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INDOLE-3-ACETIC ACID CONJUGATES:
SLOW RELEASE SOURCES OF AUXIN
FOR PLANT TISSUE CULTURE

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INDOLE-3-ACETIC ACID CONJUGATES: SLOW RELEASE SOURCES OF AUXIN FOR PLANT TISSUE CULTURE

By

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ABSTRACT

INDOLE-3-ACETIC ACID CONJUGATES:
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The auxin activities of a number of indolacetyl-amino acid conjugates have been examined in three test systems: growth of tomato hypocotyl explants (Lycopersicon esculentum Mill. cv. Marglobe); growth of tobacco callus cultures (Nicotiana tabacum L. cv. Wisconsin 38); and ethylene production from pea stems (Pisum sativum L. cv. Alaska). The activities of the conjugates differ greatly from the activity of free indoleacetic acid and also differ greatly among themselves depending on the amino acid moiety. Indoleacetyl-L-alanine supports rapid callus growth from the tomato hypocotyls while inhibiting growth of shoots and roots. Indoleacetylglycine behaves in a similar manner but is somewhat less effective in supporting callus growth and in inhibiting shoot formation. Other amino acid conjugates tested (glutamic acid, leucine, methionine, phenylalanine, proline and threonine) support shoot formation without supporting much callus growth, or root growth. Conjugates with aspartic acid, asparagine and

serine support shoot formation and root formation without much callus growth. The tobacco system, which forms abundant shoots in the presence of free indoleacetic acid, produces only rapid unorganized growth in the presence of indoleacetyl-L-alanine and indoleacetylglycine. Other conjugates inhibit shoot formation weakly if at all. Most of the conjugates are active in the ethylene production assay and again the activities differ depending on the conjugating moiety. The conjugates which are most potent in inducing ethylene formation are those which favor callus growth in the tissue culture assays. Conjugates which are very active with one species are not always active with other species.

The biological activities of the conjugates are due in part, at least, to the fact that they serve as continuous sources of free indoleacetic acid via enzyme-catalyzed hydrolysis. This conclusion is based on the following observations: 1) The conjugates are active for a much longer time than free indoleacetic acid; 2) Frequent renewals of free indoleacetic acid can mimic some of the effects of the more active conjugate, indoleacetyl-L-alanine; 3) Free indoleacetic acid and the active conjugates are converted into some of the same metabolites; 4) The rates of decarboxylation of the conjugates (an indirect measure of hydrolytic production of free indoleacetic acid) correlate with their biological activities; and 5) Conjugates with the D-isomers of the amino acids, which are probably not susceptable to enzymatic hydrolysis, are inactive as auxins.

The metabolism of indoleacetic acid by tumorous and non-

tumorous tissues of Parthenocissus tricuspidata and Helianthus annuus was examined to determine whether indole-acetic acid conjugates were involved in tumorogenesis. The data indicate that the kind of metabolite produced is more closely correlated with the plant species than with the tumorous condition.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

INTRODUCTION

The significance of plant cell cultures for crop improvement is a subject which has received a great deal of attention in recent years (cf. Sala et al., 1980). The usefulness of cell cultures depends on our ability to manipulate, in a controlled manner, the nature of the growth of the cultured tissues. Two types of manipulations which are central to the successful application of plant cell cultures for crop improvement are: 1) Routine development of rapidly growing suspension cultures from the crop plants in question. Microbiological techniques can then be used for modification and analysis of the cells of higher plants; 2) Regeneration of entire plants from the cultured cells. The cultured material can then be used by plant breeders. Regeneration of plants from cells also makes possible the rapids propagation of superior genotypes. Unfortunately, these two manipulations can not yet be carried out with some of our most important plant species.

The components of plant tissue culture media which are most critical in determining the nature of the growth are the auxins[1] and cytokinins. Commercially available auxins differ very much in their stability, in their effectiveness, and in their influence on organogenesis. Indoleacetic acid (IAA) is destroyed rapidly by many plant tissues (Bender and

Newman, 1978; Epstein and Lavee, 1975) and is often not very effective in supporting the growth of cultured plant tissues. However, when it is effective it is the least likely of the auxins to inhibit organogenesis. In contrast, the stable auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) is very active in stimulating the growth of cultures but it strongly represses organized growth and usually only callus in produced (Murashige, 1975). Because 2,4-D is so effective in supporting the growth of many plant tissues it is probably the most widely used auxin in tissue culture studies but, unfortunately, it can cause problems if one desires to regenerate plants from the tissues. Other commercially available auxins, such as napthaleneacetic acid (NAA) and indole-3-butyric acid (IBA), are also effective with some plant cultures but not with others. But, as already indicated, many economically important plants still cannot be induced to form, reversibly, callus tissues or suspension cultures, despite the variable activities of the commercially available auxins.

In the 1940's it was shown that IAA can exist in bound forms in plant tissues (Berger and Avery, 1944; Gordon, 1964; Hatcher, 1943; Thimann and Skoog, 1940; Van Overbeek, 1941). Since these first observations a number of bound forms of IAA have been identified as metabolites of exogenous IAA or as natural products (Andreae and Good, 1955; Bandurski, 1978; Hattori and Marumo, 1972; Zenk, 1961). Careful measurements of IAA and its derivatives have shown that most of the IAA in a plant is bound, sometimes through amide or through ester linkages, and that free IAA occurs in extremely low amounts

(Bandurski and Schulze, 1974, 1977). Conjugates of IAA are protected from peroxidative destruction (Cohen and Bandurski, 1978) and can act as reserve sources of IAA (Bandurski, et al., 1977; Epstein et al., 1980; Nowacki and Bandurski, 1980).

The occurrence of stored, stable IAA conjugates which can act as reserve sources of IAA in plant tissues suggested that exogenous supplies of IAA conjugates might be able to serve as slow-release sources of the natural auxin, IAA, for plant tissue cultures. IAA conjugates have previously been shown to support the growth of soybean tissue cultures (Feung et al., 1977) and to affect organogenesis of tomato and tobacco tissue cultures (Peterson, 1978).

Much of the work reported in this thesis was carried out to evaluate any possible advantages of using IAA conjugates in plant tissue cultures. The IAA conjugates were found to have a great diversity of activities which makes them valuable alternatives to the commercially available auxins. The different activities of the different conjugates probably will make possible control of the growth of plant cultures in ways which have not been possible with the traditional auxins.

The hypothesized mode of action of the conjugates — that the conjugates act as slow-release sources of free IAA — leads to several testable predictions. The hypothesis suggests that: 1) exogenous conjugates will be active for longer periods of time than exogenous free IAA; 2) frequent additions of new free IAA will mimic single additions of conjugates; 3) at least some of the metabolites of the

conjugates will be the same as the metabolites of free IAA; 4) the rates of hydroloysis of the conjugates will correlate with the biological activities; and 5) conjugates with the D-isomers of the amino acids will be largely inactive since the hydrolytic enzymes capable of splitting amide bonds are nearly always stereospecific. These predictions were all verified experimentally.

Of course the significance of the IAA conjugates may not be limited to their usefulness as slow release sources of IAA for plant tissue culture. Some IAA conjugates are naturally occurring in plants (cf. Bandurski, 1979) but their physiological role is not completely understood. Feung et al. (1976) claimed that applied IAA was metabolized into five IAA-amino acid conjugates by Parthenocissus tricuspitata crown-gall tissue. Glycine, alanine and valine conjugates were the major metabolites whereas the aspartic acid and glutamic acid conjugates were the minor metabolites. However, most other plant tissues have been reported to accumulate primarily the aspartic acid conjugate. Feung et al. (1976) suggested that the types of conjugates found in the crown-gall tissues might be characteristic of tumor tissues. Unfortunately, non-tumorous tissue of P. tricuspidata was not analyzed.

In the studies reported in this thesis, the alanine, the glycine and the valine conjugates of IAA were found to have the greatest capacity, of all the IAA-amino acids tested, for inducing de-differention of plant tissues. The putative occurrence of these very active conjugates in the P. tricuspidata tumor tissue suggested that the biosynthesis of these

conjugates might actually play a role in plant tumorogenesis. However, a comparison of the IAA metabolites from tumorous and non-tumorous tissues from different species indicates that the kind of metabolite produced is more closely correlated with the plant species than with the tumorous condition.

LITERATURE REVIEW

I. Bound Auxins

Occurrence of IAA conjugates. In 1934, Thimann put forth the hypothesis that a portion of the IAA in plants is in a bound form. This hypothesis was based on the observation that when some tissues are placed on agar they yield little "diffusilbe" auxin, whereas considerable amounts of auxin are obtained by ether extraction. Several years later van Overbeek (1941) found approximately 90% of the auxin in Zea and Avena coleoptiles to be in a non-diffusible "precursor" form. Avery et al. (1941) found 90 to 95% of the auxin bound in dormant kernals of Zea. Several investigators found that the bound form of auxin in cereals was alkali-labile (Avery et al., 1941; Berger and Avery, 1941; Haagen-Smit et al., 1942) and the auxin produced by alkaline hydrolysis was identified as IAA (Haagen-Smit et al., 1942). Evidence that the complex was an ester was obtained by Haagen-Smit et al. (1946). In 1965, Labarca et al. confirmed that the IAA complexes in the cereals are, in fact, esters of IAA.

The structures of many of the indolyic compounds in kernels of Zea mays, down to a level of 10 ug/kg dry wt, have been identified (Labarca et al., 1965; Ueda and Bandurski, 1974). They include such compounds as indoleacetic acid, indoleacetylinositols, indoleacetylinositol-arabinosides,

indoleacetylglucopyranoses, 5-O-β-L-glactopyranosyl-2-O-indoleacetylinositol and indoleacetyl-β-1,4-glucan. An indoleacetylglucoprotein from Avena seeds has been described (Percival and Bandurski, 1979). Indoleacetyl-myo-inositol has been isolated from rice (Hall, 1980). Indoleacetyl-rhamnose has been reported to occur in Peltophorum ferrugineum (Ganguly et al., 1974).

Bandurski and Schulze (1977) have measured the IAA content of a number of plant tissues. The IAA compounds were classified as free, "ester" and "amide" forms depending on their lability in alkali. Their data show that the cereals contain mainly ester IAA and that the legumes contain mainly amide-linked IAA. The amide conjugate, IAA-aspartate is known to be formed from exogenous IAA by many plant tissues and, in fact, was the first IAA conjugate to be structually characterized (Andreae and Good, 1955). There are several reports which claim that IAA-aspartate is a natural product present in plants which have not been exposed to exogenous IAA (Klambt, 1960; Row et al., 1961; Olney, 1968; Lantican and Muir, 1969; Tillberg, 1974). However, the identification in each case was based only on chromatographic evidence and needs to be confirmed by more rigorous techniques. The widespread occurrence of an auxin-inducible enzyme system for IAA-aspartate synthesis and the high level of specificity of the induction for active auxin (Andreae and Van Ysselstein, 1956; Sudi, 1964, 1966; Venis, 1964, 1972) strongly suggests that amide conjugates of IAA do occur naturally. In fact, in a rigorous study by Hattori and Marumo (1972), monomethyl-4-chloroindoyl-

3-acetyl-L-aspartate was identified at low levels in immature seeds of Pisum sativum [where the chloroindoleacetic acid seems to be the natural auxin (Marumo et al., 1968)].

The principal indole compound present in the Brassicaceae family is the thioglucoside of indoleacetonitrile named glucobrassicin (Kutacek and Prochazka, 1963; Gmelin, 1964; Kutacek and Kefeli, 1968). Early reports of the presence of ascorbigen (indoleacetonitrile-ascorbic acid) in the Brassicaceae are probably due to the hydrolysis of glucobrassicin during extraction (Gmelin, 1964).

Biological formation of IAA conjugates. Kopewicz et al. (1974) reported on the synthesis of esters of IAA by kernels and by an enzyme preparation from Zea. The enzyme preparation required ATP and CoA and could synthesize IAA esters of myo inositol, glucose and glucans. A second pathway to IAA-myo-inositol has been reported by Michalczuk and Bandurski (1980). This system involves the enzymatic synthesis of IAA-β-1-D-glucose from IAA and UDPG followed by transacylation from IAA-β-1-D-glucose to myo-inositol to form IAA-myo-inositol. Two separate enzymes for the two reaction steps were demonstrated.

Although the in vivo formation of IAA-aspartate from applied IAA has been known to occur for 25 years (Andreae and Good, 1955), its synthesis in vitro has not been adequately demonstrated. Lantican and Muir (1969) reported the presence, in vitro, of an enzyme system which formed IAA-aspartate from IAA and aspartic acid. However, this report has been criticized by others who have failed to find any convincing

demonstration of cell-free synthesis using highly sensitive assay methods (Venis, 1972). Another more recent report has appeared which claimed *in vitro* synthesis of IAA-aspartate (Higgins and Barnett, 1976). Unfortunately, the report was only preliminary and without details. Since a detailed report has not yet appeared, the report must be assumed to be unsubstantiated.

Despite the lack of direct evidence for enzymatic synthesis of IAA-aspartate, there is strong evidence from *in vivo* studies for the existence of the appropriate enzyme system. The aspartate-conjugating mechanism has clearly been demonstrated to be induceable (Andreae and Van Ysselstein, 1957; Sudi, 1964, 1966; Venis, 1964, 1972). The inductive effect has been shown to be dependent on protein and RNA synthesis and thus synthesis of a new enzyme protein (Venis, 1964, 1972). The use of a variety of related compounds as inducers has demonstrated a specific requirement for compounds with auxin activity (Sudi, 1966; Venis, 1972).

Physiological roles of IAA conjugates. The physiological significance of the chemical conjugation of IAA is not yet understood. A number of roles for the bound auxins have been proposed (Thimann, 1934; Berger and Avery, 1941; Andreae and Good, 1955; Feung et. al., 1976; Cohen and Bandurski, 1978; Liu et al., 1978; Nowacki and Bandurski, 1980). These include a function as reservoirs for auxin release, a detoxification mechanism, a role in transport and a role in tumorigenesis.

The role favored by most researchers is that of providing

a reservoir for the release of free IAA. Hatcher (1943) observed that as the auxin disappeared from maturing kernals of rye an inactive compound was formed which developed high auxin activity upon alkaline hydrolysis. Most of the bound auxin in seeds of Zea mays was found in the endosperm (Hemberg, 1955). The amount of bound auxin decreased concurrently with an increase in the amount of free auxin during germination (Hemberg, 1955; Ueda and Bandurski, 1969). More recently, the role of IAA conjugates as reserve forms used in the homeostatic control of free IAA concentrations in maize seedlings has been demonstrated through the use of modern analytical methods (Bandurski et al.,1977; Epstein et al., 1980; Nowacki and Bandurski, 1980). This storage role for IAA conjugates is plausable because they are protected from peroxidase-catalyzed oxidation while free IAA is not (Andreae and Good, 1955; Rekoslavskaya et al., 1974; Hamilton et al., 1976; Cohen and Bandurski, 1978). Recently, IAA conjugates were shown to act as slow-release sources of IAA in plant tissue cultures (Hangarter et al., 1980).

Another proposed function of the IAA conjugates is in auxin transport. Nowacki and Bandurski (1980) have shown that IAA-myo-inositol is transported from the endosperm, in Zea, into the shoot at rates 400 times faster than the rate of transport of free IAA from the endosperm to the shoot and 40 times the rate of transport of tryptophane-derived IAA. They also present evidence for the conversion of IAA-myo-inositol to free IAA in the shoot. These results show that

IAA-myo-inositol meets the requirements of a "seed auxin precursor" which moves from the seed to the shoot where it is converted to free IAA. The free IAA should then be able to move basipetally and control the growth of the shoot. The amide conjugate, IAA-aspartate, appears to be an imobilized form of IAA in intact pea plants (Morris et al., 1969). IAA-L-alanine and IAA-glycine do not seem to be actively transported in either the basipetal or the acropetal direction in pea stem segments (Hangarter et al., 1980).

The formation of amide conjugates of IAA has often been attributed to an unspecific detoxification mechanism (Andreae and Good, 1955; Zenk, 1974). An analogy with similar conjugation reactions in animals has been made (Andreae and Good, 1955). However, most of IAA-amino acids have been shown to be biologically active in bioassays (Feung et al., 1976; Hangarter et al., 1980) and therefore, any "detoxification" cannot be wholly effective. More telling, and quite contrary to the concept of non-specificity is the observation that the conjugating mechanism is specifically induced by active auxins but not by inactive analogs (Sudi, 1966; Venis, 1972). Glucosidic bound auxins are also biologically active (Bandurski, 1979). The biological activities of the various IAA conjugates and the demonstration that IAA-myo-inositol can be converted to free IAA in Zea (Nowacki and Bandurski, 1980) favors the storage role for control of hormone homeostasis.

Liu et al. (1978) showed a correlation between the levels of bound auxin and tumorigenis in hybrids of Nicotiana glauca x N. langsdorfi. Feung et al. (1976) claimed to have found

conjugates of IAA in crown-gall tissues of Parthenocissus tricuspidata which they suggested might be unique to tumor tissue. Unfortunately, Feung et al. did not investigate non-tumorous tissue of the same species. Much more work needs to be done before a role in tumorigenesis can be assigned to any auxin conjugate.

Biological activities of IAA conjugates. The effects of IAA on the growth of numerous plant tissues have been extensively studied since its discovery as a plant hormone. Despite the fact that as early as 1941 (Berger and Avery) most of the IAA in plants was known to be in a bound form, relatively little is known about the growth regulating properties of bound forms of IAA. Knowledge about the growth responses of plant tissues to IAA conjugates has some importance if we are to evaluate the physiological significance of conjugate formation.

The biological activity of IAA-aspartate, the first conjugate to be identified (Andreae and Good, 1955), has been studied in a number of bioassay systems (Table 1). The reported activities of IAA-aspartate range between none and the same as free IAA. Most of the variation is seen among species but in some cases, the reported activities in the same assay system varied markedly (cf. Andreae and Good, 1955; Lantican and Muir, 1969; Feung et al., 1977).

A number of IAA-amino acids were tested for growth activity on slit pea stems by Jerchel and Staab-Muller (1954). They found the glycine and cysteine conjugates to be nearly inactive while IAA-alanine was as active as IAA. Feung et al.

Table 1. Reports on the biological activity of IAA-aspartate.

Bioassay	Activity	Reference
Slit pea curvature	1/1000 of IAA	Andreae and Good,1955
Pea stem elongation	1/1000 of IAA	"
Avena curvature	equal to IAA	"
Avena straight growth	equal to IAA	"
Tomato root growth	similar to IAA	Thurman and Street,1962
Avena curvature	nearly inactive	Lantican and Muir,1969
Avena straight growth	nearly inactive	"
Ethylene induction in mung bean	totally inactive	Lau and Yang,1973
Ethylene induction in mung bean	18% of IAA	Sakai and Imaseki,1973
Growth of potato, soybean, and tobacco suspension cultures	weaker than IAA	Rekoslavskaya and Gamburg,1976
Soybean callus growth	less than IAA	Feung et al.,1977
Avena straight growth	similar to IAA	"
Ethylene induction in pea stems	14% of IAA	Hangarter et al.,1980
Growth of tomato and tobacco tissue	less than IAA	"
Senescence of Ipomoea	nearly inactive	Hurter et al.,1980

(1977) surveyed the activities of 20 amino acid conjugates in tow bioassay systems. In the Avena straight growth test, a short-term assay, they found that nearly all of the conjugates were less active than IAA. On the other hand, in the long-term effects on soybean callus growth, several conjugates were found to be more active than IAA. Similar observations with short-term vs. long term assays were made by Hangarter et al. (1980). A number of IAA-amino acids are useful for controlling growth and morphogenesis of plant tissue cultures (Peterson, 1978; Hangarter et al., 1980).

A great deal is known about the occurrence and concentrations of IAA esters in plants but much less about IAA amides. However, very little is known about the biological activities of the esters. Keglevic and Polorny (1969) found that 1-0-indoleacetyl-ß-D-glucopyrnose was more active in plant-section elongation tests than was IAA. IAA-myo-inositol was slightly less active than IAA in the Avena straight growth test (Nicholls, 1967) but more active than IAA in the stimulation of tomato callus growth (Hangarter et al., 1980).

Nearly all of the investigators who have studied the biological effects of IAA conjugates have suggested that their activities are due to the tissues' ability to hydrolyze them. This could explain the variation in the activity of IAA-aspartate with different species (Table 1). IAA-myo-inositol is hydrolyzed to free IAA by Zea (Nowacki and Bandurski, 1980). Hangarter et al. (1980) report good evidence for the activity of the conjugates being the result of prior hydrolysis.

Conjugates with other hormones. Conjugates of gibberellins,

cytokinins and abscisic acid have been found in a number of plant tissues. The literature concerning these conjugates was recently reviewed (Cohen, 1979). It has been suggested that gibberellins, cytokinin and abscisic acid conjugates may, like conjugates of IAA, serve as transport forms of the hormones or as stable reservoirs involved in the homeostatic regulation of hormone levels. In any case, the observation that the major plant hormones can occur as conjugates suggests that hormone conjugation may play an important part in the hormonal regulation of growth of the higher plants.

II. Roles of Indoleacetic Acid in Plant Development.

The growth and differentiation of plant cells is typically under hormonal control. Some growth responses result from the interaction of IAA effects with the effects of one or more of the other plant hormones. However, in keeping with the main topic of this thesis, the involvement of IAA in plant growth and development will be emphasized in this discussion. Evidence suggests that IAA is a controlling factor in cell elongation, tropisms, cambial cell division, vascular differentiation, fruit growth, apical dominance, flowering, root and shoot initiation and growth, and tumorigenesis. In other words, IAA is involved in nearly all phases of the growth, differentiation and development of plants.

Cell enlargement and tropisms. Probably the most extensively studied effect of applied IAA is the increased growth of isolated plant parts, such as coleoptile segments, pea stem segments and sunflower hypocotyl segments (cf. Evans, 1974). The increased growth of thses tissues is due almost

entirely to cell enlargement (Thimann, 1976). The effect of IAA on cell enlargement is the basis of the Avena curvature and straight growth bioassays which made possible much of the early studies on auxins (cf. Went and Thimann, 1937).

IAA-induced cell enlargment is important in the early stages of seedling growth when the shoot is pushing its way up through the soil to reach the light. The IAA is produced by the tip and transported downward causing the cells below the apex to expand. Cutting of the apex stops elongation. Replacing the apex with a solution of IAA causes the growth to resume (Went and Thimann, 1937).

The various tropic responses of plants are due to differential growth rates on opposite sides of the stimulated organ. Tropic behavior is generally thought to be regulated by the controlled transport of IAA (cf. Thimann, 1976). However, some investigators have presented evidence which questions the involvement of IAA transport in tropic responses (Wilkins and Wain, 1975; Bandurski, et al., 1977; Digby and Firn, 1979). The role of IAA in tropic responses has been extensively reviewed recently (Cohen, 1979).

Cambial activation. The role of auxin in cell division was first observed in the cambium of Helianthus hypocotyls (Snow, 1935). IAA-induced cambial cell division in woody plants appears to be a natural process (Avery, et al., 1937). The resumption of cambial activity which occurs in the spring is at first localized under regions of auxin synthesis, such as developing buds (Avery, et al., 1937). The wave of

activation moves downward, as auxin does, and at about the observed rate of auxin transport (Digby and Wareing, 1966a). Application of IAA to such systems stimulates division of the cambial cells in a manner similar to the seasonal induction (Digby and Wareing, 1966b).

Differentiation of xylem and phloem. IAA has been shown to be involved, in a quantitative manner, in the differentiation of xylem strands (Jacobs, 1952; Jacobs and Morrow, 1957). When a transverse cut severs the xylem in a herbaceous stem new xylem differentiates from cortical cells to remake the connection around the cut. The differentiation moves downward and is closely related to the presence of actively growing young leaves (sources of IAA and many other substances) above the severed region. In decapitated Coleus shoots, the number of xylem elements resulting is proportional to the amount of synthetic IAA applied. Similar effects of IAA on xylem differentiation have been observed in peanut internode tissue, in tomato stems (Thompson and Jacobs, 1966; Thompson, 1967) and in callus cultures of Syringa where IAA was applied to the upper surface (Whetmore and Rier, 1963). The induction of xylem differentiation appears to require that the IAA is transported basipetally (Thomposon, 1967).

The differentiation of phloem can also be controlled by IAA. In Coleus stems whose phloem has been severed, applications of IAA can induce the differentiation of new phloem tissues (La Motte and Jacobs, 1963). Whetmore and Rier (1963) found that the application of auxin to Syringa callus would

initiate phloem formation when high concentrations of sugar were applied at the same time. Phloem differentiation in tomato is also controlled by IAA (Thompson and Jacobs, 1966).

Fruit growth. The extensive growth of the ovary into a fruit is another IAA-stimulated event. Normally the growth of the ovary occurs, after pollination, through an increase in both cell number and cell size. Early work showed that extracts of pollen could bring about the swelling of the ovary of some plants (Gustafson, 1936, 1942). Many fruits can be grown to normal size with auxin application in the absence of pollination (cf. Crane, 1964).

Perhaps the most straight-forward example of IAA-stimulated fruit growth is the case of the strawberry (Nitsch, 1950). The growth of the strawberry receptacals was correlated with a rise in the auxin content of the achenes. The amount of growth of the receptacals is proportional to the number of fertilized achenes, and the number and position of the achenes will alter the shape of the strawberry. Replacing the achenes with lanolin paste containing auxin will cause the receptacals to grow close to normal size.

Flowering. Flower induction involves the differentiation of a vegetative meristem into the flowering state. There are only a few cases known where IAA may play a role in flower induction. The effect is probably indirect and may actually be due to auxin induced ethylene production (Thimann, 1972).

Another aspect of flower development that may be

controlled by IAA is the sex expression of the flower in many monecious plants. In Cannabis sativa, axils which would normally form male flowers will change to produce female flowers in response to IAA (Heslop-Harrison, 1956). IAA also favors female flowers in acorn squash (Nitsch et al., 1952). However, this response to IAA occurs in relatively few species.

Tumorigenesis. Many plant tumors, whether they have been induced by a pathogen (i.e., Agrobacterium tumefaciens), or have a genetic basis as with some hybrids (i.e., Nicotiana glauca X N. langsdorfii), or have arisen spontaneously from callus cultures (auxin-habituated) are similar in that they are capable of growing in the absence of exogenous auxin. The capacity of cultured tumors to grow without auxin has led to a number of studies comparing hormone levels in auxin-dependent normal callus and in auxin-independent tumorous callus.

Henderson and Bonner (1952) found more free auxin in crown-gall tissue than in normal callus of Helianthus annuus. Detectable levels of IAA were found in crown-gall tissues of Parthenocissus tricuspidata but not in normal P. tricuspidata tissue (Nitsch, 1955). Davies and McDaniel (1977) have reported observations from metabolic studies which they claim to be evidence that crown-gall tissues contain "excess" auxin. A study by Pengelly (1980), using a very sensitive and specific radioimmunoassay provides further evidence that production of IAA plays an important role in the growth of crown-gall tumors (ruling out the alternative,

that the tumorous tissue has somehow lost their requirement for auxin). Feung et al. (1976) claimed to have identified five IAA-amino acids in P. tricuspidata crown-gall tissues. They suggested that three of them might be unique to crown-gall tissue and therefore, their production might be involved in tumorigenesis. However, they did not report on the IAA conjugates present in normal P. tricuspidata tissue.

Tissue cultures derived from tumor-prone hybrids of Nicotiana glauca X N. langsdorfii, N. sauveolens X N. langsdorfii and N. debneyi-tabacum X N. longiflora do not require an auxin to maintain a high growth rate (Schaeffer and Smith, 1963; Ahuja and Hagen, 1966). On the other hand, tissue of the non-tumorous parental species or the non-tumorous mutant of N. glauca X N. langsdorfii do require auxin. Cheng (1972) showed that an "IAA synthetase" in tumor-prone hybrid plants could be activated by exposure to exongenous IAA but could not be activated in the non-tumorous N. glauca. Liu et al. (1978) found that tissue from the tumor-prone N. glauca X N. langsdorfii contained more bound IAA, after treatement with exogenous IAA, than did the non-tumorous parents or a non-tumorous mutant hybrid. This observation led them to hypothesize that the release of IAA from bound form plays an important role in tumorigenesis.

Auxin-habituated tissues arise occasionally from tissues which initially required auxin for growth. Such auxin-habituated tissues often behave in a similar manner to crown-gall and genetic tumor tissues and seem to produce more auxin than non-habituated tissue (Butcher, 1977). It appears from

these studies and several other lines of evidence (see Pengelly, 1980) that auxin production is involved in plant tumorigenesis.

Root and shoot formation. Root initiation appears to be controlled by a number of factors (Hess, 1969). Of these factors the presence of IAA is probably the most critical. In some early work, leaves and buds were shown to have an essential role in the formation of root primordia on plant cuttings. This was attributed to their capacity to synthesize auxin (Went and Thimann, 1937). Using a quantitative root formation assay, Thimann and Kowpfli (1935) found that IAA was a fully effective root initiating hormone. As in other auxin respones, the regulation of root growth and development seems to be a consequence of the basipetal transport of IAA (Scott, 1972). The application of auxins to induce root formation on cuttings has now become a common practice among horticulturists (Hartman and Kester, 1959).

The classic experiments by Skoog and Miller (1957) showed that root and shoot initiation from tobacco callus is regulated by the interactions between IAA and cytokinin. While both IAA and cytokinin are both required for tissue growth, their relative concentrations control the type of organ produced. A high auxin to cytokinin ratio favors root formation and represses shoot initiation. In contrast, shoot initiation is induced by a low auxin to cytokinin ratio and root initiation is repressed. Unfortunately, the principal of regulation of organogenesis by alterations of the auxin to cytokinin ratio cannot be generalized (Reinert et al.,

1977), but it does hold for many different plants (Murashige, 1974).

Although auxin by itself does not seem to regulate shoot initiation, the fact that it is required by regenerating cultures of many plants (Murashige, 1974) suggests that it can play an essential role in shoot initiation. This is probably because it is required for survival of the tissues. The observation that high concentrations of auxin suppresses shoot initiation (Skoog and Miller, 1957) indicates an inhibitory role. Experiments with the alleged auxin-antagonists, N-1-naphthylphthalamic acid (Feung and Linck, 1970) and 2,4,6-trichlorophenoxyacetic acid (Newcomb and Wetherell, 1970) show that they can promote shoot induction. The auxin-antagonists are thought to be acting by depressing the effective auxin concentrations. These results are consistent with an inhibitory role for auxin in shoot formation.

Apical dominance. The overall morphology of a plant is determined, at least partially, by the differential growth of the many meristematic regions of the shoot. The development of lateral buds in many plants is inhibited by a growing apical bud. Removal of the terminal bud results in the development of one or more of the lateral buds into branches. Stem-girdling treatments were shown to result in the growth of lateral buds situated below the girdle (Snow, 1925). This suggested that inhibition by the lateral buds is due to a downward moving substance originating in the apex. IAA, which is synthesized in growing buds, was found to be capable of substituting for the apical bud itself in maintaining

inhibition of the axillary buds in bean plants (Thimann and Skoog, 1933). Numerous studies have confirmed these observations and most investigators believe that the primary component of apical dominance is the synthesis of auxin in the shoot apex and its transport down the stem (Phillips, 1975).

Auxins in plant tissue culture. The practice of plant tissue culture is largely an empirical science, many practices being arrived at by trial and error. This empirical element has resulted in numerous advances, but very few systematic studies have been conducted on the factors controlling the nature of the growth of plant tissues. The control of growth by the auxin-cytokinin ratios (Skoog and Miller, 1957) is thought by some investigators to be a general phenomenon among plants (Murashige, 1974), but as already pointed out, this is probably an oversimplified view (Reinert et al., 1977). However, if any growth is to occur, and therefore if there is to be any potential to produce either roots or shoots, a minimum level of auxin is usually required (Linsmaier and Skoog, 1965).

The most commonly used auxins in plant tissue cultures are 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), indoleacetic acid (IAA) and indolebutyric acid (IBA) (Murashige, 1974). These differ very much in their stability and in their influences on tissue growth. The most widely used of these is probably 2,4-D. It is very potent, presumably because it is not readily metabolized. It is used for initiating and maintaining callus cultures.

Unfortunately, the persistance and potency of 2,4-D makes it very inhibitory to organogenesis (Murashige, 1974). On the other hand, IAA is rapidly metabolized by plant tissues, most notably by cultured plant tissues (Epstein and Lavee, 1975; Bender and Neumann, 1978). The rapid metabolism renders it relatively ineffective for supporting tissue growth. However, when it is effective, it is the least likely of the auxins to inhibit organogenesis. In fact, it often seems to be required for organogenesis (Linsmaier and Skoog, 1965). The other commonly used auxins, NAA and IBA, are intermediate between 2,4-D and IAA in the stability and effectiveness.

The natural auxin, IAA, is the preferred auxin for regeneration studies because it shows minimum inhibition of organogenesis (Murashige, 1974). IAA conjugates, which appear to release IAA slowly, have been shown to be useful for growing and controlling morphogenesis of tissue cultures (Hangarter et al., 1980). The conjugates offer a wide range of activities depending on the conjugating moiety, and to some extent on the plant species. They should greatly increase the usefulness of the natural auxin, IAA, in plant tissue culture if only by providing an extremely wide range of steady-state concentrations of IAA.

It is apparent from this brief suvey that the natural auxin, indoleacetic acid, is a crucial compound in plant growth and development. The involvement of the bound forms of IAA in plant growth is not clear, yet as much as 95% of the IAA in a plant is usually in a bound form (cf. Avery

et al., 1941; Bandurski and Schulze, 1977). The diversity of the bound forms which are found in plants (cf. Bandurski, 1979) suggests that the conjugation and subsequent release of IAA may have important functions in plant development. Unfortunately, very little is known about the biological effectiveness of the natural conjugates. The remainder of this thesis deals with research on the biological activities and mode of action of a number of auxin conjugates (some of which probably occur naturally), with an emphasis on their potential use in plant tissue culture.

MATERIALS AND METHODS

Synthesis of IAA-amino acids. Most of the amino acid and dipeptide conjugates were synthesized by the mixed anhydride methods of Weiland and Horlein (1955). The synthesis of IAA-L-alanine is described as an example: Ten mmol of IAA were dissolved with stirring in 30 ml of tetrahydrofuran plus 10 mmol of triethylamine in a vessel cooled in an ice bath. Over a period of 30 s, 10 mmol of ethylchloroformate were added dropwise with rapid stirring. After 5 min of stirring, 10 mmol of the sodium salt of the L-alanine in 8 ml of water were added. The mixture was allowed to warm to room temperature with continuous stirring. The reaction mixture was separated into two phases by the addition of water (50 ml) and chloroform (50 ml). The aqueous phase was adjusted to pH 4 with HCl and extracted 3 times with 50 ml of chloroform. The aqueous phase was then further acidified to pH 3 with more HCl and extracted three times with 50 ml of 1-butanol. The butanol phases were pooled and concentrated at reduced pressure until a viscous residue remained. This residue was dissolved in approximately 15 ml of 2-proponol-water (1:1,v/v) then purified by chromatography on a Sephadex LH-20 column (4 X 30 cm) using 2-propanol-water (1:1,v/v) as the eluting solvent. The fractions containing most of the product were pooled and concentrated at reduced pressure.

The product was then crystalized from ethyl acetate by the addition of hexane and recrystalized from the same solvent.

Isolation of the other IAA-amino acid conjugates differed somewhat depending on the amino acid used. The glycine, threonine, valine, leucine and the dipeptide conjugates precipitated directly from the aqueous phase on acidification. The phenylalanine conjugate remained in the organic phase and was then extracted into bicarbonate solution. It crystalized on acidification of the bicarbonate solution. These conjugates were recrystalized from ethanol and water. The methionine and proline conjugates were prepared and isolated in the same way as the L-alanine conjugate. IAA-glucosamine was prepared and isolated as was IAA-L-alanine and identified as a single spot on TLC, yielding indole and reducing color tests on chromatograms. IAA-serine, IAA-taurine and IAA-tricine were also prepared in the same way as the alanine conjugate except the purified compounds were stored in 2-propanol-water (1:1,v/v).

IAA-L-aspartate and IAA-L-glutamate were synthesized by the method of Mollan et al. (1972). First, the p-nitrophenyl ester of IAA was prepared by dicyclohexylcarbodiimide coupling. This "active ester" was then reacted with the amino acid in the presence of tetramethylguanidine in aqueous methanol. The products were purified as was IAA-L-alanine. IAA-L-glutamate was crystalized from water and IAA-L-aspartate was stored in 2-propanol-water (1:1,v/v). The propanol was distilled at reduced pressure prior to the use of IAA-L-aspartate in biological experiments.

The yields of purified conjugates from both methods were usually betwwen 40 and 50%. Thin layer chromatography in chloroform: methanol: acetic acid (75:20:5) using the Van Urk-Salkowski reagent (Ehmann, 1977) for detection of indoles showed that the products were not contaminated with free IAA or other indole compounds in detectable amounts (less than 0.05%).

It should be pointed out that IAA-alanine and IAA-phenylalanine were the only two conjugates whose structures were verified (M. A. Venis, personal communication). Since the methods of synthesis are well established and the two conjugates that were checked had the proper structures there is little chance of the other conjugates being something else.

Radioactive IAA-L-alanine and IAA-glycine were synthesized by the above described mixed anhydride procedure (Weiland and Horlein, 1955) with the following modifications. [2-^{14}C] IAA, specific activity 49 mC/mmol (New England Nuclear) or [1-^{14}C]IAA, specific activity 57.6 mC/mmol (Amersham) was diluted to a specific activity of 10 mC/mmol by the addition of unlabelled IAA and stored in acetonitrile. One umol of the [^{14}C]IAA was added to a 250 µl reaction vessel and the acetonitrile distilled at reduced pressure until about 50 µl remained. To this was added 1 µmol of triethlamine in 10 µl of acetonitrile in an ice bath. One µmol of ethylchloroformate in 10 µl of acetonitrile was added with stirring. After 5 min of stirring 1 µmol of the sodium salt of the amino acid in 30 µl of water was added. The mixture was allowed to warm to room temperature with continuous stirring.

The acetonitrile was removed at reduced pressure and 150 µl of 2-propanol-water (1:1,v/v) was added. This was acidified with phosphoric acid and the [^{14}C]IAA-amino acid conjugates were purified by chromatography on a Sephadex LH-20 column (1 X 30 cm) with elution by 2-propanol-water (1:1,v/v). The purified [^{14}C]IAA-amino acids were stored on 80% ethanol. Yields were again around 50%.

All of the IAA conjugates were stored in dark bottles in a freezer.

Tomato tissue culture. Tomato seeds (Lycopersicon esculentum Mill cv Marglobe) were sterilized by soaking them for 30 sec in 95% ethanol followed by 30 min in 0.5% sodium hypochlorite solution containing 0.01% sodium larylsulfate. The seeds were then rinsed several times in distilled water and germinated in the dark on a solid agar medium in Petri dishes. Seven days after planting, the hypocotyls were excised asceptically, cutting them 1 cm below the cotyledons and 1 cm above the radical. The hypocotyls were then cut into 5 mm segments. The segments, randomly selected with respect to treatment, were placed on agar incubation media. Nine cm (five segments) or 4.5 cm (three or four segments) disposable Petri dishes were used. All experiments contained four to six replicates and were done several times. Typical results are shown in the photographs.

Tobacco shoot induction. Tobacco callus, Nicotiana tabacum L. cv. Wisconsin 38 was used. This callus had been established from pith explants and maintained in culture for over two years. Two pieces of callus (approximately 300 mg

each) were placed onto the appropriate experimental medium in 4.5 cm disposable Petri dishes three weeks after their last transfer onto the maintenance medium. Again, all experiments contained four to six replicates and were done several times. Typical results are shown in the photographs.

Growth media. Slightly modified Murashige and Skoog culture media (MS) were used for all tissue culture experiments (Murashige and Skoog, 1962). For tomato germination the MS medium contained sucrose (29 mM), thiamine (3 uM), pyridoxine (4.9 uM) and nicotinic acid (8.1 uM). For tomato hypocotyl experiments the medium contained sucrose (88 mM), thiamine (3 uM), pyridoxine (4.9 uM), nicotinic acid (8.1 uM), benzyladenine (8.9 uM) and the particular auxin being tested. Tobacco shoot induction experiments were carried out on MS medium containing sucrose (88 mM), thiamine (3 uM), 6-δ-δ-dimethylallylaminopurine (49 uM) and the indicated auxin source. All media were solidified with 0.9% (w/v) Difco Bacto Agar. Prior to autoclaving all constituients, including hormones, were added and the pH was adjusted to 6.0 with KOH.

Conditions for tissue culture experiments. Growth experiments were conducted at 22$\pm$ 1 C on a 16 h light 8 h dark cycle under GE Delux cool-white fluorescent light at a distance to give 40-54 microeinsteins/m^2/s measured with a LiCor Radiometer LI-185A.

Auxin-induced ethylene production. Pea seeds (Pisum sativum L. cv. Alaska) were soaked overnight in running tap

water. They were sown in Vermiculite and grown in the dark at 25 C for six to seven days. Tomato seeds were sown directly in Vermiculite and grown in the dark for six to seven days. Stem sections from just below the plumlar hook were cut under a green safelight and incubated for 16 h on filter paper soaked with distilled water in 9 cm Petri dishes. The segments were transferred to 25 ml erlenmeyer flasks containing 3.5 cm filter paper disc and 1 ml of 5 mM potassium phosphate buffer or the buffer containing the indicated auxin (pH 6.5). Each flask was flushed with ethylene-free air, closed with a serum-vial cap and incubated in darkness at 25 C. Ethylene was measured by gas chromatography as described by Kende and Hanson (1976).

Auxin transport. Auxin transport was measured in pea stems by the agar block technique. Blocks of 1.5% agar (1 cm X 1 cm X 2 mm) were prepared with the appropriate [^{14}C]-auxin (10 mC/mmol) at 5 μM or without auxin (donor and receiver respectively). Etiolated peas were grown as for the ethylene production experiments. Stem sections (3.5 mm) were cut with spaced razor blades 1 cm below the apical hook and placed in the indicated orientation (12 segments/block). Donor blocks were positioned carefully so that the free stem ends came into uniform contact with the donor block. The transport was allowed to proceed in a humid chamber in darkness. After the appropriate time, the receiver blocks were placed into scintillation vials containing Tritosol scintillation fluid (Fricke, 1975) and allowed to extract overnight. Radioactivity was determined on a Packard TriCarb scintillation

counter with a counting efficiency of 83%.

"Fingerprints" of auxin metabolites. Pea seeds (Pisum sativum L. cv. Alaska) were sterilized by soaking them for 15 min in 0.5% sodium hypochlorite solution containing 0.01% sodium laurylsulfate. The seeds were then rinsed several times in distilled water and germinated in the dark on water-agar (0.6%). Six to seven days after planting the epicotyls were excised aseptically, cutting them just below the plumular hook and 2 cm above the seed. The epicotyls were then cut into 1 cm segments. Approximately 10 g of segments were transferred into 125 ml flasks containing 25 ml of autoclaved nutrient medium (MS without phytohormones) and 1.0 µC of [1-^{14}C]IAA (57.6 mC/mmol) or [1-^{14}C]IAA-amino acid (10 mC/mmol). The tissue was incubated in the dark at room temperature, with shaking for 72 h.

Crown-gall tissue of Parthinocissus tricuspidata, obtained from Dr. R. H. Hamilton, Penn. State Univ., was maintained on MS medium without phytohormones. Crown-gall, habituated and normal callus cultures of Helianthus annuus cv. Mammoth Russian were obtained from Dr. J. D. Kemp, Univ. of Wisconsin. The crown-gall and habituated tissues were maintained on MS medium without phytohormones. The normal callus of H. annuus was maintained on MS basal medium supplemented with 50 µM NAA and 50 µM kinetin. Approximately 10 g of three-week old tissue were treated with labelled auxin as were the pea stem segments.

The treated tissues were placed in a beaker with 95% ethanol (2 ml/g for pea segments; 4 ml/g for callus).

Appropriate unlabelled IAA and IAA conjugates were added at this time (100 µg of each) in order to provide carrier and chemically detectable internal chromatographic standards. The tissues were boiled for 3 min in the ethanol then homogenized with a mortar and pestle. The homogenate was filtered with suction on a Buchner funnel and the residue rinsed several times with 80% ethanol. The combined ethanol fractions were concentrated to a viscous residue at reduced pressue and then resuspended in 2-propanol-water (1:1,v/v). This solution was passed over a DEAE-Sephadex-acetate column (1 X 10 cm). The column was then eluted with a linear gradient of 0 to 3% H_3PO_4 in 2-propanol-water (1:1,v/v). The fraction containing the acidic indoles (eluting between 20 and 25 ml) was collected and the propanol evaporated at reduced pressure. The remaining aqueous fraction was immediately extracted 3 times with 25 ml diethyl ether. The ether fractions were pooled and washed once with 2 ml water. The ether fraction was evaporated to dryness at reduced pressure and resuspended in 0.5 ml 95% ethanol. This alcohol solution, containing the acidic, ether-soluble metabolites was used for TLC.

Thin layer chromatography was carried out on Silica Gel G plates. The solvent system most frequently used for separation of the acidic indoles was chloroform:ethyl acetate: formic acid (35:55:10, v/v/v). Occasionally, chloroform: methanol:acetic acid (75:20:5, v/v/v) and methyl ethyl ketone: ethyl acetate:ethanol:water (3:5:1:1, v/v/v/v) were used (as indicated in the results).

Autoradiography of TLC plates was performed with Kodak X-ray film (NS-5T). Film was developed for 5 min in Kodak X-ray developer and fixed for 5 min in Kodak Rapid Fixer.

Unlabelled standards were made visible on the chromatograms after autoradiography with the Van Urk-Salkowski reagent (Ehmann, 1977) or by quenching of UV-induced fluorescence.

Partial purification of Pathinocissus tricuspidata metabolite. The P. tricuspidata derived sample from the DEAE step (in 95% ethanol) was streaked in a 10 cm band at the origin of a 20 X 20 cm Silica Gel G plate. Guide spots of the extract were made on both sides of the streak. After chromatographic development (methyl ethyl ketone: ethyl acetate: ethanol:water, 3:5:1:1, v/v/v/v), the central portion of the plate was covered with a piece of cardboard and the guide spots made visible by illumination with short-wave UV. The silica gel from the area containing the major metabolite was scraped from the plate and eluted with 3 times 5 ml of ethanol-water (1:1, v/v). The ethanol extracts were pooled and concentrated to dryness at reduced pressure and redissolved in 0.5 ml of 2-propanol-water (1:1, v/v). This sample was then applied to a 1 X 30 cm column of Sephadex LH-20 and eluted with 2-propanol-water (1:1, v/v). The fraction containing the radioactivity was evaporated to dryness at reduced pressure and redissolved in 1 ml of 2-propanol-water (1:1, v/v). The UV absorption spectrum of this sample was obtained with a Cary 15 spectrophotometer measured against a 2-propanol-water (1:1, v/v) blank.

Decarboxylation of [1-^{14}C]IAA-amino acids. Peas were grown aseptically as described above. Stem sections (1 cm) were cut from just below the plumular hook, as for the ethylene assay. Twenty segments were transferred to the outer well of a sterilized Warburg flask containing filter sterilized potassium phosphate buffer (5 mM, pH 6.5) and the indicated radioactive auxin. A 1.5 cm^2 piece of filter paper saturated with 5 N KOH was placed in the center-well and the flask closed with a serum vial cap. The flask was then held in the dark at room temperature. At the indicated times, the filter paper was removed and excess liquid removed from the center-well with a dry piece of filter paper. Both pieces of filter paper were placed into a scintillation vial. A new, KOH saturated piece of filter paper was placed into the center-well and the flask closed again. This process was repeated until the experiment ended. All of the manipulations were carried out in such a manner that the interior of the flasks remained aseptic. Radioactivity was determined as for the transport experiments. Counting efficiency was 74%. No significant decarboxylation was found in the absence of tissue.

Enzyme assays. The amidase activity of Papain was verified by following the hydrolysis of benzoyl-D,L-arginine-p-nitroanalide hydrochloride at pH 7.5 according to the method of Arnon (1970). Cysteine and EDTA were added for enzyme activation. Protease and α-chymotrypsin were checked for activity as was Papain, except that cysteine and EDTA were not added. The activity of Acylase I was checked by following

the hydrolysis of N-acetyl-L-glutamic acid at pH 7.0 using ninhydrin for detection of the free amino acid. The hydrolysis of N-carbobenzoxy-L-phenylalanine at pH 6.8 was followed to verify the activity of Carboxypeptidase Y according to Hayashi (1976), again using ninhydrin for detection of the free amino acid. All of these enzymes were obtained from Sigma Chemical Company.

IAA-L-alanine, IAA-L-valine, IAA-L-phenylalanine and IAA-glycine were tested as substrates with each enzyme under conditions where the enzymes were found to be active on the above mentioned substrates. The presence of free amino acids in the reaction mixtures was determined with ninhydrin. The resulting IAA compounds were followed by TLC of the reaction mixture on Silica Gel G plates in chloroform:ethyl acetate:formic acid (35:55:10) and detected with the Van Urk-Salkowski reagent (Ehmann, 1977).

RESULTS

I. Growth responses of tomato hypocotyl explants (Figures 1-9; Table 2).

In the absence of any exogenous auxin, the excised hypocotyl sections usually failed to grow and often died, although the medium contained an abundant carbon source, vitamins and cytokinin. The addition of free IAA caused the growth of callus, roots and shoots, but concentrations of IAA high enough to favor good callus growth always strongly inhibited shoot formation (Fig. 1). Similar concentrations of 2,4-D allowed some growth of callus but totally inhibited organogenesis. It should be noted, however, that all of the concentrations of 2,4-D employed in the experiment depicted in Figure 1 were much to high for good growth of tomato tissues. The concentrations of NAA were also somewhat excessive and totally inhibited shoot formation, but NAA concentrations low enough to permit good callus growth also permitted considerable root development.

The responses to the IAA conjugates were quite different. None was inhibitory to growth at any of the concentrations used in the studies reported here. To the extent that they permitted growth, they encouraged callus or shoots and tended to inhibit roots (the results described below are summarized in Table 2). The responses to the different conjugates

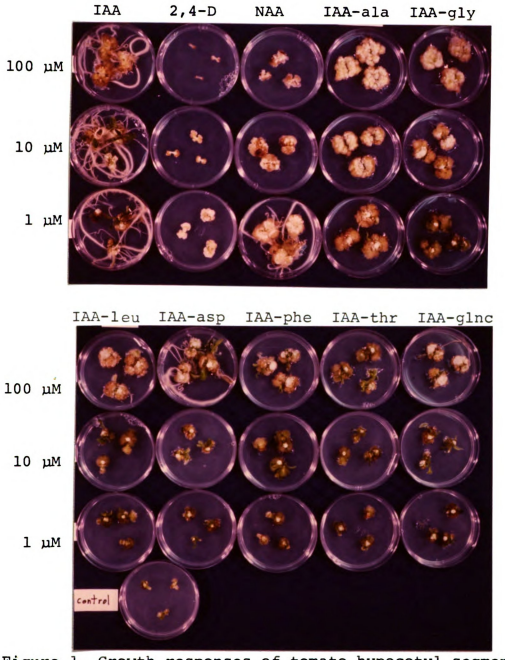

Figure 1. Growth responses of tomato hypocotyl segments treated with various IAA-amino acid conjugates, A. Seven-day-old hypocotyl segments were placed on nutrient agar containing 8.9 µM benzyladenine and the indicated auxin. The photograph was taken after 30 days. L-isomers of the amino acids were used: glnc, glucosamine.

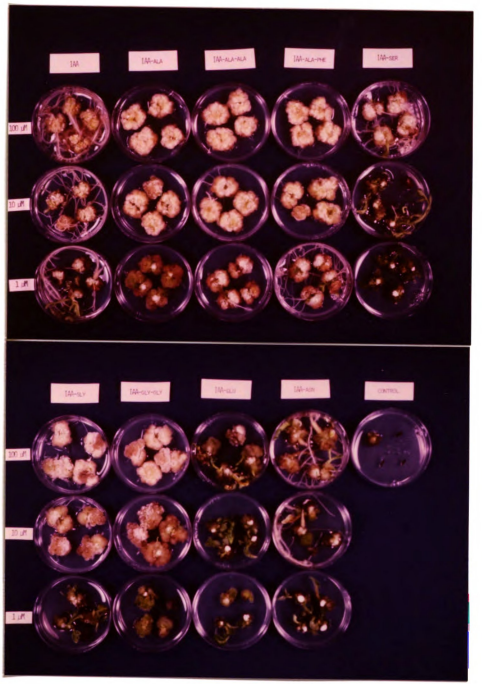

Figure 2. Growth responses of tomato hypocotyl segments treated with various IAA-amino acid conjugates, B. Conditions as in Figure 1.

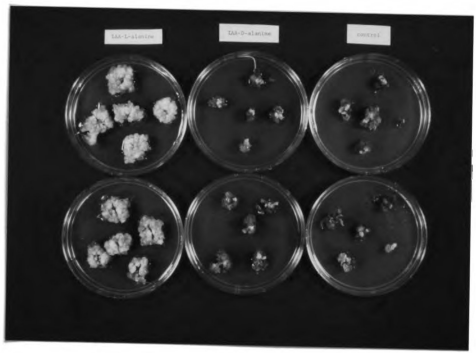

Figure 3. Influence of D- and L- stereoisomers of IAA-alanine on the growth of tomato hypocotyl segments. Conditions as in Figure 1 except that the auxin concentration was 10 μM.

varied greatly depending on the conjugating moiety;

a) IAA-L-alanine supported vigorous callus growth, even at low concentrations (Figure 1). At very low concentrations (1 μM) the L-alanine conjugate sometimes permitted a few shoots to develop but root growth was almost never observed. IAA-D-alanine was almost but not completly inactive; with the conjugate containing the unnatural amino acid there was slightly more growth at high concentrations (100 μM) than in controls without auxin (data not shown), but at lower concentrations the growth was no different from the controls (Figure 3). The dipeptide conjugates, IAA-L-alanyl-L-alanine and IAA-L-alanyl-L-phenylalanine elicited growth responses which were quite similar to those elicited by the single amino acid conjugate, IAA-L-alanine (Figure 2). However, at the lower concentrations, some root growth occurred.

b) IAA-glycine was only slightly less active in supporting callus growth than was the L-alanine conjugate and it was not quite as effective in inhibiting organogenesis (Figure 1). At lower concentrations (1 μM) the callus tended to become green, shoots were regularly formed and, in a few instances, there were some roots. The dipeptide conjugate IAA-glycylglycine had the same activity as the single amino acid conjugate, IAA-glycine (Figure 2).

c) IAA-L-valine and IAA-L-methionine, although quite effective in supporting callus growth at concentrations above 50 μM, were appreciably less effective at lower concentrations (data not shown in these figures but see Table 2). They

permitted shoot growth and occasionally root growth at concentrations which were sub-optimal for callus formation (50 µM).

d) IAA-L-leucine supported good callus growth at 100 µM but even at this high concentration some shoot development occurred (Fig. 1). At lower concentrations (10 µM) callus growth was poor and shoots frequently developed. Root growth was rarely observed.

e. IAA-L-phenylalanine, IAA-L-threonine (Fig. 1) and IAA-L-glutamic acid (Fig. 2), while not eliciting identical responses, were very similar. All three supported the growth of callus and shoots at 100 µM. At lower concnetrations, they favored shoot growth. Again, root growth was very rare.

f) IAA-L-proline supported very little growth of any kind (data not shown here but see Table 2). In this respect, it was very similar to the IAA-D-alanine conjugate (Fig. 3).

g) IAA-L-aspartic acid (which is formed with exogenous IAA in most higher plants) was not very effective in supporting callus growth even at 100 µM, and did not appreciably inhibit organogenesis (Fig. 1). At 100 µM, some callus formed and shoots and roots developed. IAA-L-asparagine elicited responses which were quite similar to the responses to IAA-L-aspartate (Fig. 2). IAA-L-serine supported fairly good callus growth, root growth and some shoot growth at 100 µM (Fig. 2). At lower concentrations callus growth was poor and shoots grew readily.

IAA-L-aspartate, IAA-L-asparagine and IAA-L-serine stand out from the other IAA-amino acid conjugates. These

three stimulate root growth at high concentrations whereas the other amino acid conjugates fail to elicit root growth and, in some cases at least, seem to inhibit roots at all of the concentrations tested.

The effects of the IAA amino acid conjugates tested are summarized in Table 2.

IAA-amino acid conjugates offer a great diversity of activities in tissue culture. However, it should be emphasized that this is also true for conjugates other than those with amino acids. Experiments with several such conjugates showed that the growth responses described so far are not unique to the amino acid conjugates. IAA-glucosamine (Fig. 1) and IAA-taurine (Fig. 4) had effects which were similar to the effects of IAA-L-phenylalanine, IAA-L-threonine and IAA-L-glutamate. That is, they supported some callus growth and shoot formation at 100 μM. At lower concentrations they favored shoot growth. Root growth was not observed. IAA-myo-inositol, which occurs naturally in considerable amounts in maize seeds, gave results similar to those obtained with IAA-L-alanine, both in supporting rapid callus growth and in suppressing organogenesis. However, the inositol conjugate became inhibitory at 57 μM (data not shown). IAA-tricine was the only conjugate tested which showed activity similar to that obtained with free IAA (Fig. 4).

Of course, it is possible to increase the range of auxin conjugates even further by altering the auxin moiety. Figure 5 compares the effects of the alanine and the phenylalanine conjugates of IAA and IBA on the growth of tomato hypocotyl

Table 2. Summary of the growth responses of tomato hypocotyl segments to various IAA-amino acid conjugates.
This table summarizes in a very general manner the effects of the different IAA-amino acid conjugates on the growth and morphogenesis of tomato hypocotyl segments. The segments were treated on nutrient agar containing 8.9 μM benzyladenine and the indicated auxin.

	Growth Response		
Amino Acid Moiety	Callus	Shoots	Roots
L-alanine	++	--	--
D-alanine	0	0	0
L-alanyl-L-alanine	++		
L-alanyl-L-phenylalanine	++		
glycine	++	--	--
glycylglycine	++		
L-valine	+		
L-methionine	+	+	
L-leucine	+	+	
L-threonine	+	+	
L-glutamate	+	+	
L-proline	0	0	0
L-phenylalanine	+	+	-
L-phenylalanyl-L-alanine	+	+	
L-aspartate	+	+	+
L-asparagine	+	+	+
L-serine	+	+	+

++, strongly favors
+, favors
0, no effect
-, inhibits
--, strongly inhibits

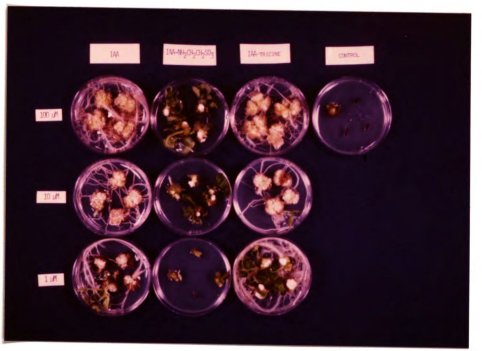

Figure 4. Growth responses of tomato hypocotyl segments treated with IAA-taurine and IAA-tricine. Conditions as in Figure 1.

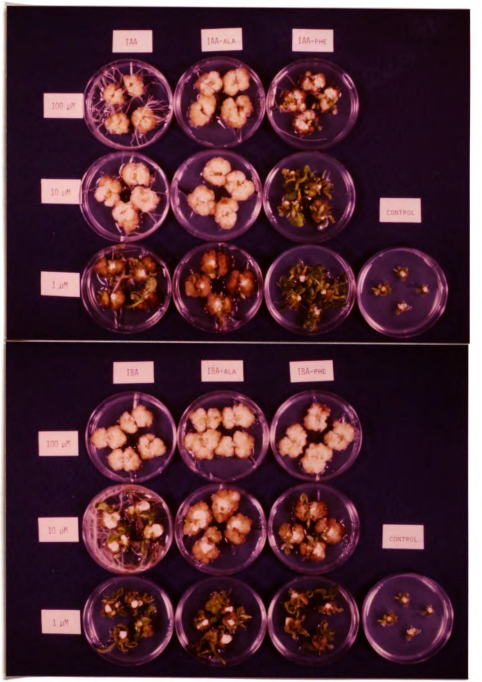

Figure 5. Comparison of IAA conjugates and IBA conjugates in the tomato hypocotyl system.
Conditions as in Figure 1.

sections. Again, with either auxin, the conjugated auxin elicits responses which are different from the responses elicited by the free auxins. The IBA conjugates tend to favor callus growth while not supporting root growth much as do the corresponding IAA conjugates. It is interesting that the very striking difference between IAA-L-alanine and IAA-L-phenylalanine (i.e., callus growth vs. shoot growth) is much less apparent between IBA-L-alanine and IBA-L-phenylalanine. See also the ethylene production data below (Table 3).

Several experiments were conducted in which the conjugating moiety in its free form (i.e., L-alanine, etc.) was added to medium along with an equal amount of free IAA. In all cases tested, such treatments were no different from treatments with free IAA itself. Figure 6 shows the results of such an experiment with L-alanine. The unique properties of the IAA conjugates must therefore be entirely due to the covalent bonding of the complex and in no way represent a contribution by amino acids or other corresponding compounds derived from the complex. The great activity of IAA-L-alanine when compared to the inactivity of IAA-D-alanine (Fig. 3) also points out the importance of the covalent bond. It should be noted that conjugates of IAA with other D-amino acids (valine, methionine, leucine and phenylalanine) were also found to have very little activity.

Exogenous IAA is notoriously labile in the presence of plant tissues, sometimes disappearing completely from the medium within a few days or even sometimes within a few

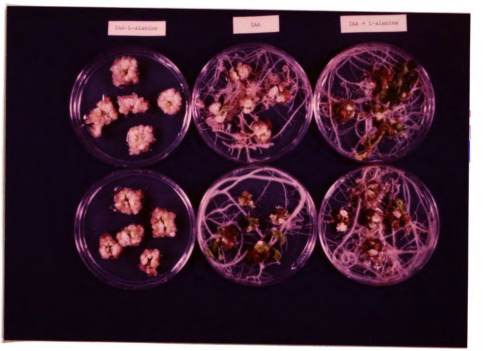

Figure 6. Role of the covalent bond in determining the activity of the conjugates.
Conditions as in Figure 1 except that the concentration of the auxins and the free amino acid was 10 μM.

hours (Epstein and Lavee, 1975; Bender and Neumann, 1978). The slow release hypothesis predicts that frequent renewals of free IAA should cause free IAA to mimic the conjugates. In an attempt to maintain a constant supply of free IAA, the tomato hypocotyl explants were transferred every four days to fresh medium containing a new supply of free IAA. This treatment resulted in vigorous callus growth and in inhibition of organogenesis, a response quite similar to the response observed with IAA-L-alanine (Fig. 7). However, it must be realized that a renewed supply of nutrients and cytokinin was also provided by the frequent transfers and that metabolic products were left behind with each transfer. Furthermore, it is not easy to explain all of the effects of IAA-L-alanine in terms of a persistent release of free IAA. If the IAA-L-alanine supports callus growth for no reason other than that it provides a continuous supply of free IAA, then the addition of free IAA to a medium containing callus-forming amounts of IAA-L-alanine should be without effect or should increase callus growth. However, the combination of IAA and IAA-L-alanine regularly produced roots in addition to vigorous callus growth (Fig. 8). Organogenesis as measured by the number of roots initiated depended on the concentration of the free IAA applied in the presence of callus-forming levels of IAA-L-alanine (Fig. 9). The callus growth, on the other hand, seemed to depend only on the IAA-L-alanine.

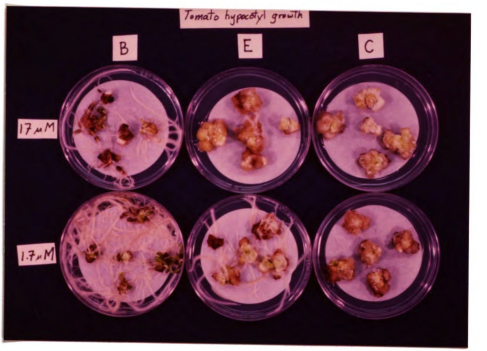

Figure 7. IAA-L-alanine as a continuous source of IAA. A comparison of a "constant" supply of free IAA to a "slow-release" source of IAA in the growth and differentiation of tomato hypocotyl segments. The hypocotyl segments were placed on nutrient agar containing 8.9 μM benzyladenine and given the following treatments: B) tissues were grown throughout on the same plate containing free IAA; E) tissues were transferred to new plates containing fresh medium with a new supply of free IAA every four days; C) tissues were grown throughout on the same plate containing IAA-L-alanine. The photograph was taken after 30 days.

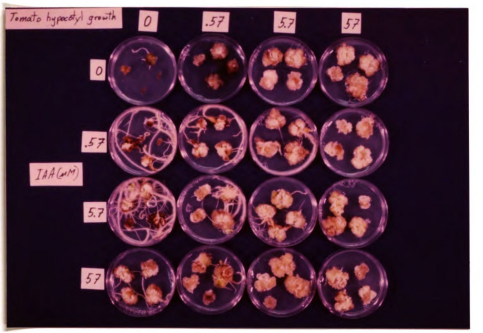

Figure 8. Interaction of free IAA and IAA-L-alanine on the growth and differentiation of tomato hypocotyl segments. Conditions as in Figure 1 except that the photograph was taken after 32 days. Note that the addition of free IAA in the presence of IAA-L-alanine gave the root initiation effect of free IAA without affecting the callus formation characteristics of the alanine conjugate. See Figure 9 for a quantitative description of callus growth and root initiation in the same experiment.

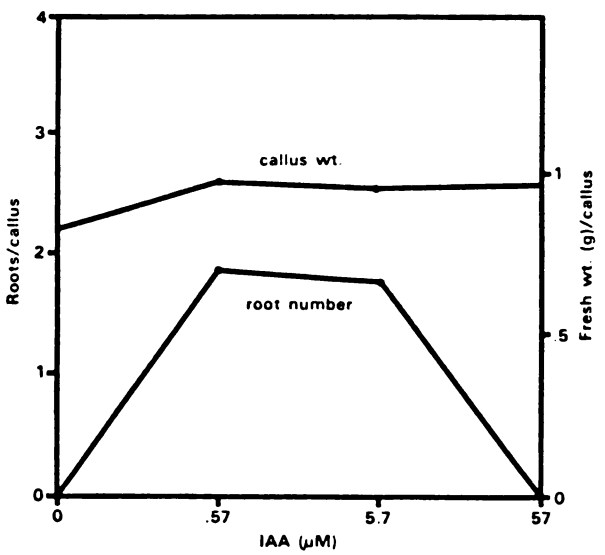

Figure 9. Influence of free IAA on root initiation in the presence of IAA-L-alanine.
Conditions as in Figure 8. After 32 days the number of roots initiated from the callus tissue was determined and the fresh weight of the callus was measured. Note again that the root initiation was a function of the concentration of exogenous free IAA while callus growth depended only on the IAA-L-alanine.

II. Shoot inductions and callus growth in tobacco cultures (Figures 10-11).

The undifferentiated tobacco callus employed in these experiments produced abundant shoots when grown on the high cytoknin medium, whether or not free IAA was added. However, the callus grew very little if at all and became brown with or without IAA. Again, the effects of the IAA-amino acid conjugates were quite different and depended very much on the amino acid moiety:

a) IAA-L-alanine supported vigorous callus growth which tended to be white and compact (Fig. 10). This was true even when the conjugate was only 0.17 µM (data not shown). At the same time, shoot induction was completely inhibited at concentrations of the conjugate down to 1.7 µM and was still greatly reduced at 0.17 µM. Very surprisingly, 1.7 µM IAA-L-alanine, which inhibited shoot formation in the absence of exogenous free IAA, permitted shoot formation in the presence of free IAA (Fig. 11). A similar type of interaction of the two auxin forms with regard to root growth has already been described in the tomato hypocotyl system (Fig. 8-9).

b) IAA-glycine also supported good callus growth but without inhibiting shoot formation quite as effectively as did the L-alanine conjugate (Fig. 10).

c) IAA-L-valine was appreciably less effective in supporting callus growth and less effective in inhibiting shoot formation than were the alanine and glycine conjugates (Fig. 10).

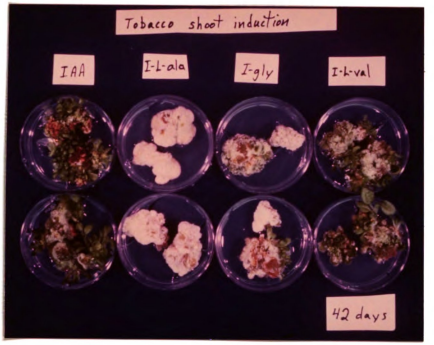

Figure 10. Inhibition of tobacco shoot induction by IAA-amino acid conjugates.
Friable tobacco callus was placed on nutrient agar containing 49 μM 6-δ,δ-dimethylallylaminopurine and the indicated auxin at 17 μM. The photograph was taken after 42 days. Duplicate plates are shown.

IAA-L-ala (µM)

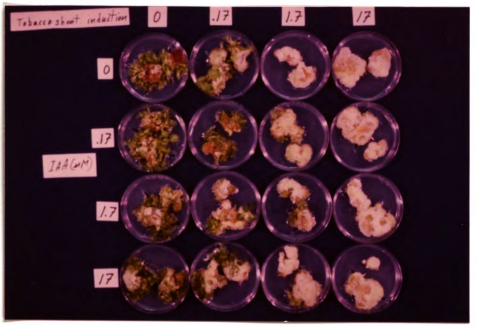

Figure 11. Interaction of free IAA and IAA-L-alanine on tobacco shoot induction.
Conditions as in Figure 10. Note that the addition of free IAA in the presence of 1.7 µM IAA-L-alanine (which by itself inhibits shoots) stimulates shoot without much affecting the callus formation characteristics of the alanine conjugate. A similar interaction, with regards to root growth, was observed in the tomato system (see Figure 8).

d) The other IAA-amino acid conjugates tested (L-methionine, L-leucine, L-phenylalanine and L-proline) were almost without effect in regard to shoot inhibition, although some of them supported a little callus growth or at least prevented the browning of the callus (data not shown.)

III. Auxin-induced ethylene production (Figure 12; Tables 3-5)

Ethylene production by plant tissues is stimulated by the addition of auxins and, at least in some cases, the amount of ethylene produced is correlated with the amount of free auxins in the tissue (Lau and Yang, 1973). Since the various IAA conjugates tested in this study are acting as auxins themselves or as sources of auxin, their abilities to induce ethylene formation in pea stem sections were investigated.

Free IAA and 2,4-D induced approximately the same rate of ethylene production in pea stems when they were both 0.1 mM (Fig. 12). However, in less than 9 h the production of ethylene in the IAA-treated stems virtually ceased unless new IAA was added. In contrast, ethylene formation by the 2,4-D-treated stems continued unabated for at least 15 h. None of the IAA-amino acid conjugates was as effective as either IAA or 2,4-D in inducing ethylene formation, although IAA-L-alanine at 1.0 mM was almost as effective as free IAA at 0.1 mM. However, the ethylene production with IAA-L-alanine did not fall off as with free IAA after 9 h, but rather continued as with 2,4-D. This persistance of ethylene production with IAA conjugates is consistent with their proposed slow-release of IAA mechanism of action.

Again, in this assay, the auxin activity of the conjugates

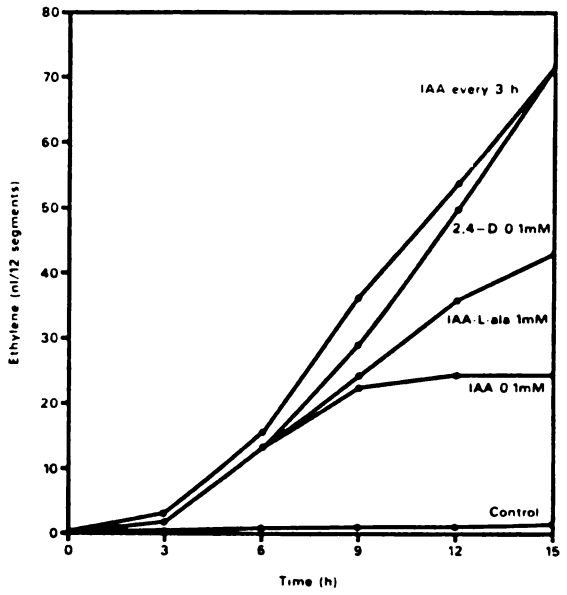

Figure 12. Time courses of the production of ethylene by pea stem segments treated with various auxins. Twelve segments were incubated in 1 ml of the auxin solutions at pH 6.5. The treatment labeled "IAA every 3 h" received additions of 17.5 µg of IAA in 20 µl aliquots every 3 h. At the time of the last addition (12 h) the final concentration of IAA would have been 0.45 mM if no IAA had been destroyed by the tissue.

Table 3. Ethylene production from pea and tomato tissues treated with IAA-amino acid conjugates.
Twelve pea epicotyl segments (1 cm) or thirty tomato hypocotyl segments (1 cm) were incubated in 1 ml of 5 mM potassium phosphate buffer and 0.1 mM auxin at pH 6.5. Ethylene was measured after a nine hour incubation. The IAA treated segments produced 15.8 nl of ethylene (pea) and 13.2 nl of ethylene (tomato).

Auxin Source	Ethylene Production	
	Pea	Tomato
	% of IAA treatment	
IAA	100	100
IAA-L-alanine	77	66
IAA-L-methionine	38	
IAA-L-threonine	32	
IAA-L-leucine	30	
IAA-L-valine	23	21
IAA-L-aspartate	14	
IAA-L-phenylalanine	9	6
IAA-glycine	8	27
IAA-L-proline	7	
IAA-D-alanine	7	
no auxin	7	6

Table 4. Production of ethylene from pea stems treated with IAA, IAA-amino acids and IAA-dipeptides.
Conditions as in table 3. The IAA treated segments produced 14.3 nl of ethylene.

Auxin Source	Ethylene Production
	% of IAA treatment
IAA	100
IAA-L-alanine	70
IAA-L-alanyl-L-alanine	67
IAA-L-alanyl-L-phenylalanine	63
IAA-L-phenylalanine	9
IAA-L-phenylalanyl-L-alanine	6
no auxin	6

Table 5. Production of ethylene from pea stems treated with IBA and IBA conjugates.
Conditions as in table 3. The IBA-treated segments produced 8.8nl of ethylene.

Auxin Source	Ethylene Production
	% of IBA treatment
IBA	100
IBA-L-alanine	78
IBA-L-phenylalanine	56
no auxin	6

depended very much on the amino acid moiety (Table 3). As in the tissue culture experiments, the most active conjugate was the IAA-L-alanine. Not surprisingly, the D-alanine caused no measurable ethylene production. There was a good correlation between the ability of the conjugates to support callus growth in the tomato system and the ability of the conjugates to cause ethylene production in the pea stems. The only discrepancy involved IAA-glycine which failed to stimulate ethylene in the pea stems but which stimulated callus growth in the tomato hypocotyls. This apparent discrepancy can be explained by the difference between pea and tomato, since in experiments with tomato stem sections instead of pea stem sections, the glycine conjugate was one of the more active in stimulating ethylene production (Table 3.)

The ability of dipeptide conjugates to induce ethylene production was compared to the abilities of single amino acid conjugates (Table 4). The activities of the conjugates are largely dependent on the amino acid which is covalently linked to the IAA. The second amino acid has very little influence on the activity. As already noted, similar observations were made in the tomato tissue culture system (Fig. 2).

Conjugates with IBA also induce ethylene production in the pea stems (Table 5). As with the IAA conjugates the activities of the IBA conjugates are different from the free auxin and from each other depending on the amino acid moiety. However, the difference between the activities of IBA-L-

alanine and IBA-L-phenylalanine is much less than the difference between IAA-L-alanine and IAA-L-phenylalanine. Again, similar results were obtained in the tomato hypocotyl system (Fig. 2).

IV. Transport of IAA and IAA-amino acid conjugates (Figure 13).

Free IAA is transported by a very specific directed transport system (Goldsmith, 1977) while there is some evidence that IAA-L-aspartic acid is an immobilized form of auxin (Morris et al., 1969). To determine whether the callus-forming IAA-amino acids are transported in the same way as free IAA, [2-^{14}C]IAA-L-alanine and [2-^{14}C]IAA-glycine were synthesized and their basipetal and acropetal movements in pea stem segments were measured. Only fee IAA was transported by the characteristic basipetal transport system (Fig. 13). The IAA-amino acids did not seem to have any specific transport and they probably only move by diffusion.

V. Metabolism of free IAA vs. the metabolism of IAA conjugates (Figures 14 and 15).

If the continuous release hypothesis for the mode of action of the IAA conjugates is correct, one would expect: 1) that the rate of hydrolysis of the conjugates, which controls the availability of free IAA to the tissues, would correlate with the biological activities of the conjugates; and 2) that the free IAA released upon hydrolysis of the applied conjugates would probably be metabolized into some of the products formed when tissues are treated with free

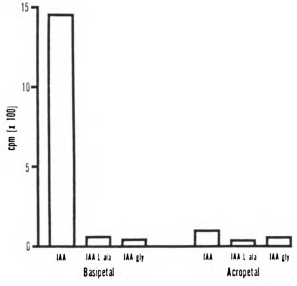

Figure 13. Transport of [^{14}C]IAA and [^{14}C]IAA-amino acid conjugates in pea stem segments.
Auxin transport is shown as the amount of radioactivity moving from donor blocks through twelve 3.5 mm pea stem segments into the receiver blocks during a 3 h period.

IAA. These two possibilities were tested by studying the metabolism of IAA conjugates that were synthesized from [1-^{14}C]IAA.

Hydrolysis of IAA conjugates. When free IAA is applied to plant tissues a fairly large portion of it is rapidly decarboxylated by enzymatically-catalyzed reactions (Epstein and Lavee, 1975). However, conjugates of IAA are protected from enzyme-catalyzed decarboxylation (Cohen and Bandurski, 1978). Therefore, the IAA moiety should be subject to decarboxylation by plant tissues only after the IAA is released by hydrolysis of the conjugates. Measurement of the decarboxylation of conjugates of IAA, containing ^{14}C in the carboxyl position of the IAA moiety, should then be an indirect measure of the rate of the hydrolysis of the conjugates.

Figure 14 shows the results of a study of the time course of $^{14}CO_2$ release from pea stem segments treated with [1-^{14}C]IAA, [1-^{14}C]IAA-L-alanine and [1-^{14}C]IAA-glycine. Free IAA is rapidly decarboxylated in the first few hours but by 12 h the decarboxylation almost stops. In contrast, IAA-L-alanine is decarboxylated at a lower but fairly constant rate for as long as 48 h. IAA-glycine shows a much lower rate of decarboxylation than IAA-L-alanine. These data agree with the data from the auxin-induced ethylene experiments (Fig. 12). The cessation of the ethylene production induced by free IAA after 9 h agrees with the observation that the decarboxylation stops by 12 h; thus, ethylene production stops when the free IAA has been metabolized (see also Lau and Yang, 1973). The low, but steady

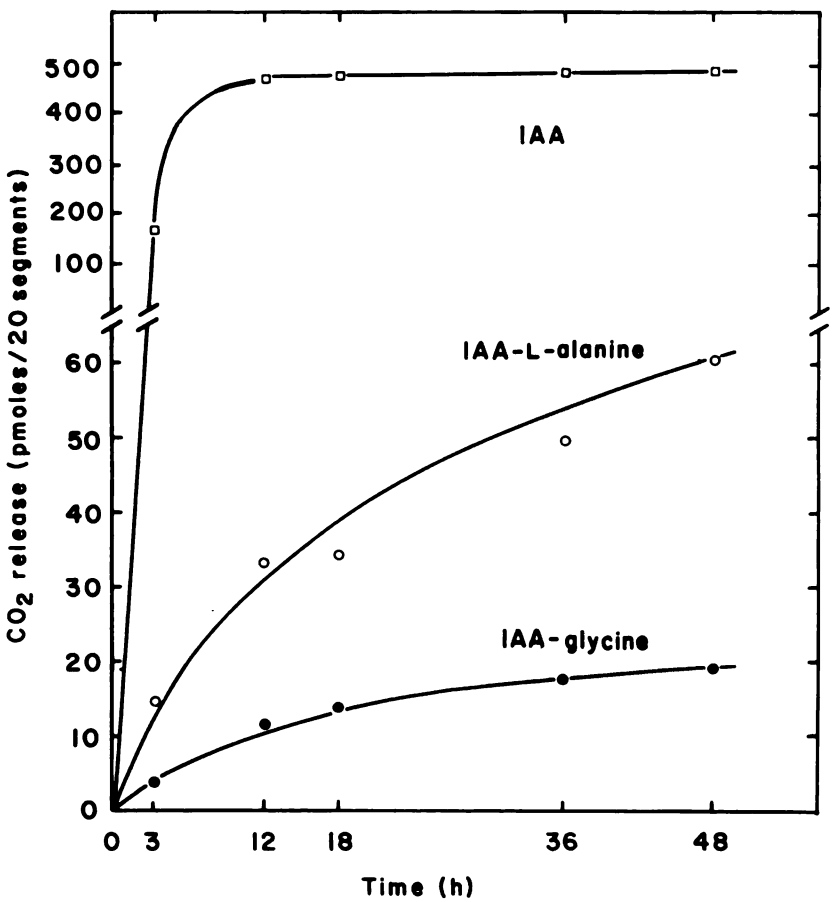

Figure 14. Time courses of $^{14}CO_2$ release from pea stem segments treated with [1-^{14}C]IAA and [1-^{14}C]IAA-amino acid conjugates.

Pea stem segments were treated with the [1-^{14}C]IAA compounds in Warburg flasks under aseptic conditions. The $^{14}CO_2$ was trapped with filter paper saturated with 5 N KOH. Counting efficiency was 74%. No significant decarboxylation was released in the absence of tissue.

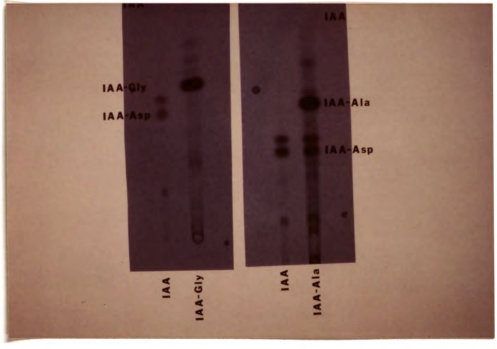

Figure 15. Autoradiograms of the metabolites of [1-^{14}C]IAA and [1-^{14}C]IAA-amino acids produced by pea stem segments. The acidic, ether-soluble metabolites were chromatographed on Silica Gel G plates in chloroform:ethyl acetate:formic acid (35:55:10, v/v/v). The autoradiograms were prepared from these chromatograms. The locations of the internal standards,which were detected colorimetrically on the TLC plates, are indicated.

decarboxylation (after hydrolysis) of IAA-L-alanine correlates with the slow steady rate of ethylene production induced by IAA-L-alanine. The very low level of decarboxylation (after hydrolysis) of IAA-L-alanine correlates with the slow steady rate of ethylene production induced by IAA-L-alanine. The very low level of decarboxylation (after hydrolysis) of IAA-glycine agrees with the inability of this conjugate to induce ethylene production by pea stems (Table 3). These data are consistent with the slow release hypothesis, showing that IAA-L-alanine, very probably, is hydrolyzed to IAA continuously over the period of the experiment.

Metabolites of IAA and of IAA conjugates. Metabolites of [1-^{14}C]IAA and of [1-^{14}C]IAA-amino acid conjugates were compared to determine whether the IAA moiety of the conjugates is metabolized into some of the same metabolic products as is free IAA. Acidic, ether-soluble extracts were prepared from pea stem segments which had been treated with the ^{14}C-labelled auxins. Thin layer chromatograms of the extracts were run on silica-gel plates and autoradiograms of the plates were developed. This method was found to produce reproducable "fingerprints" of the acidic metabolites.

"Fingerprints" of these acidic metabolites of free IAA, the very active conjugate, IAA-L-alanine, and the inactive conjugate IAA-glycine, are shown in Figure 15. The major metabolites of IAA-L-alanine are the same as those formed from free IAA. In contrast, the inactive conjugate, IAA-glycine, does not appear to be metabolized to any great

extent into acidic, ether-soluble compounds and, in particular, it does not form the same metabolites as does free IAA.

The active conjugate, but not the inactive conjugate, is indeed metabolized into some of the same products that free IAA is metabolized into. The hypothesis, that the activity of the conjugates depends on their hydrolysis to free IAA is supported by these observations, which virtually prove that at least the IAA-L-alanine is hydrolyzed to yield free IAA. Another important observation is that there is little, if any, [1-^{14}C]IAA present in any of the tissues after the 72 h incubation. However, there are still considerable amounts of unmetabolized IAA-L-alanine and IAA-glycine in the conjugate-treated tissues. This is in keeping with the proposed stability of the conjugates and the known lability of free IAA.

VI. Enzymes hydrolyzing the IAA conjugates (Table 6).

The slow release hypothesis for the mode of action of the conjugates requires the presence, in plant tissues, of an enzyme or enzymes capable of hydrolyzing the conjugates to yield free IAA. The inactivity of the conjugate with D-alanine (Fig. 6 and Table 3) and the results of the metabolism experiments (Fig. 14 and 15) imply that such enzymes do exist and that they are, as expected, stereospecific. Several different proteolytic enzymes and acylases were tested in order to obtain some information on the type of enzymes responsible for the *in vivo* hydrolysis. IAA-L-alanine, IAA-L-valine, IAA-glycine and IAA-L-phenylalanine

were tried as substrates for all of these enzymes.

Papain, a protease from Carica papaya, has a very wide specificity for both proteins and small molecular weight peptides (Arnon, 1970). However, none of the IAA-amino acids tested were hydrolyzed by papain even after prolonged incubations (up to 24 h). An unusually non-specific protease from Streptomyces griseus was also unable to hydrolyse the IAA-amino acids. Similarly, Acylase I (N-acylamino acid amidohydrolase) did not hydrolyze the IAA-amino acids. This acylase readily hydrolyzed N-acetyl-L-glutamic acid but N-indoleacetyl-L-glutamic acid remained completely intact. Carboxypeptidase Y from Baker's Yeast, which rapidly hydrolyzed N-carbobenzoxy-L-phenylalnyl-L-alanine, was unable to hydrolyze the IAA-amino acids. Several investigators have reported the release of bound auxin, in plant extracts, by the action of chymotrypsin (cf. Thimann et al., 1942; Link and Eggers, 1941). However, the IAA-amino acids tested in this study were all stable to treatment with chymotrypsin. The reported release of auxin from proteins by chymotrypsin was probably not due to covalently bound auxin.

These observations are not incompatible with the proposed mode of action of the conjugates. For the conjugates to serve as a stable, reserve sources of IAA, they should not be hydrolyzed in a non-specific manner by the more general hydrolytic enzymes found in plants, or at least not rapidly hydrolyzed by such enzymes. It would be better for a plant if the enzymes were rather specific and had a low affinity for the conjugates. The fact that the non-specific proteolytic

enzymes and acylases are unable to hydrolyze the IAA-amino acid conjugates favors the specific nature of the hypothesized enzyme.

Experiments with depeptide conjugates show that their activities are dependent on the amino acid adjacent to the IAA, the terminal amino acid having very little effect on the biological activity (Fig. 2 and Table 4). This suggests that the terminal amino acid is removed rapidly by extracellular or cell wall proteases. Otherwise differences in the activities due to differential uptake should have been observed. Since many plants contain carboxypeptidases (cf. Doi et al., 1980), the effectiveness of carboxypeptidase Y, on the hydrolysis of dipeptide conjugates, was tested. Carboxypeptidase Y rapidly removed the terminal amino acids yielding the free amino acid plus the remaining IAA-monopeptide. As already mentioned, the IAA conjugates with single amino acids do not serve as substrates (Table 6).

Some preliminary attempts were made to find the enzyme responsible for hydrolysing the IAA-amino acids to the free IAA. For these experiments, crude extracts of pea stems were used. The attempts failed to reveal any activity when looking for the release of [1-^{14}C]IAA from [1-^{14}C]IAA-alanine (10 μC/μmole). However, this was not unexpected because the rates of hydrolysis should be quite low. In the decarboxylation experiment shown in Figure 14, about 25% of the free IAA supplied to the pea stems was decarboxylated. If 25% of the IAA released from the active conjugate, IAA-L-alanine was decarboxylated then the rate of hydrolysis can be

Table 6. Effectiveness of Carboxypeptidase Y in the hydrolysis of amino acid and dipeptide conjugates of IAA. The reaction mixtures contained 50 mM sodium phosphate buffer (pH 6.8), 0.1 mM substrate and 1.0 µg enzyme/ml. The free amino acids (aa) were detected with ninhydrin. The remaining IAA compounds were followed by TLC and detected with the Van Urk-Salkowski reagent.

Substrate	Hydrolysis	Products Detected
N-CBZ-L-phe-L-ala	yes	free aa
IAA-L-ala	no	IAA-L-ala
IAA-L-phe	no	IAA-L-phe
IAA-L-ala-L-ala	yes	IAA-L-ala + free aa
IAA-L-ala-L-phe	yes	IAA-L-ala + free aa
IAA-L-phe-L-phe	yes	IAA-L-phe + free aa

estimated as four times the rate of decarboxylation. The rate of decarboxylation over the 48 h period was approximately 1.5 pmol/h/g fresh wt. The rate of hydrolysis would then be approximately 6 pmol/h/g fresh wt. With the specific activity of 10 μC/μmol, this would be only 80 dpm. Higher specific activity activity IAA-amino acids would greatly facilitate the search for the enzyme responsible for the hydrolysis of the conjugates.

VII. Metabolism of IAA by tumorous and non-tumorous tissues (Figures 16 and 17).

As already mentioned, crown-gall tissue of Parthinocissus tricuspidata was reported to make five IAA-amino acid conjugates from exogenous IAA (Feung et al., 1976). The authors suggested that three of the conjugates might be unique to tumor tissue. These three (IAA-alanine, IAA-glycine and IAA-valine) were found, in experiments reported in this thesis, to be very potent inducers of unorganized callus growth. The reported formation of these very active conjugates in the P. tricuspidata tumor tissue suggested that the biosynthesis of the conjugates might be an important factor in tumorigenesis; part of the mechanism of dedifferentiation. IAA conjugation was also suggested, by other authors, to be important in tumorigenesis of tumor-prone hybrids (Liu et al., 1978). With these reportes in mind, the following experiments were conducted to compare the metabolites of exogenous IAA in tumorous and non-tumorous tissues.

"Fingerprints" of the acidic metabolites of [1-^{14}C]IAA from Helianthus annuus tissues are shown in Figure 16. The

crown-gall tissues and the non-tumorous (hormone requiring) tissue had very similar "fingerprints." The major metabolite of the crown-gall tissues and the non-tumorous tissue co-chromatographed with IAA-aspartate. In contrast, the major metabolite from the hormone-habituated tissue was a different compound. This unknown compound and IAA-aspartate occurred in all of the sunflower tissues tested, whether callus from normal tissue, habituated callus or crown-gall derived callus. However, the abundance of the unknown was greater (when compared to IAA-aspartate) in the habituated tissue. It should be noted here that other crown-gall tissues of H. annuus, which were induced by different strains of Agrobacterium tumefaciens, all showed "fingerprints" similar to the "fingerprint" of the tissue which was induced by strain AT 1 (Fig. 16).

The "fingerprint" of [1-^{14}C]IAA metabolites from crown-gall tissue of Parthinocissus tricuspidata is shown in Figure 17. The locations of of IAA-alanine, IAA-glycine and IAA-aspartate, which were added to the tissue at the time of extraction, are also shown in the figure. Surprisingly and in contrast to the report of Feung et al. (1976), the only standard to co-chromatograph with a metabolite of IAA was IAA-aspartate. The same observation was made when the P. tricuspidata extract was chromatographed in two other solvent systems (chloroform-methanol-acetic acid, 70:20:10; and methyl ethyl ketone-ethyl acetate-ethanol-water, 3:5:1:1). Furthermore, the metabolite which chromatographed nearest to IAA-alanine was found to quench long-wave UV-induced

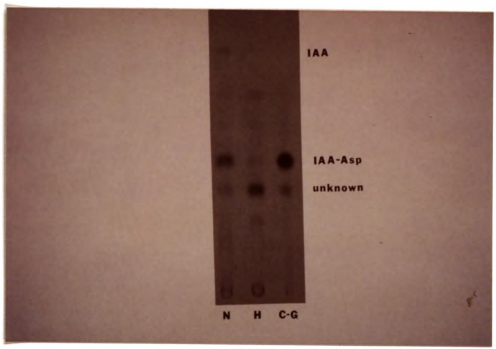

Figure 16. Autoradiogram of the acidic, [1-^{14}C]IAA metabolites from Helianthus annuus tissues.
Conditions as in Figure 15. N, normal tissue; H, auxin-habituated tissue; C-G, crown-gall tissue induced with A. tumefaciens strain AT-1.

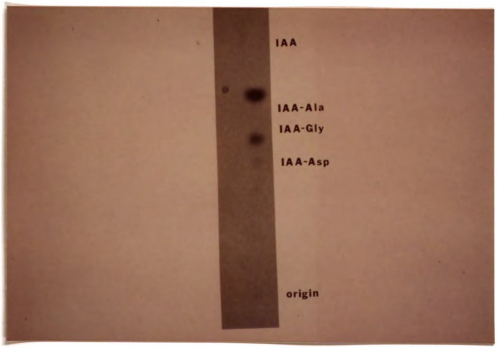

Figure 17. Autoradiogram of the acidic, [1-^{14}C]IAA metabolites from Parthenocissus tricuspidata crown-gall tissue. Conditions as in Figure 15. Crown-gall tissue was induced with A. tumefaciens strain B6.

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fluorescence rather than short-wave UV which is characteristic of indole compounds. The radioactive, long-wave UV-absorbing region did not react with indole-detecting color reagents and there was no trace of the spectrum of intact indole (no peaks near 280 nm) in the UV absorption spectrum of the compound after partial purification. These observations indicate that the major [1-^{14}C]-metabolite produced from [1-^{14}C]IAA is not an IAA conjugate. Other experiments with non-tumorous tissues of P. tricuspidata showed that the "fingerprints" of [1-^{14}C]IAA metabolites from non-tumorous tissue were identical to the "fingerprint" from the P. tricuspidata crown-gall tissue (Dr. D. Smith, personal communication).

The reason for the discrepancy between these results and the results of Feung et al. (1976) are not clear. The P. tricuspidata tissue was obtained from the same laboratory. Chromatograms of extracts which were prepared by the same procedure of Feung et al. (1976) yielded the same results as chromatograms prepared by the procedure used in this work.

The results of these experiments with H. annuus and P. tricuspidata indicate that specific IAA metabolites are not correlated with crown-gall induced tumorigenesis in the manner suggested by Feung et al. (1976). The difference observed between the hormone-habituated H. annuus and the other H. annuus tissues make it impossible, at this time, to rule out a role for IAA metabolites in some other plant tumors. The most striking difference in the "fingerprints" of the

IAA metabolites was between the different species (compare Figs. 16 and 17) and not between normal auxin-dependent and auxin-independent tumor tissue.

DISCUSSION

The presence of IAA conjugates has been known for years, but the biological activities of such compounds, other than IAA-aspartate, have been studied in only a few instances. The data reported here clearly show that a number of IAA conjugates can serve as auxins in auxin-requiring systems and that different conjugates have very different activities depending on the conjugating moiety.

Many of the effects of the IAA conjugates can be explained in terms of a slow enzymatic hydrolysis which gives a constant supply of free IAA. In long-term assays, some of the conjugates seem to be more active than free IAA, but in short-term assays free IAA is always the most active (Figs. 1 and 10; Table 3; Feung et al., 1977). The known lability of free IAA (cf. Epstein and Lavee, 1975) and the greater stability of the conjugates (cf. Cohen and Bandurski, 1978) makes this interpretation reasonable; thus, the greater activity of the conjugates in the long-term experiments is probably due to their persistence after the free IAA has been destroyed. Furthermore, constant renewals of free IAA give results which mimic those obtained with a single application of the most active of the conjugates, IAA-L-alanine (Fig. 7). These observations suggest that the conjugates are acting as they do, in part, by giving a constant supply

of free IAA.

Studies on the metabolism of free IAA and of IAA conjugates add further support to the slow release of IAA hypothesis. Free IAA and the active conjugate, IAA-L-alanine, are converted into some of the same metabolites by pea stem tissue (Fig. 15). This would be unlikely to occur unless the conjugate was first hydrolyzed to free IAA. Measurement of the decarboxylation of the IAA moiety of IAA-L-alanine indicates that the conjugate is probably hydrolyzed to IAA continuously by the tissue (Fig. 14). In addition, conjugates with the D-isomers of the amino acid are largely inactive, which indicates that the hydrolysis is enzyme-dependent (since the hydrolytic enzymes capable of splitting amide bonds are nearly always stereospecific).

The different activities of the different conjugates seem largely due to the susceptibilities of the various conjugates to the hydrolytic enzymes present in the plant tissues. This was shown in the metabolic studies where the active conjugate, IAA-L-alanine, was hydrolyzed while the inactive conjugate, IAA-glycine, was not (unlike the labelled IAA of the alanine conjugate, the labelled IAA of the glycine conjugate is neither decarboxylated very much nor transferred to aspartate).

It is possible that the uptake of the conjugates and their partitioning in the tissues and cells has some effect on their biological activities. However, the observation that both active and inactive conjugates accumulate in the tissues (Fig. 15) suggests that uptake is not likely to be a

limiting factor in determining their relative activities. Furthermore, if partitioning of the conjugates among the tissues and cells was important, there should be a correlation between the activity and the polarity of the different conjugates. No clear pattern regarding the polarity of the conjugate molecules was observed.

The regulation of the activity by hydrolytic enzymes in the tissues is the most likely cause for the species-specificity seen with some of the conjugates (i.e., Table 3, compare IAA-L-alanine and IAA-glycine in pea and tomato). In addition to the species-specificity observed in the experiments reported in this thesis, some other cases should be mentioned. In sugar beets, IAA-L-phenylalanine supports vigorous callus growth (Dr. M. Daub, personal communication), whereas in tomato it supports very little callus growth. IAA-L-alanine inhibits the proliferation of orchid protocorms (Mr. R. Greisbach, personal communication) but stimulates the proliferation of tomato callus. Similarly, indoleactonitrile must be converted to IAA for it to be active (Thimann and Mahadevan, 1958) and its activity in a particular species is related to the rate of conversion to IAA (Thimann, 1953). This variation in activities of the different conjugates with different species should be kept in mind. It makes it very difficult to predict which conjugate should be used to obtain the desired growth response when different plant species are involved.

Polar distributions of substances during development and regeneration are a striking and widespread phenomena in

biology giving rise to polarities of many kinds: electrical potentials, differential growth and differential responses. Many examples of cellular differentiation in higher plants are associated with polarity gradients within a cell or across a group of cells. The differentiation of the pollen grain, of root hairs, of xylem and phloem, and the basipetal reactivation of cambial cell divisions in the spring are but a few examples where polarity gradients are associated with differentiation.

The polar movement of IAA in plants is well documented (cf. Goldsmith, 1977) and many of the polar developmental phenomena observed in plants have been attributed to the polar movement of auxin. The IAA conjugates, on the other hand, seem to move in tissues in a non-regulated manner, presumably by diffusion (Fig. 13). Free IAA is also destroyed very rapidly by peroxidative degradation and this, in the presence of an active, directed transport probably helps maintain well-regulated concentration gradients. However, the IAA in the conjugates is probably not destroyed (Cohen and Bandurski, 1978) except as the IAA is released. As a consequence of the non-regulated transport of the conjugates, the non-destruction of the conjugates, and the slow-release of IAA from the conjugates, each cell in a tissue might well be exposed to the same steady supply of auxin as each other cell. To repeat : If auxin gradients within tissues are required for organogenesis, then the controlled transport of auxin and its rapid destruction at its target may be necessary simply to create and maintain these gradients.

If so, the marked de-differentiating effects of IAA-L-alanine are easily explained in terms of frustration of the tissues' attempts to create essential auxin gradients.

It is not impossible, however, that the IAA conjugates are auxins in their own right and that they have effects that are qualitatively different from the effects of free IAA. Thus, if IAA-L-alanine acts as an auxin only by releasing free IAA, it is not clear why it prevents organogenesis when used alone and permits organogenesis when it is added together with free IAA in the medium (Figs. 8 and 11). On the face of it, this implies that the two auxins have different actions, but such a conclusion may be premature. It remains possible that the presence of the faster moving IAA with its controlled transport can create auxin gradients even in the presence of the slowly diffusing IAA-L-alanine, since the latter may be hydrolyzed so sluggishly that it contributes a very small part of the total IAA and therefor cannot wholly obliterate the gradient formed from the additional IAA. Nevertheless, phenomena regarding the action of other of the IAA conjugates, especially their stimulations of shoot development (i.e., IAA-L-phenylalanine), remain obscure and no fully satisfactory explanation has presented itself.

Regardless of their mechanism of action, IAA conjugates offer a great diversity of effects, if only because the very wide range of conjugates available provide a wide range of steady-state concentrations of the natural auxin, IAA. The unique morphogenic responses caused by some IAA conjugates

may provide the necessary stimulus for controlling the morphogenesis of tissue cultures which have not responded successfully to other treatments.

The results reported here show that IAA-L-alanine and IAA-glycine are better auxin sources for the production of callus from tomato hypocotyl explants than the conventional auxins (IAA, 2,4-D and NAA). In the tomato hypocotyl system, where free IAA favors root development, a number of the IAA conjugates have already proven useful in several tissue culture systems. Mr. B. Martin and I were able to regenerate whole plants from tomato roots, even after the roots had been in culture for more than a year, using IAA-L-phenylalanine (unpublished). Friable callus and finely divided suspension cultures have been obtained from explants of field corn seedlings using IAA-L-alanine (Dr. S. McCormick, personal communication) and from sugar beets using either IAA-L-alanine or IAA-L-phenylalanine (Dr. M. Daub, personal communication). The glycine conjugate has proven useful in stimulating the proliferation of orchid protocorns (Mr. R. Greisbach, personal communication).

It would be interesting and potentially useful if someone were to synthesize an even wider range of auxin conjugates and evaluate the morphogenic responses that they elicit in plant tissue cultures. The identification of 4-chloroindoleacetic acid as a naturally occurring plant hormone (Marumo et al., 1968) suggests an interesting possibility for the auxin moiety. Of course, the conventional auxins (NAA, 2,4-D, IBA) could be used as well. A large number

of other auxin anaologs have been evaluated for auxin-like action over the years (Hoffman et al., 1952; Porter and Thimann, 1965; Bottger et al., 1978) and, by themselves or as conjugates, should be evaluated in plant tissue cultures.

In the studies reported here, the alanine, the glycine and the valine conjugates of IAA were found to have the greatest capacity, of all the IAA-amino acids tested, for inducing de-differentiation of the plant tissues routinely studied. However, maximum de-differentiation seems to occur with different conjugates in some other plant species. The putative occurrance of these very active conjugates in Parthenocissus tricuspidata crown-gall tissue (Feung et al., 1976) and the higher levels of IAA conjugates reported in tumor-prone hybrids of Nicotiana (Liu et al., 1978) suggested that the biosynthesis of very active conjugates might play a role in plant tumorigenesis. Unfortunately, the results reported in this thesis indicate that the kind of IAA metabolite produced by plant tissues is more closely correlated with the plant species than with the tumorous condition and there is no evidence that tumorogenesis is related to the formation of the de-differentiating IAA conjugates.

NOTES

1) Auxin is a generic term for compounds characterized by their capacity to induce elongation in short cells, when applied at suitable concentration. Auxins may, and generally do affect other processes besides elongation, but the effect on elongation is considered critical. Auxins are generally acids with an unsaturated cyclic nucleus or their derivatives (Larsen, 1954 and Tukey et al., 1954).

   In this thesis, the term "auxin" is used to include IAA and those substances which can fulfill the IAA requirement of tissue cultures in that they permit the proliferation of cells which does not occur in the absence of IAA or one of these substances.

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