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# Indole-3-acetic Acid (IAA) and IAA Conjugates Applied to Bean Stem Sections

## IAA CONTENT AND THE GROWTH RESPONSE

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### ABSTRACT

**High resolution growth recording techniques and reverse isotope dilution analysis were used to study the relationship between indole-3-acetic acid (IAA) concentration and curvature of excised bean (*Phaseolus vulgaris* L. cv Bush Burpee Stringless) first internode sections unilaterally treated with hormone. The maximum rate of curvature occurred rapidly (within 25 minutes) and was proportional to the log of the amount of applied IAA recovered in the tissue. The rate of curvature decreased after 30 minutes although little or no lateral migration of applied IAA occurred and tissue levels of IAA increased. The biologic activity of IAA-amino acid conjugates was found to be directly related to the amount of free IAA, resulting from their hydrolysis, which could be recovered from the tissue.**

In the study of plant hormones, especially auxins, many attempts have been made to examine the quantitative relationships between growth rate and hormone concentration. Typically, the methods employed were to incubate tissue segments in hormone solutions of different concentrations and relate the resultant growth obtained to the external hormonal solution concentration (2). Several assumptions and limitations are inherent in this approach. It is assumed that the hormone will penetrate rapidly into the tissue until the internal hormone levels rise to that existing in the incubation media. However, this possibility is rarely examined directly. Inasmuch as the bulk of IAA uptake is probably through the damaged ends of tissue segments, high rates of degradation may occur (24). Also, measurements are usually made after several hours and the rate of growth changes continuously during the time of the experiment (18). Finally, because of the prolonged incubation period, levels of IAA in the tissue will also change. In order to more fully understand the relationship between IAA concentration and growth, it is necessary to examine these factors more closely.

Using high resolution growth recording techniques for growth measurements and reverse isotope dilution analysis of radiolabeled IAA, we show in this report that (a) the period of maximum rate of curvature induced by an asymmetrical application of IAA to bean internode sections is transient, reaching a maximum in 20 to 25 min, whereas the level of IAA in the tissue remains relatively high for much longer periods; (b) unilaterally applied IAA remains on the side of application with little or no lateral migration; (c) tissue levels of IAA and maximum growth rate follow a log/linear relationship; and (d) the biologic activity of IAA-amino acid conjugates is directly related to the levels of free IAA found in the tissue resulting from their hydrolysis.

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### MATERIALS AND METHODS

**Plant Material.** *Phaseolus vulgaris* L. plants, cv Bush Burpee Stringless, were used in all experiments. Certified seeds were germinated in perlite-filled flats at a night temperature of 25°C and a daytime temperature of 30 to 34°C. Seedlings were grown under fluorescent light (Sylvania[2] cool white SHO-120 V-200 w) at a photoperiod of 8 h and irradiance of 4 to 8 w $m^{-2}$ (400–700 nm) and with a dark period of 16 h. The seedlings were harvested 6 to 8 d after germination when they were approximately 12 cm in length, their first internode was 4.5 to 7.0 cm long, and the length of second internode did not exceed 5 mm. First internode sections (4 cm), cut just below the second node, were used (19).

**Bioassay.** First internode sections (two/vial) were inserted, apical portion up, into 20-ml glass scintillation vials containing a piece of sponge for support and 20 ml of 1 mM sodium phosphate buffer (pH 6.4) exactly as previously described (19). Experimental solutions (usually 10 μl) were applied to 5-mm diameter paper discs backed with polyethylene film (Benchkote, Whatman Ltd), which were placed between the basal part of stem section and the sponge. Immediately after application of the disc, the angular position of the section was recorded and the experimental vials placed in a moist chamber. Bending growth response of the stem sections was determined after 1.5 and 3 h by measuring the horizontal displacement of their apical portions (19).

**Continuous High Resolution Measurement of Growth Rate.** An angular transducer with a counterbalanced arm was used for measurement of the bending of first internode sections treated with IAA or IAA conjugates. The movement of the arm of the transducer, caused by bending of a section, was monitored on a strip chart recorder (19) in such a way that a 1-mm displacement from the vertical resulted in a 50-mm pen displacement.

**IAA and IAA Conjugates Used.** Amounts of IAA (Sigma) ranging from 10 to 1000 pmol/section were used in experiments in which the growth rate of bean first internode sections was measured continuously. IAA-L-Ala[3], IAA-glycine (both from

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

[3] Abbreviations: IAA-L-Ala, indole-3-acetyl-L-alanine; IAA-L-Asp, indole-3-acetyl-L-aspartate.

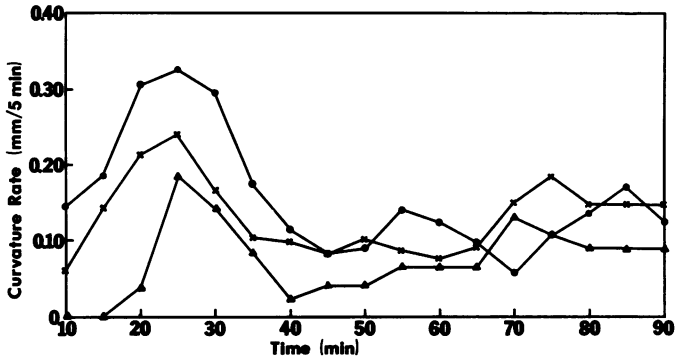


FIG. 1. Rate of the horizontal displacement of bean internode sections treated with different amounts of IAA: (●), 1 nmol; (×), 100 pmol and (▲), 10 pmol/stem section. At least four sections were used for each treatment

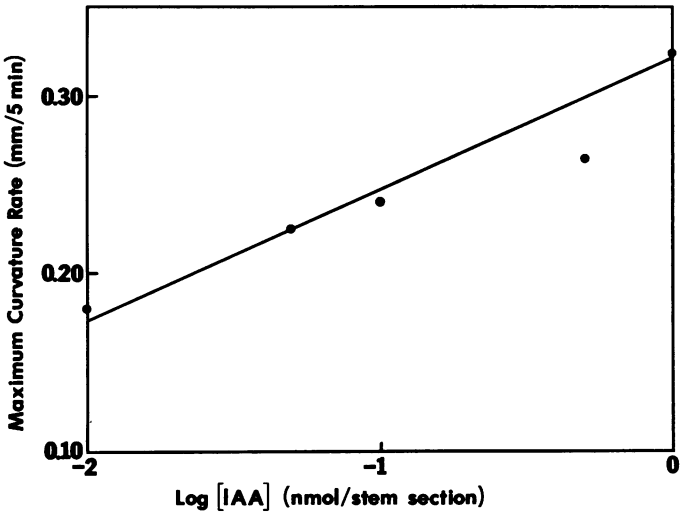


FIG. 2. Relationship between the increase in rate of curvature of bean stem sections at the maximum growth rate and the amount of IAA applied.

Table I. *Content of IAA in Bean Stem Sections at Maximum Growth Rate Treated with Different Amounts of [$^3$H]IAA*

Maximum growth rate occurred 23 min after IAA application. Twenty stem sections were used in each experiment.

| Amount of Applied IAA | | Amount of IAA Found in Stem Sections | | Residual Radioactivity on Discs |
|---|---|---|---|---|
| *mol/stem section* | *total ng /sample* | *ng/sample* | *% applied* | *%* |
| $9 \times 10^{-9}$ | 3195.5 | 23.75 ± 1.90 | 0.74 | 56.0 |
| $9 \times 10^{-10}$ | 319.5 | 2.34 ± 0.15 | 0.73 | 58.4 |
| $9 \times 10^{-11}$ | 31.9 | 0.23 ± 0.03 | 0.73 | 63.4 |

Research Organics Inc.), IAA-L-glutamate, and IAA-L-Asp (both synthetized as described previously [4]) were each tested at 1 nmol/stem section. Both labeled (see below) and nonlabeled IAA conjugates were tested by TLC (8) for free IAA levels. Chemicals utilized in these experiments had no detectable contamination, indicating they contained less than 0.1% free IAA. Commercial reagents, however, varied greatly in this regard and approximately 10% free IAA was present in one lot of commercial IAA-DL-aspartic acid which was tested. Preparations of labeled conjugates contained a few percent (<5%) of a radioactive impurity (4) which ran at the solvent front on chromatograms. A negative reaction to the Ehmann reagent (8) indicated that it was non-indolic. The conditions used for drying on cellulose paper and for 70% acetone extraction did not result in detectable levels of conjugate hydrolysis (20; Cohen, unpublished).

**Application of Labeled Compounds.** [$^3$H]IAA (22 Ci/mmol) was obtained from Amersham and 200,000 dpm was applied to each section. Prior to application, the [$^3$H]IAA was diluted with unlabeled IAA. Thus, the specific activity of the applied [$^3$H]IAA at levels of 913, 91, and 9 pmol/section was 1,240, 12,400 and 124,000 dpm/ng IAA, respectively. [$^{14}$C]IAA-L-Ala and [$^{14}$C]IAA-L-Asp were synthesized from [2-$^{14}$C]IAA using the method previously described (4). [$^{14}$C]IAA conjugates were applied without prior dilution of specific activity. Thus, 96,000 dpm (535 dpm/ng IAA) and 1.02 nmol [$^{14}$C]IAA-L-Ala (42.2 mCi/mmol) were applied to each section. The amount of applied [$^{14}$C]IAA-L-Asp (54.5 mCi/mmol) was 129,000 dpm (690 dpm/ng IAA) and 1.07 nmol/section. In some experiments, [$^{14}$C]IAA-L-Asp with specific activity of 42.2 mCi/mmol was used in amounts equivalent to [$^{14}$C]IAA-L-Ala above.

All labeled compounds were applied by paper discs backed with polyethylene film, exactly as in the internode growth experiments described above. Twenty bean internode sections were used for each experiment and all experiments were done at least twice with two replications each. Standard errors are indicated in tables. Treated discs were removed from sections at the time of their maximum growth rate as determined by high resolution growth analysis.

**Extraction, Purification, and Determination of IAA.** After removal of paper discs, the internode sections were washed in ice cold water for 30 min in order to assure removal of IAA from the tissue surface. The tissue was homogenized in a Waring Blendor in 100 ml 70% acetone containing 1 mg of IAA, left overnight, and then filtered through Eaton-Dikeman 515 paper to remove insoluble residues. The filtrate was then reduced to an aqueous phase of 20 ml *in vacuo*. This was acidified with 1 M $H_3PO_4$ to pH 2.5 and partitioned three times against an equal volume of chloroform. Pooled chloroform phases were dried over anhydrous sodium sulfate, reduced *in vacuo* to dryness, then dissolved in 1 ml of 50% 2-propanol/water (v/v) and placed on a 1.5- × 12-cm DEAE-Sephadex-acetate A-25 (Sigma) column, equilibrated with 50% 2-propanol/water (v/v). The column was washed with 50% 2-propanol/water (v/v) until the eluent was color-free, then the sample was eluted with a linear gradient of 0 to 5% acetic acid in 50% 2-propanol/water (v/v). Collected fractions were tested for IAA by silica gel 60 TLC of a 5-μl aliquot, developed in chloroform:methanol:water (85:14:1), and then using Ehmann's reagent (8) for indole detection. IAA was eluted between 17 and 27 ml. Fractions containing IAA were pooled and evaporated to dryness. The residue was dissolved in 100 μl of 50% methanol/water (v/v) and injected on to a HPLC column of 4.6 mm × 25 cm Partisil 10 ODS-3 (Whatman) with a 2.1-mm × 5-cm Co:Pell ODS precolumn. The mobile phase was 30% methanol/water (v/v) containing 1% acetic acid with a flow rate of 1 ml/min. IAA was eluted between 24 and 26 min. The amount of IAA in the pooled HPLC fractions was determined on the basis of UV absorption at 282 nm using the extinction coefficient $\epsilon = 6060$ (3). UV spectra were recorded with a Beckman model 25 spectrophotometer using 1 cm quartz 1 ml microcuvettes (Hellma). A 500-μl aliquot of the pooled fractions from HPLC was used for radioactivity determination. The samples were counted in ACS scintillation fluid (Amersham) on a Beckman 9000 liquid scintillation counter using the external standard method of obtaining dpm. We found that additional purification of the sample by silica gel TLC, followed by methylation and a second HPLC step resulted in no detectable change in specific activity of the recovered IAA.

**Calculations.** Knowing how much total IAA was recovered

Table II. *Distribution of Applied [$^3$H]IAA in Bean Stem Sections*

IAA ($9 \times 10^{-9}$ mol) was applied to each section. Twenty sections were used in each experiment.

| Time from Application | Amount of IAA Found in Tissue | | | IAA Found in Untreated Halves | Residual Radioactivity on Discs |
|---|---|---|---|---|---|
| | Treated halves | Untreated halves | Whole sections | | |
| *min* | | *ng* | | % | % |
| 23 | 19.4 ± 0.37 | 1.4 ± 0.23 | 21.3 | 6.6 | 49.6 |
| 120 | 37.5 ± 1.16 | 2.0 ± 0.23 | 39.5 | 5.0 | 23.3 |

Table III. *Response of First Internode Sections of Bean Plants to Some IAA Conjugates*

One nmol IAA or IAA conjugate was applied to each section. Values are mean ± SE for $n = 20$.

| Compound | Curvature of Stem Sections | |
|---|---|---|
| | After 1.5 h | After 3 h |
| | *Δmm* | |
| IAA | 1.75 ± 0.30 | 3.27 ± 0.34 |
| Ila-L-Ala | 1.06 ± 0.16 | 2.17 ± 0.28 |
| IAA-L-Glutamate | 1.16 ± 0.18 | 1.54 ± 0.21 |
| IAA-Glycine | 0 ± 0.20 | 0 ± 0.20 |
| IAA-L-Asp | 0 ± 0.19 | 0 ± 0.15 |

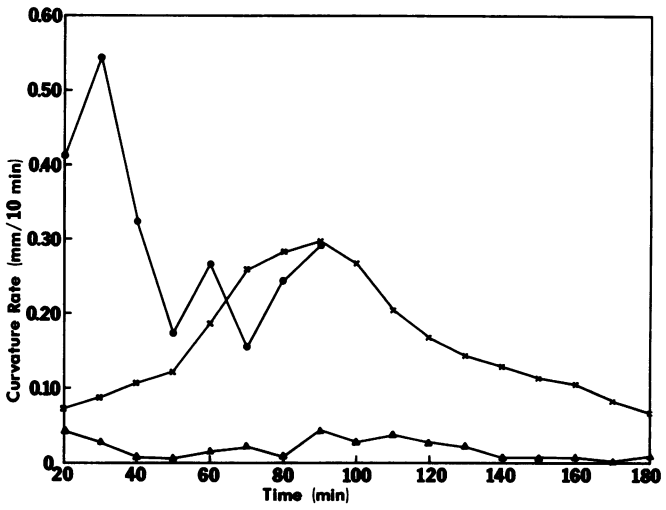


FIG. 3. Rate of the horizontal displacement of bean stem sections treated with 1 nmol/stem section of IAA (●), IAA-L-Ala (×), and IAA-L-Asp (▲). At least 10 stem sections were used for each treatment.

and the radioactivity of the sample, it was possible to make corrections for the amount of radioactive IAA lost during purification, using the reverse isotope dilution method (13). The per cent recovery of the radiolabeled IAA is identical to the per cent recovery of unlabeled carrier IAA added to the extracts. The radioactivity attributable to IAA in the bean first internode sections ($R$) was calculated from equation 1:

$$R = \frac{\text{carrier IAA added}}{\text{carrier IAA recovered}} \times \text{radioactivity of recovered sample} \quad (1)$$

## RESULTS

**Relation between Concentration of Applied IAA, Its Content in Bean Internode Sections, and the Growth Response.** The IAA-induced growth of bean internode sections, monitored continuously with a transducer, showed a maximum rate 20 to 25 min after treatment, for all levels of IAA application studied (Fig. 1). We observed also two other possibly significant peaks in the growth rate, occurring at 25- to 30-min intervals, but they were much lower in intensity than the first. The maximum growth rate was dependent on the amount of IAA applied (Fig. 2), showing a linear relation between the log of IAA dose and the growth response. Control sections without IAA showed no bending response.

Using the reverse isotope dilution method (13), we were able to show directly that the growth response of IAA-treated bean internode sections was closely related to the amount of this hormone found in the tissue. As shown in Table I, only 0.7% of applied [$^3$H]IAA was found as free IAA at the time of maximum rate of growth (after 23 min of treatment). The percentage of IAA found in the tissue was the same for the three levels of IAA used. This demonstrates that the amount of labeled IAA found in the tissue was proportional to the amount of applied IAA. After 23 min of treatment, approximately 60% of the radioactivity remained on the paper disc.

The reverse isotope dilution assay indicated that very little lateral movement of applied [$^3$H]IAA occurred in bean internode sections during the time of the experiment. Almost all [$^3$H]IAA, even after 2 h of treatment, was found in the lateral half which was treated with IAA (Table II). Thus, the decline in growth rate observed after 25 min is not due to lateral movement of the applied IAA to the untreated half.

**Relation between the Growth Response of Bean First Internode Sections to Some Amino Acid IAA Conjugates and Their Hydrolysis in Bean Tissue.** The results presented above indicate that the bean first internode bioassay used in our experiments is a good model system for studying the relationship between the amount of applied IAA and the growth response. Using this model system, we investigated the relation between the hydrolysis of some IAA conjugates and their ability to elicit a growth response.

It has been shown that IAA-amino acid conjugates can vary in biological activity based on the identity of the constituent amino acid. The activity of these compounds also differed greatly in different plant systems studied (1, 11,15, 21). For example, IAA-Asp showed high activity in the coleoptile straight growth *Avena* bioassay, but its activity in the pea hypocotyl bioassay was very low (1, 11). IAA-L-Ala seems to be one of the most active conjugates in the different plant systems which have been studied (11, 15, 16). In a preliminary experiment, we checked the activity of several IAA-amino acid conjugates in the bean first internode bioassay (Table III). Of the four IAA conjugates tested, IAA-L-Ala was most active, although its activity was lower than that of IAA. The difference was more pronounced after 3 h of treatment. IAA-L-Asp and IAA-glycine did not show any significant activity in this bioassay, although a small bending response could be detected using the growth recorder with IAA-L-Asp (Fig. 3).

To study the relationship between the growth response and the level of IAA in the tissue resulting from hydrolysis of IAA conjugates, IAA-L-Ala and IAA-L-Asp were chosen. The growth rate of bean internode sections treated with these two com-

Table IV. *Content of IAA in Bean Stem Sections at Maximum Growth Rate Treated with IAA, IAA-L-Ala, and IAA-L-Asp*

Maximum growth rate: after 25 min for stem sections treated with [$^3$H]IAA and 90 min for sections treated with [$^{14}$C]IAA conjugates. Twenty stem sections were used in each experiment.

| Compound | Amount of Applied IAA | | Amount of IAA Found in Stem Sections | | Residual Radioactivity on Discs |
|---|---|---|---|---|---|
| | *nmol/stem section* | *ng/sample* | *ng/sample* | *% applied* | % |
| IAA | 1.00 | 3500.0 | 23.7 ± 1.90 | 0.68 | 56.0 |
| IAA-L-Ala | 1.02 | 3570.0 | 8.7 ± 0.88 | 0.24 | 24.3 |
| IAA-L-Asp | 1.07 | 3745.0 | 0.8 ± 0.11 | 0.02 | 28.9 |

Table V. *Per Cent Hydrolysis of IAA-L-Ala and IAA-L-Asp in Bean Stem Sections at the Maximum Growth Rate*

A, Calculated from experiments with labeled IAA conjugates in comparison to IAA applied in the same amount of 1 nmol/stem section (Table IV). B, Calculated from growth rate curves (Fig. 4) in comparison to the log/linear relationship between amount of IAA in tissue and maximum curvature rate.

| IAA Conjugate | Released IAA | |
|---|---|---|
| | A | B |
| | % | |
| IAA-L-Ala | 36.7 | 41.8 |
| IAA-L-Asp | 3.4 | 7.4 |

pounds, monitored continuously using a high resolution growth recorder, was much lower than for sections treated with IAA (Fig. 3). Also, the maximum growth rate occurred 1 h later than for sections treated with IAA. A large difference was also observed in the magnitude of the growth rate of sections treated with the two IAA conjugates investigated. The maximum rate obtained with IAA-L-Asp treatment was much less than that seen with IAA-L-Ala treatment. Using [$^{14}$C]IAA-L-Ala and [$^{14}$C]IAA-L-Asp and the reverse isotope dilution assay, it was possible to find out directly how much IAA was found in the tissue resulting from the hydrolysis of the $^{14}$C compound applied to the bean internode sections (Table IV). The comparison of the amount of IAA found at the time of the maximum growth rate in the sections treated with IAA and IAA conjugates, permitted calculation of the relative amount of hydrolysis of the amino acid conjugates investigated assuming no degradation of the released IAA. The rate of uptake of the labeled compounds, as indicated by residual radioactivity in the filter paper discs, was similar for the three indole acids (Table I and IV). In comparison to IAA levels found following treatment with the free acid, approximately one-third as much IAA was released from [$^{14}$C]IAA-L-Ala after 90 min of treatment, and only a few percent from [$^{14}$C]IAA-L-Asp (Table VA). The percentage of hydrolysis calculated from the growth rate curves (Fig. 3), in comparison to the log/linear relationship between amount of IAA in tissue and maximum curvature rate, was very close to the amount of IAA released for both IAA conjugates studied—41.8% for IAA-L-Ala and 7.4% for IAA-L-Asp (Table VB).

## DISCUSSION

The relationship between IAA levels and growth presented in this paper differs from most prior studies in that a rapid response was measured and the amount of hormone applied was related to the maximum bending rate rather than total growth. In addition, the amount of hormone in the tissue resulting from the unilateral application through noninjured cells was determined. These results suggest that the timing of the maximum growth rate resulting from IAA application and its duration are not concentration dependent over the range examined. In addition, the decrease in growth rate after 20 to 25 min is not due to decreased levels of IAA in the tissue or to changes in the internal distribution of IAA (Table II). Thus, the response is properly described as a transient increase in growth rate, only the magnitude of which shows a log/linear relationship to the amount of IAA applied.

A differential growth response resulting from unilateral auxin application was measured in this study; thus, our experimental system differs somewhat from the straight growth response investigated by other workers (10). Nevertheless, Vanderhoef *et al.* (23), studying straight growth of soybean hypocotyls, reported a similar peak in growth rate, occurring 20 to 25 min after IAA treatment; however, only one IAA concentration was tested. A log/linear relationship between the amount of $GA_3$ applied and growth rate changes of *Avena* stem segments was shown recently by Jusaitis *et al.* (17). It is interesting that, despite the rather complex kinetics of the growth responses (Fig. 1), the total curvature obtained after only a few hours nevertheless obeys a log/linear relationship with hormone applied (19; see also 25).

Several prior studies have provided indirect evidence that the biological activity of IAA conjugates was related to the degree of hydrolysis to yield the free acid (6). Hangarter and Good (16) showed that release of $^{14}CO_2$ from [$^{14}$C]IAA-amino acid conjugates labeled in the carboxyl carbon of IAA was related to the biological activity. These results were obtained, however, for long term effects such as ethylene release (9 h incubation) and callus growth. Also, no attempt was made to show that IAA resulted from metabolism of the conjugate. Indeed, after prolonged incubation with [$^{14}$C]IAA conjugates, no radioactivity was found on the thin layer chromatogram autoradiographs corresponding to the $R_F$ of the free acid. Nowacki *et al.* (20) showed that the ester [$^{14}$C]IAA-*myo*-inositol applied to corn endosperm would yield small amounts of [$^{14}$C]IAA in the shoot, and Epstein *et al.* (9) measured the rate of [$^{14}$C]IAA formation resulting from [$^{14}$C] IAA-*myo*-inositol applied to the endosperm of germinating corn. Only a general attempt was made in these studies to relate their results to the amount of IAA required for growth based on prior literature values. Although Hamilton *et al.* (14) and more recently Haissig (12) have shown that plant tissue homogenates can hydrolyze ester conjugates of IAA, we are not aware of similar data with regard to IAA-amino acid conjugates. Davidonis *et al.* (7) showed that the conversion of 2,4-D amino acid conjugates to free 2,4-D occurs in soybean root callus; however, the relationship between this conversion and biological activity was not determined. Thus, to our knowledge, this is the first demonstration that amide-linked IAA conjugates applied to plant tissue are hydrolyzed to yield free IAA and that the amount of IAA found is closely related to the biological activity observed. This is true for both a relatively active conjugate such as IAA-L-Ala and also for IAA-L-Asp, which when applied to bean stem

sections shows very little activity. So far, the only naturally occurring amide conjugate of IAA which has been identified is IAA-L-Asp, found in seeds of soybean (5), and preliminary evidence of its occurrence in bean was provided by Tillberg (22).

Thus, we have shown that the amount of IAA in the tissue is related to the maximum rate of curvature by a log/linear relationship, that tissue levels of IAA are proportional to the amount applied, and that the biological activities of IAA-conjugates are directly related to the ability of the plant tissue to use them as a source of free IAA.

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