Inactivity of Oxidation Products of Indole-3-acetic Acid on Ethylene Production in Mung Bean Hypocotyls

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ABSTRACT

The suggestion that indole-3-acetic acid (IAA)-stimulated ethylene production is associated with oxidative degradation of IAA and is mediated by 3-methylenoxindole (MOI) has been tested in mung bean (Phaseolus aureus Roxb.) hypocotyl segments. While IAA actively stimulated ethylene production, MOI and indole-3-aldehyde, the major products of IAA oxidation, were inactive. Tissues treated with a mixture of intermediates of IAA oxidation, obtained from a 1-hour incubation of IAA with peroxidase, failed to stimulate ethylene production. Furthermore, chlorogenic acid and p-coumaric acid, which are known to interfere with the enzymic oxidation of IAA to MOI, had no effect on IAA-stimulated ethylene production. Other oxidation products of IAA, including oxindole-3-acetic acid, indole-3-carboxylic acid, (2-sulfoindole)-3-acetic acid, and dioxindole-3-acetic acid, were all inactive. 1-Naphthaleneacetic acid was as active as IAA in stimulating ethylene production but was decarboxylated at a much slower rate than IAA, suggesting that oxidative decarboxylation of auxins is not linked to ethylene production. These results demonstrate that IAA-stimulated ethylene production in mung bean hypocotyl tissue is not mediated by MOI or other associated oxidative products of IAA.

MATERIALS AND METHODS

Plant Materials. Seeds of mung bean (Phaseolus aureus Roxb.) were grown in vermiculite for 4 days in the dark at 24°C. Segments 2 cm long were cut from hypocotyls at a point 1 cm below the hook, as previously described (19). Unless otherwise specified, lots of 10 or 20 segments were incubated in 5 ml of a medium consisting of 50 mM K-phosphate buffer (pH 6), 2% sucrose, and various chemicals dissolved in water or ethanol as indicated, in a 50-ml Erlemeyer flask. A plastic center well containing 0.2 ml of 40% KOH was hung in the flask to absorb evolved CO2. The flasks were sealed with rubber serum caps and incubated in a shaker at 27°C in the dark.

Chemicals. Indole-3-acetic acid, kinetin, and peroxidase (type II, RZ = 1.3) were obtained from Sigma. M. L. Evans provided the MOI which was synthesized according to the method of Hinman and Bauman (14, 15) and purified and verified by various methods (6). MOI was rechecked in our laboratory by UV absorption spectroscopy which showed the characteristic double absorption peaks at 248 and 254 nm and a shoulder at 290 nm, as reported by other workers (6, 14). Oxindole-3-acetic acid was synthesized according to the method of Hinman and Bauman (15). (2-Sulfoindole)-3-acetic acid and dioxindole-3-acetic acid were those reported by Horng and Yang (17). IAM was a product of Sigma. [Carboxyl-14C]IAA was prepared from [carboxyl-14C]IAA as follows: IAA (2 μCi, 0.4 μmol) was first methylated with diazomethane and the resulting methyl ester was reacted with 0.5 ml of concentrated ammonia under a N2 atmosphere in a screw-capped test tube and incubated at 30°C for 18 hr in a shaker. At the end of the incubation the products were IAM and IAM, as revealed by paper radiochromatography. IAM was separated from IAA by extraction with ethyl ether.

Intermediates of Peroxidase-catalyzed IAA Oxidation. Hinman and Lang (6) reported that in the presence of peroxidase, the time required for the complete enzymic consumption of IAA at room temperature was less than 1 hr, whereas the formation of MOI, the end product of the peroxidase-catalyzed IAA oxidation pathway, continues for nearly 24 hr, indicating that the conversion of some of the intermediates (such as 3-hydroxymethylxindole) to MOI was slow. On this basis, 18 units of peroxidase were added to a 5-ml medium consisting of 50 mM K-phosphate buffer (pH 6), 2% sucrose, and 100 μM IAA and incubated at 27°C for exactly 1 hr such that all of the IAA was consumed as revealed by the Salkowski test (11), and various intermediates of IAA oxidation were accumulated without noticeable conversion of the intermediates to MOI. The progress of this reaction was followed by monitoring the UV absorption spectrum of the reaction mixture (Fig. 1) which shows that during the first hr of incubation of IAM with peroxidase, there was a rapid decrease in the A in the region (280 nm) corresponding to IAA, without an accompanying increase in the A in the region characteristic of MOI. After the 1-hr incubation there was no further decrease in the absorption peak at 280 nm.

Auxins have been well known to stimulate ethylene production in plant tissues (1). Recently, Frenkel et al. (7, 8) reported that the onset of ethylene evolution in pears was associated with oxidative degradation of IAA, and MOI, a product of IAA oxidation, was effective in initiating ripening and ethylene synthesis in pears. They therefore proposed that auxins were senescence retardants while oxindoles, products of IAA oxidation, were senescence promoters. To understand the mechanism of auxin action on stimulated ethylene production it is important to determine whether IAA-induced ethylene production is caused by IAA per se or is due to the oxidation products of IAA. We have compared the effects of IAA and its oxidation products on ethylene production by mung bean hypocotyls. We have chosen mung bean hypocotyl, a vegetative tissue, rather than fruit tissue because it is known to respond more directly to IAA and has a shorter lag period than fruit tissue. Furthermore, vegetative tissues are not complicated by the ripening processes inherent in fruit tissue where the onset of ethylene evolution is always associated with ripening.

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4 Abbreviations: MOI: 3-methylenoxindole; IAMsp: indole-3-acetylamide; IAM: indole-3-acetamide; NAA: 1-naphthaleneacetic acid.
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Fig. 1. UV absorption spectra taken at intervals of a reaction mixture of peroxidase-catalyzed IAA oxidation. The reaction was carried out in 50 mM K-phosphate buffer (pH 6) containing 2% sucrose, 0.1 mM IAA and peroxidase (3.7 units/ml) at 27 C for various lengths of time. The number on each curve represents the incubation time (in min) at start of each scanning.

peak (280 nm) for IAA, however a gradual increase in the absorption peak for MOI was observed, indicating a conversion of the intermediate of IAA oxidation to MOI. Periodic measurement of the IAA levels in the reaction mixture by the Salkowski test (11) verified that essentially all of the IAA was gone at the end of 0.5 hr.

Ethylene Determination. At indicated time intervals, 1-ml gas samples were withdrawn by hypodermic syringe from the flasks and ethylene content assayed with a gas chromatograph equipped with an alumina column and a flame ionization detector. The flasks were flushed with air and recapped for the next ethylene determination.

Analysis of $^{14}$CO$_2$. The radioactive CO$_2$ absorbed by 0.1 ml of 10% KOH solution was released by acidification with 0.2 ml of 50% lactic acid and then reabsorbed into 0.5 ml of ethanolamine-ethoxyethanol mixture (1:1, v/v) and assayed by liquid scintillation counting.

RESULTS AND DISCUSSION
In contrast to IAA, MOI, one of the products of IAA oxidation in vivo (12) and in vitro (16), was inactive in stimulating ethylene production at concentrations up to 25 $\mu$M (Fig. 2). Similarly, no synergism with kinetin was observed. The failure of this compound to stimulate ethylene production is unlikely to be due to a failure of uptake since the time course of uptake of MOI has been shown to be very similar to that of IAA (6).

Indole-3-aldehyde is another end product of IAA oxidation catalyzed by peroxidase (21). We also tested the activity of this compound in ethylene stimulation and found it to be totally inactive (data not shown). Chlorogenic acid has been shown to prevent certain auxin effects (25). As a further test of the role of MOI in IAA-stimulated ethylene production, the effects of chlorogenic acid (which inhibits the enzymic oxidation of IAA to MOI) and p-coumaric acid (which stimulates IAA oxidation) on ethylene production were also studied. However, neither chlorogenic acid nor p-coumaric acid altered the pattern.

Since peroxidase is ubiquitous in higher plants, the involvement of the intermediates of the peroxidase-catalyzed IAA oxidation (16) in stimulating ethylene production was also considered. Figure 3 shows that only IAA, but not the intermediates of IAA oxidation resulting from reacting IAA with peroxidase for 1 hr, was active in stimulating ethylene production. The amount of ethylene produced by the IAA-treated tissues with peroxidase added at zero time was not different from those treated with IAA only (Fig. 3). This might be due to the fact that the oxidative breakdown of IAA by peroxidase in the incubation mixture was comparatively much slower (16) than the uptake of IAA (4). The present results do not support the view that oxidation products of IAA are responsible for IAA-stimulated ethylene production, but do agree with the conclusions of Kang et al. (18) that peroxidase content of the tissue does not play a role in the regulation of endogenous ethylene biosynthesis and that auxin-stimulated ethylene production is not mediated by induction of peroxidase.

Fig. 2. Effects of various concentrations of IAA (O, •) or MOI (Δ, △) on ethylene production in the presence (---) or absence (-----) of 50 $\mu$M kinetin for 9 hr. Experimental conditions were as described under "Materials and Methods," except that the incubation buffer contained 1% ethyl alcohol and 20 hypocotyl segments.

Fig. 3. Inactivity of products of peroxidase-catalyzed IAA oxidation in stimulating ethylene production. Experimental conditions were as those described under "Materials and Methods"; each flask contained 20 segments. Curve 1: Control (buffer solution only). Curve 2: peroxidase (3.7 units/ml) and hypocotyls were added at the same time to the incubation buffer. Curve 3: buffer solution containing 0.1 mM IAA. Curve 4: peroxidase (3.7 units/ml) and hypocotyls were added at the same time to the buffer solution containing 0.1 mM IAA. Curve 5: 0.1 mM IAA was allowed to react with peroxidase (3.7 units/ml) for 1 hr before hypocotyls were added to the reaction mixture. The resulting reaction mixture contained intermediates of peroxidase-catalyzed IAA oxidation as revealed by Figure 1. Curve 6: buffer solution containing 0.1 mM IAA was reacted for 1 hr with peroxidase (3.7 units/ml) followed by heat denaturation of the enzyme (10-min boiling) and cooling, before hypocotyls were added. The resulting reaction mixture presumably contained largely MOI, the end product of peroxidase-catalyzed IAA oxidation (16).
Some reports indicated that MOI is responsible for the growth effect of IAA in higher plants (3, 9, 24, 25) and in microorganisms (9, 24). This suggestion has recently been refuted by various workers who have shown that purified MOI, not contaminated with unreacted IAA, shows none of the promotive effects of IAA on root initiation (2), coleoptile and stem elongation (2, 6), xylem differentiation (20), and inhibition of root elongation (5).

Several other IAA oxidation products were also tested for their activity in stimulating ethylene production. This includes oxindole-3-acetic acid, (2-sulfoindole)-3-acetic acid, dioxindole-3-acetic acid, and indole-3-carboxylic acid. None of these compounds was active in stimulating ethylene production (data not shown). Oxindole-3-acetic acid was as active as IAA in promoting elongation of pea stem (10), but was totally inactive in Avena coleoptile and first internode tests (13).

Other synthetic auxins, such as NAA and 2,4-D, are also known to stimulate ethylene production effectively (1). If auxin-stimulated ethylene production is caused by its oxidation products, it would be expected that decarboxylation of IAA, which is an obligatory process in IAA oxidation (16), and other auxins should be correlated with ethylene production. Figure 4 clearly shows that stimulation of ethylene production by IAA and NAA was not correlated with their decarboxylation rate. NAA was slightly more active than IAA in stimulating ethylene production but, unlike IAA, was decarboxylated at a very low rate. These results suggest that oxidative decarboxylation of auxins is not linked to ethylene production.

In plant tissues, IAA is metabolized mainly through oxidation and conjugation (21, 23). In mung bean hypocotyls IAAsp was identified as the major conjugation product of IAA. However, IAAsp was inactive in stimulating ethylene production, and the ethylene production rate bore no correlation with the level of IAAsp but was closely correlated with endogenous free IAA (19). IAM has also been reported as a metabolic product of IAA in some tissues (22, 23). However, IAM was ineffective in stimulating ethylene production (data not shown). Metabolism studies with indole-3-[1-14C]acetaldehyde indicated that the amide was very slowly hydrolyzed to free IAA (not detected before 10 hr) which was then metabolized as usual to form IAAsp almost as rapidly as it was formed.

On the basis of above data and discussion we conclude that IAA, but not the oxidation or conjugation products of IAA, was the active species for ethylene stimulation in vegetative tissue.

We have shown that, in vegetative tissue, it is IAA, but not the IAA oxidation products, which initiates ethylene production. However, in pear fruits Frenkel et al. (7, 8) have reported that it is the IAA oxidation products, but not IAA, which initiate ethylene production and ripening. The regulatory mechanism for the initiation of ethylene production in ripening fruit tissue is known to be different from that in vegetative tissue (1). However, it is hard to conceive that they could be exactly opposite in relation to the role of IAA and IAA oxidation products. The onset of ethylene production in fruit tissues is always associated with ripening. Hence, the effect of the IAA oxidation products as reported by Frenkel et al. with pear fruits may be best interpreted as that they directly influence the ripening process, and, as a consequence, ethylene production is indirectly initiated.

If oxidative degradation of IAA was associated with the onset of ethylene evolution and ripening fruit tissues as proposed by Frenkel and his co-workers (7, 8), one would expect that a more active oxidation of IAA would occur at the onset of ethylene evolution. In this laboratory D. O. Adams (unpublished results) has fed [carboxyl-14C]IAA to avocado fruit tissue at different ripening stages and found that IAA was metabolized more actively by conjugation (IAAsp being the major product) than by oxidation (as determined by 14CO2 production) at all ripening stages and that there is little difference in the rate of IAA oxidation throughout the ripening process. These data are not in parallel with the proposal of Frenkel and indicate that more work is needed to ascertain whether the products of IAA oxidation play an essential role in the ripening of other fruit tissues.

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