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Effect of 2,4-Dinitrophenol on Auxin-induced Ethylene Production and Auxin Conjugation by Mung Bean Tissue[1]

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ABSTRACT

Auxin-induced ethylene production by mung bean (*Phaseolus mungo* L.) hypocotyl segments was markedly inhibited by 2,4-dinitrophenol regardless of whether or not kinetin was present. Uptake of indoleacetic acid-2-^{14}C was also inhibited in the presence of 2,4-dinitrophenol. Segments treated only with indoleacetic acid rapidly converted indoleacetic acid into indole-3-acetylaspartic acid with time whereas kinetin suppressed indoleacetic acid conjugation. Formation of indole-3-acetylaspartic acid was significantly reduced when 2,4-dinitrophenol was present. The suppression of indoleacetic acid conjugation by kinetin and 2,4-dinitrophenol appeared to be additive, and the free indoleacetic acid level in segments treated with 2,4-dinitrophenol in the presence of indoleacetic acid or indoleacetic acid plus kinetin was remarkably higher than in corresponding segments which received no 2,4-dinitrophenol.

In the absence of 2,4-dinitrophenol, indoleacetic acid-induced ethylene parallels the free indoleacetic acid level within the tissue. However, in the presence of 2,4-dinitrophenol the rate of ethylene production did not correlate with the free indoleacetic acid level. These results indicate that both indoleacetic acid-induced ethylene production and indoleacetic acid conjugation require a continuous supply of ATP, the formation of which was inhibited by 2,4-dinitrophenol.

Auxins are known to stimulate ethylene production in a wide variety of plant tissue (1, 4, 6–8, 11–13). In excised tissue from legume seedlings, kinetin alone slightly stimulates ethylene production, but a remarkable synergistic effect of kinetin on IAA-induced ethylene production has been observed (4, 6, 8). It has been shown that IAA-induced ethylene production parallels the free IAA level, which, in turn, is regulated by the rate of IAA metabolism (4, 7, 8). When tissue is incubated with IAA, most of the IAA entering the tissue is rapidly converted to IAAsp[2] which has little or no auxin activity in the pea test (1) or in inducing ethylene production in mung bean (8). Kinetin markedly suppressed the conjugation of IAA and therefore increased the free IAA level and ethylene production rate of the tissue (8). The presence of IAA has been found to stimulate synthesis of a new enzyme protein responsible for IAA conjugation (16), which requires oxygen and probably proceeds via formation of indoleacetyl-CoA (18). Using a model system employing octanoate thiokinase from liver mitochondria, Zenk (18) found that ATP is required for the synthesis of indoleacetyl-CoA from IAA and CoA; the oxygen is required for ATP synthesis.

Compounds such as DNP and other halo- and nitrophenols are known to uncouple oxidative phosphorylation which leads to a depletion of ATP in the tissue. However, these agents do not uncouple substrate level phosphorylation (9). In fruit tissue DNP has been shown to inhibit ethylene production and respiration (5, 15) as well as the conversion of methionine to ethylene (Murr and Yang, in preparation). The inhibition caused by DNP could be reversed partially by ATP (5). These results suggest that at least one step in the synthesis of ethylene required ATP (5, 15; Murr and Yang, in preparation).

The present paper will describe the effect of DNP on IAA-induced ethylene production in relation to IAA conjugation by mung bean hypocotyl segments.

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 8 hr and then were grown in vermiculite for 4 days, in darkness, at 25 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 potassium phosphate buffer (pH 6) and 2% sucrose in a 50-ml Erlenmeyer flask. Where indicated, 0.15 μmole of IAA-2-^{14}C (2 μCi), 0.25 μmole of DNP, and 0.2 μmole of kinetin was included. A plastic center well containing 0.2 ml of 40% KOH was hung in the flask to absorb the CO_2 evolved. The flask was sealed with a rubber serum cap and incubated in a shaker at 25 C, in darkness.

Determination of Ethylene. At the end of the incubation, a 1-ml gas sample was withdrawn from the flask 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 IAAsp. Aliquots of incubation medium before and after incubation were assayed for total radioactivity with a liquid scintillation counter. The segments were washed with 10 μM unlabeled IAA solution and then with water. The segments were then homogenized and extracted, first with 95% ethyl alcohol and then with 80% ethyl alcohol. The combined extracts were concentrated under reduced pressure to a final volume of 2 ml. An aliquot of the concentrated extract was

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[2] Abbreviations: IAAsp: indole-3-acetylaspartic acid; DNP: 2,4-dinitrophenol.

chromatographed on paper, along with unlabeled IAA and IAAsp, using 1-butanol-3% ammonia (1:1, v/v) as developing solvent. After the chromatograms dried, they were scanned for radioactivity and the radioactivity was estimated from the peak area as previously described (8).

RESULTS

It is evident that DNP inhibits ethylene production by mung bean segments treated with IAA (Fig. 1). Concentrations as low as 1 μM markedly inhibited IAA-induced ethylene production and 90% inhibition was achieved when the DNP concentration was raised to 100 μM.

Figure 2 shows the effect of 50 μM DNP on the time course of IAA-induced ethylene production by mung bean segments in the presence or absence of kinetin. As previously reported (8), kinetin acts synergistically with IAA to stimulate ethylene production above that by segments treated only with IAA. However, DNP significantly inhibited ethylene production by about 50% regardless of whether or not kinetin was present (Fig. 2).

Because continued ethylene production requires a continuous supply of IAA (4, 6, 8) and kinetin suppresses conjugation of IAA in mung bean tissue (8), experiments were conducted to examine the effect of DNP on IAA uptake and IAA conjugation by using IAA-2-^{14}C. As shown in Figure 3, DNP inhibited the uptake of IAA into hypocotyl segments treated with IAA or with IAA and kinetin. Over the 7-hr incubation period, DNP inhibited IAA uptake about 40% in tissue treated with IAA and inhibited uptake about 30% in tissue treated with IAA plus kinetin. Hypocotyl segments treated with IAA in the absence of DNP rapidly converted IAA into IAAsp with time and by the end of the incubation period, the level of free IAA was essentially zero (Fig. 4). As previously reported (8), kinetin suppressed the conjugation of IAA, thereby increasing the level of free IAA within the tissue (Fig. 4). In the presence of DNP, the formation of IAAsp was significantly reduced whether or not kinetin was present, and as a result,

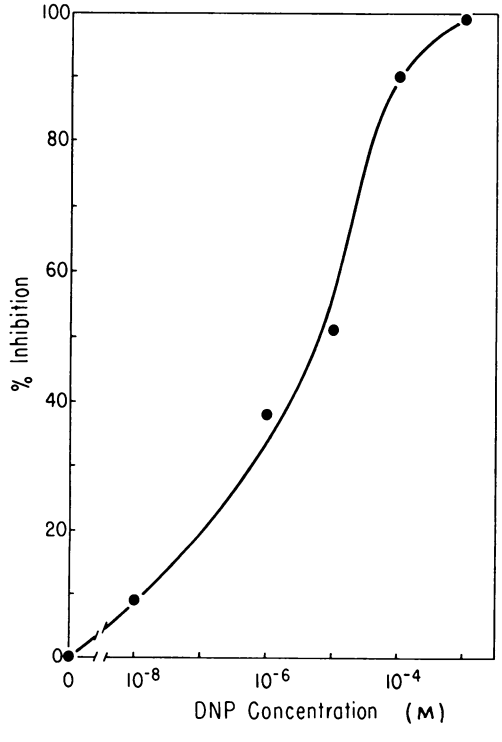

FIG. 1. Effect of DNP concentration on the inhibition of IAA-induced ethylene production by mung bean hypocotyl segments incubated in 50 μM IAA for 7 hr at 25 C.

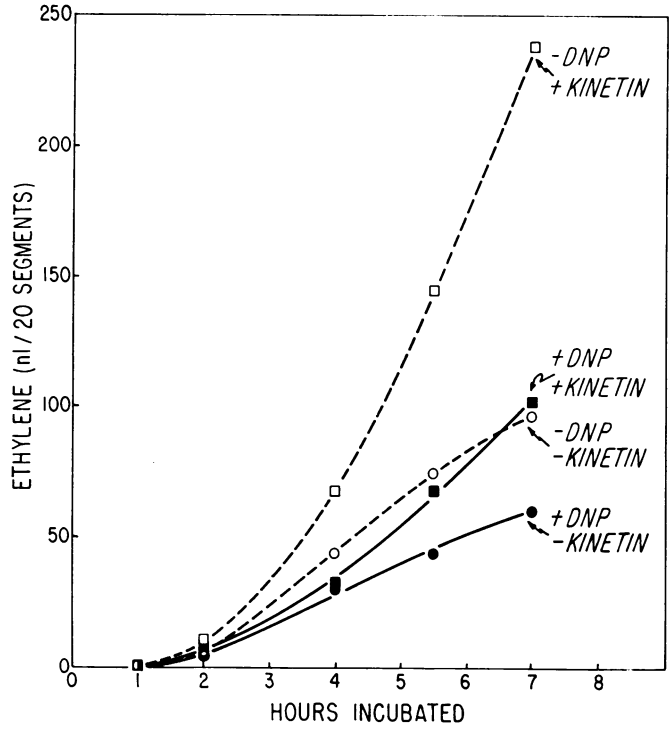

FIG. 2. Effect of DNP on the time course of IAA-induced ethylene production by mung bean segments in the absence or presence of kinetin. The incubation medium contained, in a total volume of 5 ml, 0.15 μmole of IAA-2-^{14}C (2 μCi) and, where indicated, 0.25 μmole of DNP and 0.20 μmole of kinetin.

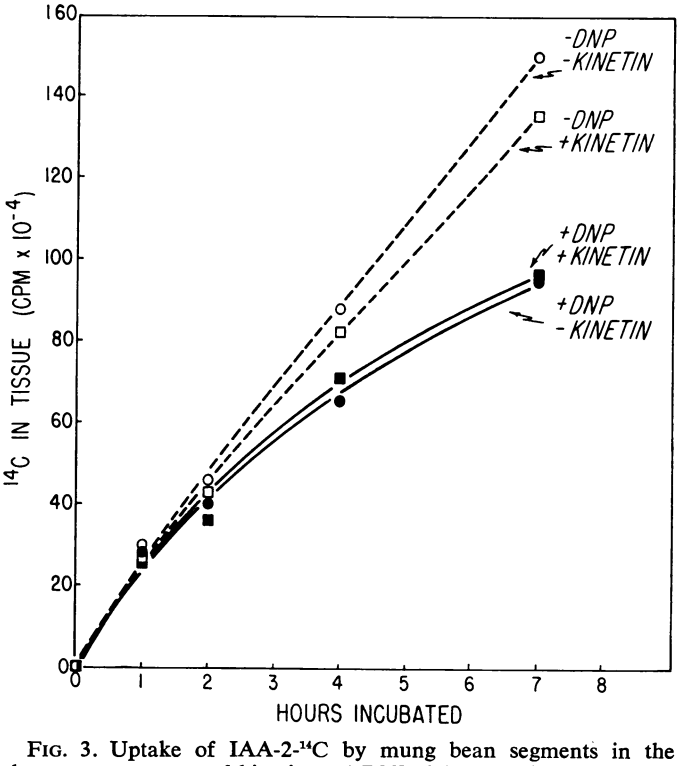

FIG. 3. Uptake of IAA-2-^{14}C by mung bean segments in the absence or presence of kinetin and DNP. The experimental conditions in the incubation medium were as described in Fig. 2.

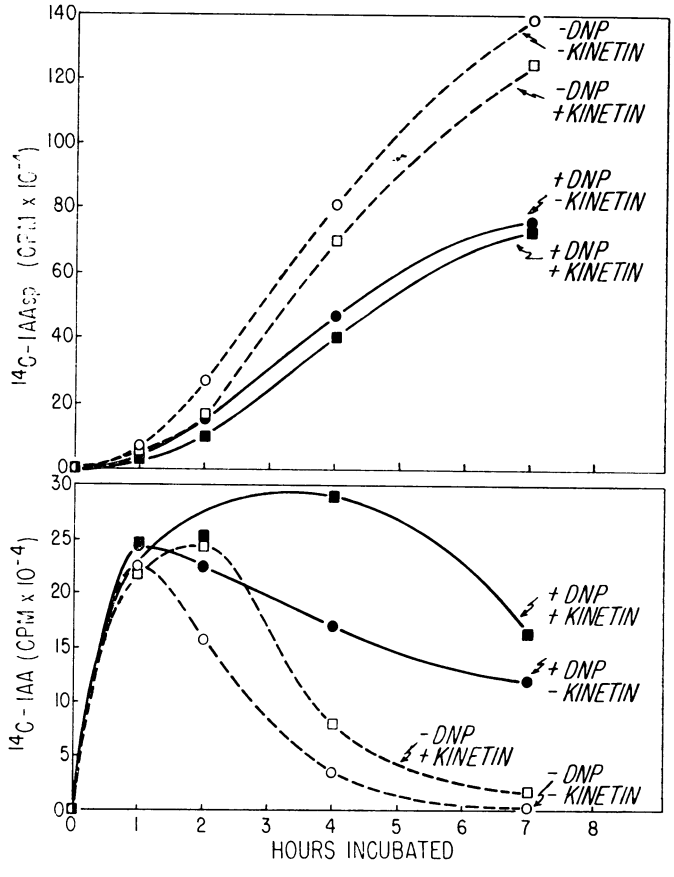

FIG. 4. Effect of DNP on the formation of ^{14}C-IAAsp and the level of free ^{14}C-IAA in hypocotyl segments treated with IAA-2-^{14}C in the absence and presence of kinetin. The experimental conditions were as described in Fig. 2.

the level of free IAA in those segments treated with DNP in the presence of IAA or IAA plus kinetin was remarkably higher than in those corresponding segments which received no DNP (Fig. 4). The suppression of IAA conjugation by kinetin and DNP appears to be additive because segments treated with both kinetin and DNP contained the highest level of free IAA.

DISCUSSION

Our results show clearly that DNP, an uncoupler of oxidative phosphorylation, strongly inhibits (*a*) the uptake of IAA-2-^{14}C, (*b*) the conjugation of IAA (the conversion of IAA into IAAsp), and (*c*) the IAA-induced ethylene production of mung bean hypocotyl segments. It has been established that IAA-induced ethylene production parallels the free IAA level, which in turn depends upon the rate of IAA conjugation and decarboxylation (4, 7, 8). However, in the presence of DNP we cannot find a correlation between the rate of ethylene production and the level of free IAA (Figs. 2 and 4). The level of IAAsp seems to reveal a close relationship with ethylene production (Fig. 4). However, it has been shown that IAAsp is not capable of inducing ethylene production in mung bean tissues (8). The synergistic effect of kinetin on IAA-induced ethylene production and the inhibition of IAAsp formation by kinetin further disproves the possibility that IAAsp may be involved in the process of promoting ethylene production (Figs. 2 and 4).

DNP is a potent uncoupler of oxidative phosphorylation (9) and has been found to uncouple phosphorylations in mature tomato fruits (10). The inhibition of ethylene production (5, 14, 15) and of fruit ripening (10, 15) by DNP suggested that a phosphorylation was involved in these processes (14, 15). The fact that the inhibition of ethylene production in fruit tissue caused by DNP was partially reversed by ATP led Burg and Thimann (5) to suggest that at least one step in the synthesis of ethylene required the energy supplied by respiration. More recent work (Murr and Yang, in preparation) indicates that the step involved is at the conversion of methionine to ethylene.

It has been suggested that ATP is required for the formation of IAAsp in a model system consisting of IAA, CoA, aspartic acid, and octanoate thiokinase, an enzyme isolated from liver mitochondria (18). Our results with DNP support this idea, because, in the presence of DNP, the formation of IAAsp from IAA in mung bean segments is reduced (Fig. 4). Formation of ethylene from methionine in fruit tissue (3) and formation of IAAsp in pea tissue (2) were observed only in the presence of oxygen. It appears that both ethylene production and IAA conjugation require a continuous supply of ATP which comes primarily from oxidative phosphorylation. The rate of ethylene production depends on the level of free IAA in the tissue (1, 4, 6, 8) when phosphorylation proceeds in the absence of an uncoupler. In the presence of an uncoupler, which inhibits both IAA conjugation and IAA-induced ethylene production, the rate of ethylene production will no longer parallel the level of free IAA in the tissue.

Based on studies using inhibitors of RNA and protein synthesis and in which a substantial lag period was observed, it was suggested that the induction of a new enzyme is involved in IAA-induced ethylene production (1) and in auxin conjugation (16, 17, 19). Because ATP is essential for amino acid activation and thus, for protein synthesis, it seems logical that a cessation of the ATP supply in the presence of DNP would slow down the synthesis of the new enzyme required for the IAA conjugation system as well as the ethylene-producing system. Our results indicate that DNP exerted a similar effect on IAAsp formation and on ethylene production.

Sakai and Imaseki (13) reported that both ethylene production and IAAsp formation were inhibited by a protein isolated from etiolated mung bean seedlings (12) and that the rate of inhibition of IAA conjugation was about the same as that of ethylene production. They suggested that the formation of ethylene and the conjugation of IAA are interrelated and regulated by the same mechanism. The effect of the proteinaceous inhibitor on IAA-induced ethylene production and on IAA conjugation reported by them (13) is quite similar to the present results with DNP. It is very likely that the proteinaceous inhibitor simply acts as an uncoupler, as DNP does, which limits the normal supply of ATP needed for the ethylene-producing and IAAsp-forming systems.

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