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INDOLE-3-METHANOL-β-D-GLUCOSIDE AND INDOLE-3-CARBOXYLIC ACID-β-D-GLUCOSIDE ARE PRODUCTS OF INDOLE-3-ACETIC ACID DEGRADATION IN WHEAT LEAF SEGMENTS

GERTRUD WIESE and HANS J. GRAMBOW*

Institut für Biologie III (Pflanzenphysiologie), Technische Hochschule, Worringer Weg, D-5100 Aachen, West Germany

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Key Word Index—*Triticum aestivum*; Gramineae; indole-3-acetic acid; indole-3-methanol-β-D-glucoside; indole-3-carboxylic acid-β-D-glucoside.

Abstract—The formation of glucosides of indole-3-methanol (IM) and indole-3-carboxylic acid (ICA) after application of radio-labelled indole-3-acetic acid (IAA) and IM to leaf segments of wheat and other members of the Gramineae is described. The results suggest that the decarboxylative pathway of IAA degradation leading to ICA via IM may occur in the leaf segments.

INTRODUCTION

In *in vitro* experiments on the degradation of indole-3-acetic acid (IAA) catalysed by horseradish peroxidase (HRP), 3-hydroxymethyloxindole (HOMOx) and indole-3-aldehyde (IAl) were found to be the major metabolites [1]. Recently, however, it has been shown that indole-3-methanol (IM) too is a not insubstantial product of this reaction [2–4]. Indeed, IM is essentially the sole product of the reaction if suitable electron donors are available as co-substrates [4]. In order to explain these results, the intermediate formation of a peroxide was postulated. In contrast to some earlier proposals, it would seem likely that indole-3-methyl hydroperoxide corresponds with this intermediate peroxide [2], and that this is then converted by peroxidase action to IM. Likewise, IM was found when IAA was degraded in the presence of wheat peroxidase isoenzyme B1 [5]. Purified wheat peroxidase shows, with respect to the degradation of IAA, the same characteristics as HRP, i.e. either HOMOx or IM is found as the primary product, depending on the reaction partners (Grambow and Wedekind, unpublished work).

In vivo application of IAA to segments from *Pisum sativum* and *Orobanche* sp. [6, 7] and to protoplasts from *Pinus sylvestris* [8, 9] also led to the formation of IM. Following *in vivo* application of [2-^{14}C]IAA to wheat leaf segments, IM was detected in very small amounts compared to the total decarboxylated IAA since IM is oxidized rapidly, via its aldehyde, to indole-3-carboxylic acid (ICA) [10]. Moreover, large amounts of polar compounds are formed from IM and ICA which hitherto have not been definitively identified or are unknown.

In the present study, evidence was obtained that these compounds are indole-3-methanol-β-D-glucoside and indole-3-carboxylic acid-β-D-glucoside. In addition, their formation was studied in different Gramineae plants. Their appearance can be taken to be indicative that IAA can be degraded via IM in all the Gramineae members tested. In this connection, it is of further interest that only very recently was the first confirmation that IM is an endogenous constituent of plants obtained using etiolated seedlings of *Pinus sylvestris* [11].

*To whom correspondence should be addressed.

RESULTS

Extraction of the polar conjugates of IM and ICA

Following application of [2-^{14}C]IAA or [^{14}C]IM to leaf segments, IAA, indolic IAA metabolites (IM, IAl, ICA) and oxindoles can be extracted by ether as well as by 80% methanol. The polar conjugates of IM and ICA, on the other hand, can be recovered only in the 80% methanol extract.

On comparing an 80% methanol extract with and without prior extraction with ether, striking differences are found in the individual metabolites: ether treatment, often used to extract auxins, leads to a markedly high recovery of IM and especially of ICA in the ether extract whereas the recovery of conjugates is reduced in the methanol extract. Similar effects have also been observed during ether extraction of IAA [12]. An explanation for these differences is that during ether extraction the conjugates are cleaved enzymatically, thus leading to the release of IM and ICA. Under the conditions of ether extraction used in this study it was indeed possible to detect β-glucosidase activity by cleavage of β-methylumbelliferyl glucoside. When a glucosidase inhibitor (δ-gluconolactone) was added to the leaf powder before extraction with ether, the recoveries of IM and ICA and of the corresponding conjugates were comparable to those obtained with direct 80% methanol extraction (data not shown in detail). For this reason, in all subsequent

experiments, extraction was performed only with methanol since the methanol extract most closely represented the content of metabolites (despite certain losses of IM due to the instability of IM).

Cleavage of conjugates of IM and ICA and identification of indolic components

The conjugates of IM and ICA were first purified by TLC (system III; IM conjugate: R_f 0.59; ICA conjugate: R_f 0.62). Both components were eluted together using methanol and were separated from one another by HPLC (system VI; IM conjugate: elution volume 83 ml; ICA conjugate: elution volume 109 ml). The appropriate fractions were located by means of the radioactivity elution profile, after which they were concentrated. No decomposition was observed after storage of the samples at $-20°$ for at least 2 weeks.

Treatment of the conjugates with a highly purified β-glucosidase preparation for 1 hr led to the release of the indole components from both compounds. In the case of the ICA conjugate, the recovery, after cleavage and chromatography, was *ca* 90% ICA. In the case of the IM conjugate, it was *ca* 70% IM. The identity of both indoles was confirmed by co-chromatography with authentic standards in the systems I (IM conjugate, R_f 0.0; IM, R_f 0.39; ICA conjugate, R_f 0.0; ICA, R_f 0.31), II, IV and V. The IM conjugate could be hydrolysed by treatment (1 hr) with 0.1 M potassium hydroxide: IM was released. Using a mixture of 0.1 M potassium hydroxide and an alcohol (HOR = methanol, ethanol, butanol), however, not IM but rather the corresponding alkyl ether (IMOR) was obtained from the conjugate. This was concluded from the fact that non-polar products were produced; with increasing chain length of the alcohol used, these showed correspondingly less polar properties in TLC (system I: KOH–MeOH, R_f 0.69; KOH–EtOH, R_f 0.73; KOH–BuOH, R_f 0.81).

Treatment (1 hr) of the IM conjugate with 0.1 M methanolic sodium methoxide similarly led to formation of the indole-3-methanol-methyl ether (R_f 0.69). When the conjugate was hydrolysed with 0.1 M potassium hydroxide, however, and then methanol added, no formation of the ether was observed, i.e. the ether was formed only from the conjugate, not from IM.

Similarly the ICA conjugate could be hydrolysed by 0.1 M potassium hydroxide with release of ICA, and the appropriate ICA esters were obtained by treatment of the ICA conjugate with 0.1 M methanolic sodium methoxide or a mixture of 0.1 M potassium hydroxide and an alcohol (ROH = methanol, ethanol, butanol) [system I: KOH–MeOH (or sodium methoxide), R_f 0.65; KOH–EtOH, R_f 0.69; KOH–BuOH, R_f 0.76].

Identification of sugar components

The conjugates of IM and ICA were prepared by application of IM (2×10^{-5} M) to wheat leaf segments (9×2.5 g in 50 ml each; experiment A). In order to monitor both the uptake of IM and the chromatographic purification of the conjugates, [^{14}C]IM was added (2.2×10^{-6} dpm). After 16 hr, 67% of the radioactivity had been taken up. Similarly, in a control experiment (B), nine 2.5 g wheat leaf segments were incubated in buffer and worked up in parallel to experiment A. After methanol extraction, the conjugates were purified by chromatography (system III) and separated from one another on a preparative column (system VI). The presence of the desired conjugate was tested for by treatment of an aliquot of the appropriate fraction with β-glucosidase. The radioactively labelled indole components released were shown to co-chromatograph with authentic standards in several chromatographic systems. To a further aliquot of the fraction (*ca* 110 000 dpm = 0.2 mg of IM), 20 μg of inositol was added as an internal standard and the mixture was then hydrolysed, reduced and acetylated. GC of the alditol acetates showed that the IM conjugate and the ICA conjugate each contained only one sugar component, namely glucose. Quantitative determination using the internal inositol standard and the specific activity of the IM added yielded the following relationships: IM conjugate: IM/glucose 1:0.85; ICA conjugate: ICA/glucose 1:0.81. These ratios indicate an equimolar relationship between the indolic components and glucose. From these results it can be suggested that the conjugates correspond to indole-3-methanol-β-D-glucoside and indole-3-carboxylic acid-β-D-glucoside, respectively.

Appearance of IM glucoside and ICA glucoside

The appearance of IM glucoside and ICA glucoside was studied in a pulse chase experiment following application of [^{14}C]IM to wheat leaf segments (Fig. 1).

IM was taken up (about 20%) and metabolized rapidly to ICA and to a lesser extent IM glucoside and ICA glucoside during the duration of the pulse of 2 hr. Following application of the chase the radioactivity contents of IM and ICA diminished rapidly whereas those of IM glucoside and ICA glucoside increased. After application of [2-^{14}C]IAA, the curves were essentially similar but with lower rates of incorporation.

The formation of the two glucosides was observed not only in wheat leaves but also in maize and oat leaves and wheat and oat coleoptile cylinders after application of [2-^{14}C]IAA and [^{14}C]IM (Table 1). Furthermore, the data show that the ratio of the two glucosides to one another after exogenous application of the substrates is different. Thus, the concentration of ICA glucoside was decidedly higher in the wheat leaf segments than in the other systems tested.

DISCUSSION

It was shown in a previous study [10] that the major metabolites formed after application of [2-^{14}C]IAA or [^{14}C]IM to wheat leaf segments are IM, IAl and ICA. In addition, the formation of a polar substance (probably a glucoside) from IM was described. This compound reacted with KOH–MeOH to form a non-polar product, which was used for the quantification of the glucoside.

In this study it has been shown that the polar substance is a glucoside of indole-3-methanol and, furthermore, that a glucoside of indole-3-carboxylic acid is formed from ICA. Both conjugates are cleaved by leaf glucosidases during ether extraction, the cleavage of the ICA glucoside occurring more rapidly than that of the IM glucoside; it is for this reason that the formation of the ICA glucoside was not detected in the earlier study [10]. The cleavage is prevented either by addition of a glucosidase inhibitor prior to ether extraction or by using 80% methanol as the extraction agent.

The glucosides exhibit some unexpected properties.

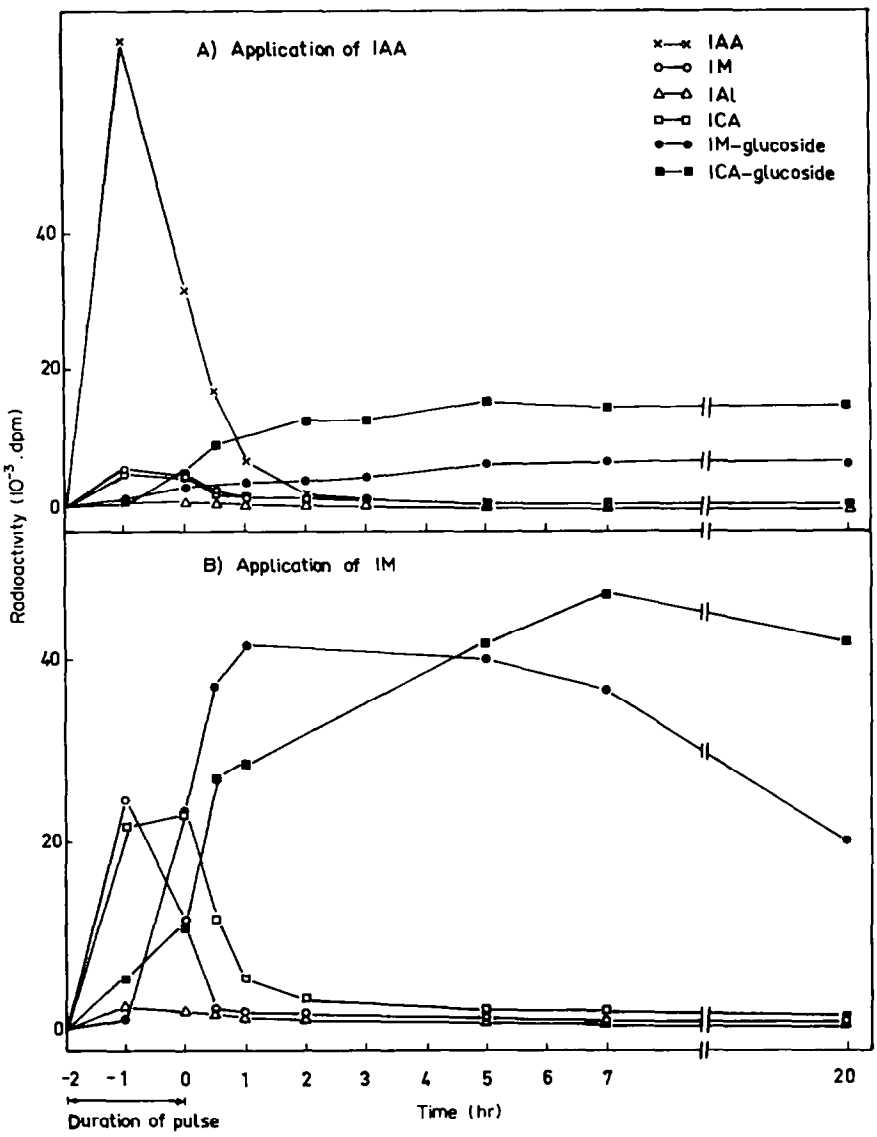

Fig. 1. Incorporation of radioactivity into products derived from IAA or IM after pulse-chase feeding of [2-^{14}C]IAA (experiment A) or [^{14}C]IM (experiment B) to wheat leaf segments [in each case, 10^{-6} M [^{14}C]-substrate (1.85 GBq/mmol), duration of pulse 2 hr, final incubation in 10^{-6} M inactive IAA or IM, respectively]. In experiment A, the non-decarboxylated products from IAA, which were produced in large amounts, are not included in the figure.

Both conjugates are converted to non-polar products with KOH–alcohol mixtures. From the ICA glucoside the corresponding alkyl ester is formed, and from IM glucoside, the alkyl ether. The latter reaction can be explained by a 1,5-fragmentation of the glucoside and a subsequent 1,4-addition of the alcohol to the intermediate Michael-type system (Fig. 2).

The concentrations of the free indoles IM and ICA in leaf segments are apparently rapidly regulated by glucosidation. Of further interest is the observation that the glucosides are taken up much more slowly when applied to wheat leaf segments than the aglyka (data not shown in detail).

The appearance of glucosides of IM and ICA in leaf segments and coleoptile segments of different Gramineae after application of [2-^{14}C]IAA has been described.

Combined with the earlier data [4], the present results permit the assumption that the peroxidase-catalysed decarboxylative pathway of IAA degradation in leaf segments leads, via IM, to IM glucoside or further to IAl, ICA and ICA glucoside (Fig. 3). Such an assumption may be of interest with regard to the proposal that the 'IM pathway' is linked to the oxidation of phenolic co-substrates (see Introduction and Fig. 3).

EXPERIMENTAL

Secondary leaves of 10-day-old wheat, oat or maize seedlings cultivated under controlled conditions (light period 16 hr, 30 W/m^2 Osram HQJ-E 400 W, humidity 60%, temp. 21°/17° day/night) were cut into segments of 4 mm, washed with H_2O

Table 1. Incorporation of radioactivity into IM glucoside and ICA glucoside in segments of different graminaceous systems after application of [2-^{14}C]IAA or [^{14}C]IM (in each case, 10^{-6} M, 1.85 GBq/mmol, incubation time 20 hr). In control experiments in which [1-^{14}C]IAA (10^{-6} M, 1.85 GBq/mmol, 20 hr) was applied, the rates of incorporation in all systems was less than 0.2%

Precursor	System	Uptake of radioactivity (% dose)	Incorporation (% total uptake)		Ratio IM glucoside /ICA glucoside
			IM glucoside	ICA glucoside	
[^{14}C]IM	Wheat leaves	60.5	20.3	22.2	0.9
	Oat leaves	55.5	37.3	3.5	10.7
	Maize leaves	43.1	48.9	5.8	8.4
	Wheat coleoptiles	35.8	38.7	5.2	7.4
	Oat coleoptiles	35.3	40.8	4.8	8.5
[2-^{14}C]IAA	Wheat leaves	76.5	3.3	4.1	0.8
	Oat leaves	65.9	10.9	4.2	2.6
	Maize leaves	60.5	4.3	1.7	2.5
	Wheat coleoptiles	28.7	16.7	5.1	3.3
	Oat coleoptiles	41.0	9.4	2.8	3.3

(2 × 10 min) and incubated in MES buffer (0.1 M, pH 5; 0.5 g leaf segments in 10 ml buffer or 2.5 g in 50 ml). Coleoptiles were cultured by allowing wheat or oat corns to swell in H_2O for 1 hr before being left for 4 days on damp filter paper in the dark at 21°. The coleoptiles were cut off and after removing the tip (4 mm), they were weighed and cut into segments of 4 mm. To reduce the endogenous IAA, these segments were left for 5 hr in MES buffer, washed for 10 min in H_2O and then incubated in MES buffer (0.5 g coleoptile segments in 10 ml buffer).

After addition of the radioactively labelled substances, all samples were incubated with gentle shaking in the dark at 20°.

Extraction. Following incubation, all leaf or coleoptile segments were washed with H_2O, dried on filter paper and pulverized in liquid N_2. For the extraction, the following procedures were performed sequentially on the powder: (a) suspended twice in Et_2O (0.5 g leaf powder/3 ml) at 4° for 70 min) and then centrifuged; (b) suspended twice in 80% aq. MeOH (3 ml, 4°, 70 min) and then centrifuged. Alternatively, the Et_2O extraction was omitted and the leaf powder suspended directly in 80% MeOH.

TLC and HPLC. The extracts were concentrated at a maximum of 30°. Aliquots were chromatographed in the following systems: (I): TLC, silica gel F_{254}; Me_2CO–$CHCl_3$–hexane (5:4:5). (II) TLC, silica gel F_{254}; EtOAc–*i*-PrOH–25% NH_4OH (9:7:4). (III) TLC, silica gel F_{254}; EtOAc–2-butanone–EtOH–H_2O–HOAc (25:15:5:5:0.2). (IV) HPLC, Lichrosorb RP 18, 25 × 8 mm; 0.02 M acetate buffer, pH 3.5–MeCN (4:1); flow rate 2.5 ml/min. (V) HPLC, Lichrosorb RP 18; H_2O–MeCN (4:1); flow rate 2.5 ml/min. (VI) HPLC, Lichrosorb RP 18; H_2O–MeCN (9:1); flow rate 2.5 ml/min. The radioactively labelled compounds were localized on the thin layer plate either by means of inactive standards or by employing a beta-camera system (Berthold BF 290 HR).

For HPLC, on occasion IM and ICA were added to the radioactive samples as inactive standards (photometric detection, 280 nm). The radioactivity of the individual fractions was determined by liquid scintillation counting.

GC and identification of glucose. The polar conjugates of IM and ICA were first purified using TLC (system III) and HPLC (system VI). Procedures to determine the sugar components were similar to those described in ref. [13]. Aliquots of the purified fractions were lyophilized, added to inositol and hydrolysed with 2 M TFA; they were then reduced with ammoniacal $NaBD_4$ soln (150 µl: 10 mg $NaBD_4$/1 ml 1 M NH_3) and finally acetylated with Ac_2O. The alditol acetates were extracted by shaking with $CHCl_3$ and after concn they were compared with standards by GC.

GC: 0.2% diethylene glycol adipate, 0.2% diethylene glycol succinate and 0.4% XF-1150 silicone oil coated on Gas-Chrom P (⅛" × 1 m, stainless steel column); N_2 60 ml/min; temp. programme: 5 min at 170°, followed by a 1°/min to 200°. The concn of sugar in the sample was calculated on the basis of the internal inositol standard using an integrator.

Determination of radioactivity. To determine the incorporation of the precursors into IM, ICA and IAA, aliquots of the extracts were chromatographed together with inactive standards (system I), the bands scraped off the DC plate and counted after suspension in a scintillation cocktail with a thixotropic gel (Cab-o-sil gelling powder; Zinsser, Frankfurt, West Germany).

To determine the incorporation into the conjugates of IM and ICA, aliquots of the extracts were separated using HPLC

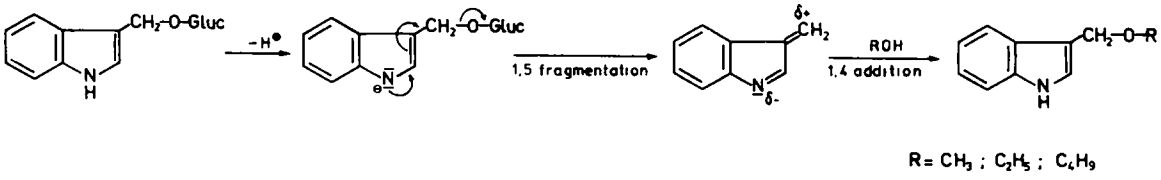

Fig. 2. Proposed reaction scheme for the formation of alkyl ethers from indole-3-methanol-β-D-glucoside by KOH–alcohol mixtures.

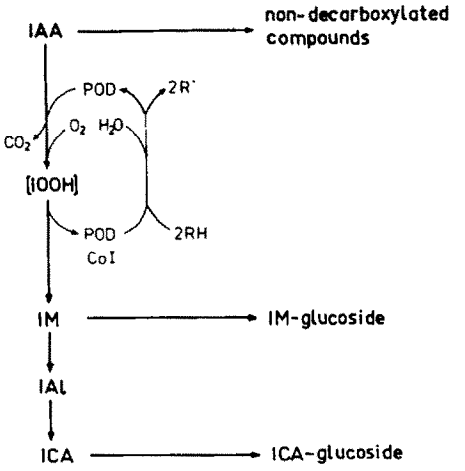

Fig. 3. Proposed pathway for the degradation of IAA in wheat leaf segments. Implicit in the scheme is the assumption that the occurrence of the 'IM pathway' may be indicative of a coupled oxygenase/peroxidase sequence of enzyme activity which involves the formation of indolic hydroperoxide (IOOH; see Introduction), the formation of peroxidase compound I (Co I), and an electron transfer from the phenolic co-substrate (RH). With regard to the mechanism of enzyme action, this concept will be discussed in more detail at a later date.

(system VI) and the radioactivity was subsequently measured in the respective fractions.

In all cases, radioactivity was measured using a Berthod BF 8000 scintillation counter coupled to a Hewlett Packard 9815 A calculator programmed for automatic quench correction.

Chemicals. IAA, IM, IAl and ICA were obtained from Sigma. [2-^{14}C]IAA (1.85 GBq/mmol) was from Amersham/Buchler, Braunschweig, West Germany. [Carbinol-^{14}C]IM with the same sp. act. was prepared from [2-^{14}C]IAA by the use of peroxidase [4]. Peroxidase grade I and β-D-glucosidase (isolated from almonds; enzyme activities other than β-glucosidase less than 0.05%) were from Boehringer, Mannheim, West Germany.

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