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Occurrence and *in Vivo* Biosynthesis of Indole-3-Butyric Acid in Corn (*Zea mays* L.)[1]

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ABSTRACT

Indole-3-butyric acid (IBA) was identified as an endogenous compound in leaves and roots of maize (*Zea mays* L.) var Inrakorn by thin layer chromatography, high-performance liquid chromatography, and gas chromatography-mass spectrometry. Its presence was also confirmed in the variety Hazera 224. Indole-3-acetic acid (IAA) was metabolized to IBA *in vivo* by seedlings of the two maize varieties. The reaction product was identified by thin layer chromatography, high performance liquid chromatography, and gas chromatography-mass spectrometry after incubating the corn seedlings with [^{14}C]IAA and [$^{13}C_6$]IAA. The *in vivo* conversion of IAA to IBA and the characteristics of IBA formation in two different maize varieties of *Zea mays* L. (Hazera 224 and Inrakorn) were investigated. IBA-forming activity was examined in the roots, leaves, and coleoptiles of both maize varieties. Whereas in the variety Hazera 224, IBA was formed mostly in the leaves, in the variety Inrakorn, IBA synthesis was detected in the roots as well as in the leaves. A time course study of IBA formation showed that maximum activity was reached in Inrakorn after 1 hour and in Hazera after 2 hours. The pH optimum for the uptake of IAA was 6.0, and that for IBA formation was 7.0. The K_m value for IBA formation was 17 micromolar for Inrakorn and 25 micromolar for Hazera 224. The results are discussed with respect to the possible functions of IBA in the plant.

The use of natural and synthetic auxins in horticultural practice is very common, and the ability of auxins to stimulate root formation is well known (16, 20). IBA[2] is the most widely used auxin for rooting purposes in agriculture (16), and although many reports deal with its use in root propagation (16), no attention has yet been paid to its biosynthesis. Several studies provided evidence for the occurrence of IBA as a natural constituent of plants (2, 6, 12–14, 24), and there are some studies on its metabolism. Epstein and Lavee (10) showed that IBA may be converted to IAA in cuttings of grapevine and olive. Andreae and Good (1) reported that IBA-treated tissues accumulated substances that were tentatively identified as indolebutyramide and IBAsp. Chromatographic data provided by Fawcett *et al.* (15) also indicated the formation of IBAsp from exogenous applied IBA. Wiesman *et al.* (27) demonstrated that IBA as well as IAA were rapidly metabolized in mung bean and that conjugation is the major pathway of IAA and IBA metabolism in this tissue. The IBA conjugates were identified as IBAsp and at least two high mol wt conjugates, in which IBA is coupled by an amide linkage (28). An IAA-peptide with a similar mol wt was also demonstrated in *Phaseolus vulgaris* (4). Our report deals with the *in vivo* conversion of IAA to IBA and the identification and characterization of the reaction product.

MATERIALS AND METHODS

Plant Material

Seeds of maize, *Zea mays* L. var Hazera 224 and Inrakorn (treated with fungicides), were soaked with water for 2 h and cultivated under sterile conditions in the presence of 0.1% streptomycin sulfate in the dark at 23°C with 90% humidity. The plant material was harvested after 8 d.

Incubation with [^{14}C]IAA and [^{13}C]IAA

Feeding experiments were carried out with either [1-^{14}C] IAA (Amersham, specific activity 1.85 TBq mol^{-1}) or with [$^{13}C_6$]IAA. The appropriate amount (25 ng [^{14}C]IAA; 500 ng [^{13}C]IAA) was added to 5 mL of 100 mM Mes-KOH buffer, pH 6. For the pH dependence studies, the buffer was brought to the appropriate pH, which was monitored during the incubation time. Leaves, roots, and coleoptiles of maize were cut into 2 mm segments and washed in substrate-free buffer. After drying the plant material on filter paper, 1 g of material was added to the incubation solution for various time intervals at 25°C. Control experiments were carried out without tissue in the feeding solution to exclude chemical conversion of IAA to IBA. Several experiments were performed in the presence of 0.1% streptomycin sulfate. After incubation, the tissue was filtered and washed with 50 mL of substrate-free buffer, homogenized with 70% acetone with an Ultra Turrax (20,000 rpm), and centrifuged for 10 min at 5000*g* and the supernatant evaporated to the aqueous phase. The aqueous residue was adjusted to pH 3.0 with 2 N HCl and extracted twice with equal volumes of ethyl acetate. The combined organic phases were dried over Na_2SO_4 and, after concentration, used directly for TLC, HPLC, and/or GC-MS analysis. The uptake of radioactivity in the tissue as well as the total recovery of radioactivity in the acetone phase after extraction were deter-

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[2] Abbreviations: IBA, indole-3-butyric acid; IBAsp; indolebutyryl-aspartic acid; RIC, reconstructed ion chromatogram.

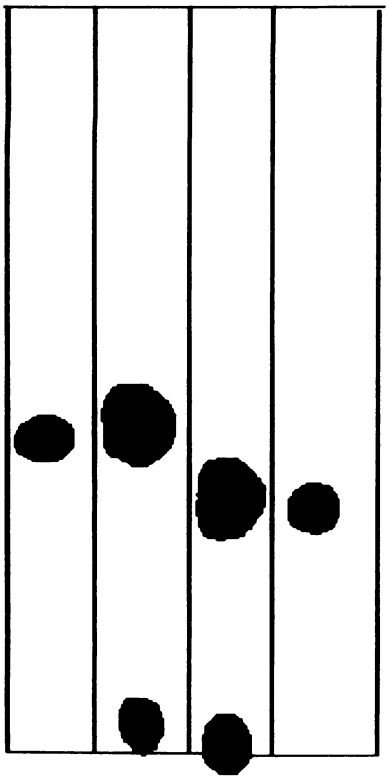

Figure 1. Thin layer chromatograph of methylated samples and of standards of authentic methylated IAA and IBA. The samples used for the TLC analysis were obtained after HPLC of the extract from the corn variety Inrakorn (total seedlings). HPLC conditions were as described in "Materials and Methods." TLC solvent was hexane:ether (60:40, v/v); visualization done with Ehmann reagent.

mined by taking appropriate aliquots and determining their radioactivity (details below).

TLC Analysis

The ethyl acetate fraction was chromatographed on silica gel F_{254} plates (Merck) with chloroform:acetic acid (95:5, v/v) as solvent. Identification was achieved by co-chromatography with authentic standards by fluorescence quenching at 254 nm (R_F values of IAA and IBA were 0.45 and 0.6, respectively). The results were confirmed on silica gel plates with benzene:dioxan (65:35, v/v) as solvent. The chromatogram was then divided horizontally in 0.5 to 1 cm zones and the silica gel was scraped off into scintillation vials. The radioactivity of the different zones was then determined after adding 1 mL methanol and 3 mL of scintillant (Szintillator 199, Packard) in a Packard 2000 CA Tri Carb liquid scintillation counter equipped with an IBM PC/XT computer. Alternatively, the sample was chromatographed on HPLC and the radioactive IAA and IBA fractions were pooled, evaporated to dryness, and methylated with diazomethane (7) prior to TLC analysis on silica gel plates with hexane:ether (60:40, v/v) as solvent. Visualization was done with Ehmann reagent (8).

HPLC Analysis

The methylated IAA and IBA from TLC plates and the ethyl acetate extract were analyzed by HPLC using a reverse-phase column (Biotronik-J., finepak SILC 18-5, Maintal, FRG; pre-column from Guard-PAK, C_{18}, Waters Associates, Milford, MA) and UV detection at 280 nm. Solvents were 55% methanol containing 1% acetic acid for the nonmethylated samples and 60% methanol for the methylated samples. Flow rate was 0.8 mL min^{-1}. Identification was achieved by co-chromatography with authentic standards. Fractions of 0.4 mL were collected and counted as above or used for subsequent GC-MS analysis.

GC-MS Analysis

GC-MS identification was performed with a Finnigan model MAT 4600 using electron impact ionization. The gas chromatograph was equipped with a Durabond-5 column, 30 m × 0.25 mm, 0.25 μm, film (J&W Scientific, Folsom, CA). Temperature program was 140°C for 2 min, following by an increase of 10°C min^{-1} to 200°C and of 20°C min^{-1} to 250°C. Under these conditions, IBA was eluted after 10.5 min. Spec-

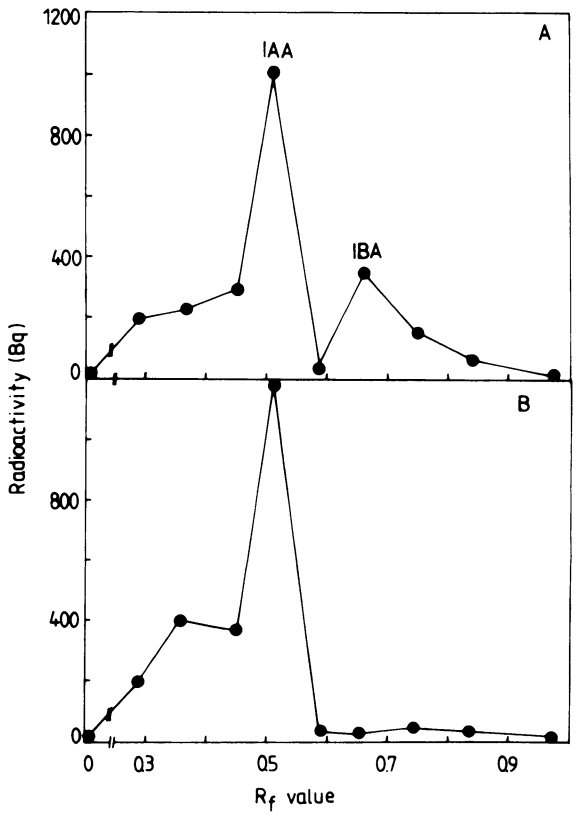

Figure 2. Distribution of [^{14}C] on TLC chromatogram of extract after 1 h incubation of corn leaves (var Inrakorn) with 5 kBq [^{14}C]IAA. Solvent was chloroform:acetic acid (95:5, v/v). A, Sample; B, control without tissue.

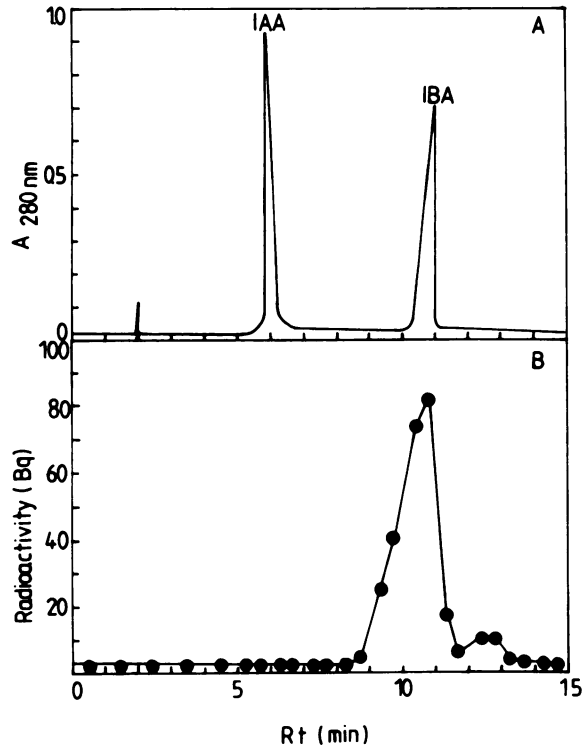

Figure 3. HPLC separation of the IBA fraction from TLC. Solvent was methanol:acetic acid:water (55:1:44, v/v). Flow rate, 0.8 mL min^{-1}. A, Standard chromatogram of IAA and IBA; B, radioactivity profile of the IBA fraction.

tra were taken by both continuous and RIC scans and by selected ion monitoring. The results of the mass spectra were confirmed by library search.

Alkaline Hydrolysis

An aliquot of the plant extract was evaporated to dryness and hydrolyzed with 7 N NaOH under N_2 at 100°C for 3 h. After hydrolysis, the solution was diluted with water to 1 mL and brought to pH 3.0 with HCl. The solution was extracted with ethyl acetate and used for TLC analysis for the determination of free IBA as described above.

Statistical Treatment of the Data and Confirmation of Results

All experiments, except GC-MS analysis, were done three to five times at the Botanical Institute in Frankfurt, Germany. Confirmation of the experiments was provided by repeating the TLC and HPLC analysis in Volcani Center, Israel, with independent cultured plants. All results present means of independent experiments. Mean SE was ≤10%. The K_m values were calculated by linear regression analysis of the data after Lineweaver-Burk transformation.

RESULTS

Identification of the Reaction Product

IBA was identified as an endogenous compound in the corn variety Inrakorn by HPLC followed by TLC (Fig. 1) and GC-MS (see Fig. 4B). All feeding experiments were carried out with both varieties (Hazera 224 and Inrakorn). Because there were no significant differences between the two varieties, representative experiments with only one variety are presented. After feeding of [1-^{14}C]IAA to 2 mm segments of corn shoots and roots, a labeled compound was detected by TLC corresponding to authentic IBA standard (Fig. 2A). The control experiments without tissue showed no significant label at the R_F of IBA (Fig. 2B). No differences were found between the experiments performed with or without streptomycin sulfate (data not shown), indicating that no bacterial activity was involved in the process. The zone corresponding to IBA was eluted from TLC and chromatographed on HPLC. The eluate was collected and the radioactivity determined and compared to a co-chromatogram with authentic IBA standard (Fig. 3A, B). Similarly, the methylated sample co-migrated with authentic methyl IBA standard on TLC (data not shown). [$^{13}C_6$]IBA was identified in the plant extract following incubation with [$^{13}C_6$]IAA (Fig. 4D). Figures 4A, B, and C show the RIC and mass spectra of methylated authentic IBA and methylated samples of the corn varieties Inrakorn and Hazera 224, respectively. Figure 4D shows the reconstructed ion

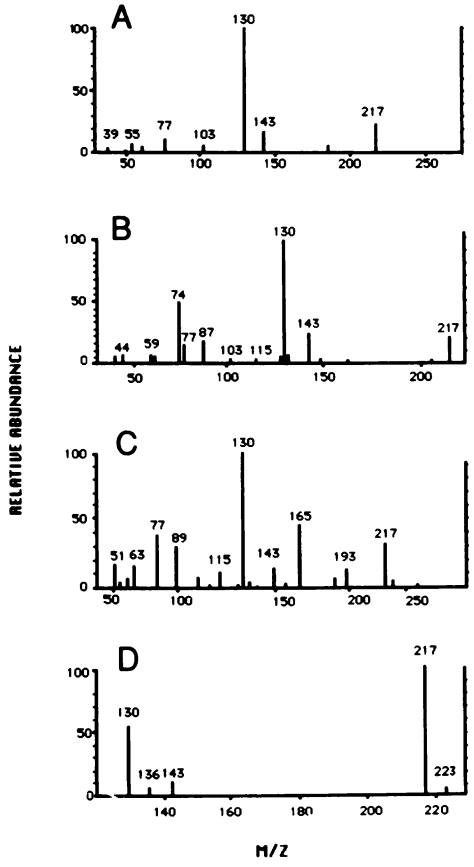

Figure 4. The 70 eV impact mass spectra of (A) methylated IBA standard, (B) methylated endogenous IBA isolated from leaves of *Zea mays* L. var Inrakorn, and (C) endogenous methylated IBA isolated from leaves of *Zea mays* L. var Hazera 224. D, Monitoring ion detector RIC of methyl[$^{13}C_6$]IBA after incubation of leaves of *Zea mays* L. var Hazera 224 with [$^{13}C_6$]IAA.

chromatography for ions 130, 136, 217, and 223 of the reaction product after the *in vivo* experiment. The mass spectrum of the RIC peak at retention time 10.5 min yielded the fragmentation pattern of typical 3-substituted indoles. The molecular ions of *m/z* 217 and 223 are those of [^{12}C]IBA and [^{13}C]IBA, respectively. The cleavage of the side chain with retention of the methylene carbon and ring expansion resulted in a base peak at *m/z* 130 and 136, the quinolinium ions of those molecules.

Characterization of the Reaction

After 8 d, the two maize varieties showed differences in root morphology (Fig. 5). The maize variety Inrakorn (Fig. 5A) possesses longer, more hairy roots, whereas the variety Hazera 224 (Fig. 5B) has little roots with a small number of root hairs under the same culture conditions. When roots, leaves, and coleoptiles of the two varieties were harvested separately and incubated with [^{14}C]IAA, they showed different rates of formation of IBA after 1 h incubation (Table I). Hazera 224 had the highest IBA formation rate in the leaves, with 4.2% of the IAA taken in by the plant, whereas in roots and coleoptiles, only 1.1% and 1.5%, respectively, of total IAA uptake was converted to IBA. Contrary to the IBA formation in Hazera 224, IAA conversion in Inrakorn was nearly the same in both leaves (2.4%) and roots (1.7%), whereas in the coleoptiles, only 0.8% of IAA taken in was converted to IBA. The time course study of IBA formation in roots and leaves of the two varieties showed a maximum after 2 h in the leaves of Hazera 224 and after 1 h in the roots of Hazera 224 and in the leaves of Inrakorn (Fig. 6). In roots of Inrakorn, IBA formation reached maximum after 2 h, but showed no decline up to 4 h incubation. Alkaline hydrolysis of the extracts from Hazera 224 leaves showed 25% more [^{14}C]IBA than without hydrolysis (data not shown). The pH optima for IAA uptake and IBA formation were at 6.0 and 7.0, respectively (Fig. 7). Both maize varieties had similar pH optima for IAA uptake and IBA formation; therefore, data of the experiment with the variety Inrakorn are representative for both varieties. After incubation with different IAA concentrations, IBA formation showed typical Michaelis-Menten kinetics. Following transformation of the data into a Lineweaver-Burk plot (Fig. 8), K_m values of 17 and 25 μM for the IBA formation were determined in leaves and roots of Inrakorn and Hazera 224, respectively.

Table I. *In Vivo Conversion of [^{14}C]IAA to [^{14}C]IBA by Various Tissues of Two Varieties of Zea mays*

One gram fresh weight of roots, leaves, and coleoptiles of *Zea mays* L. var Inrakorn and Hazera 224 were incubated with 5 kBq [^{14}C]IAA for 1 hr at 25°C. TLC (silica gel) with chloroform:acetic acid (95:5, v/v) as solvent.

Variety	Tissue	Uptake of [^{14}C]IAA	[^{14}C]IBA Formed	IBA Conversion
		Bq		%
Hazera 224	Roots	2514	28	1.1
	Coleoptiles	1225	18	1.5
	Leaves	1736	73	4.2
Inrakorn	Roots	1944	32	1.7
	Coleoptiles	1735	14	0.8
	Leaves	1540	36	2.4

DISCUSSION

In the present study, we have shown that roots and shoots of two different corn varieties are able to convert IAA to IBA. Both the ring system (indole moiety) and the side chain of the newly formed IBA were found to derive from IAA. Feeding of [$^{13}C_6$]IAA, in which the ^{13}C atoms are in the benzene ring, resulted in the formation of [^{13}C]IBA (as demonstrated by GC-MS), showing that the labeled ring system is preserved. Feeding with [^{14}C]IAA labeled in the 1-position resulted in radioactively labeled IBA, meaning that the car-

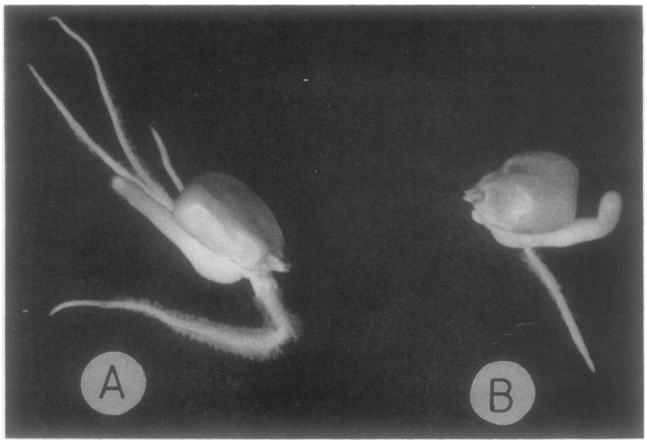

Figure 5. Eight-day-old seedlings of *Zea mays* L. var Inrakorn (A) and var Hazera 224 (B). Note the differences in root hair development between the two varieties.

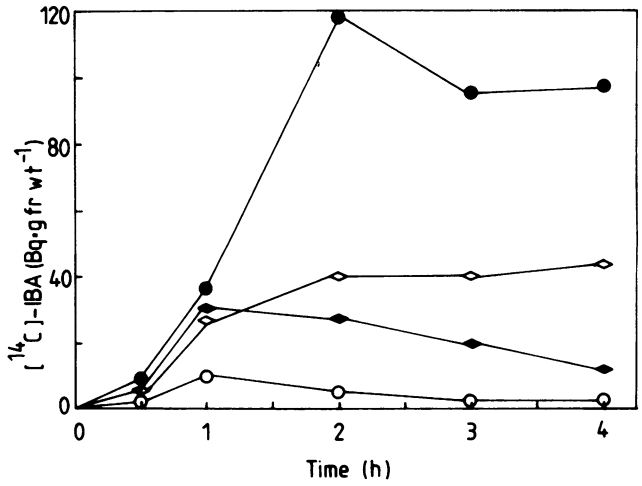

Figure 6. Time course of IBA formation from [^{14}C]IAA over a period of 4 h. (●) *Zea mays* L. var Hazera 224, leaves; (○) *Zea mays* L. var Hazera 224, roots; (♦) *Zea mays* L. var Inrakorn, leaves; (◇) *Zea mays* L. var Inrakorn, roots. All values are corrected for [^{14}C]IAA uptake.

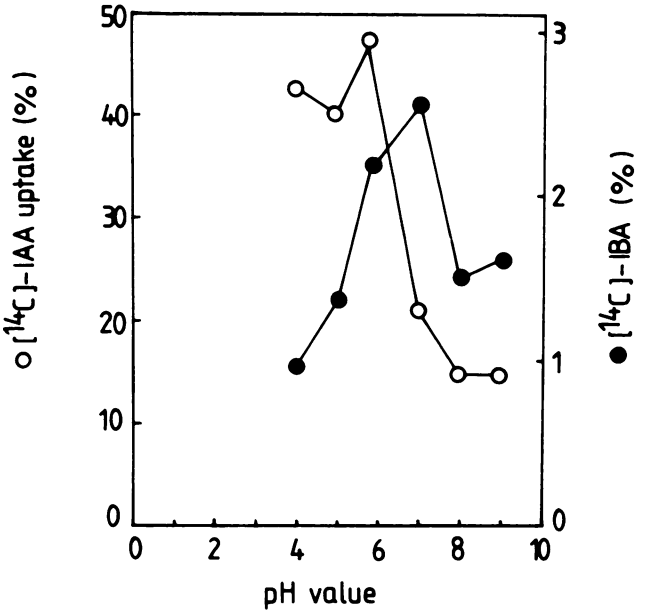

Figure 7. pH dependence of IBA formation from IAA. For every experiment, 1 g leaf tissue of *Zea mays* L. var Inrakorn was incubated for 1 h at 25°C in 100 mM Mes buffer with 5 kBq of [^{14}C]IAA at different pH values. (●) IBA formation; (O) IAA uptake.

boxyl group is maintained in the biosynthesis of IBA from IAA.

It is possible that the conversion of IAA to IBA follows the primary steps of fatty acid biosynthesis, in which acetyl moieties are transferred to an acceptor molecule (IAA) (26). Bandurski and Schulze (personal communication) demonstrated the acetylation of IAA by injecting IAA and acetyl-CoA simultaneously into corn kernels. A head-to-tail acylation of acetyl-CoA by a second molecule of acetyl-CoA resulting in acetoacetyl-CoA would build the butyryl side chain of IBA. Alternatively, two acetyl-CoA molecules are added sequentially to the IAA residue, thus forming the 4-carbon side chain. Patel and Walt (21) investigated the substrate specificity of acetyl-CoA synthetase from yeast. They found that the enzyme was not very specific and that it had some flexibility in accepting a variety of small carboxylic acids (*e.g.* CH_3-CH_2-COOH) as substrates. As there is also evidence for indole-3-propionic acid as a natural compound in plant tissue (24, 25), this might be a reasonable pathway for the biosynthesis of both compounds from IAA. So we can propose a biosynthetic pathway for the biosynthesis of IBA from IAA analogous to the first steps in fatty acid biosynthesis involving an acetyl-CoA synthetase (22), and probably also an acetyl-CoA carboxylase (17) and an acyl-CoA transferase (3) to transfer the acetyl units to IAA.

We have demonstrated that IAA was converted to IBA in the roots, coleoptiles, and leaves of corn seedlings (Table I). Overall, the highest rate of IBA formation was found in the leaves of Hazera 224 (4.2%). Significantly more IBA was found in the roots of the maize variety that formed an extensive root system (Inrakorn) than in the one with the small root system (Hazera 224). We still do not have enough experimental evidence to attach any biological significance to the conversion of IAA to IBA and its relationship to root formation. Many reports deal with the better effect of IBA *versus* IAA on the formation of lateral roots (11, 16, 20), and it was suggested that the higher rooting ability of IBA is due to the higher stability of the former compound to oxidation in the plant (16, 20). However, Wiesman *et al.* (27) were not able to find any significant differences in the rate of metabolism between these two auxins in mung bean cuttings, and there are reports that in some species, IAA even decreases rather than promotes the number of roots formed (9). One possibility is that IBA and IAA form different conjugates in the specific tissues. Epstein and Wiesman (11) demonstrated that IBA-alanine had a better effect on the number of roots induced in olive cuttings compared with free IBA. Wiesman *et al.* (28) showed that both IAA and IBA formed conjugates with aspartate in mung bean cuttings, but IBA also formed another conjugate, probably an IBA peptide.

The time course study of IBA formation in maize leaves showed a decrease of radioactivity in the IBA fraction after 1 and 2 h for the varieties Inrakorn and Hazera 224, respectively (Fig. 6). This decline might be due to conjugation of IBA during the incubation time. Higher rates of free IBA were only detected after alkaline hydrolysis with 7 N NaOH at high temperature, which cleaves all conjugated forms, but not after alkaline hydrolysis with 1 N NaOH at room temperature (J. Ludwig-Müller, unpublished results), at which auxins are only released from ester conjugates (5). These data, as well as the other data mentioned above, might prove that amide conjugates of IBA are important as a source of free auxin and, therefore, have a regulatory function in the rooting process.

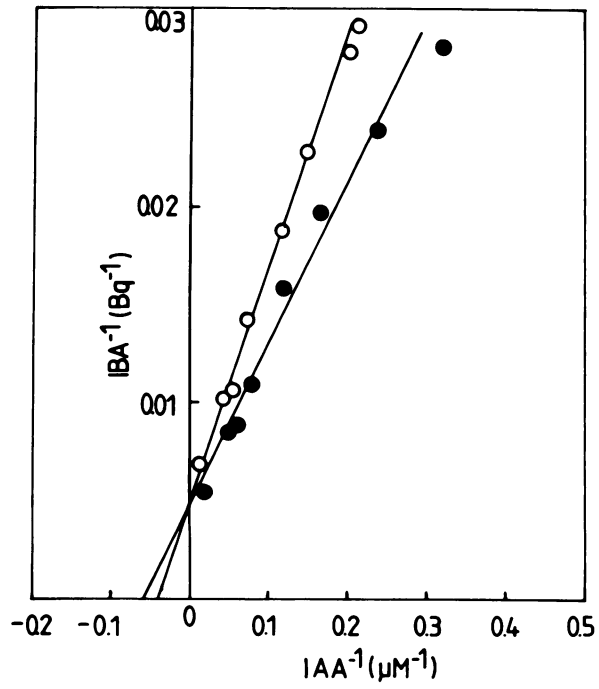

Figure 8. Lineweaver-Burk plot of substrate kinetics. (●) leaves of *Zea mays* L. var Inrakorn; (O) leaves of *Zea mays* L. var Hazera 224. The K_m values were determined as 25 and 17 μM, respectively.

However, further experiments should be carried out on this subject to generate more information about the role of these substances in the plant.

The conversion rate of IAA to IBA (2–4% of total radioactivity uptake, Table I) is in agreement with other conversion rates determined for *in vivo* reactions in the biosynthesis of IAA. Ludwig-Müller and Hilgenberg (19) found a formation rate for individual indole derivatives of 4 to 13% of total radioactivity uptake after feeding of *N*-DL-malonyltryptophan to segments of Chinese cabbage. Helmlinger *et al.* (18) found rates of 8 to 15% for the conversion of indole-3-acetaldoxime to indole-3-acetonitrile in Chinese cabbage, while Rausch *et al.* (23) determined rates of approximately 3% in benzene-soluble products and approximately 12% in water-soluble products after feeding of tryptophan to segments of *Brassica napus*. The low K_m value for the conversion of IAA to IBA, indicating high affinity for the substrate IAA (Fig. 8), allows even small amounts of IAA (*i.e.* only parts of total IAA present in plant tissues) to be converted to IBA.

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