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Inhibition of Ethylene Production by 2,4-Dinitrophenol and High Temperature[1]

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ABSTRACT

2,4-Dinitrophenol (DNP) and high temperature (35 to 40 C) are known to inhibit C_2H_4 production in various plant tissues. The present study was made to determine the step in the C_2H_4 biosynthetic pathway (methionine → *S*-adenosylmethionine [SAM] → 1-aminocyclopropane-1-carboxylic acid [ACC] → C_2H_4) at which these treatments exert their inhibitory effect. In mung bean hypocotyls the dose-inhibition curves for the effect of DNP on auxin-dependent C_2H_4 production (in which auxin exerts its effect by stimulating the conversion of SAM to ACC) and on ACC-dependent C_2H_4 production (in which ACC is directly utilized as precursor) were similar. It was concluded, therefore, that DNP at low concentrations (below 50 micromolar) exerted its effect primarily on the conversion of ACC to C_2H_4, a step which is common to both systems. This view was further substantiated by quantitative analysis of the intermediates in the biosynthetic sequence. DNP exerted little influence on the content of SAM but caused a significant increase in the ACC content and marked inhibition in C_2H_4 production, indicating that the conversion of ACC to C_2H_4 is the crossover point. At higher concentrations (above 100 micromolar), DNP inhibited the conversion of methionine to ACC and to C_2H_4, and this effect could be attributed to the inhibition of SAM synthesis.

The optimal temperature for maximal C_2H_4 production by apple tissue and mung bean hypocotyl is about 30 C. An increase in temperature to 35 C caused an accumulation of endogenous ACC, whereas C_2H_4 production was greatly reduced. These results suggest that the conversion of ACC to C_2H_4 is highly vulnerable to high temperature inhibition.

C_2H_4 is a plant hormone which regulates many aspects of plant growth and development (1). In plant tissue the C_2H_4 production rate is regulated by various physiological and environmental factors. In 1960, Burg and Thimann (9) reported that DNP[2] was an effective inhibitor of C_2H_4 production in apple tissue. Similar observations were reported for other fruit tissues and for auxin-induced C_2H_4 production in vegetative tissues (7, 15, 20). Using a tracer technique, Murr and Yang (19) concluded that DNP inhibited C_2H_4 production by interfering with the conversion of methionine to C_2H_4. Since DNP is a potent uncoupler of oxidative phosphorylation, these observations have led Burg (7) and Murr and Yang (19) to suggest that ATP is required in the conversion of methionine to C_2H_4, and that SAM, formed from methionine and ATP, may be an intermediate. The evidence for SAM serving as an intermediate in the biosynthesis of C_2H_4 from methionine was first presented by Adams and Yang (3), who fed labeled methionine to apple tissue and found that 5′-methylthioadenosine was derived from the CH_3S group of methionine molecule during its conversion to C_2H_4. The optimum temperature for C_2H_4 production by plant tissue is about 30 C. In 1942, Hansen (12) reported that pear fruits stopped production of C_2H_4 and failed to ripen when held at 40 C. In avocado, Eaks (10) found typical respiratory climacteric patterns occurring from 20 to 35 C. The respiratory climacteric maximum increased, but maximum C_2H_4 production decreased as the temperature increased from 25 to 40 C. Only trace amounts of C_2H_4 were produced at 35 C even though the tissue still showed a typical climacteric pattern. Although C_2H_4 production is more vulnerable to high temperature inhibition than is respiratory activity (8, 10, 18), the mode of the high temperature effect on C_2H_4 production has not been elucidated.

Adams and Yang (4) have elucidated the C_2H_4 biosynthetic pathway in apple tissue as follows: methionine → SAM → ACC → C_2H_4. This pathway has since been shown to be operative in auxin-induced C_2H_4 production in vegetative tissue (24) and other stress systems (21, 25). The present investigation was undertaken to determine at which step in the C_2H_4 biosynthetic sequence DNP and high temperature stress exert their inhibitory effects.

MATERIALS AND METHODS

Plant Material. Seed of mung bean (*Vigna radiata* [L.] Wilczek) were germinated and grown in Vermiculite for 3.5 days in darkness at 25 C. Twenty 2-cm long hypocotyl segments, 1 to 3 cm below the hook, were incubated in 5 ml medium consisting of 2% sucrose, 50 μg/ml chloramphenicol, 50 mM Mes buffer (pH 6.1), and, where indicated, various concentrations of IAA, IPA, ACC, DNP, or labeled methionine in a 50-ml Erlenmeyer flask. The flask was sealed with a rubber serum cap and incubated in darkness at 27.5 C with constant shaking at 60 cycles/min. Apple (*Malus sylvestris* Mill., var. Golden Delicious) plugs, 1 cm in diameter and 2 cm long, were prepared and incubated under various temperatures as described previously (3).

Chemicals. L-[3-^{14}C]Methionine was purchased from Research Products International, IAA and IPA were from Sigma, SAM was from Boehringer, and ACC was from Calbiochem.

Determination of C_2H_4. A 1-ml gas sample was withdrawn from the head space of the flask with a hypodermic syringe, and C_2H_4 was assayed on a gas chromatograph equipped with an alumina column and a flame ionization detector.

Determination of Total SAM. Mung bean hypocotyls, which had been treated for 9 h in a medium containing 50 μM IAA and various concentrations of DNP were homogenized and extracted with $HClO_4$ at a final concentration of 1.5 N. After centrifugation, the supernatant was mixed with a known amount of [methyl-^{3}H]SAM as internal standard and adjusted to pH 4.5 at 0 C by slowly adding solid $KHCO_3$. The precipitated salt was discarded, and the supernatant was passed through a sulfopropyl (SP)-Seph-

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[2] Abbreviations: DNP: 2,4-dinitrophenol; ACC: 1-aminocyclopropane-1-carboxylic acid; IPA: N^6-(Δ^2-isopentenyl)adenine; SAM: *S*-adenosylmethionine.

adex column (H^+ form) as described by Glazer and Pearle (11). After washing with 150 mM HCl until no *A* was detected at 260 nm, SAM was eluted with 500 mM HCl. The eluate was collected in fractions of 3 ml. The fractions which contained radioactivity were pooled. The concentration of SAM was determined spectrophotometrically at 260 nm, assuming a molar absorption of 15,000 cm^{-1} M^{-1}. Recovery was 55 to 60%, as calculated from the recovery of [^{3}H]SAM.

Determination of ACC. Mung bean hypocotyls or apple plugs were homogenized and extracted with $HClO_4$ at a final concentration of 3%. After centrifugation, the extracts were diluted three times with H_2O and passed through an ion-exchange resin (Dowex 50, H^+) column. Amino acids, including ACC, were eluted with 2 N NH_4OH (17). After concentration, the residues were dissolved in 1 ml H_2O. An aliquot was used for assay of ACC according to the method of Lizada and Yang (17), which is based on the chemical conversion of ACC to C_2H_4 with NaOCl reagent. For the analysis of radioactive ACC, an aliquot of the above eluate was mixed with 500 nmol authentic ACC and chromatographed on Whatman 3MM paper using 1-butanol-acetic acid-H_2O (4:1:5, v/v) as developing solvent. Radioactivity on the paper was detected by radioscanner. The radioactive region corresponding to ACC was eluted with H_2O and was degraded to C_2H_4 with NaOCl reagent (17). The C_2H_4 produced then was transferred to an evacuated 25-ml scintillation vial. A 0.5-ml gas sample was withdrawn from the vial for C_2H_4 determination by GC. The remainder of the C_2H_4 was absorbed by injecting 250 μl 0.25 M $Hg(ClO_4)_2$ reagent into the vial. The radioactivity of the C_2H_4 thus absorbed was assayed by liquid scintillation. The radioactive ACC in the sample was calculated from the following equation: radioactivity in ACC (nCi) = specific radioactivity of C_2H_4 (nCi/nmol) × 500.

RESULTS

Both auxin and ACC are known to cause a remarkable increase in C_2H_4 production in mung bean hypocotyls (22). In the ACC-dependent system, exogenous ACC was directly utilized as an C_2H_4 precursor (23). In the auxin-dependent system, however, auxin induced the synthesis of ACC synthase, which catalyzes the conversion of SAM to ACC, thus enabling the tissue to utilize endogenous methionine as C_2H_4 precursor (24). Thus, by comparing the characteristics of C_2H_4 production in auxin-dependent and ACC-dependent systems, it is possible to determine whether an inhibitor exerts its effect by interfering with a reaction prior to, or subsequent to, the formation of ACC.

DNP Effects. Figure 1 shows the effect of 0.1 mM DNP on the time course of C_2H_4 production by mung bean hypocotyls which were treated with IAA or ACC. At this concentration, DNP inhibits C_2H_4 production by about 80% in either IAA- or ACC-treated tissues. The effect of different concentrations of DNP on auxin- or ACC-stimulated C_2H_4 production is illustrated in Figure 2. Over a range of 10^{-6} to 10^{-3} M, inhibition by DNP increased progressively from 10 to 100%. The concentration of DNP required for 50% inhibition of C_2H_4 production in both IAA-dependent and ACC-dependent systems was estimated to be about 20 μM. Carbonylcyanide *m*-chlorophenylhydrazone, also a known uncoupler of oxidative phosphorylation, was found to be a more potent inhibitor than DNP. The concentration of carbonylcyanide *m*-chlorophenylhydrazone required for 50% inhibition of IAA- and ACC-dependent C_2H_4 production by mung bean hypocotyls was 5 μM (data not shown).

From the similarity of the DNP dose-inhibition curves for the IAA- and ACC-dependent C_2H_4 production systems, it is a reasonable assumption that DNP exerted its inhibitory function primarily at the step common to both systems, the conversion of ACC to C_2H_4. If the DNP inhibition is specific, there should be no, or little, interference with the conversion of methionine to ACC in the IAA-dependent system. The data of Table I, showing

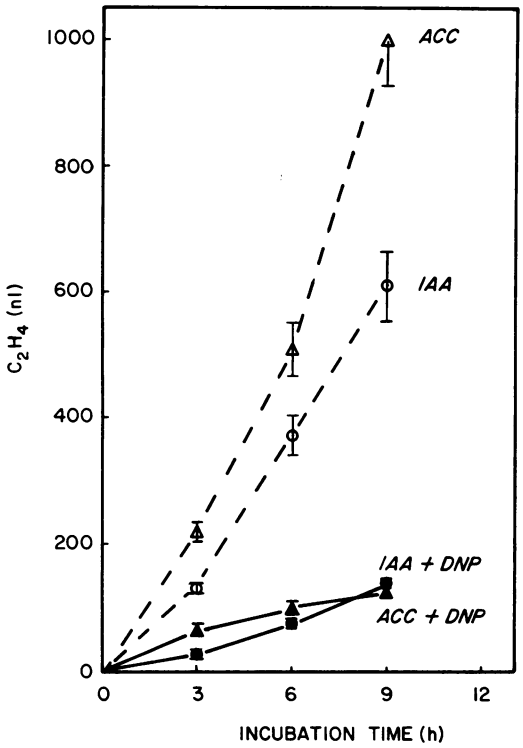

FIG. 1. Effect of DNP on the time course of IAA-dependent and ACC-dependent C_2H_4 production by mung bean hypocotyls. Twenty 2-cm long hypocotyls were incubated in 5 ml medium containing 50 μM IAA plus 20 μM IPA for the IAA-dependent system, or 0.2 mM ACC for the ACC-dependent system, in a 50-ml Erlenmeyer flask at 25 C. The concentration of DNP was 100 μM. Each treatment was performed in duplicate and the mean value ± variation is presented.

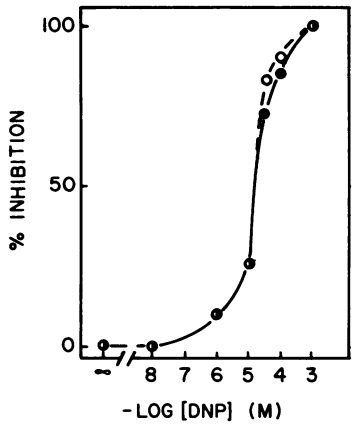

FIG. 2. Inhibition by DNP of IAA-dependent (●) and ACC-dependent (○) C_2H_4 production by mung bean hypocotyls during a 6-h incubation. Each point on the curves represents the per cent inhibition calculated as the mean of duplicate values. The incubation media and conditions were as described for Fig. 1.

the effect of DNP concentration on the incorporation of label from [^{14}C]methionine into ACC and C_2H_4 in IAA-treated mung bean hypocotyls, are generally supportive of the above assumption with respect to low concentrations of DNP. We have shown previously that, in the absence of IAA, there was little incorporation of label from methionine into ACC and C_2H_4, and there was little C_2H_4 production and little ACC accumulation (24). Depending upon the concentrations used, DNP exerted different effects on IAA-induced ACC and C_2H_4 synthesis by mung bean hypo-

Table I. *Influence of DNP on Conversion of Methionine to C_2H_4 and ACC by Mung Bean Hypocotyls*

Twenty 2-cm long hypocotyls (about 1.4 g) were incubated in 5 ml medium containing 50 μM IAA, 20 μM IPA, various concentrations of DNP, as indicated, and 1.5 μCi (30 nmol) L-[3-^{14}C]methionine. After 9 h incubation, hypocotyls were homogenized and the content of total and radioactive ACC were determined. The experiments were repeated three times and similar results and identical trends were obtained each time. The data shown below were from a single experiment.

DNP	Uptake of [^{14}C]Methionine	C_2H_4		ACC	
μM	%	nmol	nCi	nmol	nCi
0	37	90	30	88	35
10	39	73	26	140	49
50	21	39	10	145	46
100	11	28	6	84	18
500	7	4	0.2	11	0.5

Table II. *Effect of DNP on Content of ACC and SAM and on C_2H_4 Production in Auxin-treated Mung Bean Hypocotyls*

Twenty hypocotyl segments (about 1.4 g) were incubated in 5 ml medium containing 50 mM Mes buffer (pH 6.1), 2% sucrose, 50 μg/ml chloramphenicol, 50 μM IAA, 20 μM IPA and various concentrations of DNP. At the end of 9 h incubation, C_2H_4 content in the head space was determined and the hypocotyls were homogenized for assay of SAM and ACC. The data were from a single experiment.

DNP	SAM	ACC	C_2H_4
μM	nmol	nmol	nl
0	58	78	1664
10	51	124	1119
50	56	148	766

cotyls. At lower concentrations (10 and 50 μM), DNP inhibited C_2H_4 production and the conversion of labeled methionine to C_2H_4, whereas the amount of both total and labeled ACC increased. When the concentration of DNP was increased to 100 μM, C_2H_4 production was 70% inhibited but the amount of ACC remained about the same level as that of the controls. With 500 μM DNP, however, the conversion of labeled methionine to ACC and to C_2H_4 was sharply decreased. This may be due in part to the inhibition of methionine uptake by DNP. However, the level of endogenous ACC was also greatly decreased, suggesting that, at this high concentration of DNP, the synthesis of ACC was impaired. These data indicate that, at lower concentrations (10–50 μM), DNP acts by impairing the conversion of ACC to C_2H_4 but that, as the concentration is increased, DNP may also inhibit the conversion of methionine to ACC. The results with lower concentrations of DNP were somewhat unexpected because DNP, an uncoupler of oxidative phosphorylation, has been proposed to inhibit C_2H_4 production by depriving the system of ATP required for synthesis of SAM from methionine (7, 19).

It is evident that the primary effect of the lower concentrations of DNP on C_2H_4 production is inhibition of the conversion of ACC to C_2H_4. This was further substantiated by quantitative analysis of the metabolic intermediates in auxin-treated mung bean hypocotyls, as summarized in Table II. Low concentrations of DNP caused no change in the quantity of SAM but strongly increased ACC content and decreased C_2H_4 production. At a DNP concentration of 50 μM, it is apparent that accumulation of ACC and inhibition of C_2H_4 synthesis were nearly identical in extent, each varying from the control by a factor of approximately 2. These data indicate that the crossover point in the pathway caused by DNP inhibition is the conversion of ACC to C_2H_4.

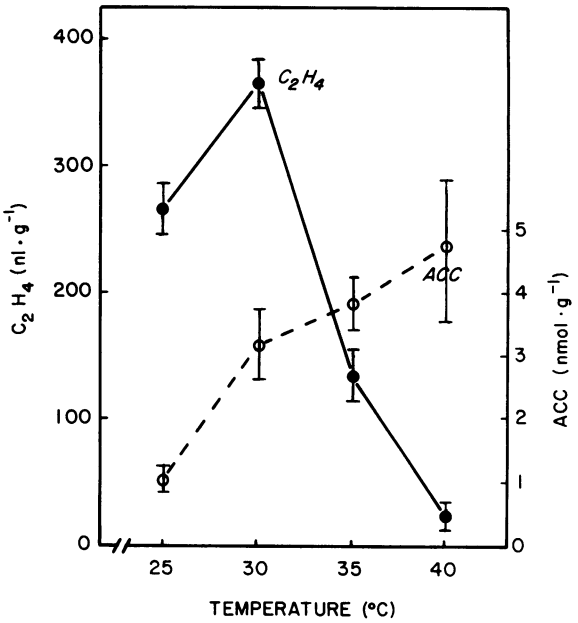

FIG. 3. Effect of temperature on C_2H_4 production and ACC content in apple plugs. C_2H_4 production was determined between 8 and 12 h incubation. The ACC content was measured at the end of 12 h incubation. Each treatment was performed in triplicate, and the mean value ± SD (bar) is presented.

High Temperature Treatment. C_2H_4 production and endogenous ACC content were determined in apple plugs and in mung bean hypocotyls incubated at temperatures ranging from 25 to 40 C (Figs. 3 and 4). The optimal temperature for C_2H_4 production by both tissues was 30 C. Temperatures higher than 30 C caused the rate of C_2H_4 production to decline and this inhibition became more pronounced during the later periods of incubation. Only a trace amount of C_2H_4 was produced from tissues incubated at 40 C. In apple tissue incubated 12 h, ACC content increased progressively as temperature was increased from 25 to 40 C, even though C_2H_4 production rates declined at temperatures above 30 C (Fig. 3). These data are consistent with the proposal that the conversion of ACC to C_2H_4 is the primary site of high temperature inactivation. This view was further supported by the observation that the effect of temperature on ACC-dependent and on auxin-dependent C_2H_4 production in mung bean hypocotyls was similar; C_2H_4 production in both systems was greatly impaired when the incubation temperature was raised to 40 C (Fig. 4). These observations indicate that the reaction converting ACC to C_2H_4, which is common to both systems, is vulnerable to high temperature inhibition. In auxin-treated mung bean hypocotyls, ACC content increased progressively as the temperature was raised from 25 to 35 C (Fig. 4, bottom), although the C_2H_4 production rates reached a maximum at 30 C (Fig. 4, top). At 40 C, both C_2H_4 production and ACC content decreased dramatically. This was in contrast to the results observed in apple tissues in which ACC content did not decrease at 40 C.

The higher rates of C_2H_4 production and higher levels of ACC at 30 C, as compared to those at 25 C, indicate that both ACC synthesis and degradation of ACC to C_2H_4 were increased as temperature was raised but that the increase in ACC synthesis exceeded the degradation of ACC to C_2H_4, resulting in a net increase of ACC. In contrast, when the temperature was increased from 30 to 35 C, C_2H_4 production decreased but the ACC content increased, indicating that the conversion of ACC to C_2H_4 is more sensitive to high temperature inactivation than is the synthesis of ACC.

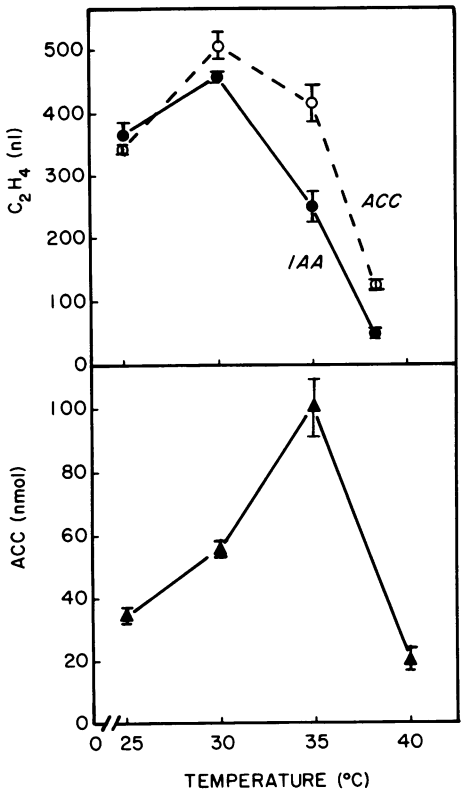

FIG. 4. Influence of temperature on C_2H_4 production and ACC content in mung bean hypocotyls. The incubation media and conditions were as described for Fig. 1. At the end of 5 h incubation at 25 C, C_2H_4 content of each flask was determined. C_2H_4 produced by the auxin-dependent and ACC-dependent systems was 253 and 343 nl, respectively. After flushing with fresh air, the flasks were resealed and transferred to various temperatures as indicated. At the end of 3 h incubation, C_2H_4 production and ACC content were assayed. Each treatment was performed in duplicate, and the mean value ± variation (bar) is presented.

DISCUSSION

DNP has long been known to inhibit C_2H_4 production in various plant tissues (7, 9, 15, 19, 20). Considering that DNP is a potent uncoupler of oxidative phosphorylation, Burg (7) and Murr and Yang (19) have speculated that SAM, formed from methionine and ATP, may be an intermediate in C_2H_4 biosynthesis and that DNP may inhibit C_2H_4 production by interfering with the conversion of methionine to SAM. Although the speculation that SAM might be an intermediate has been established (3, 4), the theoretical basis upon which the speculation was made has proven to be incorrect. The present results clearly show that DNP interferes with C_2H_4 production, not by inhibiting the conversion of methionine to SAM but by inhibiting the conversion of ACC to C_2H_4 in both auxin-dependent and ACC-dependent systems in mung bean hypocotyls (Tables I and II). This conclusion was further supported by results obtained with apple tissue, in which DNP caused inhibition of C_2H_4 production but not of the conversion of methionine to SAM (2). If the mode of action of DNP is associated with the supply of ATP, it may be that ATP is required for the conversion of ACC to C_2H_4, either serving as substrate or as effector. Although the enzymic formation of C_2H_4 from ACC has been recently reported by Konze and Kende (14), many of the reported characteristics of the enzyme did not match those obtained from the *in vivo* data. Considering the proposed reaction sequence for the conversion of ACC to C_2H_4 (21), it is difficult to rationalize the requirement for ATP as substrate. Since the synthesis of C_2H_4 from ACC was more vulnerable to DNP inhibition than was the synthesis of SAM from methionine, one may further speculate that, when ATP supply is impaired by DNP, the former reaction will be inhibited first. At higher DNP concentrations, however, both reactions will be blocked.

In addition to its role as an uncoupler of oxidative phosphorylation, resulting in the depression of ATP formation, DNP has also been suggested to influence the integrity of biological membranes (16). Work by Imaseki (13), Anderson *et al.* (5), and others (16) has suggested that some part of the C_2H_4-forming system is associated with membranes. ACC synthase has been isolated and it appears to be localized in the cytosol (6, 22). If any part of the C_2H_4-forming system is membrane-associated, it is very likely to be the enzyme involved in the conversion of ACC to C_2H_4. If so, one may further speculate that the inhibition of C_2H_4 production by DNP may be due mainly to disruption of the membrane integrity essential for the conversion of ACC to C_2H_4.

The failure of fruit to ripen normally at temperatures above 30 C is well known, and this has been attributed to the reduction or inhibition of C_2H_4 production at these temperatures. Our results show that the primary site of high temperature inhibition of C_2H_4 production is the step in which ACC is converted to C_2H_4. The mechanism by which the high temperature inhibition of C_2H_4 production occurs is unknown.

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