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Carbohydrates Stimulate Ethylene Production in Tobacco Leaf Discs[1]

I. INTERACTION WITH AUXIN AND THE RELATION TO AUXIN METABOLISM

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ABSTRACT

Various naturally occurring carbohydrates, applied at a concentration range of 1 to 100 mM, stimulated ethylene production for several days in indoleacetic acid (IAA)-treated or untreated tobacco (*Nicotiana tabacum* L. cv 'Xanthi') leaf discs. The lag period for this sugar-stimulated ethylene production was 8 to 12 hours after excision in the untreated leaf discs, but less than 2 hours in the IAA-treated ones. Among the tested carbohydrates, 12 were found to increase synergistically ethylene production, with D-galactose, sucrose, and lactose being the most active; mannitol and L-glucose had no effect. The extent and duration of the increased ethylene production was dependent upon the type of sugar applied, the tissue's age, and the existence of both exogenous IAA and sugar in the medium. Sucrose appeared to elicit a continuous IAA effect for 48 hours, as expressed by increased ethylene production, even when IAA was removed from the medium after a 4-hour pulse. Sucrose stimulated both the uptake and decarboxylation of [1-^{14}C]IAA, as well as the hydrolysis of the esteric and amide IAA conjugates formed in the tissue after application of free IAA. This gradual hydrolysis was accompanied by a further accumulation of a third IAA metabolite. Moreover, synthetic indole-3-acetyl-L-alanine increased ethylene production mainly with sucrose, and this effect was accompanied by its increased decarboxylation and turnover pattern suggesting that release of free IAA was involved. An esteric IAA conjugate, tentatively identified by GC retention time was found to be the major component (84%) of the naturally occurring IAA conjugates in tobacco leaves. Accordingly the sucrose-stimulated ethylene production in tobacco leaves can be ascribed mainly to the sucrose-stimulated hydrolysis of the esteric IAA conjugate.

Intact leaves as well as leaf discs of tobacco, incubated in plain water or buffer, produced very small amounts of ethylene (2). When 2% sucrose was added to tobacco leaf discs as a medium constituent, a remarkable increase in ethylene production, which lasted for several days, was observed. This occurred both in IAA-treated and untreated leaf discs, with the increase being much more pronounced in the former (3). A carbohydrate-stimulated ethylene production has been described so far in mung bean hypocotyls treated with D-galactose (11) and recently in citrus leaf discs treated with mannitol (21). None of the other common carbohydrates tested could elicit a stimulating effect in either of those systems, nor in pea epicotyls (8), and it was therefore suggested that the increased ethylene production in these tissues might be related to the toxicity of galactose (11) or stress effect of mannitol (21).

Our recent study (3) established that sucrose could stimulate IAA-induced ethylene production in a synergistic action. This occurred only in tissues which were responsive to IAA with respect to ethylene production.

A possible involvement of IAA in the sugar-stimulated ethylene production could be related to ACC[2] formation from SAM, which is the rate-limiting step in ethylene biosynthesis and was found to be stimulated both by IAA (26) and sucrose (19).

Regarding the well documented IAA activity exhibited by various IAA conjugates in inducing physiological phenomena (9, 14–16), as well as the prolonged carbohydrate stimulation of ethylene (3, 19), it is plausible that IAA conjugates may play an important role in this interaction of IAA with carbohydrates. Recent studies of auxin metabolism in tobacco leaves (4, 18) have suggested that the rate of ethylene production in the IAA-treated tissue is dependent on the formation of IAA conjugates and their ability to release free IAA (15). In this respect, it was reported that the synthetic IAA conjugate, IAA1a, which can induce ethylene production in vegetative tissues (15, 16), was much more active when applied with sucrose (3).

In this study we present data showing that many naturally occurring sugars, including galactose, sucrose, and glucose, stimulate ethylene production in IAA-treated and untreated tobacco leaf discs. The study provides evidence supporting the hypothesis that sucrose stimulates ethylene production by enhancing hydrolysis of IAA conjugates, either exogenously supplied or endogenously produced, thereby causing a slow release of free IAA which, in turn, promotes ethylene production in the leaf.

MATERIALS AND METHODS

Plant Material and Treatments. Tobacco (*Nicotiana tabacum* L. cv 'Xanthi') was grown in a greenhouse under LD conditions (18 h light) at temperatures between 20° and 30°C. Fully expanded mature leaves were washed under running tap water, surface-sterilized by soaking for 30 s in a 0.5% (v/v) dilution of commercial NaOCl (a 10% solution) in water, and rinsed several times with sterile distilled H_2O, as described previously (2). All subsequent handling of the tissue involved sterile techniques. Discs, 1 cm in diameter, were excised from leaf blades with a

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[2] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; IAA1a, indole-3-acetyl-L-alanine; IAAsp, indole-3-acetyl-aspartic acid; SAM, *S*-adenosylmethionine.

cork borer and either floated on water with the abaxial surface down in open Petri dishes for 1 h until treatment, or treated immediately after excision where indicated. Samples of eight or ten leaf discs, weighing about 90 or 110 mg, respectively, were blotted dry and incubated abaxial surface down, on filter paper in 50-ml Erlenmeyer flasks with 2-ml medium containing 50 mM Na-phosphate buffer (pH 6.1) and 50 μg/ml chloramphenicol.

When indicated, 0.1 mM of IAA (Sigma) or IAA1a (Research Organics Inc.), 1 to 200 mM D-glucose or sucrose, and 50 mM of D-galactose, lactose, maltose, raffinose, D-mannose, D-fructose, D-xylose, D-arabinose, inositol, *myo*-inositol, D-sorbitol or D-mannitol (BDH Chemicals Ltd.), or 100 mM L-glucose (Sigma) were included in the medium. Additions of [1-^{14}C]IAA (Amersham/Searle Corp., 59 mCi/mmol) and [1-^{14}C]IAA1a (synthesized from labeled IAA) were included when indicated.

Two plastic center wells (Kontes Glass Co.), hung in each flask, contained filter paper wicks wetted with 0.1 ml of 0.25 M $Hg(ClO_4)_2$ reagent for ethylene absorption (27) in one, and with 0.1 ml of 10% KOH for CO_2 absorption in the other. In such a system, an ethylene- and CO_2-free atmosphere (<0.03% CO_2) could be obtained. The flasks were then sealed with rubber serum caps, incubated in darkness at 30°C, and ethylene production was assayed periodically. After sampling for the different determinations at the indicated incubated intervals, the $Hg(ClO_4)_2$ and KOH solutions were renewed for subsequent incubation periods.

Determination of Ethylene. To monitor ethylene production rates during the incubation periods of the leaf discs, the plastic center wells containing the $Hg(ClO_4)_2$ solution were transferred to 50-ml Erlenmeyer flasks which were then sealed with rubber serum caps. The ethylene absorbed in these center wells was released from the $Hg(ClO_4)_2$ complex by a hypodermic syringe injection of 0.2 ml saturated LiCl. The flasks were allowed to stand for 2 h at 4°C (27), and a 1-ml gas sample was withdrawn from each flask with a hypodermic syringe for ethylene determination by a Packard gas chromatograph equipped with an activated alumina column and a flame ionization detector. Rate of ethylene production for zero time was determined by enclosing leaf discs for 30 min, immediately after excision. The rates generally were between 1.0 and 2.0 nl $(g \cdot h)^{-1}$.

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 the radioactivity was determined by a Kontron liquid scintillation counter. The counting efficiency was 85%.

Synthesis of Indole-3-[1-^{14}C]Acetyl-L-Alanine. Radioactive IAA1a (10 mCi/mmol) was synthesized by the mixed anhydride procedure (25), as modified by Hangarter *et al.* (16). Separation of the resultant [1-^{14}C]IAA1a from [1-^{14}C]IAA was carried out by TLC on Silica Gel G plates, using chloroform-methanol-acetic acid (75:20:5, v/v) as the solvent system, and a single peak of [1-^{14}C]IAA1a was obtained. Yields were around 25%.

Extraction and Chromatography of Labeled Auxins. At the end of the appropriate incubation period with [1-^{14}C]IAA or [1-^{14}C]IAA1a, leaf discs were rinsed with sterile H_2O and hand-homogenized with 2 ml of 70% (v/v) ethanol. The extraction procedure was performed as detailed by Aharoni and Yang (4), suspending the concentrated extract in a final volume of 1.2 ml. A 50-μl aliquot was taken from the extract and chromatographed on Whatman 3MM paper using isobutanol-acetic acid-H_2O (4:1:4, v/v) as the solvent system. In one experiment, where indicated, isopropanol-8 N NH_4OH (8:2, v/v) was employed. Authentic samples of unlabeled IAA, IAA1a, and IAAsp (Research Organic Inc.) 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.

Extraction and Assay of Endogenous Auxins. For extraction of the endogenous auxins, fully expanded tobacco leaves (67 g) were homogenized with 70% (v/v) acetone and further treated according to the method of Epstein and Cohen (12), except that an ether extraction was employed instead of chloroform. The resulting extract was separated into three samples, two of which were subjected to weak or strong base hydrolysis (6), in order to release free IAA from esteric or amide IAA conjugates, respectively. The two hydrolysates as well as the unhydrolyzed sample were purified by HPLC and then analyzed by GC for free IAA (12). The method for analysis of the extracted free IAA is based on preparation of a pentafluorobenzyl ester of IAA (12), which is convenient for gas chromatographic analysis using electron capture detection.

RESULTS AND DISCUSSION

Dose Response Curves for Glucose and Sucrose. Both glucose and sucrose stimulated ethylene production in tobacco leaf discs, which were incubated with or without IAA for 2 d (Fig. 1). In all cases, as little as 5 mM of each sugar could increase significantly the rate of ethylene production. The dose response curves indicate a synergistic effect between IAA and the carbohydrates tested. This synergistic effect became much more evident with 30 or 50 mM sugar, reached its peak at 100 mM, and declined afterwards, with 200 mM exhibiting a supra-optimal sugar concentration. However, without IAA in the medium, 200 mM sugar did not reduce the rate of ethylene production.

Effect of Stereoisomers and Type of Carbohydrates. Unlike D-glucose, the corresponding L-isomer which was applied at 100

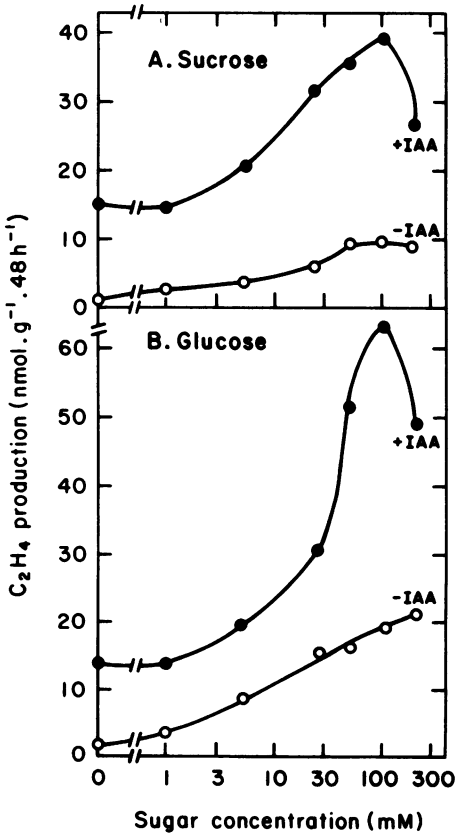

FIG. 1. Effect of increasing concentrations of sucrose (A) and D-glucose (B) on ethylene production rates by tobacco leaf discs in the presence or absence of 0.1 mM IAA. Ethylene production was measured daily during 2 d of incubation.

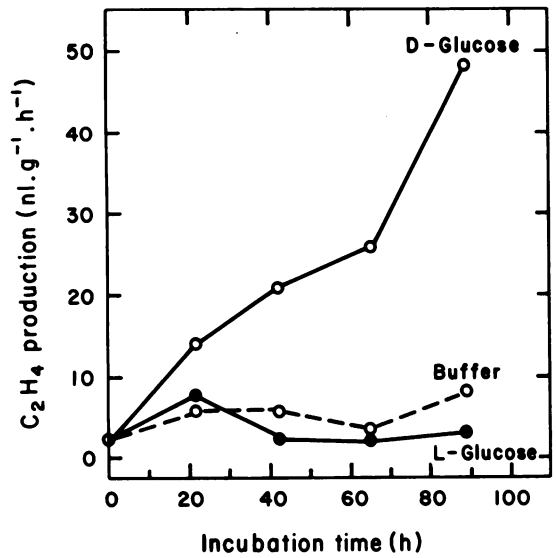

FIG. 2. Effect of D- and L-glucose on average ethylene production rates by tobacco leaf discs. The sugars concentration was 100 mM.

mM to tobacco leaf discs, did not increase ethylene production during the 4 d of incubation (Fig. 2). While D-glucose considerably increased the rate of respiration (Fig. 6 in reference 19), the L-isomer was ineffective (data not shown). This finding indicates that the D-glucose-stimulated ethylene production is not an osmotic effect and is probably related to sugar metabolism.

To clarify whether there is any sugar specificity in the carbohydrate-stimulated ethylene production, different naturally occurring carbohydrates and some of their derivatives were applied to tobacco leaf discs in the presence or absence of 0.1 mM IAA. The amount of ethylene production was measured daily during the 4 d of incubation. The accumulated levels of ethylene, produced in response to the different sugars, are depicted in Figure 3 in descending order for the IAA-treated leaf discs. Among the 14 tested carbohydrates, only two, arabinose and mannitol, were ineffective in stimulating ethylene production (data not shown), while galactose was the most active. Relatively high activity was found in the most common disaccharides: sucrose>lactose>maltose. The trisaccharide raffinose stimulated ethylene production in the presence of IAA, more or less like its constitutive monosaccharides, glucose and fructose, but less than galactose. The sugar alcohol analogs, *myo*-inositol, and sorbitol were the least effective. It is noteworthy that these analogs, when applied without IAA, stimulated ethylene production during 4 d of incubation even more than IAA did by itself (Fig. 3).

Kinetics of the Stimulatory Effect. Figure 4 illustrates typical time courses of sugar-stimulated ehtylene production in the presence or absence of IAA. The most effective sugars, galactose and sucrose (Fig. 3), when applied to untreated discs, did not significantly increase wound ethylene production, which peaked at 4 h and then sharply declined (Fig. 4A). In most of the kinetic experiments performed so far with this system, the lag time for the sugar-stimulated ethylene production was around 8 to 12 h, which is after partial subsidence of wound ethylene. Application of IAA markedly stimulated wound ethylene production, which was further enhanced by sugars (Fig. 4B). This enhancing effect of the sugars in the presence of IAA was evident by 2 h after excision. When wound ethylene had partially subsided (6 h after excision), the IAA-induced ethylene production again increased markedly, reaching another peak at 24 h of incubation (Fig. 4B). This second burst of ethylene production was markedly enhanced by galactose or sucrose but in different manners: with sucrose, there was a third rise of ethylene production, which generally lasted for several days. On the other hand, in the course of long term incubation, galactose sharply increased the rate of ethylene production, which peaked at 24 h (Fig. 4B) or 48 h (Fig. 4A) in the IAA-treated or untreated leaf discs, respectively, and then immediately declined.

Effect of Leaf Aging. In previous work, it was found that the response of the leaf tissue to added IAA decreased with leaf age (2). Figure 5 demonstrates a similar response of tobacco leaf discs to added sucrose. The highest stimulation of ethylene production was observed when sucrose was applied immediately after excision or 24 h later. Thereafter, addition of sucrose became less effective. The inset of Figure 5 demonstrates that 45-h-old leaf discs could not respond to added IAA as did fresh ones. Nevertheless, a remarkable response could be obtained with the same age leaf discs when IAA was added simultaneously with sucrose. We also observed that the presence of sucrose in the medium was essential for maintaining continuous increased ethylene production.Thus, after transferring the leaf discs to a sugar-free medium, the sugar effect diminished (data not shown). These findings may suggest an interaction between auxin and sugars, thereby increasing the rate of ethylene production in the senescing leaf.

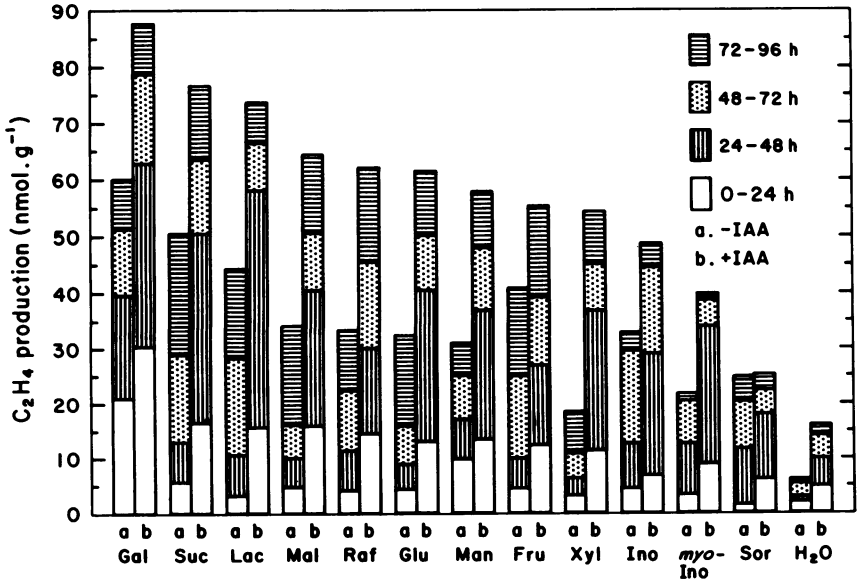

FIG. 3. Effect of various carbohydrates on ethylene production rates by tobacco leaf discs in the presence or absence of 0.1 mM IAA. Each carbohydrate was tested at a concentration of 50 mM. Ethylene production was measured daily during 4 d of incubation. Gal, D-galactose; Suc, sucrose; Lac, lactose; Mal, maltose; Raf, raffinose; Glu, D-glucose; Man, D-mannose; Fru, D-fructose; Xyl, D-xylose; Ino, inositol; Sor, D-sorbitol

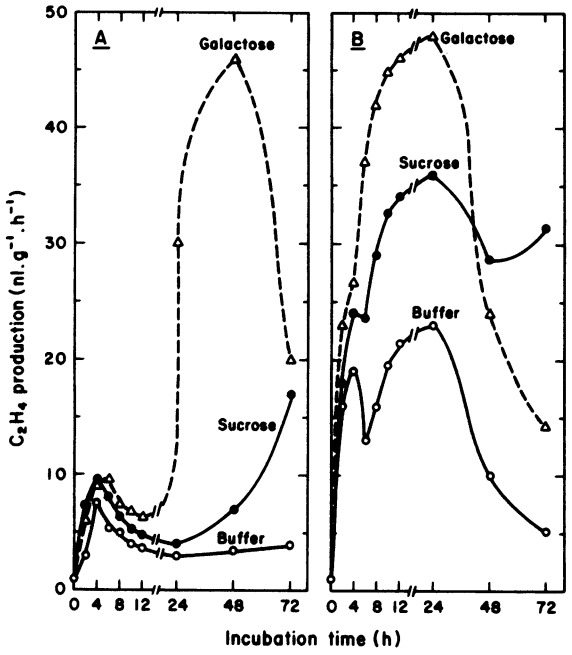

FIG. 4. Time course of ethylene production rates by tobacco leaf discs incubated with carbohydrates in the absence (A) or presence (B) of 0.1 mM IAA. Leaf discs were incubated immediately after excision in 50 mM of either D-galactose or sucrose with or without IAA.

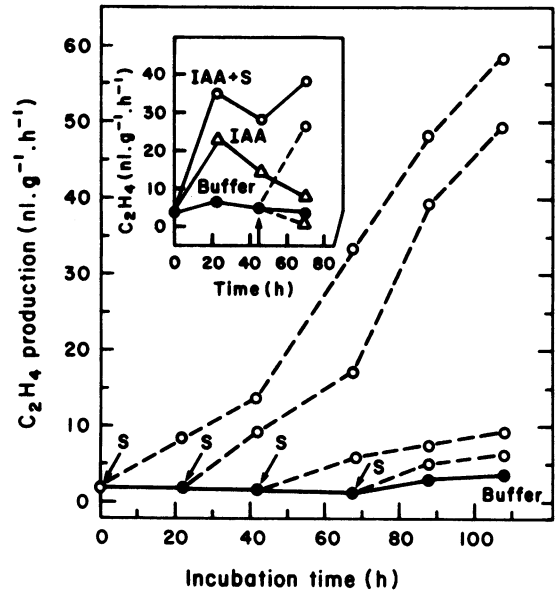

FIG. 5. The capability of sucrose to stimulate ethylene production in aged tobacco leaf discs. Fifty mM sucrose (S) was either present in the medium from the beginning of the incubation or added to buffer-treated discs at the indicated intervals. Ethylene production rates were then assayed periodically. Inset, Arrow indicates addition of either 0.1 mM IAA (Δ) or 0.1 mM IAA plus 50 mM sucrose (O) to the buffer-treated discs.

Possibility of a Toxic Effect. It is well known that increased ethylene is produced in response to toxic chemicals (1). In this respect, the carbohydrates-stimulated ethylene production found so far in vegetative tissues could be ascribed to the toxicity of galactose (11) or stress effect of mannitol (21). In the present study, when excluding galactose, it is unlikely that the sugar-stimulated ethylene production occurs due to a chemical toxicity or osmotic effect because of the following observations: (a) sugar concentrations as low as 5 mM (Fig. 1) and 1 mM (19) stimulated ethylene production significantly; (b) a relatively high concentration (100 mM) of L-glucose was found to be ineffective in stimulating ethylene production (Fig. 2); (c) the stimulatory response of the active sugars was initially very slow, then it increased progressively and lasted for several days (Figs. 2, 4, and 5) without any visible injury to the leaf tissue; and (d) the increased ethylene production was sharply reduced after transferring the tissue to a sugar-free medium. In these respects, only the galactose-stimulated ethylene production was exceptional, because of its drastic increase followed by a sharp decrease (Fig. 4). It is therefore concluded that, except for galactose, which seems to be toxic (11), all the other naturally occurring sugars exert a physiological stimulating effect rather than serving as toxic agents.

IAA Pulse Experiment. The continuous presence of sucrose in the medium of IAA-treated discs greatly extended the IAA-induced ethylene production (Figs. 3–5). To examine whether the continuous presence of IAA in the medium is likewise necessary for interaction with sucrose, a pulse experiment was performed. Thus, after a 4-h pulse of IAA, the discs were transferred to an IAA-free medium in the absence (Fig. 6A) or presence (Fig. 6B) of sucrose. Without sucrose in the medium, ethylene production by the leaf discs decreased sharply within 4 h after the IAA pulse (Fig. 6A), whereas in the presence of sucrose, increased ethylene production continued for several days to an extent similar to that with a continuous supply of IAA (Fig. 6B). Hence, it may be suggested that sucrose can elicit a continuous IAA effect, as expressed by increased ethylene pro-

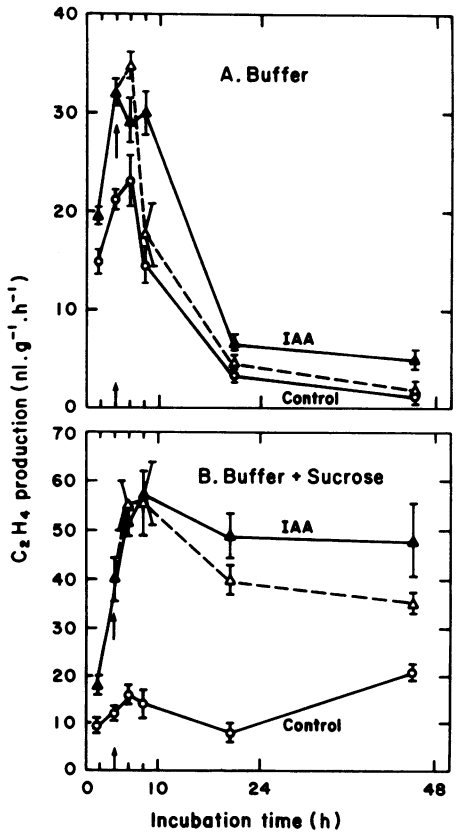

FIG. 6. Effect of continuous supply and 4-h pulse of IAA on ethylene production by tobacco leaf discs in the absence (A) or presence (B) of sucrose. Samples of ten discs were incubated for 48 h with 0.1 mM IAA in the presence or absence of 50 mM sucrose. (---), IAA-treated discs were removed after 4 h (as indicated by the arrow) from the IAA medium to each of the control solutions. The bars indicate 2 SE of three or four replicates.

duction for 48 h, even when IAA was removed from the medium after 4 h. Such an extended IAA effect might occur by activation of a bound IAA source, since free IAA applied to tobacco leaves remains in very low concentrations (4, 18), due to its metabolic removal via decarboxylation and conjugation (4), as has been also reported for pea epicotyls (5).

Effect of Carbohydrates on Uptake and Metabolism of [1-^{14}C]IAA. To elucidate the mechanism by which carbohydrates can stimulate IAA activity, we studied IAA uptake, decarboxylation, and the possibility of reversible IAA conjugation. Table I shows that both sucrose and galactose increased uptake of [1-^{14}C]IAA during different periods of incubation, when galactose was more effective at 24 h. Both sugars also stimulated significantly [1-^{14}C]IAA decarboxylation to a higher extent than their stimulatory effect on [1-^{14}C]IAA uptake (data not shown). Thus, per cents of decarboxylation after 24 h incubation were 50 ± 1.3, 60 ± 1.4, or 72 ± 2.5 of the total [1-^{14}C]IAA taken up in control, sucrose-, or galactose-treated discs, respectively. These high rates of IAA decarboxylation, observed in the presence of sucrose and galactose during the first days of incubation, may explain the fact that, in spite of their increased [1-^{14}C]IAA uptake, the sugar-treated discs contained less radioactivity in their extracts than did the untreated discs (data not shown).

In addition to their effects on IAA uptake and decarboxylation, carbohydrates were tested for their possible effect on the content of IAA conjugates and metabolites. Accordingly, tobacco leaf discs were treated with 10 μM [1-^{14}C]IAA in the presence or absence of sucrose, for different incubation periods. After 24 h, the extracts of sucrose-untreated (Fig. 7A) or -treated (Fig. 7B) leaf discs revealed three major metabolites: metabolite-1 (0.2–0.4 R_F zone), metabolite-2 (0.55–0.70 R_F zone), and metabolite-3 (0.72–0.86 R_F zone), but almost no detectable amounts of free IAA (18). After 2, 4, or 8 h incubation, there were higher levels of free IAA but the patterns of the three IAA metabolites were similar except that they were at lower levels (data not shown). No difference in the level of IAA and IAA metabolites could be observed in the sugar-treated or untreated discs. These results confirmed previous observations (5) that IAA conjugation required at least 2 h to become apparent. According to their ability to liberate free IAA by either weak or strong base hydrolysis (6), two of the IAA metabolites were characterized preliminarily as an esteric IAA conjugate (metabolite-2) and an amide IAA conjugate (metabolite-3) (18). These metabolites are apparently not IAGlu and IAAsp albeit the similarity in their R_F zone (18). Metabolite-1 was decomposed by strong base hydrolysis, but no concomitant release of free IAA could be detected, suggesting that it may be an IAA catabolite, apparently not oxindole-3-acetic acid (18). On the other hand, metabolite-1 still exhibited auxin activity in inducing ethylene production and elongation (18); its nature, therefore, remained unknown. Figure 7A illustrates that in untreated discs the level of IAA conjugate-3 (amide-linked) decreased after 72 h by about 50% and then leveled off for up to 120 h of incubation. This decrease of conjugate-3 during 24 to 72 h of incubation was accompanied by a similar increase of metabolite-1, while conjugate-2 (ester-linked) did not undergo any changes in the course of 120 h incubation. On the other hand, in sucrose-treated discs (Fig. 7B), conjugates-2 and -3 were almost completely hydrolyzed during 120 h of incubation and this was accompanied by a further accumulation of metabolite-1. The results indicate that sucrose has no visible effect on formation of the three IAA metabolites but enhances the hydrolysis of conjugates-2 and -3.

Our findings (Figs. 3 and 4) showed that galactose was the most effective in stimulating ethylene production during the first 2 d of incubation, with or without IAA. We therefore examined the effect of galactose on the metabolism of [1-^{14}C]IAA, and found that this sugar enhances, even more than sucrose, the hydrolysis of IAA conjugates-2 and -3 followed by accumulation of metabolite-1 (data not shown).

To eliminate the effect of sugars on [1-^{14}C]IAA uptake and

Table I. *Effect of Sucrose and Galactose on [1-^{14}C]IAA Uptake by Tobacco Leaf Discs*

Uptake was calculated from the residual radioactivity in the medium (radioactivity at zero time = 1740 × 10^3 dpm). Radioactive IAA employed was 1 μCi and the concentration of sugars was 50 mM.

Incubation Time	Total Uptake		
	Buffer	Sucrose	Galactose
h	*dpm × 10^{-3}/10 leaf discs*		
4	223 ± 9	292 ± 6	ND[a]
8	516 ± 16	636 ± 10	ND
24	833 ± 38	1070 ± 30	1326 ± 64
48	1233 ± 54	1591 ± 48	1585 ± 45

[a] Not determined.

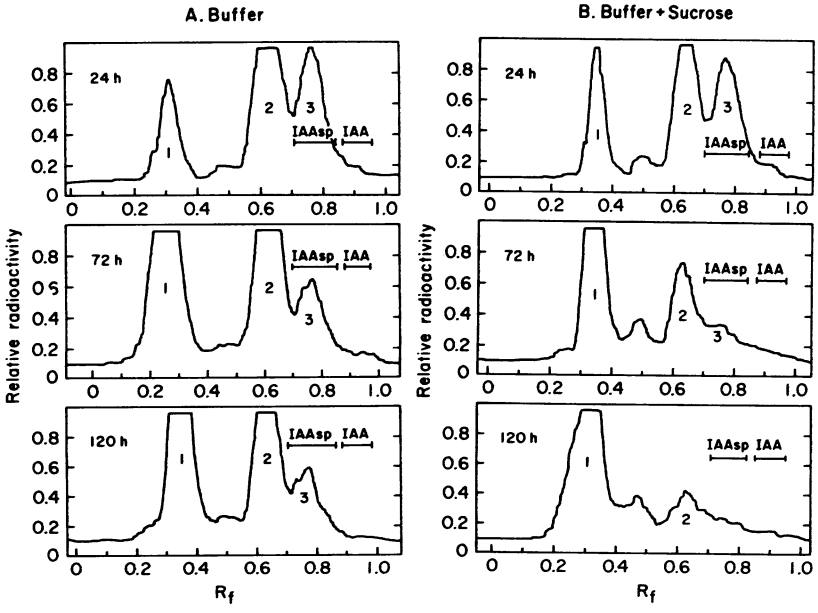

FIG. 7. Radiochromatogram scans of ethanolic extracts prepared from tobacco leaf discs incubated with 10 μM [1-^{14}C]IAA (1 μCi) in the absence (A) or presence (B) of 50 mM sucrose. Radioactive extracts were obtained from leaf discs incubated at the indicated periods; 50-μl aliquots, containing about 2 × 10^4 dpm, were applied on each chromatogram. Numbers indicate the three IAA metabolites obtained. Locations of authentic IAAsp and IAA are designated by bars.

decarboxylation, the leaf discs were preincubated only with 0.1 mM [1-^{14}C]IAA for 24 h and then transferred to the different sugar media. Also, under these conditions, both sucrose and galactose stimulated hydrolysis of IAA conjugate-2 and -3, as well as accumulation of metabolite-1 and ethylene production (data not shown).

It was found previously (4, 18) that the two IAA conjugates, as well as metabolite-1 (Fig. 7), could induce increased ethylene production when reapplied to tobacco leaf discs in the presence of sucrose. This continuous ethylene production was accompanied by a release of $^{14}CO_2$, a process which is indicative of the hydrolysis of these conjugates to free IAA (15). This newly released free IAA was then subjected to enzyme-catalyzed decarboxylation (9). It was also found that conjugates-2 and -3, whose metabolism was markedly stimulated by sucrose (Fig. 7B), underwent an IAA-like turnover pattern in the tissue, reyielding the three typical IAA metabolites (18). Hence, these results suggest that sucrose can extend the IAA effect in the tissue, as exhibited by the prolonged ethylene production after a 4-h pulse of IAA (Fig. 6B), due to its stimulatory effect on the hydrolysis of IAA conjugates (Fig. 7B).

Effect of Sucrose on Metabolism of [1-^{14}C]IAA-L-Alanine. It was reported previously that IAA1a, a synthetic IAA conjugate, exhibited rather high auxin activity in various systems (3, 7, 14, 16). This activity of IAA1a presumably stems from its enzymic hydrolysis in the tissue, which results in a slow release of free IAA (7, 10, 15). IAA1a induced ethylene production in pea stems (15) and mung bean hypocotyls (3). Its activity in tobacco, tomato, and cotton leaf discs was markedly stimulated by sucrose (3). It was, therefore, of interest to examine in detail the interaction between sucrose and IAA1a-induced ethylene production. Figure 8 demonstrates that application of IAA1a to tobacco leaf discs increased ethylene production only slightly. However, when applied simultaneously with sucrose, IAA1a caused an immediate and remarkable increase in ethylene production, which lasted for 7 d. Unlike the uptake of [1-^{14}C]IAA, which was stimulated by carbohydrates (Table I), the uptake of [1-^{14}C]IAA1a, which

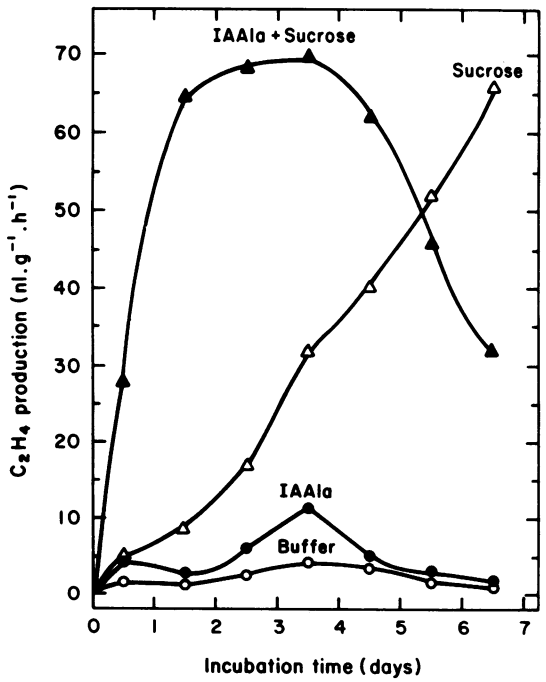

FIG. 8. Effect of sucrose on the daily average ethylene production in IAA1a-treated tobacco leaf discs. Fifty mM sucrose and 0.1 mM IAA1a were employed. IAA1a was dissolved in 70% ethanol and therefore 0.7% ethanol was included in all comparable treatments.

Table II. *Effect of Sucrose on Uptake and Decarboxylation of [1-^{14}C] IAAla in Tobacco Leaf Discs*

Samples of ten discs were incubated with 10 μM [1-^{14}C]IAAla (0.1 μCi) in the presence or absence of 50 mM sucrose. Uptake was calculated from the residual radioactivity in the medium.

Incubation Time	Total Uptake		Decarboxylation	
	Buffer	Sucrose	Buffer	Sucrose
h	%		*dpm/10 leaf discs·h*	
0–8	22	24	102	133
8–22	23	25	42	70
22–46	28	30	18	28
46–72	50	51	12	782

increased with incubation time, was almost unaffected by sucrose (Table II). Nevertheless, sucrose markedly stimulated the decarboxylation of [1-^{14}C]IAA1a during 46 to 72 h of incubation (Table II), indicating that in the presence of sucrose the hydrolysis of IAA1a proceeds at an accelerated rate, thereby exposing more free IAA to oxidation.

This conclusion was further confirmed when the effect of sucrose on the metabolism of [1-^{14}C]IAA1a was studied. Figure 9A demonstrates that, after 72 h of incubation with buffer only, more than 60% of the [1-^{14}C]IAA1a taken up was converted into IAA conjugate-3 and a certain amount to metabolite-1 (Fig. 9A), suggesting that the free IAA released after IAA1a hydrolysis is rapidly reconjugated and therefore inactivated. This is consistent with the observation that IAA conjugate-3, which was characterized as an amide IAA conjugate (18), could also undergo a certain hydrolysis after 72 h, even without sucrose in the medium (Fig. 7A). However, due to the slow rate of its metabolism, as well as the rapid reconjugation to conjugate-3 (Fig. 9A), the amount of free IAA which remained in the tissue might not be enough to induce ethylene production. On the other hand, in the presence of sucrose, all the [1-^{14}C]IAA1a taken up disappeared, presumably via hydrolysis, within 72 h and the newly released free IAA was remetabolized to its three typical IAA metabolites (Fig. 9A), with a preferable formation of metabolite-1 (Fig. 7; Ref. 18). Indeed Bialek *et al.* (7) have demonstrated recently that the biological activity of amide IAA conjugates depends on their rate of hydrolysis in the tissue.

Since both IAA1a and IAA appeared in the same R_F zone when isobutanol-acetic acid-H_2O (4:1:4, v/v) was used as the solvent system (Fig. 9A), the extracts were also chromatographed with isopropanol-8 N NH_4OH (8:2, v/v) (Fig. 9B) and with chloroform-methanol-acetic acid (75:20:5, v/v) on TLC plates (data not shown), in order to separate these two compounds. The data of Figure 9B demonstrate that when using isopropanol-8 N NH_4OH, IAA1a was separated better from IAA and was completely metabolized in the presence of sucrose. The results of Table II and Figure 9 imply that sucrose could stimulate the hydrolysis of an exogenously supplied synthetic IAA conjugate, IAA1a, as well as that of endogenous IAA conjugates. This can explain the remarkable IAA1a-induced ethylene production in the presence of sucrose (Fig. 9; Ref. 3) and lends further support to previous data of this report.

Endogenous Level of Auxins in Intact Tobacco Leaves. When the content of endogenous IAA and IAA conjugates in freshly excised tobacco leaves was determined using the gas chromatographic retention time of pentafluorobenzyl esters to tentatively verify identity, most of the putative IAA (84%) appeared as esteric IAA conjugate and only 4% remained as free IAA (Table III). This corresponds to the putative esteric IAA conjugate, formed after the IAA application, which seems to be the most important conjugate in our system, for the following reasons: (a) it undergoes the most remarkable promotion of hydrolysis by

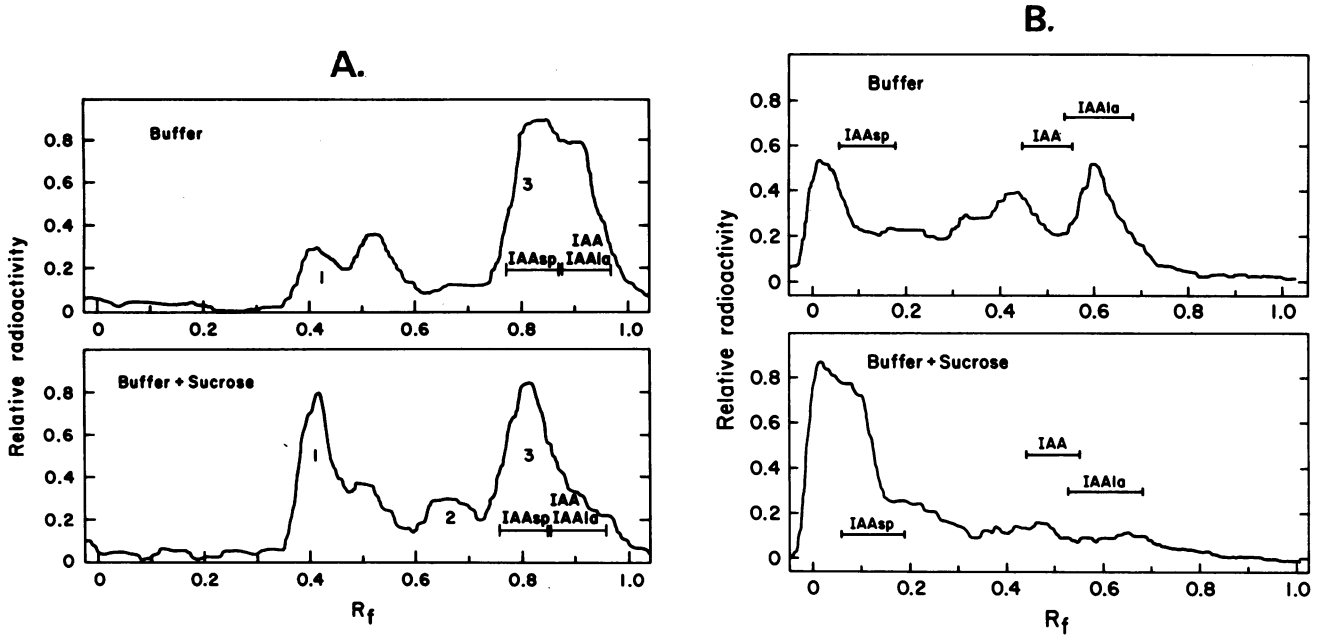

FIG. 9. Metabolism of [1-^{14}C]IAA1a in sucrose-treated or untreated tobacco leaf discs.Ten μM of [1-^{14}C]IAA1a (0.1 μCi) was applied for 72 h in the presence or absence of 50 mM sucrose. Aliquots of the radioactive extracts, containing 12×10^3 dpm, were applied on each of the chromatograms, which were developed either with isobutanol-acetic acid-H_2O (4:1:4, v/v) (A) or with isopropanol-8 N NH_4OH (8:2, v/v) (B). Numbers indicate the three IAA metabolites obtained. Locations of authentic IAA, IAAsp, and IAA1a are designated by bars.

Table III. *Content of Free IAA and IAA Conjugates in Tobacco Leaves*

Tobacco leaves, weighing about 67 g, were homogenized in 70% (v/v) acetone. All extraction and assay procedures were according to Epstein and Cohen (12). Identity of IAA based on the detection of the pentafluorobenzyl ester of IAA by electron capture GC of the retention time of an authentic standard.

Type of Auxin	Auxin Content	
	μg/g fresh wt	%
Free IAA	0.114	4
Esteric IAA	2.236	84
Amide IAA	0.320	12

sucrose (Fig. 7); (b) it appears at the highest level in the whole leaf (Table III), as well as in the epidermis tissue (18) which presumably is an important site of ethylene production; and (c) it induces the highest ethylene production when applied exogenously to leaf discs (18). Thus, we may conclude that the main effect of sucrose in leaves can be related to the existence of endogenous esteric IAA conjugates rather than to the peptidic ones.

Possible Involvement of Carbohydrates in IAA-Mediated Phenomena. The findings presented in this paper can explain results reported previously about the effects of sugars in various IAA-mediated phenomena. These include stem growth, callus embryogenesis, and leaf senescence. Sucrose was found to stimulate growth of pea epicotyls (20) and *Avena* coleoptiles (22). Similarly, various sugars also enhanced embryogenesis in callus of *Citrus* cultivars (17). When the pea tissue was supplied with sucrose, the 1-h exposure to IAA could be as effective for growth as a continuous 20-h exposure to IAA (20). Bandurski and Schulze (6) have already demonstrated that the *Avena* and pea stems, like many other tissues, contained most of their endogenous IAA as IAA conjugates (82% and 57%, respectively). Thus, their sucrose-stimulated growth could be an IAA-mediated process, as follows: sucrose might enhance hydrolysis of the endogenous IAA conjugates, thereby allowing release of free IAA which, in turn, promotes growth. It is noteworthy that although sugars, when applied individually, can induce growth of pea epicotyls (20) and embryogenesis of callus (17), their simultaneous application with IAA inhibited these phenomena (17, 20). A similar inhibition of both growth and embryogenesis was obtained also with supraoptimal IAA concentrations (17, 20). Hence, this inhibitory effect may be ascribed at least partially to the IAA-induced ethylene production, as suggested previously (11).

When referring to leaf senescence, it was found that this process is accompanied, in its advanced stages, by increased ethylene production and respiration, which rise in a climacteric-like pattern (2). Additionally, an increase in free IAA level was found in the course of leaf senescence so far in tobacco (13), oat (23), and bean (24) leaves. In the last system, the increase in free IAA level was accompanied by a decrease in the level of IAA conjugates (24). Recently, J. Riov (personal communication) found that initiation of the abscission process in citrus leaves is accompanied by an increased level of free IAA in the abscission zone. In view of these findings, it is possible that, during senescence of leaf blades and petioles (2), carbohydrates—released due to degradative processes—may stimulate hydrolysis of the endogenous IAA conjugates. Hence, these accumulated data support the postulated hypothesis that endogenous carbohydrates can control IAA level in the plant tissue, thereby affecting ethylene production and other IAA-mediated processes.

Although the findings reported herein and elsewhere (3, 4, 18) suggest IAA mediation in the sucrose-stimulated ethylene production, other mechanisms to account for the effect of sugars should also be taken into consideration (19).

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