The Auxin Conjugates

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Phytohormones occur in bound and free forms. The bound form is designated by some as conjugated if the hormone is covalently linked to a small molecule and as bound if it is attached to a macromolecule or cell particle (39). However, the terms bound and conjugated will be used interchangeably here, as they are in many other treatises. There is more information published on the relationship between free and conjugated 1*H*-indole-3-acetic acid (IAA) than is available on similar relationships of other hormones. Free IAA is the active form of the hormone. This activity was seen, for example, when IAA conjugates were applied to the first internode section of bean (*Phaseolus vulgaris* L.). The promotion of curvature was traceable to the amount of free IAA released from the conjugates (4). Excellent reviews of conjugated auxins are available (3, 9). It is the purpose of this communication to acquaint the reader with some of the broader aspects of conjugated auxin and then to focus on the use of auxin conjugates in tissue culture.

The study of bound auxin had its beginnings several decades ago, but the identification of IAA-aspartate (IAAsp) as an amide-linked metabolite in pea (*Pisum sativum* L.) seedlings following the application of IAA (1) launched what has been aptly called the "chemical era" of research on this subject (9). The report in 1955 of IAAsp in a legume was followed a year later by its identification in several other species (19). Since then, studies posing different questions about the physiological role of IAAsp have confirmed, through a variety of methods, the natural occurrence of this chemical (12, 37, 41). Definitive chemical methods, such as combined gas chromatography–mass spectrometry, have been used more recently to show the presence of IAAsp in soybean (*Glycine max* L.) seed (8) and in pea root nodules (2). IAAsp is one of the more common naturally occurring amide conjugates of IAA (Fig. 1).

IAA-glutamate (IAGlu) is another common conjugate in plants, occurring not only when the plant receives exogenous IAA (33, 35), but also as a natural constituent (40). It was thought previously that in vivo formation of both IAAsp and IAGlu was primarily a means of detoxifying excess IAA. It now appears, however, that this idea should be placed within a broader context. The fact that the enzyme acylaspartate synthetase is specifically induced by auxin with the probable involvement of RNA synthesis (42) suggests that there is a genetic control of the homeostasis of IAA levels during growth and development of the plant. Specificity of reactants also is seen in the predominately higher biological activity of L-amino acid conjugates as compared to D-enantiomers (18), and the L-enantiomer was confirmed as the naturally occurring form of IAAsp in seeds of *Glycine max* (8).

The hypothesis that there is a complex, genetically controlled homeostasis of IAA concentration in plants gains support when the relationships of free to esterified IAA in cereal grains is examined. Esterified IAA is at a much higher concentration than free IAA in the endosperm and young seedlings of *Zea mays* L. (9). The esters serve as storage components that release IAA as needed. IAA-*myo*-inositol-galactoside, for example, serves up both IAA and IAA-*myo*-inositol to the shoot, the latter chemical being provided at about twice the amount of the former (24). Some galactose is also provided to the shoot. Extracts have shown a specific esterase fraction that hydrolyzes IAA-*myo*-inositol (21). From this and other work of Bandurski and associates, it can be concluded that an elaborate control system is involved that provides an equilibrium between free and esterified IAA. Although the metabolism of the amide conjugates has not been studied as intensively, it is reasonable to assume that evolution has resulted in the analogous mechanisms for conjugation–deconjugation of amide conjugates in the maintenance of workable levels of IAA.

CONJUGATE AUXINS IN TISSUE CULTURE

The use of conjugate auxins in tissue culture has progressed slowly.

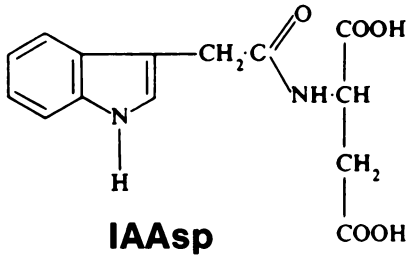

Fig. 1.—Structure of 1*H*-indole-3-acetylaspartic acid.

This is a little surprising when one considers that bound IAA is protected against enzymic inactivation (10). The free carboxyl group of IAA has been shown to be essential for the activity of IAA oxidase (26), and the mechanism by which peroxidase may function as IAA oxidase has been described (30).

Decarboxylation, if imposed by peroxidase, has a great bearing on tissue culture practices, since cutting the tissue for transfer probably activates wall-bound peroxidases in some manner. It is known that much of the exogenous IAA can be oxidized at the surface of the cultured tissue (14). It would thus appear that auxin conjugates might be good candidates for bypassing the pathway of enzymic inactivation through decarboxylation, if only long enough to provide the excised tissue with more of the active hormone as it is released from the conjugate. Selected studies that tend to affirm this supposition will be described.

IAA-alanine (IAAla) was observed to increase greatly the growth of callus from tomato (*Lycopersicon esculentum* Mill.) hypocotyl segments when compared to IAA at the same molarity, and both IAAla and IAA-glycine (IAGly) had this effect on tobacco (*Nicotiana tabacum* L.) pith callus (23). IAAla and IAGly were the only conjugates of the several tested that either completely inhibited organogenesis or greatly reduced it at all concentrations tested (1, 10, and 100 μM) in favor of callus production. The conjugates valine, leucine, aspartic acid, threonine, methionine, phenylalanine, and proline all supported shoot formation in the tomato hypocotyl segments and only weakly inhibited shoot formation in tobacco callus. Free IAA strongly inhibited shoot formation when used at high concentrations that gave more callus. It was suspected that the great increase in callus growth caused by IAAla might be due to the gradual release of free IAA over the length of the experiment. An

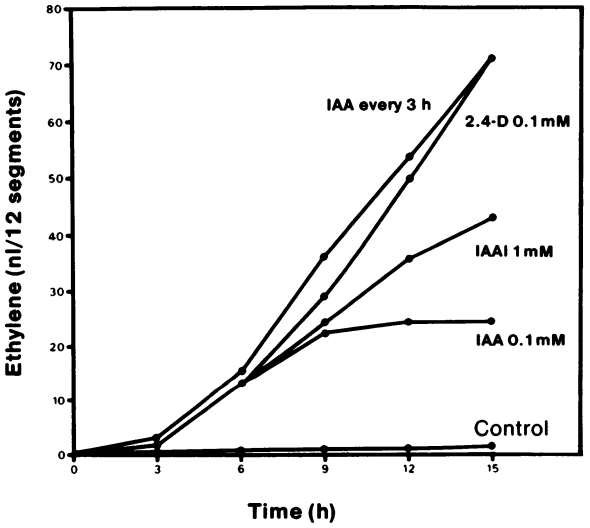

Fig. 2.—Ethylene production in pea stem segments treated with various auxins (IAA, 2,4-D, IAAla, IAA-L-alanine) (23).

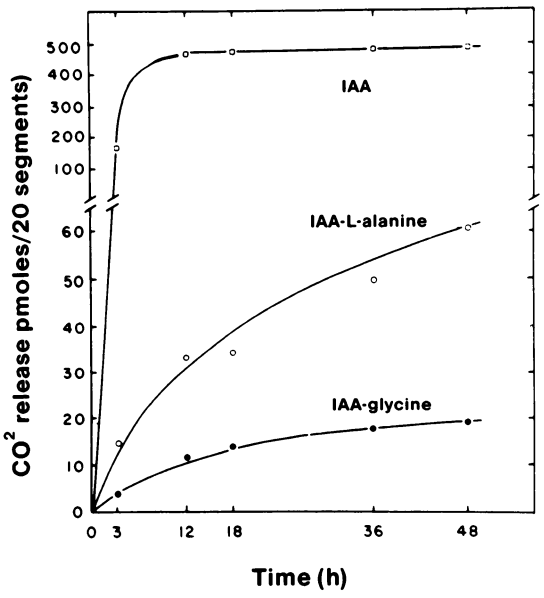

Fig. 3.—Carbon dioxide released from pea stem segments treated with radiolabeled IAA or IAA conjugates (22).

indirect test of this release was done by transferring tomato hypocotyl segments to fresh media containing IAA every 4 hr. This treatment resulted in vigorous callus growth resembling that obtained when IAAla was used; which was taken as morphological evidence that IAA was slowly released from IAAla. The alanine conjugate also showed strong activity in the growth of soybean callus in a separate study, as did the conjugates with serine, glycine, leucine, proline, and threonine (16). IAGly was one of a few conjugates reported in *Parthenocissus tricuspidata* (Sieb. and Zucc.) Planch. crown gall callus, a nondifferentiated system that had been supplied with exogenous auxin (17).

Ethylene production, which reflects the general amount of free IAA through its promotion of ACC synthetase (44), also supported the conclusion that IAA was released from IAAla (23). Pea stem segments incubated with free IAA produced ethylene for only 9 hr, unless they were given new IAA, in which case the specimens continued producing ethylene (Fig. 2). IAAla, however, did not cease to stimulate ethylene production at 9 hr, but continued to do so by what was interpreted as a slow release of IAA (23). Ethylene production also was used as a marker for the timed release of IAA from extracted IAA metabolites in tobacco leaf disks (27).

Since free IAA is susceptible to decarboxylation as one avenue of its enzymic modification (see refs. 31 and 34 for an alternative pathway), it has been possible to use [1-^{14}C]IAA linked to a conjugate in order to monitor the evolution of $^{14}CO_2$ and hence the amount of labeled IAA that is released from the conjugate. This experiment was done using pea stem segments that were incubated with IAA, IAAla, and IAGly (Fig. 3) (22). Free IAA given to pea stem segments underwent rapid decarboxylation and, by 12 hr, 25% of the IAA carboxyl had been released as CO_2 (22). This response agrees rather well with the data on ethylene production from free IAA (Fig. 2), where production virtually ceased at 9 hr. The steady production of CO_2 from IAA that was released from IAAla also agrees with the continuous production of ethylene from this compound. IAGly produces extremely low amounts of ethylene in pea and also has a low rate of decarboxylation. This work presents convincing proof that the amide conjugates tested are slow-release forms of IAA in cultured plant tissues.

The influences of IAA conjugates on morphogenesis have been studied using leaf disks of tomato (32). It was found that IAAsp and IAA-phenylalanine (IAPhe), each at 10 μM and in combination with *N*-(phenylmethyl)-1*H*-purin-6-amine (BA) at 8.9 μM, promoted shoot formation when compared with free IAA at the same molarity as the conjugates (Fig. 4). Results similar to those already reported for hypocotyls (23) were seen in that IAAla and IAGly showed strong inhibition of shoot formation and marked stimulation

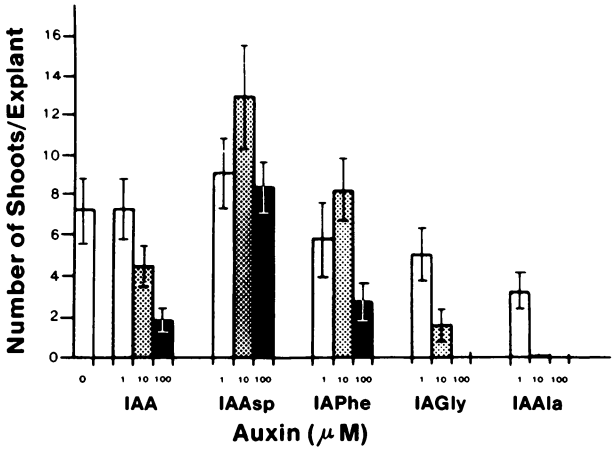

Fig. 4.—Shoot formation from tomato leaf disks grown on a nutrient medium with BA at 8.9 μM and auxins at 1, 10, and 100 μM (32).

of callus production. Recent work in our laboratory was aimed at comparing regeneration frequencies from leaf disks of an unlobed, true-breeding somaclonal variant in tomato (5). There were similarities as well as differences between the variant and normal tomato, the latter being the subject of the previously described study (32). Variant leaf disks similarly produced vigorous callus and very few shoots on IAAla; however, IAA and IAPhe both stimulated shoot production in excess of the production with BA alone. These results are different from those obtained with leaf disks of normal tomato, where IAA did not stimulate shoot production. Although these results are preliminary, they suggest that there are differences in closely allied genotypes in terms of the way in which IAA conjugates are used.

Different solubility properties have been given as a possible reason for the differences seen in response to various IAA conjugates (18). Furthermore, it has been speculated that since certain polyhydroxylated esters of IAA are highly water-soluble, they may move into the vacuole (31). Monitoring the rates of efflux of radiolabeled (2,4-dichlorophenoxy)acetic acid (2,4-D) and its conjugates under the influence of different concentrations of dimethyl sulfoxide (Me_2So, DMSO), and using neutral red stain to check for the integrity of the membrane, it was concluded that aspartic and glutamic acid conjugates as well as glycoside metabolites of 2,4-D were accumulated in the vacuoles of soybean callus cells (11). An interesting comparison was made between carrot (*Daucus carota* L.) and soybean callus in terms of efflux and conjugation rates of 2,4-D. Carrot callus becomes competent when exposed to 2,4-D. Somatic embryos subsequently develop when the tissue is exposed to a nutrient medium lacking 2,4-D. It was considered that the rapid efflux of 2,4-D into such a medium might be an important aspect of embryo formation in carrot (28). In the comparison of carrot and soybean, the latter a more recalcitrant species (7), it was found that carrot callus lost much more 2,4-D to the embryogenic medium than did soybean callus to its environment (28). Conversely, soybean callus showed much more conjugation of 2,4-D than seen in carrot.

Whereas free IAA is usually undirectional in its transport, the amide conjugates seem to move from one cell to another through a nonregulated and much slower process of diffusion (18). If there is to be a slow but steady rate of release of IAA in these cells, then the distribution of hydrolytic enzymes within a tissue or clump of cells becomes important. Other important aspects that are almost certainly related to the movement of conjugated hormone are: a) whether the affected cells are in an intact plant or are instead part of an excised tissue or organ in culture; and b) whether such cells have already been altered physiologically by prior hormone treatment. For example, auxin pretreatment of intact tomato seedlings resulted in an increased number of shoots from leaf disks that were excised and exposed to auxin and cytokinin in a nutrient medium (32). In other words, auxin inhibition of shoot initiation from leaf disks was lessened by pretreating the seedlings with auxin (Table 1). Leaf disks from seedlings pretreated with IAAla or IAGly, both of which are potent inhibitors of shoot formation in cultured tomato

Table 1. Influence of 10-μM auxin pretreatment of in vitro-germinated seedlings on shoot formation from cultured tomato leaf disks excised from the seedlings.

Germination medium (seedlings)	No. shoots: Culture medium (leaf disks)		
	No auxin	IAA	IAGly (IAAla)
No auxin	4.6 ± 0.6	0.7 ± 0.2	0.2 ± 0.2
IAA	5.1 ± 0.6	1.9 ± 0.6	0.1 ± 0.1
IAGly (IAAla)	2.6 ± 0.7	3.9 ± 0.8	0

[z]Averages are from 27 to 30 replicates; results from experiments with IAAla and IAGly were similar and were combined. Culture medium contained BA at 8.9 μM (32).

tissues, produced more than 5 times the number of shoots as disks from nontreated plants in the presence of IAA. This is not the first paradoxical problem associated with auxin conjugates. Hangarter et al. (23) observed an increased number of roots in tomato callus when IAA and IAAla were used in combination. One would have predicted an increased production of callus only under this condition based on the theory of suppression of organogenesis by excess auxin, a theory frequently invoked to explain the potent effects of 2, 4–D on callus formation.

Auxin conjugates also have been tested for their effects on the rooting of cuttings. The first two cases cited here were performed under nonsterile conditions. An enhancement of rooting in jack pine (*Pinus banksiana* Lamb.) and bean cuttings was achieved with aryl esters of auxin, such as phenyl 1*H*–indole–3–butyrate, as compared to 1*H*–indole–3–butyric acid (IBA) (20). This enhancement is especially important for a species such as jack pine, which is difficult to root. Treatment of mesquite (*Prosopis* spp.) cuttings with IBA-alanine gave a more fibrous root system and greener leaves than did treatment with IBA alone (15). Axillary buds of black walnut (*Juglans nigra* L.) seedlings were favorably disposed to rooting when the seedlings were grown on a nutrient medium containing IAPhe (6).

The relationship between IAA conjugates and other hormones needs more investigation. If the theory of auxin homeostatis is correct, increasing the concentration of IAA could give an increase in conjugates, unless, of course, all excess IAA is oxidized. It is known that cytokinin application to a tissue can give an increase in IAA content. Therefore, one might expect to see an increase in concentration of IAA conjugates following the addition of cytokinin. There is some evidence that this may be so in young bean plants when they are treated with *N*–(2–furanylmethyl)–1*H*–purin–6–amine (kinetin) (38). The opposite happened, however, when both kinetin and IAA were applied to hypocotyl segments of mung bean [*Vigna radiata* (L.) R. Wilcz], in which case there was a suppression of IAA conjugation and a concomitant increase in ethylene production (25). Cytokinins also were reported to inhibit conjugation of 2, 4–D in cultured soybean cells (29). In contrast, ethylene treatment resulted in an increase in the level of endogenous IAA conjugates in leaves of pecan [*Carya illinoensis* (Wangenh.) C. Koch] (43), in leaves and callus of olive (*Olea europea* L.) (13), and in *Citrus* leaves (36).

CONCLUSIONS

When IAA from a nutrient medium moves into a cell, at least three different things can happen (Fig. 5). An oxidation process may occur and, if so, the molecule is most often oxygenated at the number 2 position on the ring. Hence, it becomes an oxindole (OXIAA), with the likelihood of further modification. Decarboxylation of the side chain may or may not occur, which probably depends on the plant species, the type of tissue used, and the methods of handling the explant during its transfer to culture. A second possible event is that IAA may become conjugated, either through an ester or amide linkage, which is seen as IAAR in Fig. 5. The conjugate will tend to be in equilibrium with IAA (half-arrows in Fig. 5 are not meant to imply known rate constants) and will normally be hydrolyzed as IAA is needed. The third possibility is that IAA remains free and unchanged for a sufficient period of time to

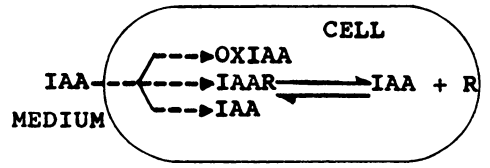

Fig. 5.—Alternative pathways for IAA as it enters a cultured cell. The rate constants for the reactions are not known (OXIAA, an oxindole; IAAR, an ester or amide cojugate of IAA).

exert its hormonal effects. Thus, the endogenous level of IAA is quite obviously important in the response of cultured cells as is the distribution of hydrolytic enzymes that release IAA from its bound form.

The auxin conjugates are not readily susceptible to enzymic inactivation and, when they move into a cell, they appear to serve as slow-release forms of the hormone. A slow and steady supply of auxin is sure to have some influence on morphogenesis, which often becomes manifested in tissue culture only after an extended period of time. Biological activity of the conjugate depends on the specific conjugate being used as well as on the innate properties of the tissue. Pretreatment of an intact plant with IAA or its conjugate also can have a marked influence on the performance of tissues that are excised from the plant and subjected to auxin treatment.

Considering these complex factors, it is perhaps not surprising that responses of cultured plant tissues to auxin can still be categorized as phenomenology. Given that there is a lack of understanding of how auxin and its conjugates control cells and tissues, a hopeful note is sounded that some of the pieces of the intriguing puzzle may soon be falling into place.

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Selected Topics on Induced Chromosome Changes in Tissue-cultured Cells

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There is ample evidence that chromosomal changes can be induced in cultured cells. These changes can involve either an increase or decrease in chromosome number or a change in chromosome structure and can be brought about by both physical and chemical agents.

Increase in chromosome number

Cells that contain more than the normal number of chromosomes are classed as polyploids or aneuploids. Polyploids contain one or more additional chromosome complements, whereas aneuploids occur through either a gain or loss of one or more individual chromosomes. There are many reasons for inducing polyploidy in cultured cells. If one is interested in gene regulation and secondary metabolism, polyploid cells might offer several advantages. Polyploid cells are larger than their counterparts of lower ploidy and, in several instances, have been shown to produce more secondary metabolites. For example, in four induced tetraploid clones of *Phlox drummondii* Hook, the activity of alcohol dehydrogenase was about two times higher than that of the diploid (14). In two other tetraploid clones, however, the enzyme activity was slightly less. Polyploidy also can change end products of biochemical pathways. In *Phlox drummondii*, 15 induced tetraploid clones were analyzed for glycoflavones (15). In 14 clones, flavonoids that were not present in the diploid were found in the tetraploid. The opposite was true in eight clones; flavonoids present in the diploid were absent in the tetraploid. In addition, many changes in tissue-specific expression were found. In *Brizia media* L., induced tetraploids produced only C-glycosyl-luteolin derivatives, whereas the parental diploids produced only C-glycosyl-apigenin derivatives (19). Hybrids between the two produced only luteolin derivatives. These limited examples demonstrate that induced polyploidy can disrupt and change regulatory mechanisms associated with gene expression.

The previous discussion on the effects of polyploidy center on autopolyploids, which contain one or more extra sets of homologous chromosomes. Another class of polyploids, allopolyploids, also can be quite useful. These contain one or more extra sets of homoeologous chromosomes from different parental genomes. Allopolyploid hybrids are quite important in plant improvement. Wide hybrids,