Influence of Cobalt on Leaf Expansion and Oxidative Phosphorylation

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The cobaltous ion, as well as many other chemicals, particularly the purine analogs, are known to affect the growth of etiolated leaves in the presence or absence of photomorphogenically active light (11, 18, 19). Additionally, cobalt has been shown to promote growth of other etiolated tissue such as the oat (Avena) coleoptile (2, 21) and pea roots (6). There have been different explanations of the mode of action of Co++. Busse (2) presented evidence that Co++ delays the deposition of secondary cell wall material, thereby prolonging the action of IAA. Galston (6) observed that the rate of peroxide formation in etiolated tissue is lowered by the addition of Co++, which in turn results in a decreased peroxidative destruction of IAA. Thimmann (21) by using specific inhibitors came to the conclusion that Co++ modifies some step in oxidative metabolism which makes energy available for growth by diverting it from other metabolic roles.

The present investigation arose from an observation that leaf disks given Co++ plus DNP responded quite differently from those given BAP plus DNP. In view of the diversity of opinion on the mechanism of Co++ action, it was of interest to obtain information about the primary site of action of Co++. The main emphasis during the present investigations was placed on the way in which Co++ might modify oxidative phosphorylation.

Evidence is presented showing that Co++-induced leaf expansion is not susceptible to inhibition by DNP. The amount of ATP in leaf tissue decreases sharply in the presence of DNP, but remains unchanged when Co++ is supplied in addition to DNP. Furthermore, experiments with mitochondria isolated from sweet potato tubers show that Co++ increases the yield of oxidative phosphorylation. Results from

other experiments indicate that Co++ has an inhibiting effect on ATPase activity.

Materials and Methods

Material. Bean plants (Phaseolus vulgaris, var. Burpee's dwarf stringless green pod) were grown in the dark for 6 days. Disks of 5 mm diameter were taken from the leaves under a dim green safelight (22), and placed in petri dishes containing filter paper and 5 ml of a nutrient solution as used by Miller (13). Each dish contained 20 leaf disks. After incubation in the dark for 48 hours, the diameter of the leaf disks was measured with the aid of a binocular microscope fitted with an ocular micrometer. In later experiments fresh weight, protein content (12), and ATP concentrations were also determined. Leaf disks were treated with chemicals only and not exposed to red light unless so stated.

Source of Chemicals. ATP, ADP, and DPN were purchased from the Sigma Chemical Co., diphosphothiamine chloride was obtained from California Corporation for Biochemical Research; and hexokinase (6,000 K.M. units per g at 5°C) was purchased from the Nutritional Biochemical Corp.

ATP Determination. The plant material was extracted as described by Sisler and Klein (20). The amount of ATP was determined by the luciferin-luciferase method using luciferase isolated from dehydrated firefly tails according to the procedure of Green and McElroy (8). The reaction mixture containing 50 μmoles of glycylglycine buffer (pH 7.8), 10 μmoles MgSO4, and 0.1 ml of the luciferase preparation in a total volume of 2.3 ml was put into a 3 ml Beckman cuvette and placed into a light-tight box. One-half ml of the ATP-containing sample was injected into the mixture with a syringe. The light emission was measured by a photomultiplier tube which was connected with an amplifier and voltage recorder, the deflection of which was directly proportional to the amount of light emitted by the sample.

The system was standardized with known concentrations of ATP (99% pure). Other nucleotides (ADP, UTP, UDP and mixtures thereof) were tested to assay for transphosphorylase present in the luciferase preparation. Nucleotides other than ATP did not give an appreciable response at concentrations up to 1 × 10−4 M. The method is suitable to measure ATP at concentrations as low as 10−5 M.

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3 Published with the approval of the Secretary of the Smithsonian Institution.
4 Present address: Chief, Biology Branch, United States Atomic Energy Commission, Washington 25, D.C.
5 Abbreviations: ATPase, adenosine triphosphatase; BAP, benzylamino purine; DNP, 2,4-dinitrophenol; DPN, diphosphopyridine nucleotide; DPT, diphosphothiamine.
Isolation of Mitochondria. Oxidative phosphorylation and ATPase activity were studied on isolated mitochondria. It was not possible to use mitochondria from etiolated bean leaves, since the various isolation procedures gave mitochondria with good O₂ uptake, but with only low phosphorylative activity. Therefore, the mitochondria were isolated from sweet potatoes which were purchased daily from local markets. The isolation procedure as employed by Hackett et al. (9) was modified as follows: The isolation medium contained ascorbic acid (500 mg/300 ml) instead of cysteine. The washing medium consisted of 0.5 M sucrose and 0.05 M Tris buffer (pH 7.0). After resedimentation, the mitochondrial pellet was resuspended in 0.5 M sucrose without any additions. All operations were conducted at approximately 0°C.

Oxidative Phosphorylation. The following reaction medium was used (final concentrations): sucrose, 0.5 M; glucose, 2 × 10⁻² M; MgSO₄, 7 × 10⁻³ M; DPT, 7 × 10⁻⁶ M; DPN, 4 × 10⁻⁴ M; phosphate buffer (pH 7.0), 27 × 10⁻² M; Tris buffer (pH 7.0), 4 × 10⁻³ M; hexokinase 1 mg/sample; substrate (as specified in individual experiments), 2 × 10⁻² M; plus 0.5 ml mitochondrial suspension in a total volume of 3.0 ml. The following supplements were made when required: ADP, in concentrations as specified; Co⁺⁺, 2 × 10⁻⁴ M; DNP, 1 × 10⁻⁴ M.

These experiments were performed in Warburg vessels at 25°C. Oxygen uptake was measured manometrically. Phosphorylation was determined in preliminary experiments by measuring the disappearance of inorganic phosphate (1). In subsequent experiments, the accumulation of the end product was measured. The original procedure (9) yielded glucose-6-phosphate; however, the spectrophotometric test for glucose-6-phosphate (5) could not be applied since the yellow color of DNP interfered with it. By omitting glucose and hexokinase from the reaction medium, the end product was changed to ATP. This permitted the use of the luciferase method (8) to determine the phosphorylative activity of isolated mitochondria. The following procedure was employed for sample collection: at zero time and at different time intervals thereafter, 1.0 ml samples were taken, placed into 2.0 ml of ice cold 5% perchloric acid. The excess amount of perchloric acid was neutralized with KOH and the precipitate removed by centrifugation. The supernatant was decanted and diluted fivefold with ice cold water. The samples were kept near 0°C during these steps. All treatments were done in duplicate or triplicate.

ATPase Activity. The ATPase activity of sweet potato mitochondria was determined according to Forti (4) to give a measure of maximal activity. Accordingly, after the first centrifugation the mitochondria were washed in 0.25 M sucrose plus Tris buffer and resedimented. The pellet was resuspended in 0.1 M sucrose. The reaction medium consisted of: sucrose, 0.1 M; MgSO₄, 7 × 10⁻³ M; phosphate buffer (pH 7.3), 4 × 10⁻² M; Tris buffer (pH 7.3), 3 × 10⁻² M; Co⁺⁺, 2 × 10⁻⁴ M; DNP, 1 × 10⁻⁴ M; ATP, between 1 and 5 × 10⁻⁴ M; 0.5 ml of mitochondrial suspension in 0.1 M sucrose, plus water to make a total volume of 3.0 ml. Initial and final concentrations of ATP in the reaction mixture were determined by the luciferase method.

Results

Effect of Co⁺⁺ on Leaf Expansion. The growth response of etiolated leaf disks to different concentrations of Co⁺⁺ is shown in figure 1. The optimal

![Fig. 1. Effect of Co⁺⁺ on the expansion (mm) of etiolated leaf disks within 48 hours of growth. Controls kept in the dark. Others exposed to 10 minutes of red light, then kept in the dark for 48 hours.](image)

![Fig. 2. Effect of DNP on expansion (mm) of etiolated leaf disks within 48 hours of growth. O——O, no further additions; X——X, BAP (4 × 10⁻⁵ M) added; △——△, Co⁺⁺ (1.5 × 10⁻⁴ M) added.](image)
concentration of Co++ is between 3.0 and 4.5 x 10^-4 M in the dark, as well as after exposure to a saturating dose of red light. These findings agree with results of Miller (14), and Scott and Liverman (19).

The Effect of DNP. The expansion of etiolated leaf disks was inhibited by DNP, and, as expected, the inhibition increased with increasing concentrations of DNP (fig 2, Control). Leaf disks incubated with Co++ were also subject to inhibition by DNP (fig 2, Cobalt). However, the difference in growth between Co++-treated disks and control samples remained constant; i.e., the growth promotion due to Co++ did not change even with increasing concentrations of DNP.

In order to find out whether this phenomenon also holds for other growth promoting substances, or whether it is peculiar to Co++, BAP was tested in the same way. BAP is known to promote the expansion of etiolated leaf disks (19), with an optimal concentration of approximately 4 x 10^-5 M. Leaf disks incubated with BAP were very susceptible to the inhibitory effect of DNP as shown in figure 2. The growth promotion due to BAP declined sharply with increasing concentrations of DNP. The difference in growth between BAP-treated disks and controls was large at low concentrations of DNP, whereas at higher concentrations of DNP, the growth of BAP-treated disks rapidly approached that of the control disks receiving DNP alone. This observation held true for concentrations of BAP from 1 x 10^-3 M to 2 x 10^-3 M.

Effect of Co++ on the ATP Content of Leaf Tissue. The primary product of oxidative phosphorylation is ATP, and uncoupling substances such as DNP result in a decrease of ATP in the treated tissue. However, since the growth promoting effect of Co++ was not reduced by DNP, one might expect differences in the concentration of ATP in leaf tissue which has been incubated with DNP in the presence or absence of Co++.

Leaf disks which had been kept in the dark were assayed at various times for ATP. In figure 3, the results are summarized with reference to fresh weight. Essentially, the same pattern was observed when these values were referred to protein content. The concentration of ATP in control samples decreased slightly during the period of 48 hours. Co++ by itself did not appreciably affect the amount of ATP in leaf tissue except to cause a slight decrease below controls, perhaps due to the intensive growth of the Co++-treated disks. DNP, as expected, caused the ATP level to be reduced drastically. This effect of DNP became more pronounced with time. Supplying Co++ in addition to DNP did not prevent an initial decrease of ATP (dotted line in fig 3); however, after 12 hours, the ATP content of these samples no longer differed markedly from the controls. Thus, the addition of Co++ to the DNP-containing medium apparently prevented (except for the first 6 hrs) the loss of ATP, which occurred if DNP alone was present.

Effect of Co++ on Oxidative Phosphorylation of Mitochondria. The enzymic activity of mitochondria isolated from etiolated bean leaves was inadequate for testing the effect of Co++. In trying different plant organs known to yield mitochondria with high phosphorylative activity, sweet potatoes were found to be a good source. Therefore, the following experiments were done with mitochondria isolated from sweet potatoes.

The influence of Co++ and DNP on the P:O ratio of isolated mitochondria was tested. Table I gives the results of a representative experiment. More than 20 experiments of this kind were done using citrate, succinate, and a-ketoglutarate as substrates. The results show that the addition of Co++ (2 x 10^-4 M) has no significant effect on respiration or phosphorylation of mitochondria. In the presence of DNP (1 x 10^-4 M), the P:O ratio of mitochondria decreased to about 50% of the control, mainly as a

Table I. Effect of Co++ and DNP on the P:O Ratio in Sweet Potato Mitochondria

<table>
<thead>
<tr>
<th>Additions</th>
<th>P1</th>
<th>O2</th>
<th>P: O</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.8</td>
<td>7.2</td>
<td>2.33</td>
</tr>
<tr>
<td>Co++ (2 x 10^-4 M)</td>
<td>17.6</td>
<td>7.0</td>
<td>2.51</td>
</tr>
<tr>
<td>DNP (1 x 10^-4 M)</td>
<td>8.1</td>
<td>6.4</td>
<td>1.26</td>
</tr>
<tr>
<td>Co++ (2 x 10^-4 M)</td>
<td>8.9</td>
<td>6.9</td>
<td>1.29</td>
</tr>
<tr>
<td>DNP (1 x 10^-4 M)</td>
<td>8.5</td>
<td>6.9</td>
<td>1.29</td>
</tr>
</tbody>
</table>

Fig. 3. Time course of the changes in the amount of ATP in etiolated leaf tissue as affected by Co++ (3 x 10^-4 M) and DNP (1.5 x 10^-4 M), with reference to constant fresh weight.
result of inhibited phosphorylation. When both DNP and Co\(^{++}\) were added to the mitochondrial suspension, surprisingly enough the degree of inhibition of the phosphorylative activity was almost the same as with DNP alone. In order to evaluate the significance of this unexpected finding, the method of determining phosphorylative activity was changed, i.e., rather than determining the disappearance of P\(_{i}\), the formation of ATP was measured directly. The following modifications had to be made in the incubation mixture: the phosphate trapping system (glucose and hexokinase) was omitted, thereby yielding ATP as the end product of the reaction. In addition, the concentration of ADP was decreased from \(1 \times 10^{-5}\) M to \(1 \times 10^{-5}\) M, to prevent its interfering with the ATP determination by the luciferase method. In other experiments, ADP was omitted entirely, relying only on the endogenous nucleotides contained in the mitochondria. The experiments on the P:O ratio quoted above had shown that the rate of oxygen uptake in isolated mitochondria is not affected by the addition of Co\(^{++}\). Therefore, only the phosphorylative activity was determined in the following experiments.

Table II summarizes experiments with mitochondria in the presence of low ADP concentration (\(1 \times 10^{-5}\) M). The data represent averages of 2 separate determinations of ATP accumulated within 30 minutes of incubation. The formation of ATP in Co\(^{++}\)-treated samples is expressed on a percentage basis relative to the control. Since the uncoupling effect of DNP varied between experiments, even though it was supplied at a constant concentration, the influence of Co\(^{++}\) in DNP-incubated samples was expressed relative to the DNP-containing sample, not to the basal control. This comparison eliminates the variation in DNP effectiveness. On the average, Co\(^{++}\)-incubated samples yielded about 40% more ATP than the control. The effect of Co\(^{++}\) became even more pronounced in the presence of DNP, where the addition of Co\(^{++}\) increased the yield of ATP by 70%.

The same observation was made when no ADP was added (Table III). No ATP could be detected at the beginning of the reaction. The ATP concentration at the end of the reaction was only about one-third of that observed in experiments of Table II. But again, Co\(^{++}\)-incubated samples contained more ATP than the controls, regardless of whether DNP was present or not. The results of these experiments suggested that the yield of ATP in isolated mitochondria was increased in the presence of Co\(^{++}\).

**Effect of Co\(^{++}\) on the Activity of ATPase.** Mitochondria are a site of ATP synthesis; however, they also contain enzymes which decompose ATP (4). It was shown by Pullman (16), and Penešký (15) that there is a common enzymic basis in mitochondria for the synthesis and for the decomposition of ATP. In the experiments reported above, the addition of Co\(^{++}\) increased the yield of ATP in oxidative phosphorylation. It was of interest to see whether Co\(^{++}\) might have this effect by modifying the activity of ATPase. Mitochondria from sweet potatoes were also used for these experiments, but the isolation procedure had to be changed slightly to obtain maximal ATPase activity (see Materials and Methods). Mg\(^{++}\) was added to all samples.

The results of these experiments are given in Table IV. The loss of ATP in samples without Co\(^{++}\) (controls) was considered 100%. Less ATP was

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**Table II. Effect of Co\(^{++}\) on ATP Formation in Sweet Potato Mitochondria in the Presence of ADP**

<table>
<thead>
<tr>
<th>m(\mu) moles ATP formed per ml</th>
<th>% increase due to Co(^{++})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Co(^{++}) 2 (\times 10^{-4}) M</td>
</tr>
<tr>
<td>14.0</td>
<td>19.8</td>
</tr>
<tr>
<td>18.9</td>
<td>31.5</td>
</tr>
<tr>
<td>6.2</td>
<td>7.3</td>
</tr>
</tbody>
</table>

**Table III. Effect of Co\(^{++}\) on ATP Formation in Sweet Potato Mitochondria in the Absence of added ADP**

<table>
<thead>
<tr>
<th>m(\mu) moles ATP formed per ml</th>
<th>% increase due to Co(^{++})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Co(^{++}) 2 (\times 10^{-4}) M</td>
</tr>
<tr>
<td>2.4</td>
<td>4.8</td>
</tr>
<tr>
<td>2.5</td>
<td>5.6</td>
</tr>
<tr>
<td>5.6</td>
<td>7.2</td>
</tr>
<tr>
<td>7.6</td>
<td>8.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>m(\mu) moles ATP formed per ml</th>
<th>% increase due to Co(^{++})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP</td>
<td>Co(^{++}) 1 (\times 10^{-4}) M</td>
</tr>
<tr>
<td>2.1</td>
<td>3.6</td>
</tr>
<tr>
<td>1.6</td>
<td>3.3</td>
</tr>
<tr>
<td>6.9</td>
<td>6.6</td>
</tr>
</tbody>
</table>
decomposed in the presence of Co++. On the average, the difference amounted to about 20%. In other words, Co++ retarded the destruction of ATP. This effect was observed both in the absence and presence of DNP. Under both conditions Co++ appeared to inhibit the activity of ATPase in mitochondria.

The accumulation of ADP during the hydrolysis of ATP interferes with the activity of ATPase (3,7). The accumulation of ADP can be avoided by the addition of an ATP-regenerating system (7). Phosphoenolpyruvate and pyruvate kinase were used to regenerate ATP following the procedure of Pullman et al. (16). The results of a representative experiment are given in table IV (experiment 2). In the presence of the ATP-regenerating system, the inhibition of ATPase activity due to Co++ became even more pronounced.

### Discussion

The experiments reported in this paper were performed to find an explanation for the phenomenon that Co++-induced growth in etiolated leaf disks is relatively insensitive to DNP.

The data on the ATP content of leaf tissue show that DNP had only a slight effect on ATP in the presence of Co++. However, this does not hold true at the beginning of the experiment. During the first 6 hours after DNP and Co++ had been added, the ATP level dropped considerably, but after 12 hours it was again as high as in the samples without DNP (fig 3). No ready explanation is available for this drop and eventual recovery of the ATP level in leaf tissue. Perhaps DNP was able to reach the phosphorylating site faster than Co++, thus resulting in a temporary decrease of ATP, before Co++ finally compensated the action of DNP. A possible explanation for this and some other Co++ effects might be that Co++ tied up DNP in a complex, which had no uncoupling properties anymore. If such a complex was formed, no inhibition at all should be observed. However, in the expansion of leaf disks (fig 2), as well as in the phosphorylative activity of isolated mitochondria (table I), there is still a noticeable inhibition even when DNP was added together with Co++. Also, the absorption spectrum in visible light was the same for individual solutions of DNP and Co++ as for a combined solution of the two. Therefore, the formation of a physiologically inactive complex is unlikely. Another possibility was that Co++ either promoted the formation or inhibited the hydrolysis of ATP.

This led to experiments testing whether the addition of Co++ might also balance the uncoupling effect of DNP in isolated mitochondria. Preliminary results, obtained by determining the disappearance of P_i indicated that the addition of Co++ to the inhibited system resulted only in a slight (but persistent) improvement of oxidative phosphorylation. Results with the luciferase method showed that Co++ caused about a 40% increase in ATP above the controls without Co++ in preparations of isolated mitochondria. It is recognized that omitting the phosphate-trapping system (glucose and hexokinase) yielded data which do not represent the absolute amount of ATP formed within a given time. The results are only relative values reflecting an equilibrium of anabolic and catabolic processes. However, since all samples were treated in the same manner, the values within one experiment can be compared. The individual experiments became comparable by expressing observed differences on a percentage basis.

Since Co++ counteracted the inhibition of DNP in oxidative phosphorylation, we further examined the possibility that Co++ may have an effect on the activity of ATPase. The results indicate that the addition of Co++ reduces the activity of ATPase by about 20%. This can account at least partially for the increased yield of oxidative phosphorylation in isolated mitochondria. In these experiments DNP had little or no influence on the activity of ATPase. This can be explained, perhaps, by the observation that DNP lacks this stimulating effect if the mitochondria are undamaged (10,16), even though DNP usually is reported to stimulate ATPase (17).

Our observations that Co++ influences the ATP

### Table IV. Effect of Co++ on ATPase Activity in Sweet Potato Mitochondria

<table>
<thead>
<tr>
<th>Initial amount of ATP (μmoles per ml)</th>
<th>Incubation time (min)</th>
<th>Amount of ATP decomposed (μmoles/ml)</th>
<th>% inhibition due to Co++</th>
<th>Amount of ATP decomposed (μmoles/ml)</th>
<th>% inhibition due to Co++</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control 2 × 10⁻⁴ M</td>
<td></td>
<td>DNP 1 × 10⁻⁴ M</td>
<td></td>
</tr>
<tr>
<td>2500</td>
<td>30</td>
<td>890</td>
<td>780</td>
<td>910</td>
<td>710</td>
</tr>
<tr>
<td>4000*</td>
<td>30</td>
<td>1580</td>
<td>1170</td>
<td>1430</td>
<td>900</td>
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<td>650</td>
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<td>850</td>
<td>5</td>
<td>255</td>
<td>190</td>
<td>235</td>
<td>210</td>
</tr>
</tbody>
</table>

* 5 μmoles of phosphoenolpyruvate and 30 μg of pyruvate kinase were added as ATP regenerator.
level in isolated mitochondria lead us to the same conclusion as Thimann (21); namely, that Co\textsuperscript{2+} in some way modifies the plant's energy metabolism. On the basis of our data, we suggest that this modification is related to the effect of Co\textsuperscript{2+} to increase ATP production by retarding the activity of ATPase. The effect of Co\textsuperscript{2+} on the ATP level in leaf tissue, and on the yield of phosphorylating mitochondria from sweet potatoes, reveals a certain conformity. This suggests that the same system operates in both tissues, although our data do not demonstrate this directly.

It is not clear whether the above mentioned mechanism is also involved in the antiperoxidative action of Co\textsuperscript{2+} as reported by Galston and Siegel (6). Further experimentation is necessary to determine whether the stimulating action of Co\textsuperscript{2+} in other plant tissues is related to the capacity of Co\textsuperscript{2+} to increase the yield of oxidative phosphorylation.

**Summary**

The growth promoting effect of cobalt ions in etiolated bean leaf tissue is independent of growth inhibition by 2,4-dinitrophenol. Cobalt ions do not affect the content of adenosine triphosphate in bean leaf tissue, but cobalt ions prevent a decrease of adenosine triphosphate in the presence of 2,4-dinitrophenol.

Oxidative phosphorylation in isolated sweet potato mitochondria, as determined by disappearance of inorganic phosphate, does not appear to be influenced by cobalt ions. However, when adenosine triphosphate is determined by the luciferase technique, cobalt ions increase the yield of oxidative phosphorylation in both the presence and absence of 2,4-dinitrophenol.

The activity of adenosine triphosphatase in sweet potato mitochondria is lower in the presence of cobalt ions than without them.

**Acknowledgments**

We wish to express our appreciation to Dr. W. H. Klein, Smithsonian Institution, for his interest and helpful criticism. Thanks are also due Miss Rebecca Gettens for her capable assistance in certain phases of the work.

**Literature Cited**

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