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Ethylene-Enhanced Catabolism of [^{14}C]Indole-3-Acetic Acid to Indole-3-Carboxylic Acid in Citrus Leaf Tissues[1]

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ABSTRACT

Exogenous [^{14}C]indole-3-acetic acid (IAA) is conjugated in citrus (*Citrus sinensis*) leaf tissues to one major substance which has been identified as indole-3-acetylaspartic acid (IAAsp). Ethylene pretreatment enhanced the catabolism of [^{14}C]IAA to indole-3-carboxylic acid (ICA), which accumulated as glucose esters (ICGlu). Increased formation of ICGlu by ethylene was accompanied by a concomitant decrease in IAAsp formation. IAAsp and ICGlu were identified by combined gas chromatography-mass spectrometry. Formation of ICGlu was dependent on the concentration of ethylene and the duration of the ethylene pretreatment. It is suggested that the catabolism of IAA to ICA may be one of the mechanisms by which ethylene reduces endogenous IAA levels.

IAA is subjected in plant tissues to several metabolic pathways. It may be converted to conjugates with amino acids, glucose, *myo*-inositol, or it may be catabolyzed to various products by decarboxylative and nondecarboxylative oxidation (1, 7, 8, 26). Several authors suggested that these processes serve to regulate the level of IAA in plant tissues (7, 8, 26). Since ethylene has been reported to reduce endogenous IAA levels (2, 6, 13, 16, 17), its effects on IAA metabolism have been examined in several studies. In most of these studies, ethylene has been shown to increase the conjugation of both exogenous and endogenous IAA (6, 12, 13, 18, 20, 21, 30). Also, ethylene increased the decarboxylation of IAA but the products of this reaction have not been investigated (3, 4).

Previous data from our laboratory indicated that ethylene increases the conjugation of [^{14}C]IAA in citrus leaf tissues to unidentified low and high mol wt metabolites (20, 21). The present paper reports that ethylene enhances the catabolism of labeled IAA in citrus leaf tissues to ICA[3] which accumulates in the tissues as glucose esters. A preliminary report of these results was presented elsewhere (22).

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[3] Abbreviations: ICA, indole-3-carboxylic acid; IAAsp, indole-3-acetylaspartic acid; ICGlu, indole-3-carboxyl-β-D-glucose; PVPP, polyvinylpolypyrrolidone; TMS, trimethylsilyl; EI, electron impact; m/z, mass per number of charges; IAGlu, indole-3-acetyl-β-D-glucose; IM, indole-3-methanol; R_t, retention time.

MATERIALS AND METHODS

Plant Material and Treatment

Leaves from the current year flush were taken from established trees of *Citrus sinensis* [L.] Osbeck, cv Shamouti orange. Leaves were treated with 25 μL L^{-1} ethylene by means of a flow system. When the effect of ethylene concentration was studied, leaves were incubated in sealed 20 L containers and ethylene was injected directly into the containers. IAA metabolism was studied in leaf discs, 6 mm in diameter, and in 1 mm long midrib sections. Experiments were repeated at least four times and treatments were triplicated.

IAA Metabolism

Three hundred mg of either leaf discs or midrib sections were incubated in 1 mL 20 mM K-phosphate-citric acid buffer (pH 4.5) containing 3.6 μM [2-^{14}C]IAA (1.02 GBq $mmol^{-1}$, Amersham, England). Incubation was carried out at 25°C in the dark with constant shaking. At the end of the incubation period, the tissue was rinsed with distilled water and ground with a mortar and pestle in 2 mL 80% (v/v) ethanol. The mortar and pestle were washed with additional 2 mL 80% ethanol and the wash was combined with the initial extract and centrifuged at 5000g for 10 min. Analysis of IAA metabolites in the supernatant was performed by either TLC or HPLC.

Thin-Layer Chromatography

TLC was performed with silica gel PF_{254} plates (Merck, Darmstadt, FRG). Aliquots were applied together with authentic IAA and IAAsp (Scheme 1) and the plates were developed in chloroform:ethyl acetate:formic acid (35:55:10, v/v) (solvent system A) and ethyl acetate:methyl ethyl ketone:formic acid:water (5:3:1:1, v/v) (solvent system B). After drying, the plates were viewed under short UV light for location of standards, and then 10 separate R_F zones were scraped into counting vials and the radioactivity in each zone was determined by means of a scintillation counter.

High Pressure Liquid Chromatography

HPLC analysis was performed by a Varian 5500 Gradient Liquid Chromatograph equipped with a Rheodyne high pressure loop injector, a Varian UV-100 variable wave length detector, and an analytical column (4 × 250 mm) packed with 5 μm LiChrosorb RP-18 (Merck, Darmstadt). The solvents were water (A) and acetonitrile (B). Solvent program

was: 0 to 10 min, 5% (v/v) B; 10 to 12 min, a linear gradient from 5 to 40% B; 12 to 25 min, 40% B. The flow rate was 1 mL min^{-1}. One mL fractions were collected, and the radioactivity in each fraction was determined.

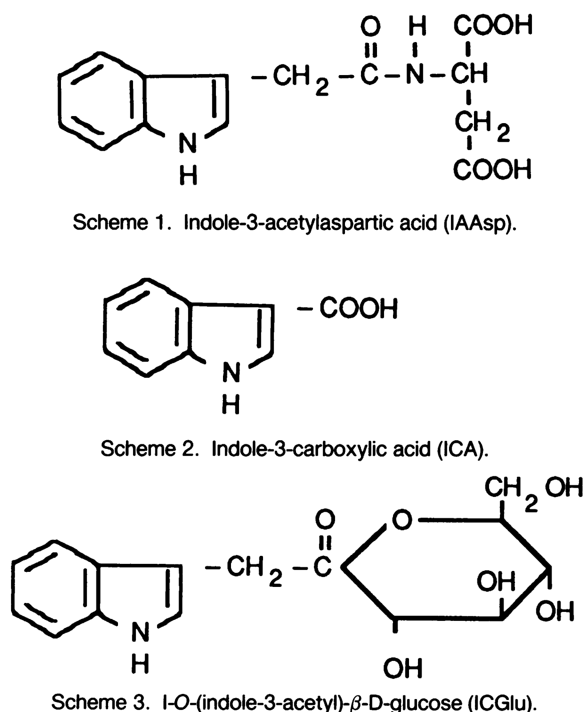
Gas Chromatography-Mass Spectrometry

GC-MS of ICA (Scheme 2) and IAAsp was performed on a Finigan MAT 4600 Mass Spectrometer. Capillary GC was carried out on a 15 m × 0.32 mm i.d. DB-5 column (J & W Scientific, Folsom, CA) with a film thickness of 0.25 μm. The helium flow rate was 1.2 mL min^{-1} and the injector temperature was 250°C. For ICA, a temperature program of 130 to 220°C at 4°C min^{-1} was started upon injection, whereas for IAAsp the temperature was programmed from 150 to 250°C as above. Mass spectra were recorded at an ionizing voltage of 70 eV with a source temperature of 250°C.

GC-MS analysis of ICGlu (Scheme 3) was performed with a VG-ZAB-2FHF High Resolution Mass Spectrometer (Shell Development, Modesto, CA). Capillary GC was performed on a 30 m × 0.25 mm i.d. DB-5 column with a film thickness of 0.25 μm. The helium flow rate was 1 mL min^{-1} and the injector temperature was 250°C. The column was at 50°C with 1 min isothermal hold followed by a programmed increase of 20°C min^{-1} to 220°C and 2°C min^{-1} from 220 to 290°C. Mass spectra were recorded at an ionizing voltage of 70 eV with a source temperature of 300°C.

Isolation of IAAsp

IAAsp was isolated from 180 g of midrib tissue incubated for 8 h in 600 mL of 20 mM K-phosphate-citric acid buffer (pH 4.5) containing 50 μM cold IAA and 40,000 dpm of ^{14}C-IAA. The tissue was extracted three times with 80% (v/v) ethanol to yield a total volume of 1 L. The extract was filtered and then centrifuged at 10,000*g* for 10 min, and the supernatant was reduced to an aqueous solution on a rotary evaporator at 45°C. The aqueous solution was adjusted to pH 8.5 and extracted three times with ethyl acetate. The ethyl acetate fraction was discarded and the aqueous fraction was adjusted to pH 2.5 and extracted with three equal volumes of ethyl acetate. The ethyl acetate fraction containing the IAAsp was reduced to dryness. The gum obtained was dissolved in a small volume of 30 mM K-phosphate-citric acid buffer (pH 4.0) and chromatographed on a PVPP column (1.8 × 16 cm) equilibrated with the same buffer. Fractions containing IAAsp were pooled and, after adjusting the pH to 2.5, IAAsp was extracted with three equal volumes of ethyl acetate. The ethyl acetate solution was evaporated to dryness, and IAAsp was dissolved in a small volume of 50% (v/v) ethanol and chro-



Scheme 1. Indole-3-acetylaspartic acid (IAAsp).

Scheme 2. Indole-3-carboxylic acid (ICA).

Scheme 3. 1-*O*-(indole-3-acetyl)-β-D-glucose (ICGlu).

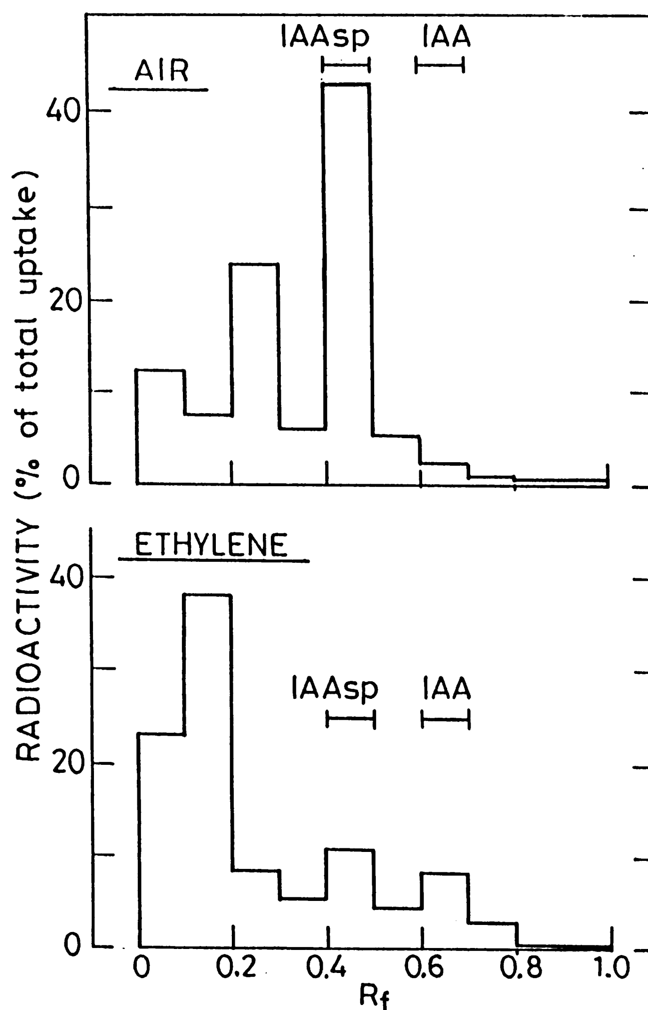


Figure 1. [^{14}C]IAA metabolism in midrib tissue excised from citrus leaves pretreated with either air (top) or 25 μL L^{-1} ethylene (bottom) for 48 h. The tissue was incubated in labeled IAA for 8 h and then the labeled metabolites were extracted with 80% (v/v) ethanol. Analysis was performed by TLC using solvent system A. The R_F regions of authentic IAA and IAAsp run on the same chromatogram are designated by bars.

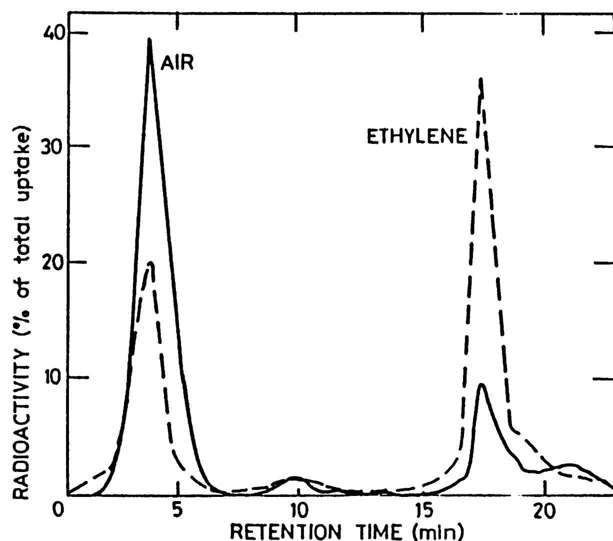


Figure 2. Analysis of the same extracts described in Figure 1 by HPLC. Elution was performed as described in "Materials and Methods." Under these conditions IAAsp had a R_t of 3.7 min.

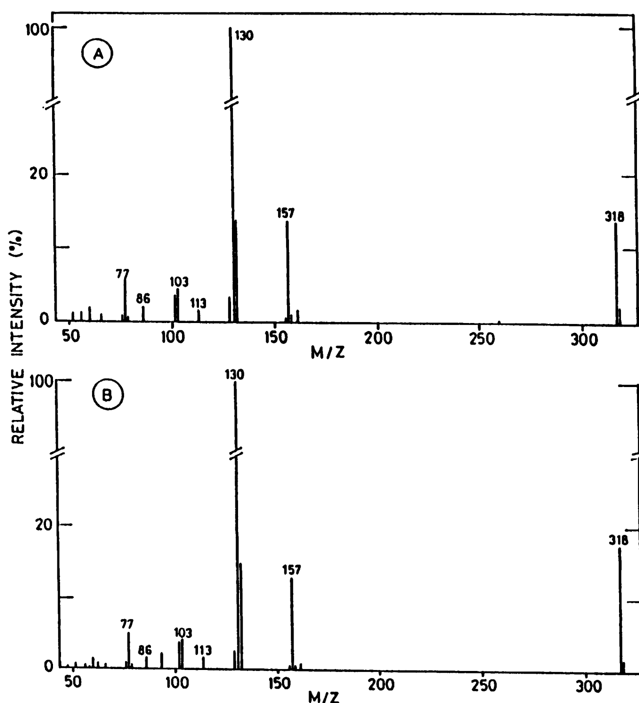


Figure 3. 70 eV EI mass spectra of (A) authentic bis-methyl-IAAsp and (B) a methylated sample of putative IAAsp isolated from midrib tissue of citrus leaves incubated for 48 h in air. Ions characteristic of *bis*-methyl-IAAsp are m/z 318 (m^+), 157, and 130 (base peak).

matographed on a 1.2 × 10 cm Sephadex LH-20 column with the same solvent. Fractions containing IAAsp were evaporated to dryness and IAAsp was methylated with diazomethane (25). The methylated IAAsp was purified on silica gel PF_{254} plates, 1 mm thick, developed with chloroform:ethyl acetate (2:1 v/v) solvent system. After drying, the zone corresponding to methylated IAAsp was scraped off and eluted with ethyl acetate. The ethyl acetate solution was reduced to a small volume and further purified by HPLC as above except that elution was performed with a 35-min linear gradient of 35 to 65% (v/v) methanol in water with a flow rate of 0.8 mL min^{-1}. Fractions containing the conjugate were collected, dried *in vacuo*, and dissolved in ethyl acetate for GC-MS analysis.

Isolation of ICGlu

Leaves were pretreated with ethylene for 48 h and then 150 g of midrib tissue were excised for isolation of ICGlu. Incubation and extraction procedures were similar to those described for IAAsp, except that the tissue was incubated in 200 μM cold IAA. The aqueous solution obtained after removal of the ethanol was adjusted to pH 2.5 and extracted with three equal volumes of diethyl ether. The etheric fraction was discarded, and the aqueous solution was adjusted to pH 8.5 and extracted with three equal volumes of 1-butanol. The butanol fraction containing the ICGlu was reduced to dryness. The gum obtained was dissolved in a small volume of methanol, loaded on a 1.5 × 25 cm column of PVPP, and eluted with methanol. Fractions containing ICGlu were pooled, reduced to a small volume, and further purified on silica gel PF_{254} plates, 1 mm thick, using chloroform:methanol:water (80:19:1, v/v) followed by three successive runs with ethyl:ethanol: water (85:6:9, v/v). After each TLC analysis, the acetate zones corresponding to ICGlu were eluted with methanol. ICGlu was silylated prior to GC-MS analysis with *N,O*-bis(trimethylsilyl)trifluoroacetamide (11).

RESULTS

IAA Metabolism in Air- and Ethylene-Pretreated Tissues

Ethanol extracts of tissues excised from mature leaves pretreated with either air or ethylene were analyzed by TLC. Midrib tissue of air-pretreated leaves metabolized [^{14}C]IAA to several substances (Fig. 1). The major metabolite (R_F = 0.4–0.5) cochromatographed with authentic IAAsp. Ethylene pretreatment increased the formation of polar metabolite(s) (R_F = 0–0.2) with a concomitant decrease in the other metabolites. The changes in IAA metabolism induced by ethylene were verified by HPLC analysis. For separating the metabolites by HPLC, we first employed acidic conditions. Under these conditions, IAAsp had the same R_t as the polar metabolite(s). Therefore, we had to use a neutral solvent system which allowed a good resolution between IAAsp and the polar metabolite(s) (Fig. 2), although the first peak eluted too fast and was relatively wide. However, since after elution there were only two major labeled compounds, the resolution adequately shows that their relative amounts were changed by ethylene. Leaf blade tissue showed similar patterns of IAA metabolism but the level of the polar metabolite(s) was lower than that in midrib tissue (data not shown).

Identification of IAAsp and ICGlu

A methylated sample of the purified putative IAAsp isolated from midrib tissue of air-pretreated leaves cochromato-

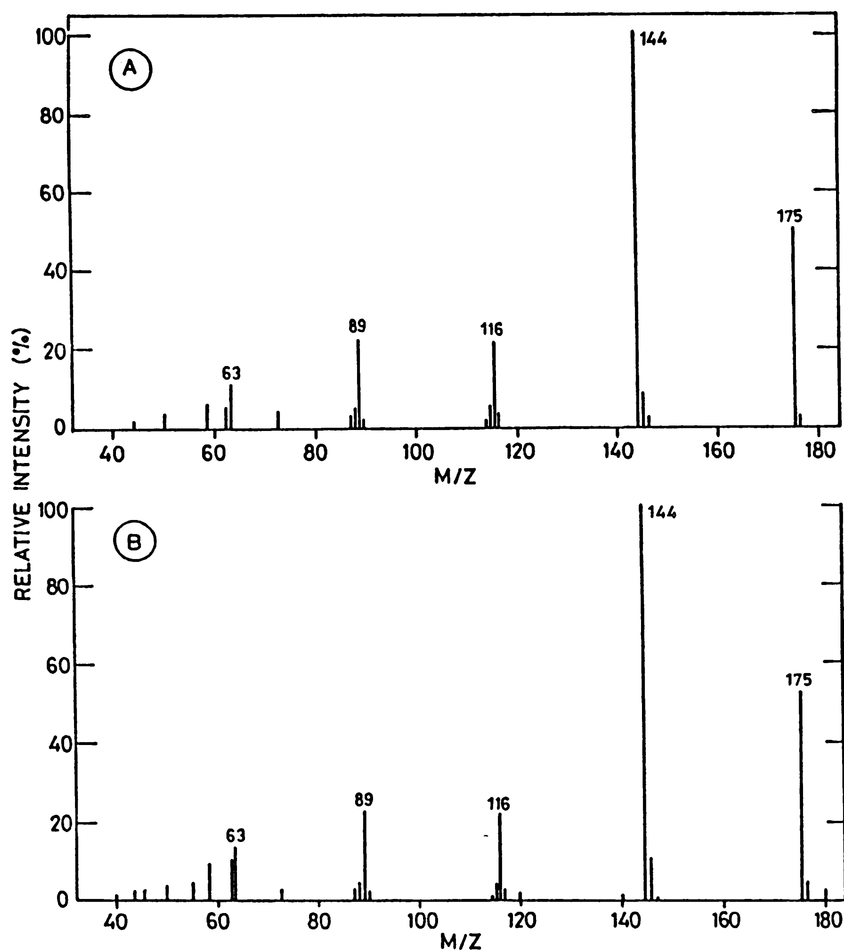


Figure 4. 70 eV EI mass spectra of (A) authentic methyl-ICA and (B) a methylated sample of putative ICA obtained by hydrolysis of ICGlu. ICGlu was isolated from midrib tissue of citrus leaves pretreated with 25 μL L^{-1} ethylene for 48 h. Ions characteristic of methyl-ICA are m/z 175 (m^+), 144 (base peak), and 116.

graphed with an authentic sample of bis-methyl-IAAsp on TLC using chloroform:ethyl acetate (2:1) solvent system (R_F = 0.38) and coeluted with authentic sample in HPLC (R_t = 10.4 min). The identity of the putative IAAsp was confirmed by combined GC-MS (Fig. 3).

The purified polar fraction isolated from midrib tissue of ethylene-pretreated leaves gave a single spot with Ehmann's reagent (10) with TLC analysis. The color obtained, yellow-orange turning to red after a few minutes, is characteristic to ICA (10). Hydrolysis of the purified metabolite with 1 N NaOH at room temperature or with β-glucosidase yielded one spot with Ehmann's reagent as described above. The indolic moiety obtained by hydrolysis cochromatographed with ICA on TLC with solvent system A (R_F = 0.76). After methylation, the putative ICA cochromatographed with authentic sample of methylated ICA on TLC using chloroform:ethyl acetate (2:1) solvent system (R_F = 0.72). The identity of the putative ICA was confirmed by combined GC-MS (Fig. 4).

GC-MS analysis of the purified ICA conjugate isolated from ethylene-pretreated leaves revealed a major peak having a molecular ion at 683 which corresponds to TMS-ICGlu (Fig. 5). The presence of two additional peaks having the same molecular ion (data not shown) suggest the presence of three isomeric forms of ICGlu. The EI spectrum of TMS-ICGlu show the ions 117, 129, 147, 204, 247, 291, 303, 319, and 450, which are characteristic for TMS ethers of carbohydrates (19). The base peak at 216 probably results from TMS-indolecarbonyl. As expected, the TMS-quinolinium ion at 202, derived by cyclization and enlargement of IAA (28), was not present.

Effect of Ethylene on ICGlu Formation

The effect of the duration of ethylene pretreatment on ICGlu formation was studied. Ethylene pretreatment for 8 h already increased significantly the formation of ICGlu (Fig. 6). The greatest stimulation occurred after 48 h of ethylene exposure. In air controls, some radioactivity was found at the R_F corresponding to ICGlu but it remained more or less constant during incubation.

When leaves were subjected to various concentrations of ethylene, a positive correlation between ethylene concentration and ICGlu formation was observed (Fig. 7). A slight increase in ICGlu was detected with 1 μL L^{-1} ethylene. Above this concentration, ICGlu formation increased progressively with the rise in ethylene concentration, reaching a maximal level at 50 μL L^{-1} ethylene.

DISCUSSION

The present paper demonstrates that IAAsp is the major metabolite of [^{14}C]IAA in citrus tissues (Figs. 1 and 2). Ethylene alters the normal pattern of IAA metabolism by increas-

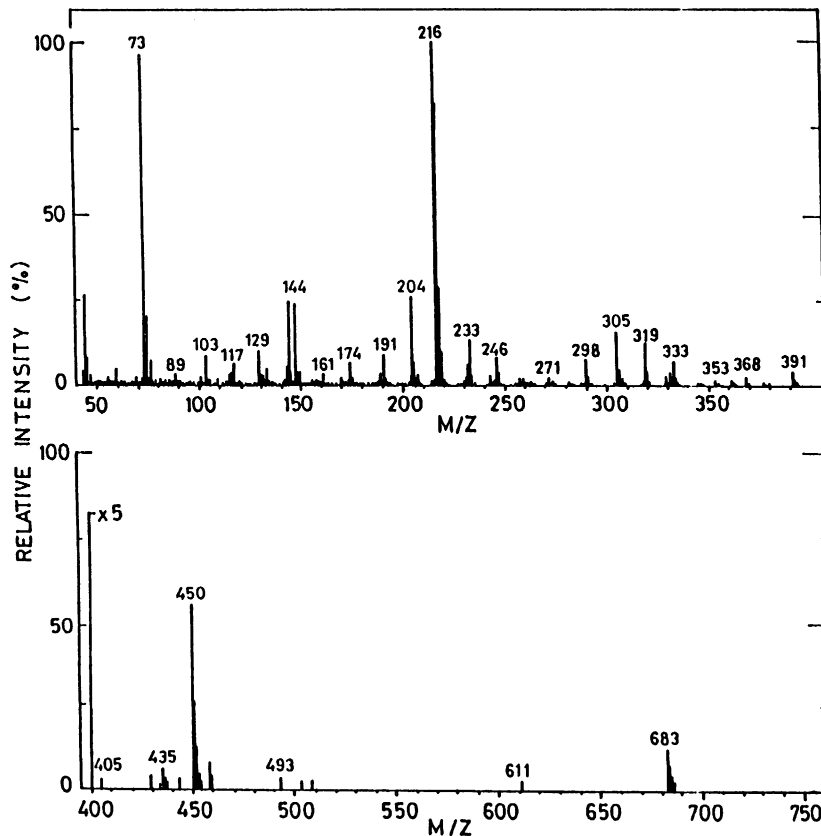


Figure 5. 70 eV EI mass spectra of a silylated sample of putative ICGlu. ICGlu was isolated from midrib tissue of citrus leaves pretreated with 25 μL L^{-1} ethylene for 48 h.

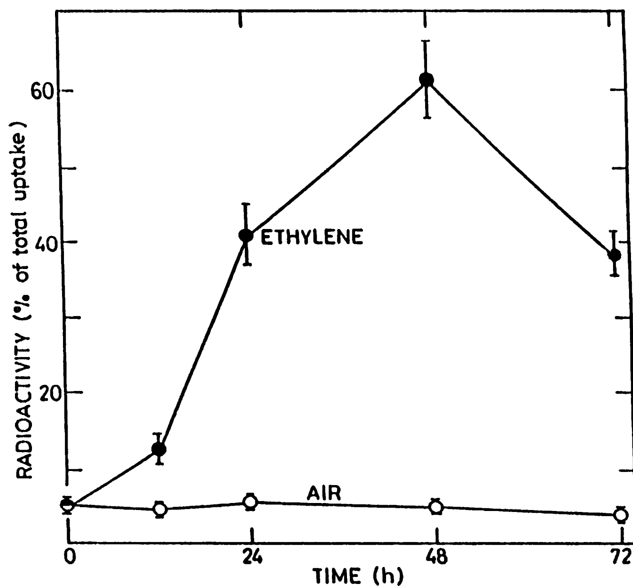


Figure 6. Effect of the duration of 25 μL L^{-1} ethylene-pretreatment on formation of ICGlu in midrib tissue of citrus leaves. Leaves were treated with ethylene for various periods and then the midrib tissue was excised and treated with [^{14}C]IAA for 8 h. Labeled ICGlu was extracted with 80% (v/v) ethanol and analyzed by TLC using solvent system B. Bars indicate 1 SE.

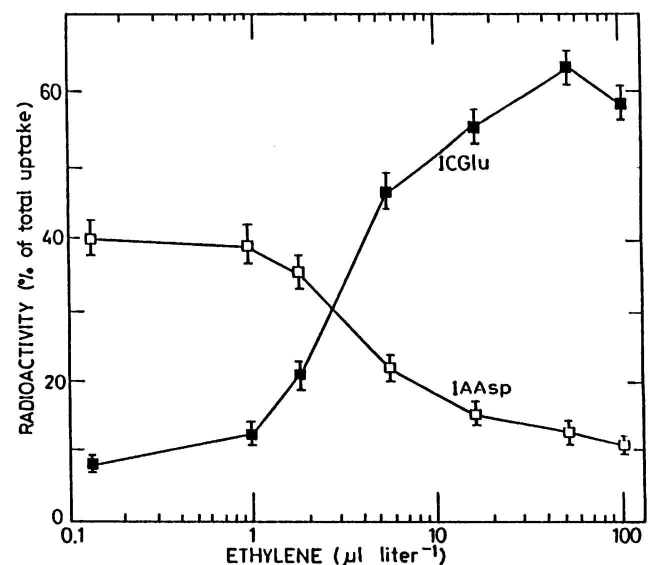


Figure 7. Effect of ethylene concentration on formation of IAAsp and ICGlu in midrib tissue of citrus leaves. Leaves were incubated in various concentrations of ethylene for 28 h and then the midrib tissue was excised and treated with labeled IAA for 8 h. Labeled metabolites were extracted with 80% (v/v) ethanol and analyzed by TLC. IAAsp was analyzed by solvent system A and ICGlu by solvent system B. Bars indicate 1 SE.

ing its catabolism to ICA. The ICA formed is rapidly conjugated with glucose to form ICGlu, and therefore no free ICA could be detected. GC-MS analysis indicates that ICGlu exists in three isomeric forms, the nature of which has not yet been investigated. Other IAA esters also exist in several isomeric forms (9).

Hydrolysis of ICGlu by β-glucosidase suggests that it corresponds to ICA-β-D-glucose. This conjugate was previously identified as one of the products of IAA catabolism in wheat leaves (29). Previous reports have claimed that ethylene induces an increase in the conjugation of IAA to IAGlu (20, 21). This claim was based on tentative identifications by TLC using solvent system A. Since IAGlu and ICGlu have the same R_F on TLC with this solvent system, it is possible that the conjugate identified in earlier reports as IAGlu was in fact ICGlu.

Increased formation of ICGlu from [^{14}C]IAA in ethylene-pretreated tissues was followed by a concomitant decrease in IAAsp formation (Figs. 1, 2, and 7). At present it is not clear whether ethylene has a direct inhibitory effect on the conjugation of IAA with aspartic acid. It is more likely that reduced formation of IAAsp resulted from a lower availability of IAA due to its catabolism.

There are two major pathways of IAA catabolism in plants: (a) oxidative decarboxylation yielding several metabolites and (b) a nondecarboxylative pathway which produces oxindole-3-acetic acid and its hydroxy derivatives (1, 7, 26). *In vitro* studies of the oxidative decarboxylation of IAA catalyzed by horseradish peroxidase or cell-free systems indicate that 3-methyleneoxindole and indole-3-aldehyde are the major metabolites (26). However, if a suitable electron donor is added to the reaction mixture, a substantial amount of IM is formed (14). Recently, IM and ICA have been established as natural constituents in pine tissue (24, 27) and these indoles or their glucose esters are formed after application of labeled IAA to plant organelles and excised tissues (5, 15, 23, 29). ICA is thought to be derived from IM *via* IAA aldehyde (15). The present study supports the idea that ICA may be an important product of *in vivo* IAA decarboxylation, suggesting that this pathway may be under hormonal control. It has been claimed that ICA may be an artefact, originating during IAA extraction (24). This claim does not apply to the present study because ICA was detected in the form of glucose esters which could be formed only *in vivo*.

It has long been suggested that ethylene reduces endogenous IAA levels (2, 13, 16, 17). There are data indicating that ethylene increases the conjugation of IAA to IAAsp and other unidentified conjugates (6, 12, 13, 18, 20, 21, 30). The present paper provides evidence that increased IAA catabolism may be an additional mechanism by which ethylene reduces IAA levels, as previously suggested (3, 4). It should, however, be mentioned that most studies, including the present paper, employed [^{14}C]IAA for studying the effect of ethylene on IAA metabolism. It remains to be determined whether ethylene also induces the catabolism of endogenous IAA to ICA. ICA has been found as a natural constituent of plant tissues (24), and it is most likely that the same enzyme systems are operating on both exogenous and endogenous IAA, suggesting that ethylene may also induce the catabolism of endogenous IAA.

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