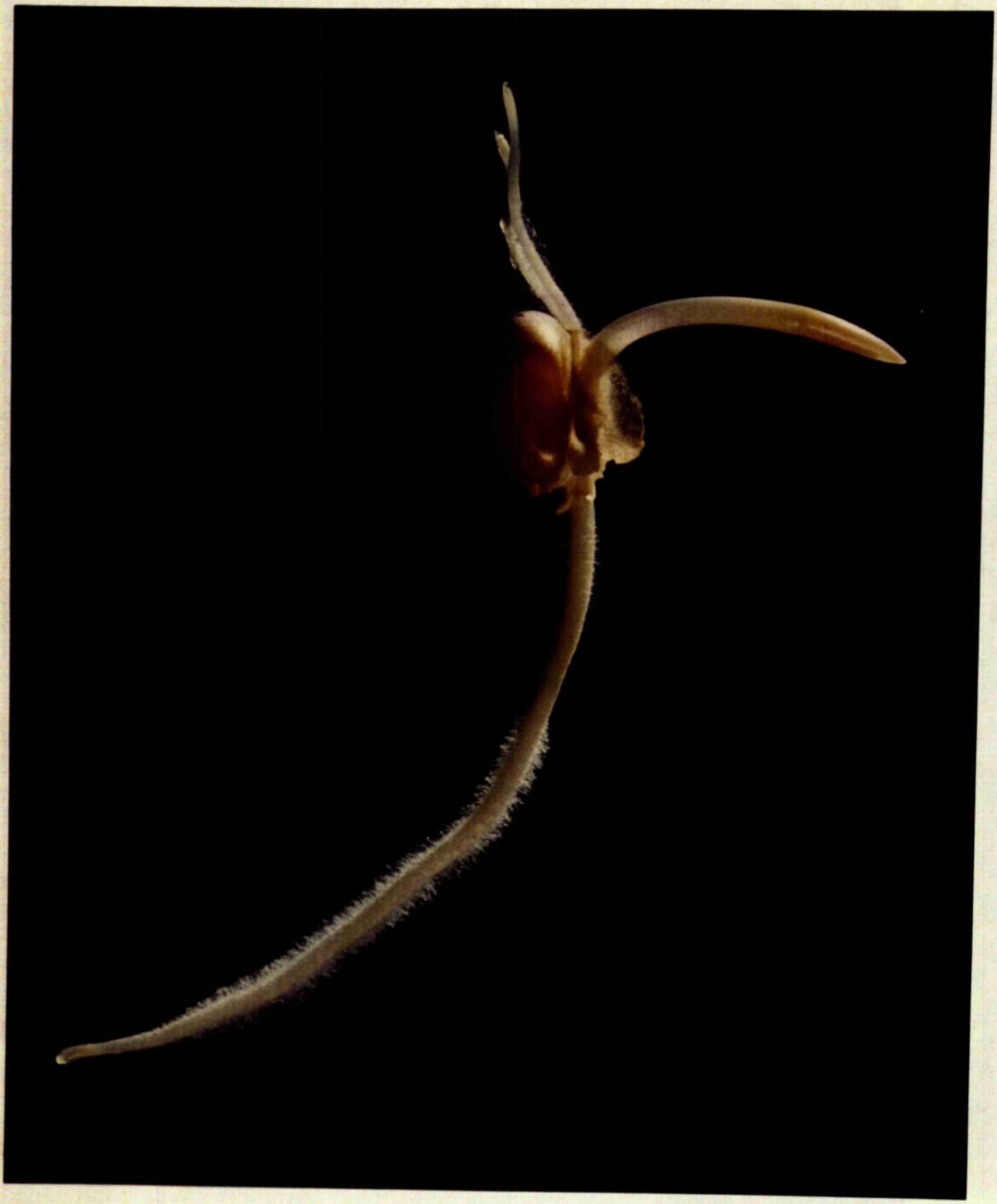

FRONTISPIECE A 3-day-old Zea mays seedling

ABSTRACT

The metabolism of exogenous indole-3-acetic acid (IAA) was examined in root and coleoptile segments taken from dark-grown seedlings of Zea mays L. cv. Fronica. The rate of disappearance of IAA, and the chemical nature of the total spectrum of products in methanolic extracts of plant material which had been incubated with IAA for 2h were investigated. The difference in IAA metabolism in separated cortex plus epidermis, and stelar tissues of the root was monitored. Two methods of supplying IAA to the segments were employed, and the effect of the mode of application on the metabolism pattern noted. The metabolism of IAA, transported through coleoptile and root segments was also studied. In each experiment, the uptake of exogenous IAA was noted and compared with published measurements of endogenous IAA levels. Finally, experiments were carried out to investigate the effect of IAA on coleoptile and root elongation.

Segments were usually supplied with IAA by floating them in aqueous solutions of the radioactively-labelled compound. Alternatively, agar blocks containing ^{14}C-IAA were placed at the apical ends of coleoptile segments, or on protruding portions of stele at the basal ends of root segments. All incubations were carried out in darkness. Crude methanolic extracts of plant material were analysed using a gradient-elution, reverse-phase high-performance liquid chromatograph with an on-stream homogeneous radioactivity monitor. Sample purification was kept to a minimum to avoid selective loss of metabolites. Control experiments showed that degradation of IAA did not occur during sample preparation or analysis. Information on the chemical nature of metabolites was obtained from high-performance liquid chromatograph retention times, use of IAA labelled with ^{14}C at different positions on the molecule, examination of the products of base-catalysed hydrolysis and methylation with diazomethane, UV spectrophotometry, and

co-chromatography with standards of IAA derivatives. The identity of the IAA remaining at the end of experiments was confirmed by combined gas chromatography-mass spectrometry.

IAA was metabolised rapidly by both roots and coleoptiles to a large number of products; at least 11 in roots. All metabolites, in methanolic extracts of tissue incubated for 2h, were more polar than IAA and did not involve decarboxylation. The main product in both roots and coleoptiles appeared to be oxindole-3-acetic acid. Other tentative identifications included 5-hydroxyindole-3-acetic acid, indole-3-acetyl glycine and an ester of IAA possibly with a sugar or myo-inositol. The appearance of several products, in similar quantities, after very short incubation times (10 min), indicated that metabolism probably did not take a single, linear pathway.

Experiments using sterile tissues confirmed that metabolism was taking place within the plant cells. At the lowest external concentrations used, the amounts of ^{14}C-IAA taken into the plant represented only a small fraction of endogenous levels (measurements obtained from published data). Increasing the concentration of IAA over two orders of magnitude did not alter the pattern of metabolism. The products observed were thus unlikely to represent results of a detoxification process.

Comparison of IAA metabolism by cortical and stelar tissues of the root indicated that the majority of metabolism took place in the cortex. IAA present in the stele, the site of the majority of endogenous IAA in the root, appeared to be protected from metabolism.

The mode in which IAA was supplied to roots had a substantial effect on the metabolism pattern. IAA applied in agar blocks to the stele at the basal end of segments was metabolised more slowly than that taken up from solution. The relative proportions of products also differed.

Although substantial metabolism took place in all experiments, radioactivity transported through both root and coleoptile segments, and collected

in agar blocks, remained exclusively associated with the IAA molecule. This provided further evidence for a transport system specific for IAA.

Preliminary experiments were also carried out to study the effects of IAA on segment elongation. Coleoptile segments incubated in 10^{-2} mol m^{-3} IAA showed maximal growth stimulation. On the other hand, IAA (10^{-3} mol m^{-3}) was shown to inhibit root elongation. Uptake at this concentration was of the same order of magnitude as endogenous levels. IAA at lower concentrations could not be shown to promote growth. The effect of IAA on roots was dependent on the presence of oxygen. Growth experiments were always carried out using 1h incubation times, as growth of segments decreased rapidly after this time. The proportion of exogenous IAA which was metabolised also increased with time.

These results have several implications for research on IAA physiology. The complexity of the metabolism pattern observed highlights the need for definitive identification of the total spectrum of metabolites. Co-chromatography with standards in thin-layer or paper systems was clearly shown to be inadequate for this purpose. The lack of decarboxylation products calls into question the rôle of IAA-oxidases in the in vivo metabolism of IAA. The fast rate of metabolism, the substantial effect of mode of IAA application on its metabolism, and the rapid decrease in growth rate of segments, all confound research on the effects, transport and binding of exogenous IAA.

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ABBREVIATIONS

A.E.S.	Automatic External Standard
BSTFA	N,O-bis-(trimethylsilyl)trifluoroacetamide
cpm	Counts per minute
et al.	et alia
f.s.d.	Full scale deflection
f.wt.	Fresh weight
GC-MS	Gas chromatography-mass spectrometry
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
I.D.	Internal diameter
m/e	Mass-to-charge ratio
Me-IAA	Methyl indole-3-acetate
NMR	Nuclear magnetic resonance
ODS	Octadecylsilane
PPO	2,5-diphenyloxazole
R_f	Retention factor
TLC	Thin-layer chromatography
T_R	Retention time
UV	Ultra-violet
v/v	Volume for volume
TMSi	Trimethylsilyl derivative

Where practicable Sl units have been employed, however, for measurement of time, minutes, hours and days were used.

CONTENTS

METABOLISM OF INDOLE-3-ACETIC ACID IN SEEDLINGS OF *ZEA MAYS* L.

By

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for the degree of Doctor of Philosophy

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INTRODUCTION

Intercellular communication is a property of fundamental importance to multicellular organisms. As the physiological and morphological complexity of the organism increases, there is a corresponding requirement for efficient and adaptive coordination mechanisms. The plant or animal must be able to integrate directly the activities of cells which may be considerable distances apart.

Specific Requirements for Intercellular Communication in Higher Plants

The sedentary habit of vascular plants requires them to be adaptable to changing environmental conditions. As they are nearly all autotrophic, plants have relatively simple nutrient requirements. They are, however, dependent on having an adequate supply of water, light and mineral nutrients and must respond to a changing external situation in a way which will ensure that this supply continues. Plants must also be responsive to seasonal changes in temperature and daylength, and should be able to react to adverse circumstances such as allelochemicals and predators in a way which will ensure their survival. For any such reaction to environmental conditions the sensing mechanisms of the plant must be able to communicate with the other organs and tissues.

Intercellular communication is also required to integrate the cell-division activity of separate meristems and to control subsequent cell growth and differentiation. The localisation of cell-division activity in these embryonic regions, which are present throughout the life of the organism, is a unique feature of plants. At any time the majority of these areas are dormant, and their activity governs the morphological organisation of the plant. In addition certain plant cells have the ability to dedifferentiate and may regain the capacity for cell division.

The existence of mechanisms which control cell division, growth, and differentiation may be demonstrated using undifferentiated callus tissue, which can be produced from a variety of plant species. This tissue can be induced to form meristems from which whole plants may be regenerated.

Once an organism has reached a certain size, diffusion is no longer an adequate means of distributing nutrients and metabolites. Thus higher plants, in common with other complex multicellular organisms, must possess one or more efficient transportation systems. The movement of water and nutrients within the plant, while a facet of communication in its own right, may also carry information concerning the activity of one part of the plant to another. For example, if the rate of photosynthesis in the leaves is reduced, the level of sugars in the phloem sap supplying the root system is also likely to be depressed. In this thesis the transport of nutrients will only be discussed with respect to this latter rôle.

Possible Modes of Intercellular Communication

Research into methods of communication in higher animals has revealed two main systems (e.g. Metzler, 1977). The message may take the form of electrical impulses as in the nervous system, or it may be chemical in nature. Hormones are examples of chemical messengers.

Evidence for these two modes of communication has also been investigated by plant physiologists. Electrical impulses or action potentials have been observed in certain plants which exhibit very rapid responses to environmental stimuli (e.g. Dionaea and Mimosa pudica; see Bentrup, 1979). The generality of such nerve-like electrical signals is uncertain, however, and there is considerable scope for more research.

A wide variety of "chemical messengers" are theoretically possible. The active molecules might vary in size from simple compounds such as ethylene to complex proteins which could carry a large amount of information.

As in animals there is the possibility that two or more substances with opposite effects might interact. The active compounds could be present in minute quantities or may be relatively abundant and ubiquitous nutrient molecules or ions. It is also feasible that the gradient of a substance across a cell or tissue may be more important than its absolute amount.

There are several possible routes for the transmission of a chemical message. For rapid communication across considerable distances the mass flow transportation systems of the xylem and phloem might be used. For shorter distances, the substance could move by diffusion in the symplast or apoplast or even through air spaces. There is also evidence that specific active-transport mechanisms for certain compounds (e.g. indole-3-acetic acid; see Goldsmith, 1977) may exist at cell interfaces.

The Discovery of Phytohormones

Despite the large variety of possible forms of chemical message, the majority of research on the means of intercellular communication in plants has concentrated on a few small organic molecules which show physiological activity when present in minute quantities. The earliest evidence for the movement of an "influence" from one part of the plant to another, came from the work of the Darwins (1880). They discovered that a coleoptile could be prevented from responding phototropically by covering the tip with an opaque cap, although the growing cells producing the curvature were located further down the organ. Indications that the "influence" was chemical in nature came from the work of Boysen Jensen (1910), who showed that the stimulus could travel through a block of gelatin, and from Páal (1919). When Páal excised the tips of Avena coleoptiles and replaced them asymmetrically, he observed a greater rate of growth in cells directly below the apex, producing a curvature

resembling that induced by a unilateral light stimulus. He concluded that the coleoptile tip secreted a growth-promoting substance, the distribution of which could be controlled in response to light. This work was further confirmed by Went (1928) who obtained growth-active diffusate from coleoptile tips. Curvature could be induced by placing a block of agar, containing diffusate, asymmetrically on a decapitated coleoptile. The angle of curvature thus obtained was proportional to the number of apices which had been placed on the agar block. Went also showed that when an excised coleoptile tip was given a directional light stimulus, more growth-promoting activity diffused out of the side away from the light source. While studying geotropism, Dolk (1930) demonstrated a redistribution of growth-promoting substance in a horizontal coleoptile tip. He further showed that the total growth and total amount of growth-promoting substance did not change in response to the gravity stimulus.

The first studies of the chemical identity of the growth-promoting substance, which became known as auxin, were carried out on extracts of human urine which had been shown to possess considerable auxin activity in bioassay. In 1934, Kögl *et al.* isolated crystalline indole-3-acetic acid from this source and demonstrated that this compound had considerable auxin activity. Indole-3-acetic acid (IAA) was subsequently extracted from *Rhizopus suinus* (Thimann, 1935) and corn seeds (Haagen-Smit *et al.*, 1946). It was not until 1972, however, that IAA was conclusively identified from *Zea mays* coleoptile tissue (Greenwood *et al.*).

IAA has now been identified from a large number of plant species using a range of analytical techniques, the accuracy of which is discussed extensively by Reeve and Crozier (1980). Table 1 gives a selection of the plant tissues from which IAA has been identified conclusively by obtaining a full-scan mass spectrum.

Since the discovery of IAA, a number of other groups of compounds, capable of affecting growth and development when present in small

Table 1: A Selection of Plants from which IAA has been Extracted and Identified by Full-Scan Mass Spectra

Species	Tissue	Selected Reference
Zea mays	coleoptile tips	Greenwood et al. (1972)
	roots : stele & cortex	Bridges et al. (1973)
	roots (sterile)	Elliott and Greenwood (1974)
Ricinus communis	xylem & phloem sap	Hall and Medlow (1974)
Avena sativa	etiolated seedlings	Bandurski and Schulze (1974)
Prunus cerasus	seeds	Hopping and Bukovac (1975)
Citrus unshiu	fruit	Igoshi et al. (1971)
Gossypium	ovules	Shindy & Smith (1975)
Phaseolus vulgaris	shoots	McDougall & Hillman (1978)
Picea sitchensis	cambium	Little et al. (1978)
Pinus sylvestris	seedlings	Sandberg et al. (1981)
Fragaria x ananassa	immature fruits	Swartz and Powell (1979)
Malus pumila	shoot tips	Swartz and Powell (1979)
Undaria pinnatifida	young thalli	Abe et al. (1972)

quantities, have also been extracted from vascular plants. There are five generally accepted groups of "phytohormones" or "plant growth substances": the auxins, the gibberellins, the cytokinins, abscisic acid and ethylene (for a discussion of the terminology for such compounds see Weyers, 1978; and Hewitt, 1980). Several other substances whose physiological activity is less well established, have also been studied (e.g. various phenolics, fatty acids and terpenoids). A selection of the better documented responses to phytohormone application is shown in Table 2.

The Rôle of Phytohormones in Higher Plant Physiology

The term hormone was first used by Bayliss and Starling (1902 and 1904) to describe the action of secretin, a molecule secreted by the duodenum that stimulates the activity of the pancreas. They defined hormones as "chemical messengers produced in one organ and transported in the bloodstream to a site of action in another". Plant growth substances have traditionally been assumed to operate in a similar manner. Thimann (1948) defined a phytohormone as "an organic substance produced naturally in higher plants, controlling growth or other physiological functions at a site remote from its site of production, and active in minute amounts". A physiological response was believed to be induced by a change in the amount of a substance binding to receptor sites in the cells of a target tissue.

Early experiments on the rôle of auxin in the geotropism and phototropism of Avena coleoptiles supported this theory. Auxin appeared to be synthesised in one place, i.e. the coleoptile tip, and transported to a site of action in another, i.e. the zone of curvature. There is, however, an increasing body of evidence that the function of phytohormones may be more complex than the traditional theories suggest, and several alternative hypotheses have been proposed.

Table 2: Physiological Responses to Plant Growth Substances (Generally Species-Specific)

a. Effects on the Fundamental Processes of Cell Division, Growth and Differentiation

Phenomenon	Growth Substance	Effect	Example Reference
Cell Division	Cytokinins	Stimulation	Letham (1963)
	IAA	Stimulation of cambial cell division	Snow (1935)
Cell Growth	IAA	Stimulation of shoot growth	Went & Thimann (1937)
		Inhibition of root growth	Went & Thimann (1937)
	Gibberellins	Promotion of growth of green shoots	Brian and Hemming (1955)
	Cytokinins	Promotion of cotyledon enlargement	Esashi and Leopold (1969)
	Abscisic acid	Growth inhibition	Addicott et al. (1964)
	Ethylene	Growth inhibition	Pratt and Goeschl (1969)
Cell Differentiation	IAA	Stimulation of xylem and phloem differentiation	Jacobs (1952) LaMotte and Jacobs (1963)
Organ Morphogenesis	IAA	Control of callus tissue morphogenesis	Miller and Skoog (1953)
		Promotion of root initiation	Avery and Johnson (1947)
	Cytokinins	Control of callus tissue morphogenesis	Skoog and Miller (1957)

b. Some Examples of Responses to Plant Growth Substances concerning Aspects of Correlative Control of Plant Development and Response to the Environment

Phenomenon	Growth Substance	Response	Example Reference
Seed Dormancy	Gibberellins	Break dormancy	Suzuki and Takahashi (1968)
	Cytokinins	Break dormancy	Miller (1956)
	Abscisic acid	Inhibits germination	Aspinall et al. (1967)
	Ethylene	Breaks dormancy	Vacha and Harvey (1927)
Seedling Morphology	Ethylene	Maintains plumular hook	Kang et al. (1967)
Geotropism	IAA	IAA transported to lower side of shoot	Shaw et al. (1973)
Phototropism	IAA	Transported to darkened side of shoot	Gardner et al. (1974)
Bud Dormancy	Gibberellins	Break dormancy	Brian et al. (1955)
	Cytokinins	Break dormancy	Benes et al. (1965)
	Ethylene	Breaks dormancy	Pratt and Goeschl (1969)
Apical Dominance	IAA	Replaces apical bud	Thimann and Skoog (1933)
	Cytokinins	Release lateral buds from apical dominance	Sachs and Thimann (1964)
Juvenility	Gibberellins	Reduce period of juvenility	Pharis and Morf (1968)
Stomatal Movement	Abscisic acid	Causes stomatal closure	Mittelheuser and Van Steveninck (1969)
	Cytokinins	Promote stomatal opening	Kuiper (1972)
Leaf Senescence	IAA	Delays senescence	Sacher (1959)
	Gibberellins	Delay senescence	Fletcher and Osborne (1965)
	Cytokinins	Delay senescence	Richmond and Lang (1957)
	Ethylene	Accelerates senescence	Burg (1968)
Leaf Abscission	IAA	Inhibits abscission	La Rue (1935)
	Abscisic acid	Promotes abscission in explants	Bornman et al. (1967)
	Ethylene	Promotes abscission	Zimmerman et al. (1931)

Phenomenon	Growth Substance	Response	Example Reference
Flowering	IAA	Promotes flowering in Bromeliads	Clark and Kerns (1942)
	Gibberellins	Induces flowering in long day and cold requiring plants	Lang (1956)
	Cytokinins	Promotes flowering, some short-day plants	Ogawa (1961)
	Abscisic acid	Promotes flowering, some short-day plants	El-Antably and Wareing (1966)
	Ethylene	Promotes flowering in Bromeliads	Rodriguez (1932)
Fruit Setting	Gibberellins	Allows development of parthenocarpic fruit	Crane (1964)
	IAA		Crane (1965)
	Cytokinins		Crane (1965)
Fruit Ripening	Ethylene	Stimulates fruit ripening	Lyons and Pratt (1964)
	IAA	Defers ripening	Kendrell (1969)
	Gibberellins	Retards ripening	Coggins and Lewis (1962)
Fruit Abscission	Abscisic acid	Stimulates abscission	Addicott et al. (1964)
	Ethylene	Stimulates abscission	Pratt and Goeschl (1969)

In the specific example of coleoptile phototropism and geotropism, Firn and Digby (1980) and Trewavas (1981) have pointed out that amounts of IAA moving laterally across the organ may not be sufficient to produce the observed change in growth rate. The effect of IAA on coleoptile elongation is proportional to the log of its concentration (Bentley and Bickle, 1952) with relatively large changes in hormone levels being required to invoke a noticeable response. However, the amount of exogenous IAA transported laterally in response to a gravity or light stimulus (Shaw et al., 1973 and Gardner et al., 1974) was no more than 56% and 15.3% respectively. Curry (1969) also showed that the tip was not the only region of the coleoptile capable of detecting a directional light stimulus, although it was somewhat more sensitive than regions lower down. Similarly, geosensitivity is found along the length of the organ (Firn and Digby, 1980). These results have led the above authors to question whether IAA has any rôle in shoot phototropism or geotropism. In the case of geotropism they suggested that the epidermal cells of the elongation zone may both detect the gravity stimulus and respond directly by changing their rate of growth.

The logarithmic relationship between exogenous concentration and effect is common to the majority of bioassays. On the other hand, the largest observed endogenous changes in amounts of phytohormones are the 40-50 fold increases in the abscisic acid content of some leaves subjected to water stress; a two- to five-fold increase is more commonly recorded (see Trewavas, 1981). On this basis Trewavas proposed that changes in levels of plant growth substances are not normally responsible for physiological changes, but that they might serve to integrate physiological processes, ensuring that growth and differentiation occur in an evenly distributed fashion throughout a group of cells. It must be recognised, however, that measurements of plant hormone levels are frequently made on whole organs or large areas of tissue. Different conclusions might be

reached if the movement of substances between individual cells or even between different compartments within cells was considered. It has been shown (Loveys, 1977; and Weyers, 1978) that movement of abscisic acid from the leaf mesophyll to the guard cells of the epidermis occurs in response to water stress and precedes the increase in total abscisic acid levels (Walton et al., 1977). Furthermore, the relationship between exogenous hormone concentration and response may not reflect accurately the effect of the endogenous substance. The influence of rates of uptake and metabolism on the activity of the applied compound must be considered.

The effective levels of a plant growth substance might also be altered by regulating the activity of putative receptor sites. Trewavas (1981) replotted the results of Wright (1961 and 1966) on the change in sensitivity to auxin and the growth rate of wheat coleoptiles, as a function of the developmental age. The graph showed a substantial increase in sensitivity which preceded the rise in growth rate. Osborne (1978) discussed a similar development in the flower buds of Ecballium elaterium. Only buds which had passed a certain stage in development could be induced by ethylene to separate from the pedicel. However, as the ovary is not usually shed until after the ovule has been pollinated and the fruit ripened, development of ethylene sensitivity cannot be the normal trigger for abscission.

Another alternative theory suggests that gradients of plant growth substances may in some cases be responsible for inducing cell differentiation. Wetmore and Rier (1963) presented evidence that gradients of IAA and sucrose within a cylinder of callus induced the formation of nodules of vascular tissue. The relative proportions of IAA and sucrose also affected the ratio of xylem and phloem.

While phytohormones have many dramatic effects on excised tissues and organs they are frequently ineffective when applied to intact plants. For example, the application of IAA to intact coleoptiles led to growth increases of only some 25% (Went and Thimann, 1937). This could be due

to problems of penetration and/or metabolism before the compound reaches its site of action but might also suggest that phytohormones, while necessary for plant growth and development, are not usually present in limiting amounts.

Table 2 shows that a large variety of physiological events appear to be controlled by the five known groups of phytohormones. Individual compounds all influence at least one of the fundamental processes of cell division, growth and differentiation; IAA appears to effect all three. Phytohormones also have a wide variety of specific effects in different tissues at various stages of the plants' development, with each process usually being affected by at least two substances. It is difficult to envisage how simple changes in the levels of these five groups of compounds alone can supply enough information to regulate such a wide variety of growth and developmental phenomena. The problem might be partly solved if it could be shown that a unique combination of substances was required for a specific process. Osborne (1978) suggested that the variety of responses might be attributable to there being more than one class of target cell on which a plant growth substance might act, with different results.

In addition, there are several situations in which the existence of a transported message is implied which cannot be explained in terms of the known phytohormones. For example, in many plants where flowering is controlled by photoperiod, it can be shown (see Zeevaart, 1976) that daylength is detected by the mature leaves although the responding tissue is the apex. Grafting experiments implicate the existence of a translocated signal, however all attempts to isolate "florigen" have failed. The nature of the inhibitor responsible for preventing the outgrowth of lateral buds, in the presence of an intact apex, remains unsolved. Similarly, the identities of "senescence factor" and the dormancy-inducing stimulus are unknown.

In the case of the flowering stimulus there is a need for genes to be

activated and deactivated, in order to effect the fundamental changes taking place when a plant is converted from the vegetative to the reproductive phase. There is evidence that steroid hormones in animals have this property (e.g. O'Malley et al., 1977) but the alteration of gene activity is not thought to be the initial response to plant growth substances. At least some effects of phytohormones are much too rapid to involve the mediation of gene-activated protein synthesis. For example, IAA can alter the rate of coleoptile elongation within 15 min (Ray and Ruesink, 1962). It is therefore possible that other substances have yet to be discovered.

In conclusion, the exact rôle of phytohormones remains unclear. It has been shown that these compounds have many effects when applied to plant tissues. The levels at which growth substances are present in intact plants are also important in certain situations. There are several examples of disease symptoms being caused by excessive hormone production by, or in response to, a pathogenic organism. The "foolish seedling" disease of rice is caused by the synthesis of large quantities of the gibberellin GA_3 by the fungus Fusarium moniliforme (Cross et al., 1961). Dwarfism in certain varieties of Zea is believed to be a result of inadequate gibberellin production (Phinney, 1960). Nevertheless, it is by no means clear whether changes in the levels of plant growth substances are normally responsible for physiological changes, or whether the translocation of a phytohormone constitutes a chemical message. Although they are necessary, phytohormones may not normally be limiting factors controlling plant growth and development.

Rationale Behind the Present Study on the Metabolism of Indole-3-acetic Acid

If an understanding of the rôle of plant growth substances is to be achieved, certain fundamental questions concerning the biochemistry of these compounds must be answered, viz.:

1. Where is each substance synthesised?

2. How are they distributed within the plant at a cellular and intracellular level, and how does this distribution change at various stages of the plants' development?

3. How are the levels of substances controlled? Several methods are possible:

   (a) Control of synthesis and catabolism.

   (b) Reversible activation and inactivation.

   (c) Intra- and intercellular movement.

4. What is the nature of the "receptor sites"?

5. What are the primary effects of each substance?

For the purposes of this present investigation, one of these aspects was chosen. The metabolism of indole-3-acetic acid in the root and coleoptile tissues of dark-grown *Zea mays* seedlings was studied. The rates of catabolism and conjugation of IAA may influence the levels present in the tissue. It is also possible that IAA metabolism might be directly linked in some way to its physiological activity. Research on the effects, transport and binding of IAA has often assumed that metabolism of the applied IAA during the course of the experiment is negligible (e.g. Batra *et al.*, 1975; Moloney and Pilet, 1981) although adequate evidence to support this contention is rarely provided. A detailed investigation of the extent to which exogenous IAA is metabolised is therefore essential.

The Plant System

Zea mays seedlings have been used extensively for research on the metabolism, transport, receptor sites and effects of IAA (e.g. Reinecke and Bandurski, 1981; Wilkins and Scott, 1968; Moloney and Pilet, 1981; Shaw *et al.*, 1973). *Zea* is the third most important cereal crop in the world, with an annual production of 362,971,000 tonnes in 1978 (Encyclopedia Britannica 1980 Year Book). Commercially available varieties have the advantage of rapid and even germination, reaching a size suitable for experimentation within three to five days. Both the coleoptile and the primary root are unusually large in diameter when compared with other cereal species. When grown in the dark, the lack of chloroplastic components, and products of photosynthesis, reduces the complexity of extracts. While IAA causes substantial stimulation of coleoptile growth, similar amounts inhibit root growth (e.g. Pilet, 1976). Thus the project allows a comparison of IAA metabolism in organs with different responses to the phytohormone. As *Zea mays* roots can easily be divided into cortex and stele (Greenwood *et al.*, 1973) the metabolism of IAA by these tissues could also be compared.

IAA in *Zea mays* Seedlings

The main source of IAA in young *Zea mays* seedlings appears to be the endosperm. Bandurski and co-workers have shown that this tissue contains large quantities of IAA, most of which is esterified to either *myo*-inositol or high molecular weight glucans (Ueda *et al.*, 1970; Piskornik and Bandurski, 1972; Bandurski and Schulze, 1977). They have further demonstrated that a sufficient quantity of IAA-*myo*-inositol is exported to the shoot where it can be hydrolysed to supply the coleoptile with its full requirement for IAA (Nowacki and Bandurski, 1980). IAA-*myo*-inositol is also transported to the root (Epstein *et al.*, 1980). On the other hand, there is some evidence that the root may be able to synthesise IAA (Elliott,

1977; Feldman, 1980).

The movement of IAA in Zea mays roots, as in many other plants, is predominantly acropetal (e.g. Scott and Wilkins, 1968; Shaw and Wilkins, 1974). The majority of IAA in the root is found in the stele, which also shows more efficient transport of IAA (Bowen et al., 1972). Quantities of IAA measured using mass spectrometry in the variety Giant White Horsetooth were as follows (Bridges et al., 1973):

stele	53.3 µg IAA/Kg fresh weight
cortex	4.8 µg IAA/Kg fresh weight
root tip	29.0 µg IAA/Kg fresh weight

Pilet and Elliott (1981) have, however, warned that quantities of IAA might vary substantially between varieties.

In Zea mays shoots, the transport of IAA is polarised in a basipetal direction (Hertel and Leopold, 1963). The quantity of IAA present in coleoptiles from the variety Anjou 21 was measured as 10 pmol /2.5 mm tip by Weiler et al. (1981) using a radioimmunoassay.

IAA has been shown on many occasions to cause substantial stimulation of coleoptile elongation (e.g. Thimann, 1969). Its effect on root growth is, however, less clear. Exogenous IAA concentrations of around 10^{-2}mol m^{-3} which produce maximum growth rates in mesocotyl and coleoptile segments inhibit the growth of root sections (e.g. Batra et al., 1975; Greenwood and Yčas, 1975). While some workers have found no evidence of IAA-induced promotion of root growth (e.g. Greenwood and Yčas, 1975) others claim to have observed a small and often transient stimulation at very low concentrations of between 10^{-4}mol m^{-3} and 10^{-7}mol m^{-3} (e.g. Batra et al., 1975; Edwards and Scott, 1977; Elliott, 1977; Evans et al., 1980). Pilet and co-workers (Pilet et al., 1979; Pilet and Elliott, 1981) presented evidence that root segments, depleted of IAA by diffusing onto agar for periods of up to 8h, will show significant IAA-induced growth promotion. They suggested that in the intact plant the roots have a saturating level of IAA.

It has been shown (Rayle & Cleland, 1970) that low pH can stimulate shoot growth in a similar manner to IAA. This led Marré and co-workers (1974) to put forward the "acid-growth" theory, in which they suggested that IAA stimulates growth by causing protons to be pumped out of the cell into the wall, where the lowered pH causes wall-loosening. In support of this hypothesis was the observation that fusicoccin, a fungal toxin, could stimulate shoot growth by such a proton-pump mechanism (e.g. Lado et al., 1973). Edwards and Scott (1974) and more recently Moloney et al. (1981) showed that the growth of roots could also be stimulated by low pH. Furthermore, fusicoccin can cause proton extrusion in roots and thereby promote elongation (Lado et al., 1976). Pilet (1976), however, has pointed out that fusicoccin and IAA cannot be acting by a common mechanism in the root, as the optimum concentration of fusicoccin for growth promotion was 10^{-2}mol m^{-3}, with stimulation also found at 10^{-3}, 10^{-4} and 10^{-1}mol m^{-3}. IAA at any of these concentrations produced growth inhibition.

Batra, Edwards and Scott (1975) used a different approach to investigate the relationship between acid growth and the effect of IAA on root elongation. They studied the effect on growth, of IAA in buffered agar of varying pH. An IAA concentration of 10^{-4}mol m^{-3} caused growth inhibition at pH 4 while promoting extension at pH 7. In a later paper, Edwards and Scott (1977) found that 10^{-4}mol m^{-3} IAA would promote growth only in a buffered solution at pH 7 and not in an unbuffered solution at the same pH. The authors suggested that their observations might be explained in terms of pH effects on IAA transport as they are not easily compatible with the acid-growth theory. The results are also complicated by the influence of the buffering substance itself on growth (Edwards and Scott, 1976; Gabella and Pilet, 1978). Thus under most experimental conditions, IAA appears to inhibit root growth. Evidence for growth promotion is inconclusive, but the acid-growth theory is not thought to apply. Evans and co-workers (Evans and Mulkey, 1980; Evans et al., 1980) have recently presented evidence that IAA may inhibit root growth by

causing protons to be pumped into the cells, in the reverse direction to shoots, thereby raising the wall pH. Such a change in pH was measured in the bathing solution surrounding maize root segments incubated in 2×10^{-3} mol m^{-3} IAA. The start of the pH change almost coincided with the time at which the growth rate began to fall rapidly.

On the other hand, the effect of exogenous IAA may not be a reliable indication of the rôle of the endogenous compound. The use of segments necessarily involves the presence of cut surfaces. Both wounding, and the application of auxin can induce ethylene production (e.g. Burg, 1962; Abeles, 1966). Chadwick and Burg (1967) and more recently Mulkey et al. (1981) have suggested that the inhibition caused by exogenous IAA is due to this stimulation of ethylene biosynthesis.

In conclusion, the existence, origin and transport of IAA in Zea mays seedlings are well established, as is the effect of the exogenous compound on the growth of the coleoptile and mesocotyl. However, much work is needed before its rôle in the control of root elongation is understood.

Metabolism of IAA

The importance of IAA metabolism was realised as early as the 1950s when Kamerbeek (1956) and Galston (1957) hypothesised that there might be a causal relationship between dwarfism, endogenous levels of IAA, and the activity of IAA-oxidising enzymes. Since then dwarfism has been found to be more readily explained by other means, such as gibberellin content or sensitivity to plant growth substances (see Jones, 1973). Interest in IAA metabolism as a possible means of regulating the levels of this phytohormone has remained, however, and much research has been carried out on this topic.

Three main approaches have been used in the study of IAA metabolism:

1) The partial characterisation of enzyme preparations capable of oxidising IAA, and identification of the reaction products.

2) The application of IAA, usually labelled with ^{14}C or ^{3}H to plant tissues, followed by the extraction and analysis of its metabolites.
3) The extraction, and identification with varying degrees of certainty, of endogenous indolic compounds.

The substances found as a result of these lines of investigation are listed in Table 3 and their chemical structures shown in Fig. 1. Table 4 compares the detection limits, specificity and information content of the various techniques used to identify IAA and its metabolites. According to Reeve and Crozier (1980) the conclusive identification of an unknown component of a complex plant extract requires either a minimum of 140 bits of information, or a demonstration that the specificity of the detector is adequate, using the successive approximation method. A comparison of Tables 3 and 4 indicates that in many cases identification of compounds has not been sufficiently rigorous.

Products formed by IAA-oxidase preparations fall into two groups: 3-methylene oxindole and related compounds, and indole-3-aldehyde. In both cases the reaction involves decarboxylation with loss of carbon-1 of the side chain as CO_2. Various pathways leading to these compounds have been proposed (e.g. Hinman and Lang, 1965; BeMiller and Colilla, 1972; Ricard and Job, 1974; Suzuki and Kawarada, 1978). Fig. 2 shows a simplified scheme from Sembdner et al. (1980). The same enzyme can produce either 3-methylene oxindole or indole-3-aldehyde; Ricard and Job (1974) demonstrated that the proportions of these two substances varied with the relative amounts of enzyme and substrate, and with the pH of the medium.

In vivo studies of IAA metabolism give a completely different picture, however. The most common and abundant metabolites appear to be IAA conjugates with amino acids, sugars or myo-inositol (e.g. Reung et al., 1976; Kopcewicz et al., 1974). Reports of decarboxylation products such as 3-methyleneoxindole or indole-3-aldehyde have usually been based on somewhat inadequate chromatographic and colorimetric identifications.

Table 3: IAA Metabolites

a. Products of *In Vitro* IAA Metabolism

Compound	Enzyme	Selected Reference	Method of Identification
3-methylene-oxindole	Horseradish peroxidase	Hinman and Lang (1965)	1b, 6
	Zea mays IAA-oxidase	BeMiller and Colilla (1972)	1a, 6
	Wheat peroxidase B_1	Zmrhal and Macháčková (1978)	1a, 6
	Turnip peroxidases P_1 and P_7	Ricard and Job (1974)	6
3-hydroxymethyl-oxindole	Horseradish peroxidase	Suzuki and Kawarada (1978)	6, 7, 8
	Zea mays IAA-oxidase	BeMiller and Colilla (1972)	1a, 6
indole-3-aldehyde	Horseradish peroxidase	Ricard and Job (1974)	6
	Turnip peroxidases P_1 and P_7	Ricard and Job (1974)	6
	Zea mays IAA-oxidase	BeMiller and Colilla (1972)	1a
indole-3-methanol	Wheat peroxidase B_1	Zmrhal and Macháčková (1978)	1a, 6
3,3' diindolyl-methane	*Zea mays* IAA-oxidase	BeMiller and Colilla (1972)	1a, 5, 7
	Horseradish peroxidase	Suzuki and Kawarada (1978)	6, 7, 8
3-acetoxyindole	Horseradish peroxidase	Suzuki and Kawarada (1978)	6, 7, 8
3-(indol-3-yl-methyl)oxindole			
3[(2″-indol-3″-ylmethyl) indol-3′-ylmethyl]oxindole			
3-hydroxymethyl-3(indol-3-ylmethyl)oxindole			
3-hydroxymethyl-3-[(2″-indol-3″-ylmethyl)indol-3′-ylmethyl]oxindole			
2-(indol-3-ylmethyl)indolyl-3-acetic acid			

b. Products of the In Vivo Metabolism of Exogenous IAA

Compound	Tissue	Species	Selected Reference	Method of Identification
Indol-3-acetyl aspartate	crown gall tissue	Parthenocissus tricuspidata	Feung et al. (1976)	1c, 3, 7
	shoot bark	Pinus pinea	Riov and Gottlieb (1980)	8, 9
	stem sections	Pisum sativum	Andreae and Good (1955)	2a, 3, 6
	callus	Citrus sinensis	Epstein et al. (1977)	1c
	stem segments	Phaseolus vulgaris	Davies (1972)	1c, 4
	leaves	Scrophularia arguta	Miginiac et al. (1978)	1c
	leaves	Olea europa	Epstein and Lavee (1977b)	2c
Indole-3-acetyl glycine	crown gall callus tissue	Parthenocissus tricuspidata	Feung et al. (1976)	1c, 3, 7
Indole-3-acetyl alanine				
Indole-3-acetyl valine				
Indole-3-acetyl glutamate				1c, 3
Indole-3-acetyl glucose	needles	Pinus pinea	Riov and Gottlieb (1980)	1c, 8
	epicotyls	Pisum sativum	Zenk (1961)	2a,2c,3,5,6
	caryopses	Zea mays	Kopcewicz et al. (1974)	1a,1c,3,5
	whole plants	Orobanche ramosa	Magnus et al. (1982)	1a, 3
Indole-3-acetyl myo-inositol	caryopses	Zea mays	Kopcewicz et al. (1974)	1a,1c,3,5
Indole-3-aldehyde	stem sections	Pisum sativum	Magnus et al. (1971)	1a
	leaves	Olea europa	Epstein and Lavee (1977b)	2c, 4
	leaves	Scrophularia arguta	Miginiac et al. (1978)	1c
	inflorescence	Orobanche lutea	Magnus et al. (1982)	1a, 6, 8
	whole plants	Orobanche ramosa	Magnus et al. (1982)	1a, 6, 8

Compound	Tissue	Species	Selected Reference	Method of Identification
Indole-3-carboxylic acid	stem sections	Pisum sativum	Magnus et al. (1971)	1a, 5
	leaves	Olea europa	Epstein and Lavee (1977b)	2c, 4
	whole plants	Orobanche ramosa	Magnus et al. (1982)	1a, 6, 8
Indole-3-methanol	stem sections	Pisum sativum	Magnus et al. (1971)	1a, 5
	inflorescence	Orobanche lutea	Magnus et al. (1982)	1a, 5
3-Methyleneoxindole	callus	apple	Epstein et al. (1975)	2a,2b,2c,5
	coleoptiles	Avena sativa	Menschick and Hild (1976)	1c
	callus	Olea europa	Lavee and Epstein (1976)	2c, 4
3-Hydroxymethyloxindole	etiolated seedlings	Pisum sativum	Tuli and Moyed (1967)	1b, 1d, 6, 5
	stem segments	Pisum sativum	Davies (1972)	1c, 4
	stem segments	Phaseolus vulgaris	Davies (1972)	1c, 4
	callus	apple	Epstein et al. (1975)	2a,2b,2c,4
	callus	Olea europa	Lavee and Epstein (1976)	2c, 4
3-Methyloxindole	incubating solution surrounding callus	Parthenocissus tricuspidata	Hamilton et al. (1976)	1c
Oxindole-3-acetic acid	endosperm	Zea mays	Reinecke and Bandurski (1981)	1c,7,9,10

c. Endogenous Compounds which may be IAA Metabolites

Compound	Tissue	Species	Reference	Method of Identification
Indole-3-acetyl aspartate	seedlings	Phaseolus vulgaris	Tillberg (1974)	1a, 3
Indole-3-acetyl aspartate	seed	Glycine max	Cohen (1981)	7
2-O-(indole-3-acetyl)-D-glucose 4-O-(indole-3-acetyl)-D-glucose 6-O-(indole-3-acetyl)-D-glucose	caryopses	Zea mays	Ehmann (1974)	7
Indole-3-acetyl myo-inositol	caryopses	Zea mays	Ehmann and Bandurski (1974)	7,1a,5,9
	shoots	Zea mays		
	seeds	Oryza sativa	Hall (1980)	1a,5,7,9
IAA-myo-inositol-arabinosides	caryopses	Zea mays	Ueda et al. (1970)	1a, 7, 9
IAA-myo-inositol-galactosides	caryopses	Zea mays	Ueda et al. (1970)	1a, 7, 9
Methyl(oxindole-3-acetate)	rice bran	Oryza sativa	Kinashi et al. (1976)	6, 7, 8
Methyl(3-Hydroxyoxindole-3-acetate)				
Methyl(5-hydroxy-oxindole-3-acetate)				
3,5-Dihydroxyoxindole-3-acetic acid				
Methyl(3,5-Dihydroxyoxindole-3-acetate				
Oxindole-3-acetate	endosperm	Zea mays	Reinecke and Bandurski (1981)	7, 9, 10

d. Key to Techniques Used in the Identification of Metabolites

1. Co-chromatography with standards in thin-layer system(s); detection of spots by (a) colour reactions
   (b) UV absorbance
   (c) radioactivity
   (d) bioassay

2. Co-chromatography with standards in paper system(s); detection of spots by (a) colour reactions
   (b) UV absorbance
   (c) radioactivity
   (d) bioassay

3. Susceptibility to, and products of hydrolysis.

4. Comparison of metabolism of IAA labelled in different positions.

5. Other chemical tests.

6. UV Absorption spectroscopy

7. Mass spectrometry (full scan).

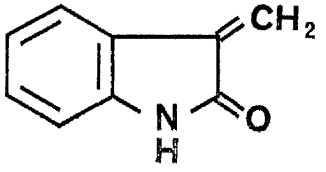
8. NMR spectroscopy.

9. Co-chromatography with standards on gas chromatograph.

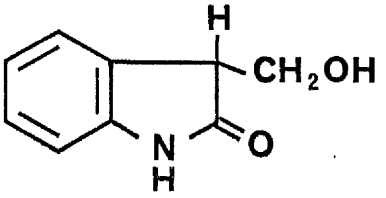
10. Co-chromatography with standards on high-performance liquid chromatograph.

Fig.1. Chemical Structures of Putative IAA Metabolites

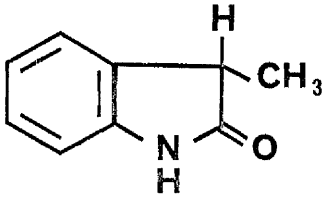
(a) Oxidation Products



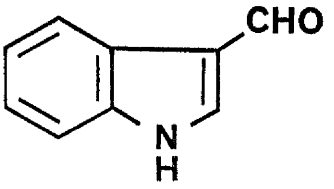
3-Methyleneoxindole



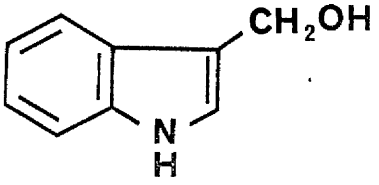
3-Hydroxymethyloxindole



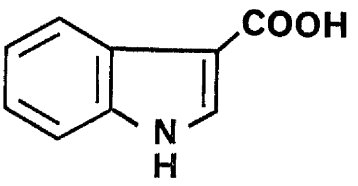
3-Methyloxindole



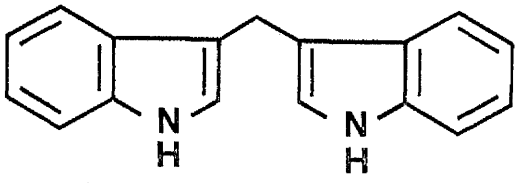
Indole-3-aldehyde



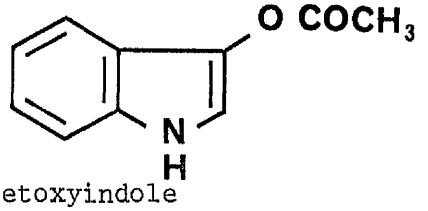
Indole-3-methanol



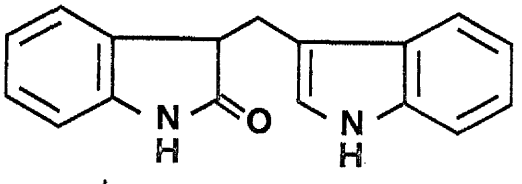
Indole-3-carboxylic acid



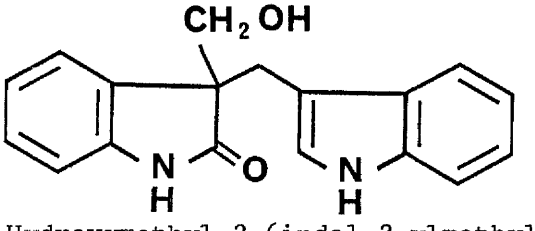
3,3'-di-indolylmethane



3-Acetoxyindole



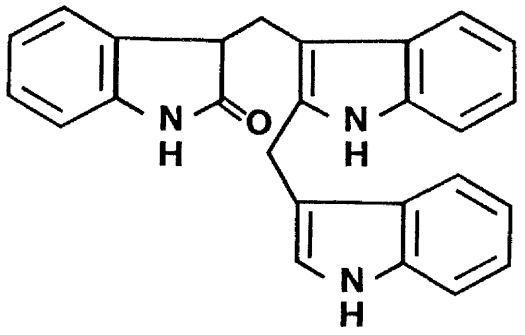
3-(Indol-3'-ylmethyl)oxindole



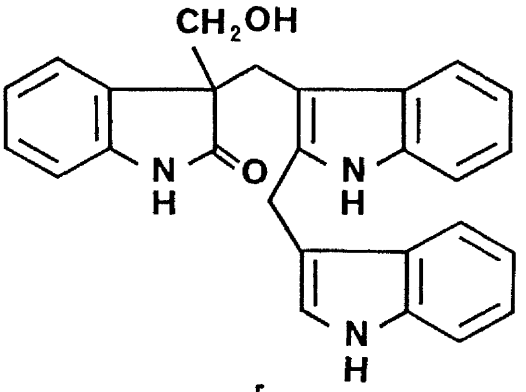
3-Hydroxymethyl-3-(indol-3-ylmethyl)-oxindole

Fig.1. (cont'd) Chemical Structures of Putative IAA Metabolites

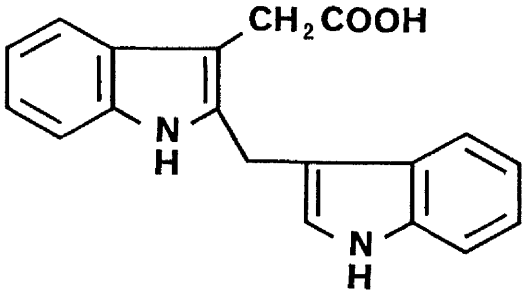
(a) Oxidation Products



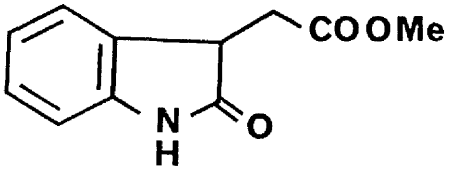
3-[(2"-Indol-3"-ylmethyl)indol-3'-yl-methyl]oxindole



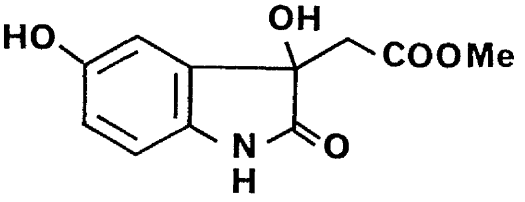
3-Hydroxymethyl-3-[(2"-indol-3"-yl-methyl)indol-3'-ylmethyl]oxindole



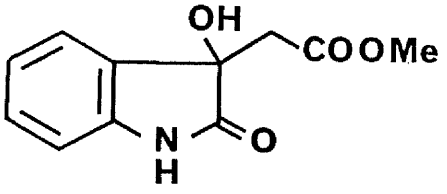
2-(Indol-3-ylmethyl)indole-3-acetic acid



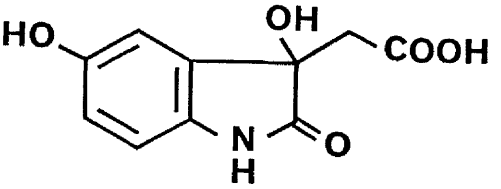
Methyl Oxindole-3-acetate



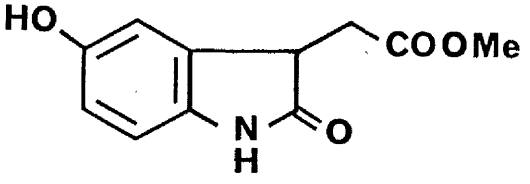
Methyl 3,5-Dihydroxyoxindole-3-acetate



Methyl 3-Hydroxyoxindole-3-acetate



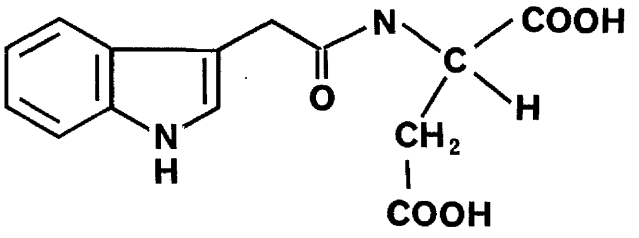
3,5-Dihydroxyoxindole-3-acetate



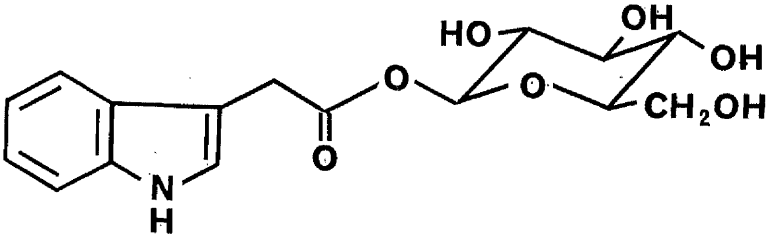
Methyl 5-Hydroxyoxindole-3-acetate

Fig.1. (cont'd) Chemical Stuctures of Putative IAA Metabolites

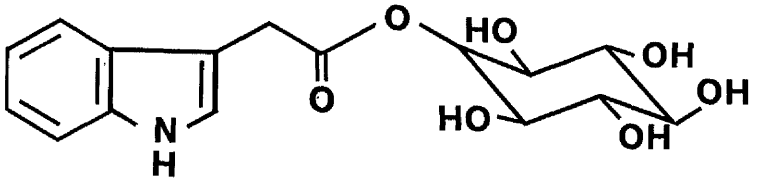
(b) IAA Conjugates



Indole-3-acetyl Aspartate



1-O-(Indole-3-acetyl)-β-D-Glucose



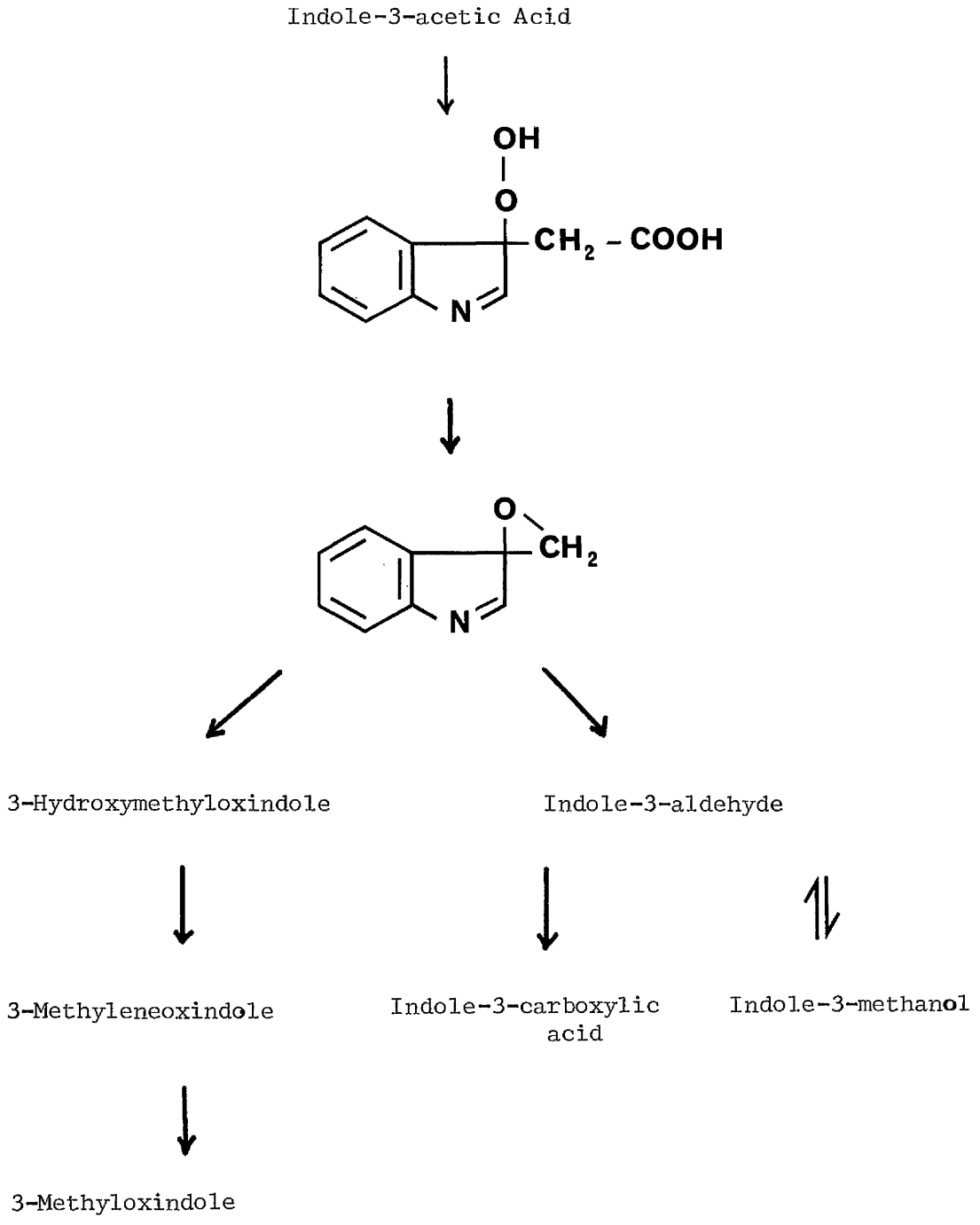
2-O-(Indole-3-acetyl)-myo-inositol

Table 4: Assessment of Methods Used to Identify IAA and its Metabolites in Complex Plant Extracts (Modified from Reeve & Crozier, 1980)

Technique	Detection Limit	Reference	Selectivity	Information Content (bits)
Bioassay e.g. Avena straight growth assay	20ng	Eliasson (1969)	medium	4
Colour Reactions				
Ehrlich's	50ng	Stahl (1965)	medium	4
Salkowski's	2μg	Tang and Bonner (1947)	medium	4
Spectrofluorimetry				
fixed	10pg	Reeve and Crozier	high	4
scan	1ng	(1980)	medium	32
Electrochemical	50pg	Sweetzer and Swartfager (1978)	medium	4
GLC Detectors				
Flame ionisation detection	10ng	Reeve and Crozier (1980)	low	4
Alkali flame ionisation detection	5ng	Swartz and Powell (1979)	medium	4
Electron capture detection	10pg	Seeley and Powell (1974)	high	4
Mass Spectrometry				
scan	100ng	Reeve and Crozier (1980)	low	2,000
single ion monitoring	100pg	" "	high	4
Infra-Red Spectroscopy	10μg	Powell (1967)	low	320
Spectrofluorometry of indole α pyrone derivative	1ng	Stoessl and Venis (1970)	high	4
Radioimmunoassay	100pg	Weiler et al. (1981)	high	4
UV Spectrometry (scan)	100ng	Reeve and Crozier (1980)	medium	24
Liquid Scintillation Spectrometry	depends on specific activity	———	high	4
^{1}H NMR spectroscopy	10 μg	Reeve and Crozier (1980)	low	124

Note: Where these techniques are used to detect compounds following chromatographic analysis, the information content quoted does not include that provided by the chromatogram. Although not easily quantified, this will be high in the cases of HPLC and capillary column GC.

Fig.2. Proposed Pathway of IAA Oxidation Catalysed by IAA Oxidases (Sembdner et al., 1980)



Moreover, Epstein et al. (1980) have failed to find any evidence for the evolution of $^{14}CO_2$ from carboxyl-labelled IAA. Instead Reinecke and Bandurski (1981) have identified oxindole-3-acetic acid as the major product of IAA catabolism in Zea mays caryopses. This compound must be formed by a quite different oxidation reaction and is unlikely to be catalysed by any of the IAA-oxidases studied. An alternative sequence of reactions to the decarboxylation pathway (see Fig. 3) was proposed by Kinashi et al. (1976) who extracted the methyl esters of oxindole-3-acetic acid and several related compounds from rice bran.

Analyses of endogenous indolic compounds have indicated that IAA conjugates may be important metabolites, on a quantitative basis. A large proportion of the IAA present, particularly in tissues such as seeds, with a storage function, is conjugated (e.g. Bandurski and Schulze, 1977). Dry Zea caryopses contained 70-90 mg IAA/g tissue as "alkali-labile" esters (Ueda and Bandurski, 1969) while the amounts of "free" IAA varied between 1 and 10% of this figure. The seedlings also contained the majority of their IAA in the form of esters (Bandurski and Schulze, 1974). In the caryopses about half of the esterified IAA was conjugated to high molecular weight glucans, most of the other half being esters of IAA with myo-inositol. The structures of the IAA-myo-inositol isomers have been verified using mass spectrometry (Ueda et al., 1970). Several esters of IAA with glucose were also identified from the same tissue (Ehmann 1974). Kopcewicz et al. (1974) fed Zea mays caryopses with labelled IAA and showed that it became esterified to the same compounds found in the tissue. An enzyme preparation, which was later purified (Michalczuk and Bandurski, 1980), was found to catalyse the esterification of IAA by the following route.

IAA + UDP-Glucose $\longrightarrow$ IAA-Glucose + UDP

IAA-Glucose + myo-Inositol $\longrightarrow$ IAA-myo-Inositol + Glucose

IAA-myo-Inositol + UDP-Galactose $\longrightarrow$ IAA-myo-Inositol-Galactoside + UDP

Several functions for the esters of IAA have been proposed. The large

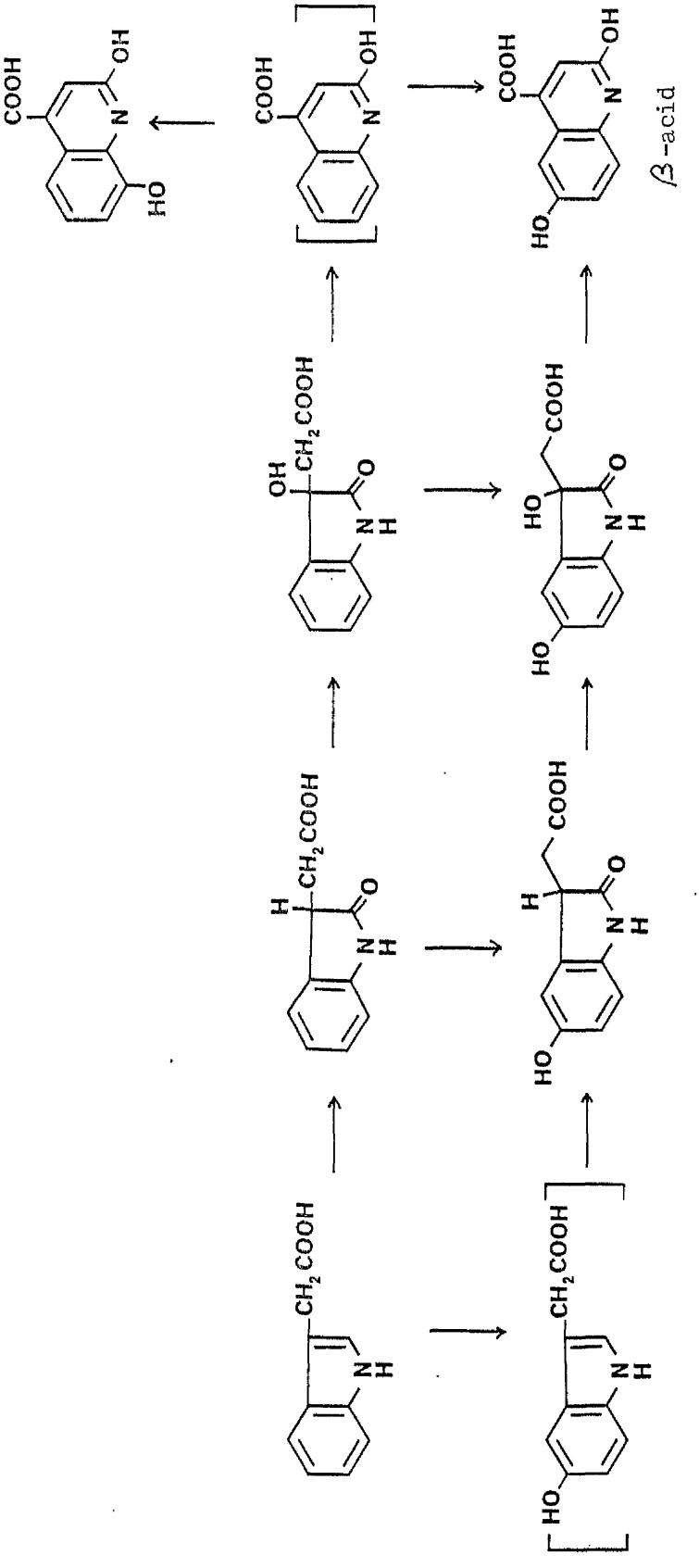


Fig.3. Scheme proposed by Kinashi et al. (1976) for the oxidation of IAA to β-acid

Compounds in brackets were not identified in the extracts from rice bran

decrease in high molecular weight esters of IAA during germination, accompanied by a small increase in free IAA, observed by Ueda and Bandurski (1969), suggested that these compounds have a storage function. Quantitative studies of the turnover of indolic compounds in Zea caryopses (Epstein et al., 1980) strongly indicated that high-molecular-weight esters are the principal source of IAA in the young seedling. Nowacki and Bandurski (1980) further demonstrated that IAA-myo-inositol was transported into the shoot, where it could be hydrolysed at a rate sufficient to fulfil the requirements for IAA. An enzyme preparation capable of catalysing this reaction has recently been extracted from Zea mays shoots (Hall and Bandurski, 1981).

In other species, labelled IAA has been shown to form conjugates with the amino acids glycine, valine, alanine, aspartic acid and glutamic acid (Feung et al., 1976). Endogenous IAA-aspartate has also been extracted recently from seeds of Glycine max (Cohen, 1981). The function of these conjugates is thought to be similar to that of esters of IAA, i.e. storage. Feung et al. (1977) and Hangarter and co-workers (Hangarter et al., 1980; Hangarter and Good, 1981) studied the physiological activity of various amino acid conjugates. The majority of compounds were apparently growth active although slightly less so than IAA. Activity of the conjugates outlasted that of IAA and was also related to the rate of decarboxylation, suggesting that these compounds were acting as slow release sources of IAA. Conjugates with amino acids or sugars have also been identified as products of the metabolism of the synthetic auxin 2,4-dichlorophenoxyacetic acid (Scheel and Sanderman, 1981).

The lack of evidence for decarboxylation of IAA in vivo casts some doubt on whether traditional IAA-oxidases are involved in the catabolism of endogenous IAA. It is now known that plants often contain several peroxidase isoenzymes which frequently possess varying degrees of IAA-oxidase activity and have different cofactor requirements (e.g. McCune,

1961; Sequeira and Mineo, 1966; Ockerse et al., 1975). Schneider and Wightman (1976) have described four classes of IAA-oxidase/peroxidase enzymes:

1. Typical peroxidases that catalyse peroxidation reactions at a very high rate while also oxidising IAA more slowly (e.g. Horseradish peroxidase).
2. Enzymes that have both high peroxidase and high IAA-oxidase activity (e.g. Turnip peroxidase P_7, Ricard et al., 1972).
3. Peroxidases that show no IAA-oxidase activity (e.g. from pea roots, Collet, 1968).
4. IAA-oxidases that display no peroxidase activity (e.g. Sequeira and Mineo, 1966; Bryant and Lane, 1975). The non-peroxidative enzyme isolated by Bryant and Lane did not possess a heme group.

Conflicting opinions exist as to whether IAA-oxidase activity and peroxidase activity can be separated (c.f. Lee, 1976; Laurema , 1974). Stonier et al. (1979) proposed that high molecular weight IAA-oxidase inhibitors, known as auxin protectors, which do not inhibit peroxidase activity, may contaminate enzyme preparations to varying degrees giving the appearance that IAA-oxidase and peroxidase activity is due to different enzymes. Siegel and Galston (1967) suggested that there might be different active sites for IAA-oxidase and peroxidase activity on the same enzyme, a view also shared by Stutz (1957). Removal of the heme prosthetic group with a mixture of cold acetone and HCl destroyed the peroxidase activity, although the apoenzyme retained its IAA-oxidase activity. This result was not, however, obtained by Ku et al. (1970) who found that both IAA-oxidase and peroxidase activity stopped when the heme group was removed by a different method.

Auxin protectors are reported (Stonier, 1976) to be derivatives, complexes or polymers of o-dihydroxyphenyl propanoids. O- and p-dihydric phenols and polyphenols are also generally found to be inhibitory to in vitro IAA oxidation (Ray, 1958; Sirois and Miller, 1972). The latter compounds

affect the oxidation rate, rather than induce a lag period (Stonier, 1970). In contrast, certain monophenols and m-dihydric phenols e.g. 2,4-dichlorophenol, are often required as cofactors (Ray, 1958). Mn^{2+} ions are also frequently needed. Maclachlan and Waygood (1956 a,b) have suggested that IAA may not be directly oxidised by the enzyme, but indirectly by Mn^{3+} formed in the mixture. Earlier, Kenten (1955) had demonstrated that peroxidase could oxidise Mn^{2+} to Mn^{3+} in the presence of certain phenols. Maclachlan and Waygood were able to decarboxylate IAA using a Mn^{3+} - EDTA solution. This was subsequently confirmed by BeMiller and Colilla (1972) who claimed that the products of this reaction were identical to those of the enzymic process. Some highly purified enzymes may, however, oxidise IAA in the absence of cofactors and several publications now favour a more intimate association of the enzyme with IAA, at least in the case of horseradish peroxidase (e.g. Gelinas, 1973; Yamazaki and Yamazaki, 1973; Nakajima and Yamazaki, 1979). The exact mechanism of the reaction is not fully understood, with several conflicting hypotheses being put forward on the basis of kinetic studies. However, it seems clear that the reaction involves the formation of free radicals (e.g. Parups, 1969; Meudt, 1971; Horng and Yang, 1975). Hydrogen peroxide is usually a requirement for peroxidase-catalysed IAA oxidation (e.g. Kokkinakis and Brooks, 1979) and, where H_2O_2 is not needed, it is thought that a bound form of peroxide is produced and used during the reaction (Mackáčková et al., 1975).

To summarize, much work has been confined to the details of the mechanism of IAA oxidation by a few enzymes, such as horseradish peroxidase, although the in vivo significance of this reaction is questionable. In addition to the lack of evidence for IAA decarboxylation products in vivo, the results of these studies show several points which bring further doubt on the rôle of IAA-oxidases:

1. There is a general lack of specificity shown by IAA-oxidising peroxidase enzymes (Butt, 1980).

2. The intimacy of the relation between substrate and enzyme has been questioned.

3. Certain inorganic species and UV radiation will catalyse IAA oxidation by the same pathway as IAA-oxidases.

(see Sembdner et al. 1980)

4. Peroxidase enzymes are known to be increased by injury and application of abnormal levels of growth substances (e.g. Birecka and Miller, 1974). Briggs et al. (1955) found that application of a drop of 0.005 M KCN to the cut ends of Osmunda cinnamomea fronds considerably increased the yield of diffusable auxin. It is possible that the IAA-oxidase/peroxidase system is produced in response to injury and its activity is predominantly artifactual.

5. Finally, the activity of tissue homogenates might sometimes result from the meaningless mixing of enzyme, phenolic compounds and inactive protein from different cells or organelles, and which are not normally in contact with one another.

On the other hand, there is some evidence to indicate that IAA-oxidases may have a rôle in vivo. A few reports have been published which suggest that IAA levels may be regulated by IAA-oxidase activity. An inverse relationship between enzyme activity and endogenous IAA content has been reported during abscission in bean seedlings (Jain et al., 1969) and light-induced hook opening of pea seedlings, by precise kinetic studies (De Greef et al., 1977). More recently, Saleh (1981) found that kinetin treatment increased the levels of extractable IAA in the roots of young Phaseolus vulgaris, Zea mays and Avena sativa plants. This treatment also decreased the amount of IAA-oxidase activity detectable. Nevertheless, the quantitative correlation was not good. The fall in IAA-oxidase activity was greatest in oat roots, where kinetin had very little effect on IAA levels, but was rather small in bean roots, where kinetin treatment

significantly raised the amount of IAA. Lee (1980) has published evidence that certain phenolic compounds which act as cofactors or inhibitors of IAA-oxidase preparations may have similar effects on the *in vivo* catabolism of IAA. Although Epstein *et al.* (1980) were not able to detect any $^{14}CO_2$, several other workers, using a variety of plant material, have detected substantial quantities of labelled CO_2 following application of IAA-1-^{14}C (eg. Wilkins *et al.*, 1972b, *Zea mays* roots; Epstein *et al.* 1977, orange callus; Epstein and Lavee, 1975, apple callus; Epstein and Lavee 1977a, olive leaves; Hamilton *et al.*, 1976, *Parthenocissus tricuspidata* callus tissue). It must be borne in mind that where experiments were performed in the light, or involved contact of IAA with cut surfaces, these results could be artifactual.

In conclusion, in order to understand whether IAA-oxidase has a rôle in the catabolism of endogenous IAA, there must be further definitive studies of IAA metabolism *in vivo*. In the past such work has been seriously hampered by the use of inadequate identification procedures and lack of controls, together with a disregard for the quantitative importance of the products studied. It is therefore proposed that ideally in future, the following precautions should be considered.

1. Where exogenous compounds are used, their initial purity must be proved.
2. Care should be taken to include controls with dead tissue and to check for breakdown of compounds during purification and analysis of extracts.
3. Identification of products must be rigorous and as far as possible quantitative (see Reeve and Crozier, 1980).
4. Exogenous IAA should not be added to *in vivo* systems in amounts which far exceed natural levels.
5. The site of application must be such that entry to the tissue mimics as closely as possible the endogenous system.
6. Contact of the IAA with wounded tissue is to be avoided as far as possible and must be considered when interpreting results.

7. It must be determined whether or not isotopic discrimination occurs in labelling experiments.
8. The possibility that surface microorganisms may be metabolising significant quantities of the applied substance should be borne in mind (see Libbert and Rische, 1969).
9. Experiments are based on the assumption that IAA itself is a biologically important compound. The growth activity and general physiology of metabolites needs to be considered.

With these guidelines in mind, this thesis presents a study of the metabolism of radioactively-labelled IAA in the root and coleoptile tissues of dark-grown Zea mays seedlings. Initial experiments were performed using segments floated in solutions of ^{14}C-IAA. Methanolic extracts of plant material were analysed by the traditional method of thin-layer chromatography in three solvent systems and by the more efficient separatory technique of gradient-elution reverse-phase high-performance liquid chromatography. Virtually no sample purification was carried out, to allow analysis of the whole spectrum of IAA metabolites. Controls using boiled tissue were included, and small-scale experiments were carried out using sterile roots to ensure that the metabolism observed was not due to contamination. The effect of external IAA concentration on the metabolism pattern was monitored; uptake of exogenous IAA was compared with endogenous levels. The changes in the pattern of metabolites were followed during time-course experiments and quantities of label remaining associated with the IAA at each time were estimated. Root segments could be divided into epidermis plus cortex and stele, and the metabolism of the two different tissues compared. In later experiments IAA was supplied to root segments via the stele, from agar blocks. This method was also used to supply IAA to the apical ends of coleoptile segments. In this way, metabolism of IAA from a source more closely resembling the endogenous supply could be monitored. The metabolism of the IAA transported through the segments was also studied. Finally, some information

on the chemical structures of metabolites was gained from comparison of ^{14}C-IAA labelled in different positions, tests for methylation and hydrolyis, co-chromatography with standards of putative IAA metabolites, and UV spectrometry.

A few preliminary experiments were carried out to study the effect of IAA on root and coleoptile elongation.

MATERIALS AND METHODS

1. PLANT MATERIAL

Dark-grown seedlings of Zea mays L. cv. Fronica were used for the majority of experiments although preliminary studies were carried out using the variety Kelvedon 33. Caryopses were obtained from Sinclair McGill (Scotland) Ltd. and stored at 4°C prior to sowing. Plants were grown on damp tissue paper in plastic seed trays covered with a glass sheet, in darkness at 22°C. After 3-4 days, seedlings with roots between 35 and 55mm in length were used as a source of root tissue (Frontispiece), whereas coleoptiles between 15 and 25mm in length were selected from 5-day-old plants.

For metabolism experiments root segments, 20mm in length, were taken from 1mm behind the tip unless stated otherwise. Sections 5mm long were used for experiments on the effect of IAA on growth. This region contains the elongation zone, but excludes the root cap and the majority of the meristem (Erickson and Sax, 1956; and Pilet and Senn, 1980). Coleoptile segments, 10mm in length, were taken from 2mm behind the apex and the leaf tissue was removed from the centre. Sections were cut either in dim green light or in the laboratory, as stated in each experiment.

The physiological safe light was obtained by covering a 40W Tungsten bulb with red and green Cinnamoid filter. This allowed light of between 520 and 590 nm to be transmitted. Tests for physiological activity of this light, using Avena sativa and Zea mays coleoptiles proved negative.

Sterile Plants

Seeds were surface-sterilised by soaking in a 10% Chloros solution (Industrial Grade; ICI, Durham Chemicals Distributors Ltd.) for 30min. After washing thoroughly with autoclaved water, they were transferred to sterile glass petridishes lined with moist sterile filter paper and allowed to grow for 4 days. Manipulation of seeds and cutting of root segments were carried out in a sterile air-flow cabinet. To test for fungal and bacterial contamination, portions of the roots were incubated at 20°C for 2 weeks on either nutrient agar (pH 7.4) or potato dextrose agar (pH 6).

Nutrient agar was prepared by dissolving 'Oxoid' nutrient agar (14g) in distilled water (500 cm^3). The recipe for potato dextrose agar was as follows: potato (100g) was boiled in water (300 cm^3) and strained through muslin. Dextrose (10g) was added, and the volume brought up to 500 cm^3 before dissolving agar (10g). Both media were autoclaved for 15min at 120°C and 103 KPa. After cooling, 50mg of Streptomycin was added to the molten potato dextrose agar.

2. CHEMICALS

Chemicals were obtained from the following sources:

a. Solvents

methanol*	Lothian Chemicals, Edinburgh
diethyl ether*, chloroform	May and Baker Ltd.
propan-2-ol*, toluene	A & J Beveridge Ltd.
methyl acetate, methoxyethanol, ethyl acetate*	British Drug Houses Ltd.
acetic acid, formic acid, xylene	Hopkin and Williams Ltd.

Solvents denoted * were routinely glass distilled before use.

b. Gases

Oxygen free nitrogen, 2% propane/98% Argon	British Oxygen Company Ltd.

c. Bulk Chemicals

2,5-diphenyloxazole, 2-phenylethylamine, ammonia	Koch Light Laboratories Ltd.
Triton-X 100	A & J Beveridge Ltd.
agar (technical), nutrient agar	Oxoid Ltd.
NaOH, KOH, HCl	May and Baker Ltd.
dextrose	Hopkin and Williams Ltd.

d. Fine Chemicals

indole-3-acetic acid, indole-3-aldehyde, indole-3-carboxylic acid	Sigma Chemical Company Ltd.
N-methyl-N-nitro-p-toluene sulphonamide	
N,O-bis-(trimethylsilyl) trifluoroacetamide	Pierce & Warriner Ltd.

e. Radiochemicals

All radioactively labelled chemicals were obtained from Amersham International Ltd.

compound	specific activity
IAA-1-^{14}C	2.18 GBq mmol^{-1}
IAA-2-^{14}C	2.11 GBq mmol^{-1}
Na $^{14}CO_3$	2.21 GBq mmol^{-1}
^{14}C-hexadecane	18.6 KBq g^{-1}

Solutions of IAA

Unlabelled IAA was dissolved in a small volume of methanol and diluted to the required concentration with distilled water. For growth experiments the same amount of methanol (i.e. 2 mm^3 per 100 cm^3 of solution) was added to each IAA concentration and to the control. Aqueous solutions of IAA were stored in darkness at 4°C for no more than 48h.

^{14}C-IAA was stored in methanolic solution at -15°C. Aqueous solutions were made up when required.

3. EXPERIMENTAL PROCEDURES

(a) Metabolism experiments

Incubation

For metabolism experiments, root segments were incubated in groups of 50 in

50mm diameter glass petridishes containing 2 cm^3 aqueous IAA solution. Coleoptile segments were incubated in groups of 20 or 40. All experiments were carried out in darkness at 22°C. After incubation the tissue from each dish was washed 5 or 6 times with 25 cm^3 distilled water for 1min each, by which time the efflux of radioactivity had reached a steady value (see Fig. 5).

Blocks of agar containing IAA for experiments E and G were prepared as follows. Approximately 2 cm^3 of a hot 1.5% agar solution with 100 µl of methanol containing IAA-2-^{14}C was poured into moulds (25 x 21 x 3 mm) and allowed to set. The agar was divided into 8 blocks of approximate dimensions 12 x 5 x 3 mm, each of which was used to supply IAA to 5 root or coleoptile segments.

Preparation of Extracts

Segments were extracted overnight with redistilled methanol at 4°C (25 cm^3 methanol per 50 segments). Extracts were then filtered. Two alternative methods of sample preparation were used:

1. Small root extracts were reduced to dryness using a Buchii rotary evaporator. After being redissolved in 1 cm^3 of methanol and centrifuged at 10,000 x g, samples were stored at -15°C in darkness until required for analysis.

2. The majority of coleoptile extracts, and root extracts from more than 100 segments were reduced in vacuo to the aqueous phase. They were then diluted with ammonium acetate buffer (pH 3.5, 20 mol m^{-3}) and loaded onto C_{18} Sep-pak cartridges (Waters Associates Inc.) using a 5 cm^3 glass syringe. Cartridges were wetted prior to use by pumping through 5 cm^3 methanol followed by 2 cm^3 buffer. Radioactive compounds in the extracts were eluted from the cartridges with 60% methanol in ammonium acetate buffer (2 cm^3). The methanol was blown off under a stream of N_2 and the samples freeze dried. Extracts were redissolved in 1 cm^3 methanol for storage until required for analysis.

Immediately prior to HPLC analysis all samples were blown dry under N_2

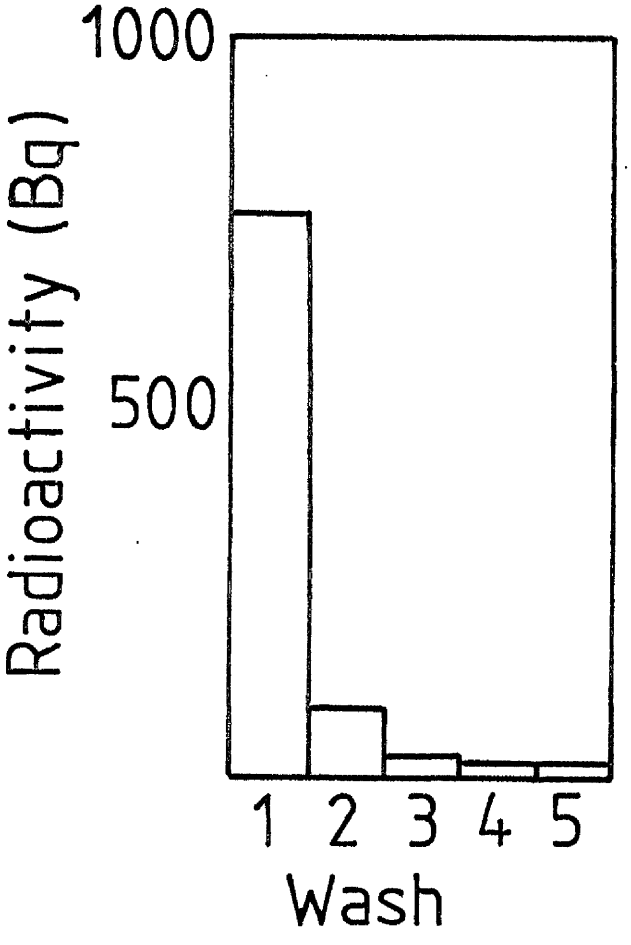


Fig. 5. Radioactivity present in successive washings of root segments incubated for 2h in IAA-2-^{14}C (10^{-3} mol m^{-3})

and dissolved in 100 mm^3 10% methanol in ammonium acetate buffer (pH 3.5, 20 mol m^{-3}).

(b) Growth Experiments

Incubation

Root segments, 5mm in length, were incubated in groups of 10, in 50mm diameter glass petridishes containing 2 or 5 cm^3 aqueous IAA or control solution. Incubation usually took place in darkness at 22°C. Roots in some experiments were incubated in the laboratory at 18-20°C.

Measurement of Growth

Tissue segments were measured using the shadowgraph method. Sections were placed on a glass plate and a photographic enlarger used to produce images of x5 magnification which were then measured using a ruler with 0.5mm divisions. Growth of intact plants was determined by placing spots of indian ink 1mm and 6mm from the root tip. The distance between the spots after incubation was measured using a ruler.

4. LIQUID SCINTILLATION SPECTROMETRY

The amount of radioactivity present in samples was assayed using Packard model 3380 liquid scintillation spectrometers. Samples were placed in 20 cm^3 plastic vials with 10 cm^3 scintillation cocktail. They were routinely counted for 5min.

Scintillation cocktails

A. For non-aqueous samples:

4 g cm^{-3} PPO (2,5-diphenyloxazole) in toluene

B. For aqueous samples:

2 parts A plus 1 part Triton-X 100

C. For both aqueous and non-aqueous samples in later experiments:

Packard Emulsifier-Scintillator 299

D. For trapping $^{14}CO_2$ in sample oxidiser

7g PPO

50 cm^3 H_2O

220 cm^3 methanol

330 cm^3 2-phenylethylamine

400 cm^3 toluene

Counting Efficiency

The instruments used comprised an external standard radiation source, the counting efficiency for which was automatically recorded for each sample. Actual counting efficiency for ^{14}C was obtained from a calibration graph in which the counting efficiency for a known quantity of ^{14}C hexadecane was plotted against the A.E.S. (Automatic External Standard) ratio. A quantity of around 20 mm^3 of labelled hexadecane was weighed accurately in a scintillation vial and 10 cm^3 of scintillation cocktail added. The number of counts and the A.E.S. ratio were recorded as an appropriate "quenching agent" was added dropwise to the vial. Separate calibration curves were prepared for different scintillants. Three quenching agents were used: water, a methanolic root extract and a methanolic coleoptile extract.

Oxidation of Tissue for Radioassay

After methanolic extraction, the quantity of radioactivity remaining in the tissue was measured. The plant material was combusted using an Intertechnique IN 4101 Sample Oxidiser. The $^{14}CO_2$ produced was trapped by phenylethylamine in the scintillation cocktail and the radioactivity assayed by liquid scintillation spectrometry.

5. THIN-LAYER CHROMATOGRAPHY

Glass TLC plates, precoated by Camlab (Macharey-Nagel and Co.) with a 0.25mm layer of silica gel were used. Chromatograms were developed for a distance of 15cm in one of three solvent systems:

1. Methyl acetate : propan-2-ol : 25% ammonia (45 : 35 : 20 v/v)
2. Chloroform : methanol : glacial acetic acid (75 : 20 : 5 v/v)
3. Chloroform : ethyl acetate : 90% formic acid (35 : 55 : 10 v/v)

Solvents used for TLC were either redistilled in glass, or Analar grade. The position of radioactive compounds was found by scanning the plates using a Panax thin-layer radiochromatogram scanner. Alternatively the silica gel from each of ten zones was scraped off, and the quantity of radioactivity determined by liquid scintillation spectrometry.

6. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The HPLC system used is shown in diagrammatic form in Fig. 5a. Solvents were delivered at flow rates of either 1 or 0.75 cm^3 min^{-1} by an Altex Model 332 gradient liquid chromatograph (Altex Scientific Inc.). A Shandon reverse-phase column (I.D. 250 x 5mm) packed with 5 μm ODS Hypersil (Shandon Southern Products Ltd.) was used. Samples were introduced off column *via* a Perkin-Elmer Model 7105 sample valve. The mobile phase consisted of a mixture of HPLC grade methanol (Rathburn Chemicals Ltd.) and ammonium acetate buffer (pH 3.5, 20 mol m^{-3}) The buffer was cleaned prior to use by passing through a 250 x 10mm I.D. column packed with 10 μm ODS silica gel. Three solvent gradients were used:

A. 10 to 60% methanol over 20 min

B. 10 to 60% methanol over 30 min

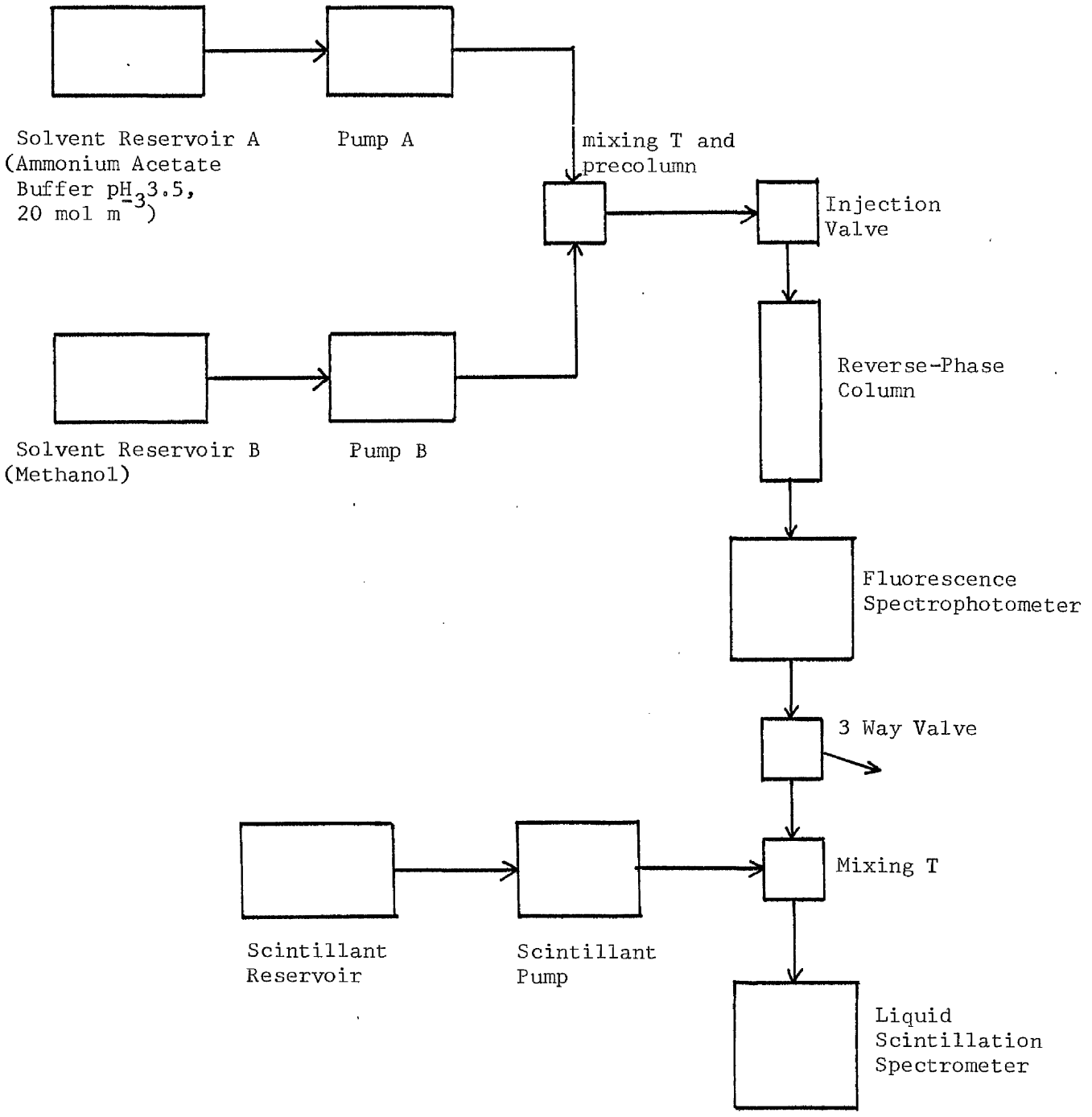
C. 50 to 100% methanol over 20 min.

The percentage of methanol was then increased to 100% over 5min and the column cleaned for a further 10min. The solvent composition was returned to the initial value and the column equilibrated for 10min prior to injecting the next sample.

Two detectors were used in series. The column was connected to a Perkin-Elmer 650-10S Fluorescence Spectrophotometer fitted with a 16 mm^3 flow cell. The excitation and emission wavelengths were set at 280 and 350 nm respectively (the fluorescence λ_{max} for IAA; Crozier *et al.*, 1980) Eluant leaving the fluorimeter was mixed with scintillant and passed through a 400 mm^3 spiral glass flow cell in an adapted ICN Tracerlab manual scintillation spectrometer (see Reeve and Crozier, 1977). The scintillant comprised 10g 2,5-diphenyloxazole, 330 cm^3 Triton X-100, 670 cm^3 distilled xylene and 150 cm^3 methanol. A 3:1 scintillant/eluant ratio was compatible with all HPLC mobile phase conditions and gave a *circa* 65% counting efficiency for ^{14}C solutes.

During analysis of unlabelled indole standards the fluorescence detector

Fig, 5a. HPLC Apparatus



was substituted with a Pye Unicam LC 871 UV absorbance detector.

The HPLC column gave 15,000 theoretical plates with an ODS test mixture comprising benzamide, acetophenone, benzophenone and biphenyl. With IAA this value was considerably lower (circa 4,000 plates). This is the result of ionisation of a proportion of the molecules at the surface of the stationary phase.

The resolution of radioactive peaks was reduced due to mixing in the connecting tubing (which was kept to a minimum) and the fluorimeter flow cell, and also due to the size of the radioactivity monitor flow cell. The width of the fluorescence peak attributable to IAA, eluting isocratically in 50% methanol at a flow rate of 1 cm^3 min^{-1} was approximately 28s compared with 57s for the radioactive peak.

7. PREPARATION OF DERIVATIVES

(a) Trimethylsilylation

Samples were dried thoroughly, dissolved in 50 or 100 µl N,O-bis-(trimethylsilyl) trifluoroacetamide and heated at 60°C for 2h. This procedure was shown by McDougall (1978) to give 99% yield of the bis-trimethylsilyl derivative of IAA.

(b) Methylation

Compounds were methylated using an etherial solution of diazomethane prepared using a modification of the method of Schlenk and Gellerman (1960). Approximately 0.5g N-methyl-N-nitro-p toluene sulphonamide was placed in a 100 cm^3 conical flask with side arm attachment. To this was added 10 cm^3 diethyl ether, 1 cm^3 methoxyethanol, and 3 cm^3 concentrated potassium hydroxide. The flask was stoppered and heated in a hot water bath. The open end of the side arm was placed under ca. 10 cm^3 diethyl ether in a flask cooled in ice. On heating the reaction mixture, diazomethane gas and

diethyl ether distilled over and dissolved in the cold ether. When an intense yellow colour was observed, the solution was ready for use.

Samples were dissolved in 100 mm^3 methanol. After addition of 0.5 cm^3 of diazomethane solution they were stored at 4°C for at least 2h before blowing dry under a stream of nitrogen. This method converted ^{14}C-IAA to a single peak which co-chromatographed with IAA-methyl ester on the HPLC column.

8. COMBINED GAS CHROMATOGRAPHY-MASS SPECTROMETRY

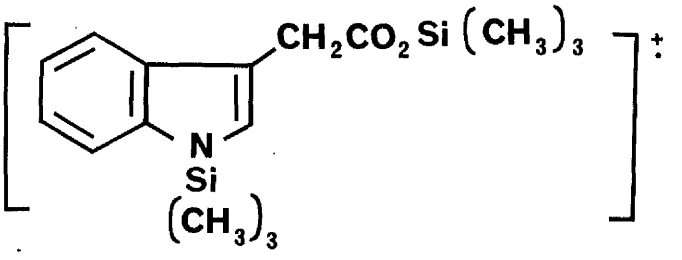
The GC-MS instrumentation comprised a single beam, double focusing AEI-MS 30 mass spectrometer coupled *via* a single stage glass jet separator to a Pye 104 gas chromatograph. For selected ion monitoring an AEI Multipeak Selectro WF-055 multipeak monitor acted as an accelerating voltage alternator. Up to 6 ions could be monitored at one time. The tuning of the instrument was checked using a perfluorokerosene standard which gave a reference peak at *m/e* 181. Gas chromatograph and mass spectrometer operating conditions are stated separately for each experiment.

For selected ion-monitoring detection of IAA the instrument was tuned to detect ions of *m/e* 202, the most prominent peak in the mass spectrum of bis-trimethylsilyl IAA (see Fig. 6).

9. UV SPECTROPHOTOMETRY

UV absorbance spectra were obtained using a Unicam SP 8000 UV recording spectrophotometer. Samples were dissolved in methanol, placed in 1cm quartz cuvettes and scanned from 200-450 nm.

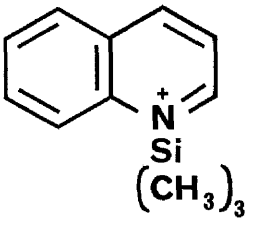
Fig. 6a. Fragmentation Pattern of IAA-TMSi



m/e 319 (M^+)

m/e 304 = $(M - CH_3)^+$

m/e 202 =



m/e 73 = $\overset{+}{Si}(CH_3)_3$

m/e 75 = $(CH_3)_2 Si = \overset{+}{O}H$

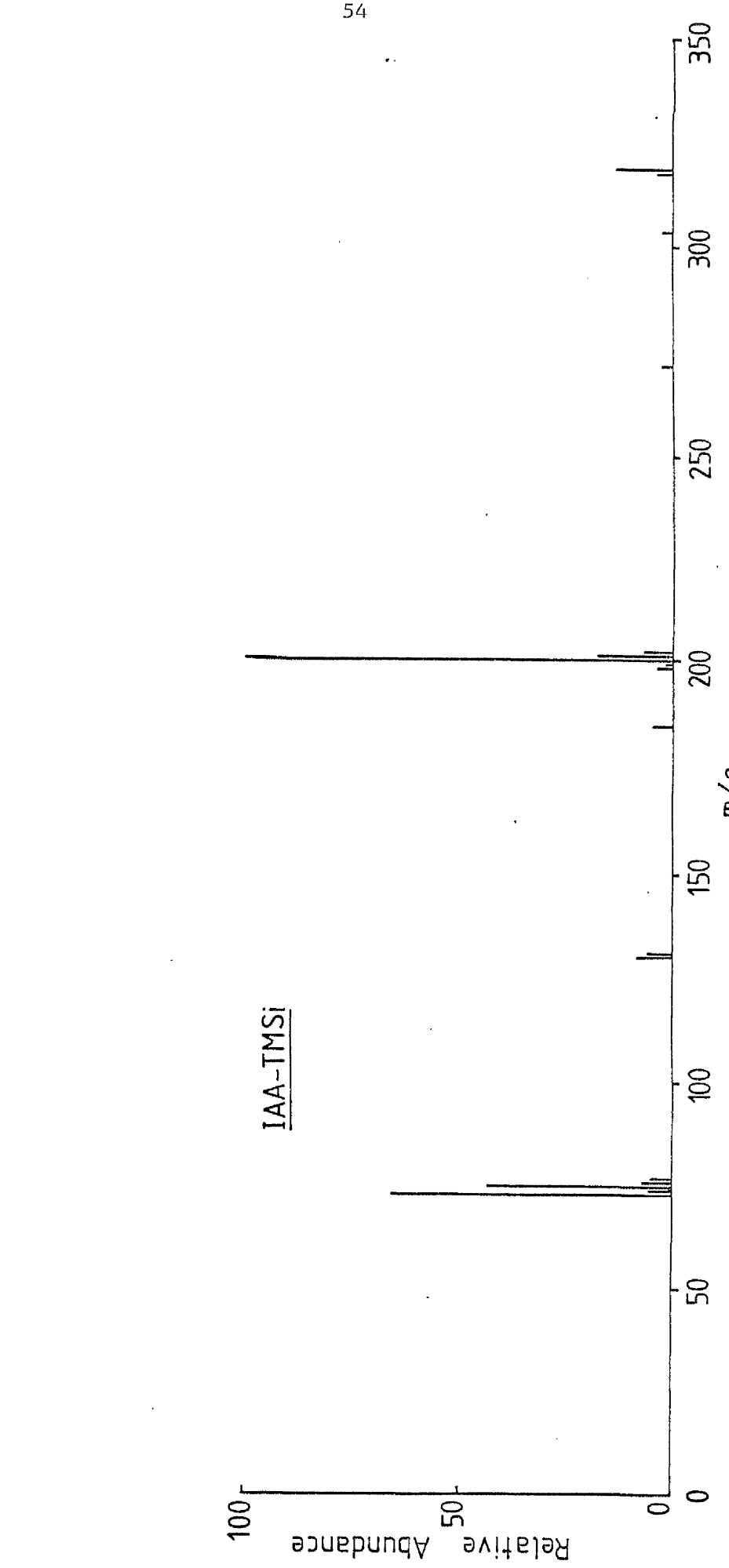


Fig. 6. Mass spectrum of IAA-TMSi
GC Conditions: column; 5ft x ¼" I_3 (1.5m x 6.4mm) 3% Dexil-300. temperature; 190°C.
flow rate; 20 $cm^3 min^{-1}$. T_R 5 min.
MS Conditions: 70ev; source temperature 280°C. separator temperature 275°C.

10. SYNTHESIS OF IAA DERIVATIVES

Conjugates of IAA with glycine, valine and alanine

Conjugates of IAA with the amino acids glycine, valine and alanine were prepared as follows using a modification of the methods of Mollan *et al.* (1972) and Hangarter *et al.* (1980). The tetramethylammonium salts were prepared by adding 50 or 100 mmol of each amino acid to 25% aqueous solutions of tetramethylammonium hydroxide. After dissolving, using additional water when necessary, the salts were freeze dried. Each compound was then suspended in dimethyl formamide (5 cm^3) and the mixed anhydrides prepared. IAA (0.88g) was dissolved in a mixture of tetrahydrofuran (20 cm^3) and triethylamine (0.7 cm^3) and stirred in a flask cooled in an ice bath. Over a period of 30s, ethyl chloroformate (0.48 cm^3) was added dropwise with rapid stirring. Stirring was continued for 10 min. The white precipitate was then filtered off and the filtrate added dropwise to the suspension of the amino acid salt. Stirring was continued for 2h at room temperature.

Water (30 cm^3) and diethyl ether (30 cm^3) were added to the reaction mixture and the whole was shaken. The ether layer was separated off and the aqueous phase acidified to pH 5 with HCl. This was then extracted twice with ether before further acidification to pH 2.

The glycine and L-valine conjugates were precipitated on acidification to pH 2. The L-alanine conjugate was obtained by freeze drying the acidified (pH 2) aqueous phase. The residue was dissolved in methanol or ethyl acetate, filtered and evaporated to dryness. All products were analysed by GC-MS and found to contain the amino acid conjugate plus some unreacted IAA (see Experiment H.8).

Oxindole-3-acetic Acid

This was prepared according to the methods of Hinman and Bauman (1964) and Reinecke and Bandurski (1981). N-Bromosuccinimide (0.712 g) was added to a solution of IAA (0.7 g) in tertiary butyl alcohol (25 cm^3) at room temperature and stirred. After 2h the solution was concentrated under reduced pressure at room temperature, to give a thick syrup. Water (30 cm^3) was then added and the mixture extracted three times with ethyl acetate (8 cm^3). The combined extracts were washed with saturated salt solution, dried over Na_2SO_4 and evaporated at room temperature. Residual ethyl acetate was entrained by repeated evaporation with acetone *in vacuo*. The residue was taken up in a mixture of acetone (0.5 cm^3) and benzene (8 cm^3). This was left for 2 days at -20°C. The resulting yellow solid was filtered and recrystallised twice from acetone/benzene before drying in a vacuum desiccator. The identity of the product was confirmed by mass spectrometry (see Experiment H.8).

RESULTS

This part of the thesis is divided into two sections:

1. An investigation of the metabolism of IAA in coleoptile and root tissues of dark-grown Zea mays seedlings.
2. A study of the effect of exogenous IAA on the growth of these tissues.

The aim of the first section was to assess quantitatively the metabolic fate of exogenous IAA, supplied to root and coleoptile segments. Reverse-phase high-performance liquid chromatography combined with a continuous radioactivity monitor was used for most analyses, and was compared with the older technique of thin-layer chromatography. A number of methods, including combined capillary-column gas chromatography-mass spectrometry were used to obtain information on the chemical structures of the products. Measurements of the uptake of IAA and its effect on growth confirmed that the amount of phytohormone entering the tissue was comparable to the endogenous content and was physiologically active.

SECTION 1 - METABOLISM OF IAA

A. Comparison of Separatory Techniques Used in the Analysis of IAA Metabolites in Plant Extracts

Traditionally, the techniques of thin-layer and paper chromatography have been used extensively in the separation and characterisation of IAA metabolites (e.g. Tuli and Moyed, 1967; Davies, 1972; Epstein and Lavee, 1977b; Magnus et al., 1982). Recently various forms of high-performance liquid chromatography (HPLC) have been used to analyse phytohormones in plant extracts (e.g. Durley and Kannangara, 1976; Crozier and Reeve, 1977; Sweetser and Swartzfager, 1978) and also to purify their metabolites (e.g. Summons et al., 1980; and Reinecke and Bandurski, 1981). HPLC has the advantage of potentially much greater resolution than thin-layer or paper chromatography. The following three experiments illustrate the usefulness of a reverse-phase HPLC system in the separation of IAA metabolites, when compared with thin-layer chromatography (TLC).

A.1 TLC Analysis of IAA Metabolites

Methanolic extracts containing the products of ^{14}C-IAA metabolism were prepared from groups of 50 root segments which had been incubated in aqueous solutions of IAA-2-^{14}C (10^{-2}mol m^{-3}) for 2h in darkness. After drying in vacuo and redissolving in a small volume of methanol, extracts were spotted onto silica gel TLC plates and developed in three different solvent systems:

1. Methyl acetate : propan-2-ol : 25% ammonia (45 : 35 : 20 v/v)
2. Chloroform : methanol : acetic acid (75 : 20 : 5 v/v)
3. Chloroform : ethyl acetate : 90% formic acid (35 : 55 : 10 v/v)

The positions of radioactive compounds were detected using a thin-layer radiochromatogram scanner (Fig. 7). The number of distinct peaks varied from two to five according to the solvent system. The most

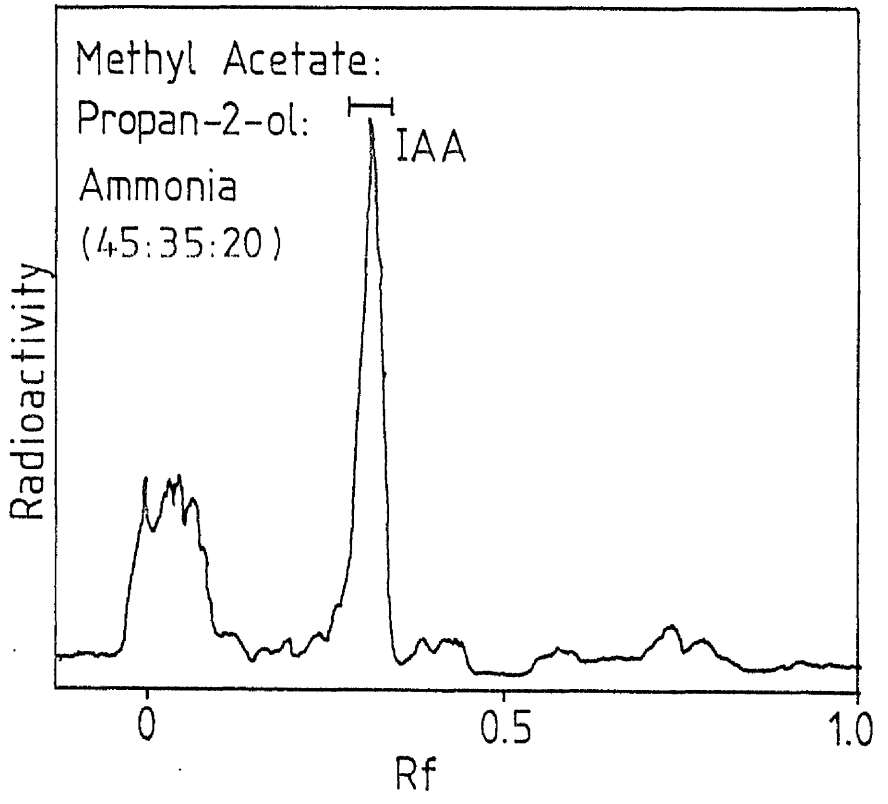


Fig. 7. TLC analysis of IAA-2-^{14}C metabolites from extracts of Zea mays roots. Root segments were incubated in IAA-2-^{14}C (10^{-2} mol m^{-3}) for 2h. Traces represent typical results from 3 replicate experiments.

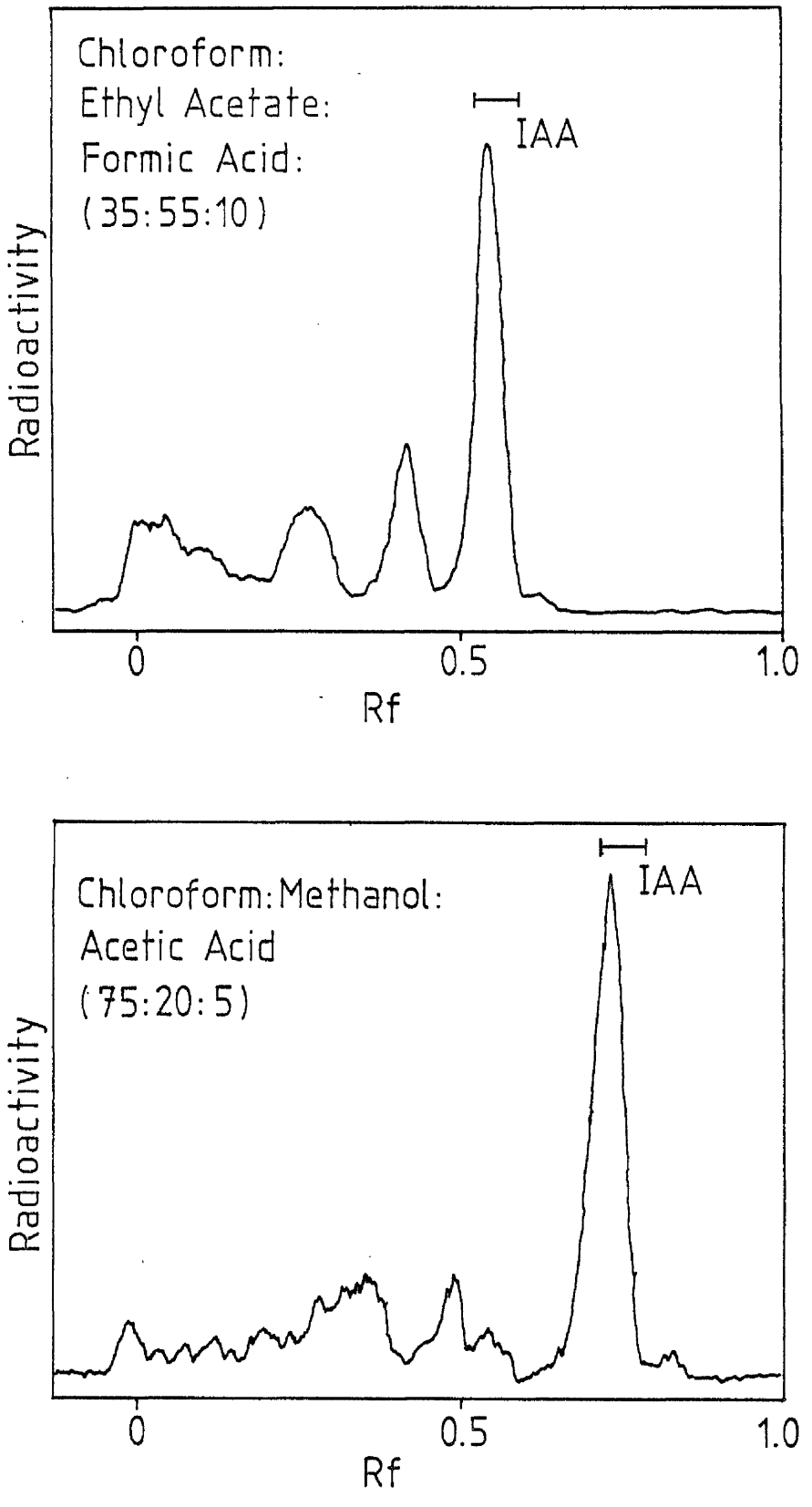

Chloroform:
Ethyl Acetate:
Formic Acid:
(35:55:10)
IAA
Radioactivity
0
0.5
1.0
Rf
Chloroform:Methanol:
Acetic Acid
(75:20:5)
IAA
Radioactivity
0
0.5
1.0
Rf

Fig. 7. Cont'd

prominent peak always had the same Rf value as IAA.

A.2 HPLC Analysis of IAA Metabolites

Extracts were prepared in the same manner, from root segments which had been incubated with IAA-2-^{14}C (10^{-3}mol m^{-3}) for 2h. Samples, dissolved in 100 mm^3 of 10% methanol in ammonium acetate buffer (pH 3.5, 20 mol m^{-3}), were injected onto the reverse-phase HPLC column. A solvent gradient of 10 to 60% methanol was run over 30 min. Fig. 8 shows the profile of radioactive peaks eluting from the column (typical result from 6 replicate experiments). A large peak with a retention time identical to that of IAA was apparent, together with at least 11 metabolite peaks all eluting before IAA. When six identical extracts were analysed during a single day these 12 peaks were consistently present and the variation in retention time of each peak was small (Table 5) giving a strong indication that the same compounds were consistently present. The metabolite peaks shown in Fig. 8 were thus labelled 1 to 11 for identification. In order to achieve an objective comparison of extracts from different experiments, in the absence of definitive identification of compounds, samples from roots incubated in 10^{-3} mol m^{-3} IAA for 2h were used as standards for numbering of peaks throughout the thesis. Peaks 7 and 8, and 1 and 2 eluted close together and were not always resolved. When this was the case the peaks were labelled 7/8 and 1/2.

A.3 Use of HPLC and TLC in a Preparative Mode

Although the column used for HPLC was of a narrow bore it could be used in a preparative mode to purify metabolite peaks. Samples of peaks 4-11 and IAA were collected after separation on the column, dried and rechromatographed. Peaks 7, 8, 9, 10 and IAA remained unchanged (Table 6). Peaks 4 and 5 appeared to be interconverted, while a proportion of radioactivity from peak 6 appeared with the retention time of peak 7. Some of peak 11 was converted to a new product.

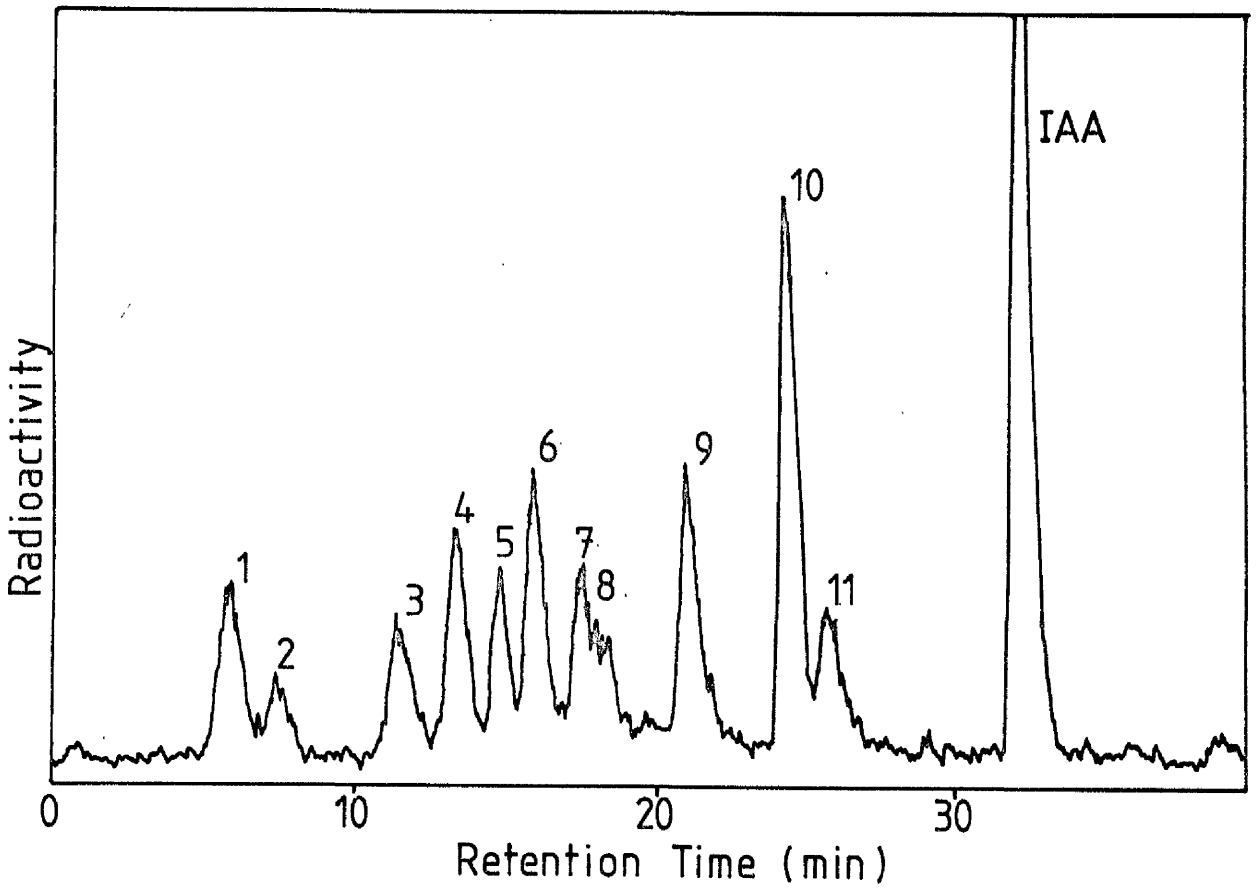


Fig. 8. HPLC Analysis of a Zea mays root extract containing metabolites of IAA-2-^{14}C. Root segments were incubated in 10^{-3} mol m^{-3} ^{14}C-IAA for 2h and extracted in methanol. The trace represents a typical result from 6 replicate experiments. Solvent gradient : 10-60% methanol over 30 min. Flow rate : 0.75 $cm^3 min^{-1}$. Detector : homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time constant.

Table 5 : Variation in retention times (min) of radioactive peaks representing ^{14}C-IAA metabolites in extracts of Zea mays roots, chromatographed on the same day

Peak No	Replicate 1	2	3	4	5	6	Mean ± standard error
1	5.7	6.0	6.2	5.8	6.2	5.9	6.0 ± 0.08
2	7.4	7.2	7.0	6.8	7.2	6.8	7.1 ± 0.10
3	11.2	11.4	11.0	10.8	11.0	10.6	11.0 ± 0.12
4	13.4	13.2	12.4	12.7	12.6	12.3	12.8 ± 0.18
5	14.7	14.8	14.0	14.1	14.0	13.8	14.2 ± 0.17
6	15.9	15.8	15.3	15.3	15.3	15.2	15.5 ± 0.12
7	17.6	17.4	16.8	17.0	17.0	16.6	17.1 ± 0.15
8	18.2	18.2	17.6	17.6	17.7	17.6	17.8 ± 0.12
9	21.0	21.0	20.1	20.4	20.4	20.2	20.5 ± 0.16
10	24.3	24.3	23.4	23.8	23.8	23.7	23.9 ± 0.14
11	25.8	25.8	25.0	25.2	25.2	25.1	25.4 ± 0.15
IAA	32.1	32.1	31.2	31.5	31.4	31.5	31.6 ± 0.15

Table 6 : Degradation of IAA and its metabolites during preparative purification by HPLC. Peaks 7 and 8 were not completely resolved and had to be collected together.

Peak No	Initial T_R on HPLC (min)	T_R during rechromatography after purification by HPLC (min)
4	9.9	9.4 (44%) + 11.1 (56%)
5	11.2	9.8 (28%) + 11.2 (72%)
6	12.6	12.4 (56%) + 14.0 (44%)
7/8	14.2 + 15	14.4 + 15.1
9	17.8	18.1
10	18.5*	18.7*
11	24	24.6 (69%) + 25.6 (31%)
IAA	30	29.6

* Peak 10 was analysed on a separate day

On the other hand, TLC was found to cause substantial sample degradation. Individual peaks, collected from the liquid chromatograph were loaded onto silica gel TLC plates, which were then developed in chloroform : ethyl acetate : 90% formic acid (35 : 55 : 10 v/v). Zones of radioactivity were collected and eluted, and the compounds rechromatographed on the HPLC. In each case at least some degradation had occurred. Peaks 4, 10 and IAA had undergone a small amount of breakdown, while peaks 5, 6, 7, 8 and 9 appeared to have changed completely. Peak 11 was not analysed.

A. Conclusions

The resolving power of HPLC is of considerable value in separating the large number of IAA metabolites present in methanol extracts of *Zea mays* root segments which had been incubated in IAA-2-^{14}C. Although it is possible that a single peak on the HPLC trace might represent more than one compound, TLC was clearly unable to separate individual metabolites. Furthermore, while degradation of compounds occurred during TLC separation, most metabolites could be recovered unchanged from the HPLC column (degradation of metabolites during TLC analysis might be reduced in other solvent systems). The reverse-phase HPLC column was ideal as all the metabolites appeared to be more polar than IAA.

B. Quantitative Fate of IAA-2-^{14}C Supplied to Zea mays Root Segments

In the past, metabolites of IAA which were analysed sometimes represented only a small fraction of the IAA supplied to the tissue (e.g. Tuli and Moyed, 1967; Magnus et al., 1971). The aim of this study was to quantify the amount of IAA metabolised and to record the relative production of the whole spectrum of IAA-2-^{14}C metabolites produced by Zea mays root segments. Control experiments were designed to confirm that the reactions were carried out by the living plant cells and to assess the effect of experimental conditions on the metabolism pattern. To answer the possibility that the compounds might represent products of a detoxification process unrelated to the normal pathways of endogenous IAA metabolism, the amount of IAA taken up by the roots was compared with published measurements of endogenous quantities (Bridges et al., 1973). The effect of varying the amount of IAA supplied, on the pattern of metabolism was also studied.

B.1 Metabolism of IAA-2-^{14}C by Root Segments during a 2h Incubation Period

Groups of 50 root segments from 3 to 4-day-old, dark-grown Zea mays seedlings, were incubated in aqueous solutions of IAA-2-^{14}C (10^{-3} mol m^{-3}; 2h ; darkness). All manipulations of plant material were carried out under dim green light. After following the washing procedure, the tissue was extracted overnight with methanol and samples prepared for HPLC analysis. When the column was routinely cleaned with tetrahydrofuran, it was possible to analyse relatively crude extracts, thereby eliminating the need for lengthy purification procedures, during which products might be lost. Root extracts were simply filtered, dried in vacuo, redissolved in 1 cm^3 methanol and centrifuged at 10,000 x g (method 1). Samples were stored at -15°C in this form until required. Prior to analysis, each sample was dried under a stream of N_2 and redissolved in 100 mm^3 of 10% methanol in

ammonium acetate buffer (pH 3.5, 20 mol m^{-3}). The average dry weight of an extract prepared in this manner was 25.4 mg. Aliquots for measuring radioactivity were taken from extracts at two stages: after filtration and after drying in vacuo. Substantial and variable losses (up to 36% of the sample; Table 7) occurred during drying in vacuo. Large losses at this stage were also noted by Mann and Jaworski (1970). The amounts of radioactivity lost during drying under N_2 and redissolving in 100 mm^3 10% methanol in buffer, and also due to centrifugation, were small. Radioactivity present in the incubating solutions at the end of the experiment, and in the washings was also measured by counting aliquots. The amount of label remaining in the roots after methanol extraction was assessed by combusting the tissue in a sample oxidiser and counting the $^{14}CO_2$ trapped by phenylethylamine.

The fate of IAA-2-^{14}C supplied to the root segments is presented in Table 7 (column 1). An average of 31% of the radioactivity supplied (44-45 Bq $segment^{-1}$) was taken into the tissue. Methanol extraction removed 93% of the ^{14}C from the roots (similar extraction efficiencies were found in all subsequent experiments). Losses of radioactivity during incubation were small (4.2% on average).

Fig. 9 shows the profile of radioactive compounds eluting from the HPLC column during the analysis of a typical methanolic root extract. Twelve peaks were consistently present (Table 8). The largest in each case co-chromatographed with IAA and also produced a fluorescence peak when the excitation and emission wavelengths of the fluorescence spectrophotometer were set at 280 and 350 nm respectively. Further evidence for the identity of this peak is presented in experiment H. After 2h incubation, an average of 46% of the radioactivity present in the methanol extracts remained associated with the IAA peak (calculated from the peak integrator on the chart recorder). Eleven metabolite peaks were present in each replicate and were numbered for identification as described in experiment A.2. The largest was always peak 10, which accounted for a mean of 25% of the radio-

Table 7 : Analysis of the Fate of IAA-2-^{14}C supplied in Aqueous Solution to *Zea mays* Root Segments. Root tissues (50 segments) were incubated for 2h in 10^{-3} mol m^{-3} IAA-2-^{14}C. Extracts designated (S) were purified using Sep-pak C_{18} cartridges. Radioactivity is expressed in Bq. The experiment was carried out in triplicate.

TREATMENT	Root segments cut in dim green light	Root segments cut in the laboratory	Sterile root segments	Dead root segments
1. Initial Radioactivity	7130 7190 7530	7210 7840 7900	32,000 21,400 23,500	6070 6350
2. Radioactivity remaining in incubating solution	3430 3620 3660	3500 3430 3490	- 17,000 13,100	3410 3300
3. Radioactivity in methanol extract	2110 2060 2130	2230 2250 2210	5,340 2,190 2,260	241 156
4. Radioactivity remaining in tissue after extraction	154 161 132	171 177 153	161 55 221	14 12
5. Radioactivity in washings	1180 1110 1160	1130 1600 1370	-	2520 2360
6. Final total of radioactivity	6880 6950 7080	7030 7450 7220	-	6190 5840
7. Loss of radioactivity during incubation	244 240 447	175 387 677	-	- 518

8. 7 expressed as a percentage of 1	3.4% 3.3% 5.9%	2.4% 4.9% 8.6%	-	- 8.2%
9. Dry weight of tissue (mg)	40.1 38.4 39.7	33.6 36.5 37.5	62.6 30.7 50.1	32.3 32.5
10. Uptake of radioactivity (Bq/segment)	45 44 45	48 48 47	-	5.1 3.4
11. Extraction Efficiency	93% 93% 94%	93% 93% 94%	97% 98% 91%	95% 93%
12. Radioactivity in methanol extract after preparation for HPLC	1440 1310 1370	1900 1680 1830	- 1640(S) 2020	229 149
13. 12 expressed as a percentage of 3	68% 64% 64%	85% 75% 83%	- 75% 89%	95% 96%
14. Radioactivity in sample of incubating solution after preparation for HPLC	2760 2940 3030	2820 2800 3020	-	2780 2920
15. 14 expressed as a percentage of 2	80% 81% 83%	81% 82% 87%	-	82% 82%

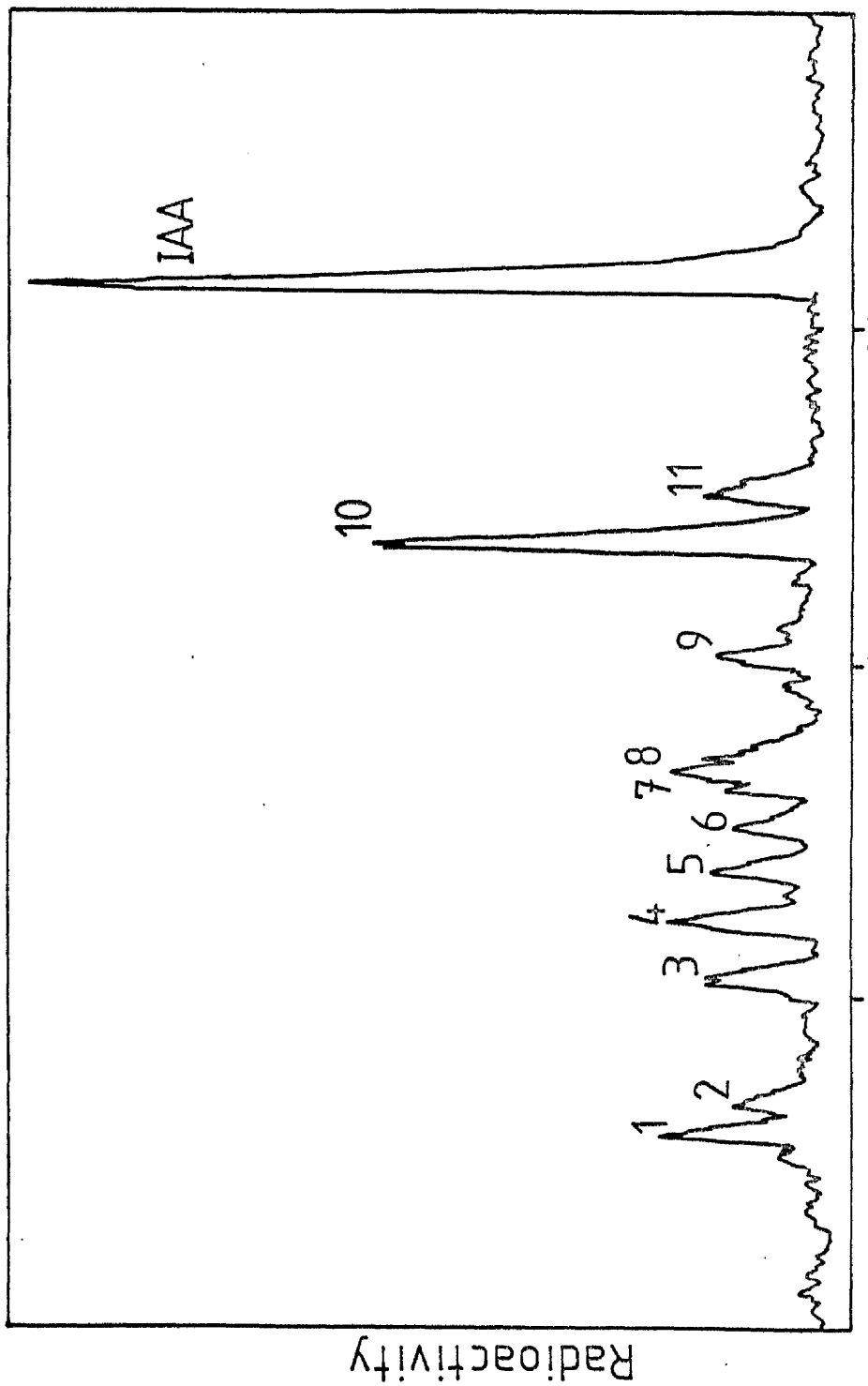


Fig.9. HPLC analysis of methanol extracts from root segments (cut in dim green light) incubated in IAA-2-^{14}C (10^{-5} mol m^{-3}) for 2h. Trace represents a typical result from 3 replicates. Solvent gradient: 10-60% methanol over 30 min. Flow rate: 0.75 cm min^{-1}. Detector: Homogeneous radioactivity monitor; 30cps full scale deflection; 10s time constant.

Table 8 : IAA Metabolites present in methanol extracts of root segments incubated in IAA-2-^{14}C for 2h. Metabolite peaks in extracts of non-sterile roots were numbered 1-11 (see Fig. 8) and used as standards for the other extracts (1). The experiment was carried out in triplicate.

TREATMENT	Root segments cut in dim green light	Root segments cut in the laboratory	Sterile root segments	Dead root segments
Radioactive peaks in methanol extracts in order of decreasing height (1)	IAA,10,1,7,9,6,4,5,8, 11,2,3	IAA,10,9,7,6,5,4,3, 1,11,8,2	10,8,IAA,2,4,5,7,1, 11,9,3,6	IAA only
	IAA,10,5,6,1,9,7,3,4, 8,2,11	IAA,10,9,6,1,5,7,8, 4,3,2,11	7,8,5,4,10,IAA,6, 9,2,1,3	IAA only
	IAA,10,1,4,7,3,8,11,5, 9,6,2	IAA,10,9,6,7,5,1,4, 3,8,2,11	10,IAA,8,7,5,4,9,1, 11,3,6,2	
% radioactivity in methanol extract, associated with IAA peak	49 40 48	34 29 30	11 11 14	100 100
% radioactivity in methanol extract, associated with peak 10	21 31 23	21 19 18	16 12 23	0 0
% radioactivity in incubating solution, associated with IAA peak	98 93 97	93 98 95	- 92 -	98 95

activity in each sample. The relative prominence of the other peaks varied.

Samples of the incubating solution were also analysed to determine the amount of IAA breakdown occurring during the experiment. Solutions were dried *in vacuo* and taken up in 1 cm^3 of methanol. Before analysis they were dried again under a stream of N_2 and redissolved in 100 mm^3 of 10% methanol in ammonium acetate buffer (pH 3.5, 20 mol m^{-3}). An average of 95% of radioactivity remained associated with the IAA peak (Table 8, Fig. 10) indicating that very little degradation had taken place.

IAA Breakdown during Sample Preparation

Three control experiments were carried out to confirm that the compounds obtained were in fact products of IAA metabolism by the plant tissue. To test whether any breakdown of IAA was occurring during the preparation of extracts, six groups of 50 root segments were extracted in methanol, aliquots of IAA-2-^{14}C were added and samples processed for HPLC analysis. Samples 1-3 were prepared according to method 1, while Sep-pak C_{18} cartridges (method 2) were used to purify samples 4-6. Typical HPLC traces from each group are presented in Fig. 11. The amount of breakdown in samples 1-3 was negligible although a small amount of peak 10 was observed in samples 4-6. Losses of radioactivity during sample preparation varied between 17 and 33% (Table 9) and were similar to those for extracts containing metabolites of IAA-2-^{14}C.

Metabolism of IAA by Killed Root Segments

Groups of 50 root segments which had been boiled for 2min were incubated for 2h in aqueous solutions of IAA-2-^{14}C (10^{-3} mol m^{-3}). After following the washing procedure, the tissue was extracted with methanol and samples prepared for HPLC analysis using method 1. No metabolism of IAA was apparent (Fig. 12; Table 8). A single radioactive peak with the same retention time as IAA was present and coincided with a peak for fluorescence.

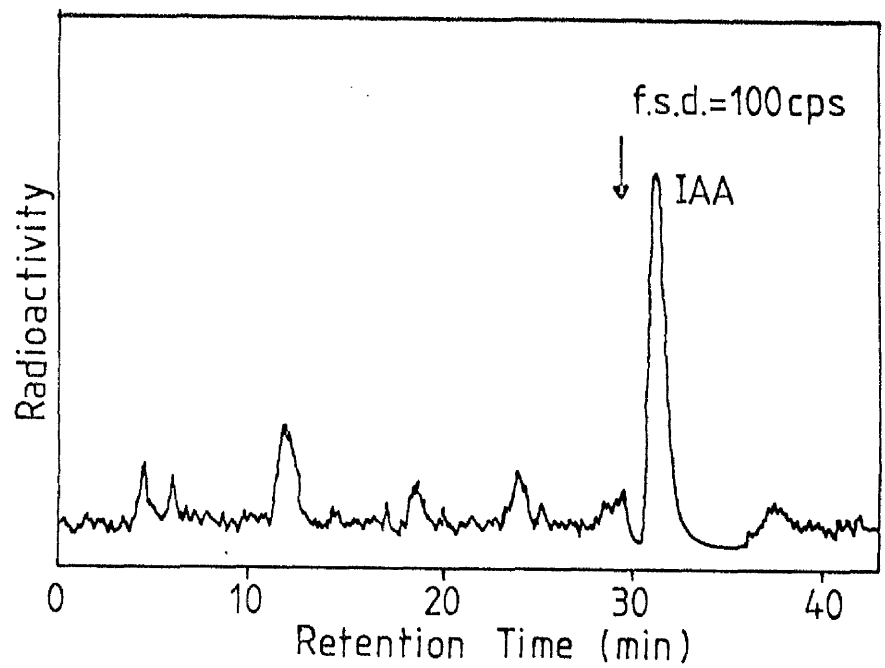


Fig. 10. HPLC analysis of radioactive compounds remaining in the bathing solution after a 2h incubation of root segments in an aqueous solution of IAA-2-^{14}C (10^{-3}mol m^{-3}). The trace represents a typical result from 3 replicate experiments. Solvent gradient : 10-60% methanol over 30 min. Flow rate : 0.75 $cm^3 min^{-1}$. Detector : homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time constant.

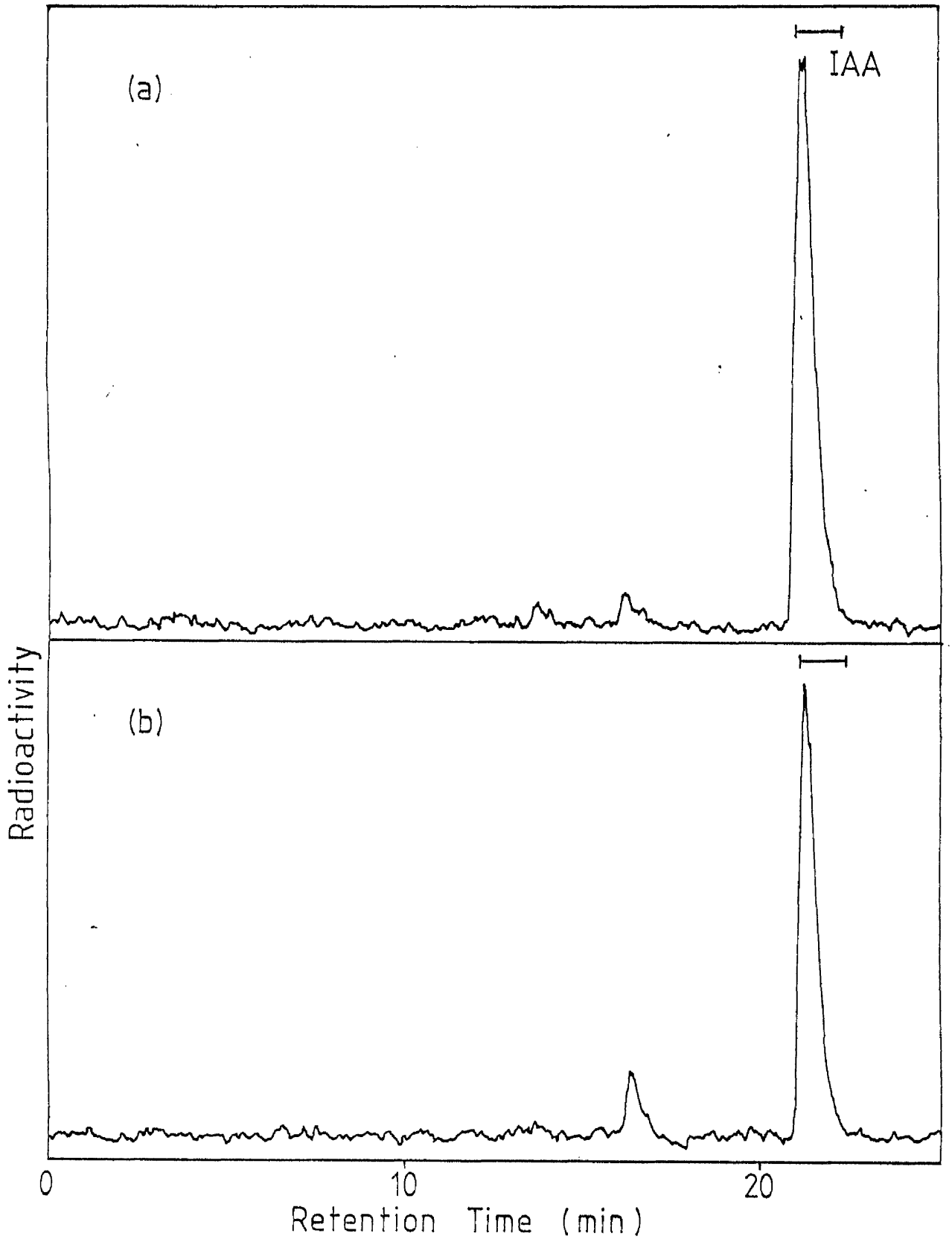


Fig. 11. Control experiment to test for IAA degradation during sample preparation. IAA-2-^{14}C was added to methanolic root extracts and samples prepared for HPLC analysis. (a) Extracts prepared by filtration and drying *in vacuo* (method 1). (b) Samples purified using Sep-pak C_{18} cartridges (method 2). Each trace represents a typical result from 3 extracts. Solvent gradient : 10-60% methanol over 20 min. Flow rate : 1 $cm^3 min^{-1}$. Detector : homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time constant.

Table 9 : Loss of IAA-2-^{14}C during preparation of control root extracts for HPLC analysis. Samples 1-3 were filtered, dried *in vacuo*, redissolved in 1 cm^3 methanol and centrifuged (method 1) while samples 4-6 were purified using Sep-pak C_{18} cartridges (method 2).

REPLICATE	1	2	3	4	5	6
1. Initial radioactivity in extract (Bq)	1180	1150	1100	759	859	821
2. Radioactivity in extract after preparation for HPLC analysis (Bq)	784	885	849	500	614	679
3. 2 expressed as a percentage of 1	67%	77%	76%	66%	71%	83%

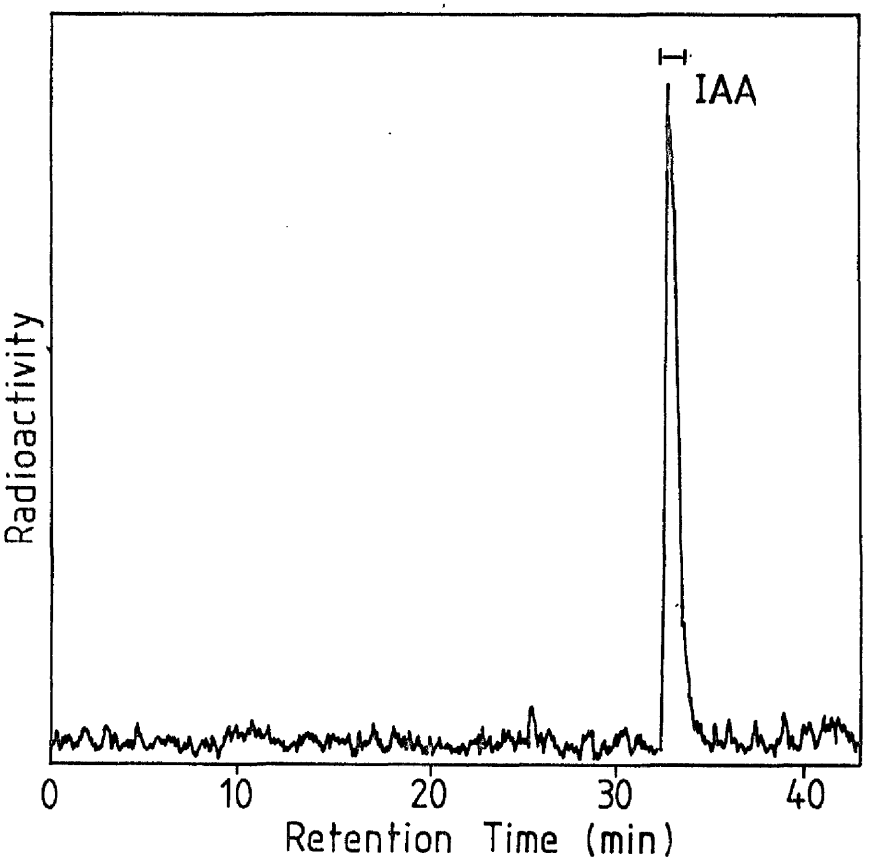


Fig. 12. Metabolism of IAA-2-^{14}C by boiled root segments (2h incubation). The trace represents a typical result from 4 replicate experiments. Solvent gradient : 10-60% methanol over 30 min. Flow rate : 0.75 cm^3 min^{-1}. Detector : homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time constant.

Efficiencies of extraction and preparation procedures were comparable to those for living tissue although uptake of radioactivity was reduced to circa 10% (Table 7).

B.2 Metabolism of IAA by Sterile Roots

Libbert et al (1966) demonstrated that surface bacteria are often capable of large-scale production of IAA. Much of the earlier research on the biosynthesis of IAA in higher plants is of questionable significance because account was not taken of the contribution of epiphytic microorganisms. On the other hand, Libbert and Rische (1969) have published results indicating that degradation of IAA by bacteria on pea plants is negligible. To confirm that the metabolism of IAA by Zea mays seedlings observed in previous experiments was in fact due to plant cells, the fate of IAA-2-^{14}C supplied to sterile roots was studied.

Sterile plants were grown in groups of 10 in glass petridishes. Roots from each dish were incubated in IAA-2-^{14}C (2cm^3 of 10^{-3} mol m^{-3}) for 2h, washed and extracted separately. The sterility of plants in each dish was tested by incubating portions of root for 2 weeks on potato dextrose agar and nutrient agar. Extracts from plants for which tests for fungal and bacterial contamination proved negative were then combined, and samples prepared for HPLC analysis according to methods 1 or 2.

The metabolism pattern observed in three replicate experiments (Fig. 13; Table 8) was closely similar to that for non-sterile roots. The majority of peaks 1 to 11 were present in each case, strongly indicating that metabolism of IAA was taking place in the plant tissue. The amount of IAA remaining in the extracts was reduced (an average of 12% compared with 31% for non-sterile root segments cut in the light). Uptake, extraction efficiences and losses of radioactivity were all similar to those in experiments with non-sterile tissue (Table 7).

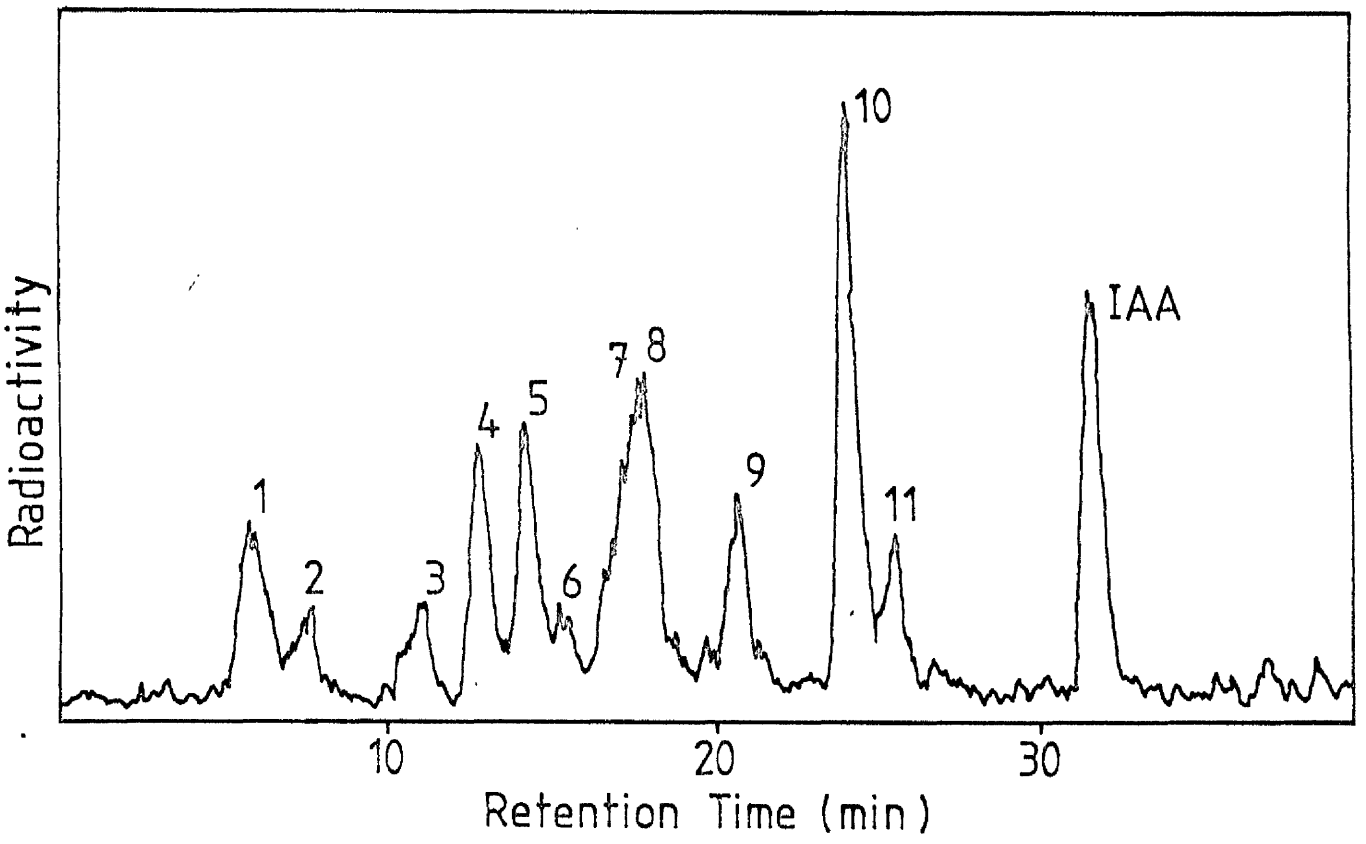


Fig. 13. Metabolism of IAA-2-^{14}C by sterile root segments (2h incubation). The trace represents a typical result from 3 replicate experiments. Solvent gradient : 10-60% methanol over 30 min. Flow rate : 0.75 $cm^3 min^{-1}$. Detector : homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time constant.

B.3 Effect of Exposure to Light on the Metabolism of IAA by Zea mays Root Segments

Root segments for sterile experiments, and for preparation of large-scale extracts, were cut in the laboratory. The effect of exposure to light on the metabolism of IAA was therefore studied.

Groups of 50 root segments were incubated in aqueous IAA (10^{-3} mol m^{-3}) for 2h. Plant material was both grown and incubated in darkness, but section cutting was performed in the laboratory.

Analysis of methanolic extracts, prepared using method 1, revealed a pattern of metabolism closely similar to that obtained in experiment B.1 with root segments cut under dim green light (Table 8; compare Figs. 8 and 9). The same peaks were present in each of three replicates although the proportion of radioactivity associated with the IAA peak was somewhat reduced (31% compared with 46%). The exposure to light had no significant effect on uptake, extraction efficiency or the amount of radioactivity lost during incubation (Table 7).

B.4 Effect of Varying the IAA Concentration in the Incubating Solution on its Metabolism by Root Segments

The rationale for this experiment was two-fold. Firstly, when applying plant growth substances to plant tissue the question always arises as to how much apparent metabolism actually represents detoxification of abnormally large amounts of the exogenous compound. A partial answer to this question might be gained by examining the metabolism of different external concentrations of IAA. Secondly, the metabolism of higher concentrations of IAA than those previously used was investigated with a view to preparing large extracts for studies on the chemical structures of metabolites.

Root segments (cut in the laboratory) were incubated in groups of 50 in four different concentrations of IAA-2-^{14}C (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} mol m^{-3}) for 2h. After following the washing procedure, tissue was extracted overnight

in methanol. Samples were prepared according to method 1 and analysed by HPLC. The pattern of metabolism in segments incubated at the three lowest concentrations was similar, with the majority of peaks 1-11 present in each case (Fig. 14; Table 10). The proportion of radioactivity in extracts, remaining associated with the IAA peak was also similar (25%, 25% and 29% for incubating solutions of 10^{-4}, 10^{-3} and 10^{-2} mol m^{-3} IAA respectively). The percentage of IAA metabolised in tissue incubated in 10^{-1} mol m^{-3} IAA was reduced and only metabolite peaks 4, 5 and 10 were consistently present. The average uptake of radioactivity from a ^{14}C-IAA concentration of 10^{-4} mol m^{-3} was equivalent to 2.3 pmol $segment^{-1}$. Given that the mean fresh weight of a root segment was 17.6mg and that an average of 25% of the radioactivity in the tissue remained associated with the IAA peak, the approximate amount of exogenous IAA present in the roots after 2h incubation could be calculated as 5.7μg kg^{-1}. This is substantially less than the endogenous IAA content of Zea mays (cv. Giant White Horsetooth) root tips measured as 29μg kg^{-1} by Bridges et al. (1973).

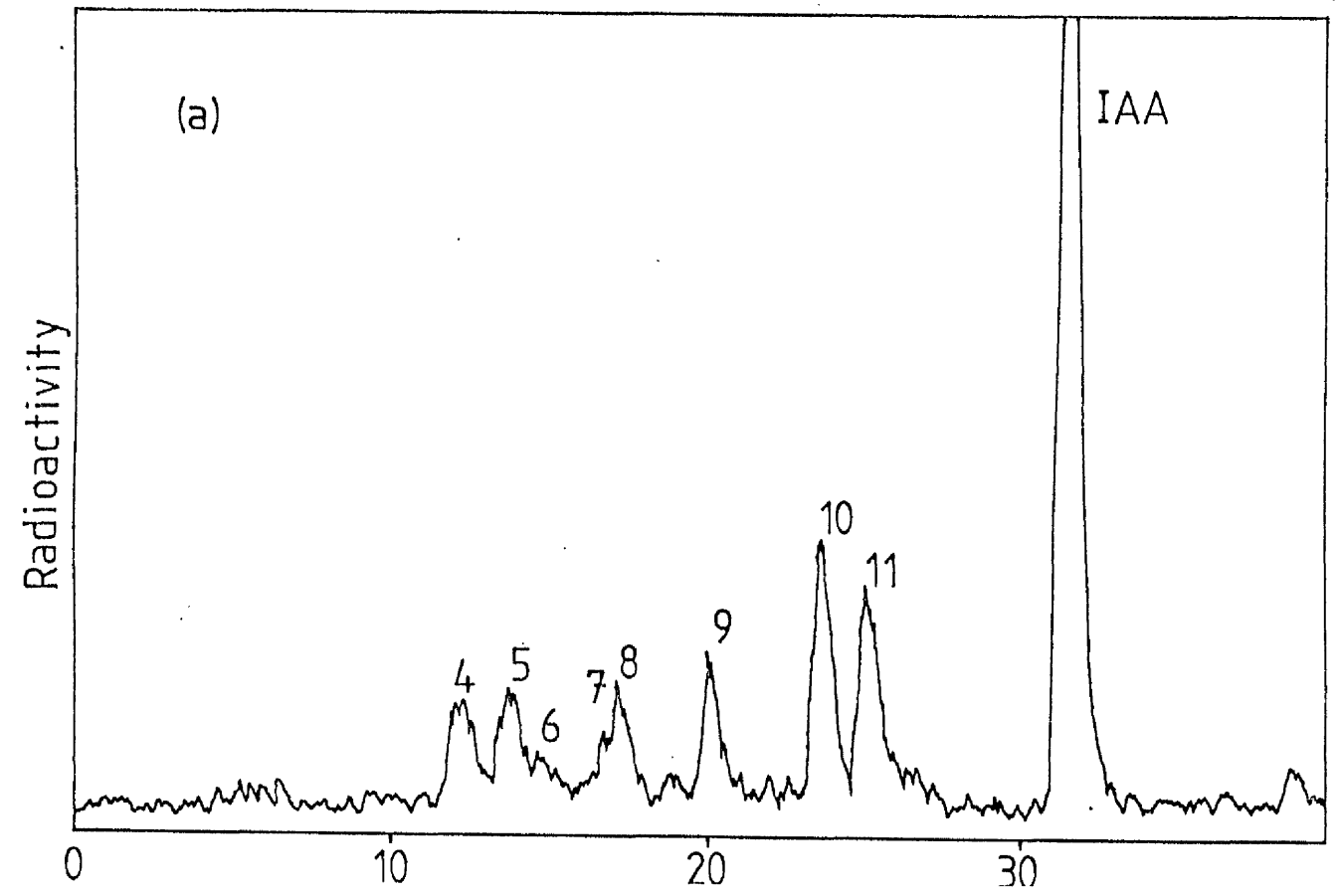
B.5 Use of Sep-pak C_{18} Cartridges in Preparation of Samples for HPLC

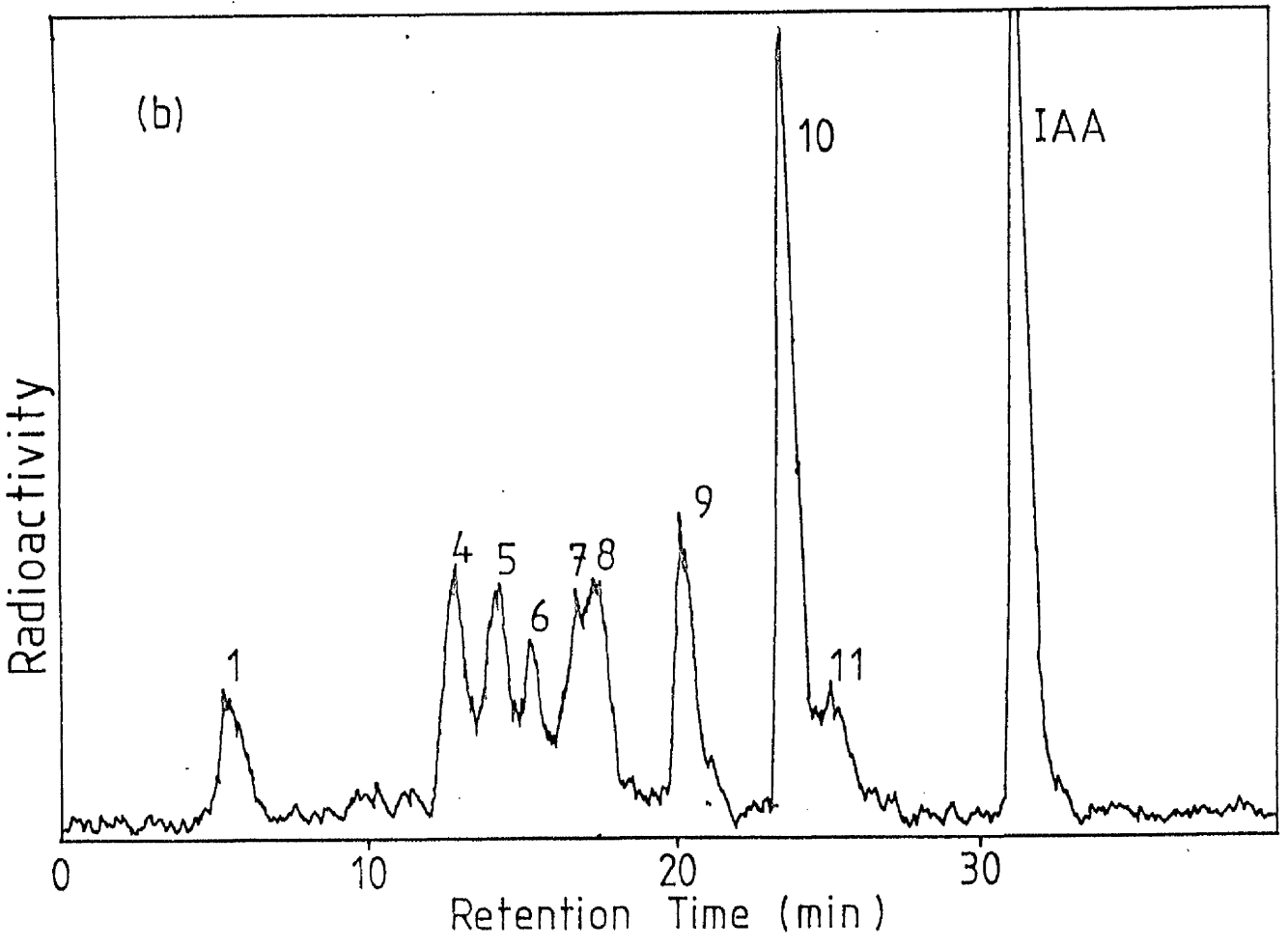
When large extracts were prepared for HPLC analysis, it was necessary to employ a purification step which would reduce the dry weight of samples, with minimal loss of IAA metabolites. Sep-pak C_{18} cartridges were found to be suitable for this purpose. An experiment was carried out to record the effect of this procedure on the profile of radioactive peaks observed during HPLC analysis of root extracts containing ^{14}C-IAA metabolites.

Root tissue (2 groups of 50 segments) which had been incubated in IAA-2-^{14}C (10^{-2} mol m^{-3}) for 2h was washed and extracted in methanol. Extracts were filtered and reduced to the aqueous phase in vacuo. After diluting with 10 cm^3 ammonium acetate buffer (pH 3.5, 20 mol m^{-3}) samples were loaded onto wetted Sep-pak cartridges. Radioactivity was eluted with 2 cm^3 60% methanol in ammonium acetate buffer. Both the buffer passing

Fig. 14. Metabolism of IAA-2-^{14}C supplied at 4 different concentrations to Zea mays root segments (2h incubation). (a) 10^{-1} mol m^{-3}. (b) 10^{-2} mol m^{-3}. (c) 10^{-3} mol m^{-3}. (d) 10^{-4} mol m^{-3}.

Each trace represents a typical result from 3 replicate experiments HPLC Conditions: Solvent gradient; 10-60% methanol over 30 min. Flow rate; 0.75 $cm^3 min^{-1}$. Detector: homogeneous radioactivity monitor; 30cps full scale deflection; 10s time constant.


(a)
IAA
Radioactivity
4
5
6
7
8
9
10
11
0
10
20
30


(b)
IAA
Radioactivity
1
4
5
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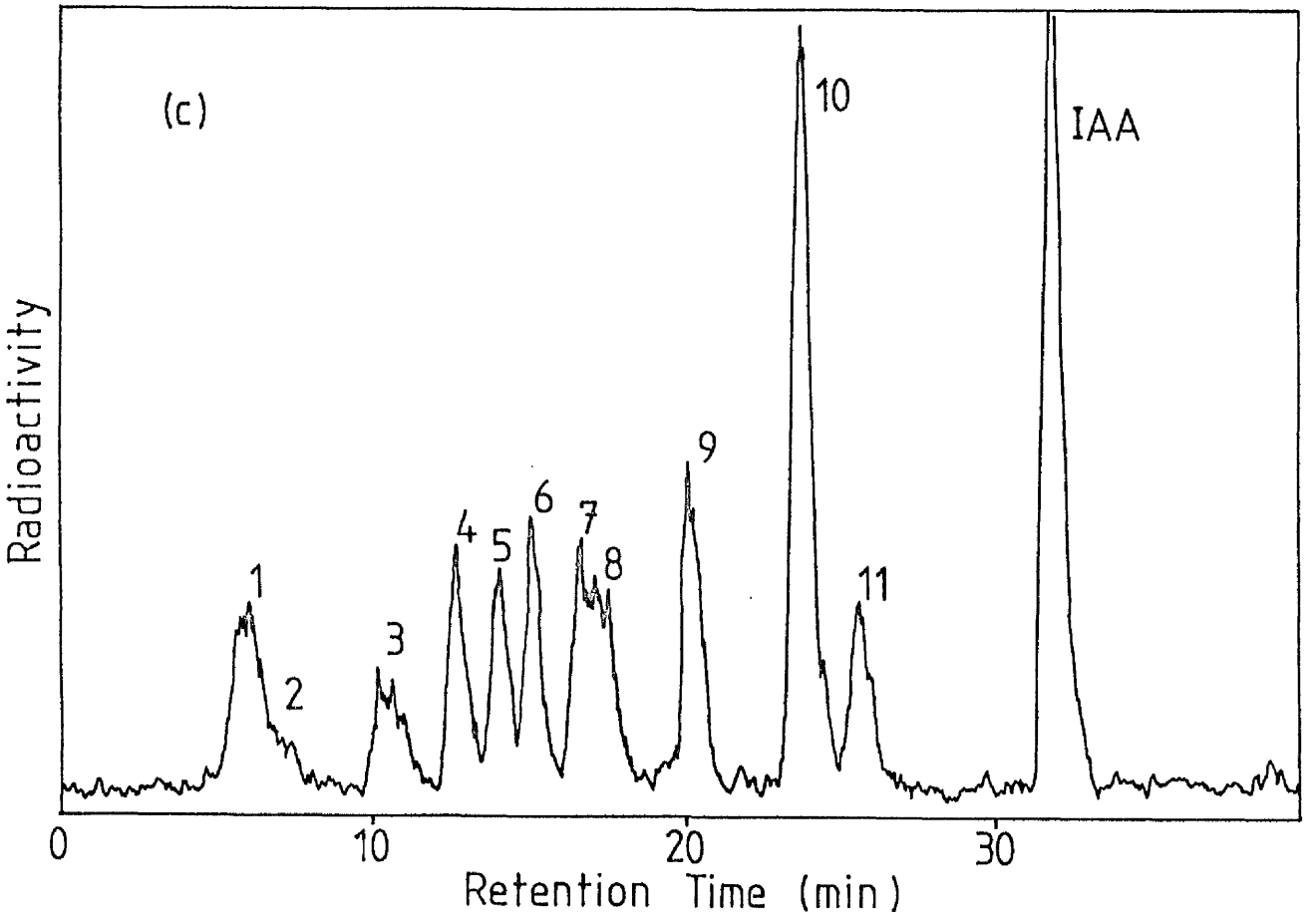
7
8
9
10
11
0
10
20
30
Retention Time (min)


(c)
Radioactivity
1
2
3
4
5
6
7
8
9
10
11
IAA
0
10
20
30
Retention Time (min)

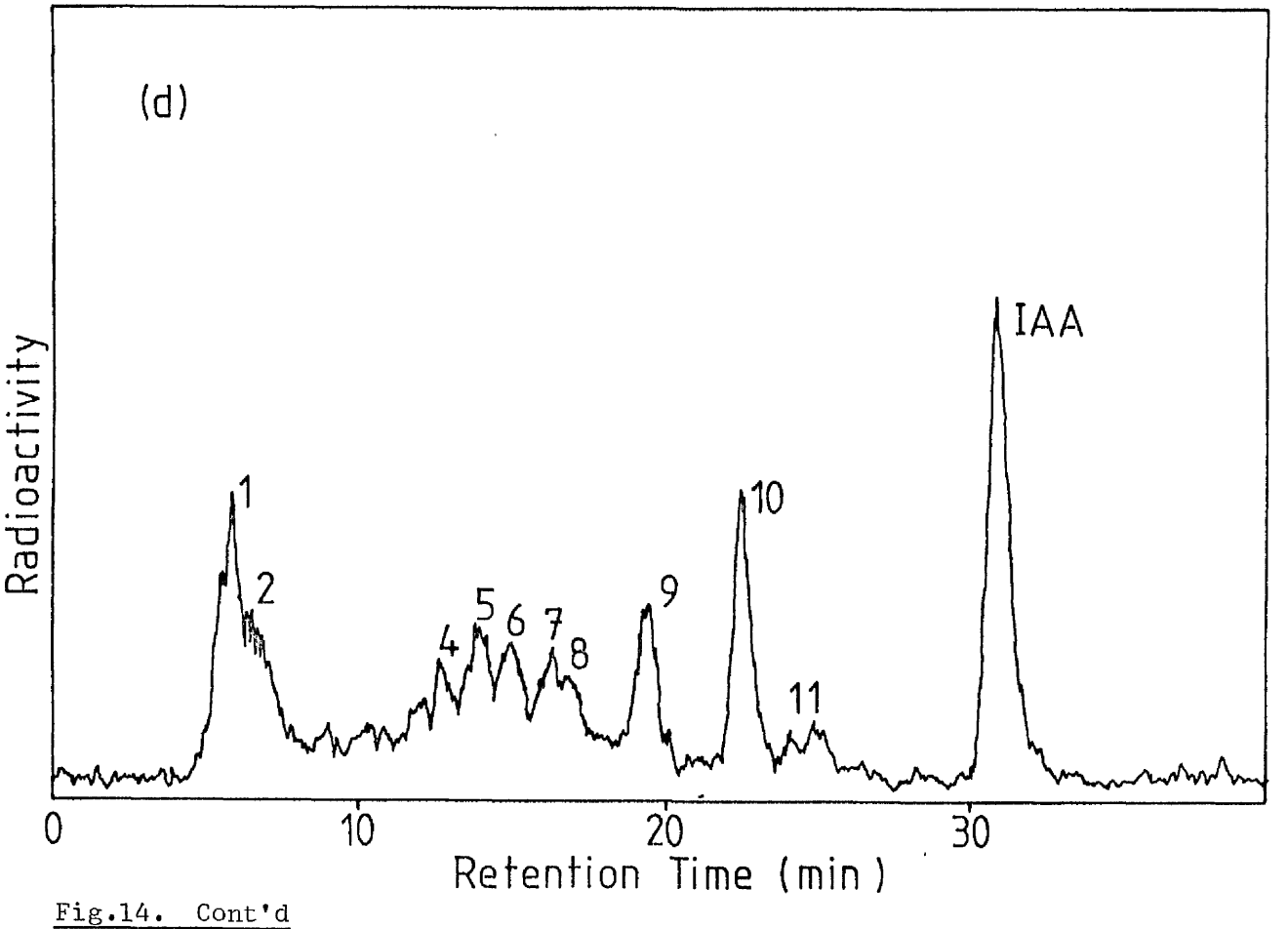

(d)
Radioactivity
1
2
4
5
6
7
8
9
10
11
IAA
0
10
20
30
Retention Time (min)

Fig.14. Cont'd

Table 10 : Uptake and Metabolism of IAA-2-^{14}C supplied at four concentrations to Zea mays root segments (2h incubation).

Metabolite peaks in extracts of roots supplied with IAA at 10^{-3} mol m^{-3} were numbered 1-11 (see Experiment A.2, Fig. 8) and used as standards for other extracts (1). The experiment was carried out in triplicate.

Concentration of IAA in incubating solution	10^{-4} mol m^{-3}	10^{-3} mol m^{-3}	10^{-2} mol m^{-3}	10^{-1} mol m^{-3}
Uptake of IAA ($pmol\ segment^{-1}$)	2.7 1.9	17 23 22	129 90 103	1000 740 765
Radioactive peaks in methanol extract in order of decreasing height (1)	IAA,10,7/8,9,1,6,4,3, 11,5,2 IAA,10,1,9,2,5,6,7/8, 4,3,11	IAA,10,7/8,9,1/2,4,5, 6,11,3 IAA,10,4,5,6,7,9,8, 1,3,2 IAA,10,9,6,7,4,5,8 11,1,3,2	IAA,10,4,5,7/8,6,9 IAA,10,4,5,8,9,7,6,11 1,3 IAA,10,9,4,7/8,5, 6,1,11	IAA,11,10,4,5 IAA,10,4,5,9,8,7,6 IAA,10,11,9,7/8,5,4
Percentage radioactivity associated with IAA peak	24 26	23 23 29	33 26 27	62 57 66
Percentage radioactivity associated with peak 10	20 12	23 20 22	23 19 24	10 11 11

through the cartridge and the 2 cm^3 of 60% methanol were dried and analysed by HPLC.

A substantial proportion of peak 1 had been lost from the purified extract (Fig. 15a). Most of this was found in the buffer passing through the cartridge (Fig. 15b).

Conclusions

Metabolism of IAA-2-^{14}C by root segments taken from dark-grown Zea mays seedlings is rapid and extensive. Non-sterile roots, cut under dim green light and incubated for 2h in darkness in 10^{-3} mol m^{-3} ^{14}C-IAA, had metabolised an average of 54% of the radioactivity extracted with methanol. At least 11 products were formed, one of which, peak 10, was always more prominent than the others. The efficiency of the extraction procedure was high, at least 93%, thus only a small fraction of the radioactivity escaped analysis in this way. However, substantial and variable amounts of label, up to 36% of samples, were lost during drying of extracts in vacuo. Control experiments confirmed that no significant breakdown of IAA occurred during sample preparation. Sterile roots yielded an apparently similar range of products to non-sterile indicating that metabolism was taking place in the plant tissue.

Evidence that the compounds observed probably did not represent products of a detoxification mechanism to eliminate abnormally high hormone levels was obtained from experiments in which the external IAA concentration was varied. The pattern and rate of IAA metabolism did not change significantly when uptake of IAA increased from 2.3 to 107 pmol $segment^{-1}$. Furthermore, the amount of exogenous IAA present in the tissue after a 2h incubation in 10^{-4} mol m^{-3} IAA-2-^{14}C was substantially less than published measurements of the endogenous IAA content.

Exposure of root segments to light for a short period resulted in a decrease in the amount of radioactivity associated with the IAA peak, but did not affect the nature of the metabolites produced, i.e. there was an

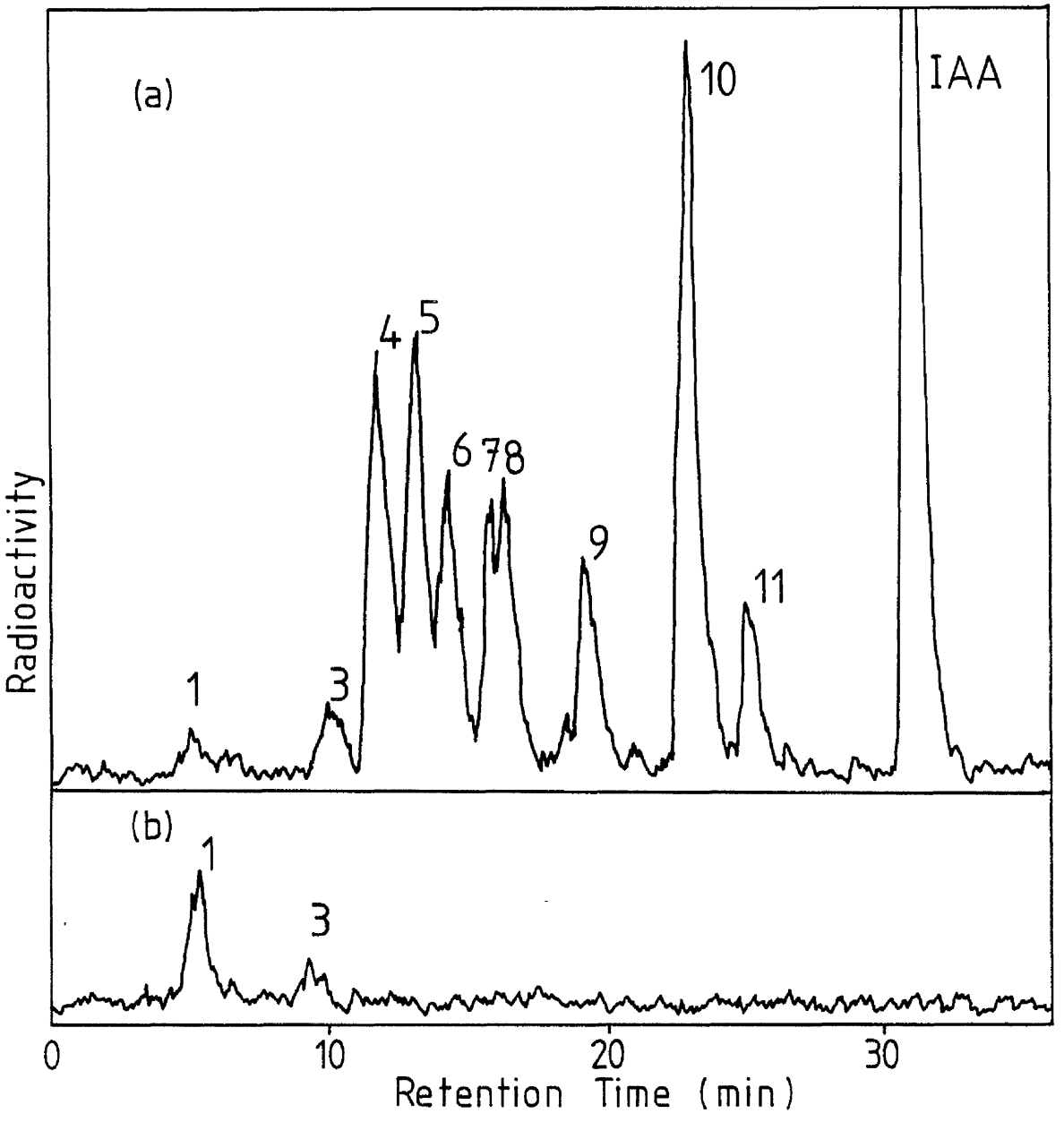


Fig. 15. HPLC of root extract purified using Sep-pak C_{18} cartridges. (a) Analysis of radioactive compounds eluted from the cartridge in 2 cm^3 60% methanol in ammonium acetate buffer (pH 3.5, 20mol m^{-3}). (b) Radioactive compounds passing through cartridge during loading of extract in 10 cm^3 ammonium acetate buffer (pH 3.5, 20mol m^{-3}). Solvent gradient : 10-60% methanol over 30 min. Flow rate : 0.75 $cm^3 min^{-1}$. Detector : homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time constant.

apparent stimulation of IAA metabolism. The increased proportion of IAA metabolised by sterile roots could be due to the increased exposure to light during section cutting in the sterile air-flow cabinet.

C. Time-Course of IAA Metabolism in Root Segments

Research, using root tissues, on the effects, transport and binding of IAA to putative receptor sites has often been performed with little consideration of the metabolism of exogenous IAA during the course of the experiment (e.g. Batra *et al.*, 1975; Moloney and Pilet, 1981). The results in section B of this thesis suggest that *Zea mays* root segments are capable of extensive metabolism of IAA. A knowledge of the amount of exogenous IAA remaining in the tissue after varying incubation times is therefore essential. It was also hoped that this study would yield information on the sequence in which metabolite peaks were produced.

Seven groups of 50 root segments (cut in the laboratory) were placed in aqueous solutions of IAA-2-^{14}C (10^{-3} mol m^{-3}) and incubated for various times : Table 11.

Table 11 : Incubation protocol for time-course experiment of IAA metabolism in root segments

Dish	Time incubated in IAA-2-^{14}C	Time incubated in distilled water	Total incubation time
1 and 2	10 min	-	10 min
3	1 h	-	1 h
4	1 h	1 h	2 h
5	1 h	3 h	4 h
6	1 h	7 h	8 h
7	1 h	23 h	24 h

Root segments incubated for periods longer than 1h were washed thoroughly (Materials and Methods) before transferring to dishes of distilled water. Methanol extracts were prepared for HPLC analysis according to method 1.

Metabolism of IAA was rapid; after incubating segments for only 10min,

an average of 43% of the radioactivity was no longer associated with the IAA peak (Table 12; Fig. 16). The amount of IAA in extracts continued to decline until only around 1% of the radioactivity co-chromatographed with IAA after 24h. The extraction efficiency declined only slightly during the course of the experiment (from 93% for a 10min incubation to 88% after 24h)

At least 3 and sometimes up to 8 metabolite peaks were apparent after 10 min (the variation appeared to result from difficulty in distinguishing small metabolite peaks from fluctuations in background radiation). The appearance of several products after such a short time indicates that there is probably not a single linear pathway of IAA metabolism. The apparently complex nature of the reaction sequence presented difficulties in interpreting relationships between different peaks. Some consistent trends were visible, however. The most prominent metabolite at short incubation times was peak 9. This declined as there was an increase in the height of peak 10 which became the largest metabolite after 2h and 4h before decreasing in size. After 24h the most prominent compounds were the components of peaks 7 and 8, and peaks 1 and 2. Precise information on the interconversions of individual metabolites, however, could not be deduced from these observations. This might be achieved by supplying labelled IAA metabolites to root tissue and extracting and analysing the radioactive products.

In a single experiment, root tissue remained in IAA-2-^{14}C for the whole incubation time, up to 24h. Although radioactivity continued to be taken up during this period and there was no significant breakdown of IAA in the incubating solution, the proportion of IAA in the tissue decreased at the same rate as in root segments transferred to water after 1h (Table 13). This would indicate that radioactivity taken up after longer periods was more rapidly metabolised.

Table 12 : Uptake and metabolism of IAA-2-^{14}C by Zea mays root segments over a time-course. Tissue was incubated in IAA-2-^{14}C (10^{-3}mol m^{-3}) for up to 1h, then washed and transferred to distilled water. The experiment was carried out in triplicate. Metabolite peaks in extracts of roots supplied with IAA-2-^{14}C for 2h were numbered 1-11 (see experiment A.2, Fig. 8) and used as standards for comparison of products (1).

INCUBATION TIME	10 min	1 h	2 h	4 h	8 h	24 h
Uptake of radioactivity (Bq segment^{-1})	8.6 9.6 6.3	19 23 18	19 22 15	18 27 16	19 25 20	21 31 16
Radioactive peaks in methanol extract in order of decreasing height (1)	IAA,9,7,8,5 IAA,9,7/8,4,5, 1,10,11,8 IAA,9,7/8,10, 11	IAA,9,7/8,1,10,5, 6,4,11,3 IAA,9,7/8,10,1,5, 4,11,6,3 IAA,9,7/8,10,4,1, 11,5,6	IAA,9,10,7/8,6,1, 11,3,5,4,2 IAA,10,9,7/8,1,6, 4,3,5,2 IAA,10,7/8,6,9	10,2,IAA,7/8,4,1 , 9,6,4,5,11 10,8,2,IAA,3,7,11, 6,9,1,4,5 10,2,IAA,3,11,7/8 9,6,1,4,5	8,2,7,10,3,1b,9, 6,IAA,4,5 2,8,10,3,7,11,9,6, IAA,4,9,5 2,10,7/8,11,3,9,4, 6,IAA,5	2,7/8,3,9,10,11,IAA, 4,5,1b,6 1,7/8,10,1b,3,9,4, 6,11,IAA,5 8,2,7,3,10,11,9, 4,6,5
% radioactivity in methanol extract associated with IAA peak	64 56 50	28 35 30	31 25 17	14 9 11	2.8 3.5 3.0	1 1 0
% radioactivity in methanol extract associated with peak 10	- 2.9 -	8 8 7	9 11 9	16 20 14	10 14 11	2 6 4

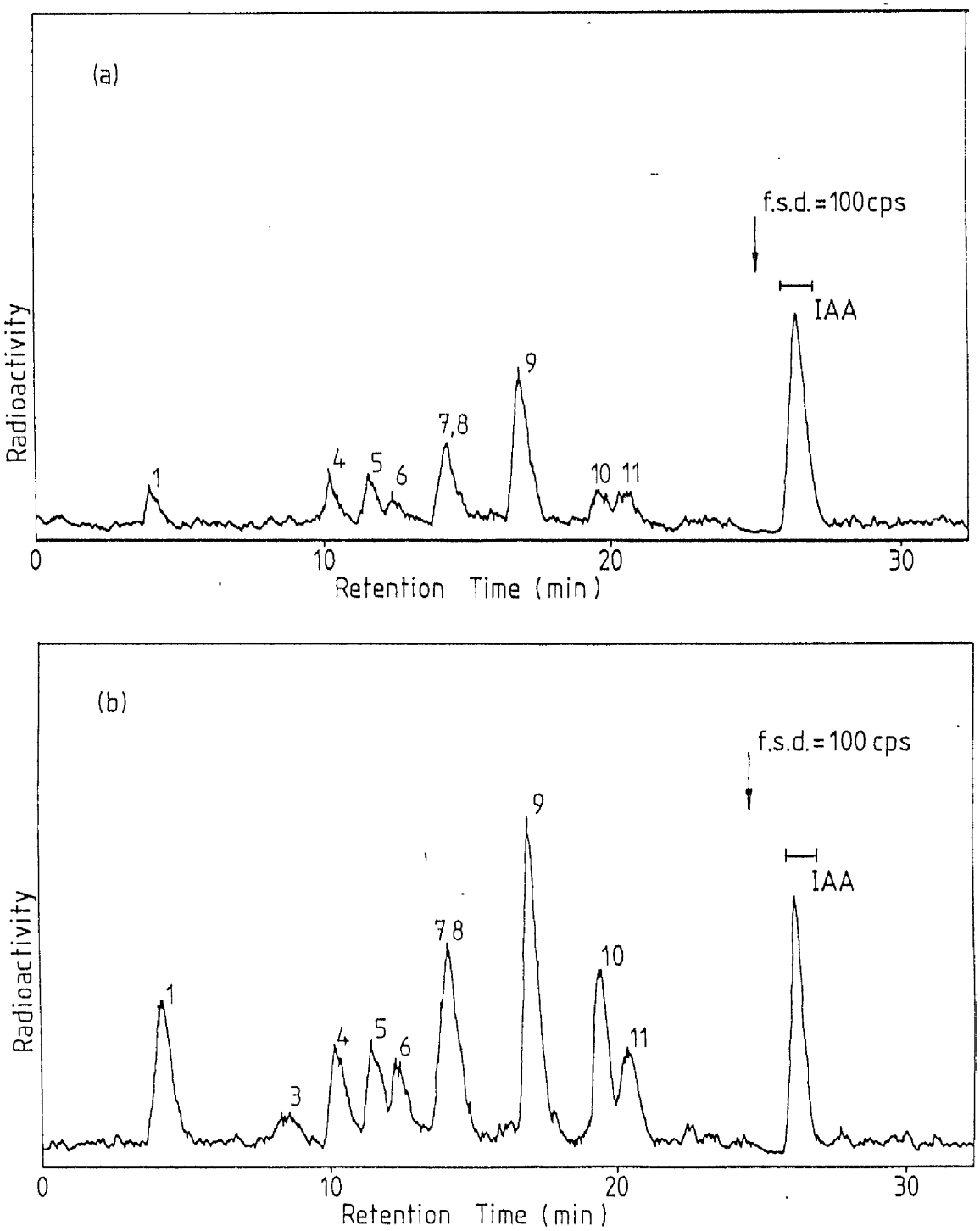


Fig. 16. Time-course of IAA-2-^{14}C metabolism by Zea mays root segments. Tissue was incubated in ^{14}C-IAA (10^{-3} mol m^{-3}) for up to 1h, then washed and transferred to distilled water. (a) 10 min incubation, (b) 1h, (c) 2h, (d) 4h, (e) 8h, (f) 24h. Each trace represents a typical result from 3 replicate experiments. Solvent gradient: 10-60% methanol over 30 min. Flow rate: 1 cm^3 min^{-1}. Detector: homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time constant.

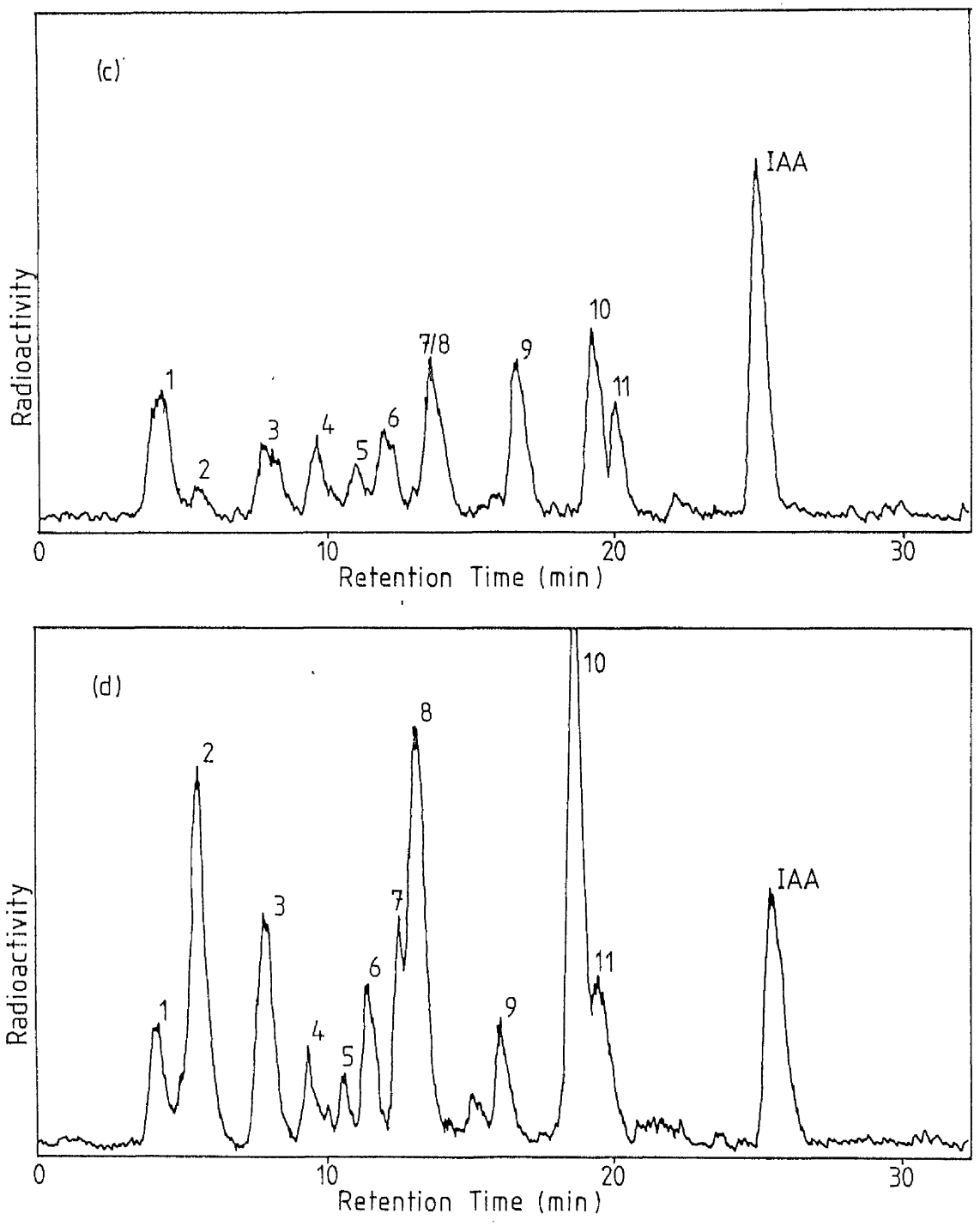

(c)
Radioactivity
1
2
3
4
5
6
7/8
9
10
11
IAA
0
10
20
30
Retention Time (min)
(d)
Radioactivity
1
2
3
4
5
6
7
8
9
10
11
IAA
0
10
20
30
Retention Time (min)

Fig. 16 cont'd

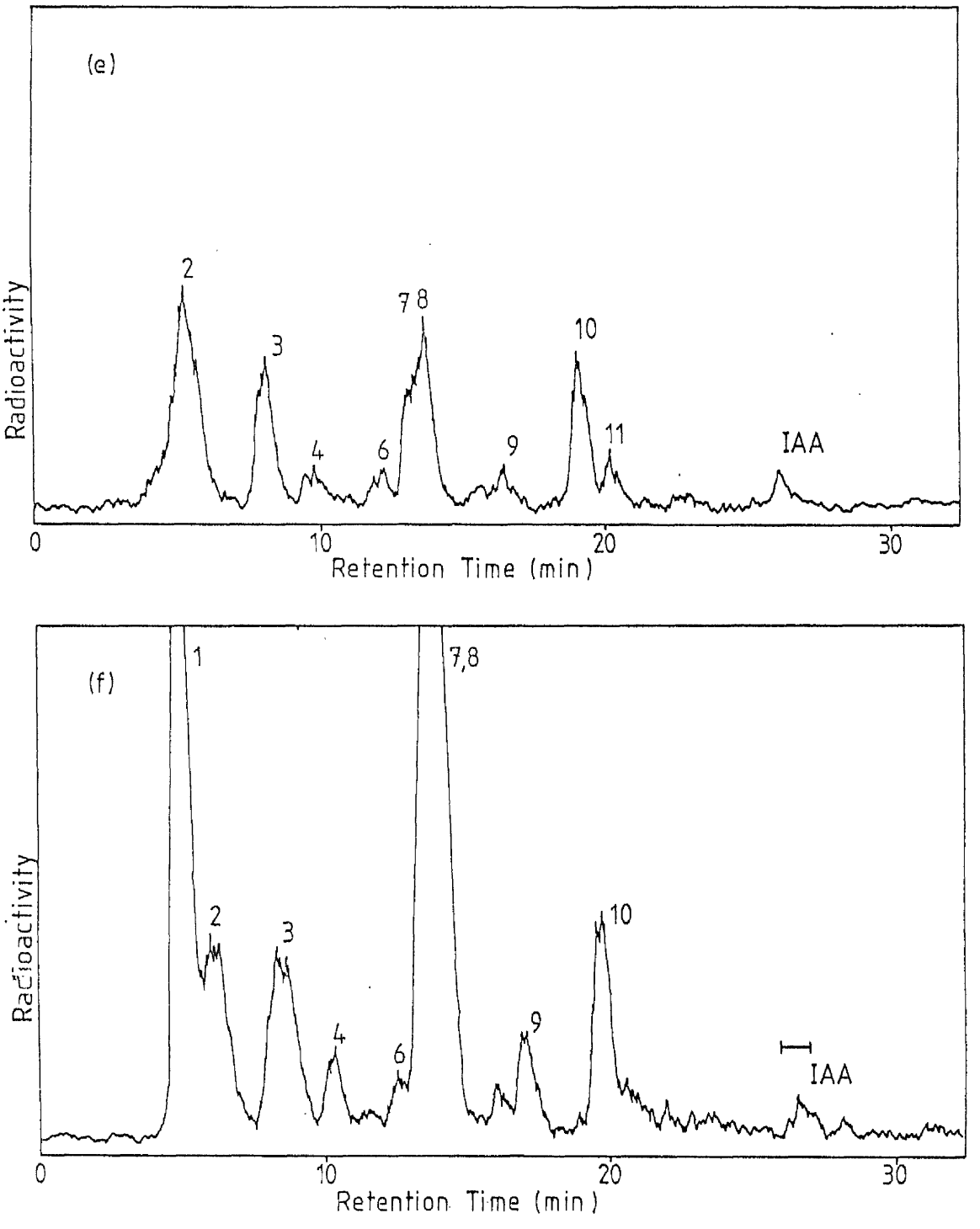

(e)
Radioactivity
2
3
4
6
7 8
9
10
11
IAA
0
10
20
30
Retention Time (min)
(f)
Radioactivity
1
2
3
4
6
7,8
9
10
IAA
0
10
20
30
Retention Time (min)

Fig. 16 cont'd

Table 13 : Metabolism of IAA-2-^{14}C supplied to root segments for up to 24h

Incubation time	Radioactivity taken up by tissue (Bq segment^{-1})	% of radioactivity in methanol extract associated with IAA peak
10 min	7.5	58
30 min	20	54
1 h	29	40
2 h	36	29
4 h	76	4
8 h	91	3
24 h	103	No detectable IAA

Conclusions

IAA is metabolised rapidly by *Zea mays* root tissue, along an apparently non-linear and complex reaction pathway. After a 24h incubation, very little IAA-2-^{14}C remained in the tissue. Similar results were obtained from experiments in which roots were removed from IAA-2-^{14}C after 1h and transferred to distilled water, as for tissue incubated in ^{14}C-IAA for the full incubation time. It appears that IAA-2-^{14}C taken up after longer periods is more rapidly metabolised.

D. A Comparison of the Metabolism of IAA by Cortical and Stelar Tissues of the Root

Measurements of the endogenous content of IAA in Zea mays roots have indicated that the amount present in the stele, per gram fresh weight of tissue, is approximately 10 times that in the cortex (Bridges et al., 1973). Wilkins and co-workers (Bowen et al., 1972; Cane and Wilkins, 1970; Shaw and Wilkins, 1974) have also demonstrated that the polar acropetal transport of IAA in Zea mays roots takes place predominantly in the stele. The products of IAA-1-^{14}C metabolism in separated cortical and stelar tissues have been examined using TLC (Greenwood et al., 1973). These studies revealed a slower rate of IAA metabolism in the stele. The major products formed by the two tissues also appeared to differ. It was decided to follow up this work by investigating the metabolism of IAA applied to separated cortical and stelar tissues, using HPLC analysis.

As the apical 5mm of the root cannot be divided into cortex and stele, segments 20mm in length were taken from 5mm behind the tip. Separated tissues were incubated in groups of 50 in aqueous solutions of IAA-2-^{14}C (10^{-3} mol m^{-3}) for 2h, 4h, 8h or 24h after which they were washed (Materials and Methods) and extracted in methanol. Samples were prepared for HPLC analysis using method 1. The results (Fig. 17 : Table 14) confirmed the observations of Greenwood et al. (1973) that metabolism took place more slowly in the stele. After 2h incubation an average of 96% of the radioactivity present in stelar extracts was associated with the IAA peak. Extracts of cortical tissue contained an apparently similar range of metabolites to those from complete root segments; only 8% of the ^{14}C appeared to represent IAA. Stele segments incubated in IAA for longer times metabolised the majority of IAA entering the tissue. As IAA was taken up from solution for up to 8h, and there was no significant breakdown of IAA in the incubating solutions, this represents an increase in the rate of IAA metabolism. This may be a result of senescence of the tissue. Without conclusive identification of the

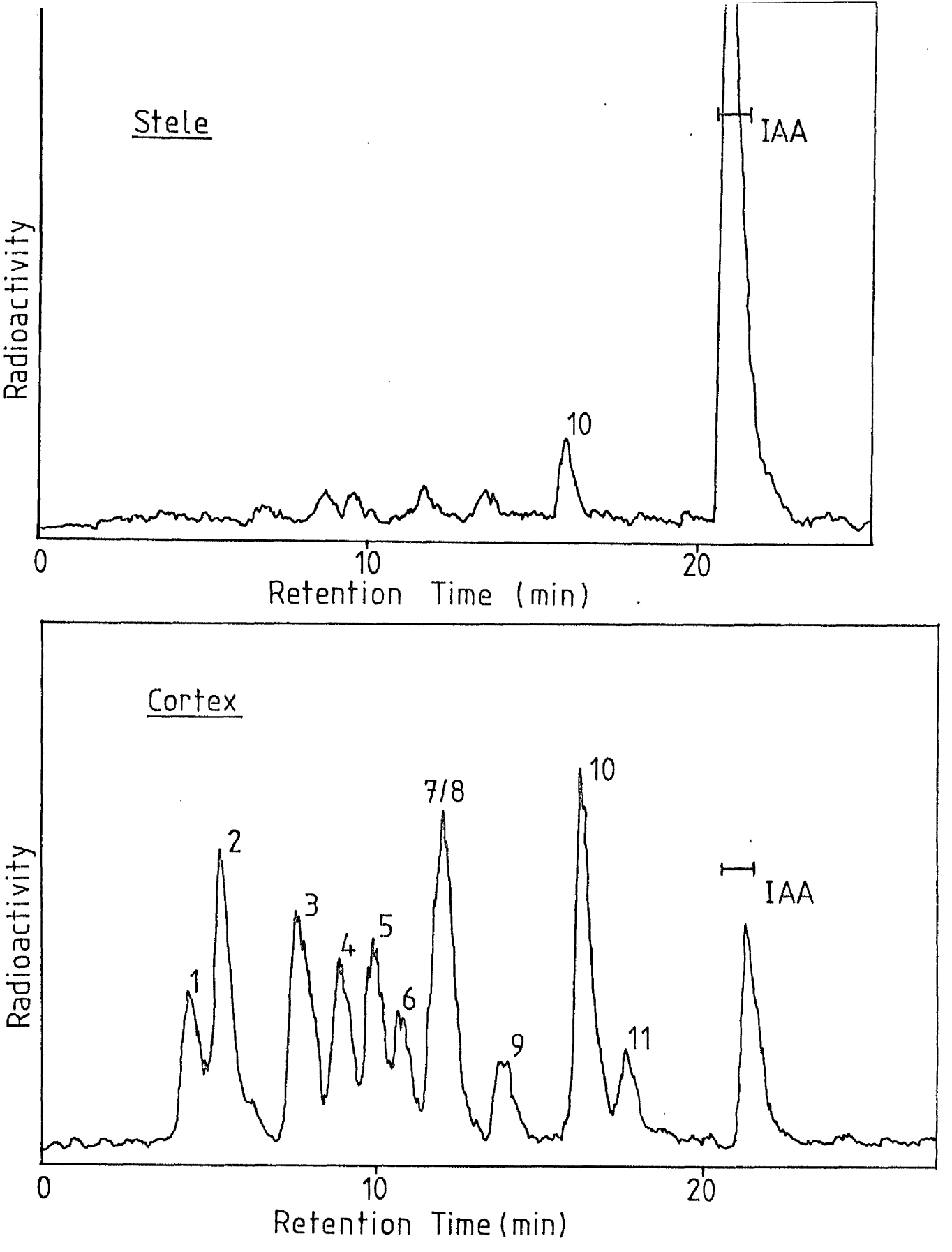


Fig. 17. Comparison of ^{14}C-IAA metabolism in separated cortical and stelar tissues of Zea mays roots - HPLC Analysis. Tissues were incubated in ^{14}C-IAA (10^{-3}mol m^{-3}) for 2h. The traces represent typical results from 3 replicate experiments. Solvent gradient : 10-60% methanol over 20 min. Flow rate : 1 cm^3 min^{-1}. Detector : homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time constant.

Table 14: Uptake and metabolism of ^{14}C-IAA by separated cortical and stelar tissues of Zea mays roots over a time-course.

Tissues (50 segments per dish) were incubated in aqueous solutions of ^{14}C-IAA (10^{-3} mol m^{-3}) for 2h, 4h, 8h or 24h. Quantitative results were obtained in 2 replicate experiments.

TREATMENT	cortical segments 2h incubation	stelar segments 2h incubation	stelar segments 4h incubation	stelar segments 8h incubation	stelar segments 24h incubation
Uptake of radioactivity (Bq segment^{-1})	39 41	18 18	21 44	54 77	25 70
Dry weight of tissue (mg)	28.0 25.2	7.5 8.0	7.2 8.8	8.4 8.7	5.5 8.9
Percentage radioactivity in extract, associated with IAA peak	8 9	99 94	61 55	14 1.8	10 1.0
Percentage radioactivity in extract, associated with peak 10	16 13	1 6	15 15	30 31	30 35

products, it was not possible to know whether the same compounds were produced in both tissues. However, a substance with the same retention time as peak 10 was the most prominent metabolite in both cortical and stelar extracts.

Conclusion

Although results in sections B and C indicate that exogenous IAA taken up from solution was rapidly metabolised, during the first 2h at least most of this appears to take place in the cortical tissues, where only a small proportion of the endogenous IAA is located.

E. Metabolism of IAA-2-^{14}C Supplied from Agar Blocks to the Stele at the Basal End of Root Segments

In order to obtain information concerning the metabolism of endogenous IAA, it is necessary to supply labelled IAA in a manner which reproduces as closely as possible its natural source in the root. Bridges et al. (1973) showed that the majority of endogenous IAA is located in the stele. Wilkins and co-workers (e.g. Bowen et al., 1972) also demonstrated that IAA is transported acropetally predominantly in the stele. In this experiment the metabolism of IAA-2-^{14}C supplied from agar blocks to the stelar tissue at the basal end of root segments was studied. As the results in experiment D showed that metabolism of IAA by the stele during a 2h incubation was slow, this means of donating the IAA would eliminate the possibility that a substantial proportion of metabolism was taking place during entry at the cut surfaces.

Sections of tissue, 13mm in length, were taken from 10mm behind the root tips of 3-day-old, dark-grown Zea mays seedlings. The cortex was removed from the basal 3mm of each segment, and the protruding stele placed in contact with a block of 1.5% agar containing IAA-2-^{14}C (see Fig. 18). Blocks of plain agar at the opposite end of each root were present to protect the tissue from drying, and to collect radioactive compounds diffusing from the segment. Eight groups of 20 segments were enclosed in glass petridishes and incubated for 2h. The protruding section of stele plus 1mm of tissue from each end of the segments was then removed and the roots extracted with methanol. Extracts were prepared for HPLC analysis according to method 1. Radioactivity present in both the donor and receiver blocks of agar was also extracted into methanol. The distribution of radioactivity at the end of the experiment, which was only performed once, was as follows:

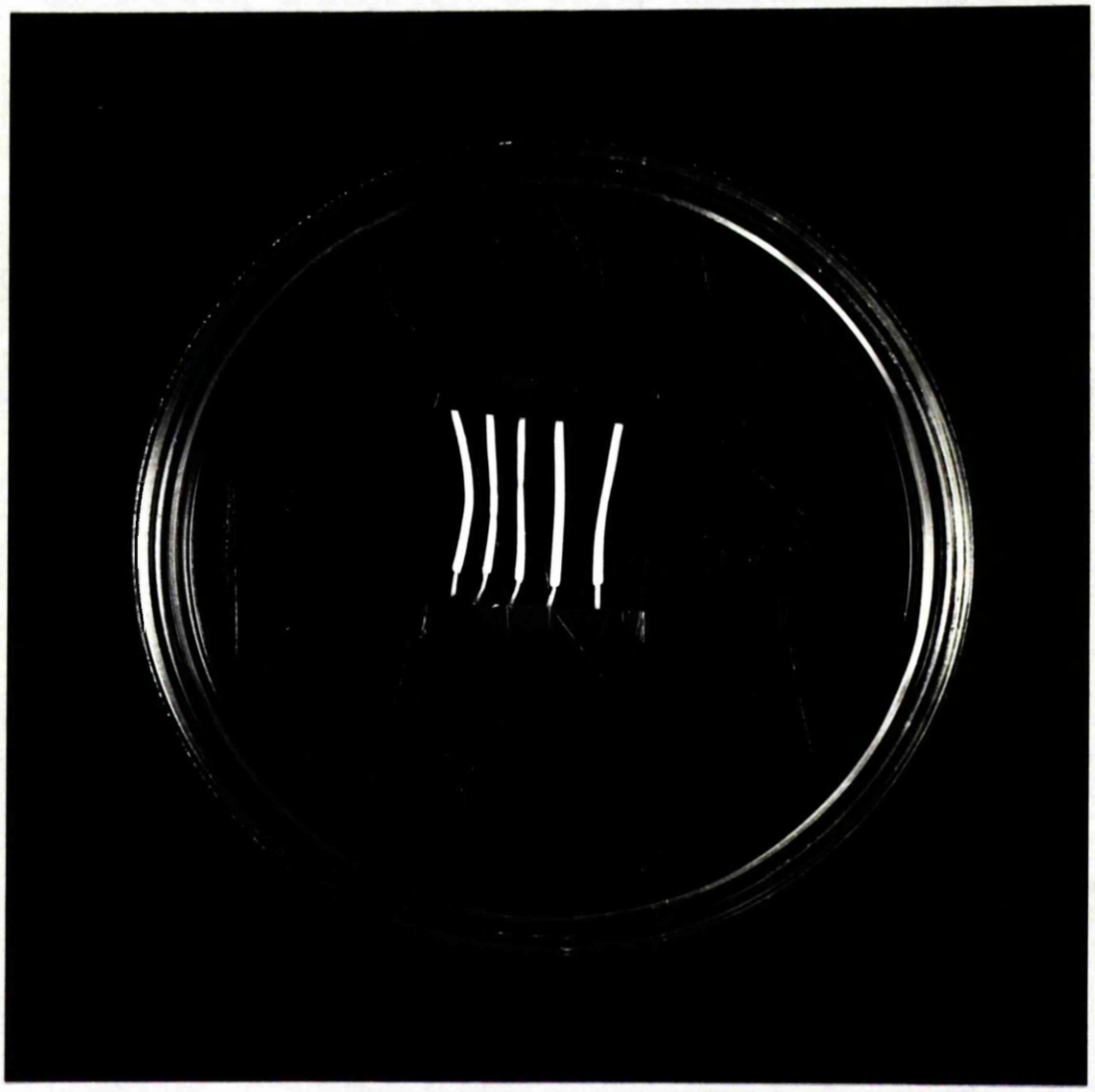


Fig. 18. Root segments supplied with IAA-2-^{14}C from agar blocks

donor block	338,000 Bq
root tissue	3,482 Bq
receiver block	1,480 Bq

The approximate amount of radioactivity present in each segment after a 2h incubation was therefore equivalent to 19.8 pmol IAA.

The proportion of IAA which had been metabolised by the root tissue was significantly less than in experiments in which segments were floated in aqueous solutions of IAA; approximately 50% of the radioactivity remained associated with the IAA peak (Fig. 19a) compared with 31% in experiment B.5. Six of the same metabolite peaks were apparently present (peaks 5, 6, 8, 9, 10 and 11). However, there was a noticeable decrease in the proportion of peak 10 which only accounted for approximately 9% of the radioactivity and was no longer the most prominent metabolite. The percentage of IAA metabolised was considerably greater than that in stelar tissues alone (experiment D), indicating that some IAA had probably moved into the cortex.

Although approximately 50% of the radioactivity in the root tissue extracts represented metabolites of IAA, extracts of the receiver block yielded only a single radioactive peak with the same retention time as IAA (Fig. 19b). This was accompanied by a fluorescence peak. When methylated using diazomethane, and analysed again by HPLC, a single peak was obtained with the same retention time as IAA - methyl ester (Fig. 20). Thus there is strong evidence that radioactivity in the receiver block was exclusively associated with the IAA molecule. HPLC analysis of donor block extracts indicated that breakdown of IAA during the experiment was negligible.

A similar experiment to that described above was carried out in which root segments were incubated for 24h. HPLC analyses of tissue extracts showed that very little IAA remained in the tissue after this time; in two out of three experiments no ^{14}C-IAA was detectable (Fig. 21). Four metabolite peaks were consistently present : peaks 3 and 10 and unresolved composit peaks 1/2 and 7/8. However, stelar tissue alone also metabolised virtually all of the IAA taken up after 24h. These results may reflect the senescence

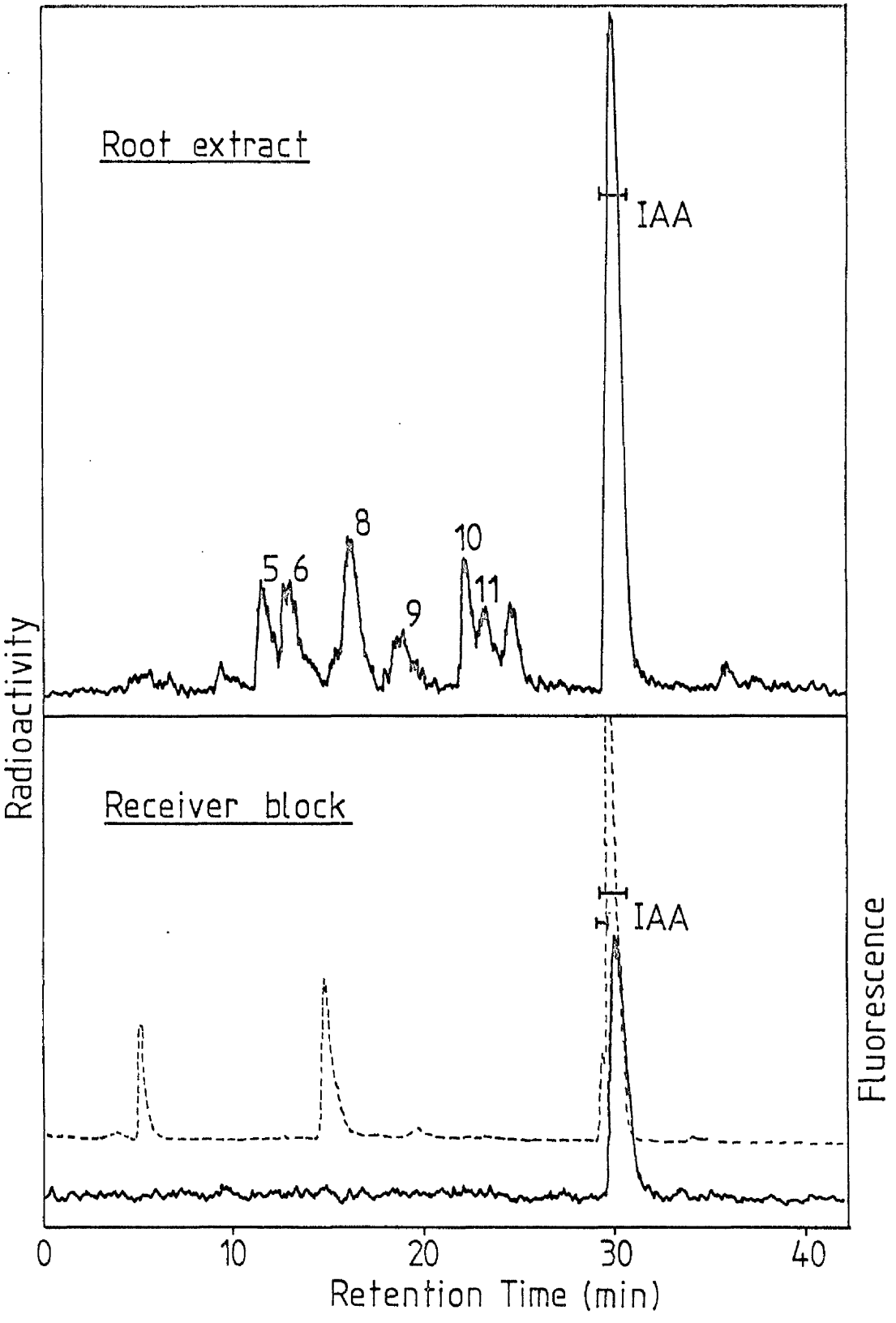


Fig. 19. HPLC analyses of extracts from root tissue and "receiver" blocks of agar. IAA-2-^{14}C was supplied to the stele at the basal end of root segments, from agar blocks (2h incubation). Traces represent results from single extracts analysed in duplicate. Solvent gradient : 10-60% methanol over 30 min. Flow rate : 0.75 $cm^3 min^{-1}$. Detector: a. (solid line) homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time scale. b. (dashed line) fluorescence spectrophotometer.

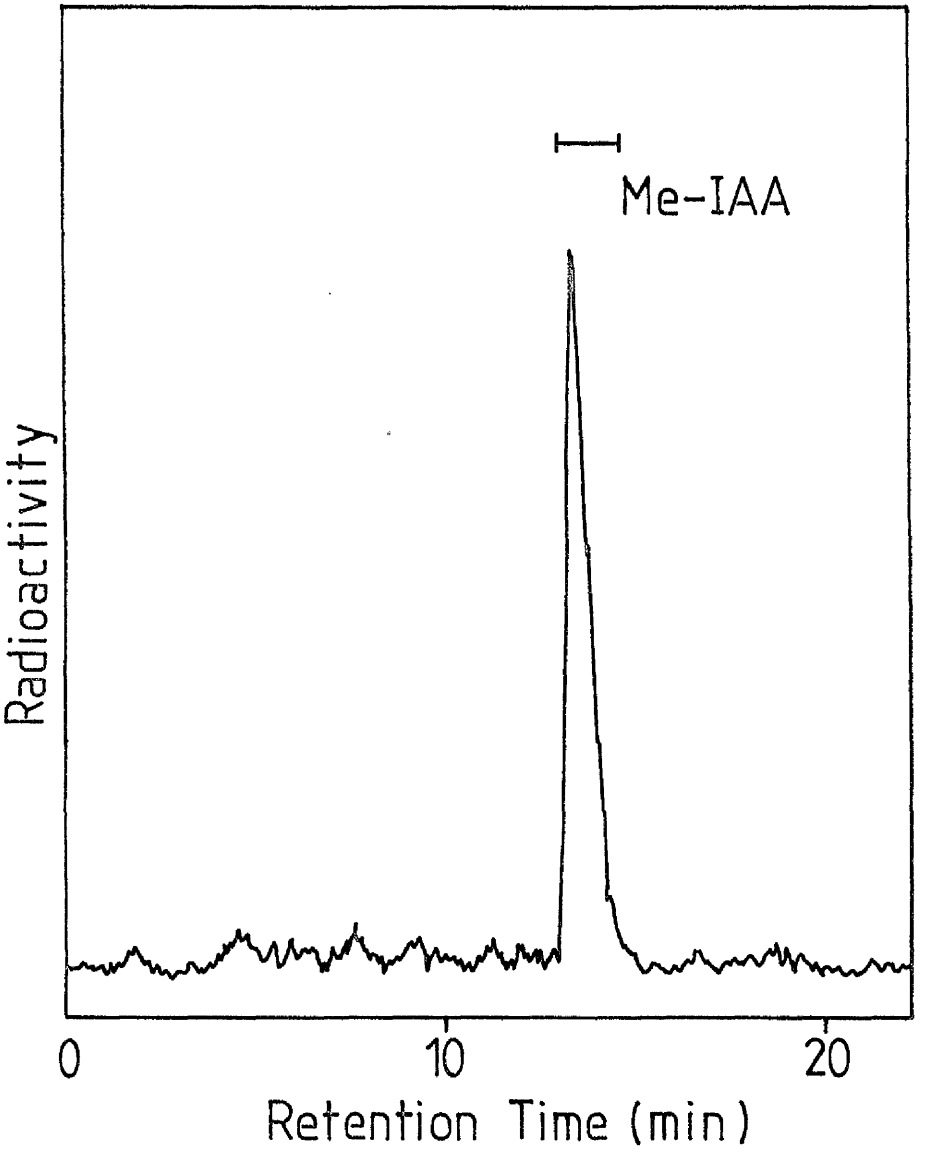


Fig. 20. HPLC analysis of radioactivity extracted from "receiver" block, after methylation using diazomethane. Trace represents results from a single extract, analysed in duplicate. Solvent gradient : 50-100% methanol over 20 min. Flow rate : 0.75 $cm^3 min^{-1}$. Detector : homogeneous radioactivity monitor; 20 cps full scale deflection; 10s time constant.

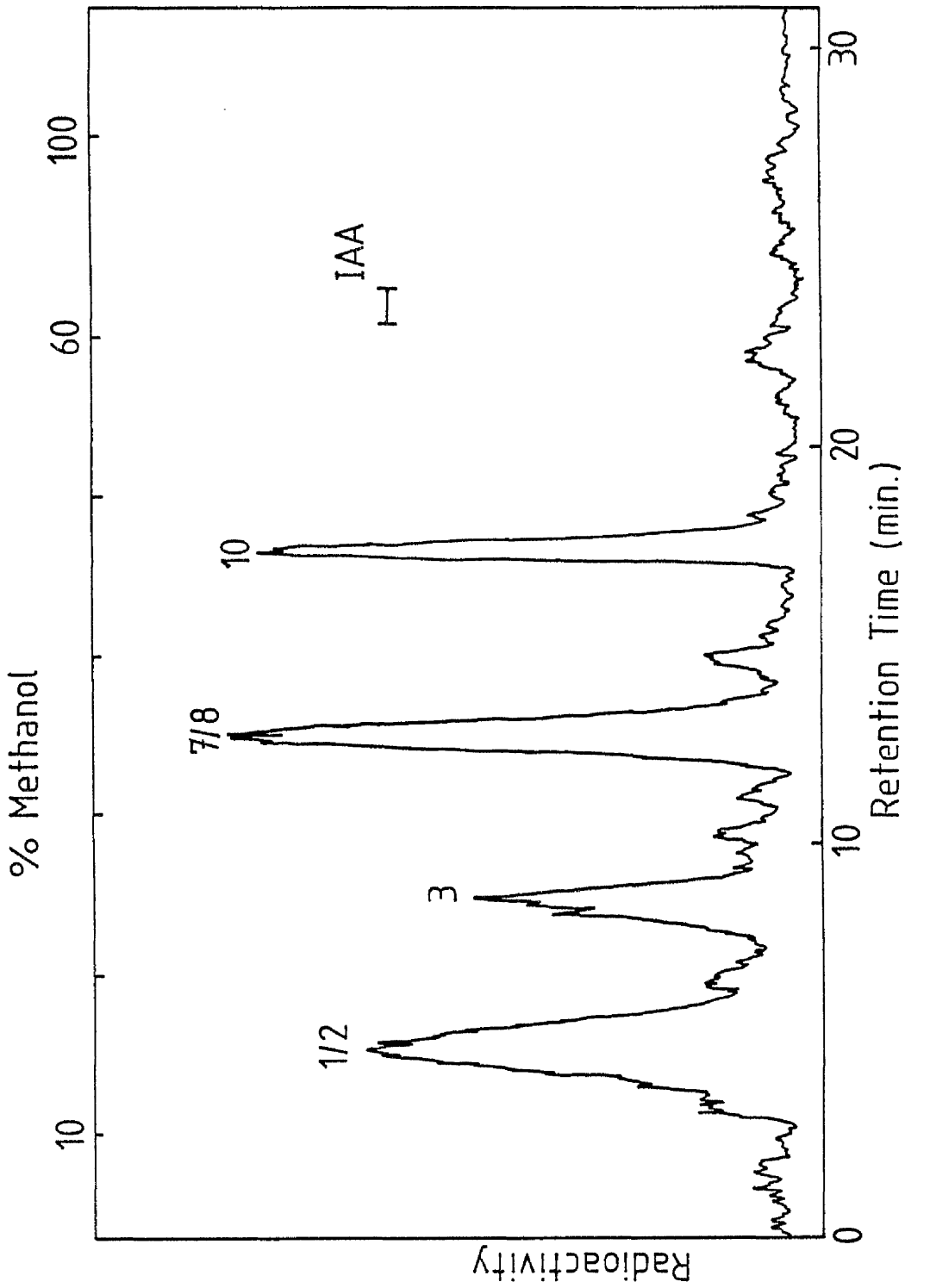


Fig. 21. HPLC analysis of methanolic extracts from roots supplied with IAA-2-^{14}C via the stele for 24h. The trace represents typical results from 3 replicate experiments. Solvent gradient : 10-60% over 20 min. Flow rate : 1 cm^3 min^{-1}. Detector : homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time constant.

of the tissues and their significance in relation to elucidating the normal pathways of endogenous IAA metabolism is questionable.

Conclusions

IAA-2-^{14}C supplied to the stele at the basal end of root segments for 2h is metabolised to at least 6 products which appear to be similar to those in extracts of tissue floated in aqueous solutions of IAA. The rate of metabolism was reduced, however, and the relative proportions of peaks differed. Radioactivity diffusing from the apical end of the segments appeared to be exclusively associated with the IAA molecule, thus providing evidence for a transport mechanism specific for IAA.

F. Metabolism of ^{14}C-IAA by Coleoptile Segments

Coleoptiles of dark-grown Zea mays seedlings have been used extensively for research on the rôle of IAA in geotropism and phototropism (e.g. Goldsmith and Wilkins, 1964; de la Fuente and Leopold, 1968; Wilkins and Whyte, 1968; Cane and Wilkins, 1969; Hertel et al., 1969; Shaw et al., 1973; Gardner et al., 1974), on the polar transport of IAA (e.g. Edwards and Goldsmith, 1980), and on binding of IAA to putative receptor sites (e.g. Hertel et al., 1972; Batt et al., 1976; Ray et al., 1977; Cross and Briggs, 1979; Moloney and Pilet, 1981). As with similar experiments on roots, the results of these studies have often been interpreted on the basis that metabolism of applied ^{14}C-IAA during the course of the experiment is negligible. In some cases this was checked using TLC (e.g. Cane and Wilkins, 1969; Shaw et al., 1973; Gardner et al., 1974; Edwards and Goldsmith, 1980). Where significant metabolism had occurred this was taken into account, although in each instance the majority of radioactivity co-chromatographed with the IAA standard. The results in section A of this thesis, however, demonstrated that TLC was not able to resolve the large number of metabolites present in methanolic extracts of Zea mays root tissue which had been incubated in IAA-2-^{14}C. The metabolism of IAA-2-^{14}C in coleoptile tissues was therefore investigated using HPLC analysis.

F.1. HPLC Analysis of IAA-2-^{14}C Metabolites from Coleoptile Segments after 2h Incubation

Coleoptile sections from 5-day-old, dark-grown Zea mays seedlings were cut under dim green light and incubated in groups of 10 or 20 in aqueous solutions of IAA-2-^{14}C (2 cm^3 ; 10^{-2} mol m^{-3}) for 2h in darkness. After following the standard washing procedure, tissue was extracted overnight with methanol and samples prepared for HPLC analysis. In one replicate the extract was prepared according to method 1, while two samples were

purified using Sep-pak C_{18} cartridges (method 2).

In each experiment a substantial proportion of IAA was metabolised (Fig. 22; Table 15, column 1); an average of 47% of the radioactivity remained associated with a peak which shared the same retention time as IAA. A corresponding fluorescence peak was also observed when the excitation and emission wavelengths were set at 280 and 350 nm respectively. All the major metabolites observed corresponded closely to peaks present in root extracts. Peak 10 was the most prominent metabolite and comprised a mean of 35% of the label. Other compounds were proportionately reduced and could not always be distinguished from background radiation. Although only metabolites 3,4,5,10 and 11 appeared consistently, peak 2 alone was absent from all coleoptile extracts.

The average amount of radioactivity taken up by the tissue was 121 Bq segment^{-1} (equivalent to approximately 57 pmol IAA). The extraction efficiencies for all coleoptile experiments were high (at least 90%; Tables 16, 18 and 20). A small and variable amount of radioactivity remained unaccounted for at the end of each experiment. Label was also lost during preparation of samples for HPLC (up to 22%). Very little breakdown of IAA took place in the incubating solution, the amount of radioactivity associated with the IAA peak never being less than 94% (Table 15).

IAA Breakdown during Sample Preparation

As with experiments on the metabolism of IAA in root tissue, controls were included to test whether breakdown occurred during the preparation of extracts for analysis. Aliquots of IAA-2-^{14}C were added to 3 separate methanolic extracts of 20 coleoptile segments each. Samples were prepared for HPLC analysis using Sep-pak C_{18} cartridges (method 2). A small amount of peak 10 was present in each trace (Fig. 23). The average proportion of radioactivity lost during sample preparation was 26% (Table 17).

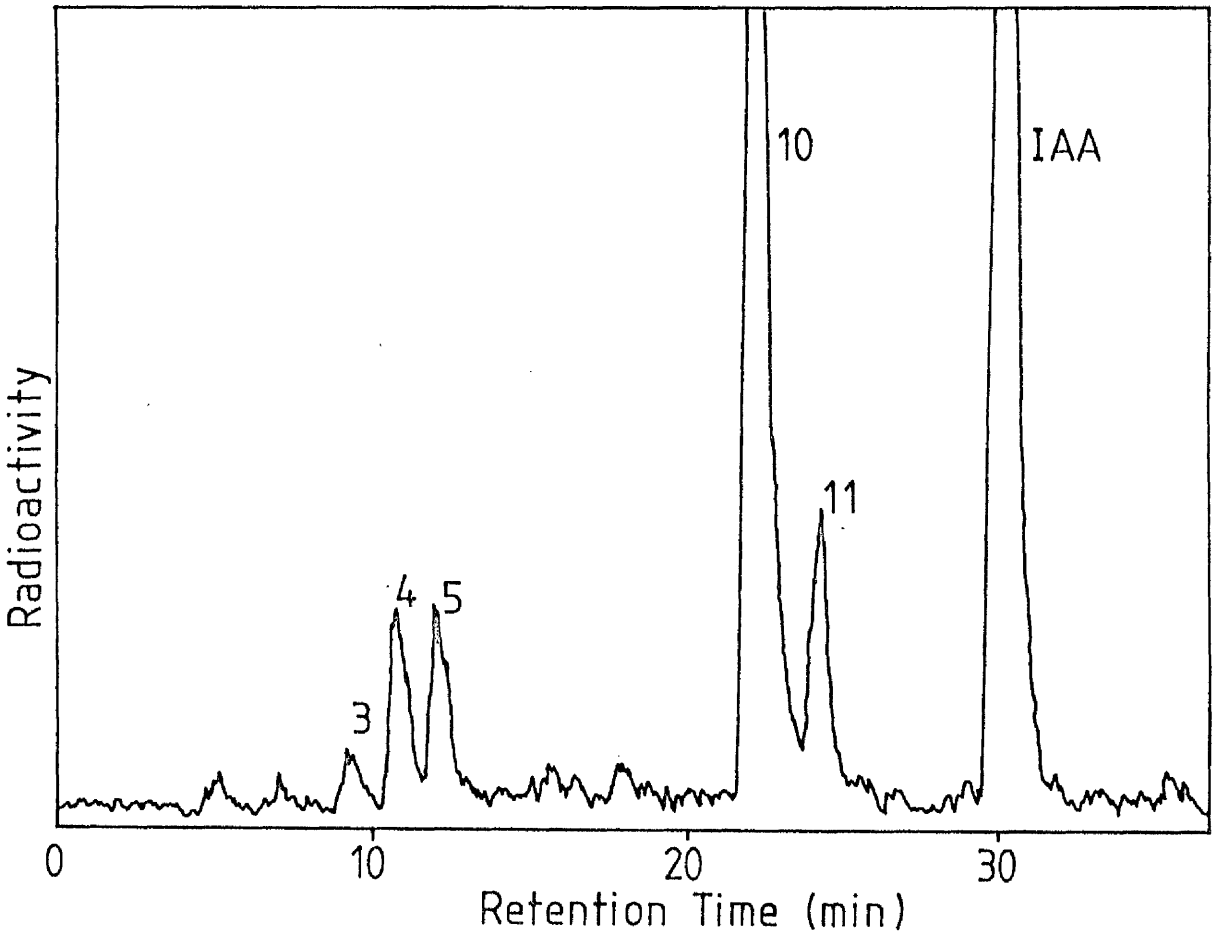


Fig. 22. Metabolism of IAA-2-^{14}C by coleoptile tissues of Zea mays seedlings : HPLC analysis. 20 segments (cut in dim green light) were incubated in 10^{-2} mol m^{-3} IAA-2-^{14}C for 2h. Trace represents a typical result from 3 replicate experiments. Root extracts containing IAA metabolites were used as standards for numbering of peaks. Solvent gradient : 10-60% methanol over 30 min. Flow rate : 0.75 $cm^3 min^{-1}$. Detector : homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time constant.

Table 15 : IAA Metabolites present in methanol extracts of coleoptile segments incubated in IAA-2-^{14}C for 2h.

Metabolite peaks were numbered, using root extracts as standards; see Expt A.2, Fig. 8 (1).

The experiment was performed in triplicate.

TREATMENT	Coleoptile segments cut in dim green light	Coleoptile segments cut in the laboratory	Boiled coleoptile segments
Radioactive peaks present in	IAA,10,4,7/8,11,5,6,9,1,3	IAA,10,11,4,5,3,1,7/8	IAA,10
methanol extracts in order of	IAA,10,11,4,5,3	IAA,10,3,4,5,1	IAA,10
decreasing height (1)	IAA,10,11,5,4,3		IAA,10
% radioactivity in methanol	63	52	96
extract remaining associated	39	44	95
with IAA peak	38		99
% radioactivity in methanol	32	36	4
extract associated with peak 10	36	44	5
	38		0.5
% radioactivity in incubating	-	99	-
solution associated with IAA peak	98	97	95
	97		96

Table 16 : Analysis of the fate of IAA-2-^{14}C supplied in aqueous solution to Zea mays coleoptile segments.

Coleoptiles (10, 20 or 40 segments from which leaves had been removed) were incubated for 2h in 10^{-2}mol m^{-3} IAA-2-^{14}C. Extracts designated (S) were purified using Sep-pak C_{18} cartridges. Radioactivity is expressed in Bq. The experiment was carried out in triplicate.

TREATMENT	Coleoptile segments cut in dim green light	Coleoptile segments cut in the laboratory	Boiled coleoptile segments
1. Initial Radioactivity	50300 49000 47800	50300 49300	45400 43300 42700
2. Radioactivity remaining in incubating solution	45500 40700 32500	42200 42600	25800 38000 35500
3. Radioactivity in methanol extract	949 2620 2500	1800 2060	6980 3280 3610
4. Radioactivity remaining in tissue after extraction	43 55 72	46 52	103 35 63
5. Radioactivity in washings	- 5170 6810	8160 4360	11800 4750 7160
6. Final total radioactivity	- 48500 41900	52200 49000	44700 46100 46400
7. Loss of radioactivity during incubation	- 451 5900	- 225	671 - -

8. 7 expressed as a percentage of 1	- 0.9% 12%	- 0.5%	1.5% - -
9. Number of segments	10 20 20	20 20	40 20 20
10. Dry weight of tissue (mg)	- 16.1 16.9	- 15.1	64.2 12.4 13.0
11. Uptake (Bq segment^{-1})	99 134 129	92 105	199 164 184
12. Extraction efficiency	96% 98% 97%	98% 98%	99% 99% 98%
13. Radioactivity in methanol extract after preparation for HPLC	- 2230(S) 2180(S)	1500 -(S)	5830(S) 2580(S) 2830(S)
14. 13 expressed as a percentage of 3	- 85% 87%	83% -	84% 79% 78%
15. Radioactivity in incubating solution after preparation for HPLC	30400 33300 28300	39300 35600	20600 32400 29200
16. 15 expressed as a percentage of 2	67% 82% 87%	93% 84%	80% 85% 82%

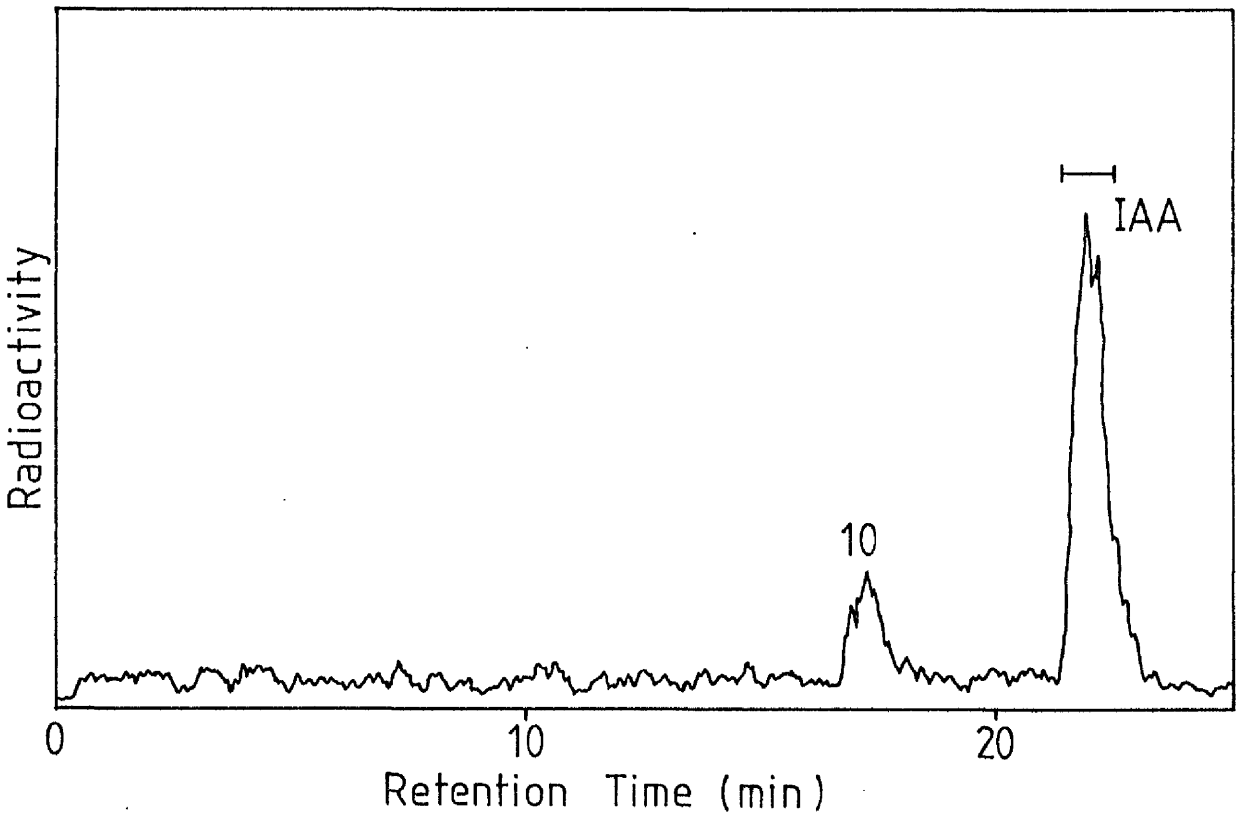


Fig. 23. IAA degradation during preparation of coleoptile extracts for HPLC analysis. IAA-2-^{14}C was added to methanolic coleoptile extracts and samples prepared for HPLC analysis using Sep-pak C_{18} cartridges (method 2). The trace represents typical results from 3 extracts. Solvent gradient : 10-60% methanol over 20 min. Flow rate : 1 $cm^3 min^{-1}$. Detector : homogeneous radioactivity monitor; 30 cps full scale, 10s time constant.

Table 17 : Loss of IAA-2-^{14}C during preparation of control coleoptile extracts for HPLC analysis

REPLICATE	1	2	3
1. Initial radioactivity in extract (Bq)	887	931	993
2. Radioactivity in extract after preparation for HPLC analysis (Bq)	626	686	761
3. 2 expressed as a percentage of 1	71%	74%	77%

Metabolism of IAA-2-^{14}C by Boiled Coleoptile Segments

Coleoptile tissue (groups of 20 or 40 segments) which had been boiled for 2 min was incubated in aqueous solutions of IAA-2-^{14}C (10^{-2} mol m^{-3}) for 2h.

After following the standard washing procedure, plant material was extracted overnight with methanol, and samples prepared for HPLC analysis using Sep-pak C_{18} cartridges (method 2).

As with the previous control, the majority of the radioactivity remained associated with the IAA peak (Fig. 24; Table 15) although a small amount of peak 10 was present, representing a mean of 3% of the radioactivity in the extract. Losses of radioactivity and extraction efficiencies were similar to those for living tissue (Table 16). In contrast to the root experiments the uptake of radioactivity had increased by a mean of 51%.

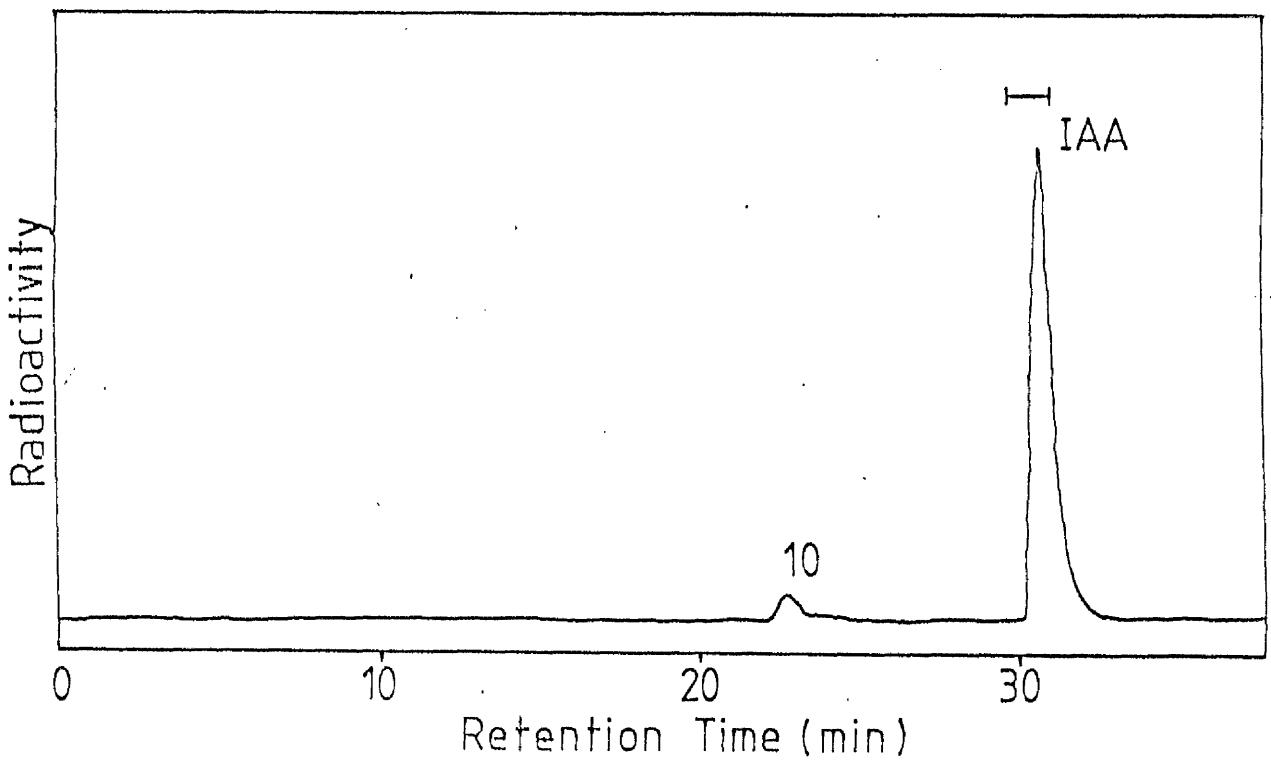


Fig. 24. Metabolism of IAA-2-^{14}C by boiled coleoptile segments (2h incubation). Trace represents a typical result from 3 replicate experiments. Solvent gradient : 10-60% methanol over 30 min. Flow rate : 0.75 $cm^3 min^{-1}$. Detector : homogeneous radioactivity monitor; 300 cps full scale deflection; 10s time constant.

F.2. Metabolism of IAA by Coleoptile Segments Exposed to Light during Sectioning

Coleoptiles for large-scale experiments were sectioned in the laboratory. An experiment was therefore carried out to determine the effect of this short exposure to light on the metabolism of IAA. Groups of 20 coleoptile segments were incubated in aqueous IAA-2-^{14}C (10^{-2} mol m^{-3}) for 2h. Plant material was both grown and incubated in darkness, but sections were cut in the laboratory. This took approximately 15 min.

Analysis of methanolic extracts, prepared according to either method 1 or 2 revealed a pattern of metabolism closely similar to that obtained in experiment F.1 with coleoptile segments cut in dim green light (Table 15). No significant differences could be found in either the amount of IAA metabolised (a mean of 52% compared with 53% for experiment F.1) or the products obtained. Uptake and extraction efficiencies were also not significantly affected (Table 16).

F.3. Effect of Varying the External Concentration of IAA on its Metabolism by Coleoptile Segments

Coleoptile segments (cut in the laboratory) were incubated in groups of 20 in three different concentrations of IAA-2-^{14}C (10^{-3}, 10^{-2} and 10^{-1} mol m^{-3}) for 2h. After washing (Materials and Methods), tissue was extracted with methanol and samples prepared for HPLC analysis using Sep-pak C_{18} cartridges (method 2).

No consistent and significant differences could be found in the metabolism patterns obtained at different concentrations (Fig. 25, Table 18). The average proportions of radioactivity remaining associated with the IAA peak were 57%, 45% and 58% for external IAA concentrations of 10^{-3}, 10^{-2} and 10^{-1} mol m^{-3} respectively. Peak 10 was always the most prominent metabolite with peak 4 also consistently present. Variation in the minor components observed, appeared to be due to the limitations of the detector sensitivity. The

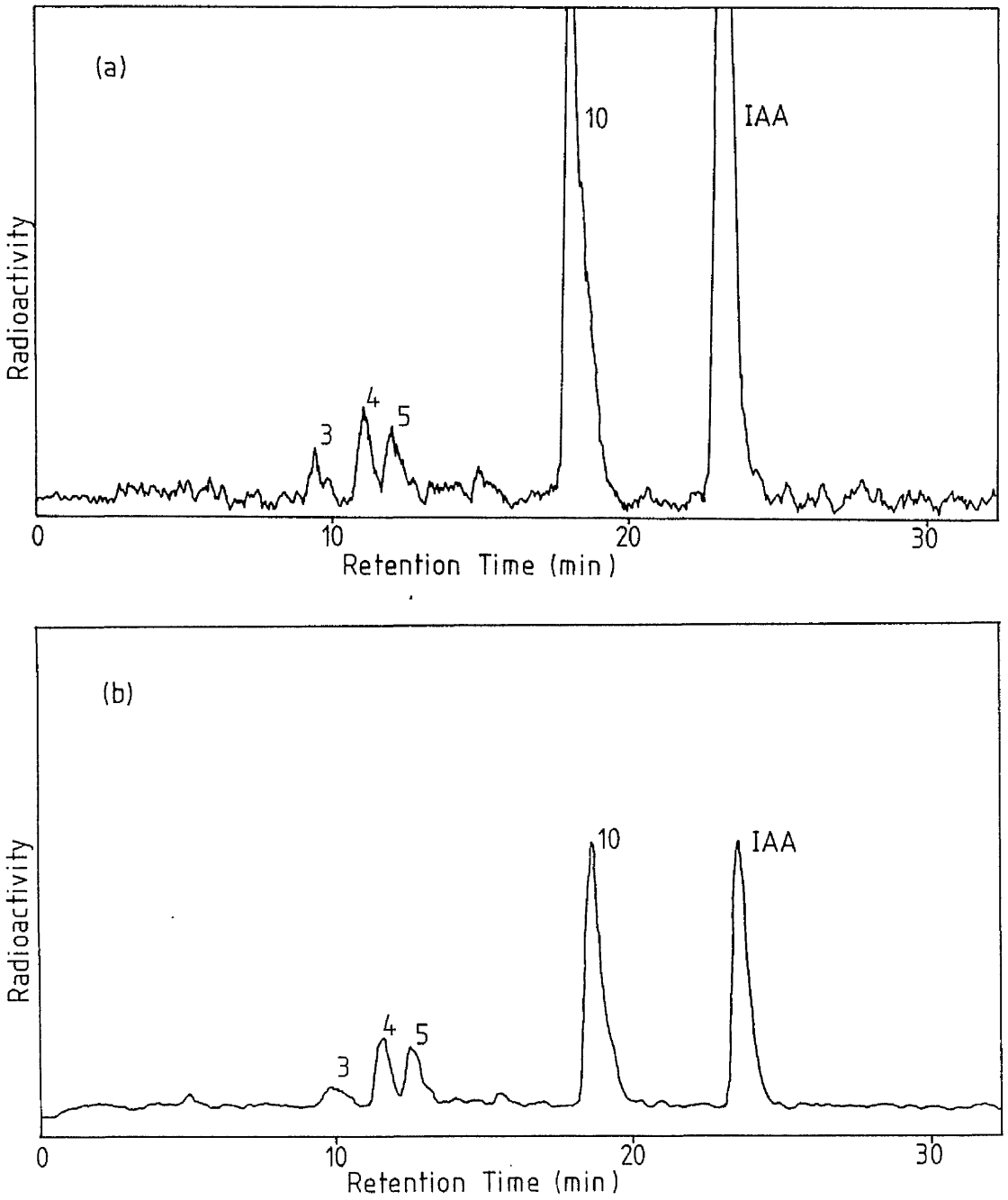


Fig. 25. Metabolism of IAA-2-^{14}C supplied at 3 different concentrations to Zea mays coleoptile segments (2h incubation). a. 10^{-3} mol m^{-3}, b. 10^{-2} mol m^{-3}, c. 10^{-1} mol m^{-3}. Each trace represents typical results from 3 replicate experiments. Solvent gradient: 10-60% methanol over 20 min. Flow rate: 1 cm^3 min^{-1}. Detector: homogeneous radioactivity monitor; 30 cps full scale deflection (a. and c.), 100 cps (b); 10s time constant.

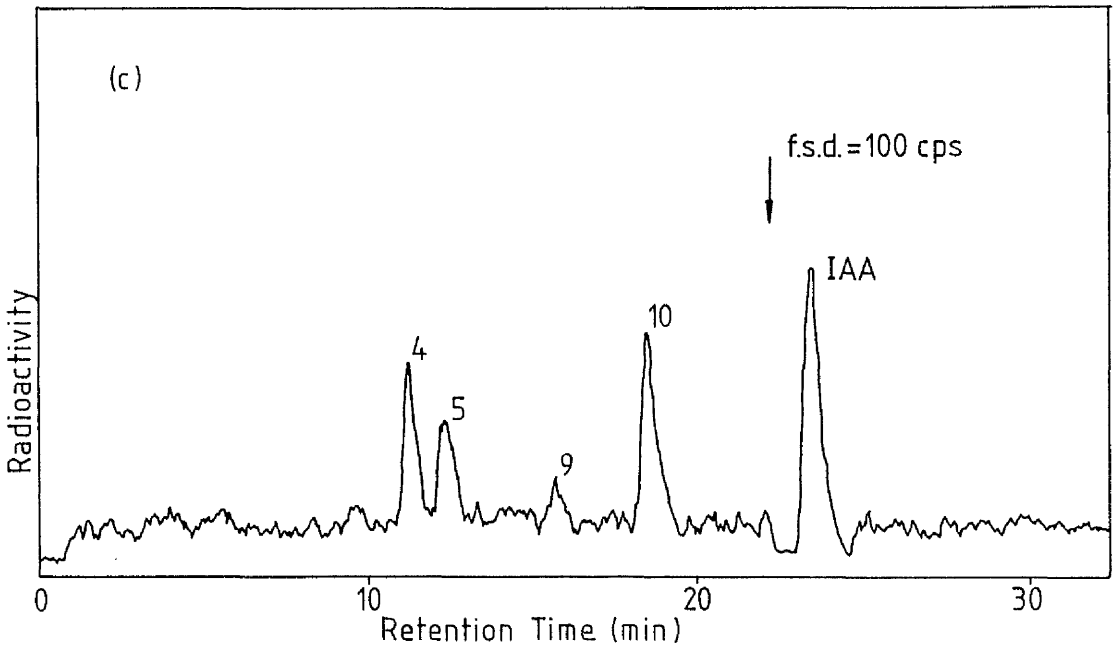

(c)
f.s.d.=100 cps
IAA
10
4
5
9
Radioactivity
0
10
20
30
Retention Time (min)

Fig. 25 cont'd

Table 18: Uptake and metabolism of IAA-2-^{14}C supplied at 3 different concentrations to Zea mays coleoptile segments (2h incubation). Metabolite peaks were numbered using extracts from root segments incubated for 2h in 10^{-3} mol m^{-3} IAA-2-^{14}C as standards: see Expt. A.2, Fig. 8 (1).

The experiment was carried out in triplicate.

Concentration of IAA in incubating solution	10^{-3} mol m^{-3}	10^{-2} mol m^{-3}	10^{-1} mol m^{-3}
Uptake of IAA (pmol segment^{-1})	7.5	38	320
	6.8	53	654
	6.8	65	551
Radioactive peaks in methanol extract in order of decreasing height (1)	IAA, 10, 4, 5, 3	IAA, 10, 4, 5, 3	IAA, 10, 4, 5, 9
	IAA, 10, 11, 4, 5	IAA, 10, 11, 4, 5, 3	IAA, 10, 4, 5
	IAA, 10, 11, 4, 3	IAA, 10, 11, 4, 3, 5	IAA, 10, 11, 4, 5
% radioactivity in methanol extract associated with IAA peak	57	39	64
	59	50	63
	56	47	48
% radioactivity in methanol extract associated with peak 10	31	38	13
	24	25	28
	25	29	27

lack of peak 1, which was present in some extracts in Experiments F.1 and F.2, was probably a result of its loss during purification of samples using Sep-pak C_{18} cartridges (see Experiment B.5). The different external IAA concentrations also had no effect on the extraction efficiency which was always at least 95%.

At the lowest concentration, i.e. 10^{-3} mol m^{-3} IAA, the average amount of radioactivity taken up over 2h was equivalent to 7.0 pmol $segment^{-1}$. Given that approximately 57% of this remained associated with the IAA peak, the amount of exogenous IAA in the tissue after this time appeared to be circa 4.0 pmol $segment^{-1}$.

F.4. Time-Course of IAA Metabolism by Zea mays Coleoptile Segments

Four groups of 20 coleoptile segments (cut in the laboratory) were placed in aqueous solutions of IAA-2-^{14}C (10^{-2} mol m^{-3}) and incubated for various times: Table 19.

Table 19 : Incubation protocol for time-course experiments on IAA metabolism in coleoptile segments

Dish	Time incubated in ^{14}C-IAA	Time incubated in distilled water	Total incubation time
1	10 min	-	10 min
2	1 h	-	1 h
3	1 h	3 h	4 h
4	1 h	23 h	24 h

Sections incubated for longer than 1h were washed thoroughly (Materials and Methods) before transferring to dishes of distilled water for the remainder of the incubation time. Methanol extracts were prepared for HPLC analysis using Sep-pak C_{18} cartridges (method 2).

Metabolism of IAA was slower than in root tissues, with no products apparent after a 10 min incubation (Fig. 26; Table 20). However, in common with root experiments very little IAA was detectable after 24h (IAA was only observed in one replicate). After 4h the IAA peak was approximately 15% of the size of peak 10. Peak 10 was the most prominent metabolite at each time. Other peaks were small and variable, although peaks 7 and 8 had consistently increased in size after a 24h incubation. As with root experiments, there were no clear indications of conversions of one metabolite into another.

Conclusions

Metabolism of IAA-2-^{14}C by *Zea mays* coleoptile segments was rapid, with approximately 47% of radioactivity remaining associated with the IAA peak after 2h incubation. During short incubation times the amount of IAA metabolised by coleoptiles was significantly less than in roots. The products of IAA metabolism in roots and coleoptiles appeared to be similar although peak 10 had greater prominence in coleoptile extracts. Controls for metabolism by dead tissue, and for breakdown of IAA during sample preparation indicated that metabolism was genuinely taking place in the living tissue. Metabolism of IAA in sterile coleoptiles was not investigated. The exposure of coleoptiles to light for a short period during section cutting did not affect the rate or pattern of IAA metabolism. Changes in the exogenous IAA concentration also did not alter its metabolism.

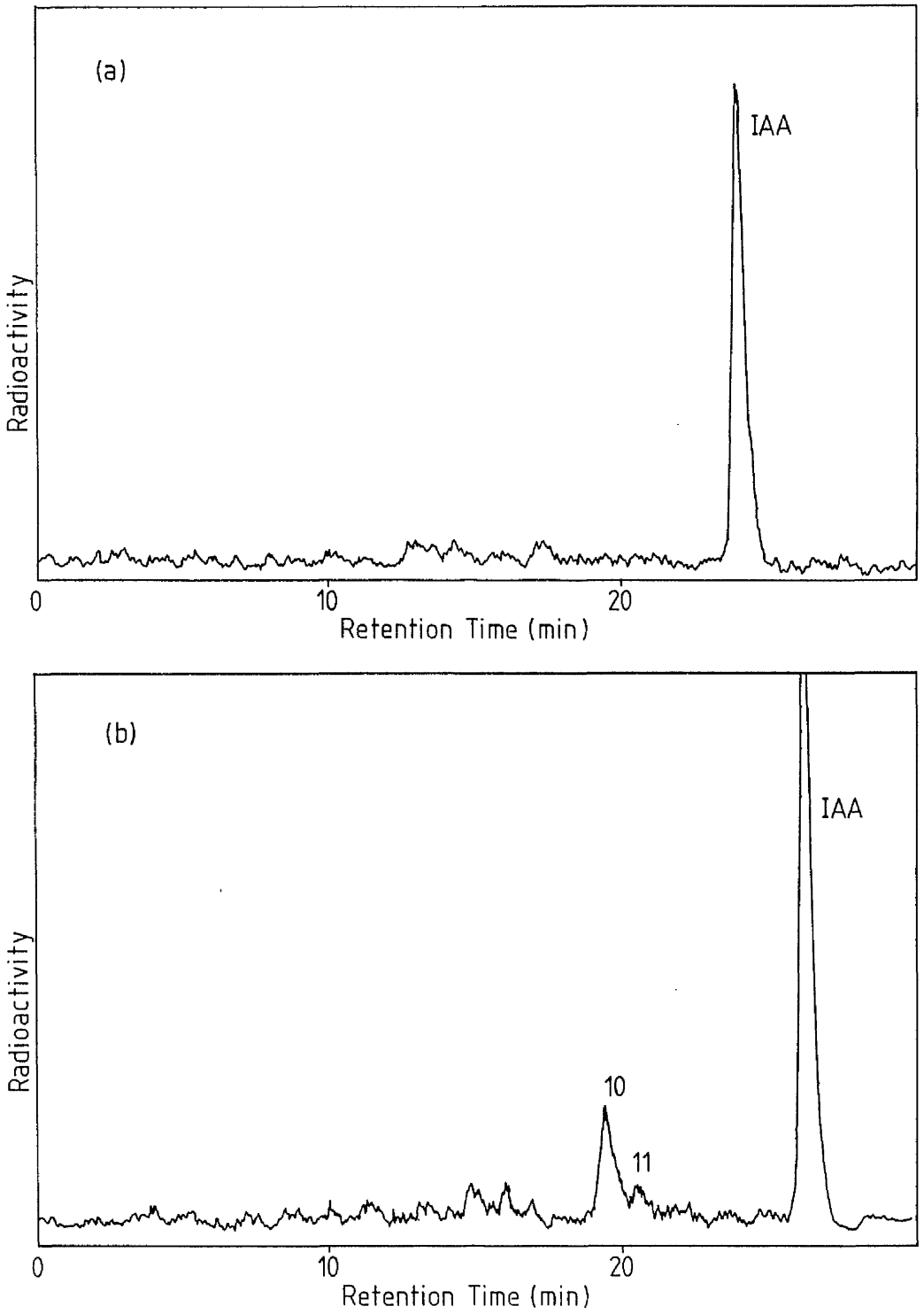


Fig. 26. Time-course of IAA-2-^{14}C metabolism by Zea mays coleoptile segments. Tissue was incubated in IAA-2-^{14}C (10^{-2} mol m^{-3}) for up to 1h, then washed and transferred to distilled water. (a) 10min incubation, (b) 1h, (c) 4h, (d) 24h. Each trace represents typical results from 3 replicate experiments. Solvent gradient: 10-60% methanol over 30 min. Flow rate: 1 $cm^3 min^{-1}$. Detector: homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time course.

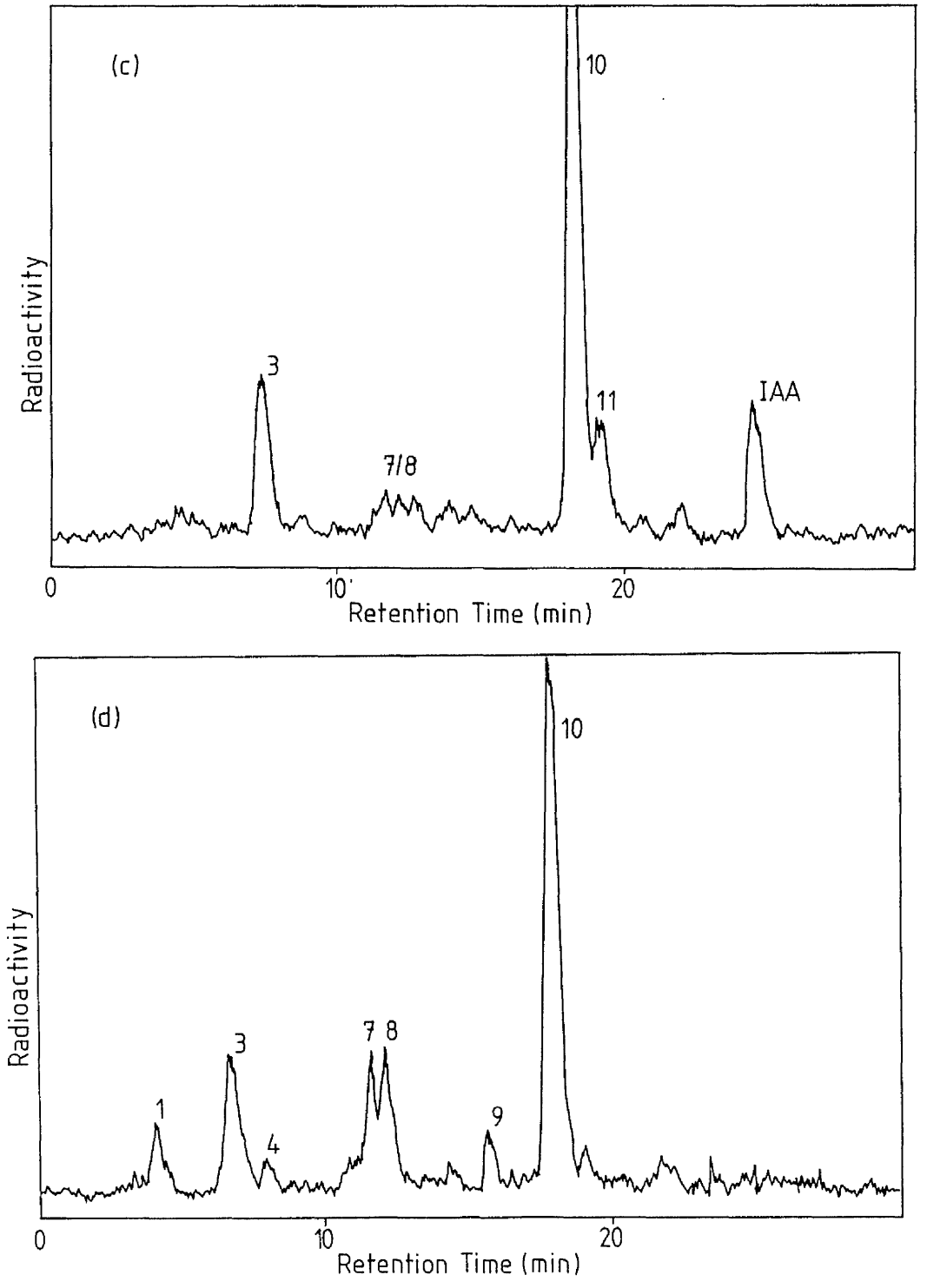

(c)
10
3
11
IAA
7/8
Radioactivity
0
10
20
Retention Time (min)
(d)
10
7 8
3
1
9
4
Radioactivity
0
10
20
Retention Time (min)

Fig. 26 cont'd

Table 20 : Metabolism of IAA-2-^{14}C by Zea mays coleoptile segments over a 24h time course. Tissue was incubated in IAA-2-^{14}C (10^{-2} mol m^{-3}) for up to 1h, then washed and transferred to distilled water. Extracts designated (S) were purified using Sep-pak C_{18} cartridges. The experiment was carried out in triplicate. Metabolite peaks were numbered using a root extracts as standards; see Expt. A.2 Fig. 8 (1).

Incubation Time	Metabolite peaks present in methanol extract in order of decreasing height (1)
10 min	IAA only
	IAA only (S)
	IAA only (S)
1 h	IAA, 10, 11
	IAA, 10, 11 (S)
	IAA, 10, 11 (S)
4 h	10, 3, IAA, 11, 7/8
	10, IAA, 11, 7/8, 3 (S)
	10, 11, IAA, 7/8, 3 (S)
24 h	10, 8, 7, 3, 1, 9, 4
	10, 8, 7, 9, 3, 2 (S)
	10, 11, 8, 7, 9, IAA (S)

G. Metabolism of IAA-2-^{14}C Transported through Zea mays Coleoptile Segments

In the previous experiment it was shown that Zea mays coleoptile tissue is capable of rapid metabolism of IAA. This has potentially important consequences for the interpretation of published results on the transport of IAA. An experiment was therefore carried out to investigate the metabolism of IAA-2-^{14}C moving basipetally along coleoptile segments.

Labelled IAA was supplied from agar blocks (donor blocks) to the apical ends of sections. Radioactivity diffusing from the basal ends was collected in agar (receiver blocks) which also served to prevent drying of the tissue. Coleoptile segments were thus incubated in petridishes, in four groups of 20, for 4h in darkness. After removing 1mm from each end of the sections, both tissue and agar blocks were extracted with methanol. Coleoptile extracts were purified using Sep-pak C_{18} cartridges (method 2) prior to HPLC analysis. Samples from agar blocks were prepared according to method 1.

Substantial metabolism of IAA took place within the plant tissue (Fig. 27; Table 21). Approximately 25% of the radioactivity remained associated with the IAA peak after 4h. Metabolites 3 and 10 are seen in Fig. 26. In a repeat experiment small amounts of peaks 6, 7 and 8 were also visible. In contrast, only a single peak, which co-chromatographed with IAA, was observed in extracts from receiver blocks. When methylated using diazomethane a compound was formed which co-chromatographed with IAA-methyl ester on the HPLC (Fig. 28). Further proof that only IAA had reached the receiver block of agar came from GC-MS analysis. When used in the selected ion-monitoring mode and tuned for ions with m/e 202 a peak was observed with the same retention time as IAA (Fig. 29).

Only a very small proportion of the IAA-2-^{14}C remaining in the donor blocks had undergone modification.

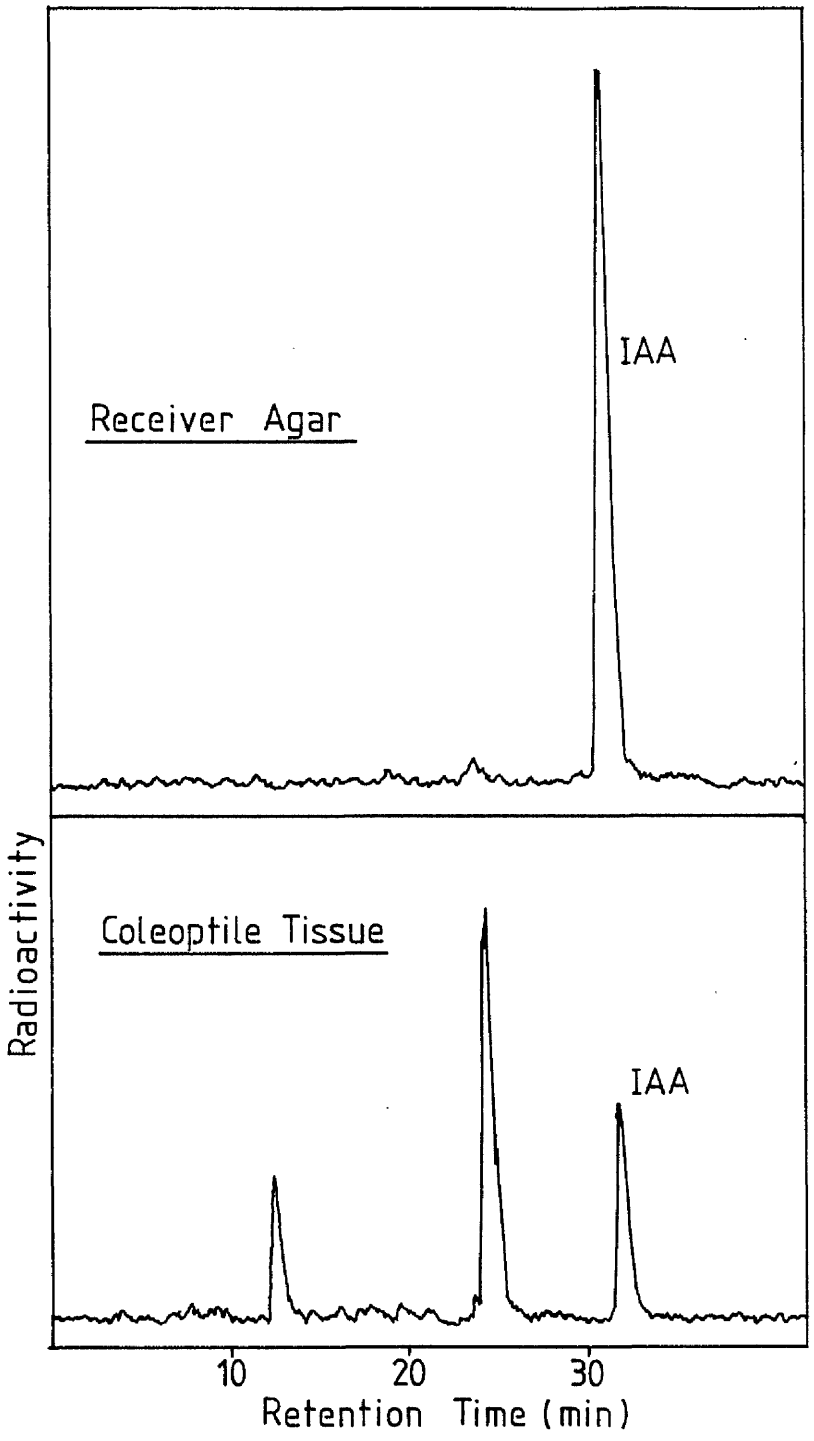


Fig. 27. HPLC analysis of methanol extract from coleoptile segments supplied with IAA from agar blocks, and of extracts from agar receiver blocks. 4h incubation. HPLC conditions: 10-60% methanol over 30 min. Flow rate: 0.75 $cm^3 min^{-1}$. Homogeneous radioactivity monitor; 30cps full scale deflection; 10s time constant

Table 21 : Metabolism of IAA-2-^{14}C transported down Zea mays coleoptile segments. Segments were supplied with IAA-2-^{14}C from agar blocks placed at the apical ends and incubated for 4h. The experiment was repeated in duplicate.

	Amount of Radioactivity (Bq)	% of radioactivity remaining associated with IAA peak
Donor block	12,600	92
	19,400	98
Coleoptile extract	1,014	26
	2,560	25
Receiver block	917	97
	1,880	100

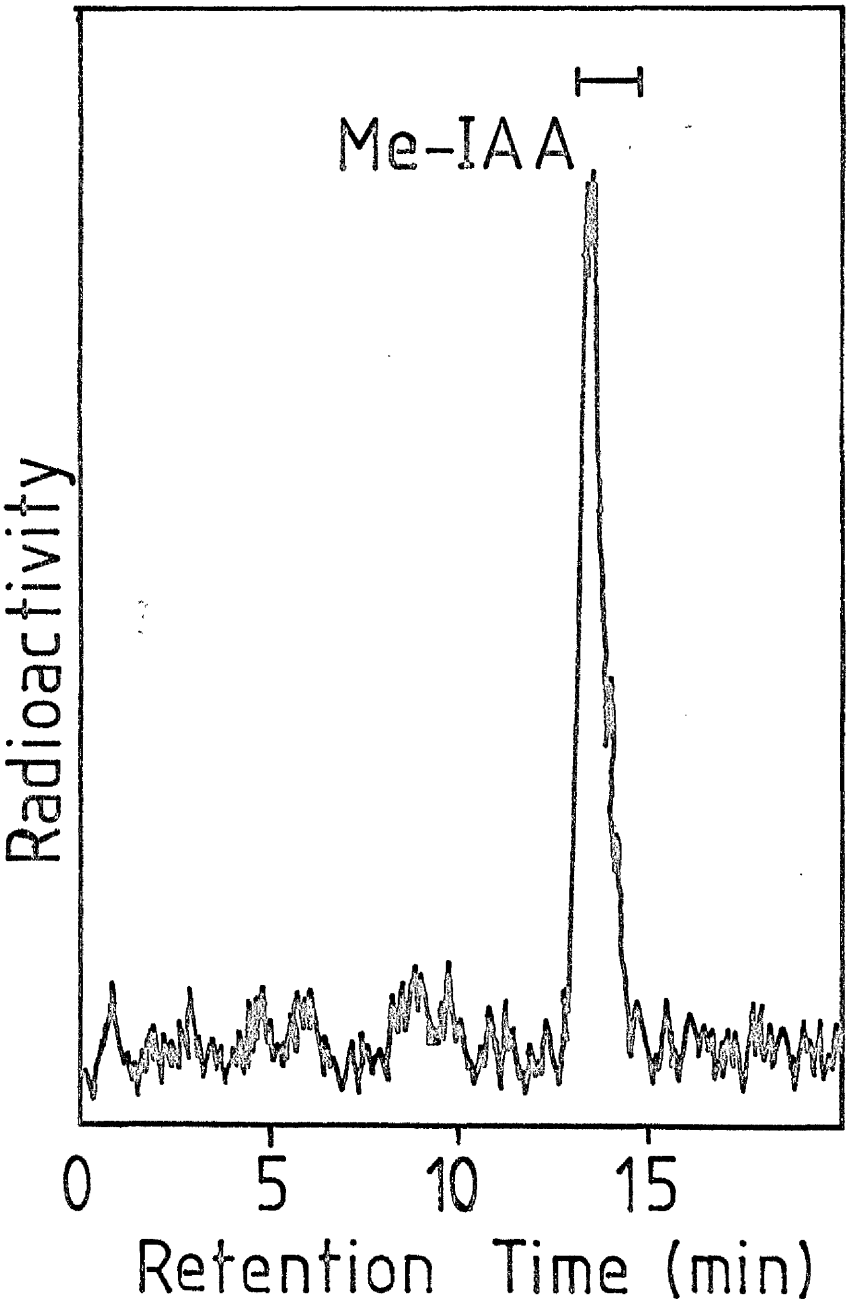


Fig. 28. HPLC Analysis of radioactivity collected in agar "receiver block", after methylation using diazomethane. Solvent gradient: 50-100% methanol over 20 min. Flow rate: 0.75 $cm^3 min^{-1}$. Detector: homogeneous radioactivity monitor; 10 cps full scale deflection; 10s time constant.

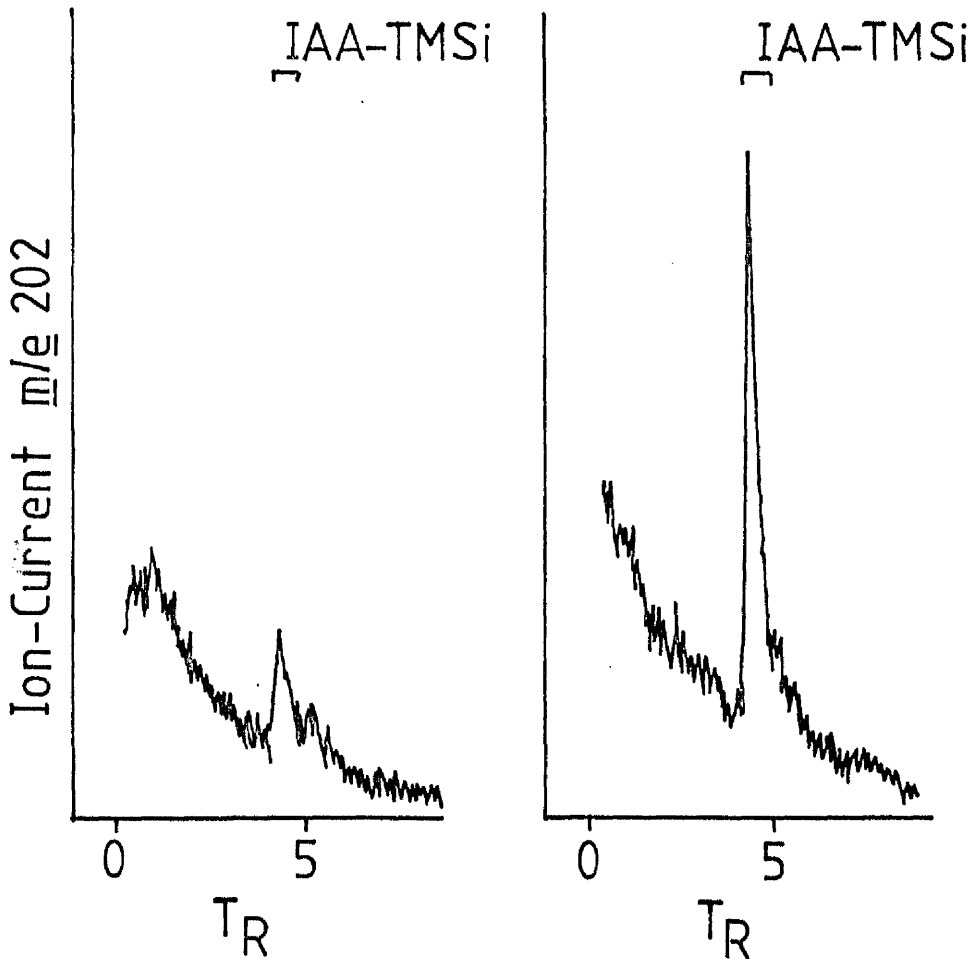


Fig. 29. GC-MS analysis of the IAA peak from methanolic extracts of "receiver blocks", after purification by HPLC, and derivatisation using BSTFA.

GC column: 5ft x ¼" (1.52m x 6.35mm) 3% Dexil-300 on Supelcoport 100-120. Temperature: 190°C isothermal. Flow rate: 20 cm^3 min^{-1}. MS Conditions: 24 eV; Source temperature 280°C; Separator temperature 250°C. (a) extract only. (b) extract plus coinjection of IAA-TMSi standard.

Conclusions

IAA-2-^{14}C supplied to coleoptile segments from apically placed agar blocks was substantially metabolised by the tissue. A comparison with Fig. 26c indicated that metabolism was somewhat slower than that taking place in coleoptiles supplied with IAA from the incubating solution.

Analyses of radioactivity in receiver blocks indicated that the IAA metabolites formed were not mobile. IAA alone was transported.

H. The Chemical Nature of IAA Metabolites

A series of experiments were carried out to investigate the chemical structures of the products of IAA metabolism. For this purpose large extracts were prepared from 10 or 15 groups of 50 root segments incubated for 2h in 10^{-2} mol m^{-3} IAA-2-^{14}C. After washing (Materials and Methods), the tissue was extracted with methanol. For the majority of studies the extracts were purified using Sep-pak C_{18} cartridges (Method 2). An aliquot of each sample was analysed using HPLC and the retention times of metabolite peaks 4-11 and the IAA peak were measured. The remainder of the extract was then loaded onto the column and metabolites collected. Peaks 1, 2 and 3 could only be collected at a very low yield and were not used. The samples were dried by first blowing off the methanol under a stream of nitrogen, then freeze-drying.

H.1. Experiment to Investigate the Number of Radioactive Compounds Represented by each Peak

Samples of individual peaks were prepared as described above. Each was then reanalysed by HPLC, firstly on a solvent gradient of 10-60% methanol over 30min to check for sample degradation, and secondly by isocratic elution. The experiment was carried out in triplicate. The results of the gradient analysis have been presented in Experiment A.3 (Table 6). The compounds giving rise to peaks 4 and 5 appeared to be interconverted. A proportion of peaks 6 and 11 had been modified while the other compounds remained unchanged.

The results of the isocratic analyses are given in Table 22. In each case no further resolution of products was observed, indicating that the radioactivity in each peak is probably associated with a single compound.

Table 22 : Results of isocratic HPLC analysis of IAA-2-^{14}C metabolites from *Zea mays* root tissue. The experiment was carried out in duplicate.

Metabolite peak	% Methanol in buffer used for HPLC analysis	No. of Peaks observed
4	12%	2 Peaks (peaks 4 and 5)
5	12%	2 Peaks (peaks 4 and 5)
6	12%	2 Peaks (peak 6 and break-down product)
7/8	18%	2 Peaks (peaks 7 and 8)
9	23%	Single peak
10	23%	Single peak
11		Not analysed
IAA	50%	Single peak

H.2. Polarity of IAA Metabolites

In a reverse-phase HPLC system compounds of greatest polarity are normally eluted first. It can be seen from the traces obtained (e.g. Figs. 8 and 22) that all metabolites from both root and coleoptile segments were eluted before IAA. This is thus a good indication that the products are all more polar than IAA itself. It must be remembered, however, that the relative polarities of compounds may be altered by the solvent system. Furthermore, in addition to the main process of solvent partitioning, other effects such as adsorption may play a part in the separation.

H.3. Experiment to Investigate whether carbon-1 of the Side Chain has been lost

An experiment was carried out to compare the metabolism of IAA-1-^{14}C with that of IAA-2-^{14}C. Groups of 50 root or 20 coleoptile segments (cut in the laboratory) were incubated for 2h in 2 cm^3 volumes of 10^{-3} and 10^{-2} mol m^{-3} IAA respectively. After following the standard washing procedure, tissues were extracted with methanol. Samples were prepared for HPLC analysis using either method 1 or method 2 as indicated in Table 23.

HPLC analyses of both root and coleoptile extracts showed metabolism patterns closely resembling those obtained in experiments using IAA-2-^{14}C (Figs. 30 and 31, Table 23). The range of metabolite peaks present appeared to be the same, as did the proportion of radioactivity remaining associated with the IAA molecule; means of 38% compared with 31% for roots and 51% compared with 48% for coleoptiles. Similar amounts of radioactivity were also associated with peak 10. No significant differences between experiments utilising IAA-1-^{14}C and IAA-2-^{14}C were found in the quantities of radioactivity lost during incubation (Table 24). It would thus appear that all the major metabolites of IAA, observed in the present experiments on *Zea mays* seedlings, do not represent products of a decarboxylation reaction.

Table 23 : Comparison of the metabolism of IAA-1-^{14}C and IAA-2-^{14}C by root and coleoptile segments from Zea mays seedlings.

Segments (50 root or 20 coleoptile segments) were incubated for 2h in ^{14}C-IAA (10^{-3} or 10^{-2} mol m^{-3}). Metabolite peaks in extracts of roots incubated for 2h in 10^{-3} mol m^{-3} IAA-2-^{14}C were numbered 1-11 (see Experiment A.3) and used as standards for the other extracts. Samples designated (S) were purified using C_{18} Sep-pak cartridges. The experiment was carried out in triplicate.

	IAA-2-^{14}C supplied to roots	IAA-1-^{14}C supplied to roots	IAA-2-^{14}C supplied to coleoptiles	IAA-1-^{14}C supplied to coleoptiles
Radioactive peaks present in methanol extracts in order of decreasing height	IAA,10,9,7,6,5,4,3, 1,11,8,2	IAA,10,7,9,8,6,1,11, 5,4,2,3	IAA,10,11,4,5,3,1, 9,7/8	IAA,10,4,5,1,3,11, 9, 7/8
	IAA,10,9,6,1,5,7,8, 4,3,2,11	IAA,10,7/8,9,4,6,1, 5,3,11,2	IAA,10,3,4,5,1(S)	IAA,10,11,4,5,1,3(S)
	IAA,10,9,6,7,5,1,4, 3,8,2,11	IAA,10,1,3,9,5,4,6, 7,11,8,2		IAA,10,11,4,5,3,1(S)
% Radioactivity in methanol extract associated with IAA peak	34	42	52	48
	29	39	44	57
	30	32		49
% Radioactivity in methanol extract associated with peak 10	21	21	17	36
	19	16	44	31
	18	21		32
% Radioactivity in incubating solution associated with IAA peak	93	98	99	97
	98	90	97	100
	95	100		94

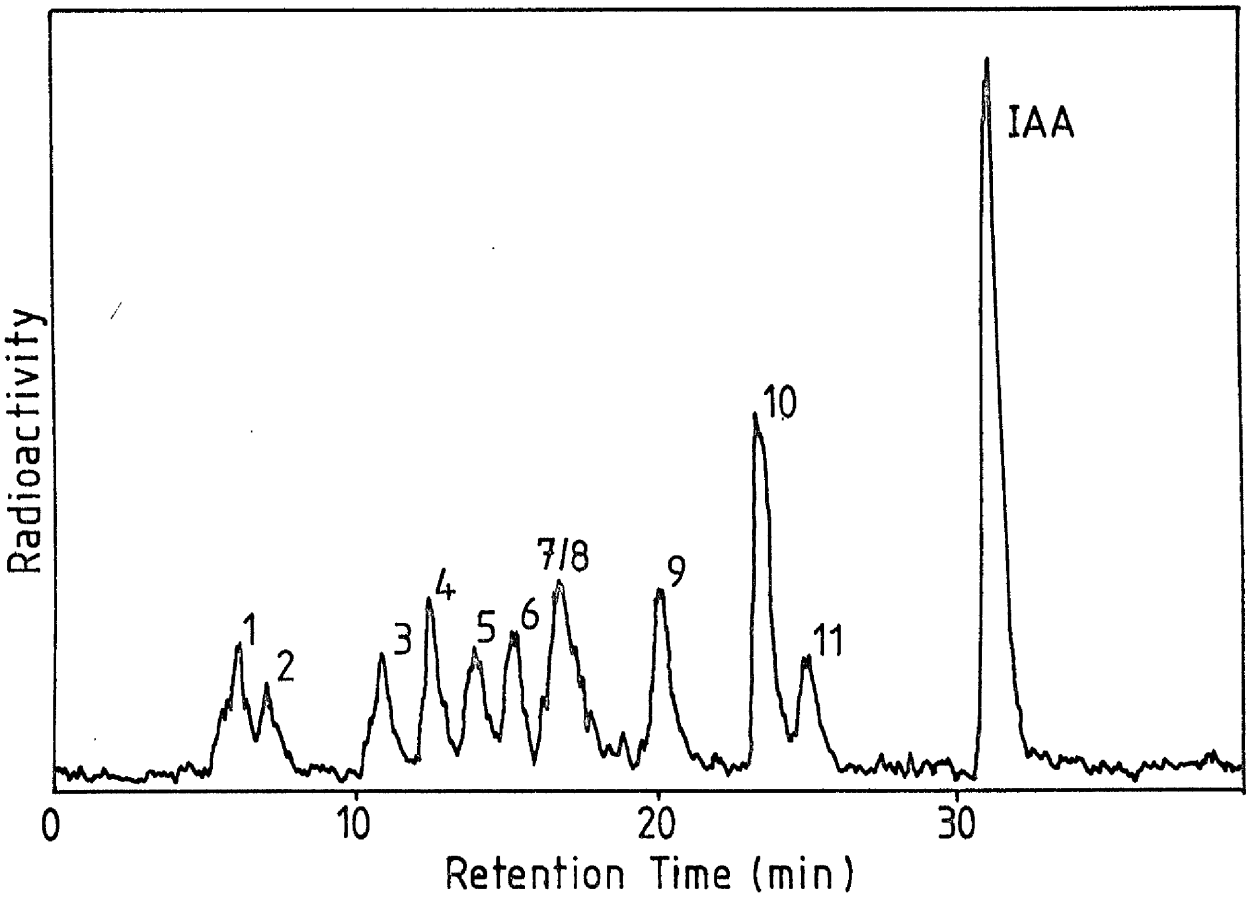


Fig. 30. Metabolism of IAA-1-^{14}C by root segments (2h incubation). A typical result from 3 replicate experiments. HPLC conditions: solvent gradient: 10-60% methanol over 30 min. flow rate: 0.75 cm^3 min^{-1}. detector: homogeneous radioactivity monitor; 30cps full scale deflection; 10s time constant.

Fig. 31. Metabolism of IAA-1-^{14}C by coleoptile segments (2h incubation)

A typical result from 3 replicate experiments.

HPLC Conditions: Solvent gradient; 10 - 60 % methanol over 30 min. Flow rate; 0.75 $cm^3 min^{-1}$. Detector: homogeneous radioactivity monitor; 30cps full scale deflection; 10s time constant.

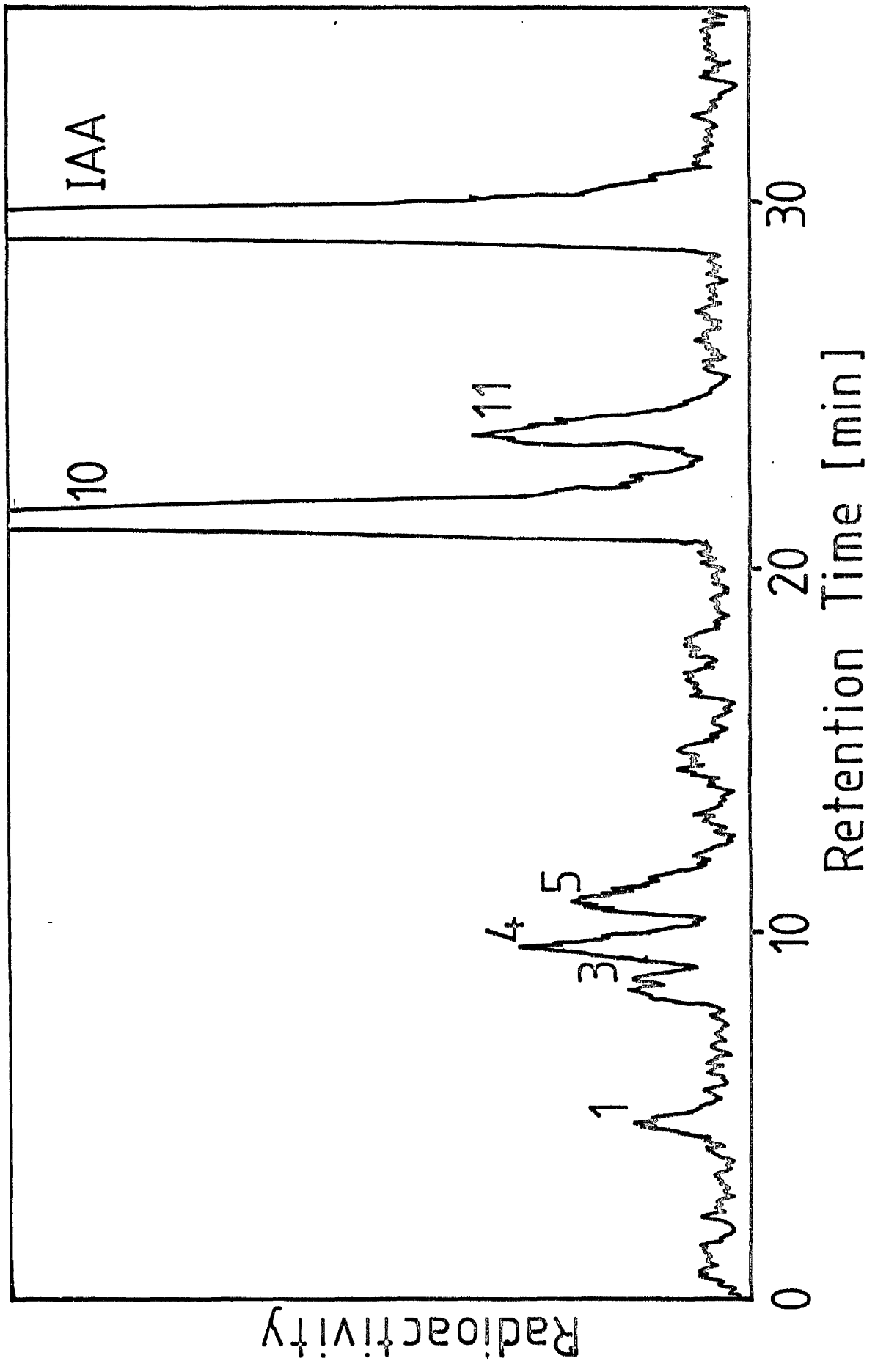

Radioactivity
0
10
20
30
Retention Time [min]
1
3
4
5
10
11
IAA

Table 24 : Comparison of the metabolism of IAA-1-^{14}C and IAA-2-^{14}C by root and coleoptile segments of Zea mays seedlings.

Segments (50 root or 20 coleoptile segments) were incubated for 2h in ^{14}C-IAA (10^{-3} or 10^{-2} mol m^{-3}). The results represent the means of 3 replicate experiments ± standard error (2 experiments in the case of coleoptile segments incubated in IAA-2-^{14}C). Radioactivity is expressed in Bq.

TREATMENT	IAA-2-^{14}C supplied to roots	IAA-1-^{14}C supplied to roots	IAA-2-^{14}C supplied to coleoptiles	IAA-1-^{14}C supplied to coleoptiles
1. Initial radioactivity	7650 ± 221	5760 ± 21	49800 ± 513	44200 ± 3660
2. Radioactivity remaining in incubating solution	3470 ± 22	2310 ± 43	42400 ± 174	37700 ± 3150
3. Radioactivity in methanol extract	2230 ± 11	1890 ± 12	1930 ± 130	1730 ± 30
4. Radioactivity remaining in tissue after extraction	167 ± 7.2	114 ± 5.2	49 ± 3	69 ± 31
5. Radioactivity in washings	1360 ± 136	900 ± 34	6257 ± 1900	4900 ± 1340
6. Final total radioactivity	7230 ± 122	5210 ± 44	50600 ± 1600	44400 ± 1810
7. Loss of radioactivity during incubation	413 ± 145	545 ± 43	-858 ± 1080	-268 ± 1850
8. 7 expressed as a percentage of 1	5.3%	9.5%	-1.7%	-0.6%
9. Uptake of radioactivity (Bq segment^{-1})	48 ± 0.4	40 ± 0.3	98 ± 6.5	104 ± 14
10. Extraction efficiency	93% ± 0.3%	94% ± 0.2%	98% ± 0%	97% ± 1%
11. Radioactivity in methanol extract after preparation for HPLC	1800 ± 63	1590 ± 170	-	1397 ± 99
12. 11 expressed as a percentage of 3	81%	84%	-	81%

H.4. UV Spectrometry

The chromophore responsible for the UV absorbance of IAA is the indole nucleus. As this is not altered directly by the conjugation of the molecule with amino acids, sugars or myo-inositol these compounds should have UV absorption spectra very similar to that of IAA (see Fig. 32). On the other hand, if the molecule is oxidised, this will alter the chromophore and thus change the spectrum very significantly. This can be seen by a comparison of the spectra of indole-3-aldehyde, oxindole-3-acetic acid and 5-hydroxyindole-3-acetic acid with that of IAA (Fig. 33).

Thus if a metabolite is found to have an absorption spectrum identical to that of IAA this would be a clear indication that it represents a conjugate of IAA. Unfortunately most of the metabolites were not sufficiently purified to give a definitive UV spectrum. However, the UV spectrum of the combined peaks 7 and 8 was very similar to that of IAA, with a broad peak at 272-282 nm and shoulders at 268 and 289 nm (Fig. 34). The sample also contained at least one other UV absorbing substance. This result was obtained in three separate experiments. Thus, at least one component of this peak appears to be a conjugate of IAA.

H.5. Methylation of IAA Metabolites

The IAA metabolites were derivatised using diazomethane which reacts rapidly with organic acids to form the corresponding methyl esters. Diazomethane also reacts with phenols to give the corresponding methyl ethers. Both types of product will be less polar than the starting material.

After methylation each IAA metabolite was analysed by HPLC. The results are summarised in Table 25. The experiment was performed once.

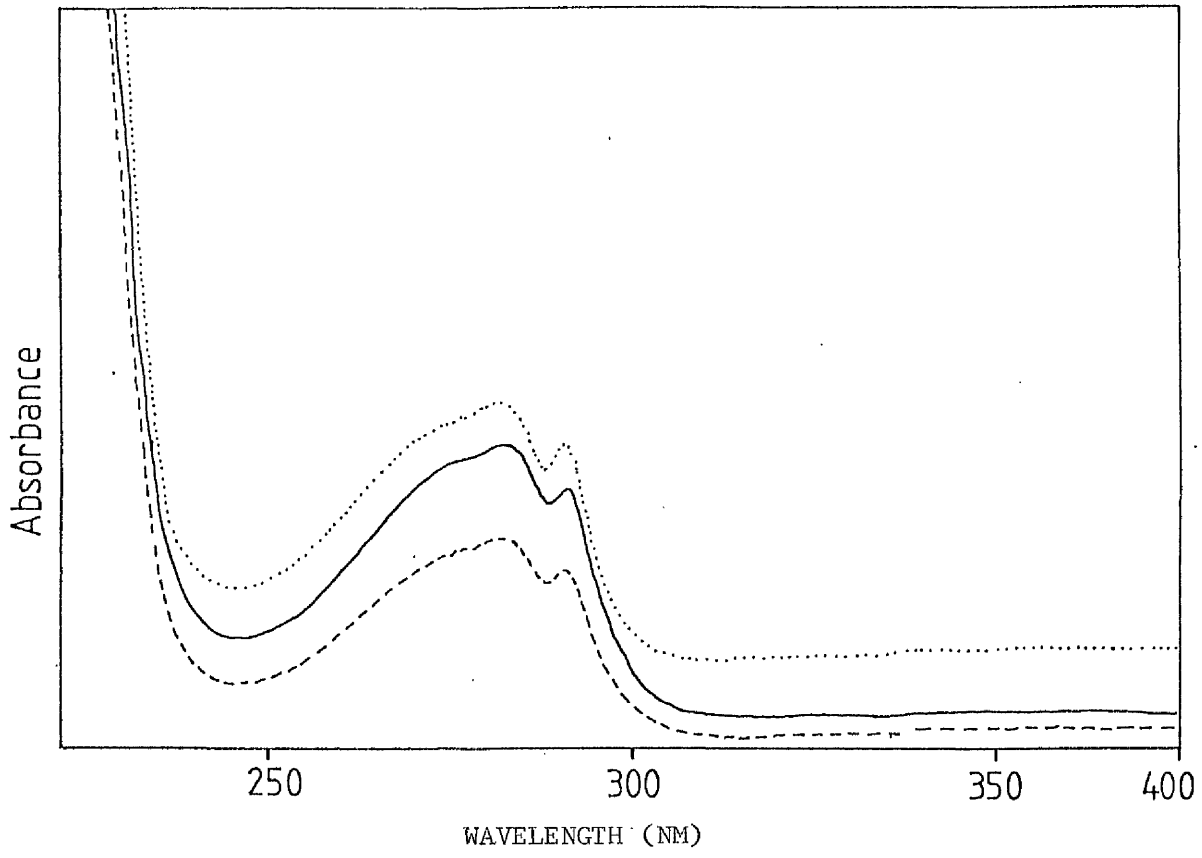


Fig.32. UV absorbance spectra of IAA and two amino acid conjugates of IAA.

——— IAA
– – – – – IAA-glycine
····· IAA-valine

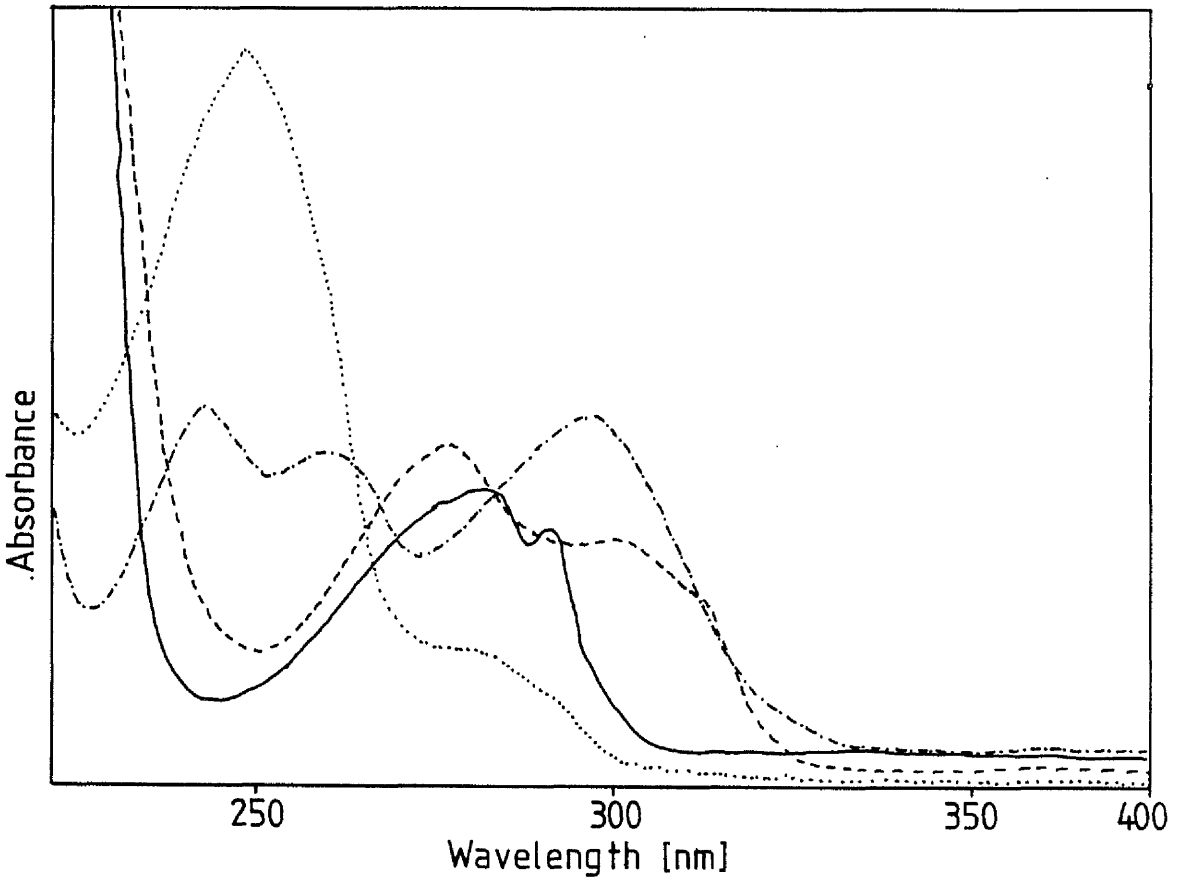


Fig. 33. UV absorbance spectra of IAA and its oxidation products.

——— IAA
- - - - - - - 5-Hydroxyindole-3-acetic acid
..... Oxindole-3-acetic acid
-·-·-·-· Indole-3-aldehyde

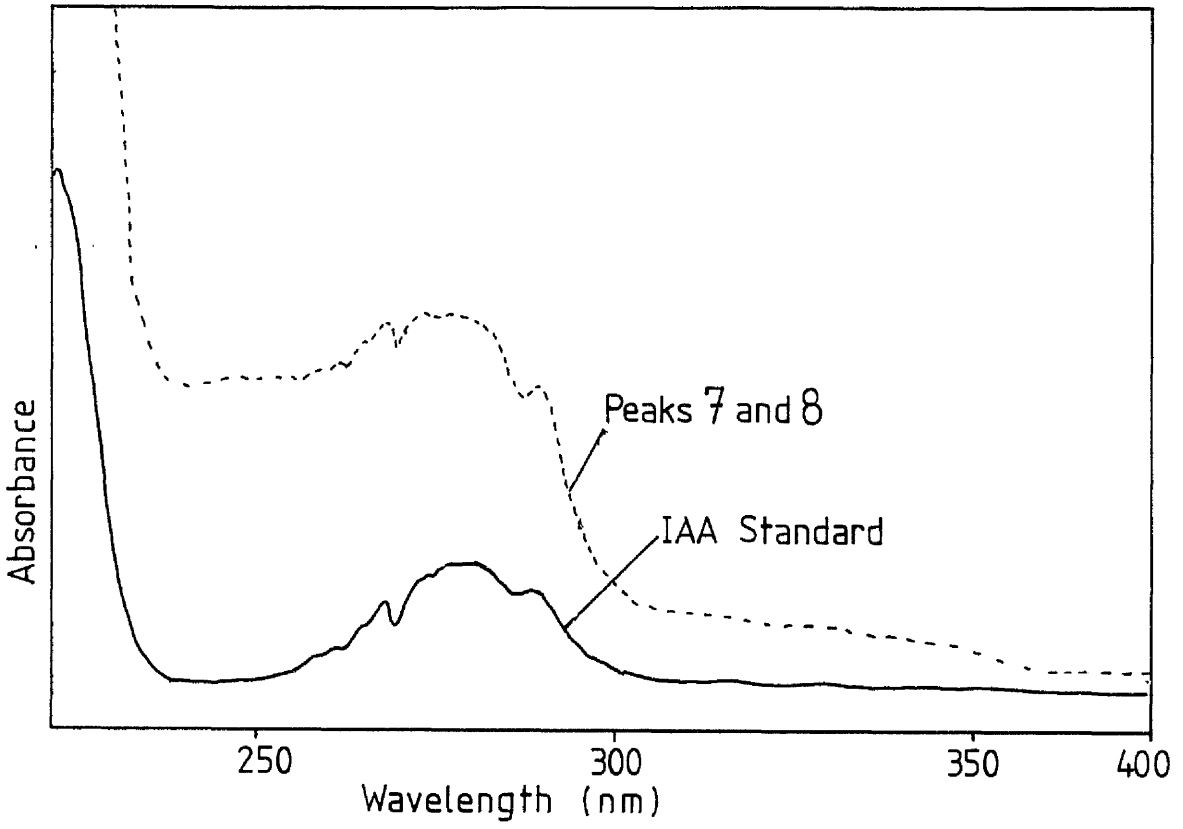


Fig. 34. UV absorbance spectrum of metabolite peak 7/8 after purification by HPLC, and compared with IAA standard

Table 25 : Results of methylating IAA metabolites

Peak No	Product of methylation
4	inconclusive
5	inconclusive
6	inconclusive
7/8	unreacted starting material plus one peak with a T_R between that of IAA and methyl-IAA.
9	single peak with T_R between that of IAA and methyl-IAA.
10	not analysed
11	single peak with same T_R as methyl-IAA
IAA	single peak with same T_R as standard methyl-IAA

The radioactive compound represented by peak 9 and a component of peak 7/8 appeared to methylate, forming products considerably less polar than the starting material. In view of the apparently high polarity of these metabolites prior to methylation, they probably possess a carboxyl group. Treatment of the peak containing putative ^{14}C-IAA with diazomethane resulted in a product which co-chromatographed with authentic IAA-methyl ester. This provided further evidence that the only radioactive component of this peak was IAA. Methylation of peak 11 also resulted in a radioactive product which had the same retention time as methyl IAA. However, the compound represented by peak 11 was somewhat labile and appeared to revert to IAA under certain conditions (see Expt H.6). It is likely that this metabolite had reverted to IAA prior to methylation.

H.6. Hydrolysis of IAA Metabolites

This work is of a preliminary nature. Two different hydrolysis conditions were used, as described by Bandurski and Schulze (1977). They reported that esters of IAA with glucose, myo-inositol and oligosaccharides could be hydrolysed using 10^3 mol m^{-3} NaOH for 1h at 22 to 25°C but that hydrolysis of peptidyl IAA required more rigorous conditions of 7×10^3 mol m^{-3} NaOH at 100°C for 3h. Hydrolysis of the compounds represented by peaks 4-11 was attempted. The effect of these conditions on IAA was also monitored. After each treatment, the reaction mixture was neutralised, and the radioactive products analysed by HPLC. The results are presented in Table 26. The experiment was performed once.

Table 26 : Results of Hydrolysis of Metabolites

Peak No	Products of Hydrolysis	
	10^3 mol m^{-3} NaOH, 22°C	7×10^3 mol m^{-3} NaOH, 100°C
4	Peak with same T_R as peak 1	-
5	Peak with same T_R as peak 1/2	-
6	Peak with same T_R as peak 1/2	-
7/8	No effect	Peak with same T_R as peak 1/2
9	No effect	Peak with same T_R as peak 1/2
10	Several peaks	-
11	Peak with same T_R as IAA	-
IAA	No effect	Peak with same T_R as peak 1/2

Only one metabolite (represented by peak 11) appeared to be hydrolysed to IAA. After treatment with 10^3 mol m^{-3} NaOH at room temperature this metabolite was converted to a product with the same retention time as IAA. Peak 11 thus probably represents an ester of IAA with a sugar or myo-inositol. The radioactive components of peaks 4, 5 and 6 were also altered by mild hydrolysis conditions. However, the products were highly polar compounds which were eluted close to the void volume. There is no need to assume that the products of each compound were necessarily identical as the separation of compounds eluting at the start of a gradient is poor. It is possible that the compounds represented by peaks 4, 5 and 6 are esters of IAA oxidation products. This interpretation is also supported by the lack of clear cut products of methylation in experiment H.5.

Unfortunately treatment with 7×10^3 mol m^{-3} NaOH at 100°C caused breakdown of IAA itself. The effect of these conditions on IAA metabolites was therefore conjectural.

H.7. Co-chromatography of Metabolites with Standards

The following putative IAA metabolites were synthesised for use as standards: IAA-glycine, IAA-alanine, IAA-valine and oxindole-3-acetic acid. The identities of these compounds were confirmed by GC-MS. The mass spectra obtained are presented in Fig. 35-38. 5-Hydroxyindole-3-acetic acid was also obtained from Aldrich Chemical Company. The HPLC retention times of the standards were compared with those of radioactive IAA metabolites. Standards were monitored using a UV absorbance detector, the wavelength of which was set at the absorbance maximum for each compound, measured from spectra shown in Figs. 32 and 33. The UV absorbance detector replaced the fluorimeter in Fig. 5a. The results are presented in Table 27.

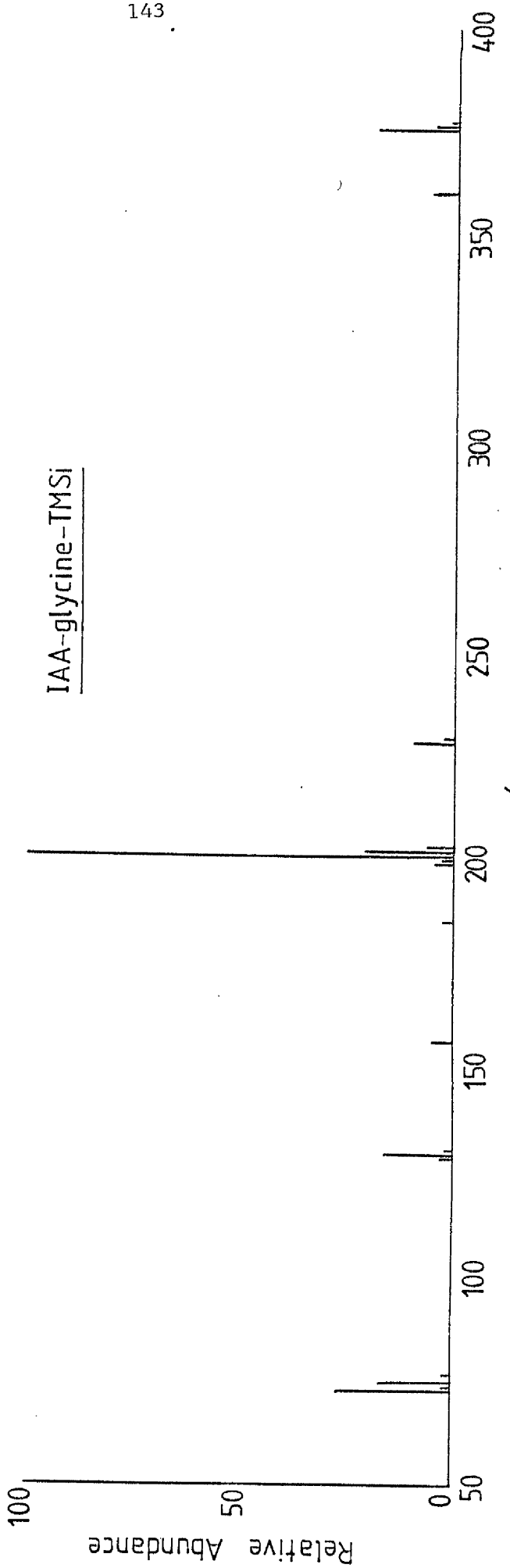


Fig. 35. Mass spectrum of IAA-glycine
GC conditions: Column; 9ft x ¼" 3% Dexil-300. Temperature; 250°C.
Flow; 20 cm min^{-1}.
MS Conditions: 70 ev; source temperature 180°C. separator temperature 275°C.

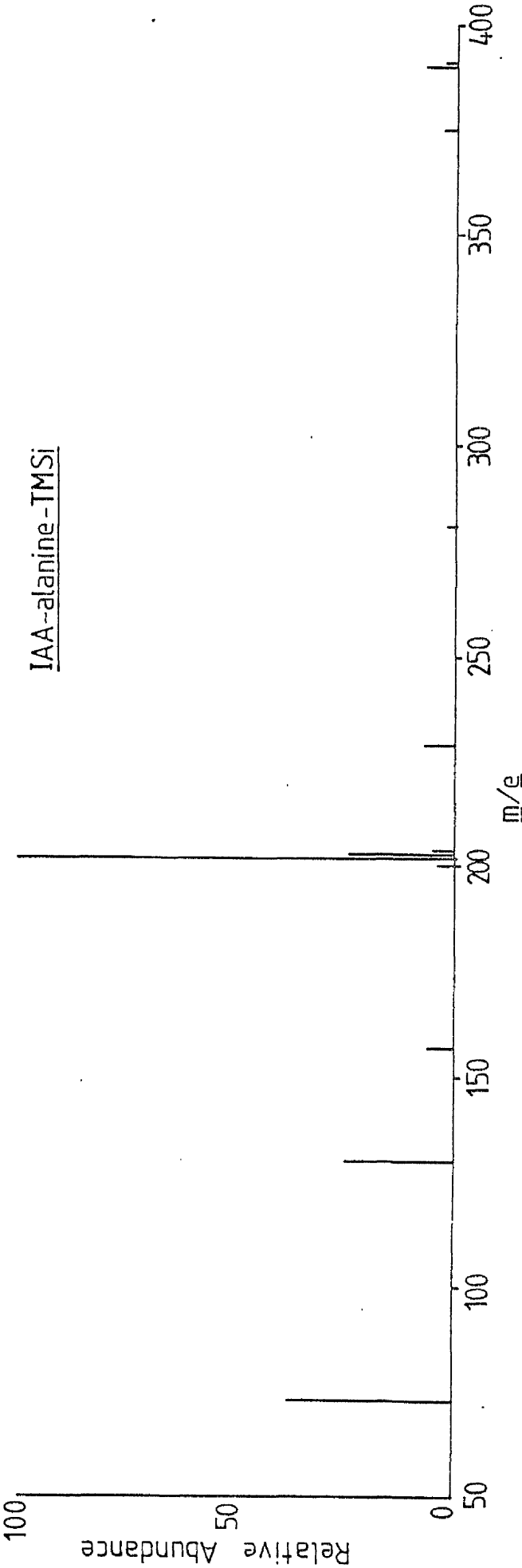


Fig. 36. Mass spectrum of IAA-alanine-TMSi
GC Conditions: column; 9ft x ¼" (2.7 m x 6.4 mm) 3% Dexil-300. temperature 250°C.
flow rate; 30 cm min^{-1}. T_R 6.6 min.
MS Conditions: 70 ev; source temperature 200° C. separator tmeperature 275° C.

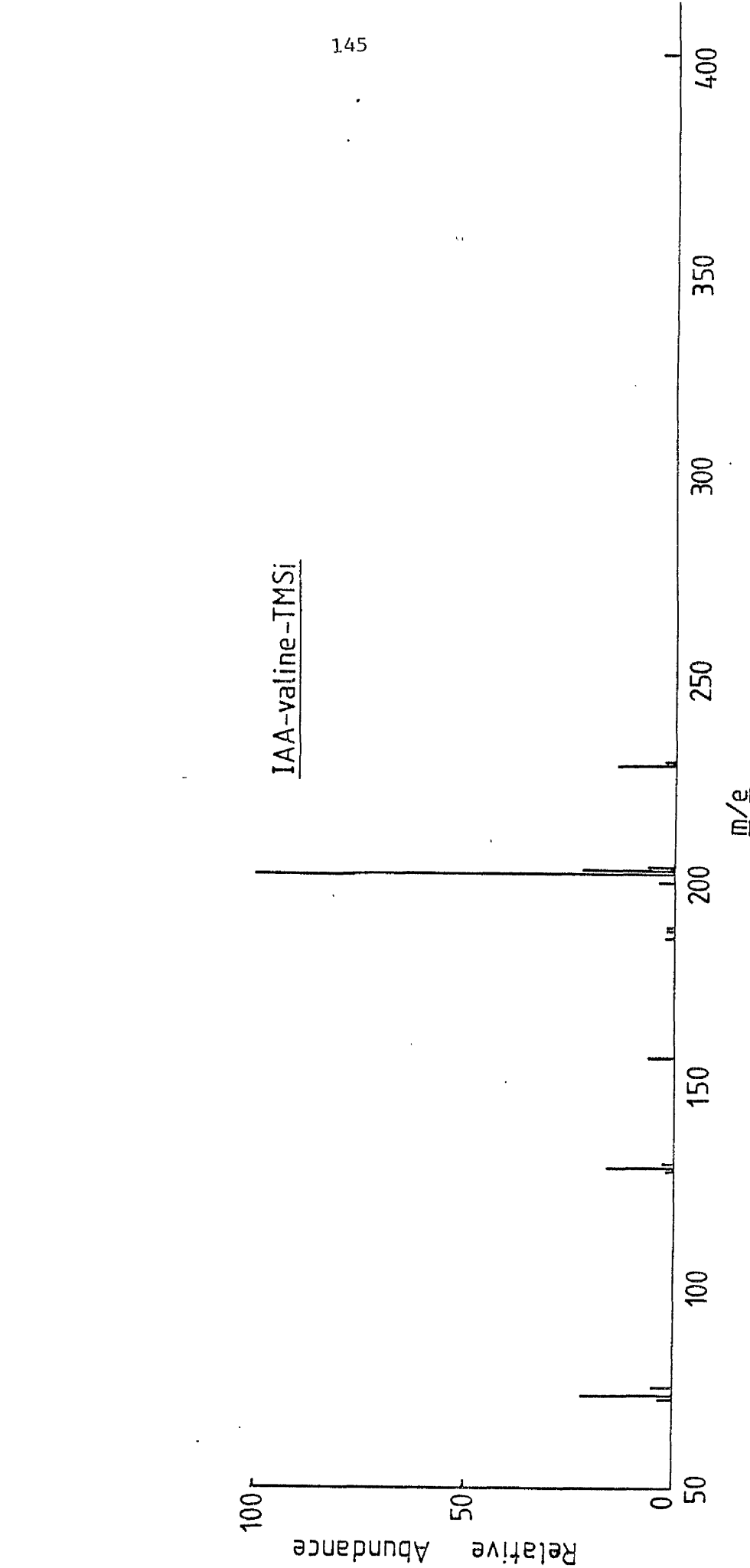


Fig. 37. Mass spectrum of IAA-valine-TMSi
GC Conditions: column; 9ft x ¼" (2.7m x6.4mm) 3% Dexil-300. temperature; 250°C.
Flow rate; 30 cm ^{3} min $^{-1}$. T_R 7.7 min.
MS Conditions: 70 ev; source temperature 280°C. separator temperature 275°C.

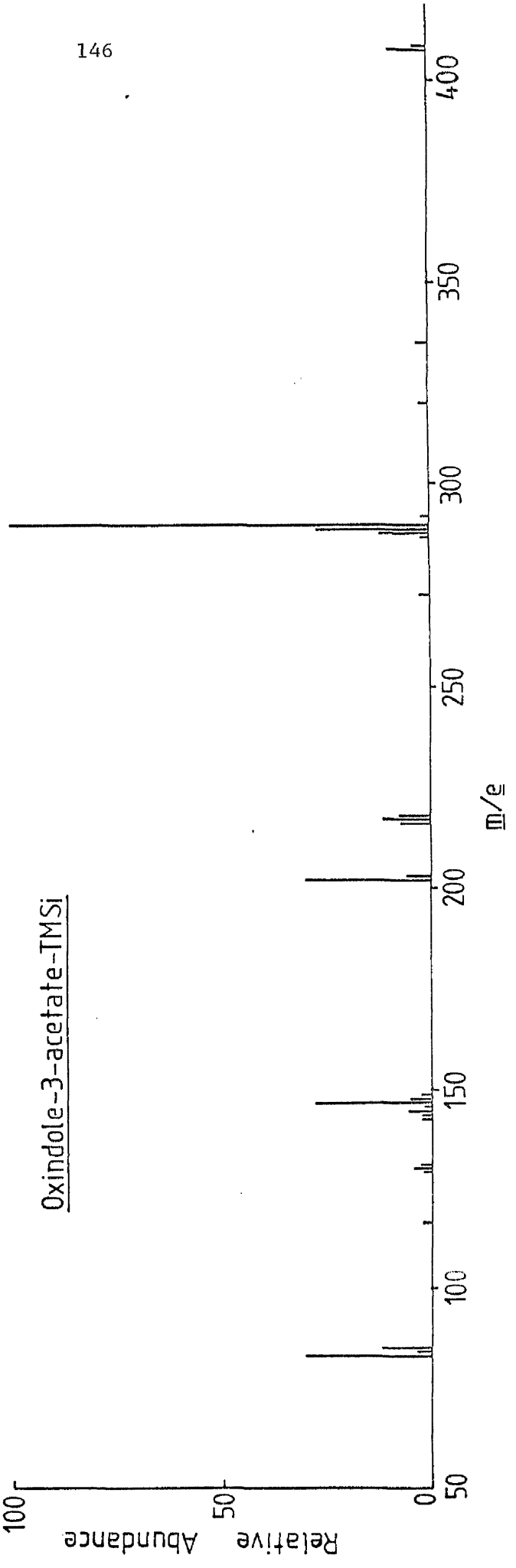


Fig. 38. Mass spectrum of Oxindole-3-acetate-TMSi

Table 27: Comparison of the retention times of IAA metabolites with those of standards. HPLC Conditions: Flow rate 1 cm^3 min^{-1}. Solvent gradient 10-60% methanol over 20 min. T_R values for standards are corrected for the time difference between detectors. Average peak width 1min. The experiment was carried out once.

Peak No	T_R	Standard	T_R
7	13.2 min	5-Hydroxyindole-3-acetic acid	12.9 min
10	17.8 min	Oxindole-3-acetic acid	17.8 min
8*	13.8 min	IAA-glycine	14.2 min

* Peak 8 was analysed on a separate occasion

Peak 10, representing the most prominent metabolite, co-chromatographed with oxindole-3-acetic acid. Peaks 7 and 8 had the same retention times as 5-hydroxyindole-3-acetic acid and IAA-glycine respectively, within experimental error. IAA-valine and IAA-alanine did not co-chromatograph with any of the metabolites.

H.8. GC-MS Analysis

Only the sample of the IAA peak was sufficiently pure to permit identification using GC-MS. The instrument was used in the selected ion-monitoring mode and was tuned to detect ions with m/e 202, the most prominent fragment in the mass spectrum of the trimethylsilyl derivative of IAA (IAA-TMSi) (see Fig. 6). The IAA peak was derivatised using BSTFA. A peak was present at the same retention time as trimethylsilyl IAA. This was confirmed by coinjection (Fig. 39).

Conclusions

The product represented by peak 10, i.e. the most prominent metabolite

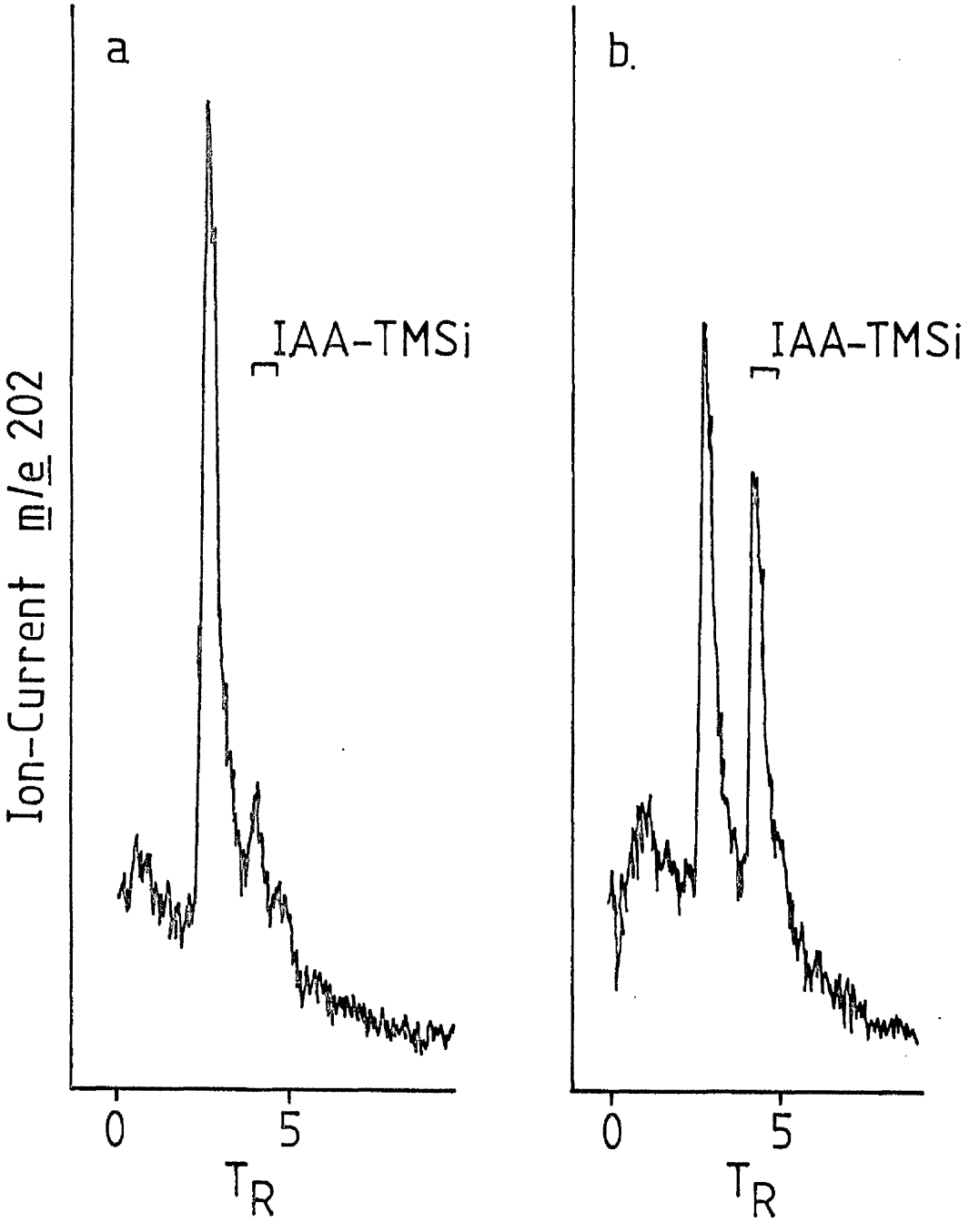


Fig. 39. GC-MS analysis of the IAA peak from methanolic extracts of root segments incubated in solutions of 10^{-5} mol m^{-3} IAA for 2h. The extract was purified by HPLC, and derivatised using BSTFA. (a) extract only (b) extract plus coinjection of IAA-TMSi standard. GC Column: 5ft x ¼" (1.52m x 6.35mm) 3% Dexil-300 on Supelcoport 100-200. Temperature: 190°C isothermal. Flow rate: 20 cm^3 min^{-1}. MS conditions: 24eV; Source temperature 280°C; Separator temperature 250°C.

in both root and coleoptile extracts, appeared to be oxindole-3-acetic acid. Evidence was provided by co-chromatography with authentic oxindole-3-acetic acid on the HPLC. Further indication that this metabolite was an oxidation product came from control analyses in Experiments B.1 and F.1. Control extracts usually contained a small amount of peak 10 (see also Experiment I). Peak 7 co-chromatographed on HPLC with 5-hydroxyindole-3-acetic acid, a compound not previously identified in plant tissue. Peak 8 had a retention time the same as that of IAA-glycine, within experimental error. Further evidence that peak 7/8 contained an IAA conjugate was provided by the UV absorption spectrum. The metabolite represented by peak 11 was converted to IAA under mild hydrolysis conditions. It is likely that this compound is an ester of IAA with a sugar and/or myo-inositol. The radioactive components of peaks 4, 5 and 6 could not be hydrolysed to IAA and are unlikely to represent sugar, myo-inositol or related esters of IAA. The compounds represented by peaks 4 and 5 were interconvertible and are probably isomeric. The metabolite represented by peak 9 could be methylated to form a substantially less polar product. This compound probably possessed a carboxyl group.

The identity of the IAA peak was confirmed by methylation and co-chromatography with IAA-methyl ester and by GC-MS (selected ion-monitoring) of the trimethylsilyl derivative.

I. HPLC Analysis of IAA Metabolites Present in the Incubating Solutions

The metabolites of IAA, present in bathing solutions after 2h incubation with Zea mays root and coleoptile segments were investigated. Samples of incubating solutions containing IAA-1-^{14}C and IAA-2-^{14}C (10^{-2} mol m^{-3} for coleoptiles; 10^{-3} mol m^{-3} for roots) were prepared for HPLC analysis. Aqueous solutions were usually dried in vacuo at 45°C and redissolved in 1 cm^3 methanol. Immediately prior to analysis, samples were dried under a stream of nitrogen and taken up in 100 mm^3, 10% methanol in ammonium acetate buffer (pH 3.5, 20 mol m^{-3}). Losses of radioactivity during this procedure are given in Tables 7 and 16. Two samples (root incubating solutions) were prepared by first acidifying the incubating solution (diluted to 10 cm^3 with water) to pH3 with HCl and partitioning three times with equal volumes of diethyl ether. The ether was then dried in vacuo and the sample dissolved in 1 cm^3 of methanol. When freshly distilled diethyl ether was used, this procedure had no significant effect on the spectrum of compounds observed. However, when diethyl ether which had not been recently distilled was used, this resulted in the oxidation of the majority of the IAA to a compound which co-chromatographed with peak 10. This was presumably due to the presence of peroxides.

The results of the analysis, which was carried out in triplicate for each treatment, are presented in Figs. 40 and 41. As stated previously (see Tables 8 and 15), in each case the majority of the radioactivity co-chromatographed with IAA. Small amounts of some of the metabolites found in methanol extracts of roots and coleoptiles were also present; peaks 3 and 10 were consistently observed. The presence of one or more compounds which were less polar than IAA, and only appeared when methylene-labelled IAA was supplied, was of particular interest. These compounds

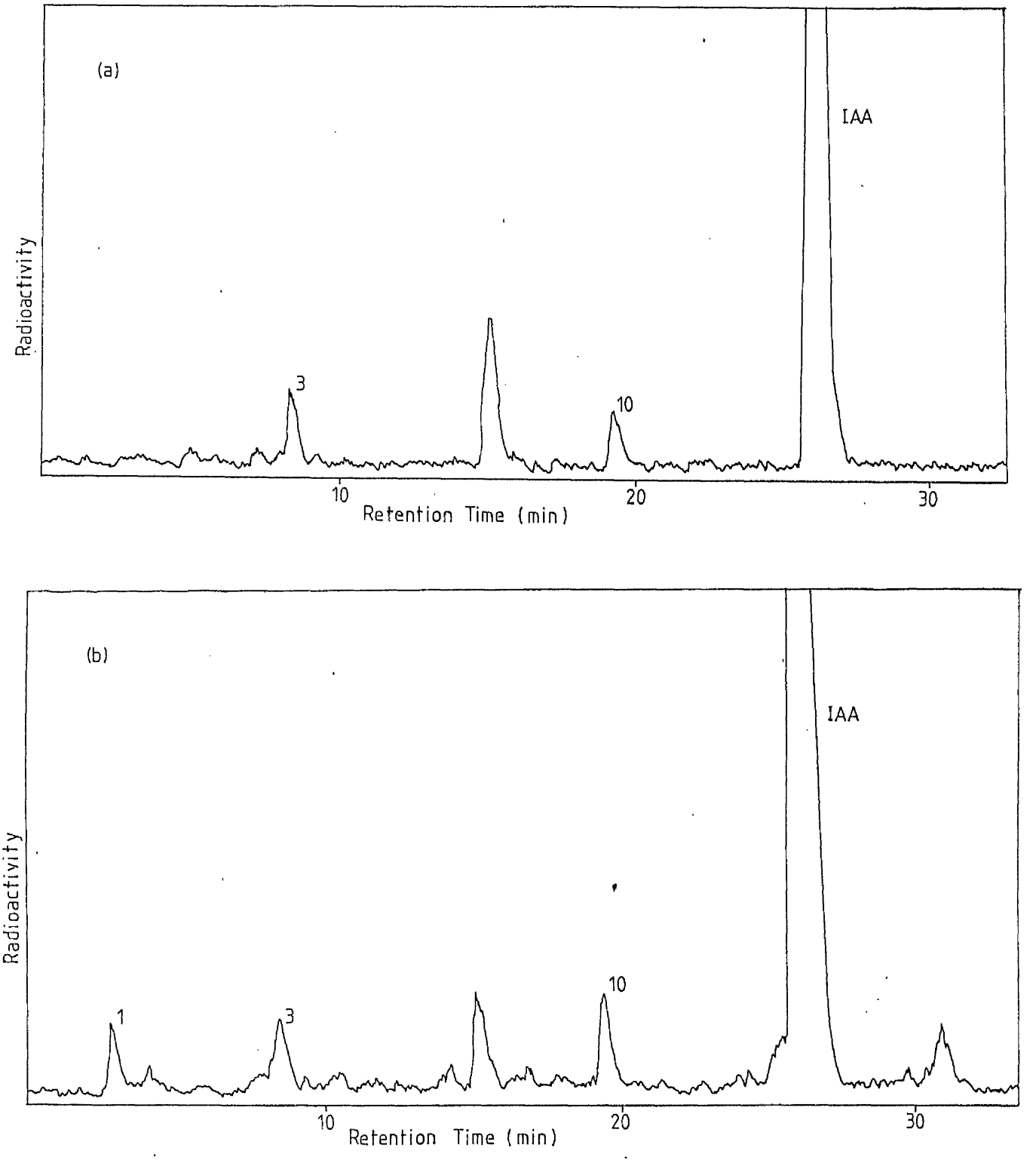


Fig.40. HPLC analysis of bathing solutions after 2h incubation with root segments. a. IAA-1-^{14}C (10^{-3} mol m^{-3}). b. IAA-2-^{14}C. Traces represent typical results from 3 replicate experiments. HPLC conditions: Solvent gradient: 10-60% methanol over 30 min. Flow rate: 1 cm^{-3} min^{-1}. Detector: homogeneous radioactivity monitor ; 30cps full scale deflection; 10s time constant.

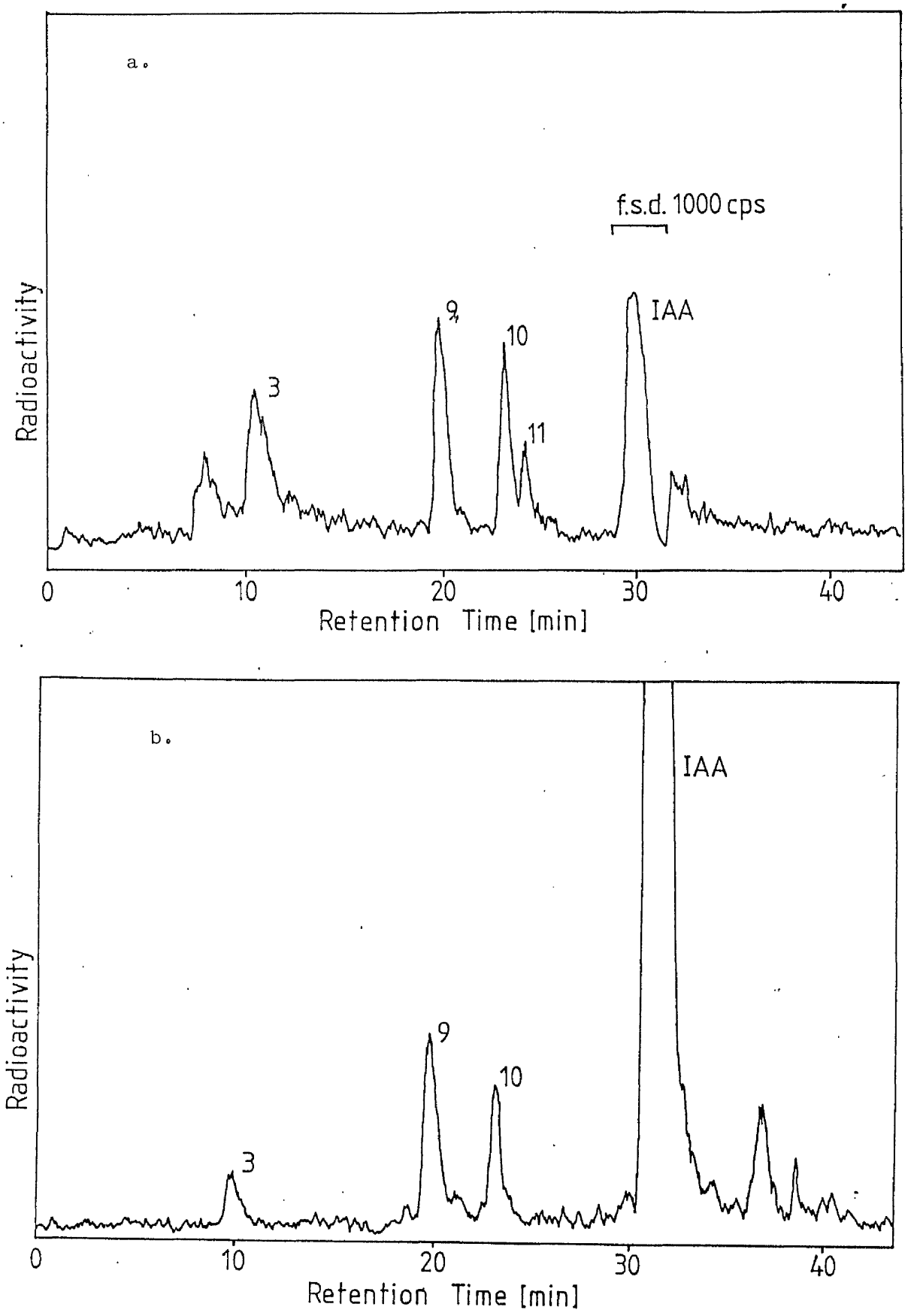


Fig.41. HPLC analysis of bathing solutions after 2h incubation with coleoptile segments. a. IAA-1-^{14}C (10^{-2} mol m^{-3}). b. IAA-2-^{14}C
HPLC conditions; Solvent gradient: 10-60% methanol over 30 mins. Flow rate: 1 $cm^3 min^{-1}$. Detector: homogeneous radioactivity monitor; 30cps full scale deflection; 10s time constant.

are probably decarboxylation products of IAA. They did not co-chromatograph with indole-3-aldehyde or indole-3-carboxylic acid, but might represent other products such as 3-methyleneoxindole.

Conclusions

Solutions of IAA, in which Zea mays root and coleoptile segments had been incubated for 2h, contained small amounts of metabolites which appeared to be decarboxylation products. As these compounds were not present in methanol extracts of plant material they may represent the products of IAA oxidation at cut surfaces. The formation of large amounts of peak 10, when aqueous solutions of IAA were partitioned against diethyl ether which had not been redistilled, provided further evidence that this compound was an oxidation product.

SECTION 2 - THE EFFECT OF EXOGENOUS IAA ON THE ELONGATION OF ZEA MAYS ROOT AND COLEOPTILE SEGMENTS

The purpose of this Section of the thesis was two fold. Firstly, it was important to confirm whether the amounts of IAA supplied to root and coleoptile segments during metabolism experiments were physiologically active with regard to cell elongation. Secondly, published data on the effect of IAA on root growth are conflicting (see Introduction); it was hoped to clarify the effect of applied IAA on the elongation of Zea mays root segments.

A. Effect of IAA on Coleoptile Elongation

The stimulation of coleoptile elongation by IAA has been well established since 1934 (Kögl et al.). A single experiment was carried out to investigate the external concentration required to give maximum growth stimulation under the experimental conditions used for metabolism studies. Coleoptile segments (5mm in length, taken from 2mm behind the apex) were incubated for 1h (darkness; 22°C) in 5 cm^3 volumes of aqueous IAA solution (0, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} mol m^{-3}). Growth was measured using the shadowgraph method.

IAA at the lowest three concentrations had no significant effect on growth when compared with the control (Fig. 42). Maximum stimulation occurred in 10^{-2} mol m^{-3} IAA.

Conclusions

Under the experimental conditions used in metabolism experiments, maximum growth was obtained in 10^{-2} mol m^{-3} IAA. This concentration was used in the majority of experiments on IAA metabolism in coleoptile tissue.

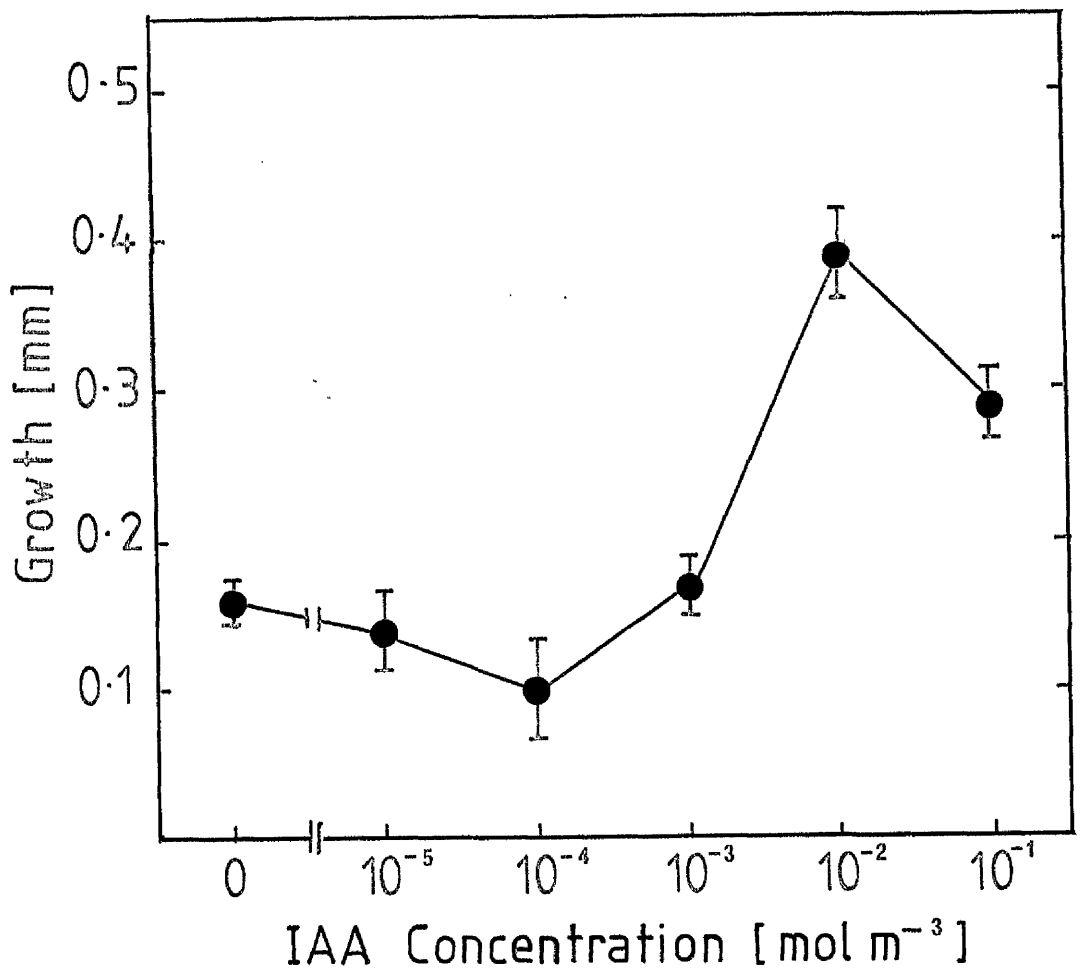


Fig. 42. Effect of IAA supplied at 5 different concentrations on the growth of Zea mays coleoptile segments during 1h after excision.

Each point represents the mean of 10 segments ± standard error. The experiment was performed once.

B. Effect of IAA on Root Elongation

B.1. Comparison of the Growth Rate of Intact Roots with that of Segments

As other scientists (e.g. Edwards and Scott, 1977; Pilet et al., 1979) appeared to have difficulty in obtaining significant effects of IAA on root growth, a preparatory experiment was carried out to investigate the optimum incubation time for such an experiment. The growth rate of intact roots was compared with that of segments at various times after excision.

Root segments 5mm in length were taken from 1mm behind the tip. Erickson and Sax (1956) and Pilet and Senn (1980) reported that this region contains the elongation zone. This was confirmed for the variety Fronica. Groups of 10 sections were incubated in water (2 cm^3) for ½h, 1h, 2h, 4h, 8h and 24h, and growth measured using the shadowgraph method. This was compared with growth of roots on intact plants, measured by recording the distance between spots of indian ink initially spaced 1mm and 6mm from the root tip.

Growth of the segments decreased rapidly with very little elongation taking place after 8h (Fig. 43). The rate of growth remained similar to that of intact plants for only circa 1h.

In a similar experiment addition of IAA at concentrations of 10^{-7}, 10^{-6} and 10^{-5} mol m^{-3} (amounts which have been reported to stimulate root elongation; e.g. Batra et al. 1975; Pilet et al. 1979) had very little effect on the growth of segments at any time (Fig. 44). Addition of IAA certainly did not allow them to maintain the rate of elongation found in intact plants.

It could thus be concluded that the decrease in growth rate of segments was due to factors other than lack of IAA, such as shortage of nutrients and other growth substances or the presence of inhibitory compounds such as ethylene. For the purposes of investigating the rôle of IAA in root growth, the tissue was therefore incubated for no longer than 1h, during which the growth rate was similar to that of intact roots.

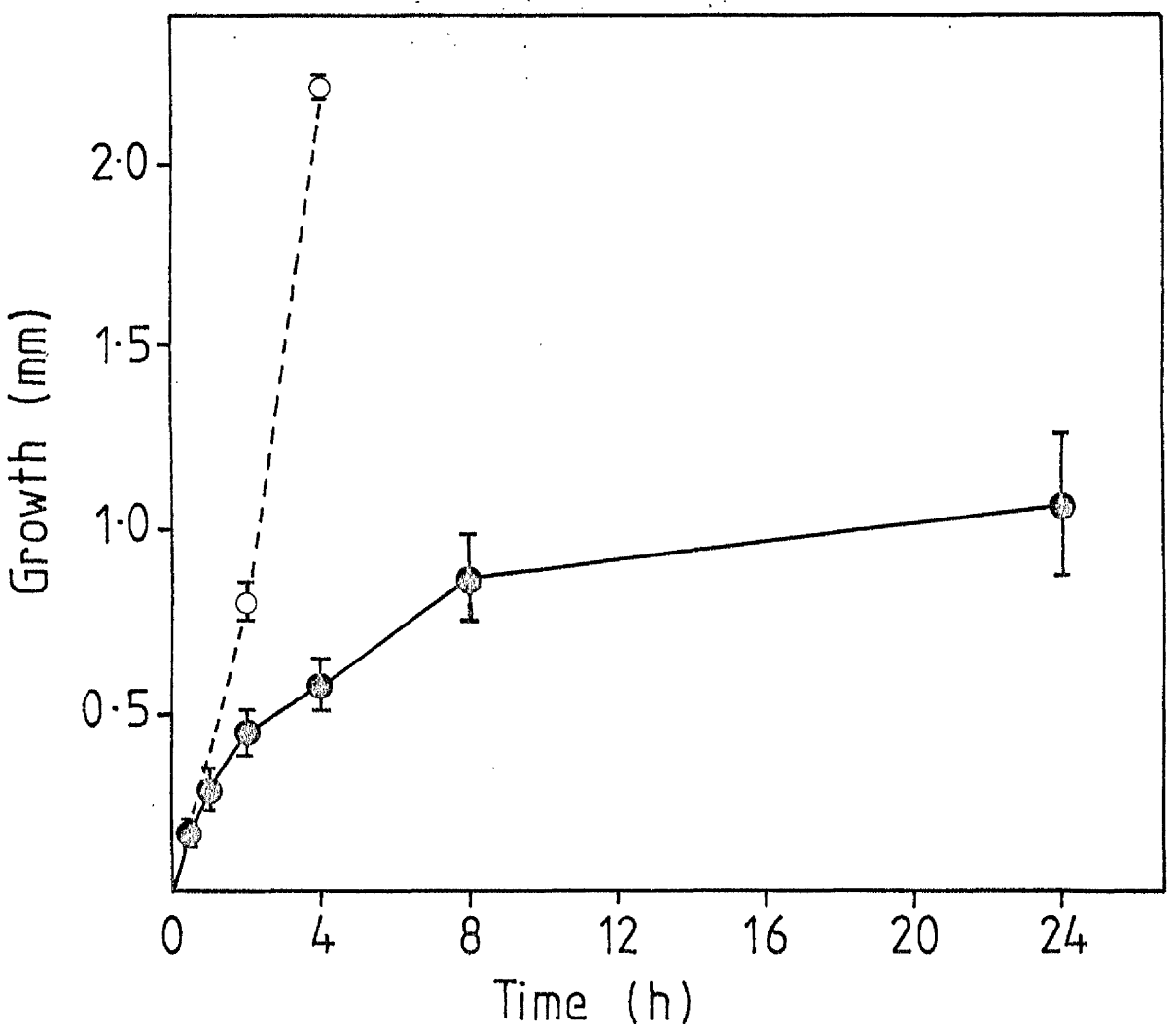


Fig. 43. A comparison of the growth of 5mm root segments (taken from 1mm behind the root tip) during 24h after excision (●) with that of the same portion of root on the intact plant (o).
Each point represents the mean of 30 plants ± standard error.
The same result was obtained in 3 replicate experiments.

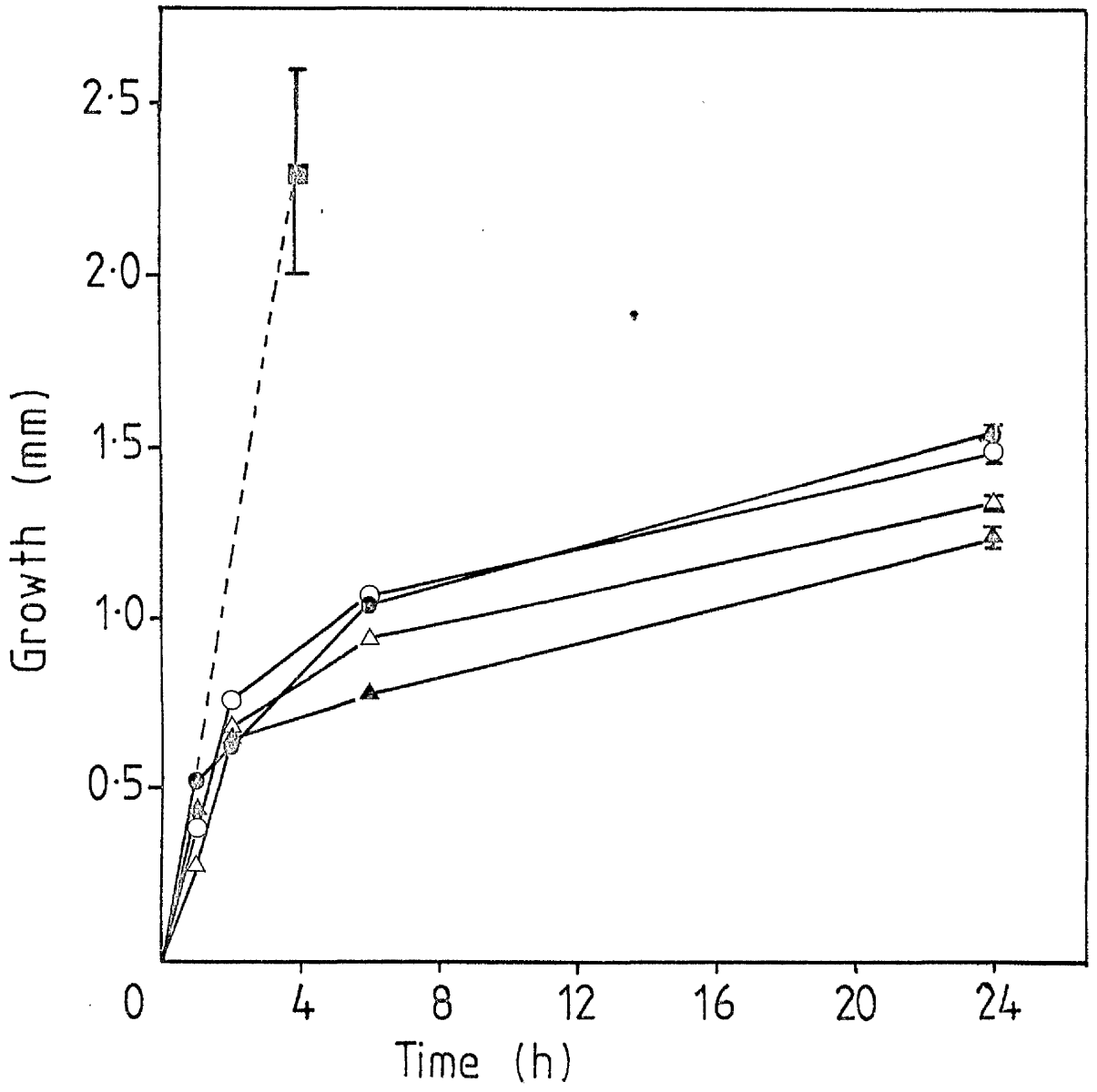


Fig. 44 . Effect of IAA at three different concentrations on the growth of root segments during a 24h time course, and compared with that of intact roots. Control (●), 10^{-7} mol m^{-3} IAA (○), 10^{-6} mol m^{-3} IAA (▲), 10^{-5} mol m^{-3} IAA (△), intact plants (■).

B.2. The Effect of IAA and Oxygen on Growth of Root Segments during a 1h Incubation

An experiment was carried out to study in greater detail the effect of different concentrations of IAA on the growth of root segments during the first 1h after excision. Root segments were incubated in groups of 10 in the laboratory in solutions of 0, 10^{-9}, 10^{-7}, 10^{-5} and 10^{-3} mol m^{-3} IAA (5 cm^3 per dish). Growth was measured using the shadowgraph method. Evans et al. (1980) have suggested that a supplementary source of oxygen is required for the optimum response to IAA. For this reason solutions were supplied with oxygen, nitrogen (20 cm^3 min^{-1}) or air (120 cm^3 min^{-1}).

The results (Fig. 45) showed no stimulation of growth by IAA at any concentration. Incubation in 10^{-3} mol m^{-3} IAA inhibited growth and this occurred only when solutions were supplied with O_2 or air. Roots incubated under N_2 did not respond significantly to IAA supplied at any concentration. The bubbling of solutions with O_2 and air caused significant increases in growth of control roots and those incubated in low concentrations of IAA. An analysis of variance was carried out on measurements of roots supplied with various concentrations of IAA under O_2 or N_2 (Table 28). This revealed a highly significant interaction between the O_2 availability and the effect of IAA on growth.

Although the experiments presented were carried out in the laboratory similar experiments in which roots were incubated in darkness showed this to have no significant effect on the growth rate.

B.3. The Effect on IAA-2-^{14}C Metabolism of Bubbling the Incubating Solution with O_2

A single experiment was carried out to discover whether the effect of addition of O_2 on the growth and response to IAA of Zea mays root segments could be attributable to an alteration in the pattern of IAA metabolism.

Root segments (50, 20mm in length) were incubated in the laboratory in

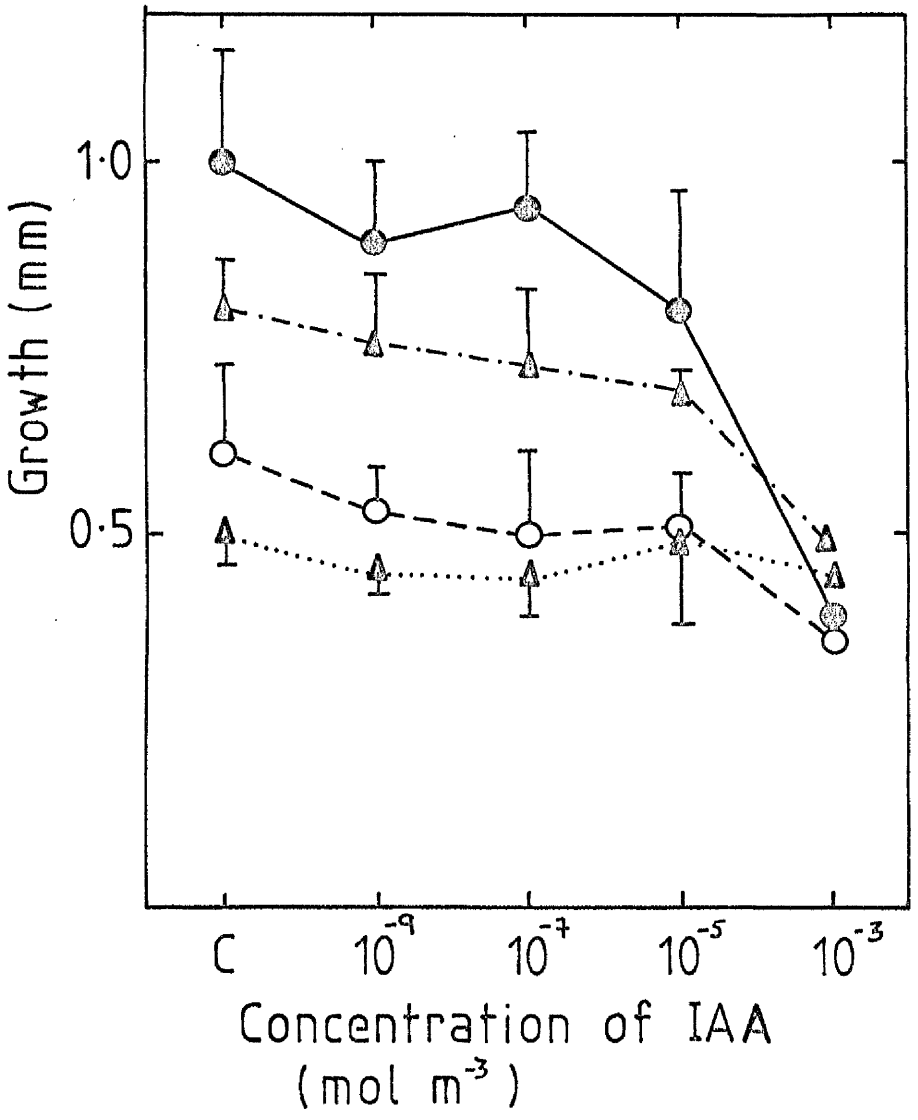


Fig. 45. Growth of root segments incubated in the laboratory for 1h in 4 different concentrations of IAA and compared with controls Roots were incubated in the absence - O - (4 dishes per concentration) or presence of supplementary oxygen - ● - (3 dishes), nitrogen - Δ - (2 dishes) or air - ▲ - (2 dishes). Each dish contained 10 segments. Results represent the means ± standard error.

Table 28 : Analysis of variance on results obtained when root segments were incubated at 5 different concentrations of IAA in the presence of O_2 or N_2

	S.Sq	D.f	Mean Sq.	F
Between concentrations of IAA	112	4	28	
Between O_2 availability	176	1	176	
Interaction	103	4	25.75	32.8
Residual	186	237	0.785	

an aqueous solution of IAA-2-^{14}C (5 cm^3 of 10^{-3} mol m^{-3}) which was bubbled with O_2 at a rate of 20 cm^3 min^{-1}. After 2h the segments were washed (Materials and Methods), extracted overnight in methanol, and a sample prepared for HPLC analysis. The results (Fig. 46) revealed a metabolism pattern similar to that for roots incubated in aqueous IAA with no supplementary source of O_2 (see Fig. 8). Peaks 1,2,3,4,5,6,7,8,9,10,11 and a peak corresponding to IAA were all present with peak 10 representing the most prominent metabolite. A detailed quantitative comparison of results is not meaningful without further repetition of the experiment and conditions were not identical to those in Section 1. The proportion of radioactivity remaining in the IAA peak was 13% compared with a value of 31% obtained in Section 1, Experiment B.3.

B.4. Changes in pH of IAA Solutions during Incubation with Root Tissue

Evans et al. (1980) have reported that the inhibitory effect of 2 x 10^{-3} mol m^{-3} IAA on root growth was associated with an increase in the pH of the incubating medium. They suggested that IAA causes H^+ influx into the cells; i.e. a proton pump operating in the reverse direction to that in shoots might be activated. An experiment was carried out to try to repeat these results.

Groups of 10 root segments were incubated in darkness in 5 cm^3 volumes of solution (0, 10^{-9}, 10^{-7}, 10^{-5} or 10^{-3} mol m^{-3} IAA) for 1h. The pH of the medium was measured before and after incubation. The experiment was carried out in triplicate.

An increase of approximately 0.5 pH units was observed after 1h incubation (Table 29); a value similar to that obtained by Evans et al. (1980). However, this increase occurred irrespective of the IAA concentration and O_2 availability, and was even observed in control dishes lacking IAA. Bubbling the solution with O_2 in the absence of root tissue produced no observable change in pH.

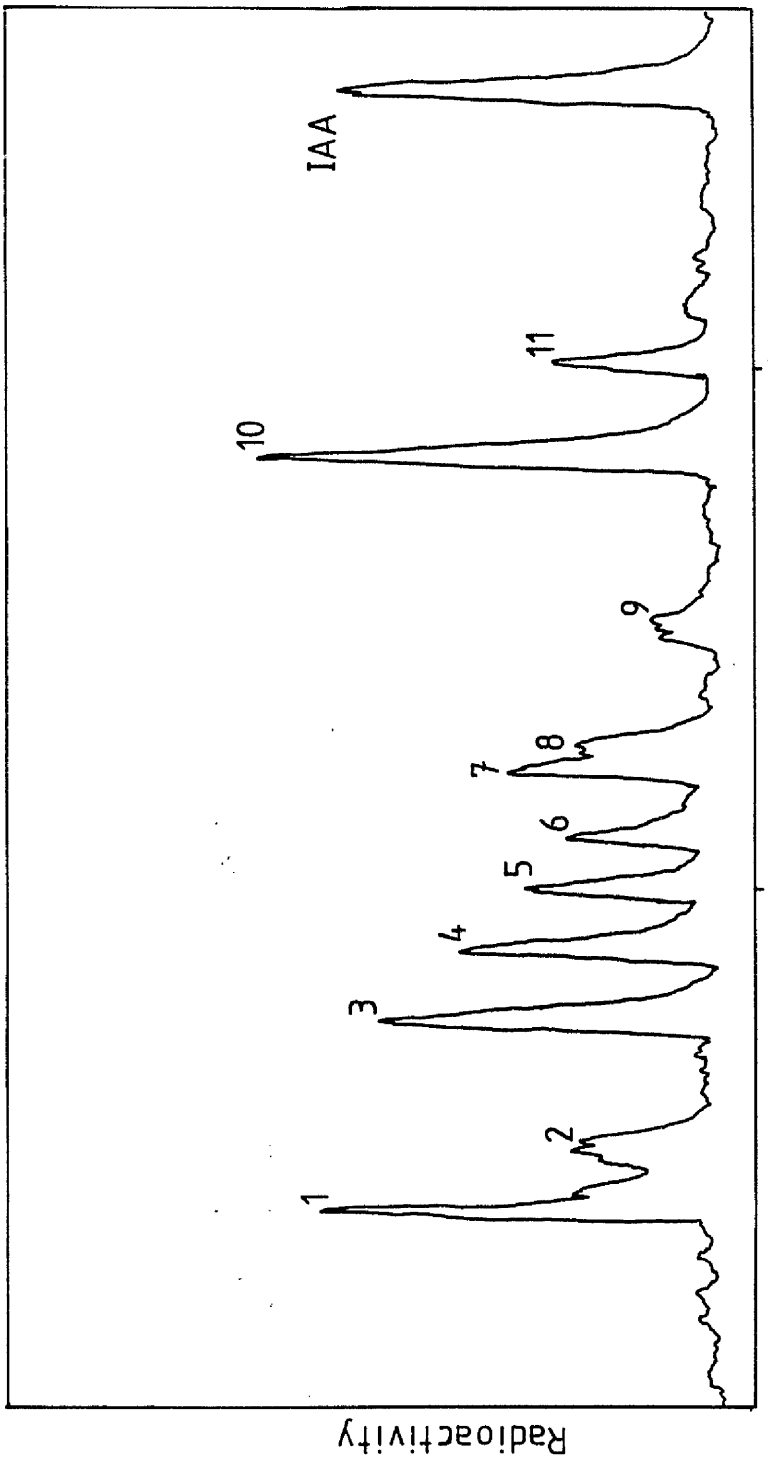


Fig. 46 Metabolism of IAA by root segments incubated for 1h in aqueous IAA-2-^{14}C solution bubbled with oxygen. Trace represents the result from a single experiment. Solvent gradient 10-60% methanol over 30 min. Flow rate 1cm min^{-1}. Detector: Homogeneous radioactivity monitor; 30 cps full scale deflection; 10s time constant

Table 29: Change in pH of bathing solution during incubation of root segments with IAA. Results represent the means of 3 dishes ± standard error.

(a) Solution bubbled with air (120 $cm^3 min^{-1}$)

	IAA concentration (mol m^{-3})				
	0	10^{-9}	10^{-7}	10^{-5}	10^{-3}
Mean pH before incubation	6.1	6.1	6.1	6.1	6.1
Mean pH after incubation	6.6	6.5	6.6	6.6	6.6
pH change	+0.5	+0.4	+0.5	+0.5	+0.5
Standard error	0.07	0	0.07	0.07	0

(b) No supplementary gas

	IAA concentration (mol m^{-3})				
	0	10^{-9}	10^{-7}	10^{-5}	10^{-3}
Mean pH before incubation	5.8	5.8	5.8	5.8	5.8
Mean pH after incubation	6.3	6.3	6.3	6.3	6.3
pH change	+0.5	+0.5	+0.5	+0.5	+0.5
Standard error	0.09	0.08	0.09	0.09	0.06

(c) Solution bubbled with oxygen (20 $cm^3 min^{-1}$)

	IAA concentration (mol m^{-3})				
	0	10^{-9}	10^{-7}	10^{-5}	10^{-3}
Mean pH before incubation	5.7	5.8	5.8	5.8	5.8
Mean pH after incubation	6.3	6.3	6.3	6.4	6.4
pH change	+0.6	+0.5	+0.5	+0.6	+0.6
Standard error	0.17	0.15	0.09	0.07	0.12

(d) Control : Solution bubbled with oxygen (20 $cm^3 min^{-1}$), no plant tissue

	IAA concentration (mol m^{-3})				
	0	10^{-9}	10^{-7}	10^{-5}	10^{-3}
Mean pH before incubation	6.0	5.9	5.9	5.9	5.9
Mean pH after incubation	6.0	5.9	5.9	5.9	5.9
pH change	0	0	0	0	0
Standard error	0	0.1	0.15	0.1	0

B.5. Uptake of IAA by Root Tissue

In order to assess the significance of experimental observations on the effects of exogenous IAA, the amounts of IAA entering the tissue must be compared with the endogenous levels. An experiment was carried out to measure the uptake of IAA from five different external concentrations. Groups of 10 root segments were incubated in ^{14}C-IAA solutions for 1h. They were then washed (Materials and Methods) and extracted in 1 cm^3 methanol. The amount of radioactivity taken up by each group of segments was assayed by liquid scintillation spectrometry and the approximate uptake of IAA calculated (Table 30).

Table 30. Uptake of ^{14}C-IAA into 5mm root segments incubated in five different concentrations for 1h

concentration of IAA (mol m^{-3})	Bq $segment^{-1}$	Uptake of IAA $\mu g\ kg^{-1}$ fresh weight of tissue	$\mu g\ kg^{-1}$ fresh weight taking into account IAA metabolism
5.9×10^{-6}	0.088	2.1	0.64
1.4×10^{-5}	0.21	48	0.15
1.0×10^{-4}	0.82	19	5.9
9.4×10^{-4}	5.3	124	38
1.0×10^{-5}	2.7	628	195

These values can be compared with measurements of the endogenous content of IAA in Zea mays roots: 30 µg/kg f.wt. (Bridges et al., 1973) and 76 µg/kg f.wt. (Rivier and Pilet, 1974).

Conclusions

The growth rate of Zea mays root segments decreased rapidly after excision

due to factors other than lack of IAA. IAA supplied at concentrations of 10^{-9}, 10^{-7} and 10^{-5} mol m^{-3} had no effect on elongation of root segments during 1h incubation. In the presence of O_2, IAA at 10^{-3} mol m^{-3} had an inhibitory effect on growth. This was not apparent under N_2. Supplementary O_2 increased the growth rate of control segments and those in low concentrations of IAA. This addition of oxygen did not have a significant effect on IAA metabolism or on the pH of the incubating solution.

A decrease of approximately 0.5 units in the pH of the bathing solution surrounding root segments was observed during 1h incubation. This change took place both in the presence and absence of various concentrations of IAA. Measurements of IAA uptake from solutions containing different concentrations of ^{14}C-IAA were compared with published values for the endogenous IAA content of Zea mays roots. The amount of exogenous IAA present in the tissue after 1h incubation was of the same order of magnitude as the endogenous content, where the external concentration was 10^{-3} mol m^{-3}. Where the IAA concentration was 5.9×10^{-6} mol m^{-3} the quantity of IAA taken up was circa 1% of published measurements of the endogenous levels.

DISCUSSION

A. The Pathway of IAA Metabolism in *Zea mays* Seedlings and its Rôle in the Regulation of IAA Levels within the Plant

If a chemical substance is to be used to regulate physiological processes, it is imperative that the organism can control accurately the amounts of that substance which are present and active at the responding tissue. There are several possible means by which it might do this. The amount of the compound itself could be regulated by one or more of 4 general ways: by controlling the rate of biosynthesis, by regulating its catabolism, by reversible inactivation of the molecule or by transport both between cells and tissues, and between different compartments within the cell. Alternatively, the effectiveness of a physiologically active compound might be controlled by changes in the levels or activity of hypothetical receptor sites. Thus to understand fully how a plant growth substance operates it is necessary to investigate the details of its metabolism and transport, as well as possible binding sites.

In this thesis the metabolism of exogenous radiolabelled IAA in the roots and coleoptiles of dark-grown *Zea mays* seedlings has been studied. Products were analysed using the high resolution technique of reverse-phase HPLC in combination with an on-stream radioactivity monitor.

In initial experiments IAA was supplied by floating root and coleoptile segments in aqueous solutions of the plant growth substance. Metabolism of IAA was rapid and extensive with a large number of products being formed. Non-sterile root segments, incubated for 2h in darkness in 10^{-3} mol m^{-3} IAA-2-^{14}C metabolised on average approximately 59% of the radioactive IAA taken up by the tissue. At least 11 products were formed and some information about their chemical nature has been obtained.

IAA metabolites described in the literature can be divided into three groups (see Introduction):

1. IAA conjugates with amino acids, sugars, myo-inositol or high molecular weight compounds.
2. Oxidation products formed by decarboxylation, with loss of carbon-1 of the side chain (e.g. 3-methyleneoxindole, indole-3-aldehyde and indole-3-carboxylic acid).
3. Products formed by oxidation of the indole nucleus (e.g. oxindole-3-acetic acid, 3-hydroxyoxindole-3-acetic acid, 5-hydroxyoxindole-3-acetic acid, and 3,5-dihydroxyoxindole-3-acetic acid).

Conditions reported by Bandurski and Schulze (1977) to hydrolyse esters of IAA with sugars and myo-inositol were used to test IAA metabolites. Only a single metabolite, represented by peak 11 was hydrolysed to IAA under these conditions. This compound may be an ester of IAA with glucose or myo-inositol. It is unlikely that the other metabolites are esters of IAA. UV absorbance data provided some evidence for the presence of a second IAA conjugate, as a component of peak 7/8. Peak 8 co-chromatographed with IAA-glycine. A comparison of HPLC retention times of IAA metabolites with those of standards indicated that IAA-valine and IAA-alanine were not present in the plant extracts.

All the major metabolites appeared to be more polar than IAA. A comparison of the metabolism of IAA-1-^{14}C and IAA-2-^{14}C also showed that the products had not been formed by decarboxylation. They therefore did not include 3-methyleneoxindole, indole-3-aldehyde, indole-3-carboxylic acid or related compounds.

It was thought probable that several of the metabolites might represent compounds from group 3, formed by oxidation of the indole nucleus. These compounds are likely to be more polar than IAA. A standard of oxindole-3-acetic acid was synthesised as this compound had been found in Zea mays endosperm by Reinecke and Bandurski (1981). When analysed by HPLC peak 10, the most prominent metabolite, co-chromatographed with oxindole-3-acetic acid. This identification was supported by evidence from IAA breakdown controls that

peak 10 represented an oxidation product of IAA. Peak 7 also co-chromatographed with 5-hydroxyindole-3-acetic acid. This compound has not been previously identified in plant extracts.

Coleoptile segments, incubated under the same conditions, had metabolised a somewhat smaller proportion of the IAA-2-^{14}C taken up by the tissue than root segments. An average of 57% of the radioactivity remained associated with the IAA peak after 2h incubation. The products obtained were apparently similar to those formed in root tissue. However, there was a much greater predominance of peak 10 (presumptive oxindole-3-acetic acid).

The products observed in either tissue did not appear to be artifacts of the sample preparation procedure. Only a very small amount of breakdown was found in control experiments to test for IAA degradation during sample preparation. Similarly boiled segments did not metabolise IAA significantly. Further tests confirmed that modification of IAA and its metabolites during HPLC analysis was small. Only two metabolites (peaks 6 and 11) showed any sign of breakdown on rechromatography after collection from the column and drying. Experiments employing sterile roots indicated that metabolism was genuinely taking place within the plant tissue and was not due to epiphytic microorganisms.

When IAA metabolism has been studied in the past, work has usually concentrated on the identification of one or two products which often represented only a small fraction of the total amount of IAA metabolised (e.g. Tuli and Moyed, 1967; Magnus et al., 1971). In the present study relatively crude extracts were analysed, eliminating purification steps during which components might be selectively lost. This was made possible by the relatively low dry weight of extracts from dark-grown tissue and by the high selectivity of a radioactivity detector. Methanolic extracts of root segments were simply filtered, dried in vacuo and redissolved in a small volume of methanol. In most cases they were then centrifuged.

The efficiency of the methanol extraction procedure was always high: usually at least 90%. Thus a relatively small proportion of IAA metabolites

escaped analysis in this way. Similarly, recovery of radioactivity after HPLC analysis was good (ca. 95%). In spite of the minimum amount of sample preparation involved, however, a significant and variable amount of radioactivity (up to 36% of the sample) was lost during preparation. Most of this occurred during drying of the sample under reduced pressure (see Mann and Jaworski, 1970). It was not possible to be sure whether some metabolites were selectively lost. This was not thought to be probable, however, for three reasons. Firstly, as the samples were dissolved in the same solvent (i.e. methanol) after drying, differential solubility was unlikely to cause losses. Secondly, although losses were variable, the same products were present in each replicate extract. Thirdly, similar losses occurred during the preparation of control extracts which contained IAA alone. It was thus tentatively concluded that HPLC traces gave a reliable representation of the total spectrum of ^{14}C-IAA metabolites extracted into methanol from Zea mays root segments which had been incubated in 10^{-3} mol m^{-3} IAA-2-^{14}C for 2h.

During preparation of the majority of coleoptile samples, reduction of the extract to dryness by rotary evaporation was avoided. Samples were reduced to the aqueous phase then diluted with buffer and purified using C_{18} Sep-pak cartridges. However, as shown in experiment B.5, peaks 1 and 2 were selectively lost during this procedure.

In addition to eliminating selective purification procedures it was imperative to use an analytical technique which had high resolution. TLC was found to be inadequate for the separation of the large number of metabolites. Reverse-phase HPLC was, however, able to resolve the extract into 12 major components. Some evidence that these peaks might represent individual metabolites was obtained by isocratic chromatography. This could only be confirmed by the use of another analytical technique dependent on different properties of the molecules, e.g. ion-exchange chromatography. These results highlight the inconclusive nature of "identifications" of IAA metabolites based on co-chromatography with standards on paper or TLC systems (see Table 3).

Time-course experiments were carried out to study the interconversions of

products. However, after only 10min incubation at least 3 and sometimes up to 8 radioactive compounds, in roughly similar quantities, were observed in methanolic root extracts. These comprised a mean total of 43% of the radioactivity present in the extracts. The appearance of several products apparently simultaneously indicated that the path of metabolism in this tissue was probably complex and non-linear. Metabolism took place more slowly in coleoptile segments and also appeared less complex. After 1h only two products, represented by peaks 10 and 11 (presumptive oxindole-3-acetic acid and probably an ester of IAA with sugar or myo-inositol) were observed.

Without conclusive identification of metabolites and demonstrations of their interconversions, deductions concerning the pathway of IAA metabolism in Zea mays seedlings remain tentative. However, it would appear that root tissues are capable of very rapid metabolism of IAA by a non-linear and predominantly oxidative reaction pathway. Oxidation did not involve loss of the carboxyl group. Instead, the major product appeared to be oxindole-3-acetic acid. 5-Hydroxyindole-3-acetic acid may also have been formed. There was some evidence for the formation of two conjugates with IAA. One may have been an ester of IAA with a sugar or myo-inositol while the other co-chromatographed with IAA-glycine.

Coleoptiles appeared to metabolise IAA more slowly, at least at short incubation times. Although most of the products found in root extracts were apparently present in coleoptile samples, a single compound which co-chromatographed with oxindole-3-acetic acid was predominant. Again none of the major products involved decarboxylation of the IAA.

Certain questions must be answered before these results can be related to the metabolism of endogenous IAA. Firstly, how does the manner in which IAA is supplied to the plant affect its metabolism? When segments are floated on aqueous solutions of IAA, much of the plant growth substance is taken up at cut surfaces. Degradation might occur at this site and have very little relation to endogenous IAA metabolism. The remainder of the IAA will enter

across the epidermis and move towards the centre of the organ. In the case of roots this is likely to give rise to a distribution rather different from that in the intact plant, where the majority of IAA enters the root from, and is located in, the central stele (see Bridges et al., 1973; Bowen et al., 1972).

Experiments were carried out to investigate the metabolism of separated cortical and stelar segments. The results agree with those of Greenwood et al. (1973) in that very little IAA metabolism was found to take place in the stele during a 2h incubation. Products from cortical extracts were similar to those from whole segments and metabolism was rapid. Thus the IAA present in the stele, i.e. the bulk of the endogenous compound, would appear to be protected from metabolism. The rate at which IAA, taken up from solution, is metabolised is thus likely to be considerably greater than the overall rate of endogenous IAA metabolism.

An experiment was therefore carried out in which IAA was supplied to protruding portions of stele at the basal ends of root segments. In this way a closer approximation to the natural site of entry of IAA was obtained. As very little IAA was metabolised by stelar segments at short incubation times, this method of donation also avoided the possibility that much of the metabolism was taking place during entry at cut surfaces. IAA supplied in the above manner was metabolised more slowly than IAA taken up from solution; the proportions of radioactivity, in methanol extracts, remaining associated with the IAA peak were 50% and 31% respectively. The distribution of metabolites also differed. Peaks 5,6,8,9,10 and 11 were present as well as a small amount of a new product which was less polar than peak 11. In addition, peak 10 (oxindole-3-acetic acid) was no longer the most prominent metabolite.

Experiments were also carried out in which IAA was supplied to coleoptile segments from agar blocks placed at their apical ends. The apex is believed to be the major source of free IAA to the intact coleoptile (see Cohen and Bandurski, 1982) and transport is polarised in a basipetal direction (e.g.

Hertel and Leopold, 1963). This mode of donation is therefore a closer approximation to the natural source of IAA than that in experiments in which IAA was taken up from a bathing solution. The quantity of IAA metabolised by the coleoptiles supplied by the two different methods could not be directly compared as the experimental procedure was not identical. In both cases the most prominent metabolite was peak 10 (presumptive oxindole-3-acetic acid).

From these observations it can be deduced that the site at which exogenous IAA is supplied to the tissue may have a marked effect on its metabolism, although this was much more pronounced in the root than in the coleoptile. The results of experiments in which IAA was supplied from agar blocks were believed to give a more reliable approximation of the metabolism of endogenous IAA.

A second question which must be answered when relating the metabolism of exogenous IAA to that of the endogenous compound is, to what extent does the observed metabolism represent the detoxification of abnormally large amounts of plant growth substance? To answer this question in part, experiments were carried out to look at metabolism in the presence of different external IAA concentrations. Coleoptiles incubated in solutions of 10^{-3}, 10^{-2} and 10^{-1} mol m^{-3} IAA all metabolised similar proportions of the IAA taken up by the tissue. The nature and distribution of metabolites was also not significantly altered. Furthermore, when the effect of different IAA concentrations on segment elongation was studied, maximum growth took place in 10^{-2} mol m^{-3} IAA. The amount of IAA taken up from 10^{-3} mol m^{-3} IAA solution and present in the tissue after 2h incubation was approximately 4 pmol segment^{-1} (0.20 pmol mg^{-1} fresh weight). Coleoptile segments supplied with IAA-2-^{14}C from agar blocks over 4h contained approximately 2.2 pmol segment^{-1} (0.11 pmol mg^{-1} fresh weight) of exogenous IAA. Published measurements of the endogenous IAA content of Zea mays coleoptiles (Weiler et al., 1981; cv Anjou 21) using a radioimmunoassay are as follows: 10 pmol extractable "free" IAA per 2.5mm tip; ca. 3 pmol "diffusible" IAA

per mg fresh weight of coleoptile taken from between 2.5 and 10mm from the tip.

The amounts of IAA supplied were therefore of the same order of magnitude or lower, than the endogenous levels. Thus three lines of evidence indicated that the metabolism of IAA observed in the present experiments was unlikely to be a detoxification reaction to reduce abnormally high levels of the compound.

The same results were obtained in experiments with root tissues. Segments incubated in external concentrations of 10^{-4}, 10^{-3} and 10^{-2} mol m^{-3} IAA metabolised comparable proportions of the IAA-2-^{14}C taken up and contained a similar pattern of products, despite a large increase in uptake of IAA at higher concentrations. After a 2h incubation in 10^{-4} mol m^{-3} IAA solution, the amount of exogenous IAA present in the tissue was calculated to be approximately 5.7 μg kg^{-1} fresh weight. Measurements of endogenous quantities of IAA in similar portions of root by Bridges et al., (1973) for the variety "Giant White Horsetooth" and by Rivier and Pilet (1974) for the variety Kelvedon 33, were 29 and 76 μg kg^{-1} fresh weight respectively. Thus the amounts of IAA taken up were an order of magnitude less than endogenous quantities.

A third problem concerns whether metabolism of IAA changes due to the ageing of excised segments. Results of time-course experiments on IAA metabolism in roots indicated that IAA taken up after longer incubation times was more rapidly metabolised. Growth experiments indicated that after 1h there was a progressive decrease in the rate of elongation. It would therefore appear that short-term experiments are more likely to give an accurate representation of IAA metabolism in the intact plant. More work is required to discover whether more immediate wound responses might also have a significant effect on IAA metabolism.

Fourthly, it should be considered whether the ^{14}C label will have a significant effect on the distribution of products. However, with ^{14}C at

room temperature the maximum isotope effect is ca. 10% (e.g. Alder et al., 1971).

In summary, it is difficult to relate the results of studies on exogenous IAA metabolism in excised plant tissues to the endogenous metabolism. However, the results obtained in the present study did not appear to be artifacts produced at cut surfaces or consequences of detoxification of abnormally large amounts of hormone. Experiments in which IAA was supplied from agar blocks placed in a situation to mimic the endogenous source of IAA were most likely to give reliable information on endogenous metabolism. In future experiments it would be possible to supply labelled IAA to the endosperm of intact seedlings. As IAA is transported acropetally along the root this is likely to provide an adequate means of donation to this organ. Hall and Bandurski (1978) have shown, however, that when labelled IAA was supplied in this way, 98% of the radioactivity moving into the shoot had been metabolised. This method would therefore not be a feasible means of supplying IAA to the coleoptile. Instead, IAA could be applied to the coleoptile tip of intact seedlings using a micropipette.

Without the results of such studies, considerations of the potential of pathways of IAA metabolism to regulate its endogenous levels are speculative. Nevertheless, it would appear that both root and coleoptile tissues have the capacity for rapid metabolism of IAA. It is likely that the free IAA in the plant has a relatively rapid turnover. Whether or not the metabolism of IAA is a limiting step in regulating its levels, this property would allow the plant to change the amount of IAA present within a short time.

If the level of IAA is controlled by the rate of its metabolism, the latter might be expected to rise as the amount of exogenous IAA taken up is increased. In fact the proportion of IAA metabolised by both root and coleoptile segments was independent of the amount of exogenous IAA taken up over a range of two orders of magnitude. Thus there is some evidence that IAA levels might not be controlled solely by the rate of metabolism. The results of experiments D and E indicated that IAA present in the stele of the root is

largely protected from metabolism, while IAA in the cortex disappears rapidly. The levels of IAA in the root might therefore be regulated by the amount of lateral movement from the stele into the cortex.

Bandurski and coworkers (e.g. Cohen and Bandurski, 1978) have proposed that the reversible conjugation of IAA with sugars or myo-inositol may be important in regulating IAA levels in Zea mays seedlings. The results of this thesis indicated that IAA conjugates comprised only a small fraction of the total spectrum of metabolites of exogenous IAA. It is possible that conjugation may be more important in storage tissues such as the endosperm.

Experiments in which the metabolism of IAA-1-^{14}C and IAA-2-^{14}C were compared indicated that none of the major products had been formed by decarboxylation. Reinecke and Bandurski (1981) also presented evidence that the initial oxidation of IAA in Zea mays endosperm does not involve the loss of carbon-1 of the side chain. These results call into question the rôle of traditional IAA-oxidases in the in vivo oxidation of IAA in Zea mays seedlings, as these enzyme preparations have been shown to catalyse the decarboxylation of IAA to form either 3-methylen eoxindole or indole-3-aldehyde (e.g. Hinman and Lang, 1965; Ricard and Job, 1974).

IAA-oxidase preparations, usually peroxidases, (see Introduction), frequently show low substrate specificity (Butt, 1980). They have been extracted from a wide range of plant species (Schneider and Wightman, 1974). The possibility that these enzymes might be important in regulating IAA levels in higher plants has been studied extensively. Various types of correlations between IAA-oxidase activity and endogenous IAA levels have been reported (e.g. Frenkel, 1972; Atsumi and Hayashi, 1978; Saleh, 1981; Rao et al., 1982). None of these investigations has shown conclusively that IAA-oxidase can regulate endogenous IAA levels. Briggs et al. (1955) reported no relation between extractable IAA-oxidase activity and the disappearance of IAA in vivo in tissues of the fern Osmunda cinnamomea L.

Despite the lack of evidence for decarboxylation in the present study,

other researchers have found evidence for the decarboxylation of IAA. Substantial quantities of $^{14}CO_2$ may be produced when IAA-1-^{14}C is supplied to a variety of plant species (e.g. Wilkins et al., 1972b; Grochowska, 1974; Ryugo and Breen, 1974; Epstein and Lavee, 1975; Hamilton et al., 1976; Menschick and Hild, 1976; Epstein and Lavee, 1977a).

There are at least three possible explanations for this discrepancy. Firstly, decarboxylation might be occurring at cut surfaces catalysed by wound-produced peroxidase enzymes. Evidence for products of decarboxylation in the incubating medium rather than the plant tissue have been found by Andreae and Collet (1968) and Hamilton et al. (1976). In the present study there was some evidence for the presence of decarboxylation products in the incubating solution. One or sometimes two peaks, which were less polar than IAA, and only appeared when methylene-labelled IAA was supplied, were observed on HPLC traces.

Secondly, it is probable that there may be differences in IAA metabolism between species. Evidence for such differences in the nature of the IAA conjugates formed is substantial. Bandurski and Schulze (1977) studied the endogenous IAA conjugates present in the seeds from several plant species. They discovered that in cereals much of the IAA was esterified, while in legumes peptidyl IAA was more abundant. More conclusive identifications of endogenous IAA conjugates indicate the same trend. Esters of IAA with sugars and myo-inositol have been extracted from Zea mays (Ehmann, 1974; Ehmann and Bandurski, 1974) and Oryza sativa (Hall, 1980) while IAA-glycine was found in soybean (Cohen, 1981). Similar variation may be found in oxidation pathways.

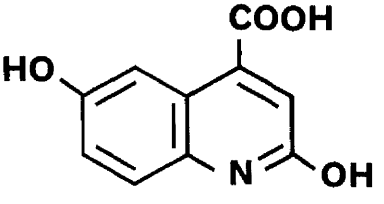
Thirdly, while the initial oxidation of IAA may not involve decarboxylation, this must occur at some stage in its catabolism. In most of the publications quoted, the majority of $^{14}CO_2$ evolution occurred after incubating the tissue for longer than 2h. Indeed preliminary experiments in the present study (results not given) indicated that $^{14}CO_2$ was evolved by Zea mays root segments and intact seedlings incubated in aqueous solutions of IAA-1-^{14}C for longer periods.

In conclusion, until it is shown conclusively that products such as indole-3-aldehyde and 3-methyleneoxindole are actually formed *in vivo* the rôle of IAA-oxidases remains conjectural.

B. Implications of Results on IAA Metabolism for Research into other Aspects of IAA Physiology

In this thesis it has been shown that exogenous IAA is rapidly and extensively metabolised by the roots and coleoptiles of *Zea mays* seedlings. As a large proportion of research into the rôle of IAA in higher plants is carried out using exogenous IAA, it is imperative that metabolism during the course of such experiments is taken into consideration.

A large variety of responses may be elicited when IAA is applied to various plant tissues (Table 1). It is possible, however, that metabolites of IAA may also have physiological activity. Indeed, IAA itself might not be the active molecule. A series of papers by Tuli and coworkers (e.g. Tuli and Moyed, 1967 and 1969) presented evidence that 3-methyleneoxindole was responsible for at least some effects of IAA. However, a number of other scientists (e.g. Skytt Andersen *et al.*, 1972; Evans and Ray, 1973; Roberts, 1974; Evans, 1976 a and b; Hamilton *et al.*, 1976; Sabater *et al.*, 1976; Lau *et al.*, 1978) have since found 3-methyleneoxindole to be devoid of auxin activity and the theory has now been discounted. Nevertheless, other IAA metabolites may well be physiologically active. Kinashi *et al.* (1976) suggested that the growth-inhibitory substance "β-acid" found in rice bran is



β-acid

an oxidation product of IAA. Hangarter and coworkers (e.g. Hangarter et al, 1980, and Hangarter and Good, 1981) and Feung et al. (1977) have examined the physiological activity of various amino acid conjugates of IAA: however, results indicated that activity was dependent on their hydrolysis.

The metabolism of IAA must also be considered when planning the duration of experiments on the effects of IAA. After 24h, Zea mays root and coleoptile segments incubated in IAA-2-^{14}C had metabolised at least 99% of the radioactive IAA taken up by the tissue. The metabolism of IAA in roots was markedly affected by the site of its donation. This may also have a bearing on experiments on the effects of exogenous IAA. When root segments are supplied with IAA from solution, most will enter the tissue through the cortex where it is rapidly metabolised. This must be borne in mind when interpreting the results of experiments such as those of Steen and Hild (1980) and Schurzman and Hild (1980) in which IAA was supplied from agar blocks placed on the side of maize roots for a 4h or 6h period. Firn (1982) noted that IAA, supplied to sunflower hypocotyl sections via the cut surfaces, produced a very weak response. On the other hand, IAA entering through the epidermis induced characteristic rapid growth promotion. The difference in effectiveness of IAA supplied at two different sites could reflect alterations in the rate or pattern of metabolism.

Zea mays roots and coleoptiles have been used extensively in experiments on various aspects of the physiology of IAA. These studies are frequently based on the premise that applied IAA is not metabolised significantly during the experiment. In particular, Zea mays coleoptiles and sometimes roots have been used to investigate IAA binding sites (e.g. Hertel et al., 1972; Batt et al., 1976; Ray et al., 1977; Cross and Briggs, 1979; Moloney and Pilet, 1981). None of these studies mention any form of test to ensure that radioactivity from labelled IAA supplied to cell preparations remains associated with the IAA molecule at the end of the experiment.

It was proposed by Went (1928) that geotropic and phototropic curvatures

in plants were mediated by the lateral transport of growth promoting substance from one side of the organ to the other. After the proposal that the chemical identity of auxin was IAA, several studies were carried out using radiolabelled IAA to investigate its lateral movement in Zea coleoptiles (e.g. Goldsmith and Wilkins, 1964; de la Fuente and Leopold, 1968; Wilkins and Whyte, 1968; Cane and Wilkins, 1969; Hertel et al., 1969; Shaw et al., 1973; Gardner et al., 1974). In some cases, tests for metabolism were carried out using TLC analysis of radioactive compounds (e.g. Cane and Wilkins, 1969; Shaw et al., 1973; Gardner et al., 1974). In the present study TLC was found not to have sufficient resolution to separate the large number of IAA metabolites. It was therefore thought possible that the amount of IAA metabolised may have been underestimated. However, Shaw et al. (1973) found that about 40% of the radioactivity extracted from coleoptile tissue after 1h, had been metabolised; i.e. a little more than recorded in this thesis. Given the differences in experimental conditions the results are in reasonable agreement. As in the present experiments Shaw et al. (1973) found only a single peak of radioactivity, corresponding to IAA, on analysis of extracts from receiver blocks of agar. This indicated that IAA metabolites were not mobile and gave further evidence for the specific transport of IAA.

Zea coleoptiles have also been used in the study of polarised basipetal transport of IAA (e.g. Hertel and Leopold, 1963; Edwards and Goldsmith, 1980; Sussman and Goldsmith, 1981). In later papers TLC analysis of extracts indicated a slow rate of metabolism (circa 5-10% of IAA supplied, per hour); in comparison with present results this would appear to be an underestimate.

In Zea mays roots, transport of IAA appears to be polarised in an acropetal direction (e.g. Wilkins and Scott, 1968; Wilkins et al., 1972a; Martin et al., 1978) and takes place predominantly in the stele (e.g. Cane and Wilkins, 1970; Bowen et al., 1972; Shaw and Wilkins, 1974). Some papers (e.g. Wilkins et al., 1972aand Shaw and Wilkins, 1974) have tested for metabolism of IAA using TLC analysis. The results indicated that although

substantial metabolism took place in the plant tissue, only IAA itself was collected in receiver blocks of agar. This result has been confirmed in this thesis using the higher resolution technique of HPLC.

The results in this thesis thus confirm the validity of the published work on IAA transport. It is suggested, however, that in future more rigorous, quantitative identification of the radioactivity at the end of experiments should be carried out.

C. The Effect of IAA on Root Elongation

As detailed in the Introduction, data on the effect of IAA on root elongation are contradictory. Some investigators have only found evidence for an inhibition of growth by IAA (e.g. Greenwood and Yčas, 1975). Others have observed a small growth promotion at very low concentrations (e.g. Batra et al., 1975; Edwards and Scott, 1977; Elliott, 1977; Evans et al., 1980; Mulkey et al., 1981; Pilet and Elliott, 1981). Experiments on the effects of IAA on root growth, described in this thesis, are of a preliminary nature. Nevertheless, some interesting observations have been made.

The growth rate of root segments decreased rapidly after excision. Experiments in this thesis were carried out using short incubation periods (1h). During this time the growth rate of segments was comparable to that of intact roots. Metabolism experiments which showed exogenous IAA to be rapidly metabolised provided a further reason for using short incubation periods.

Other scientists have frequently used longer incubation times; e.g. Batra et al., 1975 (6 and 2h); Pilet et al., 1979 (4, 8 and 12h); Pilet and Elliott, 1981 (8h). The deceleration of growth should be considered when interpreting these results. The data of Pilet et al. (1979), may be treated as an example. In this paper the effect of IAA on the growth of root segments which had been stood on agar for 8h was studied. The results were compared with those for freshly cut segments supplied immediately with IAA. Roots left on agar for 8h showed a substantial decrease in endogenous IAA. Furthermore,

low concentrations of IAA, inhibitory to the growth of freshly cut segments, were reported to stimulate growth of roots which had been left on agar for 8h. The authors hypothesised that intact Zea maysroots contained a saturating or supra-optimal level of IAA. A growth-promotory effect was therefore only observed when the endogenous IAA content was depleted. However, root segments left for 8h would show very little growth. It is noteworthy that Baehler and Pilet (1981) also noticed a decrease in the growth rate of segments after excision. Although this appeared to be somewhat slower than reported in this thesis, growth had decreased substantially 8h after excision. It is thus difficult to see how a valid comparison between growth of segments left for 8h and that of freshly cut segments, could be carried out. Furthermore, substantial changes, other than a decrease in the IAA levels, will have taken place during 8h after excision. Results presented in this thesis indicated that the decrease in growth was not principally due to lack of IAA. Incubation with various concentrations of exogenous IAA over a 24h period did not maintain elongation at the same rate as in intact roots. It was therefore concluded that the decrease in growth was due to lack of other substances (e.g. nutrients, other plant growth substances) or to the presence of inhibitory compounds such as ethylene. The same conclusion was reached by Greenwood and Yčas (1975). They monitored the growth of root segments after excision. Whole root segments were compared with those from which the stele (the main site of IAA in the root) had been removed. The growth of both groups decreased at the same rate. It is also worth pointing out that if the IAA levels in the root are limiting growth then IAA should not be present in saturating amounts.

More detailed studies of the effect of various concentrations of IAA on growth of root segments were carried out with greater replication. The availability of oxygen was also varied. A promotory effect of IAA on growth was not observed under any circumstances. On the other hand, IAA at a concentration of 10^{-3}mol m^{-3} and in the presence of oxygen inhibited root elongation. To assess the relevance of this work with respect

to the rôle of endogenous IAA, it was important to compare the amounts of IAA taken up from different external concentrations with endogenous quantities. Data on uptake of IAA-2-^{14}C indicated that with an external concentration of 10^{-3} mol m^{-3} IAA (i.e. inhibitory to growth), the amount of exogenous IAA present in the tissue after 1h incubation was approximately 38 µg per kg fresh weight of tissue. This value was of the same order of magnitude as published measurements of endogenous levels in similar portions of Zea mays root: 29 µg kg^{-1} for the variety Giant White Horsetooth (Bridges et al., 1973); 76 µg kg^{-1} for the variety Kelvedon 33 (Rivier and Pilet, 1974). Thus it is possible that endogenous IAA may have an inhibitory effect on growth.

A comparison of the IAA concentrations reported to promote root growth (e.g. 10^{-4} mol m^{-3}, Edwards and Scott, 1977; 10^{-5} and 10^{-7} mol m^{-3}, Mulkey et al., 1981; 10^{-5} mol m^{-3} Pilet and Elliott, 1981) with the results presented in this thesis indicated that the amount of IAA taken up from growth stimulating concentrations was equivalent to only a small fraction of the endogenous content. The amount of exogenous IAA present in segments incubated in 5.9×10^{-6} mol m^{-3} IAA for 1h was approximately 0.64 µg per kg fresh weight of tissue; i.e. circa 1% of endogenous levels.

Accordingly, IAA uptake should be viewed as a critical factor in assessing the significance of experiments on the effects of low concentrations of IAA on root growth. Nevertheless, it is possible that IAA may be located in more than one discrete compartment and that measurements of the total IAA content of the tissue may mask the levels of "effective" IAA. It must also be noted that published measurements of endogenous IAA were carried out using different varieties of Zea mays from that employed in the present study. Pilet and Elliott (1981) suggested that there may be substantial differences in the endogenous IAA levels of different cultivars.

Significant interaction was found between oxygen availability and the effect of IAA. IAA did not inhibit the growth of segments incubated under nitrogen. The effect of oxygen was to increase the growth of roots incubated in control solution and in low concentrations of IAA. IAA at a concentration

of 10^{-3} mol m^{-3} then reduced the growth rate to that observed for segments incubated under nitrogen at any concentration of IAA.

This dependence of the inhibitory effect of exogenous IAA on the presence of supplementary oxygen may indicate that root segments incubated in aqueous solutions rapidly become depleted of oxygen, and that the intact roots possess an oxygen-dependent component of growth which may be inhibited by IAA. Segments incubated in solutions not bubbled with gas showed a small growth inhibition in the presence of 10^{-3} mol m^{-3} IAA. On the other hand, the levels of oxygen supplied to roots bubbled with pure oxygen will be substantially higher than those encountered *in vivo* and the resulting high growth rate may be artifactual.

To summarise, the results presented in this thesis indicated that exogenous IAA is only able to inhibit *Zea* root growth. Several publications reporting a growth promotory effect used long incubation times (2h or more) during which ageing of the tissue, shortage of essential substances and possibly a build-up of wound-produced inhibitory compounds may have affected the response to IAA. In addition, calculations of uptake of ^{14}C-IAA from various concentrations indicated that the amount of exogenous IAA taken up from the low concentrations reported to promote growth, may be only a small fraction of the endogenous content.

On the other hand, it was suggested by Chadwick and Burg (1967), that the inhibition of root growth, induced by exogenous IAA may be a result of IAA-stimulated ethylene biosynthesis. This was investigated by Mulkey *et al.* (1981). Roots were pretreated with the ethylene biosynthesis inhibitor aminoethoxyvinylglycine before being placed in solutions of IAA. This treatment increased growth of controls and permitted growth enhancement by 10^{-3} and 10^{-4} mol m^{-3} IAA; higher concentrations remained inhibitory.

The site at which IAA is supplied to root segments might also affect results. Changes in the method of applying IAA-2-^{14}C resulted in variation in the rate and pattern of its metabolism. Firn (1982) also noticed that the site of IAA uptake into sunflower hypocotyls substantially affected the

growth response.

Evans et al. (1980) reported that the inhibitory effect of IAA on root growth was accompanied by an increase in the pH of the medium. This was not found in the present study. A pH increase occurred in all solutions containing root segments, irrespective of the presence or concentration of IAA. It is possible that the discrepancy is a result of small differences in experimental procedure; the above authors used buffered incubating solution, and a larger number of sections for the volume of medium.

D. Future Experiments

The present study has emphasised the need to investigate the whole spectrum of IAA metabolites produced within a single species. Conclusive identification of these products is now required. A comparison of mass spectra of metabolites with those of pure compounds is likely to prove the most suitable method of definitive characterisation. The following compounds may prove to be useful standards, in addition to those already synthesised: 3-hydroxyoxindole-3-acetic acid, 5-hydroxyoxindole-3-acetic acid, 3,5-dihydroxyoxindole-3-acetic acid and conjugates of IAA with myo-inositol and glucose.

The sequence in which metabolites are produced also requires investigation. This is likely to prove easier in the coleoptile where the metabolic pathway appears to be less complex than in the root. Radioactively-labelled IAA metabolites could be supplied to the plant tissue and the products isolated and identified.

The overall aim of a study on IAA metabolism should be to obtain information leading to an understanding of the rôle of IAA in the intact plant. The site at which IAA was supplied to plant tissue was found to have a substantial effect on the metabolism pattern in roots. It is therefore suggested that in future experiments, the metabolism of IAA supplied to the intact plant by means, and in amounts, which mimic the endogenous source should be investigated. Application to the endosperm

might serve as a suitable site for root studies, while IAA could be supplied to the coleoptile tips from micropipettes. Extractions should also be carried out to determine whether the exogenous IAA metabolites are naturally present within the plant. Possible diurnal variations in the rate and/or path of IAA metabolism should be considered (see Sandberg *et al.*, 1982).

Once the pathway of IAA metabolism has been established it should be possible to identify the enzymes catalysing these reactions. Proteins catalysing the esterification of IAA with glucose and *myo*-inositol have already been isolated (Michalczuk and Bandurski, 1980). Enzymes involved in the oxidation of IAA to compounds such as oxindole-3-acetic acid have not yet been studied. The rôle of "IAA-oxidases" requires further investigation. It would appear unlikely that this type of enzyme is normally responsible for the *in vivo* oxidation of IAA in *Zea mays* seedlings. However, further study of IAA metabolism in other species might provide evidence for an IAA-oxidase-catalysed decarboxylation reaction. An investigation of the enzymes involved in IAA metabolism might reveal correlations between their activity and endogenous levels of IAA and thus provide information as to how endogenous quantities of IAA are controlled. The subcellular location of IAA metabolism and the products should also be examined. This might be achieved by the use of autoradiography and cellular fractionation techniques. In order to understand how the activity of IAA is controlled, the physiological activities of IAA metabolites should be studied. Hangarter *et al.* (1980) and Feung *et al.* (1977) have investigated the physiological activity of several amino acid conjugates. 3-Methyleneoxindole has also been studied extensively in this context (e.g. Tuli and Moyed, 1969). The activity of compounds such as oxindole-3-acetic, however, has not been tested. Experiments using esters of IAA with *myo*-inositol and glucose would be complicated by the ease with which these compounds are hydrolysed.

Bandurski and co-workers (e.g. Cohen and Bandurski, 1978) have suggested that IAA may be oxidised as a result of its producing a physiological response. This hypothesis might be tested by examining possible correlations

between the rate of IAA oxidation and tissue sensitivity.

Experiments on the effect of IAA on root elongation have highlighted several points which should be borne in mind in future experiments: It is essential that the quantity of exogenous IAA entering the root is of the same order of magnitude as the endogenous content. The level of IAA in the tissue and Zea mays variety used should therefore be measured and compared with the uptake of IAA during growth experiments. The importance of subcellular compartmentation should also be investigated.

IAA should be applied to the root in a manner which most closely mimics the natural source of the compound. Donation to the endosperm or to a portion of stele protruding from the basal end of segments might be suitable.

The metabolic fate of IAA should be correlated with growth effects and the bioactivity of metabolites considered. The rate of IAA metabolism would be expected to influence choice of the duration of experiments.

The use of buffers is to be discouraged, as these may affect the growth in their own right (e.g. Edwards and Scott, 1976). Nevertheless the pH of solutions may be important in determining uptake of IAA (see Edwards and Goldsmith, 1980). The levels of oxygen available to the tissue presents another variable to be investigated. The effect of oxygen on IAA-controlled growth needs further study.

Finally the rôle of ethylene in the response to IAA requires clarification. This would involve the use of inhibitors of its biosynthesis and mode of action, or removal of ethylene (e.g. by mercuric perchlorate). The production of ethylene in the presence and absence of IAA should be studied, as should the response to exogenous ethylene.

The results presented in this thesis must be viewed in the context of the rôle of endogenous IAA in the correlative control of growth and differentiation in Zea mays seedlings. Early work (e.g. Went, 1928) provided evidence that auxin acted as a chemical messenger within the plant. Confirmation of this theory requires unequivocal proof that IAA is synthesised at a discrete site, from where it is transported to specific site(s) of action. An understanding of the rôle of plant growth substances is most likely to be achieved by the detailed study of a relatively simple plant system. Young Zea mays seedlings represent a compact experimental system, exhibiting most of the correlative features of vegetative plant growth and have been used extensively for research on the rôle of IAA. It is now beyond dispute that endogenous IAA is present in extracts of roots, endosperm, mesocotyls and coleoptiles of dark-grown Zea mays seedlings. Furthermore, the zone of cell expansion in the coleoptile is at least one site of action. Levels of exogenous IAA comparable with the endogenous pool size inhibit root elongation. Exogenous IAA is metabolised rapidly by coleoptiles and roots. Moreover, cortical and stelar tissues of the root metabolise IAA at substantially different rates. The exogenous compound is specifically transported in a polarised fashion in both roots and coleoptiles. Recent advances in analytical technology have made possible the conclusive identification of minute quantities of plant growth substances in complex plant extracts. Methods also exist for determining the intracellular location of compounds. These technological developments, coupled with an increasing understanding of the effects, transport and metabolism of exogenous IAA provide encouraging signs for clarifying the rôle of endogenous IAA in the life of the intact plant.

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ADDENDUM I

Subsequent to the compilation of this thesis a sample of metabolite peak 10 was purified from a methanolic extract of root segments which had been incubated in aqueous IAA-2-^{14}C (10^{-2}mol m^{-3}) for 2h and derivatised using diazomethane. The resulting methyl ester co-chromatographed on HPLC with the methyl ester of standard oxindole-3-acetic acid, thus giving further evidence that peak 10 represents this compound.

ADDENDUM II

Recently a report has been published describing HPLC analysis of IAA metabolites from Cucumis sativus L. (Purves and Hollenberg, 1982). Hypocotyl segments were incubated with ^{3}H-IAA or ^{14}C-IAA for varying lengths of time (from 0.5 to 48h) and the products extracted with methanol. Reverse-phase HPLC analysis of metabolites revealed an unspecified number of products, most of which were more polar than IAA. Two products were identified by co-chromatography as IAA-aspartate and IAAglutamate. The identity of the other products was not investigated.

Reference: Purves, W.K. and Hollenberg, S.M. (1982) Metabolism of exogenous indole-3-acetic acid to its amide conjugates in Cucumis sativus L. Plant Physiol.,70, 283-286

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