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# Inhibition of Ethylene Production by Cobaltous Ion[1]

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## ABSTRACT

**The effect of $Co^{2+}$ on ethylene production by mung bean (*Phaseolus aureus* Roxb.) and by apple tissues was studied. $Co^{2+}$, depending on concentrations applied, effectively inhibited ethylene production by both tissues. It also strongly inhibited the ethylene production induced by IAA, kinetin, IAA plus kinetin, $Ca^{2+}$, kinetin plus $Ca^{2+}$, or $Cu^{2+}$ treatments in mung bean hypocotyl segments. While $Co^{2+}$ greatly inhibited ethylene production, it had little effect on the respiration of apple tissue, indicating that $Co^{2+}$ does not exert its inhibitory effect as a general metabolic inhibitor. $Ni^{2+}$, which belongs to the same group as $Co^{2+}$ in the periodic table, also markedly curtailed both the basal and the induced ethylene production by apple and mung bean hypocotyl tissues.**

**In a system in which kinetin and $Ca^{2+}$ were applied together, kinetin greatly enhanced $Ca^{2+}$ uptake, thus enhancing ethylene production. $Co^{2+}$, however, slightly inhibited the uptake of $Ca^{2+}$ but appreciably inhibited ethylene production, either in the presence or in the absence of kinetin. Tracer experiments using apple tissue indicated that $Co^{2+}$ strongly inhibited the *in vivo* conversion of L-[U-$^{14}$C]methionine to $^{14}$C-ethylene. These data suggest that $Co^{2+}$ inhibited ethylene production by inhibiting the conversion of methionine to ethylene, a common step which is required for ethylene formation by higher plants.**

**$Co^{2+}$ is known to promote elongation, leaf expansion, and hook opening in excised plant parts in response to applied auxins or cytokinins. Since ethylene is known to inhibit these growth phenomena, it is suggested that $Co^{2+}$ exerts its promotive effect, at least in part, by inhibiting ethylene formation.**

We have previously reported that $Ca^{2+}$ or $Sr^{2+}$ synergistically stimulated ethylene production in the presence of kinetin, whereas $Co^{2+}$ or $Ni^{2+}$, in contrast, greatly inhibited ethylene production in the presence or in the absence of kinetin (16, 18). As ethylene is endogenously produced by ripening fruit tissues (1, 3, 4, 5) and as its production in vegetative tissues can be regulated by the application of auxin (1, 7, 11, 14, 19, 20), kinetin (1, 15, 16, 19), auxin plus kinetin (1, 15, 19), $Ca^{2+}$ (16, 17), kinetin plus $Ca^{2+}$ (16, 17), or $Cu^{2+}$ (1, 2, 18), it is important to determine whether $Co^{2+}$ also inhibits these ethylene-producing systems. Evidence from tracer studies has established methionine as the *in vivo* precursor of ethylene in fruit and vegetative tissues (1, 3, 4, 22, 24, 29). This paper describes the inhibitory effect of $Co^{2+}$ on ethylene production by treated mung bean hypocotyl and by apple tissues, and on the conversion of L-[U-$^{14}$C]methionine to $^{14}$C-ethylene in apple tissue.

## MATERIALS AND METHODS

**Mung Bean Experiments.** Seedlings 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 (16). 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 $Co^{2+}$, $Ni^{2+}$, $Ca^{2+}$, $Cu^{2+}$, IAA, or kinetin 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.

For uptake studies, the hypocotyls were incubated with $^{45}Ca^{2+}$ (100 $\mu$Ci, 50 $\mu$moles). At the end of incubation the tissues were washed with 10 changes of distilled $H_2O$, and then ground with a glass homogenizer in 9 ml of 80% ethanol. The debris was pelleted by centrifugation and the supernatant was collected. The pellet was 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.

**Apple Experiments.** Apples used were Golden Delicious, purchased from a local market. Cylindrical plugs of apple tissue (1 cm in diameter and 2 cm in length) were cut with a cork borer and razor blade. One hundred $\mu$l of KCl (2%), with or without various concentrations of $CoCl_2$ or $NiCl_2$, were introduced into the plug by a vacuum injection technique (3). The concentration of Co or Ni ions cited in Tables I and II and Figure 4 represents the calculated concentration of the cations within the apple plugs, assuming that they are evenly distributed within the tissues. The plugs were sealed in 25 ml-Erlenmeyer flasks and incubated for a given period of time as indicated at 25 C under laboratory illumination. For tracer studies on the conversion of methionine to ethylene, 100 $\mu$l of 2% KCl containing 1 $\mu$Ci L-[U-$^{14}$C]methionine (100 $\mu$Ci/$\mu$mole) with or without $Co^{2+}$ were similarly fed to each apple plug. Each plug was sealed in a 12-ml syringe and incubated for 2-hr intervals at 25 C.

**Gas Analysis.** At time intervals indicated, 1-ml gas samples were withdrawn by hypodermic syringe from the reaction flask or reaction syringe and ethylene was assayed with a gas chromatograph equipped with an alumina column and a flame ionization detector. The total and radioactive $CO_2$ and radioactive ethylene evolved from apple plugs which were fed with radioactive methionine were assayed by a gas chromatograph equipped with a thermal conductivity detector and connected to a proportional counter. After each determination the reaction syringes or flasks were flushed with air and recapped for the next ethylene determination.

## RESULTS

**Inhibitory Effect of Cobalt on Ethylene Production in Mung Bean Hypocotyls.** $Co^{2+}$ strongly inhibits basal ethylene production as well as that induced by the application of $Ca^{2+}$, kinetin, $Ca^{2+}$ plus kinetin, $Cu^{2+}$, IAA, or IAA plus kinetin (Fig. 1). The inhibition by $Co^{2+}$ was more pronounced during the later periods of incubation. Its effectiveness as an inhibitor of ethylene production is concentration-dependent. $Co^{2+}$ at concentrations above 10 $\mu$M exerted a significant inhibition on most ethylene-producing systems, suggesting that $Co^{2+}$ is a potent inhibitor of ethylene production.

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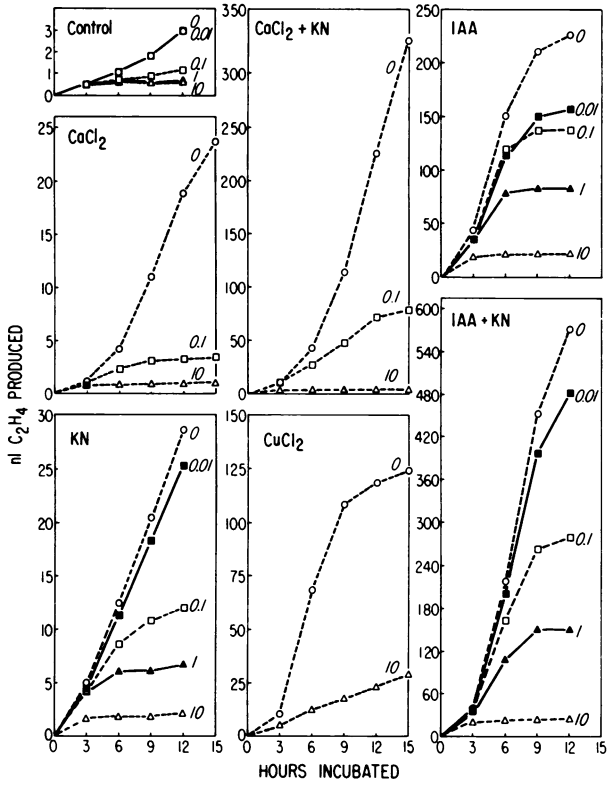


FIG. 1. Effect of various $Co^{2+}$ concentrations on the time course of basal ethylene production, and of ethylene production stimulated by the application of 10 mM $Ca^{2+}$, 0.1 mM kinetin (KN), 10 mM $Ca^{2+}$ plus 0.1 mM kinetin (KN), 10 mM $Cu^{2+}$, 30 $\mu$M IAA, or 30 $\mu$M IAA plus 0.1 mM kinetin (KN) in mung bean hypocotyl segments. The numbers on each curve represent the concentration of $CoCl_2$ employed in mM.

Fe, Co, and Ni belong to the Group VIII of the periodic table and the latter two have many features in common. $Fe^{2+}$, unlike $Co^{2+}$, had been shown previously to stimulate ethylene production (1, 18). $Ni^{2+}$, however, was found to inhibit ethylene production by mung bean hypocotyl tissues nearly as effectively as $Co^{2+}$ (Fig. 2).

We have recently shown a synergistic stimulation of ethylene production in mung bean hypocotyl segments treated with kinetin plus $Ca^{2+}$ (16, 17) or with $Cu^{2+}$ plus $Ca^{2+}$ (18). Since the synergism was closely related to enhanced uptake of $Ca^{2+}$ by kinetin (17) or by $Cu^{2+}$ (18), it was suggested that enhanced $Ca^{2+}$ uptake was responsible for the synergistic stimulation of ethylene production. Similarly, the inhibition of ethylene production by $Co^{2+}$ in the above $Ca^{2+}$-mediated systems (Fig. 1) may be explained on the basis that $Co^{2+}$ inhibits $Ca^{2+}$ uptake, thereby reducing the ethylene production rate. To test this possibility, $^{45}Ca^{2+}$ uptake in the presence of $Co^{2+}$ was assayed. $Co^{2+}$ (100 $\mu$M) inhibited $^{45}Ca^{2+}$ uptake only very slightly in either the presence or absence of kinetin (Fig. 3), but strongly inhibited ethylene production (Fig. 1). These results suggest that the inhibitory effect of $Co^{2+}$ on ethylene production cannot be explained in terms of the inhibition of $Ca^{2+}$ uptake by $Co^{2+}$. Some other mechanism(s) must be involved.

**Inhibitory Effect of Cobalt on Ethylene Production and on Conversion of Methionine to Ethylene in Apple Tissue.** Tissue of postclimacteric apple fruits, known to produce large amounts of ethylene, was used to determine whether $Co^{2+}$ and $Ni^{2+}$ inhibit ethylene production, as in the vegetative hypocotyl tissue. It is evident from Figure 4 that $Co^{2+}$, depending on concentration applied, effectively inhibited the rate of ethylene production. $Ni^{2+}$, which is sowewhat less effective than $Co^{2+}$, also strongly inhibited ethylene production in apple tissue (Table I), as in mung bean hypocotyl tissues (Fig. 2).

Tracer and inhibitor studies have indicated that methionine is the probable precursor of ethylene in fruit tissues, in auxin-treated vegetative tissues, and in leaf tissues subjected to injury by toxic compounds (1, 2, 3, 4, 20, 24, 29). In an effort to identify the biochemical step at which $Co^{2+}$ exerts its inhibitory effect on ethylene production, L-[U-$^{14}$C]methionine was introduced into apple tissue, and the total and radioactive ethylene and $CO_2$ evolved were assayed at 2-hr intervals. Table II shows that $Co^{2+}$ did not inhibit the respiration of apple tissue, as measured by the $CO_2$ output, but did effectively inhibit ethylene production and greatly inhibited the conversion of L-[U-$^{14}$C]methionine to $^{14}$C-ethylene and $^{14}CO_2$.

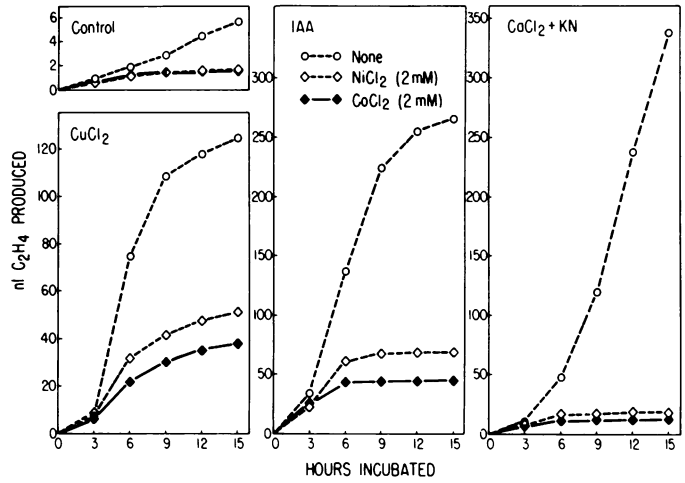


FIG. 2. Comparative effect of 2 mM $Ni^{2+}$ and 2 mM $Co^{2+}$ on inhibition of basal ethylene production (control) or on ethylene production stimulated by the application of 10 mM $CuCl_2$, 30 $\mu$M IAA, or 10 mM $CaCl_2$ plus 0.1 mM kinetin (KN) in mung bean hypocotyl segments.

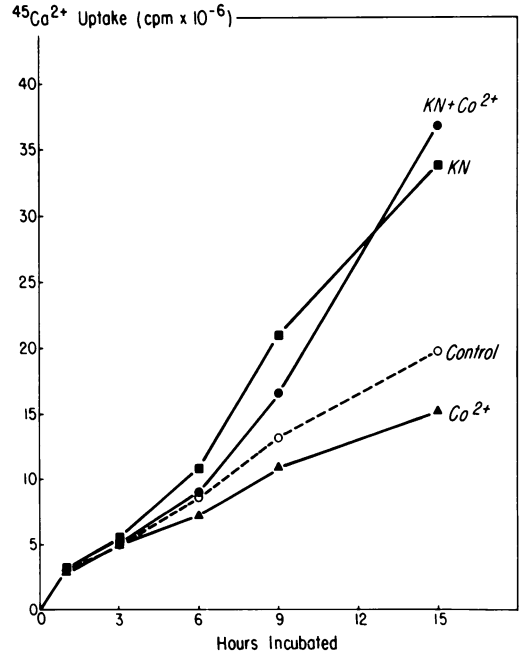


FIG. 3. Effect of 0.1 mM $Co^{2+}$ on $^{45}Ca^{2+}$ uptake in the presence or the absence of kinetin (KN) in mung bean hypocotyl segments. All flasks contained 100 $\mu$Ci and 50 $\mu$moles of $^{45}CaCl_2$ in 5 ml of incubation buffer. The additions were, where indicated, 0.1 mM kinetin (KN), 0.1 mM $CoCl_2$, or 0.1 mM kinetin (KN) plus 0.1 mM $CoCl_2$.

Table I. *Comparative Effect of $Co^{2+}$ and $Ni^{2+}$ on Inhibition of Ethylene Production by Apple Tissue*

One hundred μl of 2% KCl solution containing either $NiCl_2$ (1 or 10 μmoles) or $CoCl_2$ (1 or 10 μmoles) were fed under vacuum to each plug of apple tissue (1 × 2 cm). Ethylene produced was measured at 2-hr intervals. One or 10 μmoles of the cation should provide an internal concentration of approximately 0.7 or 7 mM, respectively, assuming the cation is evenly distributed in the tissue.

| Treatment | $C_2H_4$ Produced/Plug | | |
|---|---|---|---|
| | 0-2 hr | 2-4 hr | 4-6 hr |
| | | *nl* | |
| Control | 104 | 107 | 110 |
| $NiCl_2$ | | | |
| 0.7 mM | 86 | 85 | 79 |
| 7 mM | 64 | 43 | 30 |
| $CoCl_2$ | | | |
| 0.7mM | 86 | 78 | 58 |
| 7 mM | 65 | 36 | 18 |

Table II. *Effect of $Co^{2+}$ on Ethylene and $CO_2$ Production, and on Conversion of L-[U-$^{14}$C]methionine to $^{14}$C-ethylene and $^{14}$C-$CO_2$ by Apple Tissue*

One hundred μl of 2% KCl solution containing L-[U-$^{14}$C]methionine (1 μCi, 10 nmoles), with (1 or 10 μmoles) or without $CoCl_2$, were fed under vacuum to each apple plug (1 × 2 cm). One or 10 μmoles of $Co^{2+}$ per plug should give concentrations of approximately 0.7 or 7 mM, respectively, inside the apple tissue, assuming $Co^{2+}$ is evenly distributed in the tissue.

| Incubation Period | $[CoCl_2]$ | Ethylene | | $CO_2$ | |
|---|---|---|---|---|---|
| *hr* | *mM* | *nl* | *nCi* | *μl* | *nCi* |
| 0-2 | 0 | 107 | 38 | 69 | 22 |
| | 0.7 | 93 | 23 | 62 | 14 |
| | 7 | 55 | 5 | 59 | 6 |
| 2-4 | 0 | 147 | 78 | 64 | 49 |
| | 0.7 | 76 | 24 | 69 | 20 |
| | 7 | 27 | 4 | 62 | 8 |

## DISCUSSION

Methionine has been established as a precursor of ethylene in fruit and auxin-treated or stressed vegetative tissues (2, 3, 5, 24, 29). Since $Co^{2+}$ inhibits all ethylene-producing systems tested, including vegetative and fruit tissues (Figs. 1 and 4), it is logical to assume that the site of $Co^{2+}$ inhibition lies at a common step in the conversion of methionine to ethylene. Tracer experiments (Table II) verify the above hypothesis. $Co^{2+}$ inhibited ethylene production and the *in vivo* conversion of L-[U-$^{14}$C]methionine to ethylene, but did not affect the respiration of apple tissue (Table II). This indicates that the effect of $Co^{2+}$ on ethylene production was specific, and not a general metabolic inhibition. The ineffectiveness of $Co^{2+}$ on the respiration of other plant tissues has been reported (6, 12, 21). It should be noted that ethylene production in apple tissue was not as strongly inhibited by $Co^{2+}$ as was the conversion of radioactive methionine to ethylene (Table II). Since the volume of solution containing the radioactive methionine and $Co^{2+}$ administered to apple plugs represents less than one-fifteenth the volume of the apple plug, it is possible that during infiltration the radioactive methionine and $Co^{2+}$ were not uniformly distributed throughout the tissue, As a result, some portions of the tissue plug might receive little or no $Co^{2+}$ and radioactive methionine, and the corresponding ethylene production from the endogenous methionine would remain unaffected. However, in those regions which received $Co^{2+}$ and radioactive methionine, the conversion of methionine to ethylene would be inhibited.

The essentiality of cobalt ($Co^{2+}$) in animals is widely recognized, especially in terms of its participation as a component of vitamin $B_{12}$. All nitrogen-fixing organisms that have been thoroughly investigated appear to require cobalt for growth and for synthesis of $B_{12}$ compounds, but a $Co^{2+}$ requirement for higher plants grown in the absence of symbiotic microorganisms is not evident (8). Many workers have recognized that cobalt promotes several growth processes in excised plant parts in the presence (6, 9, 10, 13, 23, 29) or absence (10, 13, 21, 22) of IAA and/or cytokinin. A number of possible mechanisms of cobalt action have been advanced (6, 9, 21, 29), but no conclusive evidence has been shown. IAA has been found to promote elongation of pea epicotyls, plumular hook openings, and leaf-disk expansion (1, 7, 13, 14) and cytokinin to promote leaf-disk expansion (26). Cobalt further enhances these growth responses in auxin-treated (6, 9, 13, 23, 28) or cytokinin-treated tissues (26, 27) and in tissues treated with no hormones (10, 13, 21, 22), whereas ethylene inhibits these same phenomena (1, 7, 14). $Co^{2+}$, therefore, appears to promote the effect of IAA and/or cytokinin and thus to exert an influence opposite to that of ethylene. Kang and his coworkers (12, 14) proposed that $Co^{2+}$ exerts its promotive effect on hypocotyl hook opening by interfering with both the production and action of ethylene.

Auxins and cytokinins are known to stimulate ethylene production in a variety of plant tissues (1, 11, 15, 19, 24), and, as exemplified by the above phenomena, there are a number of physiological events in which the influence of an auxin may be counteracted or repressed by auxin-induced ethylene. The present report demonstrates that this feedback system may be further regulated by cobalt, and the evidence is strong that the regulation is accomplished by the inhibition of ethylene synthesis. $Co^{2+}$, at concentrations as low as 10μM, caused significant inhibition of both basal and induced ethylene production (Fig. 1). At a $Co^{2+}$ concentration of 0.1 mM or greater, inhibition was strong, and, in some cases, nearly complete at later part of the incubation. The promotive effect of $Co^{2+}$ on the above-mentioned growth phenomena may be explained, in part, as due to

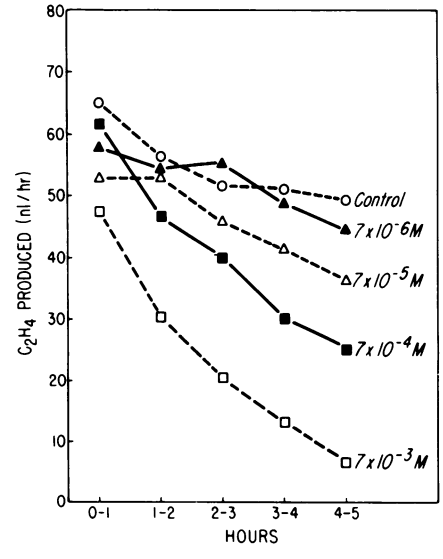


FIG. 4. Effect of various $Co^{2+}$ concentrations on the rate of endogenous ethylene production in apple tissue. Each apple plug (1 × 2 cm) received 100 μl of 2% KCl containing 0, 0.01, 0.1, 1, or 10 μmoles of $CoCl_2$, providing approximately 0, $7 \times 10^{-6}$, $7 \times 10^{-5}$, $7 \times 10^{-4}$, or $7 \times 10^{-3}$ M $Co^{2+}$, respectively, within the tissues, assuming uniform distribution.

the inhibition of basal and hormone-induced ethylene formation by $Co^{2+}$. In addition, $Co^{2+}$ may also exert its effect by blocking the ethylene action, as suggested by Kang and Ray (14). Because of the dual effects of auxins, it may be, in some cases, difficult to determine whether the growth response is caused directly by the auxin, or whether it is caused or altered by auxin-induced ethylene. This function of $Co^{2+}$ to inhibit ethylene formation may be useful in the resolution of such a question of casuality.

Fe, Co, and Ni belong to the first triad of Group VIII in the periodic table and have similar electron configurations. It is pertinent to note that $Ni^{2+}$ has been shown to promote the straight growth of pea stem sections (28) and the expansion of etiolated bean leaf disks (22), although to a lesser extent than does $Co^{2+}$. This observation is comparable to our present finding that $Ni^{2+}$ inhibits ethylene production, but to a slightly lesser extent than does $Co^{2+}$ (Table I and Fig. 2). $FeSO_4$ which belongs to the same subgroup as Co and Ni in the periodic table, was reported to inhibit the straight growth of pea stem sections at all concentrations tested (28). This is also expected because $FeSO_4$ at high concentrations stimulates stress ethylene production (1, 18), which would cause inhibition of the straight growth of pea segments or the expansion of leaf disks.

The present study shows that $Co^{2+}$ inhibits ethylene formation by interfering with the conversion of methionine to ethylene. However, the mode of its action in this process is not yet clear. A possible interaction of $Co^{2+}$ and sulfhydryl groups in plant tissues has been suggested (25, 28). Such an interaction was manifested by our observation that a dark brown complex was formed when $Co^{2+}$ was mixed *in vitro* with dithioerythritol (unpublished). However, the addition of dithioerythritol did not reverse the inhibition of ethylene production which was exerted by $Co^{2+}$ in apple plugs.

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