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Role of IAA Conjugates in Inducing Ethylene Production by Tobacco Leaf Discs

Shimon Meir, Sonia Philosoph-Hadas, and Nehemia Aharoni

Department of Fruit and Vegetable Storage, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel

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Abstract. Indole-3-acetic acid (IAA) labeled in its carboxyl group was metabolized by tobacco leaf discs (*Nicotiana tabacum* L. cv. Xanthi) into three metabolites, two of which were preliminarily characterized as a peptide and an ester-conjugated IAA. Reapplication of each of the three metabolites (at 10 μM) resulted in a marked stimulation of ethylene production and decarboxylation by the leaf discs. Similarly, these three IAA metabolites could induce elongation of wheat coleoptile segments, which was accompanied by decarboxylation. Both the exogenously supplied esteric and peptidic IAA conjugates were converted by the leaf discs into the same metabolites as free IAA. (1-^{14}C)IAA, applied to an isolated epidermis tissue, was completely metabolized to the esteric and peptidic IAA conjugates. This epidermis tissue showed much higher ethylene production rates and lower decarboxylation rates than did the whole leaf disc.

The results suggest that the participation of IAA conjugates in the regulation of various physiological processes depends on the release of free IAA, which is obtained by enzymatic hydrolysis of the conjugates in the tissue. The present study demonstrates biological activity of endogenous IAA conjugates that were synthesized by tobacco leaf discs in response to exogenously supplied IAA.

Auxins can induce ethylene production in vegetative tissue (Abeles 1973, Lieberman 1979) by stimulating the synthesis of 1-aminocyclopropane-1-carbox-

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ylic acid (ACC) synthase (Yu et al. 1979, Yu and Yang 1979, Yang et al. 1980). Kang et al. (1971) and Lau and Yang (1973) showed that the rate of indole-3-acetic acid (IAA)-induced ethylene production by stem segments of pea and mung bean was parallel to the level of free IAA retained in the tissue. However, Aharoni and Yang (1983) recently showed that the rate of IAA-induced ethylene production in tobacco leaf discs corresponded to the accumulation of some polar IAA conjugates. They also found that these IAA conjugates are capable of inducing ethylene production. A number of IAA amino acid conjugates were found to be effective in some physiological processes that are known to be IAA-mediated. These include growth of tomato hypocotyl explants and tobacco callus cultures, or induction of ethylene production by pea stems (Hangarter et al. 1980).

In the present study we describe a preliminary characterization of some IAA conjugates formed in tobacco leaf discs, following IAA treatment. We also reexamined the hypothesis postulating that the capability of IAA conjugates to induce ethylene production is related to their turnover, during which a slow release of free IAA occurs.

Materials and Methods

Plant Material

Experiments were conducted with discs taken from fully expanded leaves of tobacco plants (*Nicotiana tabacum* L. cv. Xanthi), which were grown in a greenhouse under long-day conditions (18 h light) at temperatures between 20°C and 30°C. In one experiment, where indicated, epidermis layers cut from tree tobacco (*Nicotiana glauca*) leaves were also employed. Leaves were washed under running tap water, surface-sterilized with 0.5% sodium hypochlorite solution for 30 s, and washed several times in sterile distilled H_2O. The use of NaOCl as indicated had no effect on ethylene production rates and NaOCl did not react with the IAA added to the tobacco leaves, unlike the situation previously reported for other tissues (Abdul-Baki 1974). Discs (1 cm in diameter) were cut from leaf blades with a cork borer and were floated on water for about 1 h in Petri dishes. Subsequent handling of the leaf discs involved sterile techniques (Aharoni et al. 1979).

Incubation Media

Samples of ten leaf discs weighing about 100 mg were placed on filter papers in 50-ml Erlenmeyer flasks, to which 1.8 ml of the test solution was added. The test solution contained 50 mM Na-phosphate buffer (pH 6.1), 50 mM sucrose, and 50 μg/ml chloramphenicol (Aharoni and Yang 1983). Additions of IAA (Sigma) and (1-^{14}C)IAA (Amersham/Searle Corp., 59 mCi/mmol) were included as indicated. Two plastic center wells were hung in each flask, one containing a filter paper wick wetted with 0.1 ml of 0.25 M $Hg(ClO_4)_2$ reagent to absorb evolving ethylene (Young et al. 1952), and the second with 0.1 ml of

10% KOH to absorb evolving CO_2. The flasks were sealed with rubber serum caps and then incubated in darkness at 30°C.

Measurements of Ethylene Production Rates

At the end of the incubation period, the plastic center wells containing the mercury perchlorate reagent were transferred to new 50-ml Erlenmeyer flasks sealed with rubber serum caps. The ethylene absorbed was then released from the mercury perchlorate complex by injecting 0.15 ml saturated lithium chloride, and allowed to stand in the flasks for 2 h at 4°C (Young et al. 1952). Ethylene concentration was analyzed by a gas chromatograph equipped with an activated alumina column and a flame ionization detector.

Decarboxylation of (1-^{14}C)IAA

The plastic center wells, containing the $^{14}CO_2$ absorbed by the KOH solution, were placed in scintillation vials containing 10 ml of toluene–Triton X-100 solution and assayed by a Kontron liquid scintillation counter.

Auxin Extraction and Chromatography

At the end of the incubation period, leaf discs were rinsed with sterile water and hand-homogenized with 2 ml of 70% (v/v) ethanol. The homogenizer was washed twice with 1 ml of 80% ethanol. The homogenate was centrifuged at 5,000 rpm for 10 min. The combined extract was concentrated *in vacuo* at 38°C to a final volume of 1 ml. An aliquot was taken from the extract and chromatographed on Whatman 3MM paper, using 1-butanol–acetic acid–H_2O (4:1:4, v/v), isopropanol-8 N NH_4OH (8:2, v/v), and chloroform–ethyl acetate–formic acid (5:4:1, v/v) as solvent systems. Authentic samples of unlabeled IAA, indole-3-acetyl–aspartic acid (IAAsp) (Research Organic Inc.), and 1-(indole-3-acetyl)-β-D-glucose (IAGlu) (kindly provided by Dr. J. Riov) were cochromatographed. After drying, the chromatograms were scanned with a Packard Radiochromatogram scanner, and visualized under short UV light for location of the unlabeled standards.

Elution of IAA Metabolites from the Chromatograms

The scanned chromatograms were cut into sections according to the parallel R_fs, and the sections of each peak were placed in a 150-ml glass jar. The metabolites were eluted from the paper sections by shaking the jars with 40 ml ethanol in the dark at 30°C for 24 h. Thereafter, the paper sections were washed twice with 15 ml of ethanol and the combined extract was dried *in vacuo* at 38°C. The dried extract was then treated as follows (according to the experiment required): For hydrolysis of the IAA metabolites, the extract was

brought to a volume of 1 ml with 70% ethanol and further processed as described below. For application of the IAA metabolites to the leaf discs or to the coleoptile segments, the dried samples were brought to a volume of 2 ml either with 50 mM Na-phosphate buffer (pH 6.1) or with 10 mM K-phosphate-citrate buffer (pH 5), respectively.

Hydrolysis of IAA Conjugates

Three 0.25-ml aliquots were taken from each reextracted metabolite and were diluted with H_2O to a volume of 25 ml each. One diluted sample of each metabolite was used as a control. The second sample was hydrolyzed for 1 h in 1 N NaOH at 25°C to liberate IAA from the esteric IAA conjugates (Bandurski and Schulze 1977). The third sample was hydrolyzed for 3 h in 7 N NaOH at 100°C to liberate IAA from the peptidic IAA conjugates (Bandurski and Schulze 1977). During hydrolysis, N_2 gas was blown over the surface of the hydrolysis mixture to protect free IAA from decarboxylation. The hydrolysates were acidified to pH 6.5 (5 N HCl), concentrated to about 7 ml *in vacuo*, and centrifuged at 5,000 rpm for partial desalting. The resulting supernatant was dried and dissolved in 1 ml ethanol 70%. Then aliquots were taken from each mixture for paper chromatography, using 1-butanol–acetic acid–H_2O (4:1:4, v/v) as the solvent system.

In another experiment, the hydrolysates were acidified to pH 2.5 (HCl) and extracted three times with equal volumes of diethyl ether for desalting. The organic phase was dried *in vacuo* and dissolved in 0.5 ml ethanol 70%. Aliquots were taken from each mixture for paper chromatography, using 1-butanol–acetic acid–H_2O (4:1:4, v/v) and isopropanol-8 N NH_4OH (8:2, v/v) as solvent systems, and for TLC, using chloroform-methanol–acetic acid (75:20:5,v/v), chloroform-methanol-H_2O (85:14:1, v/v), and chloroform–ethyl acetate–formic acid (35:55:10, v/v).

Wheat Coleoptile Bioassay for Auxins

The bioassay of wheat coleoptile elongation, to test the auxin activity of the IAA metabolites, was performed according to Nitsch and Nitsch (1956). Wheat seeds (var. Lachish), washed in tap water for 2 h and soaked overnight, were germinated on vermiculite at 25°C in the dark. All subsequent handling was performed under green light. Segments of 10 mm, 3 mm from the apex, were cut from 3-day-old coleoptiles and floated on distilled water for 1–1.5 h. Samples of 12 segments were then transferred to 50-ml Erlenmeyer flasks, containing 1.5 ml of 10 mM K-phosphate-citrate buffer (pH 5) with 50 mM sucrose. Where indicated, free IAA or each of the IAA metabolites at a concentration of 5×10^{-6} M was included. Three replicates of 12 segments each were used for every treatment. The coleoptile segments were incubated, as described above, in a C_2H_4- and CO_2-free atmosphere at 25°C in the dark, under continuous shaking. Elongation and decarboxylation ($^{14}CO_2$) were determined after 22 h of incubation.

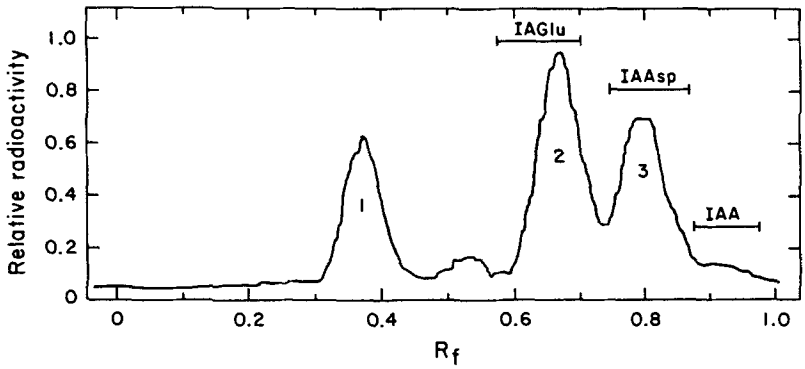

Fig. 1. Radiochromatogram scan of ethanol extract prepared from tobacco leaf discs incubated with 10 μM (1-^{14}C)IAA (1 μCi) for 24 h. An aliquot of the radioactive extract containing 2 × 10^4 dpm was applied on the chromatogram. Numbers indicate the three IAA metabolites obtained. Locations of authentic IAA, IAAsp, and IAGlu are designated by bars.

Results and Discussion

Metabolism of (1-^{14}C)IAA and Characterization of the Metabolites

Tobacco leaf discs, treated with 10 μM (1-^{14}C)IAA (1 μCi) for 24 h, absorbed about 85% of the radioactive IAA applied, when 57% of it underwent decarboxylation. Chromatographic separation of the labeled IAA metabolites from the extract of these leaf discs revealed three major radioactive peaks (Fig. 1). The first peak (0.3–0.43 R_f zone) was defined as metabolite 1, the second peak (0.6–0.74 R_f zone) as IAA metabolite 2, and the third peak (0.75–0.86 R_f zone) as IAA metabolite 3 (Aharoni and Yang 1983). All three metabolites were found to be polar compounds and were already detected in the IAA extract obtained from leaf discs after 2 h of incubation. Preliminary characterization of the IAA metabolites was done by exposing them to either a weak or a strong base to identify esteric or peptidic bonds, respectively (Bandurski and Schulze 1977).

Metabolite 1 appeared to be quite stable in a weak-base hydrolysis, but after applying a strong base for 3 h, most of this metabolite was decomposed into one compound with a peak at the 0.67–0.9 R_f zone (Fig. 2A). No release of free IAA from metabolite 1 could be detected (Fig. 2A), even after 6 h of strong-base hydrolysis (data not shown).

Metabolite 1 was also unreactive with Ehmann reagent, which identifies indole derivatives (Ehmann 1977). These may suggest that metabolite 1 could be oxindole-3-acetic acid (OxIAA), the major catabolic product of IAA (Reinecke and Bandurski 1981). However, it did not correspond to authentic OxIAA when chromatographed on TLC plates, using chloroform:methanol:H_2O (85:14:1, v/v) as solvent system (Reinecke and Bandurski 1981). On the other hand, the weak-base hydrolysis of metabolite 2 (Fig. 2B) resulted in the release of free IAA (0.82–0.93 R_f zone), suggesting that this metabolite can be an esteric IAA conjugate according to Bandurski and Schulze (1977). Although metabolite 2 corresponded to authentic IAGlu in the chromatograms developed with 1-butanol–acetic acid–H_2O (Fig. 1), the use of another solvent system, 2-propanol-8 N NH_4OH (8:2, v/v), revealed that metabolite 2 cannot be identified as IAGlu.

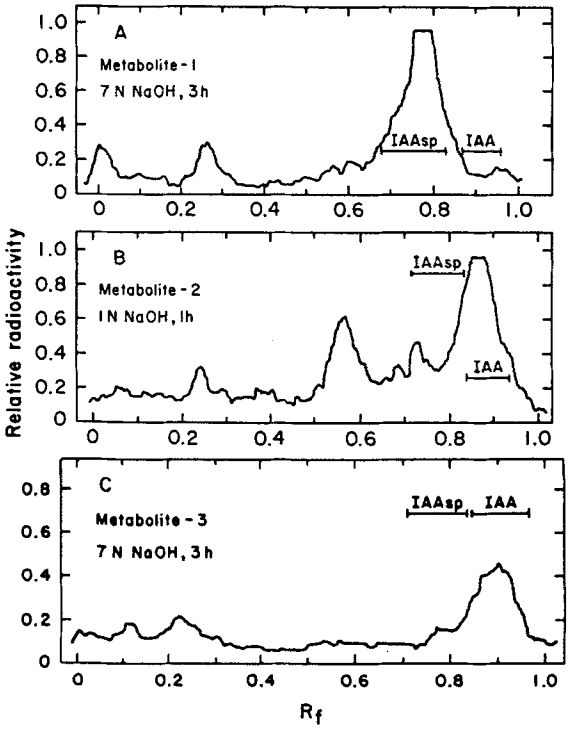

Fig. 2. Radiochromatogram scans of hydrolyzed IAA metabolites. The metabolites were obtained and chromatographically separated as described in the legend to Fig. 1. (A) and (C), hydrolysis with 7 N NaOH; (B), hydrolysis with 1 N NaOH. Aliquots containing 14.6 $\times$ 10^3 dpm (A), 12 $\times$ 10^3 dpm (B), and 5 $\times$ 10^3 dpm (C) were applied on the chromatograms. Locations of authentic IAA and IAAsp are designated by bars.

Metabolite 3 did not undergo hydrolysis by a weak base, but when applying a strong base its complete hydrolysis, followed by release of free IAA, was achieved (Fig. 2C). This suggests that metabolite 3 can be identified as a peptidic IAA conjugate. When cochromatographed with standards, IAA conjugate 3 corresponded to IAAsp in the solvent system of Fig. 1, as well as in 2-propanol-8 N NH_4OH (8:2, v/v), and in chloroform–ethyl acetate–formic acid (5:4:1, v/v) (data not shown). However, Aharoni and Yang (1983) demonstrated a high degree of both uptake and decarboxylation of this IAA conjugate 3, which was found to induce a high rate of ethylene production in tobacco leaves. Since the uptake of IAAsp by tobacco leaf discs was very low, and IAAsp was inactive in inducing ethylene production (even at a concentration of 0.1 mM) in tobacco (unpublished), pea (Hangarter et al. 1980), and mung bean (Sakai and Imaseki 1973), we may conclude that the main activity of IAA conjugate 3 cannot be attributed to IAAsp, despite their identical R_f zones. It seems, therefore, that a further separation method, in addition to paper chromatography, is required.

After their basic hydrolysis (Bandurski and Schulze 1977), both metabolites 2 and 3 were cochromatographed in five different solvent systems (see Materials and Methods) with authentic IAA. In all these systems, their released free IAA corresponded to the standard IAA, thus implying that metabolites 2 and 3 can be regarded as IAA conjugates. Furthermore, unlike metabolite 1, metabolites 2 and 3 were reactive with Ehmann reagent, indicating that they are indole compounds (Ehmann 1977).

When the three IAA metabolites eluted from the chromatogram (Fig. 1) were rechromatographed separately, each appeared in its original R_f zone, as expected (data not shown). This suggests that the additional peaks, obtained after hydrolysis of the three IAA metabolites (Fig. 2), are a result of the hydrolytic process per se rather than artifacts of the chromatography. IAA metabolite 1 differed from IAA conjugates 2 and 3 since its hydrolysis, either in weak or strong base (Fig. 2A), was not accompanied by release of free IAA, as observed for the others (Fig. 2B,C). In spite of the fact that the hydrolytic product of IAA metabolite 1 corresponded to IAAsp according to their R_f zones (Fig. 2A), it seems that this product is not a regular amide-linked IAA, since it did not undergo any further decomposition even after 6 h of strong-base hydrolysis (data not shown).

Biological Activities of the IAA Metabolites

In order to collect a large amount of IAA metabolites, the tobacco leaf discs were incubated for 48 h with 1 mM IAA and 10 μM (1-^{14}C)IAA (1 μCi), without sucrose in the medium (Aharoni and Yang 1983). At such high IAA concentrations a shifting of the IAA metabolites' formation occurred, when mainly the peptidic IAA conjugate (metabolite 3) was accumulated, and the level of IAA metabolite 1 was very low. This large accumulation of IAA conjugate 3 is apparently a result of applying a high IAA concentration (1 mM) in the medium (Andreae and Van Ysselstein 1960), which causes a quantitative rather than a qualitative change in the IAA metabolites. The three IAA metabolites were eluted from the chromatograms and were reapplied at concentrations of 10 μM to tobacco leaf discs for 4 days of incubation. These concentrations were calculated on the basis of specific activity of (1-^{14}C)IAA applied. Figure 3 shows that all three metabolites are capable of inducing ethylene production in tobacco leaf discs. Ethylene production rates in the leaf discs treated with IAA metabolite 1 and conjugate 2 were already similar to or even higher than those of IAA-treated discs (applied at the same concentration) on the first day of incubation. On the other hand, with the peptidic IAA conjugate (metabolite 3), a significant amount of ethylene production could be observed only after 2 days of incubation. This low activity could be ascribed to the low uptake of the peptide IAA conjugate during the first 2 days of incubation. Concerning the activity of IAA and IAA metabolites in inducing ethylene production as referred to the rate of their uptake by the discs (Table 1), it seems that the IAA conjugate 2 (the esteric-linked one) is the most active in stimulating net ethylene production as compared with the free IAA applied. When applying IAA conjugates 2 and 3 at concentrations of 100 μM (data not shown), ethylene production was enhanced by 40% and 55%, respectively, in comparison with 90% enhancement obtained with 100 μM of free IAA. However, ethylene production rates in leaf discs treated with IAA conjugate 3 at this concentration (100 μM) were significantly higher than those of control discs. This high level was obtained during the first day and increased further with time of incubation.

Besides, by IAA, ethylene production can also be induced by toxic chemicals in vegetative tissues (Abeles 1973, Lieberman 1979). Therefore, to exclude

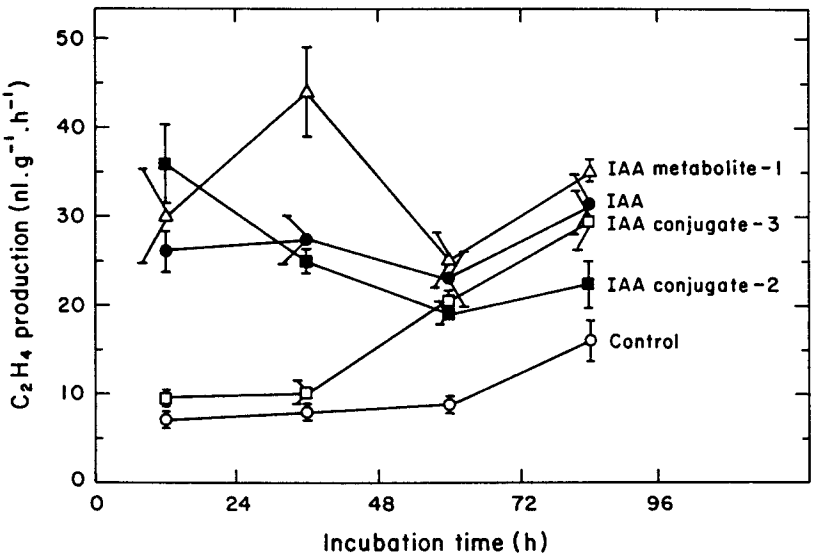

Fig. 3. Effect of IAA and IAA metabolites on the time course of ethylene production rates in tobacco leaf discs. Ten micromoles of either (1-^{14}C)IAA or each of the different IAA metabolites, eluted from the chromatogram described in the legend to Fig. 2, were used. The bars indicate two standard errors of three or four replicates.

Table 1. The activity of IAA and IAA metabolites in inducing ethylene production by tobacco leaf discs.

Treatment	Total uptake		Total $^{14}CO_2$		Net C_2H_4 production[a]
	(nmol)	(%)	(nmol)	(% of uptake)	(nmol)
IAA	14.9	97	7.45	50	4.49
IAA metabolite 1	6.7	30	0.80	12	4.69
IAA conjugate 2	5.4	22	2.05	38	6.56
IAA conjugate 3	5.4	23	1.94	36	1.43

Ten leaf discs were incubated for 70 h with 10 μM of either (1-^{14}C)IAA (1 μCi) or each one of the eluted IAA metabolite and conjugates, as shown in Fig. 3. Uptake, decarboxylation, and ethylene production were measured every 24 h. The uptake was calculated according to the residual radioactivity in the medium. Results represent accumulated nanomoles after 70 h. Control discs were incubated with buffer and 50 mM sucrose.

[a] Net nmol C_2H_4 is expressed after subtraction of nmol C_2H_4 in control discs (=2.06 nmol).

the possibility that the IAA metabolites may induce ethylene production through toxic effect rather than through auxin activity, the IAA metabolites were tested also in the bioassay of wheat coleoptile elongation (Nitsch and Nitsch 1956). The three IAA metabolites were extracted from tobacco leaf discs as described above. Table 2 demonstrates that all the three metabolites could induce elongation of wheat coleoptile segments, when IAA conjugate 3 was the most active and the two other metabolites had an activity similar to that of free IAA. These results suggest that all the three IAA metabolites have an auxin activity.

Table 2. The activity of free IAA and tobacco IAA metabolites in inducing elongation of wheat coleoptile segments.

Treatment	Elongation (mm)	Decarboxylation (% of uptake)
Control	7.6[c]	—
IAA	8.6[b]	39
IAA metabolite 1	9.3[b]	4
IAA conjugate 2	9.2[b]	19
IAA conjugate 3	10.3[a]	32

Three replicates of 12 coleoptile segments each were incubated with 5×10^{-6} M of either (1-^{14}C)IAA (50 mCi/mmol) or each of the labeled IAA metabolites (4.5 mCi/mmol), isolated from tobacco leaf discs. Control segments were incubated with buffer and 50 mM sucrose. Uptake, elongation, and decarboxylation were measured after 22 h. Means in the column "Elongation," followed by the same letter, are not significantly different according to multiple-range test (α = 0.05).

Turnover and Compartmentalization of IAA Metabolites

Table 1 demonstrates that the uptake of free IAA by tobacco leaf discs is much more rapid than that of the different IAA metabolites. Thus, 97% of free IAA has already been taken up after 2 days of incubation, whereas with the three IAA metabolites only 22–30% uptake was obtained after 3 days of incubation. Nevertheless, IAA conjugates 2 and 3 showed quite a high degree of decarboxylation, yielding 38% and 36% of uptake, respectively, compared with 50% decarboxylation of free IAA (Table 1). IAA metabolite 1 was exceptional with its low degree of decarboxylation (12% of uptake). Similar results of decarboxylation of IAA and its three metabolites were also obtained in the bioassay of wheat coleoptile elongation (Table 2). The turnover of the three IAA metabolites was examined with extracts of tobacco leaf discs which were incubated separately with each IAA metabolite for 2–3 days. The pattern of the chromatographed extracts is illustrated in Fig. 4. In the disc extract treated with IAA metabolite 1, no other metabolite could be detected besides IAA metabolite 1 itself (Fig. 4A), whereas in extracts of discs treated with IAA conjugates 2 and 3, all three IAA metabolites were obtained (Fig. 4B,C), in a similar pattern as in IAA-treated discs (Fig. 1). This pattern of the three typical IAA metabolites, together with their increased decarboxylation (Table 1), indicates that IAA conjugates 2 and 3 in the tissue did undergo hydrolysis, which was followed by release of free IAA. This newly released free IAA was in turn remetabolized, yielding the three typical IAA metabolites (Fig. 4B,C).

Free IAA was previously reported to induce ethylene production in vegetative tissues by increasing ACC synthase activity (Yu et al. 1979, Yu and Yang 1979, Yang et al. 1980). Accordingly, it may be suggested that the IAA metabolites could stimulate ethylene production (Fig. 3, Table 1) by a slow release of free IAA (Aharoni and Yang 1983) which, in turn, promotes ACC formation. Hence, the hydrolysis of IAA conjugates in the tissue may provide the free IAA for inducing increased ethylene production. The great efficiency of two of the three IAA metabolites in inducing ethylene production as compared with free IAA probably stems from the fact that the free IAA released by hydrolysis

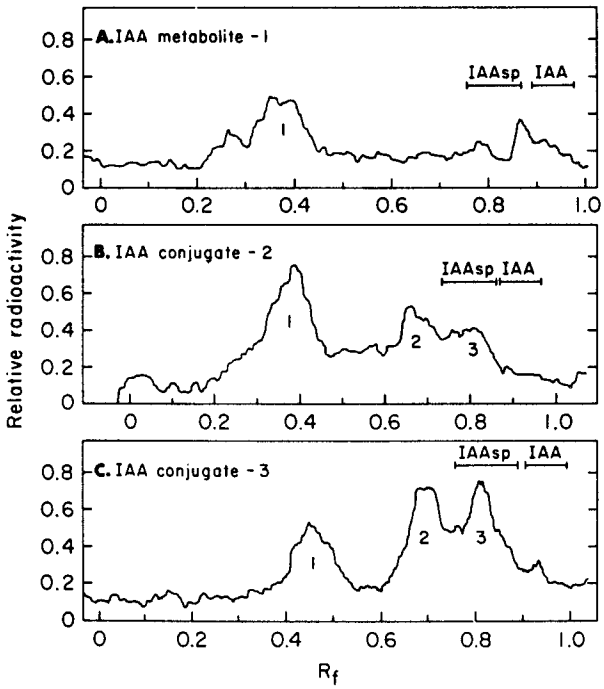

Fig. 4. Turnover of IAA metabolites. Extracts, pooled from tobacco leaf discs incubated with 10 μM of each radioactive IAA conjugate for 3 days, were scanned at a sensitivity of 3×10^2. (A) 4.5×10^3 dpm of IAA metabolite 1; (B) 12×10^3 dpm of IAA conjugate 2; (C) 15×10^3 dpm of IAA conjugate 3. Locations of IAA and IAAsp are designated by bars.

is not exposed to decarboxylation as the exogenously supplied IAA. The latter underwent 50% decarboxylation in tobacco leaves (Table 1), 39% decarboxylation in wheat coleoptiles (Table 2), and more than 73% decarboxylation in sugar beet (Aharoni and Yang 1983). The results point to a possible connection between the site of IAA conjugation in the leaf and the site of IAA release, when these two sites are probably close to the site of ethylene production.

The discrepancy in the turnover patterns of the three IAA metabolites (Fig. 4) points to their possible partitioning among the leaf tissues and cells. Accordingly, the free IAA obtained by hydrolysis of conjugates in the course of a prolonged incubation (72 h) may be released in a leaf region where it is preferably remetabolized to yield metabolite 1. Also, Imaseki et al. (1975) reported that in mung bean hypocotyls, most of the IAA conjugation to IAAsp occurred in the epidermis tissue, thus suggesting that tissues may differ in their conjugative ability. To test this hypothesis we have incubated an epidermis layer, isolated from tree tobacco (*Nicotiana glauca*), with 10 μM (1-^{14}C)IAA and sucrose for 48 h. About 90% of the labeled IAA was already taken up within 24 h, but a very low rate of decarboxylation could be observed, giving only 1% and 1.5% on the first and second days, respectively. Nevertheless, this isolated epidermis tissue showed very high rates of ethylene production, which are five times higher than the rates obtained with leaf discs: 234 nl/g/h in the first 24 h and 80 nl/g/h in the next 24 h.

When the metabolism of (1-^{14}C)IAA in the epidermis tissue was studied during the 48-h incubation, it was found that IAA was conjugated only to conjugates 2 and 3, and metabolite 1 could not be detected (Fig. 5). This is in

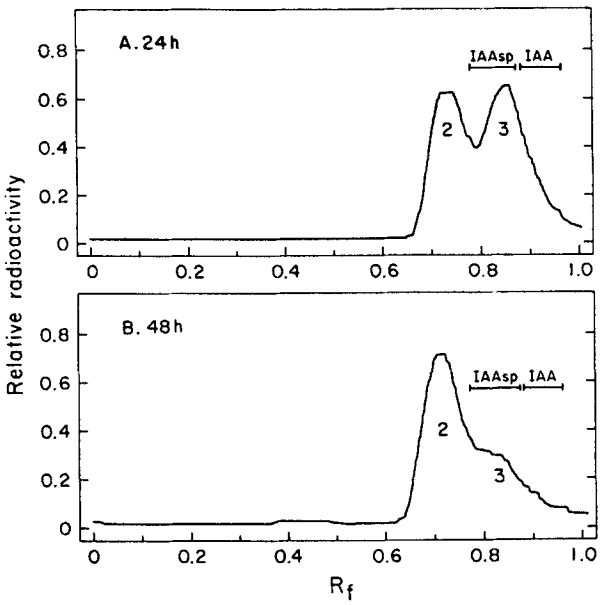

Fig. 5. Radiochromatogram scans of ethanolic extracts prepared from epidermis tissue incubated with (1-^{14}C)IAA for 24 and 48 h. Epidermis layers (0.2 g), cut from leaves of tree tobacco (*Nicotiana glauca*), were incubated with 10 μM (1-^{14}C)IAA (1 μCi), and at the indicated periods were extracted with ethanol. Aliquots of these labeled extracts, containing 21 × 10^3 dpm, were applied on each chromatogram. Locations of authentic IAAsp and IAA are designated by bars.

contrast to the metabolites' pattern of IAA obtained in the intact leaf discs where all three IAA metabolites appeared already after 2 h of incubation and were not impaired up to 24 h (Fig. 1). A similar metabolic pattern was obtained with epidermis layers isolated from *Nicotiana tabacum* L. cv. Xanthi. These results may lead to the following conclusions: (1) Ethylene production in the leaf might take place mainly in the epidermis. Indeed, Sakai and Imaseki (1973) and Imaseki et al (1975) have previously demonstrated that damaging the epidermis of mung bean hypocotyls reduced their ethylene production. Whole tobacco leaf discs exhibited lower ethylene production rates than the rates of their isolated epidermis layers, probably because the epidermis weight in the leaf is relatively small. (2) The epidermis layer may be further associated with ethylene production since it contains mainly conjugate 2 (the esteric one) (Fig. 5), which was demonstrated to induce the highest ethylene production (Table 1). (3) Lack of IAA metabolite 1 in the epidermis can result from compartmentalization of IAA conjugation in the leaf tissue. Thus, it is possible that this IAA metabolite is not formed in the epidermis but in the mesophyll.

The Role of IAA Conjugates

It was previously suggested that the IAA conjugates produced in response to added IAA have a regulatory role by sustaining the desired physiological free IAA level in the plant tissue (Bandurski et al. 1977, Cohen and Bandurski 1978). This role can also be expressed by protecting the plant against excess IAA, as proposed for IAAsp and IAGlu (Andreae and Van Ysselstein 1960, Zenk 1964). The results of the present study obtained with IAA conjugates 2 and 3, as well as those of a preceding report (Aharoni and Yang 1983), support

the suggestion that certain IAA conjugates, produced in vegetative tissue, can serve as sources of free IAA and thereby participate in the regulation of various physiological processes (Andreae and Good 1955, Bandurski and Schulze 1977, Cohen and Bandurski 1978, Epstein et al. 1980, Feung et al. 1977, Liu et al. 1978). The IAA conjugates, which are protected from oxidation (Cohen and Bandurski 1978), may be regarded as reservoirs of free IAA, which in turn are released slowly by hydrolytic enzymes. Hangarter et al. (1980) and Hangarter and Good (1981) have already shown that, in addition to the biological activity of IAA conjugates in growth regulation, these conjugates can induce ethylene production in pea seedlings.

Regarding the fact that IAA metabolite 1 was very active in inducing ethylene production, as well as elongation of wheat coleoptiles, but showed a low decarboxylation (Tables 1, 2) and, in addition, lack of turnover, one might consider the possibility that IAA metabolite 1 can induce an auxin activity by itself rather than through free IAA. Still, the 12% decarboxylation obtained with IAA metabolite 1 (Table 1) indicates that a certain degree of hydrolysis of this compound can occur, thereby exposing the released free IAA to further oxidation. Alternatively, this low decarboxylation may imply that metabolite 1 can be decomposed rather than hydrolyzed. That no free IAA was released by an *in vitro* hydrolysis of IAA metabolite 1 does not yet exclude the possibility that this IAA metabolite may be enzymatically hydrolyzed *in vivo*. Moreover, the observation that no other metabolites could be detected in the chromatogram of IAA metabolite 1 (Fig. 4A) may be explained by a different compartmentalization of this IAA metabolite. Thus, the free IAA released from IAA metabolite 1 is not available for further conjugation or for decarboxylation, and therefore cannot be detected. It seems that further investigation is required for elucidating the nature of this IAA metabolite 1.

In contrast to all previous investigations, which were performed with synthetic auxins, we show in the present work, as well as in the preceding study (Aharoni and Yang 1983), a biological activity of endogenous IAA metabolite and conjugates that were synthesized by tobacco leaf discs in response to exogenously supplied IAA. Although we have not yet identified all the IAA metabolites produced in the tobacco leaf, it is initially established that at least one of them is a peptidic IAA conjugate whereas the second is an esterified one. Further evidence about the regulatory role of these IAA conjugates in ethylene production will be presented elsewhere.

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