Plant Physiol. (1976) 57, 88-92

Stimulation of Ethylene Production in the Mung Bean Hypocotyls by Cupric Ion, Calcium Ion, and Kinetin[1]

Received for publication June 24, 1975 and in revised form September 18, 1975

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ABSTRACT

The synergistic stimulation of ethylene production by kinetin and Ca^{2+} in hypocotyl segments of mung bean (*Phaseolus aureus* Roxb.) seedling was further studied. The requirement for Ca^{2+} in this system was specific. Except for Sr^{2+}, which mimicked the effect of Ca^{2+}, none of the following divalent cations, including Ba^{2+}, Mg^{6+}, Cu^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Sn^{2+}, and Zn^{2+}, showed synergism with kinetin on ethylene production. Fe^{2+}, however, showed a slight synergism with kinetin. Some of them (Hg^{2+}, Co^{2+}, and Ni^{2+}) had a strong inhibitory effect, while others (Zn^{2+}, Mg^{2+}, Sn^{2+}, and Ba^{2+}) had a slight or no inhibitory effect on ethylene production in the absence or presence of kinetin.

Cu^{2+} alone, depending on the concentration applied, stimulated ethylene production with a lag period of about 2 hours and had no synergism with kinetin on ethylene production. When Cu^{2+} was applied with Ca^{2+}, a remarkable synergistic stimulation of ethylene production was observed. Tracer experiments indicated that Cu^{2+} enhanced the uptake of $^{45}Ca^{2+}$ into the tissues during the first few hours of incubation, and this increase of $^{45}Ca^{2+}$ uptake paralleled the enhancement of ethylene production. When Ca^{2+} was applied together with kinetin plus Cu^{2+}, both the ethylene production and the $^{45}Ca^{2+}$ uptake were greatly increased over those from the segments treated with Cu^{2+} or kinetin alone. The increase in ethylene production as a result of kinetin plus Ca^{2+} plus Cu^{2+} treatment is equal to the combined increases caused by kinetin plus Ca^{2+} and Cu^{2+} plus Ca^{2+}. A possible mechanism accounting for such cooperative effects of Cu^{2+}, Ca^{2+}, and kinetin on ethylene production is discussed.

Ethylene production *in vivo* is induced during certain stages of growth, such as germination, ripening of fruits and abscission, by wounding, disease, radiation, and other physical and chemical stresses, and by treatment with IAA and other plant growth regulators (1). Recently, we reported another ethylene-producing system in which ethylene production by mung bean hypocotyl segments was synergistically stimulated by Ca^{2+} and kinetin (14). We have found that kinetin greatly enhances the uptake of Ca^{2+} with a lag period of 6 hr which corresponds to the time required for induction of ethylene production. We suggested that the synergism between kinetin and Ca^{2+} on ethylene production is due to the enhanced intake of Ca^{2+} by kinetin into a specific site for ethylene production (15).

We have therefore examined the specificity of Ca^{2+} by substituting various divalent cations for Ca^{2+} in this system. The present paper shows that the requirement for Ca^{2+} is quite specific. Furthermore, we show that a synergistic increase in ethylene production occurred when Ca^{2+} and Cu^{2+} were applied together. Cu^{2+} has been known to induce "stress" ethylene in various tissues (2, 3, 7).

MATERIALS AND METHODS

Seeds of mung bean (*Phaseolus aureus* Roxb.) were grown in vermiculite for 3.5 days in darkness at 24 C. Segments 2 cm long were cut from hypocotyls at a point 1 cm below the hook, as previously described (14). Lots of 20 segments were incubated in 5 ml of a medium consisting of 50 mM potassium phosphate buffer, pH 6, 2% sucrose, various concentrations of different divalent cations, kinetin, or labeled $^{45}Ca^{2+}$ (100 μCi, 50 μmoles) as indicated, in a 50-ml Erlenmeyer flask. A plastic center well containing 0.2 ml of 40% KOH was hung in the flask to absorb CO_2 evolved. The flasks were sealed with rubber serum caps and incubated in a shaker at 27 C in darkness.

At time intervals indicated, 1-ml gas samples were withdrawn by hypodermic syringe, and ethylene was assayed with a gas chromatograph equipped with an alumina column and a flame ionization detector. The flasks were flushed with air and recapped for the next ethylene determination.

For $^{45}Ca^{2+}$ uptake studies, the hypocotyls, incubated for a given time interval, were washed with 10 changes of distilled H_2O, and then ground with a glass homogenizer in 9 ml of 80% ethyl alcohol. The debris was pelleted by centrifugation, and the supernatant was collected. The pellet was serially extracted three times with 5 ml of 20 mM HCl. The radioactivity in each extract and in the debris was determined with a liquid scintillation counter.

RESULTS

We have shown that Ca^{2+} and kinetin synergistically stimulate ethylene production by the mung bean hypocotyls (14). To determine the specificity of the Ca^{2+} requirement in this system, we tested the ability of various divalent cations, Sr^{2+}, Cu^{2+}, Fe^{2+}, Ba^{2+}, Zn^{2+}, Sn^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, and Hg^{2+}, in place of Ca^{2+}, to stimulate ethylene production. It is evident from Table I that except for Sr^{2+}, none of the divalent cations tested could substitute for Ca^{2+} to show a synergistic relationship with kinetin on ethylene production. As shown in Figure 1, the pattern of ethylene production in the Sr^{2+} plus kinetin system was identical to the Ca^{2+} plus kinetin system, indicating that Sr^{2+} can substitute for Ca^{2+} in the present system. The magnitude of ethylene production in response to Sr^{2+} was dependent on Sr^{2+} concentration (Fig. 2), as was the case in Ca^{2+} (14). Some divalent cations such as Ni^{2+}, Co^{2+}, and Hg^{2+} caused a strong inhibition, while others, such as Zn^{2+}, Mg^{2+}, Sn^{2+}, and Ba^{2+}, caused little or no inhibition of ethylene production either in the absence or in the presence of kinetin (Table I). An additive or slight synergistic relationship between Fe^{2+} and kinetin was observed when they were applied together (Table I). Ethylene production induced by Fe^{2+} in the presence or absence of kinetin was characterized by a lag period of about 1 hr, while the lag period of the production induced by Ca^{2+} (14) or Sr^{2+} (Fig. 1) in the presence of kinetin was about 5 hr.

Ethylene production in plants often increases following

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[1] This work was supported by National Science Foundation Grant BMS75-14444.

Table I. *Comparative Effect of Various Divalent Cations on Ethylene Production in Absence or Presence of Kinetin*

The concentration of divalent cations employed was 10 mM in chloride salts except for Fe^{2+} which was in sulfate salt.

Divalent Cation	C_2H_4 produced	
	−Kinetin	+0.1 mM Kinetin
	nl/12 hr	
None	3.5	38
Ca	15	141
Sr	12	117
Cu	93	102
Fe	19	78
Ba	1.8	23
Zn	2.9	23
Sn	2.8	12
Mg	1.9	22
Ni	1.0	4.3
Co	1.1	4.6
Hg	1.1	0.6

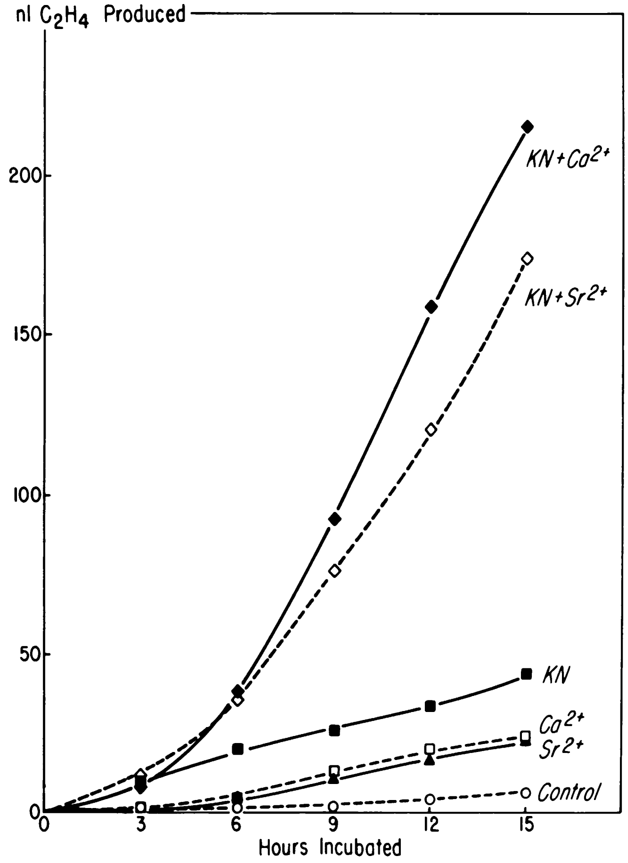

FIG. 1. Similarity between 10 mM Sr^{2+} and 10 mM Ca^{2+} to induce ethylene production in the absence or in the presence of 0.1 mM of kinetin (KN).

wounding or stress from a variety of sources (1). Several workers (2, 3, 7) have reported "stress" ethylene production by the application of Cu^{2+}. Figure 3 shows that the lag period of ethylene production induced by 10 mM Cu^{2+} was about 2 hr, which was quite different from the other ethylene-producing systems induced by IAA (about 1 hr) or by kinetin plus Ca^{2+} (about 5 hr). When 10 mM Cu^{2+} was applied along with 10 mM Ca^{2+}, a striking synergistic stimulation of ethylene production was observed (Fig. 3). Cu^{2+} at 1 mM had no synergistic effect with Ca^{2+} on ethylene production. When 10 mM Cu^{2+} was applied with kinetin and Ca^{2+}, the resulting ethylene production was equal to the sum of that in the presence of Ca^{2+} plus kinetin and that in the presence of Cu^{2+} plus Ca^{2+} (Fig. 3). This is

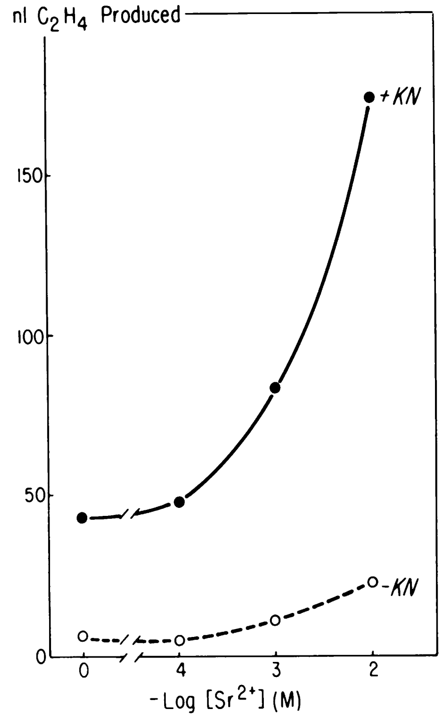

FIG. 2. Effect of various $SrCl_2$ concentrations on ethylene production in the absence or presence of 0.1 mM kinetin (KN) for 15 hr.

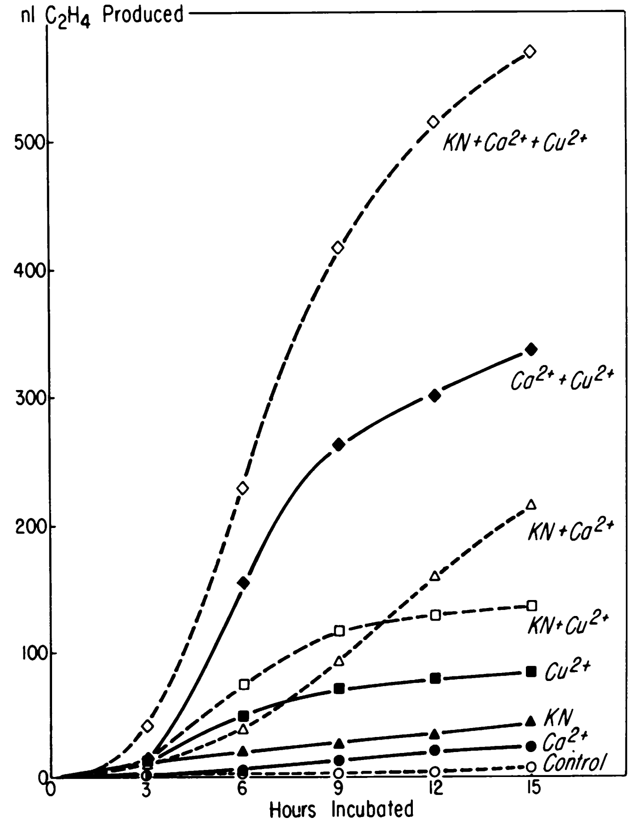

FIG. 3. Time courses of ethylene production from mung bean hypocotyls treated with 10 mM Ca^{2+}, 10 mM Cu^{2+}, 0.1 mM kinetin (KN), or their combinations as indicated.

expected because there existed a synergism between kinetin and Ca^{2+}, and between Ca^{2+} and Cu^{2+}, but not between kinetin and Cu^{2+}, on ethylene production.

The effect of Cu^{2+} concentration on ethylene production by mung bean segments incubated with Ca^{2+}, kinetin, or Ca^{2+} plus kinetin is shown in Figure 4. It is apparent that Cu^{2+} exerted little effect at concentrations lower than 1 mM.

It should be mentioned that accompanying the surge of ethylene production, serious tissue damage, with a complete loss of turgidity and skrinkage of tissue, was observed at 10 mM Cu^{2+}, but much less or no visible damage was observed at lower Cu^{2+} concentrations. Segments treated with both 10 mM Ca^{2+} and 10 mM Cu^{2+}, in the absence or presence of kinetin, showed much less tissue injury than those treated only with 10 mM Cu^{2+}, either in the absence or presence of kinetin, indicating that Ca^{2+} played a role in protecting the tissues from Cu^{2+} injury.

During the course of the study of the synergistic stimulation of ethylene by kinetin and Ca^{2+}, we found that kinetin greatly enhanced the uptake of Ca^{2+} into the tissue, with a lag period of about 5 hr, which corresponds to the time required for induction of ethylene production (15). Our results are compatible with the view that kinetin plays a role by releasing and transporting Ca^{2+} from cell wall to an intracellular site where Ca^{2+} is required for ethylene biosynthesis. Such an argument was advanced, based on the observation by LeJohn *et al.* (16, 17) that cytokinins play a role in releasing Ca^{2+} from cell wall and enhancing Ca^{2+} intake into the cell in a fungal system. It is therefore pertinent to ask whether the synergistic stimulation of ethylene production by Ca^{2+} and Cu^{2+} may be due to the enhanced uptake of Ca^{2+} by Cu^{2+}. To examine this possibility, we studied the effect of Cu^{2+} on the uptake of $^{45}Ca^{2+}$, using the same technique as we previously used for the kinetin plus Ca^{2+} system (15). As shown in Figure 5A, Cu^{2+} enhanced the uptake of $^{45}Ca^{2+}$ as early as the

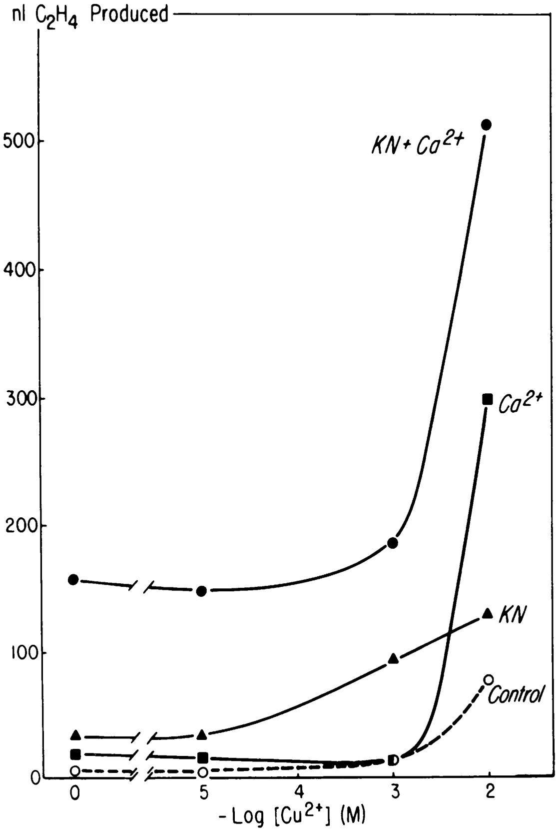

FIG. 4. Effect of various Cu^{2+} concentrations on ethylene production from control hypocotyls or hypocotyls treated with 0.1 mM kinetin (KN), 10 mM Ca^{2+}, or 0.1 mM KN plus 10 mM Ca^{2+} for 12 hr.

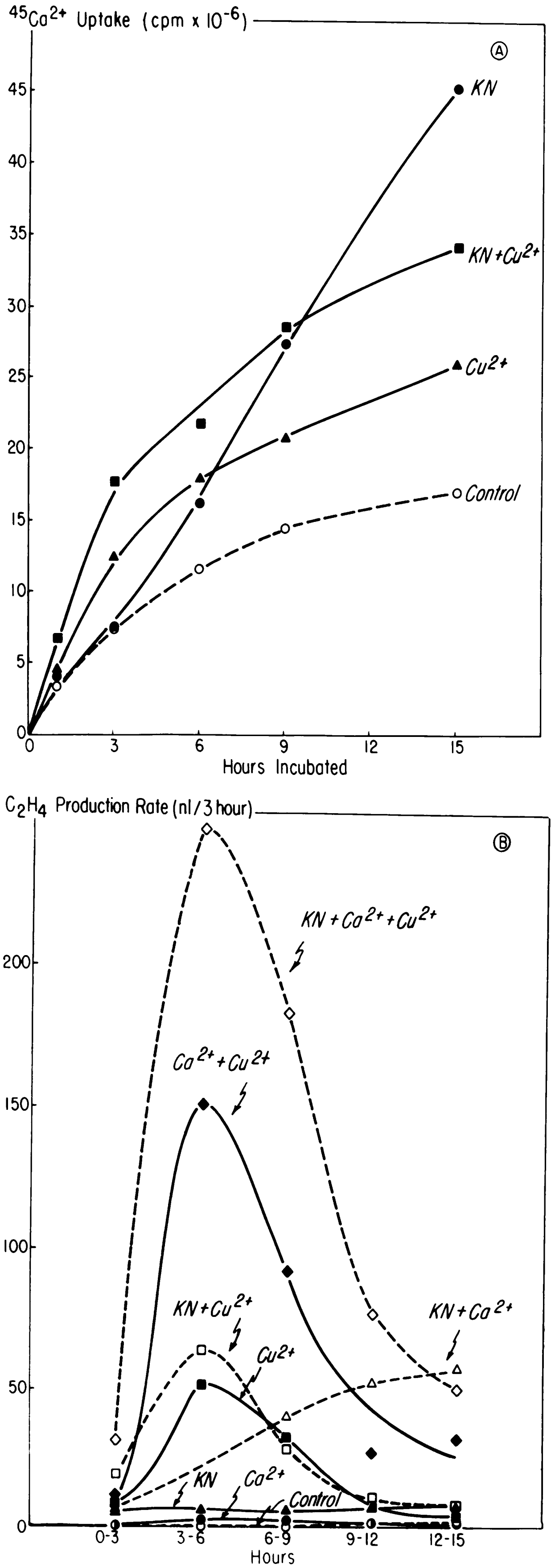

FIG. 5. Comparison of the uptake of $^{45}Ca^{2+}$ (A) and the rate of ethylene production (B) in the presence of 0.1 mM kinetin (KN), 10 mM Cu^{2+}, 10 mM $^{45}Ca^{2+}$, or their combinations.

1st hr, and throughout the entire period of incubation. Kinetin and Cu^{2+} enhanced $^{45}Ca^{2+}$ uptake (Fig. 5A) and caused a synergistic stimulation of ethylene production with Ca^{2+} (Fig. 5B).

During the early part (about 6 hr) of the incubation period the treatments, in the order of their increasing effectiveness in promoting $^{45}Ca^{2+}$ uptake, were: control, kinetin, Cu^{2+}, and kinetin plus Cu^{2+}. The order of effectiveness of these treatments on the rate of ethylene production was identical to that for $^{45}Ca^{2+}$ uptake. Later in the incubation period kinetin became the most effective promoter of $^{45}Ca^{2+}$ uptake and, again, the order of effectiveness of the treatments was the same for rate of ethylene production as for $^{45}Ca^{2+}$ uptake. Thus there is a correlation between the increase in $^{45}Ca^{2+}$ uptake and the increase in ethylene production. If ethylene production is directly dependent on Ca^{2+} available at the synthetic site, it may be assumed that the Ca^{2+} taken up in the absence of Cu^{2+} and kinetin was largely bound to cell walls, and thus unavailable for ethylene biosynthesis, while kinetin, Cu^{2+}, or Cu^{2+} plus kinetin would enhance the release and transport of Ca^{2+} to the site where ethylene is synthesized. The decline of the ethylene production rate during the later part of incubation in the presence of Cu^{2+} (Fig. 5B) was apparently due to the result of tissue injury caused by Cu^{2+}.

DISCUSSION

Except for Sr^{2+} and Fe^{2+}, none of the divalent cations tested exhibited synergism with kinetin on ethylene production (Table I). The synergistic interaction between Fe^{2+} and kinetin is, however, very slight. There was a similarity between Ca^{2+} and Sr^{2+} to interact synergistically with kinetin on ethylene production, as shown by their pattern of ethylene production (Figs. 1 and 2) as well as the dose-response curve (14). This is understandable because both Ca^{2+} and Sr^{2+} belong to the alkaline earth metal group and have similar electron configurations (8). It has been reported that growth is supported in several species of algae (22, 27) and of higher plants (24, 28) when Sr^{2+} is substituted for Ca^{2+} in the nutrient media, although complete replacement for Ca^{2+} by Sr^{2+} is not observed (24). Skeletal muscle phosphorylase kinase is stimulated by Ca^{2+} as well as by Sr^{2+} (5).

Iron ion, in the form of FeEDTA (7) or $FeCl_3$ (3), has been found to stimulate ethylene production. We also observed the same phenomenon with $FeSO_4$ in hypocotyl segments (Table I). Cupric ion, at relatively high concentrations (higher than 1 mM), stimulated ethylene production in mung bean hypocotyls (Figs. 3 and 4). Similar results were obtained from bean leaves (2), Calamondin fruit (7), and Valencia orange (3). In the present system, mung bean hypocotyls were incubated with $CuCl_2$ containing 50 mM potassium phosphate buffer at pH 6. Massive ethylene production was observed after a lag period of about 2 hr (Figs. 3 and 5). The lag period was 50 min in bean leaves to which $CuSO_4$ was applied as a spray (2). The difference in duration of the lag period is possibly due to the presence of other ions (26) and/or due to different tissues.

The basic cellular reactions leading to stimulated ethylene production by Cu and Fe ions are unknown (3). Lieberman *et al.* (18) have shown that Cu^{2+} or Fe^{2+} catalyzes the conversion of methionine to ethylene chemically in the presence of H_2O_2 and ascorbic acid. It has also been shown that ethylene produced endogenously, or induced by auxins, stress, or toxic compounds in vegetative tissues, was derived from methionine (2). Copper is an essential micronutrient for algae and higher plants and is an essential constituent of a number of plant enzymes (21). Copper at concentrations higher than 1 μM is increasingly toxic to algal and higher plant tissues (10, 23). For instance, cupric sulfate has been extensively used as an algacide since the beginning of the century (20). The cupric ion has been shown to be an inhibitor of photosynthesis in algal cells (10, 19, 26) and to inhibit photosynthetic electron transport in isolated chloroplasts (6, 12). The basis for Cu toxicity in plants is largely unknown. Copper ion has been suggested to catalyze the oxidation of sulfhydryl groups to form disulfide bridges (11) or to alter membrane integrity, which could be the focal point of Cu action (10).

In addition to a surge of ethylene production (Figs. 3–5), exposure of hypocotyls to high Cu^{2+} concentrations also caused severe visible tissue damage, as shown by the complete loss of turgidity. This suggests that Cu^{2+} may act on cell membranes (10), resulting in "stress" ethylene production. The degree of visible cellular damage seems to be interrelated with the amount of ethylene production because Cu^{2+}, at concentrations lower than 1 mM, neither stimulated massive ethylene production (Fig. 4) nor caused severe visible damage to the hypocotyls. From our time-course studies, the Cu^{2+}-treated tissues stopped producing ethylene after 12 hr of incubation (Fig. 5B) and showed severe injury. It has been noted that "stress" ethylene is a product of living tissue, since it ceases when damage is severe enough to kill (2).

Ca^{2+} is relatively immobile in cells (4), possibly because it has a high affinity for cell wall materials (25). During the course of $^{45}Ca^{2+}$ uptake studies, we found that $^{45}Ca^{2+}$ was readily extracted from the tissue homogenate by HCl (10–20 mM), but not by ethyl alcohol or H_2O. It appears that Ca^{2+} is bound and hardly available for cellular metabolism. It has been shown in animal (5) and in fungal cells (16, 17) that the presence of an appropriate agent may cause the release of Ca^{2+} from a bound form to a metabolically available free form.

We have shown that kinetin enhanced the uptake of $^{45}Ca^{2+}$, and that the increase in uptake was paralleled by an increase in ethylene production (15). We have further compared kinetin-enhanced ethylene production by mung bean seedlings which had been germinated in the presence of different levels of Ca^{2+}, and found greater ethylene production by high Ca^{2+} seedlings than by low Ca^{2+} seedlings (unpublished results). These facts suggested that Ca^{2+} at elevated concentrations could regulate ethylene production via the kinetin plus Ca^{2+} system. Data presented here support this argument, in that increased $^{45}Ca^{2+}$ uptake caused by Cu^{2+} was correlated to increased ethylene production (Fig. 5).

It is evident from Figure 5 that both kinetin and Cu^{2+} enhanced $^{45}Ca^{2+}$ uptake and caused a synergistic stimulation of ethylene production with Ca^{2+}. It should be noted that kinetin and Cu^{2+} treatments in the presence of Ca^{2+} showed quite different kinetics both for ethylene production and for $^{45}Ca^{2+}$ uptake. Kinetin required a much longer lag period than Cu^{2+} (Fig. 5). The results indicate that the mode of action of kinetin and Cu^{2+} on $^{45}Ca^{2+}$ intake may be different. Our interpretation is that kinetin acts by releasing bound Ca^{2+} from the cell wall and by facilitating its transport to the site of ethylene production. Cu^{2+}, on the other hand, may act by disrupting the permeation barrier which normally restricts entry of Ca^{2+} into the site where ethylene is synthesized.

The severe visible damage of hypocotyl tissues caused by excessive concentrations (10 mM) of Cu^{2+} was alleviated by the addition of Ca^{2+}. The mechanism by which Ca^{2+} protects tissues against Cu^{2+}-induced injury is not known. However, it has been well documentated that Ca^{2+} can counteract the adverse effect of increasing hydrogen concentration and can protect against NaCl and heavy metal toxicity (9, 13).

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