Pergamon

0031-9422(95)00259-6

Phytochemistry, Vol. 40, No. 1, pp. 61–68, 1995

0031-9422/95 $9.50 + 0.00

THE *IN VITRO* BIOSYNTHESIS OF INDOLE-3-BUTYRIC ACID IN MAIZE

JUTTA LUDWIG-MÜLLER,* WILLY HILGENBERG and EPHRAIM EPSTEIN†

Botanisches Institut, J. W. Goethe-Univ., Siesmayerstr. 70, 60054 Frankfurt am Main, Germany; †Department of Horticulture, ARO, The Volcani Centre, Bet Dagan 50250, Israel

(*Received in revised form* 16 *February* 1995)

Key Word Index—*Zea mays*; Gramineae; maize; corn; biosynthesis; acetyl coenzyme A; adenosine-5′-diphosphate; adenosine-5′-triphosphate; indole-3-acetic acid; indole-3-butyric acid.

Abstract—The *in vitro* biosynthesis of indole-3-butyric acid (IBA) was investigated in maize (*Zea mays* L.). Incubation of microsomal membranes from shoots and roots of dark-grown maize with labelled indole-3-acetic acid (IAA), acetyl coenzyme A (acetyl CoA) and ATP resulted in the formation of an unknown labelled reaction product, product X. Other coenzymes had very little effect on the formation of this product. No IBA was detected in this system. The labelled product was purified and fed to other cell fractions of dark-grown maize. Incubation with the organelle fraction using NADPH as a cofactor resulted in the conversion of product X to a new compound which had the retention time of IBA in HPLC. Attempts to identify product X led to the hypothesis that it is a conjugate of IAA with ADP. Microsomal membranes from light-grown shoots and roots converted IAA to IBA, as demonstrated by HPLC analysis. The identity of the IBA peak was confirmed by GC-mass spectrometry. Microsomal membranes from light-grown roots formed both IBA and little of product X. For the direct *in vitro* biosynthesis of IBA acetyl CoA was needed as a cofactor, and the reaction as enhanced by the addition of ATP or NADPH. The optimum pH of the *in vitro* formation of IBA was 6–7 in the roots and 5 in the shoots. Product X was formed only at neutral pH (7–8). The K_m value for IAA in the synthesis of IBA was 16 μM.

INTRODUCTION

Auxins are a class of phytohormones that play a central role in the growth and development of plants. They are involved in processes such as cell elongation and division, induction of root growth, flower and fruit development and fruit ripening [1, 2]. Indole-3-butyric acid (IBA) is used extensively as a synthetic auxin for the induction of root formation [3] and only in the last few years was it identified as a natural constituent of plants [4–8]. It was shown that IBA is conjugated with amino acids, mainly aspartic acid [9, 10] and sugars [11]. Ludwig-Müller and Epstein [5, 12] found that indole-3-acetic acid (IAA) is converted *in vivo* to IBA in maize and *Arabidopsis thaliana*. IBA was identified in both organisms by TLC, HPLC and GC-mass spectrometry after feeding [^{14}C]-IAA and [$^{13}C_6$]-IAA. The *in vitro* biosynthesis of IBA from IAA was found to be dependent on the presence of different cofactors and it seemed to involve an, as yet unknown, reaction product [13]. This paper deals with the characterization of the *in vitro* biosynthesis of IBA from IAA through a putative intermediate.

*Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

It was shown that maize seedlings [5] and sterile cultured seedlings of *Arabidopsis thaliana* [12] were able to convert IAA to IBA *in vivo*. The reaction product was identified in both cases by GC-mass spectrometry. In contrast, van der Krieken and coworkers [14] could not detect any IBA after feeding IAA *in vivo* to apple cuttings, but this might have been due to longer incubation times or to the type and age of plant material. The reverse reaction (formation of IAA from IBA) has been reported earlier [6, 14–16].

In this paper we demonstrate that IBA is synthesized from IAA seedlings via a two-step reaction *in vitro* by extracts of light- and dark-grown maize and we were able to characterize this reaction by several physiological and biochemical parameters.

Until this work, three hypotheses concerning the *in vitro* biosynthesis of IBA could be envisioned:

1. A pathway analogous to the IAA biosynthetic pathway via the tryptophan pathway (indole + serine) using glutamate-γ-semialdehyde instead of serine. The biosynthesis of IBA could then proceed by the same enzymes of IAA biosynthesis from tryptophan.
2. Chain elongation reactions similar to those found in the biosynthesis of fatty acids [17].
3. A non-tryptophan pathway similar to that demon-

strated by Wright *et al.* [18] and Normanly *et al.* [19] for IAA in a mutant of maize and *Arabidopsis thaliana*, respectively.

Our *in vivo* studies [5, 12] have shown that IAA seems to be the direct precursor of IBA biosynthesis. Therefore, the second hypothesis was tested using an incubation system with different coenzymes for the chain elongation reaction.

Formation of an unknown reaction product from IAA

Feeding of IAA to microsomal membranes of dark- and light-grown maize seedlings resulted in a different peak pattern after HPLC separation of the acidic organic phase. We found that microsomal membranes extracted from light-grown shoots were able to form one new peak which co-chromatographed with an IBA standard, whereas microsomal membranes from light-grown roots formed two new peaks after incubation with IAA. One of these peaks showed the chromatographic properties of IBA, the other did not co-chromatograph with any other auxin tested; therefore it was called product X. Microsomes from dark-grown plants (roots and shoots) showed only one new peak after incubation with IAA, which showed the same retention time as the unknown product formed by microsomal membranes from light-grown roots. This phenomenon was more thoroughly studied using dark-grown maize seedlings, because such a compound was not detected in our *in vivo* experiments [5, 12]. Feeding [^{14}C]-IAA to different cell fractions (organelles, microsomes and soluble fraction) of dark-grown maize seedlings resulted again in the accumulation of the unknown compound. This radioactive reaction product was mainly made by the microsomal fraction. No IBA could be detected in any of the investigated fractions. Different factors were tested for their ability to enhance the formation of labelled product X (Table 1). The best yield was achieved after incubation with acetyl CoA and ATP, followed by acetyl CoA alone, and acetyl CoA plus $MgCl_2$. NADPH and other reduction equivalents had no effect (data not shown). Other CoA derivatives did not enhance product X formation. Previous work showed that product X turned over very rapidly [13]. Its accumulation reached a maximum after 30 min and then decreased continuously. The product was not very stable in the ethyl acetate fraction after extraction of the reaction mixture, but following HPLC purification it was stable for a long time (J. Ludwig-Müller, unpublished results).

Table 1. Effect of different cofactors on the formation of product X from IAA by the microsomal membrane fraction of dark-grown seedlings of *Zea mays*

Cofactors	Product X (Bq)
None	48
Acetyl CoA	137
Acetyl CoA + ATP	445
Acetyl CoA + $MgCl_2$	355
Acetyl CoA + ATP + $MgCl_2$	266
Propionyl CoA	147
Acetoacetyl CoA	30
Malonyl CoA	37

Incubation was at room temperature for 1 hr at pH 7. The coenzymes were at 2 mM in the reaction mixture, and ATP and $MgCl_2$ at 6 mM.

Conversion of product X to IBA

Because no IBA was made from labelled IAA by the microsomal fraction, we tested whether other cellular fractions besides the microsomal membranes were able to convert product X to IBA. Product X was purified by HPLC and fed to the different cell fractions of dark-grown maize seedlings. The organelle fraction with NADPH as cofactor converted product X to a substance with the R_t of IBA (Fig. 1). Negligible metabolism of product X was observed after incubation with the soluble fraction, and no conversion was found by the microsomal membranes.

As our results show that IBA is converted to product X by microsomal membranes and that product X is converted to IBA in an organelle fraction, we suggest that it is an intermediate of IBA biosynthesis from IAA. It is possible that the biosynthesis of IBA from IAA, similar to fatty acid biosynthesis, occurs in different cell fractions. Synthesis of fatty acids up to C_{18} in chain length and their acyl carrier protein (ACP) derivatives and desaturation of the C_{18} chains are completed in the plastids [20], where they are either incorporated into the plastid lipids or exported into the cytoplasm, following hydrolysis to their free fatty acids. Subsequent desaturation and elongation reactions occur outside the plastids, preferably on microsomal membranes [21]. As fatty acid synthetase is a multifunctional enzyme complex [21], a part of this enzyme complex might be used for IBA biosynthesis.

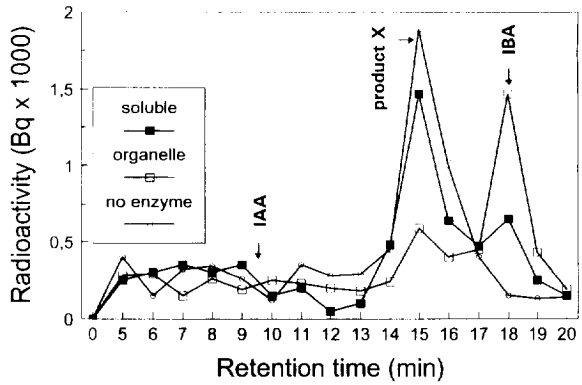

Fig. 1. HPLC chromatogram (C_{18} reversed-phase column, solvent 52% MeOH, 48% aq. 1% HOAc) of the organic phase after the conversion of product X to IBA by different fractions of dark-grown *Zea mays* seedlings (incubation time 2 hr). NADPH was included as cofactor. R_t of IAA, 9.5 min; R_t of product X, 15 min; R_t of IBA, 18 min. (■) Soluble fraction, (□) organelle fraction, (○) no enzyme.

However, the possibility of a novel enzyme, not of the fatty acid synthetase complex, cannot be ruled out.

Tentative identification of product X

After HPLC purification, product X gave a blue–green colour on TLC with Ehmann reagent, suggesting that it might be an indole. None of several other indole derivatives tested had the same retention time on HPLC as product X (Table 2). Product X showed a broad absorption spectrum with maxima at 260 and 280 nm. Alkaline hydrolysis of purified product X with 1 N NaOH for 1 hr at room temperature showed two prominent peaks on HPLC, one co-chromatographing with IAA, one with ADP (Fig. 2). Hydrolysis with 7 N NaOH gave similar results. Calculation of the peak areas according to standard curves revealed an approximate ratio of 1:1 between the putative 'ADP' peak and the IAA peak (108 μmol ADP versus 89 μmol IAA). The lower IAA value can probably be explained by degradation of IAA during alkaline hydrolysis. The ADP fraction was further analysed using a glutamate dehydrogenase assay (Table 3). In this assay NH_4^+ ions are transferred to oxoglutaric acid during synthesis of ATP from ADP and oxidation of NADPH, which was measured at 340 nm. Using the aqueous sample instead of ADP after alkaline hydrolysis resulted in an increase of NADPH oxidation, indicating that the sample contained free ADP. The identity of IAA was confirmed by GC-mass spectrometry. The chromatogram after alkaline hydrolysis showed a clear peak with the R_t of IAA and the mass spectrum revealed the characteristic fragmentation pattern of methylated IAA (*m/z* 189, 130, 103, [22]). Methylated product X showed no peak with *m/z* 130. To show that ATP or ADP is bound covalently to IAA, 20 μCi [^{32}P]-γ-ATP or [^{35}S]-α-ATP were included into the reaction mixture. The reaction product was separated on TLC with subsequent detection of radioactively labelled compounds on X-ray film. Product X was strongly labelled when α-ATP was used, whereas incubation with γ-ATP resulted in a non-specific smear on TLC. We therefore propose that product X is a conjugate of IAA with ADP. The molecular mass of product X was determined on a Sephadex-G 25 column (equilibrated with 100 mM Tris–HCl buffer, pH

Table 2. Retention times of several indole derivatives after HPLC in comparison to product X

Substance	Retention time (min)
Indole-1-acetic acid	8.4
Indole-3-acetic acid	8.5
Indole-3-butyric acid	16.7
Indole-3-carboxylic acid	7.9
Indole-3-glyoxylic acid	6.8
Indole-3-lactic acid	7.4
Indole-3-propionic acid	12.3
Indole-3-pyruvic acid	11.5
Indole-3-aldehyde	9.0
Indole-3-methanol	12.6
Methyloxindole	9.0
Product X	14.8

Column was C_{18}, and methanol: 1% acetic acid (52:48) was used as solvent. Flow rate was 0.7 ml min^{-1}. Values are averages of five runs.

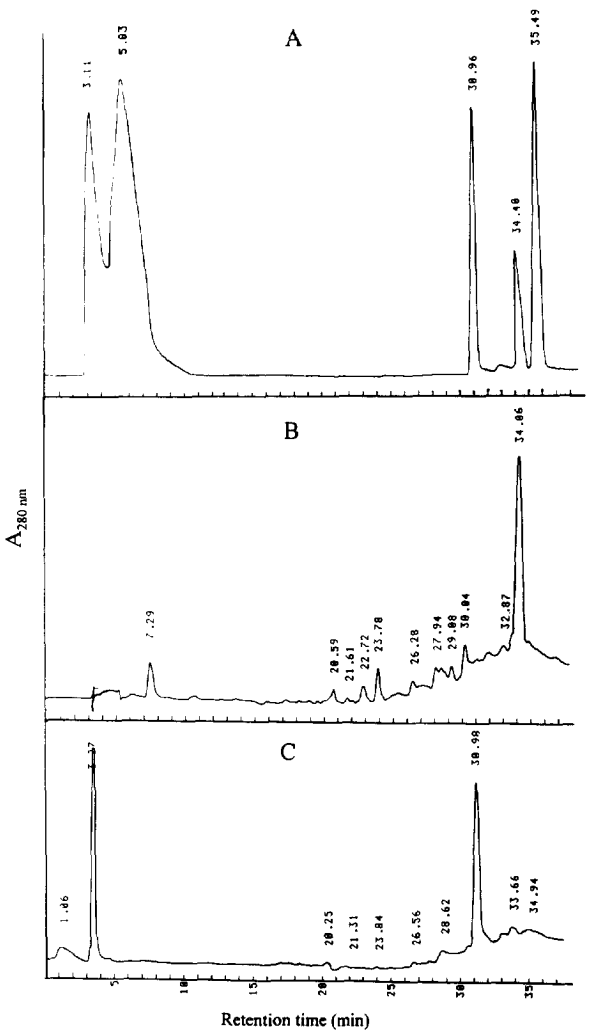

Fig. 2. HPLC chromatogram (C_{18} reversed-phase column, gradient as described in Experimental) of IAA (R_t 30.96), IBA (R_t 35.49), product X (R_t 34.40), ATP (R_t 5.83), and ADP (R_t 3.11) standards (A), purified product X before alkaline hydrolysis (B), and after hydrolysis with 1 N NaOH for 1 hr at room temp. (C).

Table 3. Tentative identification of ADP in the aqueous fraction after hydrolysis of product X with 1 N NaOH by a glutamate dehydrogenase assay

Sample	ΔE (nm min^{-1})
no ADP	0.047
'ADP'-containing sample (90 μl)	0.048
'ADP'-containing sample (160 μl)	0.061
1 mM ADP	0.124

7.0), which was calibrated with IAA (M_r 214, potassium salt), ATP (M_r 551, sodium salt), acetyl CoA (M_r 824), and streptomycin sulphate (M_r 1457) as standards (data not shown). Product X showed a molecular mass of *ca* 1500 dalton, which leads to the assumption that acetyl-CoA may also be a part of the molecule.

Attempts to characterize product X more thoroughly by GC-mass spectrometry and NMR spectroscopy were unsuccessful because insufficient amounts were available (J. Ludwig-Müller *et al.* unpublished results). However, some conclusions concerning the compound can be made.

1. This compound is thought to be indolic, because the HPLC-purified peak of product X gave a blue-green signal with Ehmann's reagent on TLC.
2. Product X could be an IAA conjugate, because alkaline hydrolysis of purified product X with 1 N NaOH for 1 hr at room temperature resulted in a peak co-chromatographing with IAA. After hydrolysis GC-mass spectrometry confirmed this peak to be IAA.
3. From its chromatographic properties product X is thought *not* to be IAA–CoA (L. Michalczuk, personal communication), a possible theoretical intermediate.
4. There is evidence that IAA is bound to ADP, because we were able to label product X after feeding [^{35}S]-α-ATP, but not after feeding [^{32}P]-γ-ATP after TLC separation. We assume that acetyl CoA might be also a part of product X, because product X was not formed in the absence of acetyl CoA.

Two possible structures for product X are presented in Fig. 3. However, only one of them would result in free IAA after alkaline hydrolysis, so only the structure given in Fig. 3B would be consistent with our findings.

Conversion of IAA to IBA

IAA was metabolized directly to IBA by microsomal fractions of light-grown maize seedlings. In contrast to dark-grown seedlings, a new peak at R_f 15.5 min was found after incubation of extracts from light grown seedlings with IAA; this co-chromatographed with IBA standard. Analysis of this putative IBA peak purified from HPLC by GC-mass spectrometry showed the characteristic ions for IBA at m/z 217 (the molecular ion of methylated IBA), m/z 143 (R–CH = CH_2), m/z 130 (quinolinium ion), m/z 103 (styryl ion), and m/z 77 (phenyl ion) [5, 11] (Fig. 4).

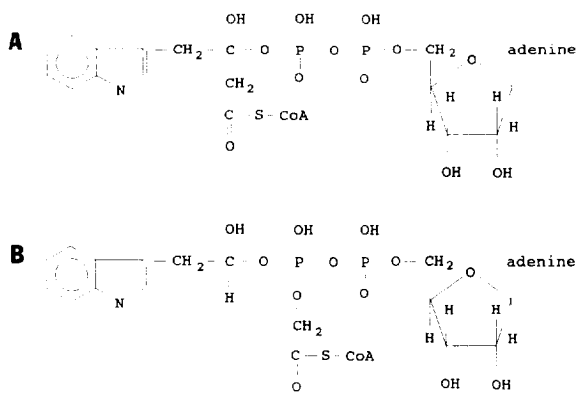

Fig. 3. Proposed possible structures for product X. Note that only the structure given in (B) would result in free IAA after alkaline treatment.

The amounts of IBA and product X formed by extracts from both roots and shoots of the dark- and light-grown seedlings are shown in Table 4. Tissues of light-grown shoots and roots of maize seedlings converted IAA to IBA, and some product X was also found as a reaction product in the extracts of light-grown roots. Extracts from tissues of dark-grown seedlings did not synthesize IBA from IAA, whereas high levels of product X were detected in those two tissues. In extracts of the dark-grown tissues, product X concentration following incubation was generally much higher than in the extracts of roots of the light-grown plants. The conversion of IAA to IBA and product X was more prominent in the roots, whereas little difference was found between shoots and kernels.

Further characterization of the factors needed for the conversion of IAA to IBA by extracts from light-grown seedlings showed that the same cofactors that produced high amounts of product X (Table 1) were also optimal for the formation of IBA (Table 5). The best incubation mixture for the synthesis of IBA from IAA, by extracts from both shoots and roots, included ATP and acetyl CoA. Some IBA formation without acetyl CoA was probably due to breakdown reactions in the membranes, where small quantities of free acetyl CoA might have been formed. ATP was also needed, but could be replaced by NADPH to some extent. UTP could substitute for ATP, GTP had no effect and CTP and TTP were inhibitory. Microsomal membranes from shoots were found to synthesize more IBA from IAA with all combinations of cofactors tested than similar preparations from roots. No formation of indole-3-propionic acid was found in the present system, possibly because, as in fatty acid biosynthesis, the even and odd chain-length compounds are synthesized by different systems [23]. These data demonstrate that IBA is made by chain elongation reactions from IAA and effectively rule out the other proposed biochemical origins, of the IBA found in maize.

A time course study showed a steady increase of IBA formation by microsomal fractions from both shoots and roots up to 4 hr with ATP and acetyl CoA as cofactors. After that time, a decline in IBA was observed in extracts of shoots. This is in contrast to root extracts, where the IBA level remained steady. IBA formation showed a pH optimum of 6–7 for root extracts and 5 for shoot extracts. Product X accumulated at pH 7 in shoot extracts and at pH 7–8 in root extracts. This may be because IBA formation under these conditions was less. The K_m values for the formation of IBA in light-grown shoots were determined at pH 7, using whole light-grown seedlings as material, to be 16 μM for IAA, 50 μM for ATP, and 40 μM for acetyl CoA (Table 6), whereas those for the formation of product X in dark-grown shoots were 100 μM for IAA, 1.3 mM for ATP, and 300 μM for acetyl-CoA.

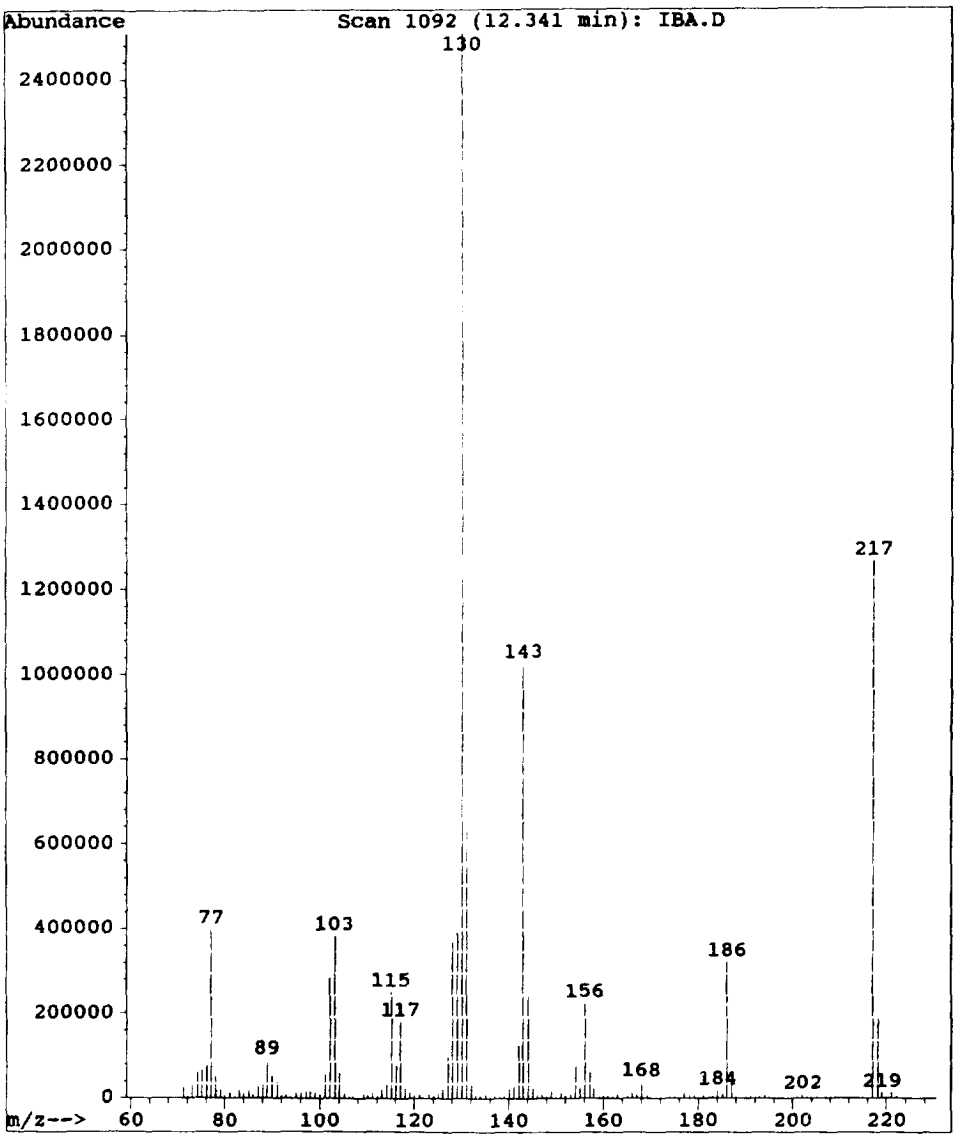

Fig. 4. The 70 eV total scan mass spectrum of enzymatically synthesized, HPLC-purified IBA isolated after incubation of microsomal membranes of light-grown seedlings of *Zea mays* with IAA, acetyl CoA and ATP. Note the typical ions for methylated IBA at *m/z* 217 (molecular ion), *m/z* 186, *m/z* 143, and *m/z* 130 (quinolinium ion).

Our findings about the synthesis of product X and IBA by extracts of light- and dark-grown seedlings are summarized in the following equations:

$$\text{IAA} + \text{acetyl CoA} + \text{ATP} \xrightarrow{\text{microsomal membranes (light grown shoots and roots)}} \text{IBA}$$

$$\text{IAA} + \text{acetyl CoA} + \text{ATP} \xrightarrow{\text{microsomal membranes (light grown roots, dark grown roots and shoots)}} \text{IAA–X}$$

$$\xrightarrow{\text{organelle fraction (dark grown roots and shoots)}} \text{IBA}$$

In contrast to dark-grown plants, the synthesis of IBA by microsomal membranes from light-grown plants of maize seems to be possible in one cellular fraction. We did not use a purified membrane preparation for these experiments, and the possibility of contamination of the microsomal fraction with plastid membranes can therefore not be ruled out.

The *in vivo* role of IBA is still not clear. Many investigations have shown that IBA has a higher ability to promote adventitious root formation than IAA, but this effect has been assumed to be due to the higher stability of IBA than IAA in the tissue [10]. Other theories on the differences between IAA and IBA on root formation have been: (1) rooting ability depends on a certain ratio IBA:IAA; (2) rooting ability depends on the ability to

Table 4. *In vitro* synthesis of product X and IBA by light-grown and dark-grown roots, shoots, and kernels of *Zea mays* seedlings

Growth conditions	Organ	IBA (ng mg^{-1} protein)	Product X (ng mg^{-1} protein)
Light-grown	Shoots	26.2	1.3
	Roots	38.7	17.8
	Kernels	20.6	1.3
Dark-grown	Shoots	n.d.	69.6
	Roots	n.d.	74.6
	Kernels	n.d.	22.7

The microsomal membrane fraction from each organ was incubated with IAA, acetyl CoA and ATP at room temperature for 1 hr at pH 7. The reaction products were separated by HPLC with C_{18} column and methanol: 1% acetic acid (52:48) as solvent.

n.d. = Not detected under these conditions.

Table 5. The effect of acetyl CoA, nucleotide triphosphates (ATP, CTP, GTP, TTP, UTP) and NADPH on the formation of IBA from IAA in light-grown roots and shoots from *Zea mays* seedlings

	IBA formation (μg ml^{-1})	
Cofactor	Shoots	Roots
None	0.40	0.24
ATP	0.58	0.49
Acetyl CoA	0.75	0.34
ATP, acetyl CoA	2.26	1.57
CTP, acetyl CoA	0.08	0.04
GTP, acetyl CoA	0.75	0.42
TTP, acetyl CoA	0.52	0.16
UTP, acetyl CoA	2.16	1.38
NADPH	1.32	0.64
ATP, NADPH	1.09	0.51
Acetyl CoA, NADPH	1.89	0.12
Acetyl CoA, ATP, NADPH	1.57	0.77

Incubation time was always 1 hr at pH 7. IAA was always at 1 mM in the test mixture. The same protein concentrations were employed both for roots and shoots.

Table 6. K_m values for the formation of product X and IBA in dark- and light-grown shoots, respectively, of *Zea mays* seedlings

	Product X	IBA
IAA	100 μM	16 μM
Acetyl-CoA	300 μM	40 μM
ATP	1.3 mM	50 μM

convert the respective conjugates to the free hormones; (3) rooting depends on the stability of the respective conjugates; (4) uptake and transport differ significantly [6]. Our studies have shown that, at least in *Arabidopsis thaliana*, most of the IAA was found in amide conjugates, whereas most of the IBA was conjugated via ester linkages [7]. Different conjugation properties of those two hormones could result in different functions in the plant. It was shown recently that both the endogenous content and the *in vitro* biosynthesis of IBA could be increased in maize seedlings by drought stress and exogenous IBA [24]. These findings might indicate a role of IBA during stress situations in the plant. Further studies on the biosynthesis of IBA *in vitro* might lead to purified enzyme preparations, which will enable us to study IBA and its possible role on a molecular level.

EXPERIMENTAL

Plant material. Maize (*Zea mays* L. cv Ascot) was cultivated under sterile conditions, as previously described [5], in the dark and under continuous illumination (33 μE cm^{-2}) at 23°. The plant material was harvested routinely after 6 days of culture.

Enzyme preparation. The plant material was harvested, separated into shoot and root sections, and homogenized with an Ultra Turrax (Janke & Kunkel) at 20 000 rpm for 5 min using 100 mM HEPES, pH 7, containing 1 mM $MgSO_4$ and 1 mM DTT. The homogenate was filtered and centrifuged at 1000g for 10 min to remove cell particles. The supernatant was then centrifuged for 20 min at 10 000g (organelle fraction), the pellet resuspended in 5 ml 100 mM HEPES, pH 7, and the supernatant centrifuged at 50 000g for 60 min (microsomal fraction). The 50 000g pellet was resuspended in 100 mM HEPES (1 ml g^{-1} fr.wt) and the supernatant was desalted on a Sephadex-G-25 column (soluble fraction) using the same buffer. All operations were carried out at 4°.

Enzyme assays for IBA formation. Formation of product X and IBA from IAA. The enzyme assay for the conversion of IAA to product X and IBA was performed as previously described [24]. The reaction mixture contained, in a total volume of 500 μl, 100 μl microsomal membranes, 2 mM acetyl-CoA, 6 mM ATP and/or 1 mM NADPH. CTP, GTP, TTP, and UTP were also added at

6 mM in the reaction mixture. The reaction was started by the addition of 1 kBq 1-[^{14}C]-IAA (Sigma, specific activity 2.2 GBq mmol^{-1}) or 1 mM of non-labelled IAA. In some experiments 20 μCi [^{32}P]-γ-ATP (Amersham, specific activity 185 TBq mmol^{-1}) or 20 μCi [^{35}S]-α-ATP (Amersham, 22 TBq mmol^{-1}) were added. The reaction was carried out at pH 7, if not otherwise indicated, and incubation time was routinely 1 hr at room temp. The reaction was stopped by adding 30 μl 1 M HCl, and the aq. phase was then extracted with 500 μl of EtOAc. The organic phase was removed, evaporated to dryness, and resuspended in 20 μl MeOH. The sample was kept in liquid nitrogen for HPLC analysis. For TLC analysis, the organic phase was evaporated to a small volume.

Conversion of product X to IBA. The reaction mixture for the conversion of the intermediate contained 50 μl enzyme extract and 1 mM NADPH in a total volume of 500 μl. The reaction was started by the addition of 2 kBq of the labelled intermediate. After incubation for 2 hr the reaction was stopped as described above and the product was extracted with EtOAc, evaporated to dryness, resuspended in 20 μl of MeOH and analysed by HPLC.

Alkaline hydrolysis. Hydrolysis of conjugated auxins was performed with 1 N NaOH at room temperature for 1 hr or with 7 N NaOH at 100° under N_2 for 3 hr as described in [25]. The hydrolysate was filtered, pH brought to 3, and an aliquot was removed for direct HPLC analysis. The auxins were extracted with equal volumes of EtOAc, the EtOAc fr. was evapd to dryness and the sample resuspended in 100 μl MeOH.

TLC Analysis. The EtOAc fraction was chromatographed on silica gel F_{254} plates. The plates were developed with $CHCl_3$–HOAc (95:5) as a solvent. Tentative identification was achieved by co-chromatography with authentic standards and visualization under UV (R_f values of IAA and IBA 0.45 and 0.6, respectively). For confirmation, the plates were treated with Ehmann's reagent [26]. For autoradiography the plates were incubated for 24 hr with X-ray film.

HPLC analysis. The total MeOH extract (20 μl) was subjected to HPLC with 52% MeOH containing 1% aq. HOAc as solvent at a flow rate of 0.7 ml min^{-1}. When ATP/ADP was simultaneously determined, analysis was carried out using a gradient (solvent A = 1% aq. HOAc, solvent B = MeOH) from 99–40% A for 30 min. Tentative identification of known substances was achieved by co-chromatography with authentic standards. Frs of 0.4 ml were collected and mixed with 4 ml scintillant and radioactivity was determined in a liquid scintillation counter. The amount of the non-labelled reaction product was determined using a standard curve.

GC-MS Analysis. The peak of putative IBA after incubation of microsomal membranes with IAA and all cofactors needed, was collected after HPLC, pooled and methylated with diazomethane [27]. GC-MS identification of all samples was performed using electron impact ionization on a Durabond-5 column, 30 m × 0.25 mm, 0.25 μm film. The temp. program was 140° for 2 min, followed by an increase of 10° min^{-1} to 200° and of 20° min^{-1} to 250°. Spectra were taken both in full continuous scan mode and selective ion monitoring mode. The sample of product X was treated likewise before and after alkaline hydrolysis for GC-MS.

Enzymatic assay for determination of ADP. The presence of ADP in unknown samples was confirmed using the glutamate dehydrogenase assay according to ref. [28], where NH_4^+ ions (from appropriate amounts of $(NH_4)_2SO_4$) are transferred to oxoglutaric acid during the synthesis of ATP from ADP. The amount of ADP present was measured photometrically by the oxidation of NADPH at 340 nm during this reaction.

Statistical treatment of the data. All experiments were repeated two to three times using different enzyme preparations. All results present means of independent experiments. Mean s.e. was ± 10%. The K_m values were calculated by linear regression analysis of the data after Lineweaver–Burk transformation.

Acknowledgements—We would like to thank Dr Christopher D. Town, Department of Biology, Case Western Reserve University, Cleveland, USA, Dr Jerry D. Cohen, Horticultural Crops Quality Laboratory, USDA, Beltsville, MD, USA for critical reading of the manuscript and Mrs K. Pieper and Mrs B. Schubert for technical assistance.

REFERENCES

1. Bandurski, R. S. and Nonhebel, H. M. (1984) in *Advanced Plant Physiology* (Wilkins M. B., ed.), p. 1, Pitman, London.
2. Davies, P. J. (ed.) (1987) *Plant Hormones and their Role in Plant Growth and Development*. Martinus Nijhoff, Dordrecht, Netherlands.
3. Hartmann, H. T., Kester, D. E. and Davies, F. T. (1990) in *Plant Propagation: Principles and Practices*, p. 246, Prentice-Hall, Englewood Cliffs, NJ.
4. Epstein, E., Chen, K.-H. and Cohen, J. D. (1989) *Plant Growth Regul.* **8**, 215.
5. Ludwig-Müller, J. and Epstein, E. (1991) *Plant Physiol.* **97**, 765.
6. Epstein, E. and Ludwig-Müller, J. (1993) *Physiol Plant.* **88**, 382.
7. Ludwig-Müller, J., Sass, S., Sutter, E. G., Wodner, M. and Epstein, E. (1993) *Plant Growth Regul.* **13**, 179.
8. Sutter, E. G. and Cohen, J. D. (1992) *Plant Physiol.* **99**, 1719.
9. Wiesman, Z., Riov, J. and Epstein, E. (1989) *Plant Physiol.* **91**, 1080.
10. Nordström, A.-C., Jacobs, F. A. and Eliasson, L. (1991) *Plant Physiol.* **96**, 856.
11. Ludwig-Müller, J. and Epstein, E. (1993) *Plant Growth Regul.* **13**, 189.
12. Ludwig-Müller, J. and Epstein, E. (1994) *Plant Growth Regul.* **14**, 7.
13. Ludwig-Müller, J. and Epstein, E. (1992) in *Progress in Plant Growth Regulation* (Karssen, C. M., van Loon, L. C., Vreugdenhil, D., eds), p. 188, Kluwer Academic Publishers, Netherlands.

14. van der Krieken, W. M., Breteler, H. and Visser, M. H. M. (1992) *Plant Cell Physiol.* **33**, 709.
15. Epstein, E. and Lavee, S. (1984) *Plant Cell Physiol.* **25**, 697.
16. Baraldi, R., Cohen, J. D., Bertazza, G., Predieri, S. and Bregoli, A. M. (1993) *Acta Hort.* **329**, 289.
17. Wakil, S. J. (1990) *Biochemistry* **28**, 4523.
18. Wright, A. D., Simpson, M. B., Neuffer, M. G., Michalczuk, L., Slovin, J. P. and Cohen, J. D. (1991) *Science* **254**, 998.
19. Normanly, J., Cohen, J. D. and Fink, G. R. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10355.
20. Slabas, A. R. and Fawcett, T. (1992) *Plant Mol. Biol.* **19**, 169.
21. Caughey, I. and Kekwick, R. G. O. (1982) *Eur. J. Biochem.* **123**, 553.
22. McDougall, J. and Hillman, J. R. (1978) in *Isolation of Plant Growth Substances*, p. 1, Cambridge University Press, Cambridge.
23. Lehninger, A. L. (1978) in *Biochemistry*, p 659, Worth Publishers, Inc., New York.
24. Ludwig-Müller, J., Schubert, B. and Pieper, K. (1994) *J. Exp. Bot.*, **46**, 423.
25. Cohen, J. D., Baldi, B. G. and Slovin, J. P. (1986) *Plant Physiol.* **80**, 14.
26. Ehmann, A. (1977) *J. Chromatogr.* **132**, 267.
27. Cohen, J. D. (1984) *J. Chromatogr.* **303**, 193.
28. Fonseca-Wollheim, F. Bergmeyer, H. U. and Gutmann, I. (1974) in *Methoden der enzymatischen Analyse*, Vol. II (Bergmeyer, H. U., ed.), p 1850, Verlag Chemie, Weinheim.