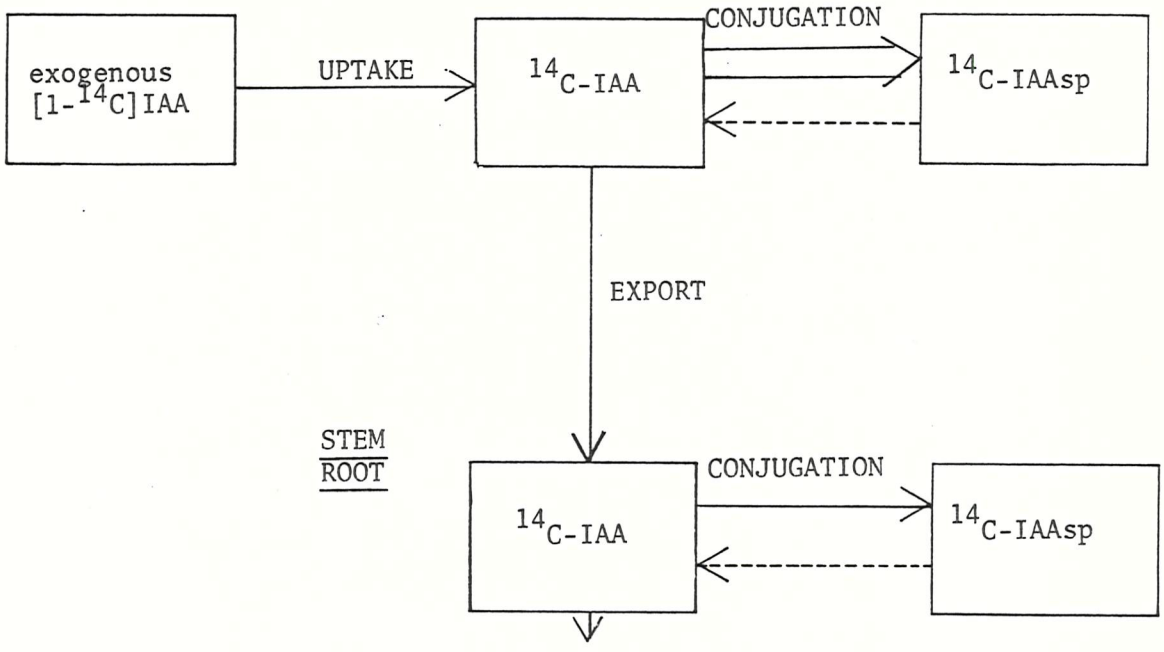
IAA and IAAsp.

In summary the uptake, metabolism and transport of [1-^{14}C]IAA applied to the apical buds of light-grown dwarf pea seedlings have been described. High concentrations of exogenous IAA are reduced in the plant by conjugation and it may be that detoxification is the major role of this process in the intact plant, although IAAsp may act as a storage compound from which IAA can later be released. The transport of ^{14}C from the stem to the root occurred to a greater extent than could be accounted for by the amount of [^{14}C]IAA in the stem. This observation suggests that IAAsp is converted to IAA, although further experiments are necessary to substantiate this hypothesis. A summary produced from the observations available is presented as Figure 41.

Time-course studies to observe the influence of light on uptake, metabolism and export of [1-^{14}C]IAA

Results

Time-course changes in uptake following the application of [1-^{14}C]IAA to dwarf peas grown and translocated in different light conditions revealed that the rate of uptake was slowest in dark-grown dark-transported plants (Fig. 42). After 5 hours the percentage of activity applied to the apical tissue which had been taken up by the plant was 21% less in etiolated plants maintained in complete darkness than in the other three treatments which received some light. Dark-grown plants transferred to a light environment prior to labelling paralleled more closely the pattern of uptake of light-grown plants, this was particularly noticable after 18 h.



UPTAKE	Maximum rate 1-5 h after application. 85% of ^{14}C-IAA applied was taken up within 5 h. Continued for 8 d.
CONJUGATION	Detectable 1 h after application. 90% of ^{14}C-IAA taken up was conjugated by 5 h. Continued while ^{14}C-IAA taken up.
EXPORT	Maximum rate 1-5 h after application. 40% of the total ^{14}C-exported from the apex (over 48 h) occurred by 5 h.

Figure 41 A summary of the observed fate of exogenous ^{14}C-IAA applied to the apical buds of light-grown dwarf pea seedlings.

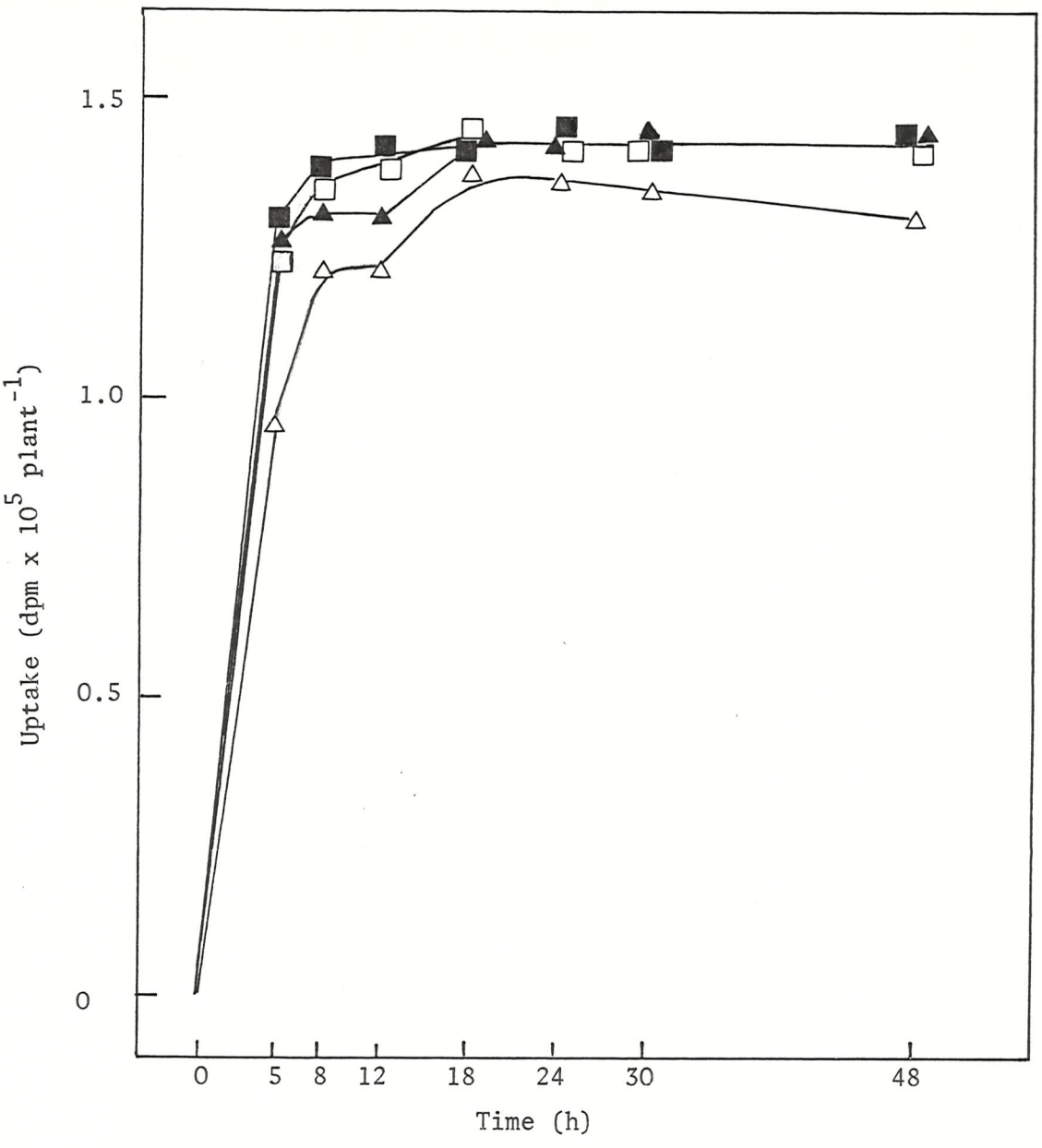


Figure 42

Time-course of changes in uptake following the application of [1-^{14}C]IAA to the apical buds of 12-day old intact dwarf peas. Uptake was estimated as ^{14}C-activity applied - ^{14}C-activity removed by washing.

□ light-grown and light-transported plants,

■ light-grown and dark-transported plants,

△ dark-grown and dark-transported plants,

▲ dark-grown and light-transported plants.

Applied activity = 146,800 dpm plant^{-1}

The transfer of light-grown plants to a dark environment before labelling had negligible effect on the uptake pattern.

Figure 43 could be interpretted as showing large differences in the apical metabolism of [1-^{14}C]IAA under different environmental conditions during plant growth. However, when the apices were surface washed prior to extraction, removing any IAA not taken up (Fig. 44), the pattern of conjugation was found to be independent of the light treatments (Fig. 45). The differences seen in Fig. 43 are accounted for by the different rates of uptake of IAA. As with the light-grown plants described in the previous sub-section five hours after labelling the majority of the IAA which entered the plant was metabolised to IAAsp. Light conditions had little effect on the metabolism of IAA after uptake, but affected uptake itself.

Figure 46 illustrates the constant percentage of [1-^{14}C]IAA applied to the apical bud that is conjugated with aspartic acid. The ^{14}C-metabolite, indistinguishable from IAAsp by chromatography, remained the major metabolite (over 80%) for the 48 h of this experiment. The compound designated 'A' running at Rf 0.20 in IAW was present in the apical extracts from all the plant treatments, appearing between eight and twelve hours after labelling. 'A' reached a maximum level within the apex at different times with varying light treatments, never exceeding 15% of the total ^{14}C-activity. The appearance of an additional metabolite, 'B', at Rf 0.60 in the same solvent system was noted after 48 h in all treatments except the dark-grown, dark-transported plants in which it was detected 30 h after labelling.

A major variation from the pattern of metabolism found in light-grown peas was the occurrence of a larger percentage of

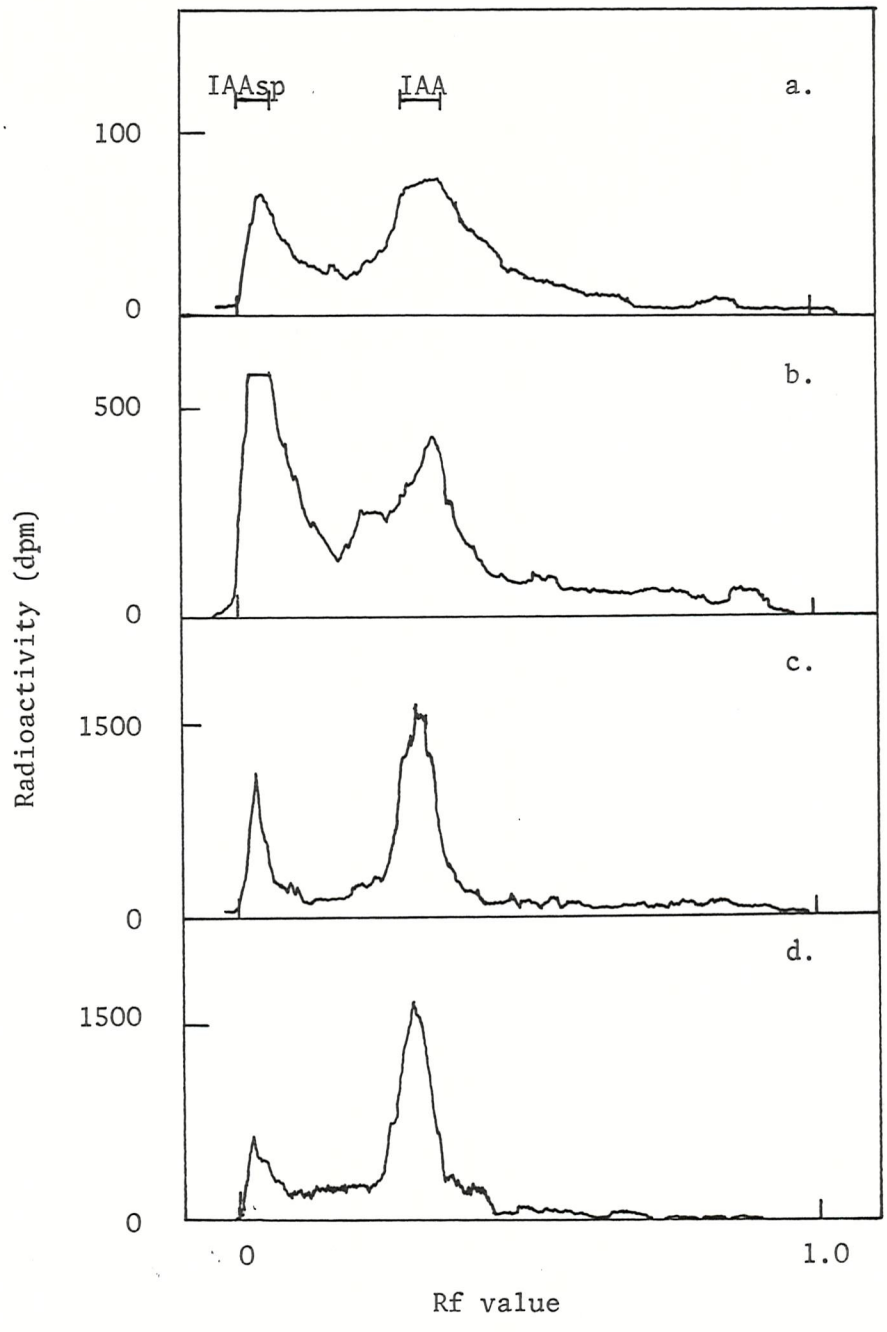


Figure 43

Radiochromatogram scans of 70% ethanol-soluble ^{14}C extracted from apices 5h after the application of [1-^{14}C]IAA to the apical buds of 12-day old intact dwarf peas. The apices were not surface washed prior to extraction. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars represent the position of authentic IAA and IAAsp.

a and b: light-grown, transported in light and dark respectively.

c and d: dark-grown, transported in light and dark respectively.

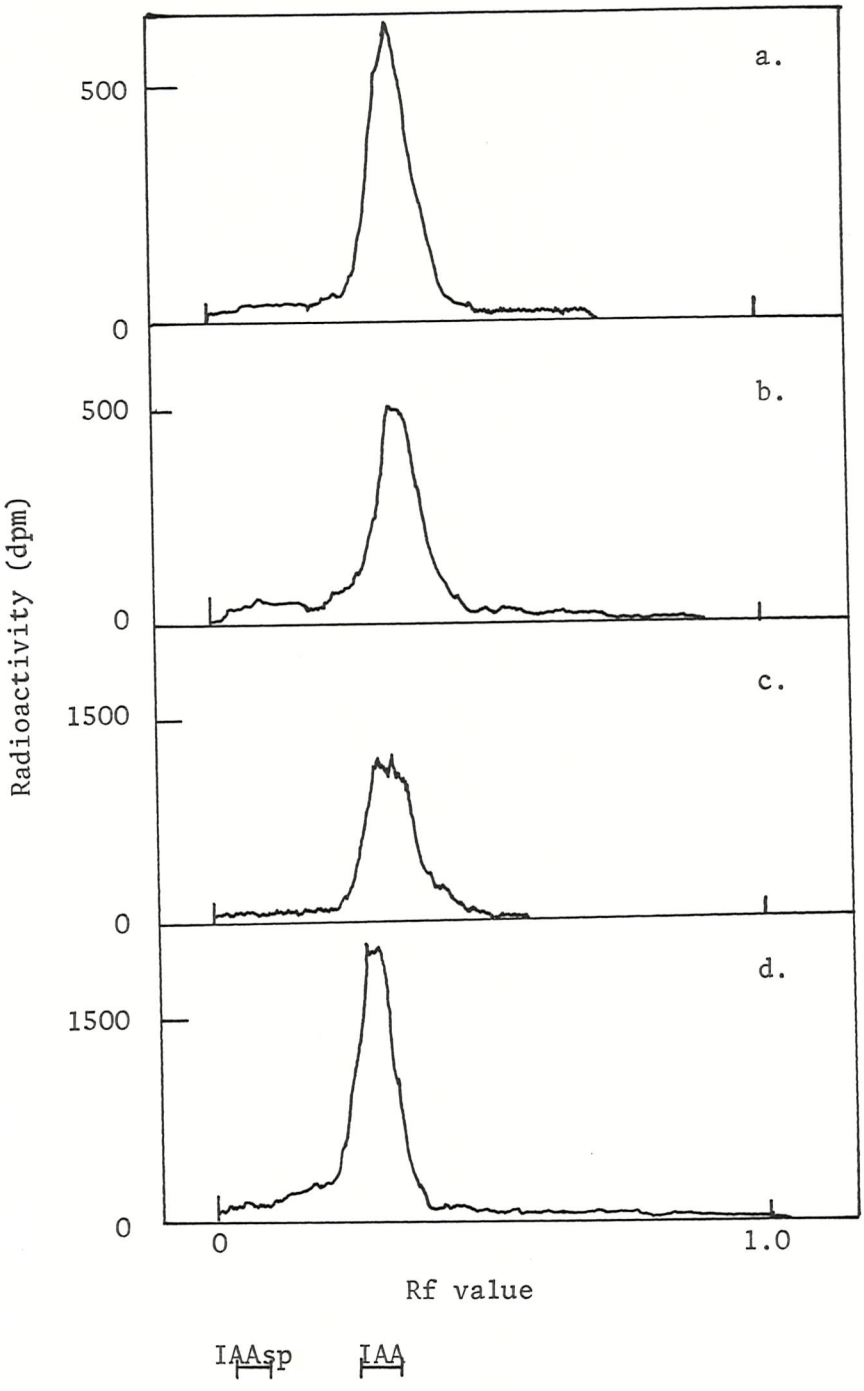


Figure 44

Radiochromatogram scans of ^{14}C washed from the apical surfaces 5h after the application of [1-^{14}C]IAA to the apical buds of 12-day old intact dwarf peas. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars represent the position of authentic IAA and IAAsp.

a and b: light-grown, transported in light and dark respectively.

c and d: dark-grown, transported in light and dark respectively.

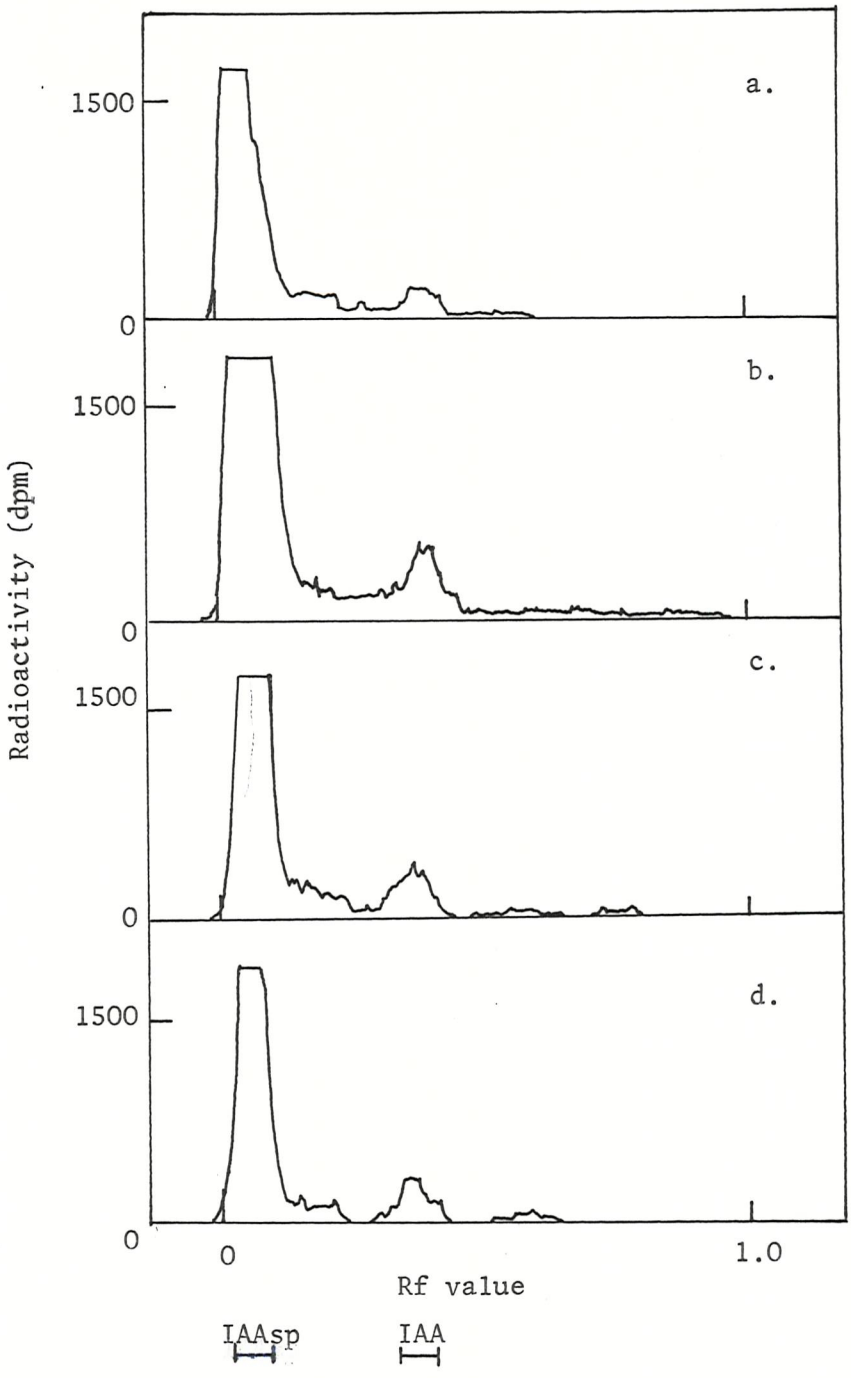


Figure 45

Radiochromatogram scans of 70% ethanol-soluble ^{14}C extracted from the apices 5h after the application of [1-^{14}C]IAA to the apical buds of intact dwarf peas. The apices were surface washed prior to extraction. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars represent position of authentic IAA and IAAsp.

a and b: light-grown, transported in light and dark respectively.

c and d: dark-grown, transported in light and dark respectively.

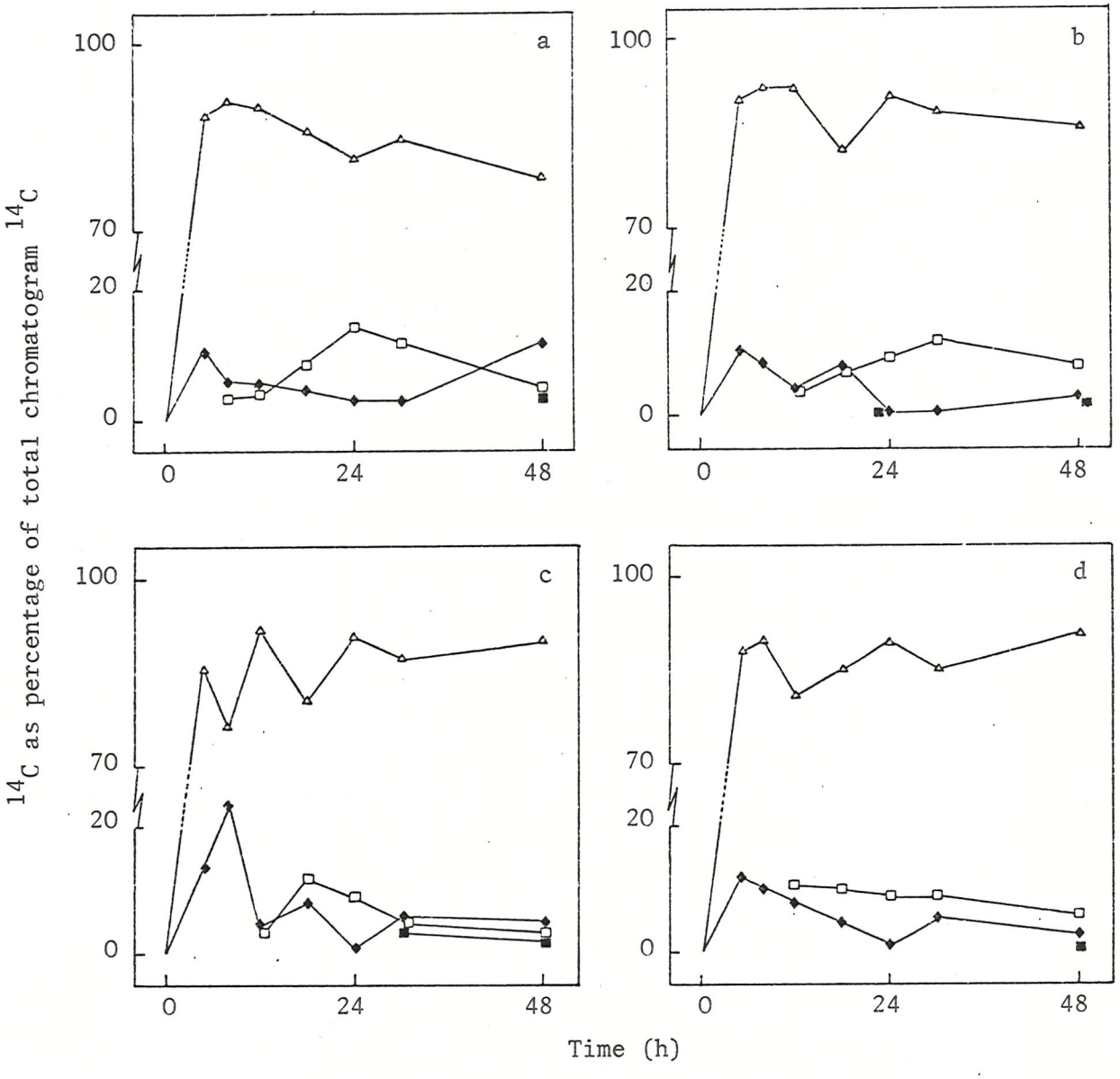


Figure 46

Changes with time in the proportions of ^{14}C-metabolites recovered by 70% ethanol extraction of washed apical buds following the application of [1-^{14}C]IAA to the apical buds of intact 12-day old dwarf peas.

a and b: light-grown, transported in the light and dark respectively

c and d: dark-grown, transported in the light and dark respectively

△ = IAAsp, ♦= IAA, □= 'A', ■= 'B'

^{14}C-IAA after eight hours in the apices of etiolated peas that were maintained entirely in dark conditions during the experiment. The amount of IAA in the apical tissue of these plants increased between 5 and 8 h after the application of [1-^{14}C]IAA. Thereafter the proportion of IAA declined. This reflected the greater increase in the rate of uptake of IAA between 5 and 8 h and the slightly slower initial conversion to IAAsp seen during the first eight to twelve hours. Additionally lower export may have contributed to higher IAA levels in the apex (Fig. 47).

A slight decline in activity present as IAAsp, together with a corresponding increase in IAA was noted between 30 and 48 h in the light-grown, but not the dark-grown, plants. This could be due to the conversion of IAAsp to IAA later in the experiment when free IAA levels may have dropped back to endogenous concentrations. However, the observations described above for the ten-day time-course experiment using light-grown plants indicated this slight decline was not continued.

The time-course of ^{14}C-export from the apices was estimated as before by measuring the total ethanol-soluble radioactivity in the remainder of the plant following the removal of the apex at each harvest period. The transfer of light-grown plants to dark conditions had little effect on the amount or the rate of export from the apices of these plants compared to the light-grown light-transported seedlings (Fig. 47). However, the amount of ^{14}C exported from dark-grown plants was far lower for the first twelve hours, after which the rate of export increased dramatically bringing the total amount of ^{14}C exported in both light- and dark-grown plants to a similar level by 24 h. Radiochromatogram scans of the ethanol-soluble ^{14}C

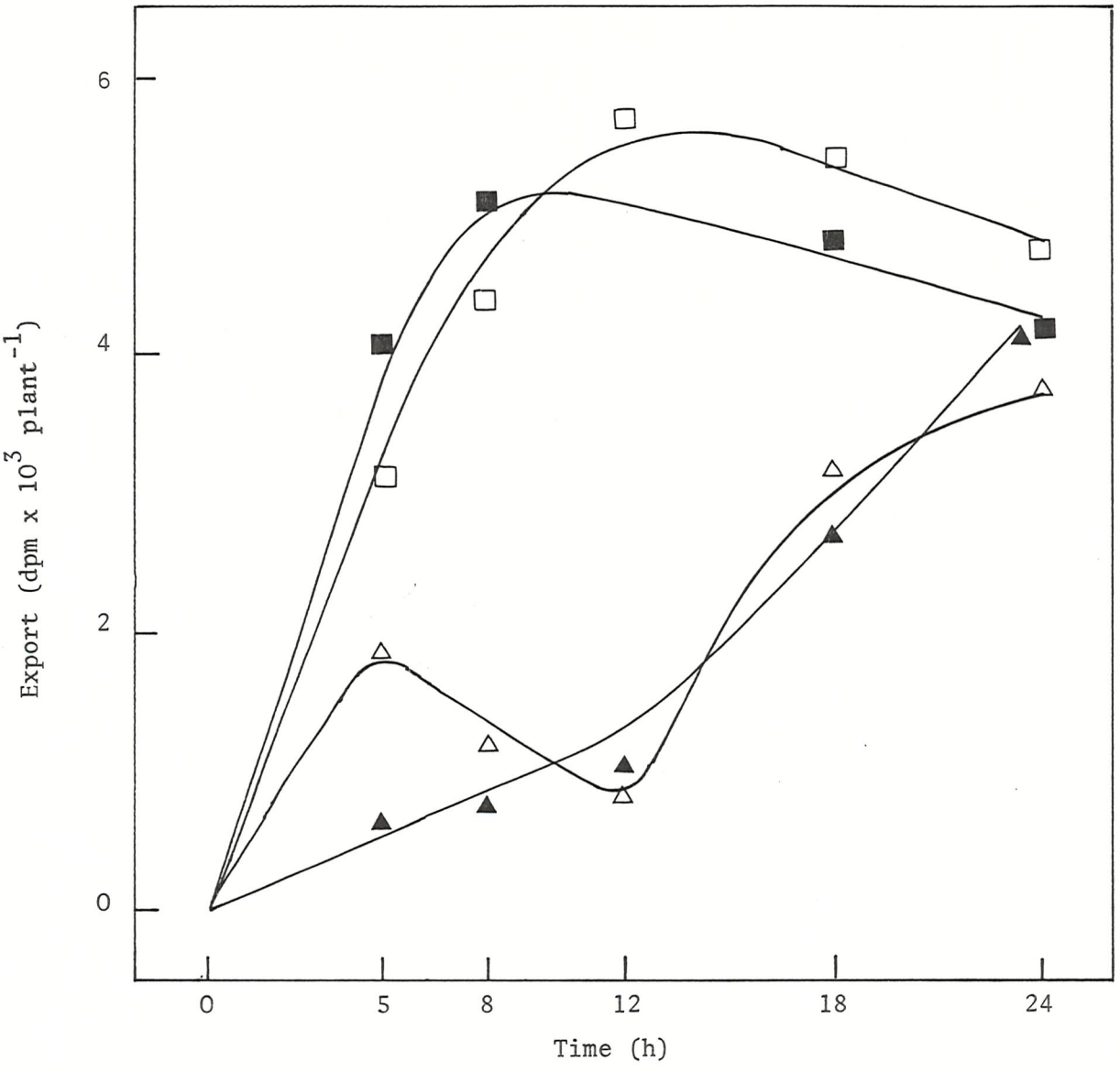


Figure 47

Time-course of cummulative export of ^{14}C from the apex to the rest of the plant following the application of [1-^{14}C]IAA to the apical buds of intact 12-day old dwarf peas.

□ and ■ represent light-grown plants, transported in light and dark respectively,

▲ and △ represent dark-grown plants, transported in light and dark respectively.

Applied activity = 146,800 dpm plant^{-1}.

extracted from the stems indicated no difference between the metabolites found in light-grown stems (Fig. 38) and from those plants grown and/or transported under other conditions. Fig. 48 shows the chromatograms of stem extracts 48 h after the application of IAA.

Discussion

This study provided evidence for the formation of IAAsp by apical tissues of P.sativum from exogenous [1-^{14}C]IAA, independent of the light environments in which the plants were grown, or subjected to, during the course of the experiment. This result is in agreement with data published by Tillberg (1974) in which she found endogenous IAAsp in both dark- and light-grown P.vulgaris stem tissue. The conjugate system was active in both light- and dark-grown plants. The results described here showed negligible differences in the activity of the conjugate system in the apical tissue of light or etiolated apices.

These results are in contrast with those published by Lantican and Muir (1969), Morris (1970) and Eliezer (1978) who found evidence to suggest that more IAAsp was formed, and that less decarboxylation occurred in light conditions than in dark. The increased activity of IAAsp synthetase in the light was demonstrated by Eliezer (1978). Lantican and Muir (1969) concluded that the formation of the IAAsp synthetase enzyme(s) in plants grown in sunlight was probably the result of induction by higher levels of endogenous IAA in the light. They were observing the behaviour of a crude enzyme preparation of P.sativum epicotyls, a method that has been criticised on several grounds by Venis (1972). Hofinger and Lis (1977) had also recorded the lack of inducible IAAsp formation in the incubated

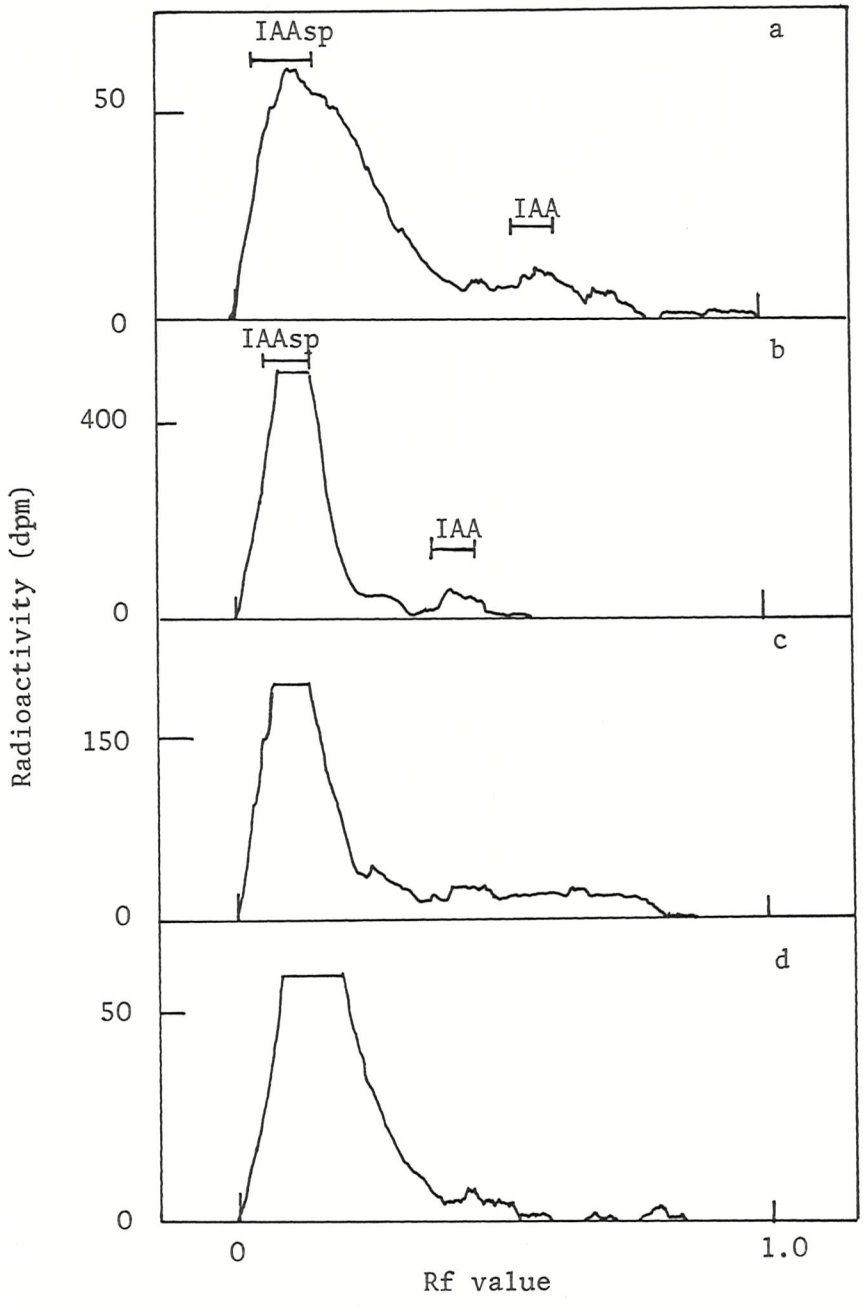


Figure 48

Radiochromatogram scans of 70% ethanol-soluble ^{14}C extracts of stems 48h after the application of [1-^{14}C]IAA to the apical buds of intact 12-day old dwarf peas. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars indicate the position of authentic IAA and IAAsp.

a and b: light-grown, transported in light and dark respectively,

c and d: dark-grown, transported in light and dark respectively.

epicotyls of dark-grown Lens culinaris.

The reasons behind the different observations are not known, however it is possible that the different concentrations of IAA used affected the conjugation system. Higher concentrations may have induced IAAsp synthetase in all tissues, overcoming any different thresholds between the plants, while lower concentrations would induce only the more "sensitive" systems. Alternatively, the varying reports may have resulted from the use of a different line of peas which responded differently to the treatments. It is possible that additional IAA was not required by the plants in this study and consequently the proposed equilibrium between IAA and IAAsp favoured IAAsp formation. This seems unlikely as some variation in auxin requirement between plants grown in different light conditions would be expected, especially in the meristematic apical region. Such variation may be outside the detection limits of the analytical procedure used. Variations in the endogenous IAA system may occur without influencing [^{14}C]IAA metabolism, such as an altered rate of synthesis (Fig. 1).

In this investigation the most dramatic effects of the light conditions upon the plants' response to exogenous IAA were in terms of uptake and export of IAA from the apex. The results produced show that the light-stimulation effect on uptake occurred even in dark-grown plants transferred to the light, and light-grown plants transferred to the dark. This may suggest that the presence of chlorophyll in the tissues is important. Thimann and Wardlaw (1963) found that light stimulated uptake in green stem segments of P.sativum was dependent on the presence of chlorophyll and tentatively suggested that the local production of oxygen during photosynthesis

might be involved. The response appeared not to be dependent on photosynthetic production of assimilate. This is not in agreement with results presented here, as uptake continued at the same rate in light-grown plants remaining in the light as those transferred to darkness and in which the production of oxygen by photosynthesis had presumably ceased. Naqvi (1974) described a sugar requirement for auxin absorption in etiolated Z.mays coleoptile segments which may explain the slower uptake in etiolated plants recorded here.

Sherwin and Furuya (1973) could find no effect of red-light upon the destruction or immobilization (as IAAsp) of exogenous IAA. They recorded a rapid increase in the rate of absorption of exogenous IAA by Oryza sativa seedlings five hours after application, and described the photoreversibility of changes in the rate of uptake caused by red light irradiation. Consideration was given to the involvement of phytochrome in the control of IAA absorption. If the light effect on uptake is a phytochrome controlled response, as Sherwin and Furuya (1973) suggest, it seems possible that an alteration in the permeability of membranes would explain the stimulation of uptake by light (Marmé, 1977). However, if this is the explanation for the pattern of uptake, the continuing high uptake by light-grown plants transferred to darkness suggests that the process is not photoreversible, in contrast with most phytochrome-mediated reactions.

In addition to absorbing more IAA than plants grown in darkness, light-grown plants were found to export IAA initially at a faster rate. This result is supported by the data published by Morris (1970) and Eliezer (1978). Work on light-grown Coleus stem segments has also shown that light promotes auxin transport (Koevenig and Jacobs,

1972). However, there is not a consistant picture in the literature concerning the effects of light on auxin transport, for example results obtained from etiolated coleoptile tissue of Avena and Zea demonstrated that light reduced auxin transport (Naqvi and Gordon, 1967; Thornton and Thimann, 1967; Shen-Miller et al., 1969).

The results of this study suggest that it was the conditions of plant growth that had the major effect on export, not the conditions during transport. The difference between light- and dark-grown tissues is not easily explained. Light-grown stems may have a higher capacity to transport IAA due to a larger number of cells available for transport, perhaps a greater development of the cambial region believed to be associated with auxin transport (Bonnemain, 1971; Bourbouloux and Bonnemain, 1973, 1974; Morris and Thomas, 1978).

From these studies the influence of light on the conjugation system of dwarf peas appeared negligible and from this the implication may be drawn that conjugation is not regulated by environmental influences. However, these results, although in agreement with some cases cited in the literature, contradict others. In view of this inconsistancy it is not possible to say whether conjugation plays a major role in the control of hormone levels in response to changing environmental or physiological conditions. The major effects of light under the conditions studied were on the absorption of exogenous [1-^{14}C]IAA by the apical tissue and its subsequent export.

A comparison of tall and dwarf pea varieties

Results

The pattern of uptake observed in dwarf peas was different to that seen in tall peas under corresponding light treatments. Tall peas took up [1-^{14}C]IAA at a slightly slower rate than dwarf peas (Fig. 49). As described for dwarf peas, those tall peas grown and transported in total darkness had taken up less [1-^{14}C]IAA than the other treatments five and eight hours after labelling. However, the amount taken up was not considerably lower than the other treatments, except the light-grown light-transported plants which took up an additional 20,000 dpm plant^{-1} (12%) during the first five hours of the experiment. In tall peas therefore a period of darkness (or the absence of light) would appear to result in decreased uptake.

A comparison of Figures 50 and 45 reveals little difference in the amount of [1-^{14}C]IAA conjugated in tall and dwarf peas 5 h after labelling. This was also the case 8 h following the application of [1-^{14}C]IAA (Fig. 51) with two exceptions. Firstly a greater percentage of IAA was found in tall peas grown and transported in continuous light, and secondly the higher proportion of IAA found in dwarf pea apices which received no light was not reflected in the tall pea apical extract from the same treatment. The influence of light on the conjugation mechanism of tall peas apices labelled with [1-^{14}C]IAA appeared negligible.

The pattern of export of ^{14}C from the apices of tall peas was similar to that observed in dwarf peas. The conditions under which the plants were grown were of paramount importance (Fig. 52).

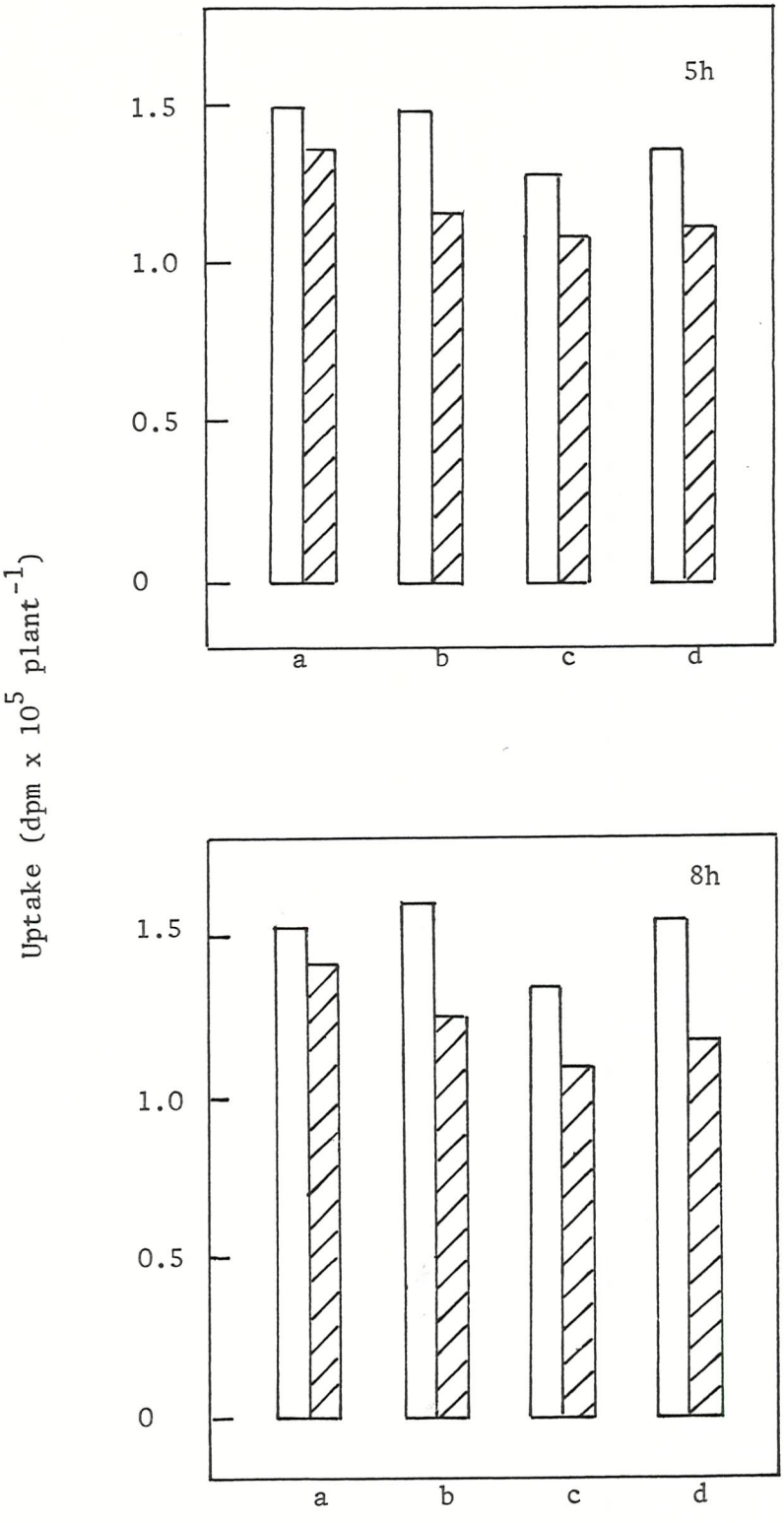


Figure 49

A comparison of uptake by tall and dwarf peas 5 and 8h following the application of [1-^{14}C]IAA to the apical buds of 12-day old plants. Uptake was estimated as ^{14}C-activity applied - ^{14}C-activity removed by apical washing. The striped blocks represent tall peas, un-striped blocks dwarf peas.

a and b: light-grown, transported in light and dark respectively,

c and d: dark-grown, transported in dark and light respectively.

The applied activity was 180,000 dpm plant^{-1}.

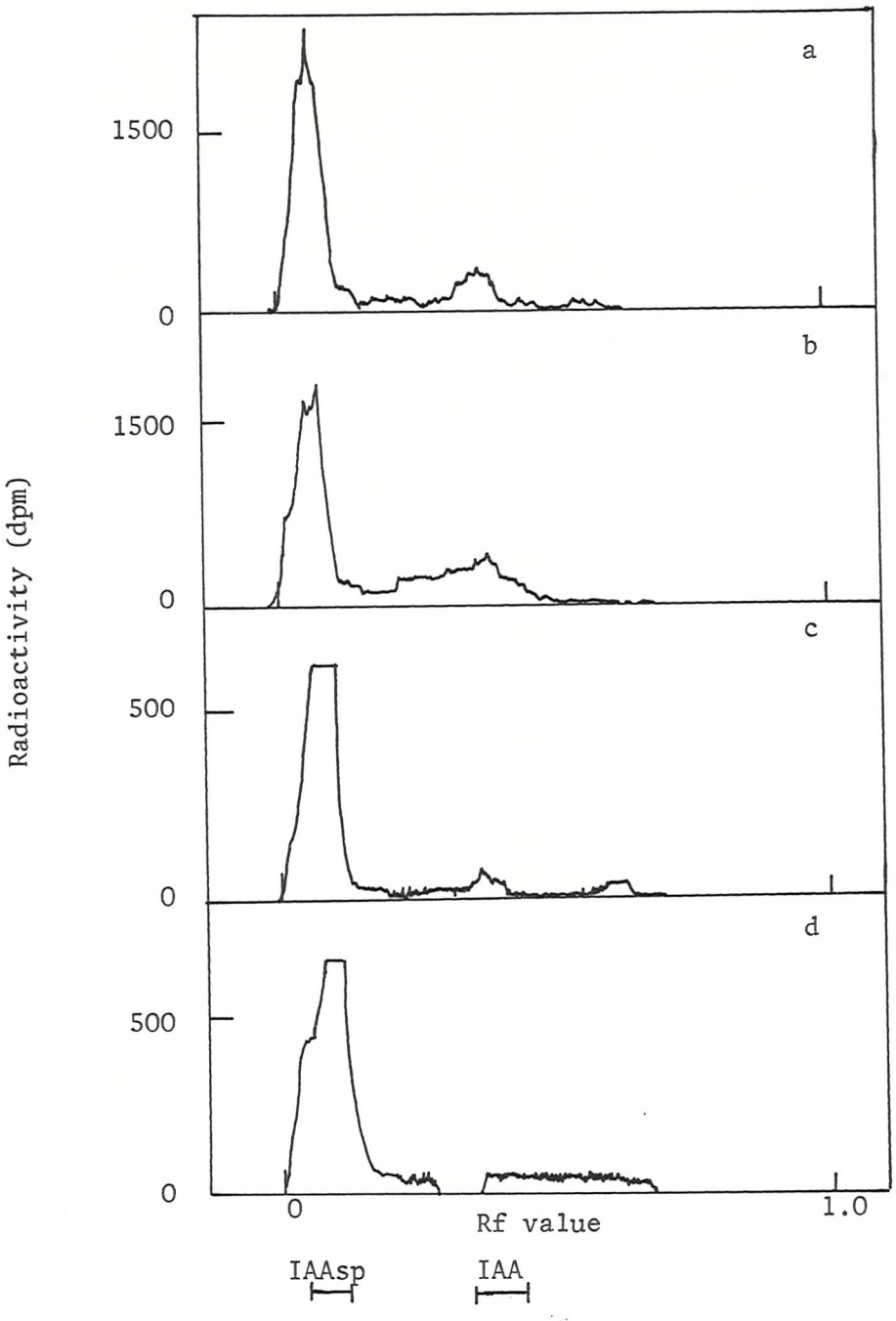


Figure 50

Radiochromatogram scans of 70% ethanol-soluble ^{14}C extracted from washed apices 5 hours following the application of [1-^{14}C]IAA to the apical buds of 12-day old intact tall peas. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars represent the position of authentic IAA and IAAsp.

a and b: light-grown, transported in light and dark respectively,

c and d: dark-grown, transported in light and dark respectively.

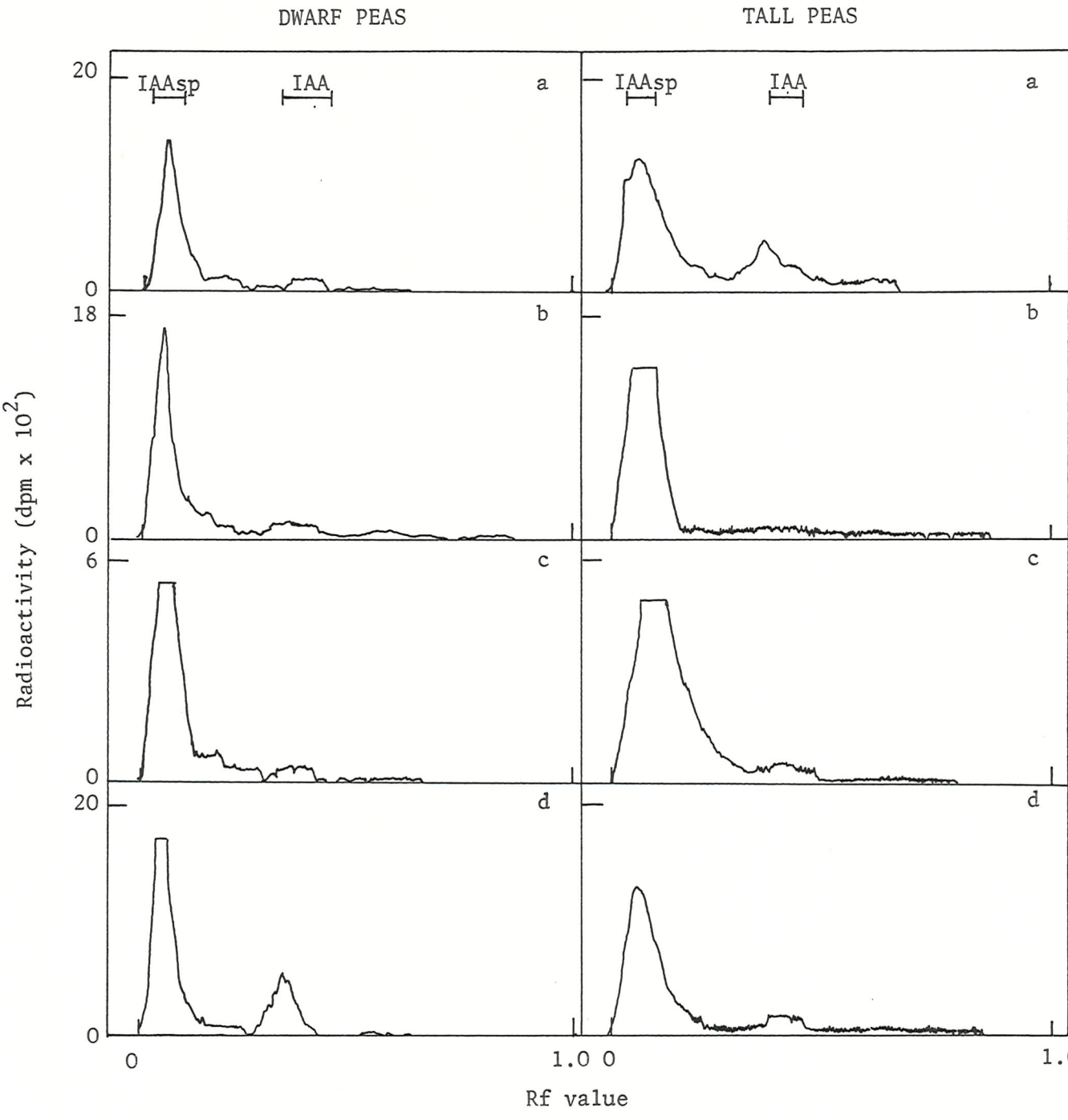


Figure 51

Radiochromatogram scans of 70% ethanol-soluble ^{14}C-extracts from the washed apical buds of dwarf and tall peas 8 hours following the application of [1-^{14}C]IAA to the apices of 12-day old intact seedlings. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars represent the position of authentic IAA and IAAsp.

a and b: light-grown, transported in light and dark respectively,

c and d: dark-grown, transported in light and dark respectively.

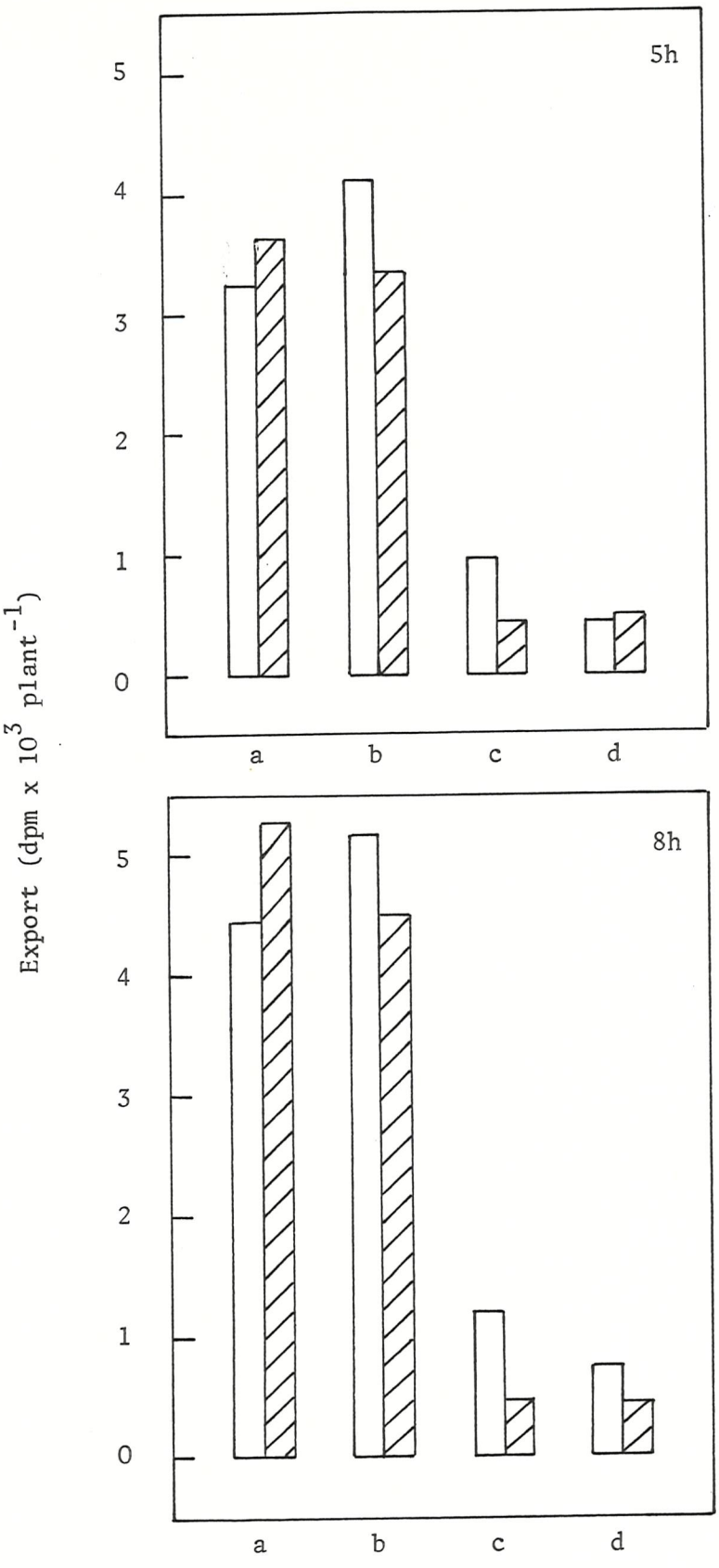


Figure 52

A comparison of ^{14}C export from the apices of tall and dwarf peas 5 and 8h following the application of [1-^{14}C]IAA to the apical buds of 12-day old intact plants. Export is measured as total ^{14}C extracted from shoot and root. Striped blocks represent tall peas, un-striped dwarf peas.

a and b: light-grown, transported in light and dark respectively,

c and d: dark-grown, transported in dark and light respectively

The applied activity was 180 000 dpm plant^{-1}.

The activity transported in dark-grown plants was 90% less than light-grown plants, irrespective of the conditions of transport.

The metabolism of [1-^{14}C]IAA by stem segments revealed less formation of IAAsp in dark-grown segments of both varieties (Fig. 53, Table 25). A greater proportion of the IAA was conjugated in the light-grown segments, approximately 50% of the ^{14}C extracted from the segments was indistinguishable from IAAsp by chromatography. Dwarf dark-grown segments conjugated more IAA than dark-grown tall segments, although still less than 20% of the ^{14}C-activity extracted. Pretreatment of tall dark-grown pea segments for eight hours with unlabelled IAA (2µg ml^{-1}) was shown to increase the amount of IAAsp formed (Fig. 54).

Discussion

Lantican and Muir (1969) explained the difference in growth of tall and dwarf peas by variations in the activity of the IAAsp synthesis system which regulated the 'levels of diffusible auxin' and therefore growth. They found that the amount of conjugate formed from [^{14}C]IAA by a crude enzyme preparation from dwarf peas was approximately twice that formed by the preparation from the tall peas on a fresh weight basis. In this experiment a difference was not found in the amount of conjugation by light-grown tall and dwarf pea stem segments. The major variation occurred between light- and dark-grown segments of both varieties. Although there was a slight increase in the proportion of IAAsp in dark-grown dwarf compared with dark-grown tall segments it was not as large as that described by Lantican and Muir (1969). Neither was the difference in the amount of conjugation between intact tall and

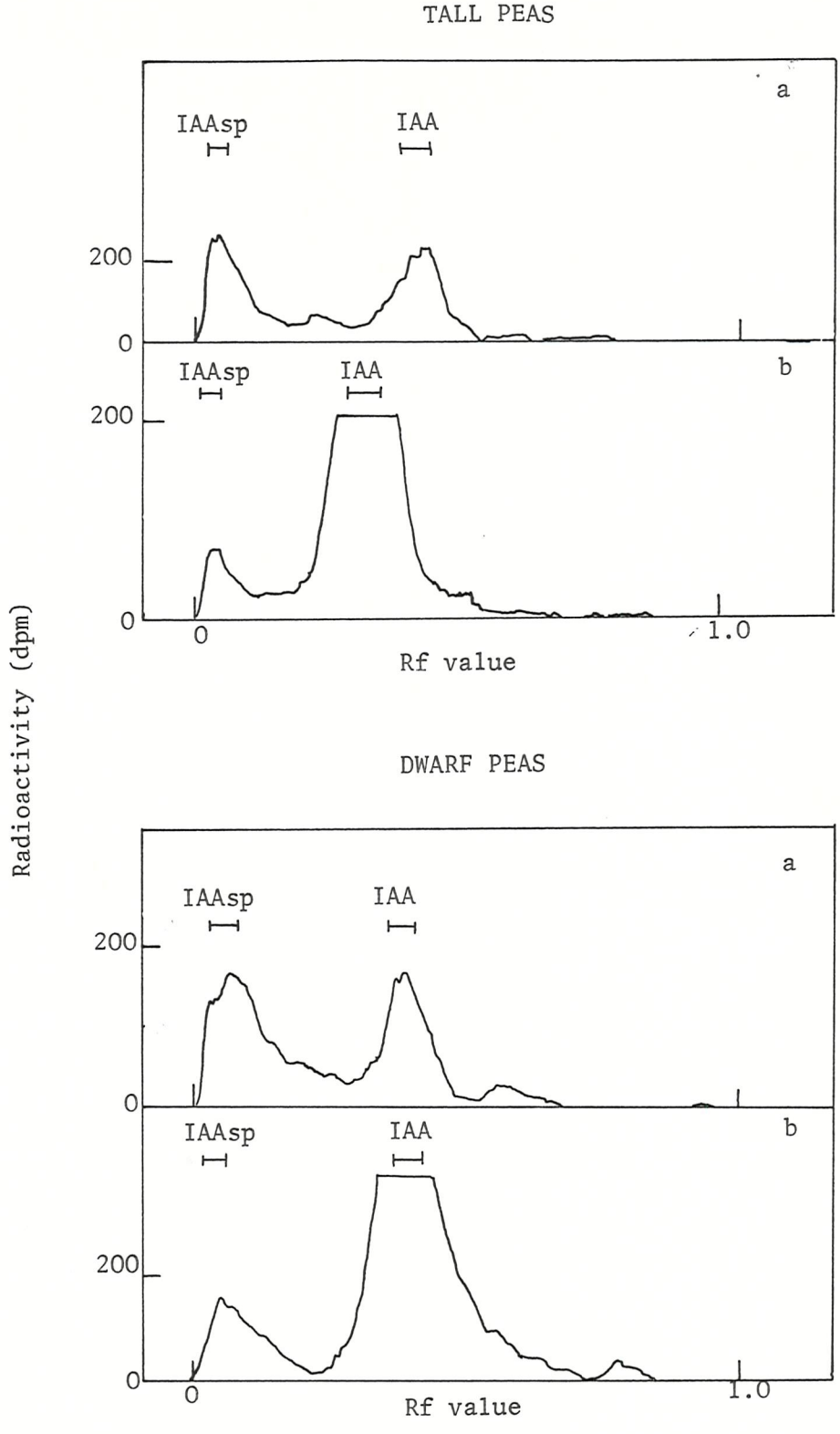


Figure 53

Radiochromatogram scans of 70% ethanol-soluble ^{14}C extracted from stem segments of tall and dwarf peas following a 4 hour incubation with [1-^{14}C]IAA. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars represent the position of authentic IAA and IAAsp.

a and b: light and dark grown plants respectively.

Table 25 The recovery of 70% ethanol-soluble ^{14}C from stem segments of tall and dwarf peas following a 4 hour incubation with [1-^{14}C]IAA. The amounts of ^{14}C-compounds extracted from the segments were estimated from the proportions of ^{14}C separated by chromatography of the extracts (isopropanol:NH_3:H_2O, 10:1:1 v/v). Applied activity = 12837365 dpm per incubation treatment (2 µg ml^{-1} [1-^{14}C]IAA).

Segments incubated	^{14}C extracted (dpm)	% ^{14}C extracted of total IAA incubated	^{14}C extracted (dpm)	
			IAAsp	IAA
Light-grown dwarf peas	319 043	2.5	163 066	155 977
Dark-grown dwarf peas	222 873	2.0	44 575	178 298
Light-grown tall peas	269 585	2.0	118 617	150 967
Dark-grown tall peas	305 086	2.5	76 271	228 815
Pretreated dark-grown tall peas	376 165	3.0	161 214	214 951

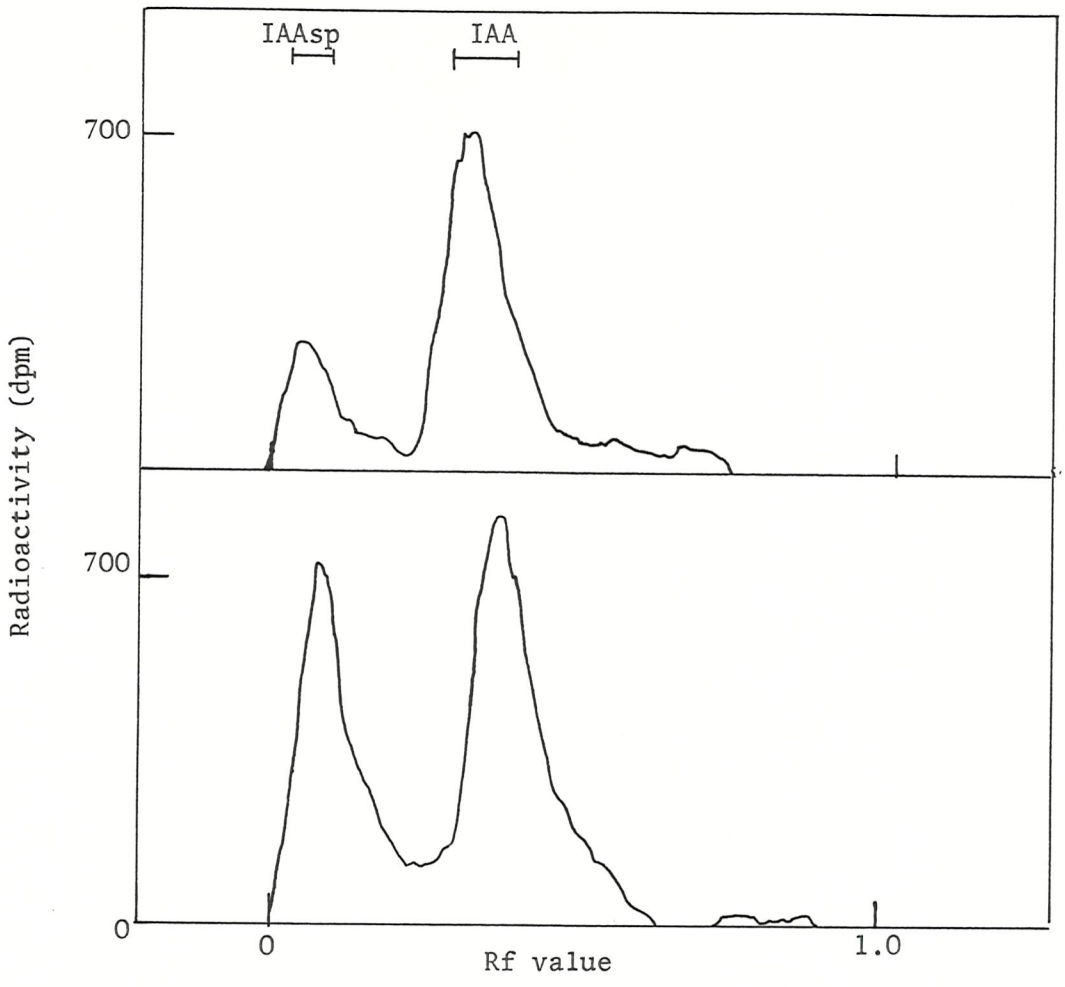


Figure 54 Radiochromatogram scans of 70% ethanol-soluble ^{14}C extracted from stem segments of dark-grown tall peas following a 4 hour incubation with [1-^{14}C]IAA. a: with no pretreatment and b: with an 8 hour incubation with unlabelled IAA prior to the incubation with [1-^{14}C]IAA. The chromatograms were developed in isopropanol:NH_3:H_2O (10:1:1 v/v). Horizontal bars represent the position of authentic IAA and IAAsp.

dwarf peas (both light- and dark-grown). It would seem unlikely from these results that IAAsp formation controlled differential IAA concentrations in the tissue, and consequently the differential growth rate of the two varieties. This may suggest that auxin levels are regulated by other systems as suggested by Moore and Shaner (1967) who found that the differences between tall and dwarf peas could be correlated with differences in the rate of IAA synthesis from tryptophan and the 'sensitivity of the tissue to auxin'.

The difference noted in conjugation between light- and dark-grown segments was not reflected in the experiments where [1-^{14}C]IAA was applied to the apex of intact plants. Eliezer (1978) reported the mean cell length of epidermal parenchyma and differentiating xylem cells was far greater in dark-grown than light-grown stems of P.sativum (cv. Meteor), P.vulgaris and Lupinus albus (L.). Extrapolation of such data would indicate a lower cell number per segment (standard length) in dark-grown plants. If the capacity of the tissue to conjugate IAA is limited by cell number, as suggested by the lower proportion of conjugate formed in segments than apices,this may explain the lower amount of IAAsp formed in the dark-grown segments. If the amounts of IAAsp formed reflect a different capacity of light- and dark-grown tissue to conjugate IAA, it is difficult to explain the absence of this difference in intact plants.

The pretreatment of dark-grown tall pea segments with cold IAA for 8 h before the addition of [1-^{14}C]IAA increased the efficiency with which these segments metabolised IAA to IAAsp. This agrees with those authors who have described auxin induction of the IAAsp synthetase system (Section I).

As light- and dark-grown apical tissue failed to show a variation in conjugation it may be suggested that the conjugation mechanism is not involved in the regulation of auxin concentration under different light conditions. Alternatively the amount of exogenous [^{14}C]IAA taken up by the apex may have overcome variation in the endogenous system. It is also difficult to explain the variation in the pattern of uptake between dwarf and tall peas and further experimentation would be necessary before conclusions could be drawn.

This study found no difference in [^{14}C]IAA metabolism or export between tall and dwarf pea seedlings that would explain the differential growth of the two varieties.

Section VI

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Among the metabolites of IAA which occur naturally in plant tissues, or which can be isolated following the application of exogenous IAA, a number of conjugates of IAA with amino acids and with sugars have been identified. Prominent among these is the compound indol-3yl-acetyl aspartic acid (IAAsp). The available evidence suggests that this compound is immobile in the polar auxin transport system and is physiologically inactive (Section I). It is formed when tissue concentrations of IAA are high and its synthesis has been regarded widely as a means of preventing the accumulation in tissues of supra-optimal levels of IAA (Section I).

Little attention seems to have been paid to the fate of auxin conjugates themselves following their formation from IAA. Both the peptide and ester bonds (formed between IAA and amino acids and sugars respectively) are likely to be susceptible to enzymic hydrolysis, raising the possibility that the formation of conjugates may not only act to prevent the accumulation of excessive concentrations of free IAA, but also may represent the storage of IAA in a protected and physiologically inactive form from which it can be released if free IAA levels in the tissues fall. The original objective of the present study was to investigate this possibility by examining the formation and metabolic fate of IAAsp following the application of IAA to intact pea apices.

In early experiments the compound assumed to be IAAsp from its chromatographic properties was isolated from pea tissue following the application of [1-^{14}C]IAA. In several of the experiments in

which the isolated, ^{14}C-labelled compound was re-applied to intact pea seedlings, a small proportion of the ^{14}C was re-isolated with free IAA, suggesting that the plants possessed the necessary enzyme systems with which to hydrolyse the peptide bond (Section II). However, before further work could be carried out it was necessary to improve the techniques for the isolation and purification of the presumed IAAsp from plant tissues. The work described in Section III was devoted to a detailed study of these isolation and purification procedures and estimates were made of losses which occurred at each stage during the methods investigated. From the results obtained the procedure giving the best yields of the pure compound was determined and this was used in subsequent experimental work.

A detailed search of the literature concerning the metabolism of IAA in plant tissues and the formation and subsequent metabolism of auxin conjugates (including IAAsp) revealed that previously published evidence for the chemical identity of the conjugates was frequently inadequate. Commonly identities were established only by comparison of the Rf values of the unknowns with the assumed authentic compounds following chromatography in a few (in some cases, only one) solvent systems (Section I). It therefore seemed essential to confirm the identity of the assumed IAAsp by acceptable chemical criteria and a large part of the work reported here was necessarily devoted to attempts to do this (Section IV). The techniques employed included paper and thin-layer chromatography in a wide range of solvent systems, mass spectroscopy, gas-chromatography/mass spectroscopy and attempts to form the dual-labelled compound by supplying ^{3}H-aspartic acid and ^{14}C-IAA to pea tissue. As a

necessary part of this work the chemical synthesis of IAAsp was undertaken, both to provide samples of the authentic compound for use as an internal standard and with a view to using the synthesis technique to produce authentic ^{14}C-labelled IAAsp for further metabolic studies. Although the compound was successfully synthesised in quantity, low recoveries and low specific activities of available ^{14}C-labelled IAA precluded use of the method to synthesise ^{14}C-labelled IAAsp.

In chromatographic studies there was a close correlation between the mobility in eight solvent systems of the assumed IAAsp isolated from pea plants and the authentic compound. However, attempts to detect the compound in plant extracts using MS and GC-MS techniques failed. Problems associated with the breakdown of IAAsp during derivitization procedures designed to increase its volatility for GC and failure of the purification procedures to provide samples of sufficient purity and concentration for mass spectroscopy may have contributed to the failure to detect IAAsp in the extracts. Until these problems have been resolved by further work on the techniques themselves, the identification of IAAsp as the principal IAA conjugate in pea tissue must remain tentative.

Although little is known concerning the influence of environmental factors on the formation and metabolism of auxin conjugates in plants, earlier work suggests that conjugation may play a role in the growth responses of internodes to changes in light intensity. It has also been suggested that differences in the rate and extent of stem elongation in light between dwarf and tall pea cultivars may be caused by differences in the extent to which free IAA levels are reduced in the stem tissues by conjugation to IAAsp. In the

final part of the work described here (Section V) these possibilities were examined. In contrast to earlier studies, the metabolism of [1-^{14}C]IAA to IAAsp was found to be similar in tall and dwarf peas and was unaffected by different light conditions. This result suggests that increased immobilization of IAA by conjugation to IAAsp cannot be the major cause of dwarfism. Furthermore, the apparent failure of light conditions to influence the extent of conjugation suggests that conjugation cannot account for the inhibition of stem elongation by light. However, it was found that when [1-^{14}C]IAA was applied to dwarf peas in light a large proportion of the applied compound was rapidly conjugated to the presumed IAAsp and remained in this form for up to 10 days with no evidence of re-mobilization as IAA. Since the amount of IAA exported from the labelled apices was very small compared with the proportion which was conjugated, it is possible that small differences in the rate and extent of conjugation of endogenous IAA between light- and dark-grown plants (and possibly between tall and dwarf cultivars of pea) may have been masked by abnormally high rates of conjugation induced by the application of exogenous IAA.

Although light conditions had no measurable effect on the extent to which IAA taken up by intact pea seedlings was conjugated to the presumed IAAsp, the rates of uptake of IAA from the applied solutions was generally greater in light-grown plants than in those grown in darkness. Initial rates of export of IAA from the apical buds also were greater in light-grown plants than in dark-grown plants regardless of the light conditions during transport itself. Previously published evidence has indicated that in the intact herbaceous dicotyledon, the vascular cambium is the major pathway

for polar IAA transport and that the transport is sensitive to changes in cambial activity. The different rates of export of IAA in light- and dark-grown plants may have reflected the known differences in cambial development in etiolated and light-grown plants.

BIBLIOGRAPHY

BIBLIOGRAPHY

Aasheim T. and T-H Iversen, 1971. Decarboxylation and transport of auxin in segments of sunflower and cabbage roots. II. A chromatographic study using IAA-1-^{14}C and IAA-5-^{3}H. Physiol. Plant. 24, 325-329.

Allen, J.R.F., A.M. Greenway, D.A. Baker, 1979. Determinations of indole-3-acetic acid in xylem sap of Ricinus communis L. using mass fragmentography. Planta 144, 299-303.

Anderson, J.A., 1968. Fractionation of indole compounds on Sephadex G-10. J. Chromat. 33, 536-538.

Andreae, W.A. and N.E. Good, 1955. The formation of indoleaspartic acid in pea seedlings. Plant Physiol. 30, 380-382.

Andreae, W.A. and N.E. Good, 1957. Studies in indoleacetic acid metabolism. IV. Conjugation with aspartic acid and ammonia as processes in the metabolism of carboxylic acids. Plant Physiol. 32, 566-572.

Andreae, W.A., G.R. Robinson and M.W.H. Van Ysselstein, 1961. Studies of 3-IAA metabolism. VII. Metabolism of radioactive 3-indoleacetic acid by pea roots. Plant Physiol. 36, 783-787.

Andreae, W.A. and M.W.H. Van Ysselstein, 1956. Studies on 3-indoleacetic acid metabolism. III. The uptake of 3-indoleacetic acid by pea epicotyls and its conversion to 3-indoleacetylaspartic acid. Plant Physiol. 31, 235-240.

Andreae, W.A. and M.W.H. Van Ysselstein, 1960. Studies of indoleacetic acid metabolism. VI. Indoleacetic acid uptake and metabolism by pea roots and epicotyls. Plant Physiol. 35, 225-232.

Bandurski, R.S. and A. Schulze, 1974. Concentrations of IAA and its esters in Avena and Zea. Plant Physiol. 54, 257-262.

Bandurski, R.S. and A. Schulze, 1977. A light induced change in indole-3-acetic acid concentrations. Plant Physiol. 59, Suppl. 6, Report 55.

Bandurski, R.S. and A. Schulze, 1977. Concentration of indole-3-acetic acid and its derivatives in plants. Plant Physiol. 60, 211-213.

Bandurski, R.S., A. Schulze and J.D. Cohen, 1977. Photo-regulation of the ratio of ester to free IAA. Biochem. and Biophys. Res. Comm. 79, 1219-1222.

Batt, S., M. Wilkins and M. Venis, 1976. Auxin binding to corn coleoptile membranes: kinetics and specificity. Planta 130, 7-15.

Beevers, L., 1976. Nitrogen metabolism in plants. Publ. Edward Arnold, 41-43.

Bender, L. and K-H Neumann, 1978. Investigations on the indole-3-acetic acid metabolism of carrot tissue cultures (Dancus carota L.). Z. Pflanzenphysiol. Bd. 88, 209-217.

Bennet-Clark, T.A., M.S. Tambiah and N.P. Kefford, 1952. Estimation of plant growth substances by partition chromatography. Nature 169, 452.

Bennet-Clark, T.A. and A.W. Wheeler, 1959. The metabolism of 3-indolylacetic acid in plant tissues. J. Expt. Bot. 10, 468-479.

Bentley, J.A., 1958. The naturally-occurring auxins and inhibitors. Ann. Rev. Plant Physiol. 9, 47-80.

Beyer, E.M. Jr. and P.W. Morgan, 1970. Effect of ethylene on the uptake, distribution and metabolism of IAA-1-^{14}C and 2^{14}C and naphthalenacetic acid-1-^{14}C. Plant Physiol. 46, 157-162.

Bogers, R.J., Z. Kulescha and K.R. Libbenga, 1978. The molecular action of auxins in tissue-culture systems: IAA metabolism and synthesis of nucleic acids and proteins in tobacco-pith explants. Federation of European Societies of Plant Physiol. Abstract 30-A, 94-95.

Boissonas, R.A., 1951. A new method of peptide synthesis. Helv. Chim. Acta 34, 874-879.

Bonnemain, J-L., 1971. Transport et distribution des traceurs apres application de AIA-2-^{14}C sur les feuilles de Vicia faba L. C.R. Acad. Sci. (Paris) (D) 273, 1699-1702.

Bourbouloux, A. and J-L Bonnemain, 1973. Transport de l'auxine-^{14}C en provenance de jeune grusses de Vicia faba L. Planta 115, 161-172.

Bourbouloux, A. and J-L Bonnemain, 1974. Transport, distribution et metabolisme de l'auxine dans la racine de Vicia faba L. apres application de ^{14}C AIA on de ^{3}H AIA sur le bourgeon. Planta 119, 169-182.

Cohen, J.D. and R.S. Bandurski, 1978. The bound auxins: protection of indole-3-acetic acid from peroxidase-catalysed oxidation. Planta 139, 203-208.

Dahlhelm, H., 1969. Studies on the mode of action of indole-3-acetic acid by means of heavy water. Planta 86, 224-234.

Davidonis, G., R.H. Hamilton and R.O. Mumma, 1978. Metabolism of 2, 4-dichlorophenoxyacetic acetic in soyabean root callus and differentiated soyabean root cultures as a function of concentration and tissue age. Plant Physiol. 62, 80-83.

Davies, P.J., 1972. The fate of exogenously applied IAA in light grown stems. Physiol. Plant. 27, 262-270.

Davies, P.J., 1973. The uptake and fractional distribution of differentially labelled indoleacetic acid in light grown stems. Physiol. Plant. 28, 95-100.

Davies, P.J., 1976. Bound auxin formation in growing stems. Plant Physiol. 57, 197-202.

Davies, P.J. and A.W. Galston, 1971. Labelled indole-macromolecular conjugates from growing stems supplied with labelled indoleacetic acid. I. Fractionation. Plant Physiol. 47, 435-441.

Davies, P.J. and P.H. Rubery, 1978. Components of auxin transport in stem segments of Pisum sativum L. Planta 142, 211-219.

DeYoe, D.R. and J.B. Zaerr, 1976. Indole-3-acetic acid in douglas fir. Analysis by gas-liquid chromatography and mass spectrometry. Plant Physiol. 58, 299-303.

Eliasson, L., 1969. Growth regulators in Populus tremula. I. Distribution of auxin and growth inhibitors. Physiol. Plant 22, 1288-1301.

Eliezer, J., 1978. Some factors influencing exogenous auxin transport in intact plants. Ph.D. thesis, University of Southampton.

Elliott, M.C., 1971. α-N-malonyl-D-tryptophan in seedling wheat roots. New Phytol. 70, 1005-1015.

Epstein, E., I. Klein and S. Lavee, 1975. Uptake and fate of IAA in apple callus tissues: metabolism of IAA-2-^{14}C. Plant and Cell Physiol. 16, 305-311.

Epstein, E., J. Kochba and H. Neuman, 1977. Metabolism of IAA by embryogenic and nonembryogenic callus lines of "Shamouti" orange (Citrus sinesis OSB). Z. Pflanzenphysiol. 85, 263-268.

Epstein, E. and S. Lavee, 1977. Uptake, translocation and metabolism of IAA in the olive (Olea europea). II. The fate of exogenously applied [2-^{14}C]IAA to detacted 'manzanilla' olive leaves. J. Expt. Bot. 28, 629-635.

Eschrich, W., 1968. Translocation of labelled indolyl-3-acetic acid in sieve tubes of Vicia faba. Planta 78, 144-157.

Feung, C.S., R.H. Hamilton and R.O. Mumma, 1973. Metabolism of 2, 4-dichlorophenoxyacetic acid. V. Identification of metabolites in soyabean callus tissue cultures. J. Agric. Food Chem. 21, 637-640.

Feung, C.S., R.H. Hamilton and R.O. Mumma, 1976. Metabolism of indole-3-acetic acid. III. Identification of metabolites isolated from crown gall. Plant Physiol. 58, 666-669.

Feung, C.S., R.H. Hamilton and R.O. Mumma, 1977. Metabolism of indole-3-acetic acid. IV. Biological properties of amino acid conjugates. Plant Physiol. 59, 91-93.

Feung, C.S., R.H. Hamilton and F.H. Witham, 1971. Metabolism of 2, 4-dichlorophenoxyacetic acid by soyabean cotyledon callus tissue cultures. J. Agric. Food Chem. 19, 475-479.

Feung, C.S., R.H. Hamilton, F.H. Witham and R.O. Mumma, 1972. The relative amounts and identification of some 2, 4-dichlorophenoxyacetic acid metabolites isolated from soyabean callus cultures. Plant Physiol. 50, 80-86.

Feung, C.S., R.O. Mumma and R.H. Hamilton, 1974. Metabolism of 2, 4-dichlorophenoxyacetic acid. VI. Biological properties of amino acid conjugates. J. Agric. Food Chem. 22, 307-309.

Field, R.J., 1973. The effect of gibberelic acid, kinetin and indolyeacetic acid on the lateral movement of labelled plant growth regulators in willow stems. New Phytol. 72, 471-478.

Field, R.J. and A.J. Peel, 1971. 1. The metabolism and radial movement of growth regulators and herbicides in willow stems. New Phytol. 70, 743-749.

Field, R.J. and A.J. Peel, 1971. 2. The movement of growth regulators and herbicides into the sieve elements of willow. New Phytol. 70, 997-1003.

Field, R.J. and A.J. Peel, 1972. The longitudinal mobility of growth regulators and herbicides in sieve tubes in willow. New Phytol. 71, 249-254.

Freychet, P., 1976. Interactions of polypeptide hormones with cell membrane specific receptors: studies with insulin and glycogen. Diabetologia 12, 83-100.

Fletcher, R.A. and S. Zalik, 1965. Effects of light of several spectral bands on the metabolism of radioactive IAA in bean seedlings. Plant Physiol. 40, 549-552.

Goldsmith, M.H.M., 1977. The polar transport of auxin. Ann. Review Plant Physiol. 28, 439-478.

Good, N.E., 1956. The synthesis of indole-3-acetyl-D,L-aspartic acid and related compounds. Canadian Jour. Chem. 34, 1356-1358.

Good, N.E. and Andreae, W.A., 1957. Malonyl-tryptophan in higher plants. Plant Physiol. 32, 561-566.

Good, N.E., W.A. Andreae and M.W.H. Van Ysselstein, 1956. Studies on 3-indoleaceticacid metabolism. II. Some products of the metabolism of exogenous IAA in plant tissues. Plant Physiol. 31, 231-235.

Goren, R. and M.J. Bukovac, 1973. Mechanisms of naphthaleneacetic acid conjugation: no effect of ethylene. Plant Physiol. 51, 907-913.

Goren, R., M.J. Bukovac and J.A. Flore, 1974. Mechanism of indole-3-acetic acid conjugation. No induction by ethylene. Plant Physiol. 53, 164-166.

Greenwood, M.S., S. Shaw, J.R. Hillman, A. Richtie and M.B. Wilkins, 1972. Identification of auxin from Zea coleoptile tips by mass spectrometry. Planta 108, 179-183.

Greenwood, M.S., J.R. Hillman, S. Shaw and M.B. Wilkins, 1973. Localization and identification of auxin in roots of Zea mays. Planta 109, 369-374.

Hamilton, R.H., R.S. Bandurski and B.H. Grigsby, 1961. Isolation of indole-3-acetic acid from corn kernels and etiolated corn seedlings. Plant Physiol. 36, 354-359.

Hattori, H. and S. Marumo, 1972. Monomethyl-4-chloroindolyl-3-acetyl-L-aspartate and absence of IAA in immature seeds of Pisum sativum. Planta 102, 85-90.

Hemberg, T. and E. Tillberg, 1978. The yield of auxin with different extraction solvents and methods. Federation of European Societies of Plant Physiol., Abstract 118-A, 255-256.

Hertel, R. and R. Flory, 1968. Auxin movement in corn coleoptiles. Planta 82, 123-144.

Higgins, S.L. and N.M. Barnett, 1976. Cell-free synthesis of indoleacetyl aspartic acid. Plant Physiol. 57, Suppl., Abstract 95, 18.

Hillman, J.R. 1978. Isolation of plant growth substances. Publ. Cambridge University Press.

Hoad, G.V., S.K. Hillman and P.F. Wareing, 1971. Studies on the movement of indole auxins in willow (Salix viminalis L.). Planta 99, 73-88.

Hofinger, M. and E.K. Lis, 1977. Control of indolylacetylaspartate by indolylacetic and indolylacrylic acids in lentil seedlings. Plant Biochem. J. 4, 72-76.

Hutzinger, O. and T. Kosuge, 1968. Microbial synthesis and degradation of indole-3-acetic acid. III. The isolation and characterization of indole-3-acetyl-E-L-lysine. Biochem. 7, 601-605.

Imaseki, H., K. Kondo and A. Watanabe, 1975. Mechanism of cytokinin action on auxin induced ethylene production. Plant Cell Physiol. 16, 777-787.

Imaseki, H. and S. Sakai, 1973. An endogenous inhibitor of ethylene production from mung bean hypocotyls. 8th Int. Conference Plant Growth Subs., 374-383.

Jacobs, W.P. and I.B. Morrow, 1957. A quantitative study of xylem development in the vegetative shoot apex of Coleus. Am. J. Bot. 44, 823-842.

Jamieson, W.D. and O. Hutzinger, 1970. Identification of simple naturally occurring indoles by mass spectrometry. Phytochem. 9, 2029-2036.

Jasdanwala, R.T., Y.D. Singh and J.J. Chinoy, 1977. Auxin metabolism in developing cotton hairs. J. Expt. Bot. 28, 1111-1116.

Johnson, M.P. and J. Bonner, 1956. Uptake of auxin by plant tissue. Physiol. Plant. 9, 102-118.

Kang, B.G., W. Newcomb and S.P. Burg, 1971. Mechanism of auxin-induced ethylene production. Plant Physiol. 47, 504-509.

Kasamo, K. and T. Yamaki, 1976. In vitro binding of IAA to plasma membrane-rich fractions containing Mg^{2+}-activated ATPase from mung bean hypocotyls. Plant Cell Physiol. 17, 149-164.

Kefford, N.P, 1955. The growth substances separated from plant extracts by chromatography. I. J. Expt. Bot. 6, 129-151.

Kendall, F.H., C.K. Park and C.L. Mer, 1971. Indole-3-acetic acid metabolism in pea seedlings. Ann. Bot. 35, 565-579.

Kenny, G., J. Südi and G.E. Blackman, 1969. The uptake of growth substances. XIII. Differential uptake of IAA through the epidermal and cut surfaces of etiolated stem segments. J. Expt. Bot. 20, 820-840.

Kirk, S.C. and W.P. Jacobs, 1968. Polar movement of indole-3-acetic acid-^{14}C in roots of Lens and Phaseolus. Plant Physiol. 43, 675-682.

Klämbt, H.D., 1961. Wachstumsinduktion und wuchssfottmetabolismus in weizenkoleoptilzylinder. Reaktionsprodukte der ies in abhängigkeit von den inkubationskonzentrationen. Planta 56, 309-321.

Koevenig, J.L. and W.P. Jacobs, 1972. Effect of light on basipetal movement of indoleacetic acid in green stem sections of Coleus. Plant Physiol. 49, 866-867.

Kögl, F., A.J. Haagen-Smit, H. Erxleben, 1934. Über ein neues Auxin ("heteroauxin") aus Harn. XI. Z. physiol. Chem. 228, 90-103.

Kopcewicz, J., A. Ehmann and R.S. Bandurski, 1974. Enzymatic esterification of indole-3-acetic acid to myo-inositol and glucose. Plant Physiol. 54, 846-851.

Krul, W.R. and G.C. Colclasure, 1977. Effect of galactose and other monosaccharides on IAA movement in bean hypocotyl segments. Physiol. Plant. 41, 249-253.

Kulescha, Z. 1971. L'utilisation des substances de croissance par les tissus de Topinambour (Helianthus tuberosus) cultivés in vitro. 96e Congrès national des societes savantes, Toulouse, Sciences, t. IV, 291-297.

Labarca, C., P.B. Nicholls and R.S. Bandurski, 1965. A partial characterization of indoleacetylinositols from Zea mays. Biochem. Biophys. Res. Commun. 20, 641-644.

Lantican, B.P. and R.M. Muir, 1969. Auxin physiology and dwarfism in Pisum sativum. Physiol. Plant. 22, 412-423.

Lau, O., D.P. Murr and S.F. Yang, 1974. Effect of 2, 4-dinitrophenol on auxin-induced ethylene production and auxin conjugation by mung bean tissue. Plant Physiol. 54, 182-185.

Lau, O-L and S.F. Yang, 1973. Mechanism of a synergistic effect of kinetin on auxin-induced ethylene production. Suppression of auxin conjugation. Plant Physiol. 51, 1011-1014.

Lau, O-L, S.F. Yang and K.H. Yung, 1973. Suppression of IAA conjugation by kinetin in relation to a synergistic effect of kinetin on IAA-induced ethylene production. Plant Growth Subs., 364-373.

Le Cam, A., P. Freychet and P. Lenoir, 1975. Degradation of insulin by isolated liver cells. Diabetes 24, 566-573.

Lee, T.T., 1971. Promotion of indoleacetic acid oxidase isoenzymes in tobacco callus cultures by indoleacetic acid. Plant Physiol. 48, 56-59.

Leep, N.W. and A.J. Peel, 1971. Patterns of translocation and metabolism of ^{14}C-labelled IAA in the phloem of willow. Planta 96, 62-73.

Lesham, Y., 1973. The molecular and hormonal basis of plant-growth regulation. Publ. Pergamon Press Ltd.

Libbert, E., E. Fischer, A. Drawert and R. Schröder, 1970. Pathways of IAA production from tryptophan by plants and their epiphytic bacteria: a comparison. II. Establishment of the tryptophan metabolites, effects of a native inhibitor. Physiol. Plant. 23, 278-286.

Libbert, E. and H. Rische, 1969. Interactions between plants and epiphytic bacteria regarding their auxin metabolism. V. Isolation and identification of IAA producing and destroying bacteria from pea plants. Physiol. Plant. 22, 51-57.

Lieberman, M. and E. Knegt, 1977. Influence of ethylene on indole-3-acetic acid concentrations in etiolated pea epicotyl tissue. Plant Physiol. 60, 475-477.

Little, C.H.A., J.K. Heald and G. Browning, 1978. Identification and measurement of indoleacetic acid and abscisic acid in the cambial region of Picea sitchensis (Borg.) Carr by combined gas chromatography - mass spectrometry. Planta 139, 133-138.

Liu, S-T, D. Gruenert and C.A. Knight, 1978. Bound form indole-3-acetic acid synthesis in tumerous and non-tumerous species of Nicotiana. Plant Physiol. 61, 50-53.

Luckwill, L.C., 1959. Factors controlling the growth and form of fruits. J. Linn. Soc. Lond. Bot. 56, 294-302.

Maan, A.C., 1978. Isolation, purification and identification of an indole-acetic acid conjugate. Publ. Botanisch laboratorium der Rijksuniversiteit et Leiden.

Mace, M.E., 1965. Isolation and identification of 3-indoleacetic acid from Fusarium oxysporum f. cubense. Phytopath. 55, 240-241.

Magnus, V., S. Iskric and S. Kveder, 1971. Indole-3-methanol - a metabolite of indole-3-acetic acid in pea seedlings. Planta 97, 116-125.

Maillard, F. and J.-P. Zryd, 1977. Métabolisme de l'acide indolyl-3-acétique dans les cultures de cellules d'Acer pseudoplatanus cultivés in vitro. Can. J. Bot. 55, 2530-2534.

Mann, J.D. and E.G. Jaworski, 1970. Minimizing the loss of indoleacetic acid during purification of plant extracts. Planta 92, 285-291.

Marmé, D., 1977. Phytochrome: membranes as possible sites of primary action. Ann. Rev. Plant Physiol. 28, 173-178.

McCune, D.C. and A.W. Galston, 1959. Inverse effects of gibberellin on peroxidase activity and growth in dwarf strains of pea and corn. Plant Physiol. 34, 416-418.

McDougall, J. and J.R. Hillman, 1978. Analysis of indole-3-acetic acid using GC-MS techniques. In Isolation of plant growth substances. Ed. J.R. Hillman. Publ. Cambridge University Press, 1-25.

McDougall, J. and J.R. Hillman, 1978. Purification of IAA from shoot tissues of Phaseolus vulgaris and its analysis by GC-MS. J. Expt. Bot. 29, 375-386.

Miflin, B.L. and P.J. Lea, 1977. Amino acid metabolism. Ann. Rev. Plant Physiol. 28, 299-329.

Minato, T. and Y. Okazawa, 1978. Effect of ethylene treatment on auxin metabolism of potato tubers. J. Fac. Agric. Hokkaido Univ. 58, 535-547.

Minchin, A. and M.A. Harmey, 1975. The metabolism of indoleacetic acid by barley grains. Planta 122, 245-254.

Mino, Y., 1968. Studies on the destruction of indole-3-acetic acid by a species of Arthrobacter I. On the induction of the enzyme responsible for the destruction. Plant Cell Physiol. 9, 169-183.

Mollan, R.C., D.X. Donnelly and M.A. Harmey, 1972. Synthesis of indole-3-acetylaspartic acid. Phytochem. 11, 1485-1488.

Moore, T.C. and C.A. Shaner, 1967. Biosynthesis of indoleacetic acid from tryptophan-^{14}C in cell-free extracts of pea shoot tips. Plant Physiol. 42, 1787-1796.

Morris, D.A., 1970. Light and the transport and metabolism of IAA in normal and albino dwarf pea seedlings. Planta, 91, 1-7.

Morris, D.A., R.E. Briant and P.G. Thomson, 1969. The transport and metabolism of ^{14}C-labelled IAA in intact pea seedlings. Planta 89, 178-197.

Morris, D.A., A.G. Thomas, 1978. A microautoradiographic study of auxin transport in the stems of intact pea seedlings (Pisum sativum L.). J. Expt. Bot. 29, 147-157.

Muir, R.M., 1970. The control of growth by the synthesis of IAA and its conjugation. In Plant Growth Substances 1970. Ed. D.J. Carr. Publ. Springer-Verlag.

Naqvi, S.M., 1974. The effect of sugar supplement on the kinetics of indoleacetic-2-^{14}C transport in Zea mays coleoptile segments. Z. Pflanzenphysiol. 71, 1-5.

Naqvi, S.M. and S.A. Gordon, 1967. Auxin transport in Zea mays coleoptiles. II. Influence of light on the transport of indoleacetic acid-2-^{14}C. Plant Physiol. 42, 138-143.

Ockerse, R. and L.M. Mumford, 1973. The regulation of peroxidase activity by gibberellin and auxin in pea stem segments. Can. J. Bot. 51, 2237-2242.

Ohwaki, Y. and S. Tsurmi, 1976. Auxin transport and growth in intact roots of Vicia faba. Plant Cell Physiol. 17, 1329-1342.

Olney, H.O., 1968. Growth substances from Veratrum tenuipetalum. Plant Physiol. 43, 293-302.

Patrick, J.W. and D.J. Woolley, 1973. Auxin physiology of decapitated stems of Phaseolus vulgaris L. treated with indol-3yl-acetic acid. J. Expt. Bot. 24, 949-957.

Piskornik, Z. and R.S. Bandurski, 1972. Purification and partial characterization of a glucan containing indole-3-acetic acid. Plant Physiol. 50, 176-182.

Ray, P.M., 1977. Auxin-binding sites of maize coleoptiles are localized on membranes of the endoplasmic reticulum. Plant Physiol. 59, 594-599.

Ray, P.M., U. Dohrmann and R. Hertel, 1977. 1. Characterization of naphthaleneacetic acid binding to receptor sites on cellular membranes of maize coleoptile tissue. Plant Physiol. 59, 357-364.

Ray, P.M., U. Dohrmann and R. Hertel, 1977. 2. Specificity of auxin-binding sites on maize coleoptile membranes as possible receptor sites for auxin action. Plant Physiol. 60, 585-591.

Rekoslavskaya, N.I. and K.Z. Gamburg, 1976. On the biological activity of IAA conjugates. Biochem. Physiol. Pflanzen. 169, 299-303.

Rekoslavskaya, N.I. and K.Z. Gamburg, 1977. Auxin induction of indolylacetyl aspartate synthesis in the auxin-independant dewberry. Fiziol. Rast. (Mosc.) 24, 1206-1210.

Riov, J., R. Cooper and H.E. Gottlieb, 1979. Metabolism of auxin in pine tissues: naphthaleneacetic acid conjugation. Physiol. Plant. 46, 133-138.

Robinson, B.J., M. Forman and F.T. Addicott, 1968. Auxin transport and conjugation in cotton explants. Plant Physiol. 43, 1321-1323.

Row, V.V., W.W. Sanford and A.E. Hitchcock, 1961. Indole-3-acetyl-D, L-aspartic acid as a naturally occurring indole compound in tomato seedlings. Contribs. Boyce Thompson Inst. 21, 1-10.

Sabater, F., J. Sanchez, C. Nunez and J. Cuello, 1978. Distribution of IAA-oxidase and peroxidase activity in hypocotyls of lupine. Federation European Societies of Plant Physiol. Abstract 231-A, 458.

Sakai, S. and H. Imaseki, 1973. Properties of the proteinaceous inhibitor of ethylene synthesis: action on ethylene production and indoleacetylaspartate formation. Plant Cell Physiol. 14, 881-892.

Schneider, E.A., R.A. Gibson and F. Wightman, 1972. Biosynthesis and metabolism of indol-3yl-acetic acid. 1. The native indoles of barley and tomato shoots. J. Expt. Bot. 23, 152-170.

Scott, T.K. and W.R. Briggs, 1960. Auxin relationships in the Alaska pea (P. sativum). Am. J. Bot. 47, 492-499.

Scott, T.K. and W.R. Briggs, 1963. Recovery of native and applied auxin from the dark-grown 'Alaska' pea seedling. Am. J. Bot. 50, 652-657.

Scott, T.K. and W.P. Jacobs, 1964. Critical assessment of techniques for identifying the physiologically significant auxins in Coleus blumei plants. Colloq. Int. Centre Nat. Rech. Sci. 123, 457-474.

Selwood, D., 1978. Undergraduate project, Dept. of Chem., University of Southampton.

Shen-Miller, J., P. Cooper and S.A. Gordon, 1969. Phototropism and photoinhibition of basipetal transport of auxin in oat coleoptiles. Plant Physiol. 44, 491-496.

Sherwin, J.E. and M. Furuya, 1973. A red-far red reversible effect on uptake of exogenous IAA in etiolated rice coleoptiles. Plant Physiol. 51, 295-298.

Stahl, E. and H. Kaldewey, 1961. Trace analysis of physiologically active, simple indole derivatives. Z. Physiol. Chem. 323, 189-191.

Stahl, E. and H. Kaldewey, 1964. The quantitative evaluation of thin-layer chromatographically separated auxins in the Avena daylight test according to Söding (Avena-silica-gel curvature test). Planta Arch. Wiss Bot. 62, 22-38.

Steen, I. and L. Elliasson, 1969. Separation of growth regulators from Picea abies Karst. on sephadex LH-20. J. Chromat. 43, 558-560.

Stowe, B.B. and K.V. Thimann, 1953. Indolepyruvic acid in maize. Nature 172, 764.

SÜdi, J., 1964. Induction of the formation of complexes between aspartic acid and indolyl-3-acetic acid or 1-napthaleneacetic acid by other carboxylic acids. Nature 201, 1009-1010.

SÜdi, J., 1966. Increases in the capacity of pea tissue to form acyl-aspartic acids specifically induced by auxins. New Phytol. 65, 9-21.

Sweetser, P.B. and D.G. Swartzfager, 1978. Indole-3-acetic acid levels of plant tissue as determined by a new high performance liquid chromatographic method. Plant Physiol. 61, 254-258.

Tambiah, M.S., 1951. The isolation and estimation of plant hormones using paper chromatography. Thesis, King's College, University of London.

Thimann, K.V., 1956. Studies on the growth and inhibition of isolated plant parts. V. The effects of cobalt and other metals. Am. J. Bot. 43, 241-249.

Thimann, K.V., 1969. The auxins. In Physiology of plant growth and development. Ed. M.B. Wilkins, Publ. McGraw-Hill, 3-45.

Thimann, K.V. and F. Skoog, 1940. The extraction of auxin from plant tissues. Am. J. Bot. 27, 951-960.

Thimann, K.V. and I.F. Wardlaw, 1963. The effect of light on the uptake and transport of indoleacetic acid in the green stem of pea. Physiol. Plant. 16, 368-377.

Thomson, B.F. and P.M. Miller, 1963. The role of light in histogenesis and differentiation in the shoot of Pisum sativum. III. The internode. Am. J. Bot. 50, 219-227.

Thornton, R.M. and K.V. Thimann, 1967. Transient effects of light on auxin transport in the Avena coleoptile. Plant Physiol. 42, 247-257.

Thurman, D.A. and H.E. Street, 1962. Metabolism of some indoleauxins in excised tomato roots. J. Expt. Bot. 13, 369-377.

Tillberg, E., 1974. Occurrence of endogenous indol-3yl-aspartic acid in light and dark grown bean seedlings. Physiol. Plant. 31, 271-274.

Udea, M. and R.S. Bandurski, 1974. Structure of indole-3-acetic acid myoinositol esters and paramethyl-myo-inositols. Phytochem. 13, 243-253.

Valdovinos, J.G., L.C. Ernest, T.E. Jensen, 1970. Studies on the action of ethylene in physiological processes of plant cells. In Plant Growth Substances, 493-501.

Veen, H., 1967. On the relation between auxin transport and auxin metabolism in explants of Coleus. Planta 73, 281-295.

Veen, H., 1972. Relationship between transport and metabolism of α-naphthaleneacetic acid, β-naphthaleneacetic acid and α-decalylacetic acid in segments of Coleus. Planta 103, 35-44.

Veen, H. and W.P. Jacobs, 1969. Transport and metabolism of indole-3-acetic acid in Coleus petiole segments of increasing age. Plant Physiol. 44, 1157-1162.

Venis, M.A., 1964. Induction of enzymatic activity by indolyl-3-acetic acid and its dependence on synthesis of ribonucleic acid. Nature 202, 900-901.

Venis, M.A., 1967. Effects of antibiotics on protein and ribonucleic acid synthesis in relation to control of bacterial growth in excised tissues. Phytochem. 6, 799-806.

Venis, M.A., 1972. Auxin-induced conjugation systems in peas. Plant Physiol. 49, 24-27.

Weaver, G.M. and H.O. Jackson, 1963. Genetic differences in leaf abscission and the activity of naturally occurring growth regulators in peach. Can. J. Bot. 41, 1405.

Wildman, S.G. and R.M. Muir, 1949. Observations on the mechanism of auxin formation in plant tissue. Plant Physiol. 24, 84-90.

Winter, A. and K.V. Thimann, 1966. Bound indoleacetic acid in Avena coleoptiles. Plant Physiol. 41, 335-342.

Zenk, M.H., 1960. Enzymatische aktivierung von Auxinen und ihre konjugierung mit Glycin. Z. Naturforschg. 15, 436-441.

Zenk, M.H., 1961. 1-(Indole-3-acetyl)-β-D,glucose, a new compound in the metabolism of indole-3-acetic acid in plants. Nature 191, 493-494.

Zenk, M.H., 1962. Aufnahme und Stoffwechsel von α-Naphthyl-essigsäure durch Erbsenepicotyle. Planta 58, 75-94.

Zenk, M.H., 1964. Isolation, biosynthesis and function of indoleacetic acid conjugates. 5th Int. Conf. Plant Growth Subs., Paris, CNRS, 241-249.

Zenk, M.H., 1966. In Biosynthesis of aromatic compounds. Ed. E. Billek, Pergamon Press, London, New York, 45.

Zenk, M.H. and G. Müller, 1964. Über den Einfluss der Wundflachen auf die enzymatioche oxydation der Indol-3-essigsäure in vivo. Planta. 61, 346-351.

Zweig, G. and J. Sherma, 1972. In CRC. Handbook of chromatography, Vol. II, 147.

APPENDIX

THE CHEMICAL SYNTHESIS OF IAAsp

THE CHEMICAL SYNTHESIS OF IAAsp

The method of Mollan et al. (1971) was followed with slight modification.

METHOD

1. The synthesis of the p-nitrophenol ester of IAA from IAA and p-nitrophenol

The IAA (5.8 g) was added to 65 ml of ethyl-acetate. The mixture was stirred. The p-nitrophenol (4.8 g) was also dissolved in 65 ml ethyl-acetate and added after stirring to the IAA mixture which was then cooled to 0°-5°C Dicyclohexylcarbodiimide (6.9 g) and 25 ml of ethyl-acetate were added gently to the stirring IAA and p-nitrophenol. The reaction mixture was stirred at 0°-5°C for 1 h and at 20° ± 3°C for a further hour. The dicyclohexylurea formed was filtered off and the solution concentrated to 25 ml by rotary evaporation (35°C), cooled and refiltered. A viscous solid remained after complete removal of the remaining solvent. IAA p-nitrophenol (Fig. 55) was crystallized and recrystallized by dissolving the solid in ethyl-acetate and light petroleum ether (b.p. 60°-80°C)(2:1 v/v) followed by heating, cooling and filtering. The yellow solid had a melting point of 105-106°C. The IAA p-nitrophenol (5.13 g) was thoroughly dried and aliquots taken for NMR and IR spectra.

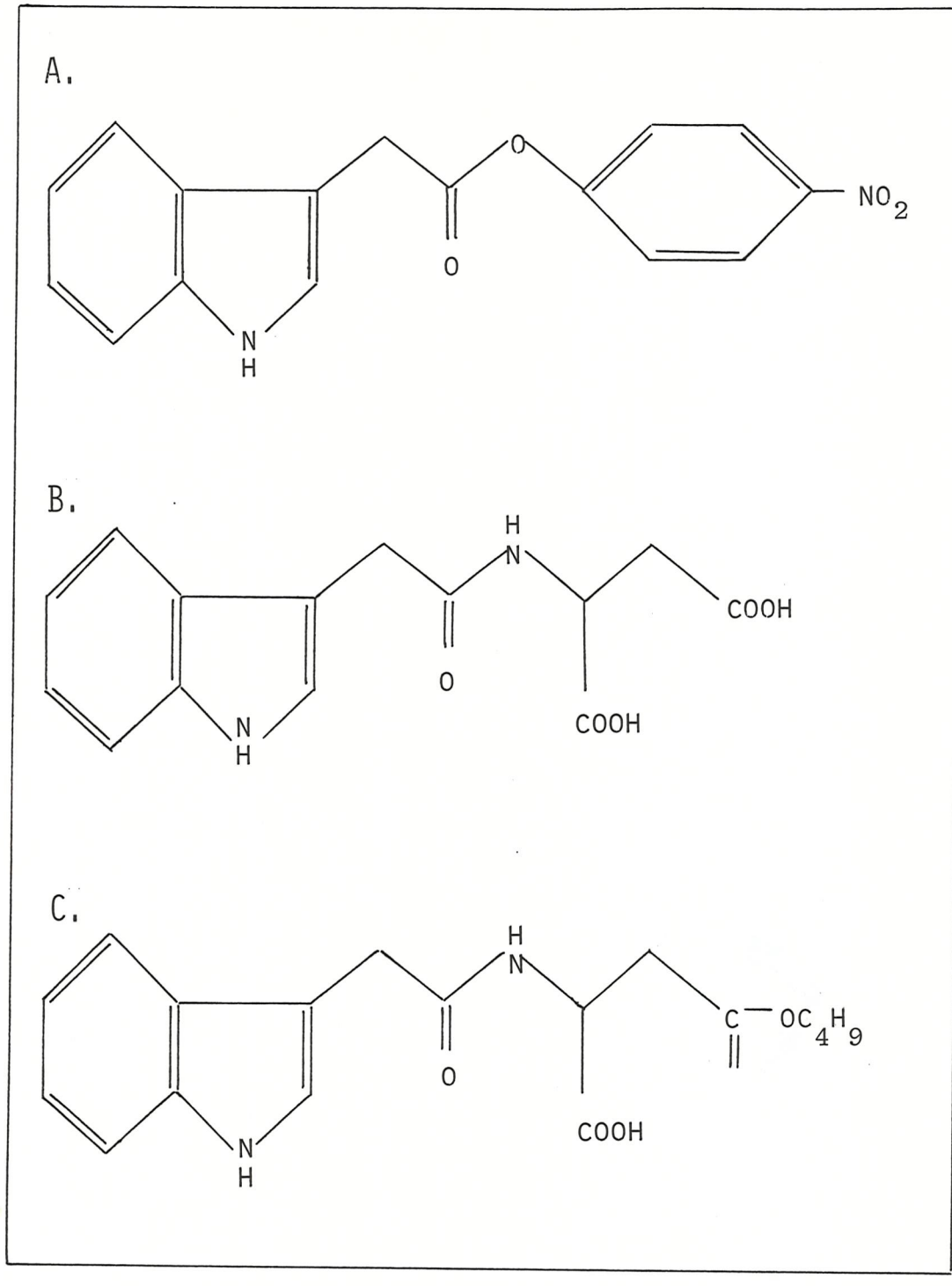


Figure 55 The molecular formulae a A = IAA p-nitrophenol ester, B = IAAsp and C = IAAsp-monobutyl ester, an intermediate, the product and a by-product of the synthesis of IAAsp.

2. The synthesis of indole-3-acetyl-L-aspartic acid from IAA p-nitrophenol ester and L-aspartic acid

L-aspartic acid (2.5 g) and tetramethylguanidine (3.91 g) were dissolved in aqueous methanol (75 ml 50% aq.) and IAA p-nitrophenol ester (5.5 g) added. The suspension was stirred for 48 h at room temperature. 400 ml H_2O was added and the mixture extracted with diethyl ether (2 x 400 ml). The aqueous phase was acidified to pH 5.0 with dilute HCl and extracted with diethyl ether (2 x 400, 1 x 250 ml). The aqueous phase was further acidified to pH 1.0 and extracted with butan-1-ol (1 x 320, 1 x 80 ml). The butanol phase was washed with water (2 x 120, 2 x 80 ml) and concentrated to dryness by rotary evaporation giving a pink glass of IAAsp (2.54 g, 47%)(Fig. 55). The addition of NH_4OH (25%, 0.88 NH_4OH, 35% NH_3) to the final butanol fraction containing the product was found to prevent the formation of IAAsp-esters. These were detected when the butanol fraction was reduced alone. TLC (isopropanol: NH_3:H_2O. 10:1:1 v/v) had shown a compound at Rf 0.52 which mass spectra and NMR data indicated to be the monoester (Fig. 55).

RESULTS

The recorded yield (moles. IAA converted to IAAsp) was 28% for the first synthesis. Subsequent synthesis increased the efficiency of the second stage from 47% to 60%.

The following spectral analysis confirmed the synthesis of IAAsp.

Spectra analysis

1. NMR

The NMR (solvent D_2O) is shown in Fig. 56. The following characteristics were recorded:-

7.6	multiplet	= α H indole	This multiplet (7.6 to 7.0) is the 5 aromatic H.
7.5 to 7.0	multiplet	= benzene H indole	
7.31	major peak		

3.76	singlet	= 2 methylene H
2.6	multiplet	= 2 methylene aspartate protons
2.65	major peak,	minor peaks 2.57, 2.52.

2. UV

IAAsp exhibits a UV spectra typical of all indoles:-

Solvent methanol, 1.46 x 10^{-14} m/e, peaks at wavelengths 289, 280, 275 and 230 (millimicrons).

3. IR

The solvent was nujol and NaCl cells were used, major peaks at 1480, 1380 cm^{-1} as can be seen in Fig. 57.

4. Mass spectra

At 15eV the major peaks were:-

272 (30%), 176 (10%), 175 (96%), 174 (9%),
132 (9%), 131 (100%), 130 (94%), 54 (9%).

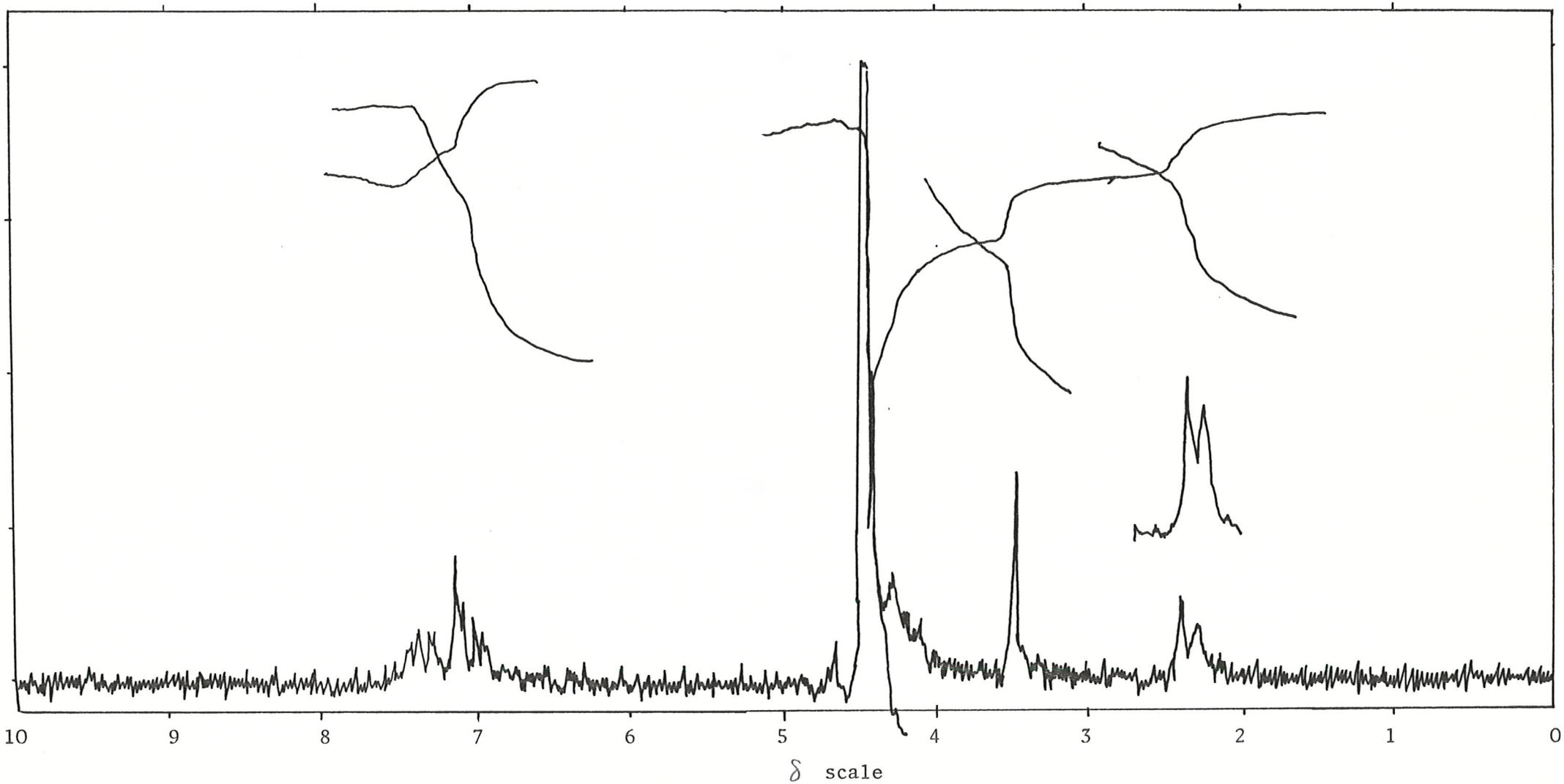


Figure 56 Nuclear magnetic resonance of indole-acetyl aspartic acid. The solvent was D_2O.

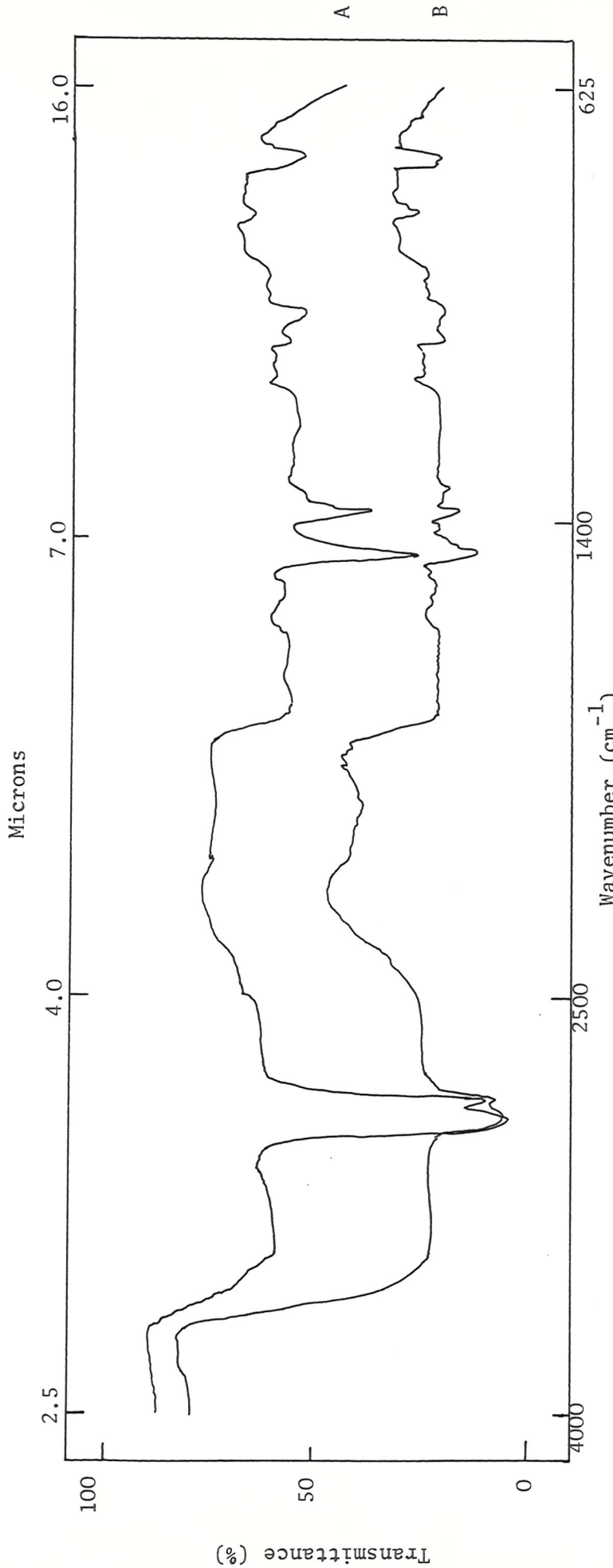


Figure 57 Infra-red spectra of indoleacetyl aspartic acid. The IAAsp was mixed in a paste with nujol and spread on NaCl cells. Scan speed A = slow, B = fast.

at 70eV (Fig. 58):-

272 (7%), 175 (8%), 131 (19%), 130 (100%),
103 (6%), 77 (10%), 44 (14%), 26 (12%).

The fragmentation pattern is illustrated by Fig. 59.

MECHANISM OF THE SYNTHESIS REACTION

The synthesis reaction is summarized in Fig. 60, and detailed in Fig. 61 and 62.

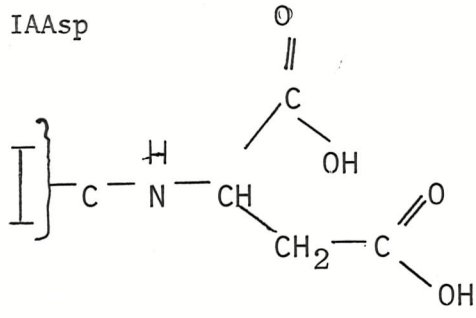
MASS	INT %B
272.64	Ø.76
271.62	6.51
27Ø.63	3.Ø2
175.9Ø	Ø.59
174.89	8.31
173.92	Ø.33
156.93	1.26
155.96	Ø.29
154.96	Ø.19
146.89	8.34
131.98	1.59
13Ø.96	19.12
129.92	-
128.95	5.22
127.98	2.Ø6
122.99	Ø.13
117.Ø2	Ø.56
1Ø5.Ø4	Ø.Ø9
1Ø4.Ø5	Ø.63
1Ø3.Ø2	6.35
1Ø1.99	4.28
1ØØ.96	1.13
98.95	Ø.Ø9
97.97	2.19
97.ØØ	1.62
88.95	Ø.26
78.Ø8	Ø.99
77.12	1Ø.2Ø
76.15	1.86
75.17	1.76
74.18	1.Ø6
73.19	Ø.Ø9
7Ø.1Ø	Ø.Ø9
69.15	Ø.43
65.8Ø	Ø.59
65.31	2.66
64.81	Ø.53
64.3Ø	Ø.53
63.28	1.52
62.24	Ø.39
57.29	Ø.Ø6
55.26	Ø.53
54.22	9.61
53.17	1.19
52.12	1.66
51.Ø3	3.89
49.93	1.72
45.26	Ø.53
44.24	14.76
43.27	1.Ø9
42.21	Ø.59
41.14	Ø.79
4Ø.Ø4	Ø.13
39.14	1.96
38.22	Ø.29
37.28	Ø.Ø9
36.28	Ø.13
32.13	Ø.Ø6
28.96	Ø.39
28.ØØ	6.58
27.Ø2	2.22
25.98	12.37
24.9Ø	1.26

Figure 58

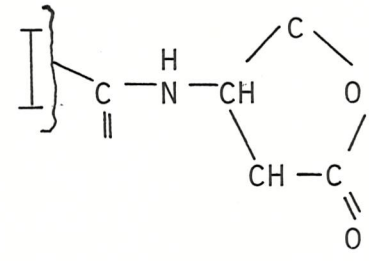
Mass spectrogram of indole acetyl aspartic acid at 70 e/v. Direct probe analysis.

Figure 59 The mass spectra fragmentation pattern of IAAsp at 70 e/v.

IAAsp

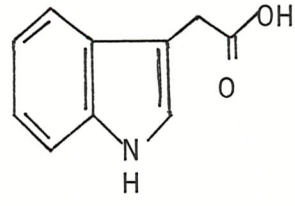


mass 272

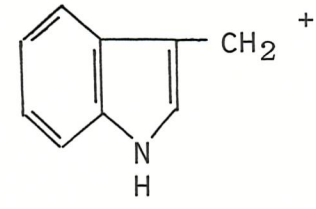


major fragments:-

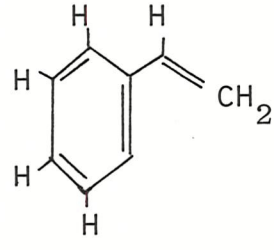
175



130



103



54 possibly C_3H_5O

44 CO_2

26 CO or HC = NH

No molecular ion formation is observed at mass 290 because the IAAsp cyclyses in the mass spectrometer losing one water molecule to give an ionic species at mass 272.

Figure 60 MECHANISMS OF THE SYNTHESIS REACTION.

A summary of the reactions.

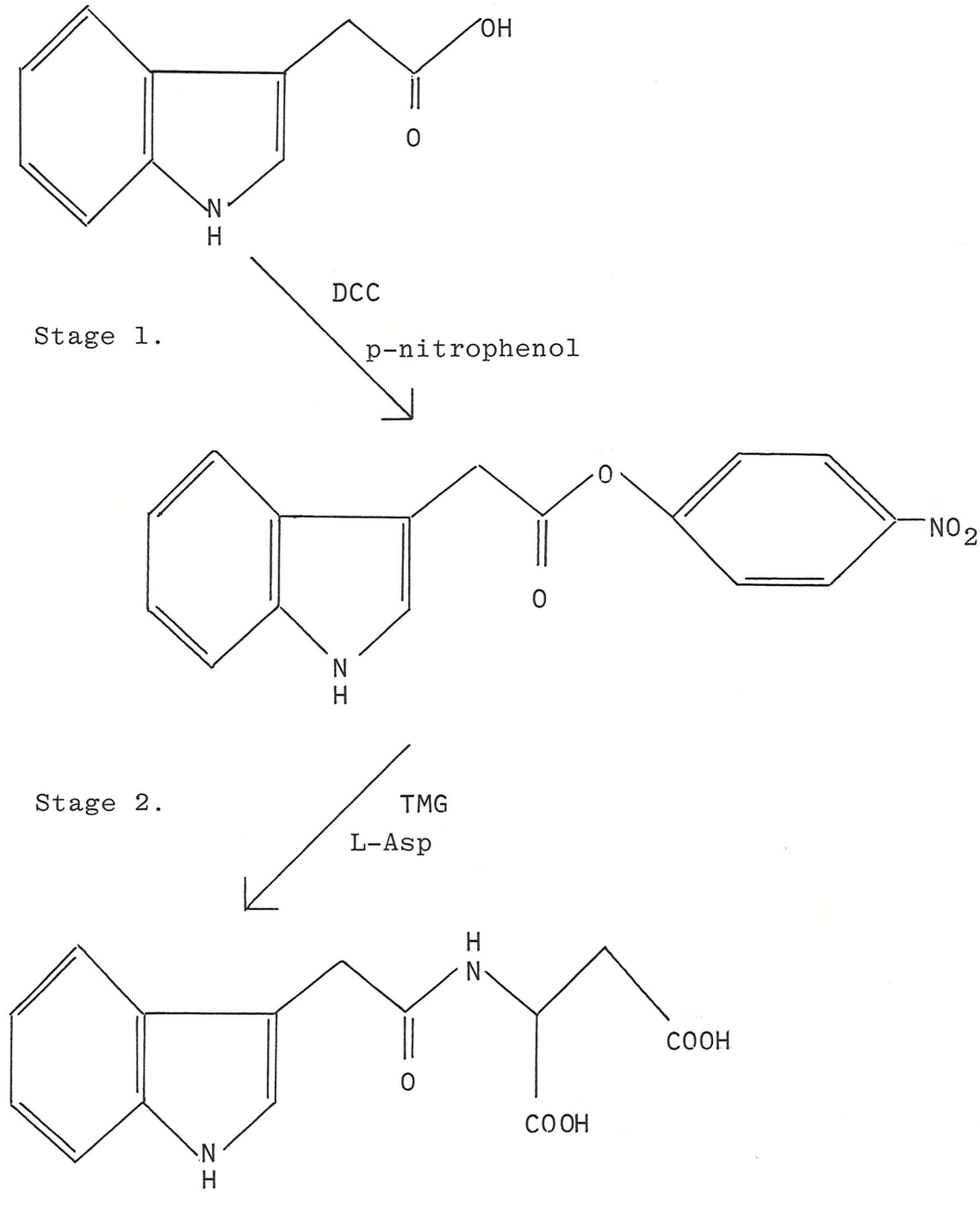


Figure 61 MECHANISMS OF THE SYNTHESIS REACTION.

Stage 1. The use of dicyclohexylcarbodiimide (DCC) as an activating agent with the reaction with p-nitrophenol

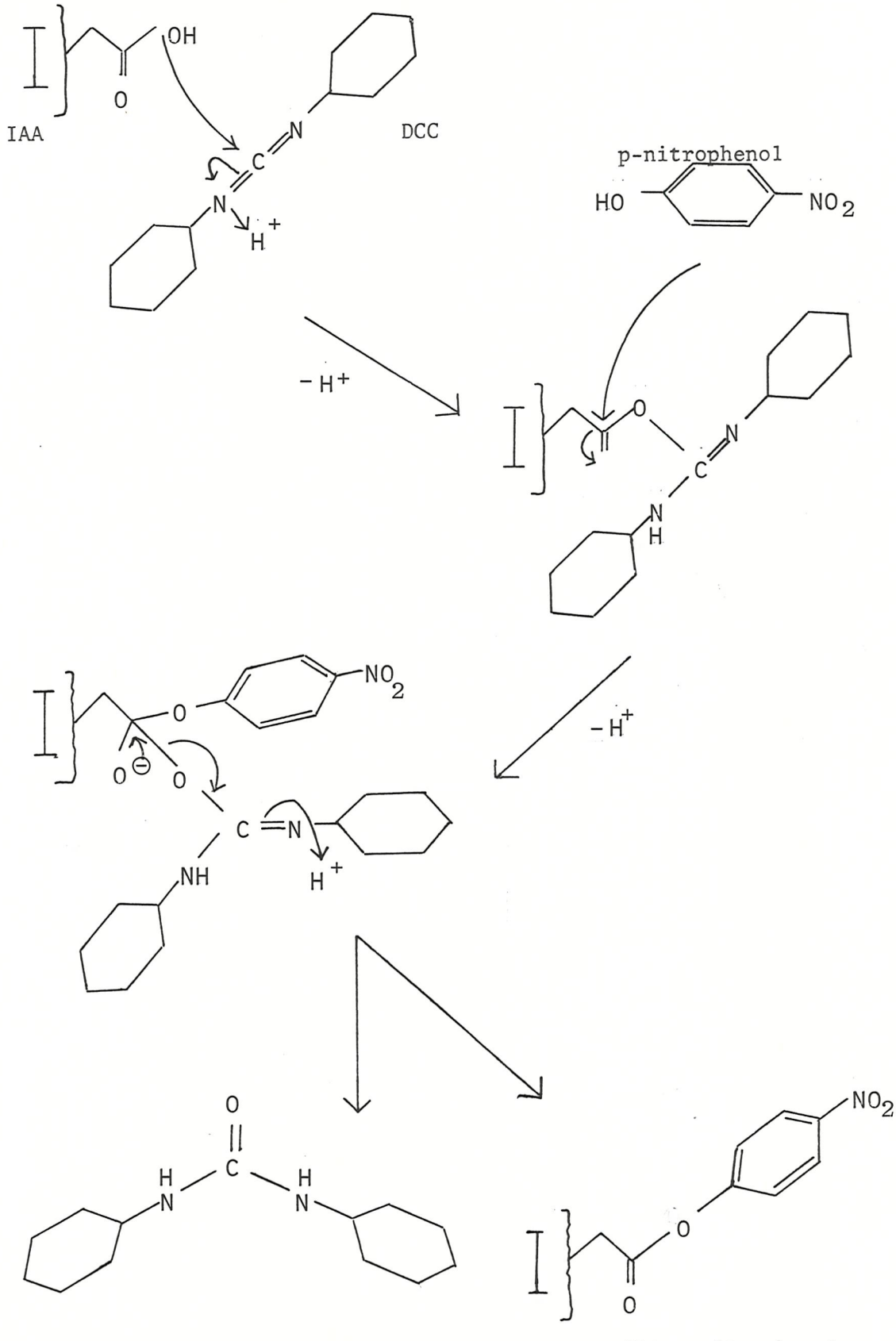
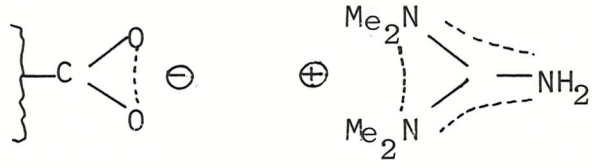


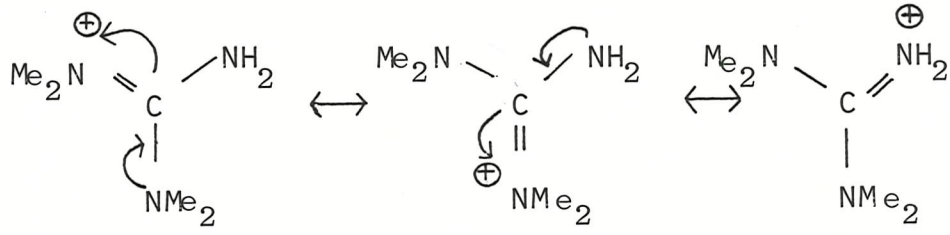
Figure 62 MECHANISMS OF THE SYNTHESIS REACTION.

Stage 2. The reaction of p-nitrophenol IAA with protected amino acid (aspartic).

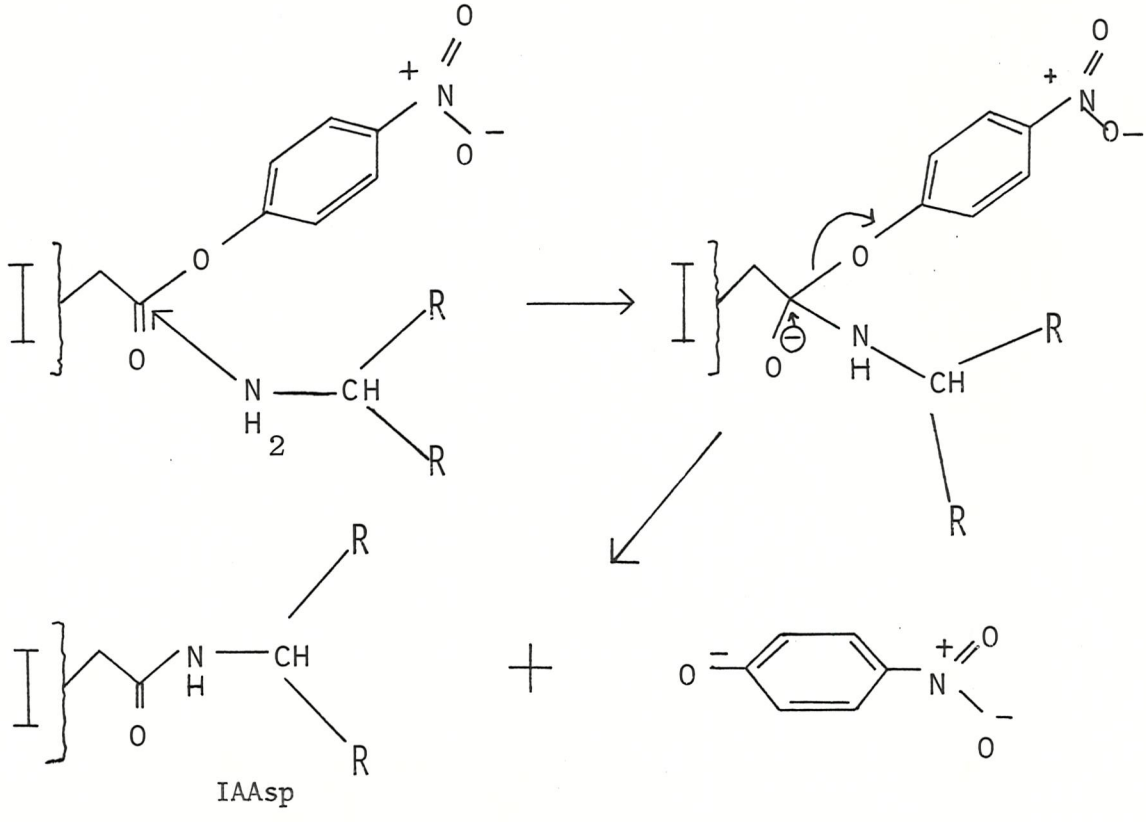
First the carboxyl groups of the aspartic acid are protected by salt formation with tetramethylguanadine (TMG).



The positive charge of the TMG is delocalized over all three nitrogen atoms.



Protected in this way the carboxyl groups of the aspartate will not form anhydrides with p-nitrophenol IAA. Nucleophilic attack by the amino nitrogen occurs to form the amide conjugate.



IAAsp

Lowering the pH cleaves the salts leaving the free conjugate.

I = indole nucleus
R = protected carboxyl groups of the aspartic acid