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Effect of Exogenous Indole-3-Acetic Acid and Indole-3-Butyric Acid on Internal Levels of the Respective Auxins and Their Conjugation with Aspartic Acid during Adventitious Root Formation in Pea Cuttings

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ABSTRACT

The influence of exogenous indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) on the internal levels of these auxins was studied during the first 4 days of adventitious root formation in cuttings of *Pisum sativum* L. The quantitations were done by high performance liquid chromatography with spectrofluorometric detection. IBA, identified by combined gas chromatography-mass spectrometry (GC-MS), was found to naturally occur in this plant material. The root inducing ability of exogenous IBA was superior to that of IAA. The IAA level in the tissue increased considerably on the first day after application of IAA, but rapidly decreased again, returning to a level twice the control by day 3. The predominant metabolic route was conjugation with aspartic acid, as reflected by the increase in the level of indole-3-acetylaspartic acid. The IBA treatment resulted in increases in the levels of IBA, IAA, and indole-3-acetylaspartic acid. The IAA content rapidly returned to control levels, whereas the IBA level remained high throughout the experimental period. High amounts of indole-3-butyrylaspartic acid were found in the tissue after feeding with IBA. The identity of the conjugate was confirmed by ^{1}H-nuclear magnetic resonance and GC-MS. IBA was much more stable in solution than IAA. No IAA was detected after 48 hours, whereas 70% IBA was still recovered after this time. The relatively higher root inducing ability of IBA is ascribed to the fact that its level remained elevated longer than that of IAA, even though IBA was metabolized in the tissue. Adventitious root formation is discussed on the basis of these findings.

Auxins and cytokinins appear to be the major endogenous factors regulating adventitious root formation. The cytokinins are considered inhibitory (6, 7), whereas the auxins are stimulatory (13, 21). Although IAA is probably the major auxin, the possibility that other naturally occurring ones like IBA, PAA, 4Cl-IAA, and IPA (12, 26) participate in regulation cannot be discounted.

In a previous work the endogenous IAA dynamics during adventitious root formation in pea was investigated (21). In this plant material well organized primordia develop during the first 4 d. The endogenous IAA level in the lowest part of the cutting base, where roots are formed, did not vary during this period. On the contrary, regulatory factors apparently maintained the IAA level at a steady state. That regulation was involved was deduced from the fact that the endogenous content of the naturally occurring IAAsp[2] started to increase on the day after cutting. This increase was believed to reflect a rapid conjugation of excessive IAA, thereby preventing its accumulation in the tissue. However, a certain level of IAA was found to be necessary for root formation. Decapitation and disbudding, which resulted in an almost complete inhibition of root formation, led to an 80% reduction in the IAA level in the root forming part.

It is well known that when supplied to the rooting solution IAA and IBA differ in their root inducing ability (17, 20, 24, 29). IBA treatment usually increases root number dramatically while simultaneously stimulating root formation along the whole stem base. IAA at the same concentration may even reduce root number, and the roots formed only appear in the lowermost part of the cutting base (10, 24). The reason for these differences in root inducing ability is not fully clear. Higher stability of IBA (15), slow and continuous release of IAA from IBA (9, 11), and release of IBA through hydrolysis of conjugates (29, 30) have been proposed as causal mechanisms.

Exogenous IAA is rapidly metabolized in the tissues, and IAAsp has been reported to be the major metabolite in pea (1, 18) as well as in other dicotyledons (8). The metabolism of IBA in the tissues is more obscure. Andreae and Good (2) found that IBA was converted to a substance identified putatively as IBAsp. Recently, in a study of the fate of exogenous ^{3}H-IBA in the tissues of *Vigna radiata,* Wiesman *et al.* (30) unequivocally identified IBAsp as the major—but not the only—metabolite.

In this contribution the effects of exogenous IAA and IBA on their internal levels and on their conjugation with aspartic acid during adventitious root formation in pea cuttings were examined.

MATERIALS AND METHODS

Plant Material

Seeds of pea, *Pisum sativum* L. cv Marma, were imbibed in tap water for 6 h and germinated in trays with garden soil.

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[2] Abbreviations: IAAsp, indole-3-acetylaspartic acid; IBAsp, indole-3-butyrylaspartic acid; BHT, butylated hydroxytoluene; CK, cytokinins.

After 10 d cuttings were taken, with the third internode acting as the stem base, and placed in black plastic pots with 1 L of modified Hoagland solution at 25% of full strength (20). Ten cuttings were placed in each pot. The growth of the seedlings as well as the rooting of the cuttings was performed in a growth chamber (20°C, RH 70%, photon flux density 250 μmol m^{-2} s^{-1} [400–700 nm]).

IAA and IBA were dissolved with an equivalent amount of KOH in water and added to the rooting solution to reach a final concentration of 10 μM. The solution was not renewed during the 4 d the experiment lasted. In rooting experiments the cuttings were supplied with fresh nutrient solution without auxins after 4 d, and the roots were counted after 13 d. Samples were collected daily.

Extraction

Thirty stem bases, 3 to 4 cm long, were excised and homogenized in liquid nitrogen using an Ultra-Turrax T 25 homogenizer. The resultant powder, about 1.5 g, was extracted for 1h at 4°C in 30 mL of 5 mM K-phosphate buffer (pH 6.5), containing 1-[^{14}C]IAA, (44,000 cpm, specific activity 11.5 MBq/mg, Amersham International) as internal standard and BHT as an antioxidant. The extract was filtered through a glass fiber filter (Whatman GF/C 1.2 μ), which was subsequently washed with 15 mL of the extraction buffer. The extract was then adjusted to 50 mL and divided into three portions, each comprising 15 mL.

Purification by Solid-Liquid Extraction

BondElut cartridges with 0.5 g C_{18} sorbent (Analytichem International) were activated with 2 × 2 mL ethanol and conditioned with 2 mL double-distilled water and 5 mM K-phosphate buffer with a pH of either 6.5 or 2.5 (see below).

The samples were first passed through the columns conditioned to pH 6.5. The columns were then washed with 6 mL 5 mM K-phosphate buffer (pH 6.5). The eluate, containing the putative IAA, IAAsp, IBA, IBAsp, and other polar acidic indoles, was acidified to pH 2.5 and applied to the C_{18} columns conditioned to pH 2.5. The columns were washed with 2 mL double-distilled water and 2 mL ethanol:acetic acid:water (v:v) 20:2:78. The washings were combined and as this fraction contained a great part of IAAsp it was collected and applied to new C_{18} columns (pH 2.5), and eluted as described below. The columns were washed with an additional 2 mL double-distilled water and 0.5 mL 100% methanol. The IAA, IBA, and their conjugates were then eluted with 1 mL 80% methanol. The solvents were evaporated *in vacuo* at 30°C and the residue dissolved in 0.5 mL 5% methanol in double-distilled water.

Quantitation by HPLC

Samples (200 μL) were analyzed on a reversed phase HPLC, consisting of an Altex pump, model 110 A, a Shimadzu spectrofluorophotometer RF-510 (excitation 291, emission 360), and a Merck Hitachi 655A-12 integrator. The column used was a Hypersil ODS, 3 μm particle size, 60 mm long (Shandon, England), and the mobile phase was 10% acetonitrile, 2% acetic acid and water with a flow of 2 mL/min. The system was operated isocratically. IAAsp eluted after 1.7 min, IAA after 4.5 min, IBAsp after 7.4 min, and IBA after 23.3 min. Quantitations were made by comparing the peak areas with known amounts of IAA and IAAsp. IAA and IAAsp were purchased from Sigma and Research Organics, respectively. For calculation of IAA recovery 200 μL of each sample were taken for scintillation counting, using a Packard TRI-CARB 460 C liquid scintillation counter. The recovery of known amounts of authentic IAAsp and IBA was approximately 80% for both.

Every experiment was performed at least three times, always in triplicate. Mean values and standard deviations were calculated from the pooled values.

The fractions from the HPLC step corresponding to IBA and IBAsp were collected and either diluted with 5 mM K-phosphate buffer (pH 2.5), applied on a C_{18} column activated to pH 2.5 as described above, and eluted with 1mL of 80% methanol, or evaporated to dryness under a stream of nitrogen. The sample containing the putative IBAsp was then analyzed by ^{1}H-NMR followed by GC-MS, while the putative IBA fraction was analysed by GC-MS.

^{1}H-NMR of IBAsp

The IBAsp sample was dissolved in D_4-methanol (CD_3OD), and the ^{1}H-NMR spectrum was recorded on a Bruker AM instrument, 400 MHz. The acquisition time was 2 h. The sample was then reduced to dryness and further characterized by GC-MS.

GC-MS

IAA and IAAsp were identified earlier (21).

The putative IBA and IBAsp were dissolved in 1 mL of 90% diethyl ether + 10% methanol and then methylated with diazomethane according to Schlenk and Gellerman (25).

GC-MS was performed on a Finnigan MAT 4600, using electron impact ionization with an ionization energy of 70 eV. Capillary GC comprised a DB1 column, 25 m and 0.25 mm i.d. The temperature was programmed from 150 to 270°C at a rate of 8°C/min. The injector temperature was 250°C and the interface temperature was 260°C. The injections were made in splitless mode.

RESULTS

IBA occurred naturally in pea shoots. Its identity was confirmed by GC-MS (Fig. 1). The mass spectra of the purified and methylated pea extract is consistent with the mass spectra of the methyl ester of IBA obtained by Schneider *et al.* (26) and Epstein *et al.* (12). Highest concentrations of IBA were found in the shoot apex (35.5 ± 4.5 ng/g fresh weight). IBA was only occasionally detected in samples from the root forming zone.

Control levels of IAA and IAAsp (Figs. 2, 3) were the same as those found earlier (21).

There were no quantitative differences in internal auxin levels between the basal 1.5 cm and the uppermost 1.5 cm of the stem base (data not shown). Therefore, the whole stem base was included in the experiments.

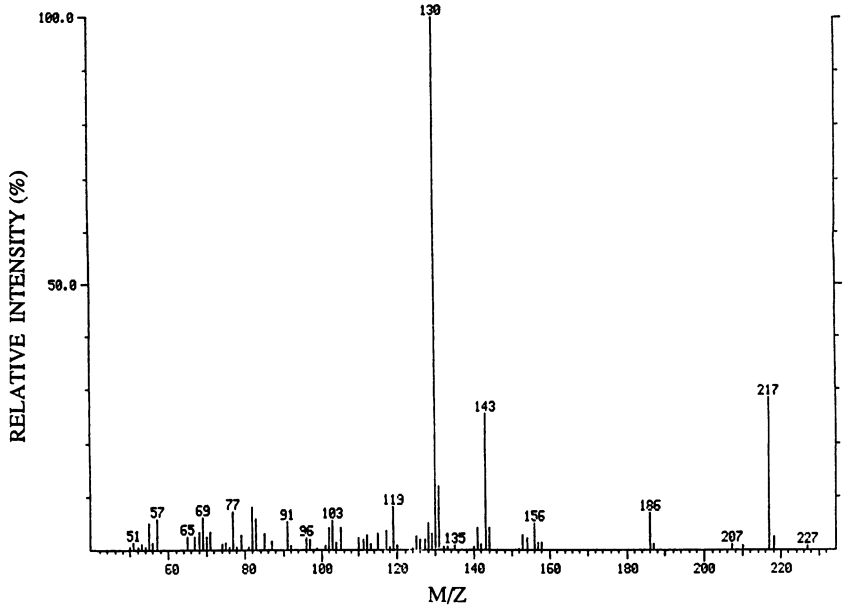

Figure 1. The 70 eV electron impact mass spectrum of indole-3-butyric acid methyl ester obtained from a purified extract of pea. Characteristic ions are m/z 217 (M+), 143 (R-CH = CH2), and 130 (quinolinium ion).

Roots in untreated as well as IAA-treated cuttings developed in the basal 1.5 cm of the cuttings. There was an average of 25 ± 1 roots in controls and 17 ± 6 in the IAA-treated cuttings. The internal IAA level in the stem bases increased 100 times during the first day of IAA treatment; thereafter it decreased, and by d 3 it was twice that of the control (Fig. 2). As the IAA level decreased, the IAAsp level showed a corresponding increase (Fig. 3). The IAAsp level declined after 3 d in both IAA-treated and untreated cuttings.

In response to the IBA treatment 176 ± 23 roots developed, which were spread out along the whole stem base. The IBA level increased to at least 30 times the IAA level of controls and remained elevated throughout the experimental period (Fig. 2). Part of the IBA taken up was probably conjugated with aspartic acid. The identity of IBAsp was confirmed by GC-MS (Fig. 4). Since no authentic IBAsp was available, its identity was verified by ^{1}H-NMR (Fig. 5). ^{1}H-NMR chemical shifts revealed the presence of the indole moiety between ∂ 6.97 and 7.53 ppm, a hydrocarbon chain, consisting of three carbon atoms adjacent to a carbonyl group, between ∂ 2.02 to 2.79, and two protonated hydrocarbons from aspartic acid at ∂ 4.70 and between ∂ 2.75 and 2.81. Protons from the acidic moiety and the nitrogen cannot be seen in the spectrum owing to proton exchange with deuterium from the solvent. The molecular ion (M+) in the GC-MS spectrum is consistent with the M+ expected for IBAsp (m/z 346). The fragmentation pattern is the same as that obtained in the GC-MS spectrum by Wiesman *et al.* (30) and is characteristic of 3-substituted indoles.

The lack of IBAsp standard also prevented us from quantifying the concentration of IBAsp. Nevertheless, it is evident from the HPLC chromatogram that IBAsp increased steadily during the 4 d studied and that it was present in microgram amounts (data not shown).

IBA treatment caused the IAA level to increase about 10 times during the first day. Thereafter it decreased until the control level was reached again on d 4 (Fig. 2). The level of IAAsp also increased after the IBA-treatment (Fig. 3).

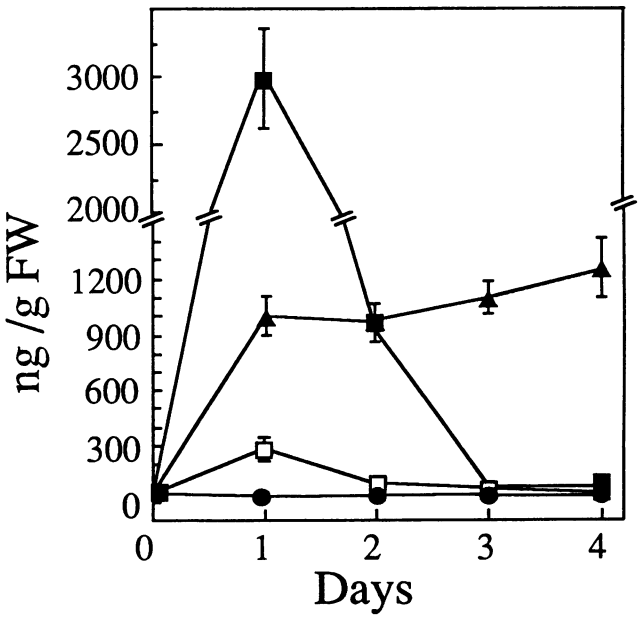

Figure 2. Internal levels of IAA and IBA in the stem bases of pea cuttings after applying IAA or IBA to the rooting solution. The rooting solution was not changed during the first 4 d of the experimental period. Mean values of at least three independent experiments with three replicates within each experiment. Horizontal bars showing SD (within symbols if not indicated). (■), Internal IAA level in IAA-treated cuttings; (□) internal IAA level in IBA-treated cuttings; (▲), internal IBA level in IBA-treated cuttings; (●), endogenous IAA level in untreated cuttings.

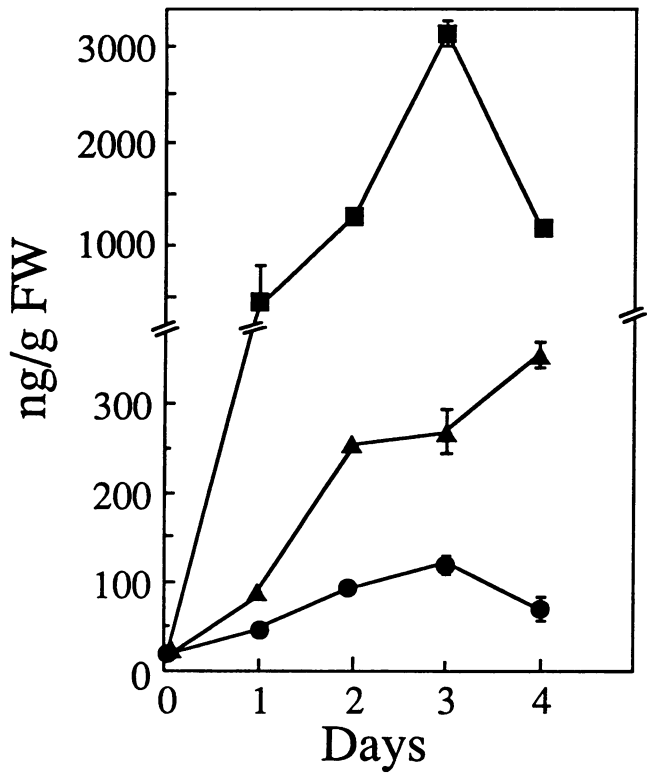

Figure 3. Endogenous levels of IAAsp in the stem bases of IAA- or IBA-treated pea cuttings. Mean values for three experiments each made in triplicate ± SD. SD bars within symbols if not indicated. (■), IAA-treated cuttings; (▲), IBA-treated cuttings; (●), untreated cuttings.

DISCUSSION

IAA, IAAsp, and IBA were found to be natural constituents in pea shoots (21; Fig. 1). Treatment with exogenous IAA and IBA brought about a dramatic increase in their internal levels (Fig. 2). The treatment also led to a rapid increase in levels of IAAsp and IBAsp (Fig. 3), indicating that the auxins are rapidly metabolized in the tissue. Our results are thus partly in accordance with those obtained by Wiesman *et al.* (29, 30).

HPLC chromatograms from untreated cuttings showed a small peak with the same retention time as IBAsp, suggesting that IBAsp appears naturally, although at low concentrations (data not shown).

The IAAsp level increased as a result of IAA treatment and continued to increase during the decrease in the IAA level, indicating that conversion to IAAsp is the predominant metabolic route for regulation of the IAA content. However, the possibility that other metabolic systems participate in this process cannot be excluded. Grambow (14) and Smulders *et al.* (27) reported that a substantial part of the IAA or NAA taken up was first glucosylated and then incorporated into other substances. Decarboxylative or nondecarboxylative oxidation might also constitute a part of the regulatory system (23). The occurrence and relative importance of these two oxidative systems is not clear. The IAAsp level decreased after 3 d, both in controls and in IAA-treated cuttings (Fig. 3). Norcini and Heuser (19) obtained a similar result. The drop in the IAAsp level during the fourth day was not accompanied by a rise in the IAA level (Fig. 2). Some IAAsp may have been hydrolyzed and the released IAA rapidly metabolized again, or IAAsp could have been catabolized directly as shown by Tsurumi and Wada (28) and Plüss *et al.* (22). It is reasonable to assume that root formation is related to the level of the free auxin. IAAsp supplied to the rooting solution had no significant effect on root formation (21, 22), and did not

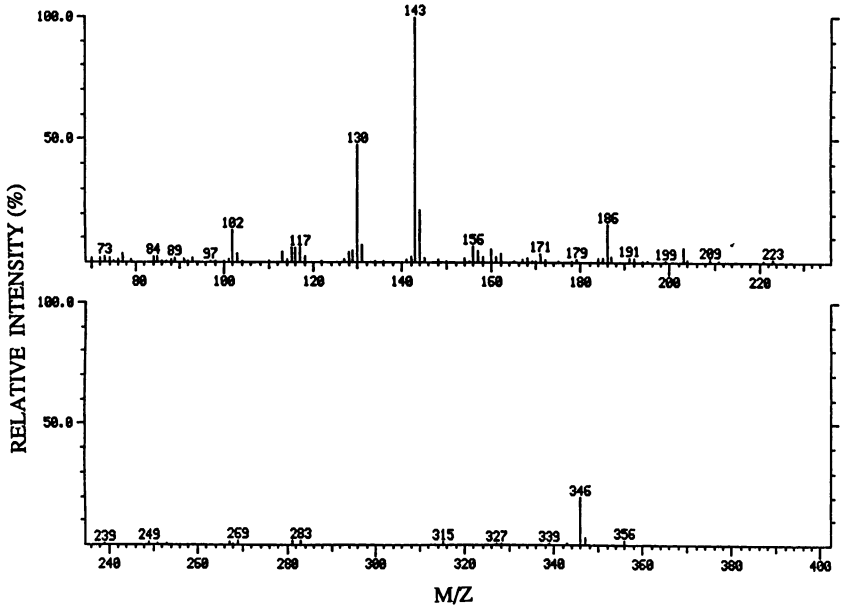

Figure 4. The 70 eV mass spectrum of IBAsp methyl ester obtained from a purified extract of pea. Characteristic ions are m/z 346 (M+), 186 (R-CH2-CH2-CH2-C = O), 156, 143(R-CH = CH2), and 130 (quinolinium ion).

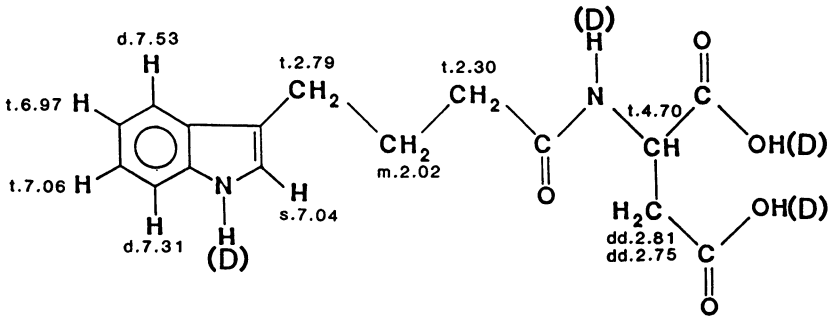

Figure 5. Chemical shifts from ^{1}H-NMR of IBAsp in deuterated methanol, CD_3OD. The acidic protons were exchanged with deuterium from the solvent. s, singlet; d, doublet; dd, double doublet; m, multiplet; t, triplet.

restore rooting in pea cuttings in which the endogenous IAA level had decreased by decapitation and disbudding (21). Several studies indicate that IAAsp is inactive and at most releases minute amounts of IAA (5, 16, 22).

Both the IAA and IAAsp level increased in IBA-treated cuttings (Figs. 2, 3). Whereas the IAA level returned to control levels within 4 d, the IAAsp level continued to increase during the period studied. Increased IAA levels after IBA treatment was also found by Dunberg *et al.* (9), and the conversion of [^{14}C]IBA to [^{14}C]IAA was demonstrated by Epstein and Lavee (11). The steady increase of the IAAsp level could be due to a continuous conversion of IBA to IAA while the excess IAA was rapidly conjugated to IAAsp. However, this remains to be confirmed by use of labeled compounds.

The higher stability of IBA in solution implies that it is present in the rooting solution for a longer period than IAA (Table I). Thus, even though a considerable portion of IBA was conjugated, or converted to IAA, the actual level of IBA in the tissue did not decrease, probably since the supply of IBA was not interrupted. Wiesman *et al.* (29, 30) demonstrated that when [^{3}H]IBA was withdrawn from the rooting solution the internal [^{3}H]IBA level decreased as rapidly as the [^{14}C]IAA level. They therefore concluded that the superior rooting ability of IBA was not due to its slower metabolism in the tissue, as suggested earlier by Gorter (15) and discussed by Hartmann and Kester (17). Instead, they proposed that the IBA conjugates, and IBAsp in particular, are a better source of free auxins than the IAA conjugates.

The IBA treatment resulted in root formation along the entire stem base of pea cuttings and in an increase in the number of roots formed. However, to achieve a considerable stimulation IBA has to be present in the rooting solution for more than 2 d (10); otherwise, roots develop only in the lowest part of the stem base, as in IAA-treated cuttings and in untreated controls, despite the fact that both IBA and IBAsp levels had increased dramatically by that time. IBAsp occurred in microgram amounts after 2 d (data not shown). This indicates the importance of a continuous supply of the auxin for several days. In pea cuttings high IBAsp levels in the tissue could evidently not substitute for continuous IBA supply. In untreated cuttings, the IAA level in the upper part of the stem base, where no roots formed, was the same as in the root forming zone (21). Thus, the IAA content was apparently not the factor limiting root formation in this tissue. A 100-fold increase in the internal IAA level that occurred during the first few days (Fig. 1) did not alter this rooting pattern. However, 1 to 2 d later high levels of IBA induced root formation along the whole stem base. By that time the IAA level had decreased, but was still about twice as high compared with the control level. For stimulation to occur obviously requires that the internal auxin level remains high during d 3 and 4 as well. Thus, the number of roots formed should be regarded as a function of both concentration and time. Root formation is believed to be regulated by a balance between the stimulatory effect of auxin and the inhibitory of endogenous factors. It has been hypothesized that CK synthesized in the root system and transported through the xylem are endogenous inhibitors of root formation in the intact shoot (6, 7). Thus, removal of the root system, which interrupts the flow of CK to the shoot, would allow adventitious root formation. In pea the CK content in the root forming tissue falls during the first day after cutting (6). Until the CK content has dropped below a certain threshold level, root initiation, which is stimulated by auxins, will not take place. This inhibition appears to be of an "all-or-nothing" nature, since in the upper part of the stem base, it cannot be counteracted by the high concentrations of IAA or IBA that occur during the first 2 d. The time factor suggests that the inhibition disappears gradually upward, followed by root initiation promoted by auxins. A concentration-dependent type of inhibition appears to remain, as indicated by the fact that neither the control level of IAA, about 36 ng (g fresh weight)$^{-1}$, nor levels twice as high in IAA-treated cuttings induced root initiation in this tissue. The IBA level, on the other hand, was about 30 times higher compared with the control level and was evidently sufficient for root initiation.

Our results indicate that the higher root inducing ability of IBA is the consequence of two important facts: First, IBA exerts its effects on its own, not through the IAA released, as

Table I. *Stabilities of IAA and IBA in Solution*

IAA or IBA was dissolved in an equivalent amount of KOH in water and diluted with water to a final concentration of 500 ng/mL. The solutions were kept in black pots at room temperature. The concentrations in the pots were estimated by HPLC at time 0 and after 24 and 48 h.

Time	IAA	IBA
h	%	
0	100	100
24	17.6	83.1
48	ND[a]	70.4

[a] Not detectable.

suggested by Dunberg *et al.* (9) and Epstein and Lavee (11). The increased IAA level after IBA treatment is probably insignificant as far as root formation is concerned. Second, the IBA level remained elevated longer than the IAA level, which may partly be due to the fact that IBA is relatively more stable in the rooting solution.

Despite early indications that IBA occurs naturally in plant tissues (4), this auxin has widely been regarded as being purely synthetic. During recent years, however, IBA has been identified in pea (Fig. 3; 3, 26) and maize (12). In our studies the highest concentrations of IBA were found in the shoot apex, the IBA content of which was somewhat higher than the IAA content (see also ref. 21). Further research is necessary to elucidate the physiological role of endogenous IBA.

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