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Mechanism of a Synergistic Effect of Kinetin on Auxin-induced Ethylene Production

SUPPRESSION OF AUXIN CONJUGATION[1]

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ABSTRACT

In hypocotyl segments of mung bean (*Phaseolus mungo* L.) seedlings, exogenously supplied indoleacetic acid was rapidly conjugated mainly into indoleacetylaspartic acid, which was inactive in inducing ethylene production. Kinetin is known to stimulate indoleacetic acid-induced ethylene production. The mechanism of kinetin action on indoleacetic acid-induced ethylene production by hypocotyl segments of mung bean seedlings was studied in relation to indoleacetic acid uptake and indoleacetic acid metabolism. Kinetin enhanced indoleacetic acid uptake during the initial 2-hour incubation and markedly suppressed the conversion of indoleacetic acid to indoleacetic acid conjugates throughout the whole 7-hour incubation. As a result, there was more free indoleacetic acid and less conjugated indoleacetic acid in the segments treated with kinetin than in those receiving no kinetin. A close relationship was demonstrated between the rate of ethylene production and the level of free indoleacetic acid, which was regulated by kinetin.

It is well known that auxins stimulate ethylene production in a wide variety of plant tissues (1–5, 8, 9, 11, 14, 20), and many of the effects of auxin are now attributed to its effects on ethylene production (13, 20). Induction of new enzymes is suggested to be involved in this process, based on the observation that inhibitors of protein and RNA synthesis retard auxin-induced ethylene production and that the stimulation of ethylene production by auxin has a substantial lag period (1, 3, 14). Apparently, the ethylene-producing system induced by auxin is labile and is characterized by a rapid turnover rate (9, 14).

In excised segments of etiolated pea shoots, Kang *et al.* (9) and others (2, 4, 5) have shown that IAA-induced ethylene production parallels the free IAA level, which in turn inversely depends upon the rate of IAA conjugation and decarboxylation.

Kinetin alone slightly stimulates ethylene production by etiolated seedlings of several species, but a remarkable synergistic effect of kinetin on IAA-induced ethylene production has been observed (4, 8, 10). It was concluded that kinetin exerts its stimulating effect upon ethylene production only in the presence of IAA (H. Imaseki, unpublished results). The mechanism of the kinetin effect has not been elucidated.

This study was undertaken to determine whether kinetin plays a causal role in the uptake and metabolism of IAA. The present paper presents evidence showing that in mung bean hypocotyl segments kinetin suppresses the conversion of IAA into IAA conjugates, the main pathway of IAA metabolism. As a consequence, a higher free IAA level is sustained and is, in turn, responsible for higher ethylene production.

MATERIALS AND METHODS

Plant Materials and Chemicals. Seeds of mung bean (*Phaseolus mungo* L.) purchased from a local market were sorted and surface-sterilized with 0.02% sodium hypochlorite solution for 10 min. After thorough washing, the seeds were imbibed and aerated for 12 hr and were then grown in vermiculite for 4.5 days in darkness at 24 C. Under dim green light, 2 cm long segments were cut from hypocotyls at a point 1 cm below the hook. Twenty segments were incubated in 5 ml of incubation medium containing 50 mM KH_2PO_4 (pH 4.5) and 2% sucrose in a 50-ml Erlenmeyer flask. Where indicated, additions were 0.15 μmole IAA-1-^{14}C (1 μc) and 0.5 μmole of kinetin. A plastic center well containing 0.1 ml of 40% KOH was hung in the flask to absorb CO_2 evolved. The flask was sealed with a rubber serum cap and incubated in a shaker at 25 C in darkness.

IAA-1-^{14}C was purchased from Amersham/Searle Corporation. IAAsp[2] was a product of Calbiochem.

Determination of Ethylene. At the end of the incubation, a 1-ml gas sample was withdrawn with a hypodermic syringe, and ethylene was assayed with a gas chromatograph equipped with an alumina column and a flame ionization detector.

Analysis of IAA and Its Metabolites. Aliquots (50 μl) of incubation medium before and after incubation were assayed for total radioactivity with a liquid scintillation counter. Decarboxylation of IAA-1-^{14}C was measured by counting $^{14}CO_2$ evolved. The $^{14}CO_2$ absorbed by KOH solution during the incubation was released by acidification and then reabsorbed into 0.5 ml of ethanolamine-ethoxyethanol mixture (1:1, v/v) and assayed by liquid scintillation counting as described previously (19).

At the end of the incubation, the segments were washed with 10 μM of unlabeled IAA solution and then with water. The segments were then homogenized and extracted first with 10 ml of 95% ethanol and then with 5 ml of 80% ethanol. The combined extracts were concentrated under reduced pressure

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[2] Abbreviation: IAAsp: indole-3-acetylaspartic acid.

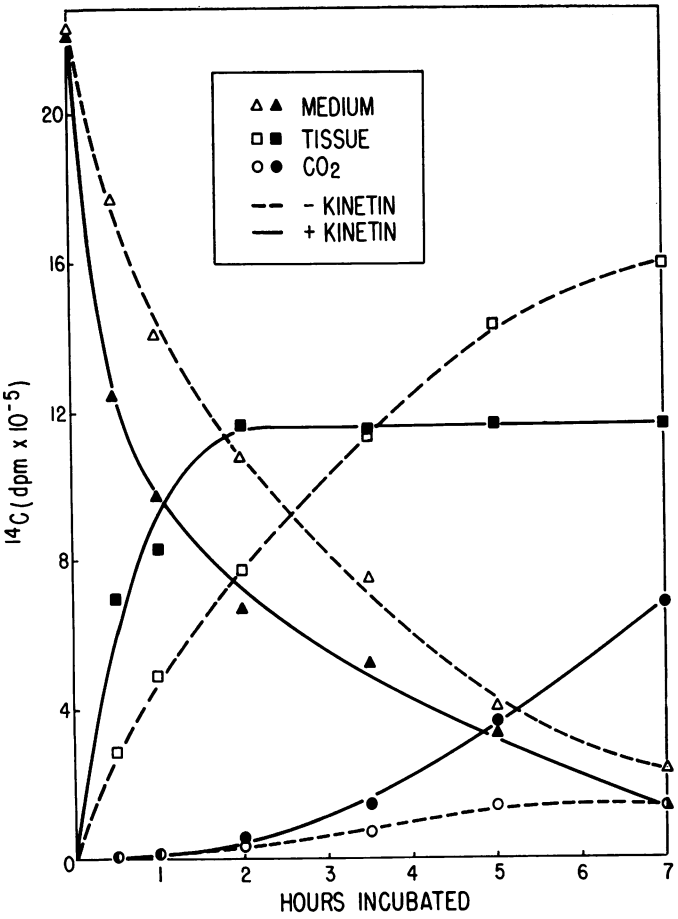

FIG. 1. Effect of kinetin on the uptake and decarboxylation of IAA and the decrease in external IAA in the medium by hypocotyl segments. Twenty segments were incubated in 5 ml of incubation medium containing 50 mM KH_2PO_4, 2% sucrose, and 30 μM IAA-1-^{14}C (1 μc) in the presence or absence of 0.1 mM of kinetin.

to a final volume of 2 ml. A 50-μl aliquot of the extract was counted for radioactivity. A 100-μl aliquot was chromatographed on paper along with unlabeled IAA and IAAsp using 1-butanol-3% ammonia (1:1, v/v) as developing solvent. After drying, the chromatograms were scanned for radioactivity, and the radioactivity was estimated from the peak area. The known IAA (R_F = 0.22) and IAAsp (R_F = 0.01) on the chromatograms were located under ultraviolet light.

RESULTS

Effects of Kinetin on the Decarboxylation and Uptake of IAA. Kinetin enhanced the decarboxylation of IAA during the 7-hr incubation period and also accelerated IAA uptake during the first 2-hr incubation. These effects were reflected by a concomitant decrease in radioactivity in the incubation media and by an increase in radioactivity within the tissue (Fig. 1). During the later part of the incubation, the segments receiving no kinetin continued to accumulate radioactivity, but there was no further net increase in radioactivity in the tissues treated with kinetin. This was apparently not due to a cessation of uptake of IAA by the kinetin-treated tissue, but due to a balance between the rate of IAA uptake and IAA decarboxylation. This view was further supported by the data showing that the sum of radioactivity recovered from the tissues and the CO_2 or the decrease in radioactivity of the incubation medium in kinetin-treated tissue was similar to that of the kinetin-free tissue during the later part of the incubation (Fig. 1). The fact that kinetin enhanced the uptake of IAA during the early part of the incubation suggests that kinetin may exert its influence on ethylene production by regulating the rate of IAA uptake.

It should be noted that the decarboxylation of IAA recorded in Figure 1 was due to metabolic rather than spontaneous degradation, since the incubation medium containing IAA-1-^{14}C but without tissues gave off no significant radioactivity in CO_2.

Effect of Kinetin on the Conjugation of IAA in Relation to Ethylene Evolution. Paper radiochromatograms of the extracts prepared from tissue which had been incubated for various periods revealed two radioactive spots (Fig. 2). One had an R_F value of 0.22 which corresponds to IAA. The other, designated as IAA-conjugates, had an R_F value of 0.01, corresponding to that of IAAsp. Changes in the levels of IAA and IAA-conjugates in tissues with time are shown in Figures 2 and 3. The kinetin-treated tissue had a much higher free IAA level but a lower IAA-conjugate level than the control tissue throughout the incubation period. The difference in free IAA levels between the two treatments was more prominent during the later part of the incubation when IAA was rapidly conjugated. At the end of 7-hr incubation, all of the IAA taken up had been converted into IAA-conjugates in both treatments. These data suggest that kinetin, in addition to the promotion of IAA uptake during the early incubation period, also acted to suppress the conversion of IAA into IAA-conjugates. As a result, a higher level of free IAA was sustained during the incubation period in the kinetin-treated tissue.

Figure 3 shows the time curves for the rate of ethylene production by the mung bean segments incubated in the ab-

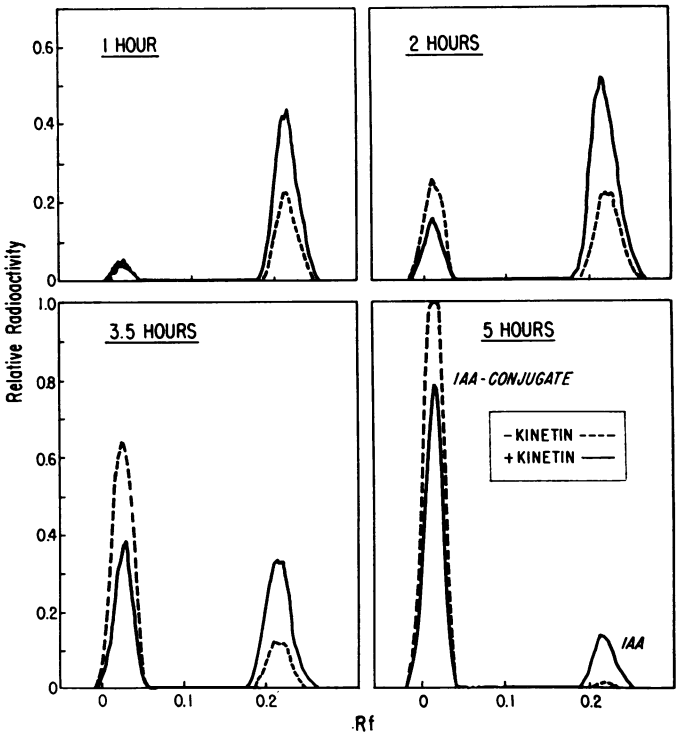

FIG. 2. Paper radiochromatograms of the extracts of tissues which were incubated with IAA-1-^{14}C or with IAA-1-^{14}C plus kinetin. Extracts prepared from those tissues used in Figure 1 were chromatographed on paper developed with 1-butanol-3% NH_3 in water (1:1, v/v). The radioactive spots with R_F of 0.01 and 0.22 are IAA-conjugate and free IAA, respectively. There were no other radioactive spots on the chromatograms.

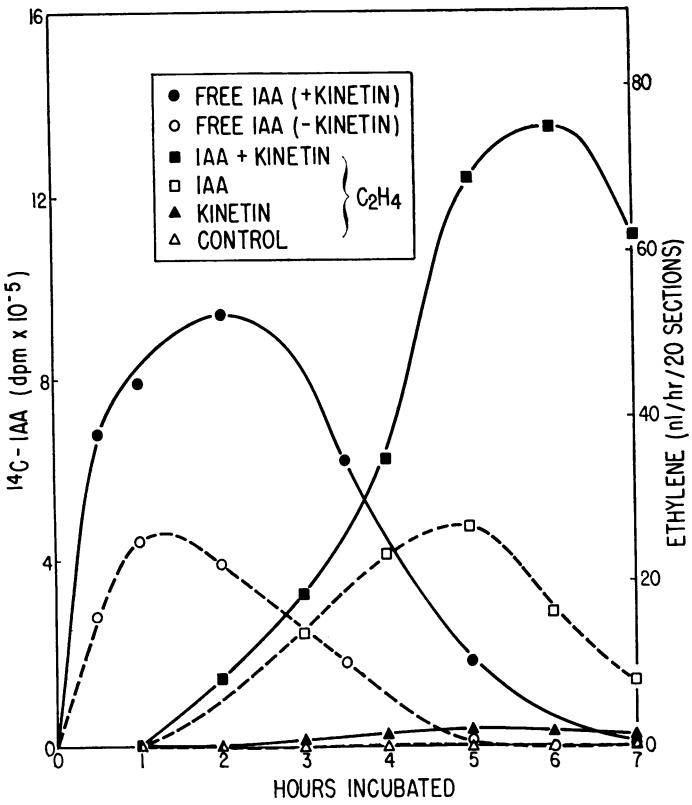

FIG. 3. Comparison of the levels of free IAA and ethylene production rate by hypocotyl segments. For the assay of radioactive free IAA, the tissues were incubated with either IAA-1-^{14}C (○) or IAA-1-^{14}C plus kinetin (●) as described in Figure 1. For the determination of the rate of ethylene production, the tissues were incubated with IAA plus Kinetin (■), IAA (□), kinetin (▲), or without any growth regulator (△).

sence of growth regulators or in the presence of 0.1 mM kinetin, 30 μM IAA, or 0.1 mM kinetin plus 30 μM IAA. A remarkable synergistic stimulation of ethylene production by kinetin in the presence of IAA was shown. Kinetin did not shorten the lag period required for IAA-induced ethylene production. It is apparent that the rate of ethylene production under the influence of IAA or IAA plus kinetin is closely correlated with the level of free IAA within the tissue, but not with the level of IAA-conjugates (Fig. 3). As will be described later, the major compound of the IAA-conjugates was identified as IAAsp. When IAAsp (0.1 mM) was tested for its effectiveness to induce ethylene production, either in the presence or in the absence of kinetin, it was found, as expected, to be totally inactive. These results fully agree with the earlier observation of other investigators (2, 4, 5, 9) that IAA-induced ethylene production in excised segments of etiolated pea shoots parallels the free IAA level in the tissue. The time lag between the maximum free IAA level and the maximum ethylene production rate was approximately 4 hr in both kinetin-treated and kinetin-free segments (Fig. 3).

Characterization of IAA-Conjugates. Extracts were prepared from tissues which had been incubated for 7 hr in the presence of either IAA-1-^{14}C or IAA-1-^{14}C plus kinetin. Paper radiochromatograms of these extracts developed with 1-butanol-3% ammonia (1:1, v/v) revealed only one radioactive spot with a R_F value of 0.01. This value corresponds to that of IAAsp. When the same extract was chromatographed with 1-butanol-acetic acid-water (4:1:4, v/v), 3 radioactive spots with R_F values of 0.05 (spot 1), 0.71 (spot 2), and 0.87 (spot 3) were observed. Spots 1, 2, and 3 contained 14, 6, and 80%, respectively, of the total radioactivity. In this system free IAA had a R_F of 0.96, while authentic IAAsp had a R_F of 0.87, which is identical to spot 3. That the majority of the radioactivity in the extract (spot 3) was indeed IAAsp was further supported by data from electrophoresis and alkaline hydrolysis. When spot 3 was subjected to paper electrophoresis at pH 2.0, the radioactivity remained at the origin as did authentic IAA and IAAsp. Upon paper electrophoresis at pH 7.0 using 0.02 M phosphate buffer, both authentic IAA and IAAsp moved toward the anode; spot 3 moved the same distance as authentic IAAsp with a mobility of 1.5 relative to IAA. Spot 1 had a mobility of 1.0 relative to IAA at pH 7.0. Further characterization of spots 1 and 2 was not attempted. When the extract was hydrolyzed with saturated barium hydroxide in water for 16 hr at 100 C and extracted with ethyl acetate after acidification with H_2SO_4 solution, it yielded a radioactive compound which contained more than 80% of the total radioactivity and had the same R_F value as IAA when cochromatographed on paper developed with 1-butanol-3% NH_3 (1:1, v/v). These results indicate that the major component of the IAA conjugate was IAAsp.

DISCUSSION

The synergistic effect of kinetin on IAA-induced ethylene production (Fig. 3) has been recognized in several laboratories (4, 8, 10). It has been suggested that kinetin may act by suppressing the degradation of IAA-induced enzymes which were responsible for ethylene biosynthesis (10). However, Imaseki (presented at the Gordon Research Conferences, Postharvest Physiology Section, Andover, N.H., 1972) has presented data showing that the role of kinetin on IAA-induced ethylene production by hypocotyl segments of mung bean seedling could not be attributed to the suppression of protein degradation. In excised leaf tissue, kinetin has been reported to delay senescence by stimulating protein and nucleic acid synthesis (12, 17), by suppressing RNase and DNase activity (17) and by retarding the decline in auxin levels (7).

Since kinetin alone stimulates ethylene production only slightly, and the IAA-induced ethylene production parallels the free IAA level in the pea tissue (2, 4, 5, 9), it is logical to speculate that kinetin may exert its effect upon ethylene production by regulating the IAA level within the tissue. In the present studies, we have shown that exogenously supplied IAA was rapidly converted through conjugation to form IAAsp, which was inactive to induce ethylene production. The present data, as well as that of Kang *et al.* (9), show that the IAA-induced ethylene production parallels the free IAA level. As shown in Figures 1, 2, and 3, kinetin enhanced IAA uptake during the early part of the incubation period and markedly suppressed the conversion of IAA to IAA-conjugates. As a result, a higher level of free IAA and a lower level of IAA-conjugates were maintained in the kinetin-treated tissue. These data led us to conclude that kinetin exerts its synergistic effect on IAA-induced ethylene production by regulating IAA uptake and IAA metabolism. However, the molecular basis of these effects of kinetin on the uptake and metabolism of IAA is unknown. The biochemistry of IAA conjugation has been reviewed (15). Kinetin has been reported to promote amino acid uptake by detached leaves (16, 18).

Although there was more decarboxylation of IAA in the kinetin-treated tissues than in control tissues (Fig. 1), this was probably due to a greater amount of free IAA available for oxidative decarboxylation in the kinetin-treated tissue. IAAsp possesses no free carboxyl group and is not expected to be susceptible to decarboxylation.

We have recently shown that ABA moderately inhibits IAA-induced ethylene production in the same tissue, and this inhibition could be ascribed to the enhanced IAA conversion to IAAsp and decreased level of free IAA (O. L. Lau and S. F. Yang, unpublished results). In this regard it is pertinent to note the report of Chang and Jacobs (6) who also showed that ABA enhanced the conversion of IAA to IAAsp and decreased the free IAA level in *Coleus* petioles.

It remains to be shown whether cytokinins also act through regulation of IAA conjugation in other plant systems where auxin and cytokinins are known to interact.

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