OXIDATIVE PHOSPHORYLATION AND GLYCOLATE OXIDATION
BY PARTICLES FROM SPINACH LEAVES

ISRAEL ZELITCH AND G. A. BARBER

It has recently been reported (2) that oxidative phosphorylation occurs when organic acids of the citric acid cycle are supplied as substrates to particles isolated from the cotyledons of etiolated lupine seedlings. The ratios of the number of moles of orthophosphate consumed to the number of atoms of oxygen taken up (P/O ratios) were found to be as high as those previously reported only for animal tissues (7). Furthermore, Ohmura (13) has shown that particles obtained from green spinach leaves are also capable of carrying out oxidative phosphorylation although he did not determine P/O ratios. This observation suggested that spinach leaves might provide an active particulate system for further study. Accordingly, such particles have been isolated by a method modified from that of Ohmura and have been found to bring about phosphorylations with high P/O ratios when substrates such as pyruvate, citrate, L-isocitrate, α-ketoglutarate, succinate, fumarate, L-malate, and reduced diphosphoryridine nucleotide (DPNH) are used.

Glycolic acid oxidase, a riboflavin phosphate-linked enzyme which catalyzes the oxidation of glycolic acid to glyoxylic acid, was isolated some years ago in a highly purified form from extracts of spinach leaves (21) and has recently been crystallized (5). Experiments performed in this laboratory suggest that glycolic acid oxidase plays a role in the respiration of leaves in sunlight (19, 20). Some glycolic acid oxidase activity has now been found to be associated with the particles obtained from spinach leaves, and a search was accordingly made for evidence of oxidative phosphorylation with glycolate as the substrate. No significant activity was found. The effect of glycolate on the oxidation and phosphorylation of other substrates by these particles has also been investigated.

Materials and Methods

Preparation of Particles: Spinach was purchased locally, and stored in a plastic bag at 5°C. occasionally for as long as several weeks, until used. Storage of leaves for this duration was found to have no effect on the activity of isolated particles. The younger pale-green leaves were taken unless otherwise noted. The midribs were removed, and a weighed quantity of leaf blade tissue was washed several times in water and chilled for a few minutes on ice before being ground in the cold room.

Grinding was done in a chilled mortar with washed sand, with the addition of two volumes of a grinding medium, similar to Ohmura's (13), of the following composition: 0.45 M sucrose, 0.05 M mannitol-borate buffer at pH 7.2, 0.03 M potassium citrate, 0.01 M sodium ethylenediamine tetraacetate (Versenate) at pH 7.5, and 0.05 M tris(hydroxymethyl)aminomethane chloride (Tris) at pH 8.3. The ground tissue, final pH 7.2, was squeezed through two layers of cheesecloth, and then centrifuged for 5 min at 600 × G at −5°C. The residue, consisting of chloroplasts and other heavy cell fragments, was discarded, and the supernatant fluid was centrifuged at 10,000 × G for 20 min. The residue of particles was gently suspended in a washing medium (1 ml per g of leaf) composed of 0.3 M sucrose, 2 × 10⁻⁴ M Versenate, and 0.05 M tris buffer at pH 7.5, and centrifuged at 10,000 × G for 20 min. The particles were finally taken up in the washing medium (1 ml per 1.8 g of leaf) for use in the experiments. Usually 0.9 ml of the suspension was used in each Warburg vessel. This is equivalent to 1.6 g of leaf tissue, and 0.5 to 0.8 mg of protein N per vessel. The suspension was still green in color, doubtless because of material originating in the chloroplasts.

Manometry: Oxygen uptake was measured by conventional techniques in 15 ml Warburg vessels in an atmosphere of air. All of the substrates used were of the highest grade commercially available. Crystalline potassium pyruvate (9), L-isocitrate (17), and sodium glyoxylate monohydrate (15) were prepared in the laboratory. Cytochrome c from horse heart, yeast coenzyme concentrate, glucose-6-phosphate dehydrogenase, and Type III hexokinase from yeast were purchased from the Sigma Chemical Co. Substrates, cofactors, and the particles suspended in the washing medium were placed in the main compartment of chilled Warburg vessels. The center well contained 0.2 ml of 5N KOH, and to the side arm glucose and an excess of hexokinase were added to convert the adenosine triphosphate (ATP) formed by oxidative phosphorylation into glucose-6-phosphate. After equilibration at 30°C for 5 min, at zero time, the contents of the side arm were tipped into the main compartment. The vessels were shaken at 120 oscillations per minute, and light was excluded with a black cloth. Under these experimental conditions, the rate of diffusion does not limit the oxygen uptake until a rate of 8 μl per minute is approached. At the end of the reaction period, the vessels were quickly transferred to an ice bath, and 1 ml of 3% perchloric acid was added to stop enzymic reaction.

1 Received June 29, 1959.
The reaction mixture in experiments 1 and 2 contained the following components in chilled Warburg vessels: sucrose, 300 μmoles; MgSO₄, 10 μmoles; substrate, 20 μmoles; potassium phosphate buffer at pH 7.0, 37 μmoles; yeast coenzyme concentrate, 1 mg; ATP, 2 μmoles; particles from 1.6 g of leaf tissue in 0.9 ml of washing medium; and water to make the final volume 2.0 ml. In the sidearm were placed glucose, 50 μmoles, and yeast hexokinase, 0.2 mg. The center well contained 0.2 ml of 5 N KOH. The reaction was stopped after 25 min at 30° C.

In experiment 3, all conditions were the same except that 6 μmoles of substrate and 19 μmoles of phosphate buffer were added, and yeast coenzyme concentrate was omitted. The reaction was terminated after 10 min.

All values are corrected for the slight uptake of oxygen (0 to 0.5 μatoms) observed in a control vessel containing all components except substrate.

**Results**

**Oxidative Phosphorylation with Organic Acids:** Evidence is presented in table I that particles isolated from young spinach leaves contain a vigorous oxidative phosphorylation system with all the members of the Krebs citric acid cycle tested. In experiment 1, the value for a-ketoglutarate (theoretical P/O = 4.0) has probably been lowered by the accompanying oxidation of the succinate formed (theoretical P/O = 2.0). In contrast to the behavior of particles obtained by Pierpoint from tobacco leaves (14), these preparations are also able to oxidize fumarate. When fumarate, citrate, or pyruvate are the substrates, a lag in the rate of oxygen uptake was always observed for the first 10 to 15 min, presumably because the fumarase and aconitase activities are limiting, after which the rates of oxidation approached those of succinate and malate. Accordingly, the figures for the oxidation during 25 min given for fumarate, citrate, and pyruvate are minimal values.

The P/O ratios obtained with succinate are equal to the highest values given in the literature (7) and serve as a criterion of the undamaged nature of the particles. This point is further demonstrated by the failure of added cytochrome c (0.5 mg per vessel) to stimulate the rate of oxidation of succinate. As has also been observed with particles from tobacco leaves (14), the rate of succinate oxidation is not influenced by added yeast coenzyme concentrate. As is demonstrated in table I, addition of fluoride is not necessary in order to demonstrate high P/O ratios (13); this indicates that the phosphatase activity of the spinach leaf particles is low.

Good agreement was achieved when the disappearance of orthophosphate was compared with the net increase in glucose-6-phosphate under conditions similar to those of table I. For example, in one experiment in which succinate was the substrate, 14.1 μmoles of orthophosphate were taken up (P/O = 1.7) and 13.0 μmoles of glucose-6-phosphate were produced (P/O = 1.6). No significant disappearance of orthophosphate has ever been observed in reactions carried out as described in table I but in the absence of substrate.

Although glycolate is oxidized by these particles, significant esterification of inorganic phosphate is not observed in the presence of this substrate. The enzyme which oxidizes glycolate could not be removed from the particles by further washing. Its activity in the particles represents about 15% of that found initially in homogenates of the leaf; the first residue from centrifugation contained 25% of the activity, and the remainder was present in the supernatant fluid.

**Requirements for Pyruvate Oxidation:** The presence of a catalytic amount of malate, which presumably serves to produce an acetyl acceptor, is required to obtain good rates of oxidation of pyruvate (table II). This requirement is commonly observed in analogous systems prepared from other sources. The particles already possess a complement of coenzymes, but oxidation is further stimulated by adding DPN and coenzyme A (CoA). The high P/O ratio obtained, 2.7, approaches the highest figure, 3.0, observed with particles from other sources (7).

**Effect of Age of Leaves on Oxidative Phosphorylation by Isolated Particles:** Particles obtained from older spinach leaves, that is, leaves inserted lower on the stem than those hitherto discussed,
were found to oxidize organic acids of the Krebs cycle less rapidly than those from younger leaves. Thus, in an experiment under the reaction conditions described in Table I with particles from older leaves, succinate elicited the uptake of 5.7 μatoms of oxygen (P/O = 1.6), and malate 6.6 μatoms (P/O = 2.0). Glycolate, on the other hand, consumed 9.2 μatoms in the same experiment. Thus glycolate was oxidized at about twice the rate by particles from older leaves than by those from younger leaves, but succinate and malate were oxidized at half the rate. The protein N in a preparation of the particles from older leaves was not less than that ordinarily present in particles from the same weight of young leaves.

**Attempts to Demonstrate Oxidative Phosphorylation with Glycolate:** The P/O ratio that could be obtained consistently with glycolate as substrate was about 0.1. This represents a smaller disappearance of phosphate than can be accurately determined by the phosphomolybdate method employed, but it could be confirmed by the considerably more sensitive assay of the glucose-6-phosphate formed. A number of attempts were made to increase the phosphorylation with glycolate as substrate without success. Among the experimental conditions that were varied were: the physiological age of the spinach leaves from which the particles were prepared; the age of the particle preparations; the centrifugal force used to obtain the particles (500 × G, 10,000 × G, 100,000 × G); the kinds and amounts of coenzymes added; the substrate concentration; the presence of additional substrates; the reaction time; the concentration of orthophosphate; the addition of dialyzed or undialyzed portions of the supernatant fluid; the addition of excess catalase; the addition of semicarbazide to combine with the glyoxylate produced in the oxidation of glycolate, and increased hexokinase concentration. It must be concluded that particles capable of carrying out oxidative phosphorylation cannot do this effectively with glycolate as the substrate.

It has been suggested that glycolic acid oxidase accounts for a significant proportion of the oxygen taken up by leaves in sunlight (19, 20). The above observations indicate, however, that this oxidase cannot provide much ATP for use by the leaf.

**Effect of Enzyme Inhibitors on Oxidation of Succinate and Glycolate:** The system which oxidizes glycolate differs from that involved in the oxidation of the other substrates tested in that there is little or no associated oxidative phosphorylation (Table I). Differences in the mode of oxygen uptake by glycolate and other organic acids of the Krebs cycle can also be demonstrated with suitable inhibitors. A series of competitive and highly specific inhibitors of purified glycolic acid oxidase has been described (18). These compounds are all α-hydroxysulfonates, and α-hydroxy-2-pyridinemethanesulfonic acid is the most effective of these compounds in vivo (20). As shown in experiment 1 (Table III), the potassium salt of this inhibitor has no effect on succinate oxidation or on phosphorylation, but at a concentration of $5 \times 10^{-4} \text{M}$ it inhibits the oxidation of glycolate to the extent of 68%. Azide, on the other hand, inhibits the oxidation of succinate by 67% and also inhibits phosphorylation to a marked degree. The stimulation of the oxygen uptake observed with

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Substrate</th>
<th>Without Inhibitor</th>
<th>α-Hydroxy-2-Pyridinemethanesulfonate ($5 \times 10^{-4} \text{M}$)</th>
<th>K Cyanide ($5 \times 10^{-3} \text{M}$)</th>
<th>Na Azide ($1 \times 10^{-3} \text{M}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Succinate</td>
<td>10.4 (1.9)</td>
<td>10.1 (1.7)</td>
<td>3.4 (1.2)</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>Glycolate</td>
<td>3.8</td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Succinate</td>
<td>9.1 (1.5)</td>
<td></td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycolate</td>
<td>5.2</td>
<td></td>
<td>6.2</td>
<td></td>
</tr>
</tbody>
</table>

* Figures in parentheses represent P/O ratios observed.

Leaf particles and reaction mixtures were prepared and treated as described in Table I. Cyanide was also placed in the center well (11) in experiments with this inhibitor. The reaction was stopped after 25 min in experiment 1, and after 20 min in experiment 2.
glycolate, on the addition of azide, occurs presumably because the catalase in the particles is inhibited and hence more oxygen is taken up per mole of glycolate oxidized (8, 21). Thus the pathway of electron transport by these particles in the presence of succinate is undoubtedly through the cytochrome system, whereas with glycolate a flavoprotein oxidase is involved. This conclusion is further borne out in experiment 2, in which cyanide was found to inhibit oxidation of succinate completely, but oxidation of glycolate was stimulated (16).

**Inhibition of Organic Acid Oxidations by Glycolate and Glyoxylate:** Some unusual effects have been observed when certain organic acids of the Krebs cycle are oxidized by particles from spinach leaves in the presence of glycolate, or its oxidation product glyoxylate. Table IV shows that the simultaneous oxidation of glycolate and either succinate or citrate inhibits the normal oxygen uptake achieved by each acid alone. Similar mutual inhibitions have been observed by Pierpoint (14) when two substrates of the Krebs cycle were supplied to particles from tobacco leaves. This was ascribed to the saturation of some intermediate reactant in the oxidation system common to both substrates. In the oxidation of glycolate, however, where no common intermediate exists (table III), some other mechanism must obtain.

Glyoxylate is oxidized only slowly by these preparations (table IV). In spite of the inhibitions of the oxidation of succinate and citrate caused by glycolate and glyoxylate, no lowering of the P/O ratio for either of these substrates occurs.

**Discussion**

The rates of oxidation, and the efficiency of oxidative phosphorylation in particles from spinach leaves compare favorably with the highest figures reported for particles from other plant parts (2), and for various tissues of animal origin (7). There is little doubt, therefore, that these leaves are capable of classical Krebs cycle activity and accompanying synthesis of ATP. The question of what part this scheme plays in the metabolism of the leaf is, of course, not answered by information of this kind. It should be emphasized that the isolated particles oxidize succinate at a rate (table I) found to be only 20% of the endogenous dark respiration by a weight of intact leaf equal to that from which the particles were obtained. Nevertheless, for many studies of leaf biochemistry, the system described here is a convenient one. The plant material used is readily available, can be stored, and the activity of the particulate preparations is high and reproducible.

It has been suggested that pyruvate is oxidized in photosynthesizing tissues less rapidly in the light than in the dark (1, 6), so that differences apparently exist in the mechanism of respiration under these two conditions. Since glycolate is produced by leaves almost exclusively in the light, and has been shown in this study to inhibit the oxidation of substrates of the Krebs cycle, it is tempting to ascribe the effect of light on respiration to such a mechanism. A way in which glyoxylate may specifically inhibit the Krebs cycle is suggested by evidence that this compound and oxaloacetate may react to form an inhibitor of the oxidation of citrate (3).

**Summary**

The isolation from green spinach leaves of particles which carry out oxidative phosphorylation with high P/O ratios is described. Conditions are given

**Table IV**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Total O₂ Uptake IN μATOMS</th>
<th>O₂ Uptake of Citrate or Succinate IN μATOMS</th>
<th>O₂ Uptake of Glycolate or Glyoxylate IN μATOMS</th>
<th>Orthophosphate ESTERIFIED IN μMOLES</th>
<th>P/O for Succinate or Citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>14.2</td>
<td>8.1</td>
<td></td>
<td>5.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Citrate</td>
<td>8.1</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Glycolate</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td>Succinate + glycolate</td>
<td>12.8</td>
<td>9.9</td>
<td>2.9</td>
<td>18.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Succinate + glyoxylate</td>
<td>9.6</td>
<td>8.9</td>
<td>0.7</td>
<td>15.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Citrate + glycolate</td>
<td>7.9</td>
<td>5.2</td>
<td>2.7</td>
<td>14.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Citrate + glyoxylate</td>
<td>6.3</td>
<td>5.6</td>
<td>0.7</td>
<td>14.2</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Reaction mixtures were prepared as described in table I, and 5 μmoles of glyoxylate or 20 μmoles of glycolate were added where indicated. Oxygen uptake of glycolate in the presence of succinate or citrate was estimated from measurement of the glyoxylate formed based on the observed stoichiometry of the reaction: glycolate $+\frac{1}{2} O₂$ $\rightarrow$ glyoxylate $+ H₂O$. The total oxygen uptake less that quantity derived from the glyoxylate determination was considered to be the oxygen consumed by citrate or succinate. Oxygen uptake given for the Krebs cycle substrates in the presence of glyoxylate represents the total oxygen consumed less that utilized in the control for the oxidation of glyoxylate alone. The reaction was stopped after 30 min.

Copyright © 1960 American Society of Plant Biologists. All rights reserved.
for the oxidation of pyruvate, citrate, L-iso-citrate, a-ketoglutarate, succinate, fumarate, L-malate, and reduced diphosphopyridine nucleotide. Some of the glycolate oxidizing activity of the leaf is also associated with this particulate system, but this reaction is not accompanied by significant synthesis of adenosine triphosphate.

Studies with enzyme inhibitors indicate that oxygen uptake is mediated by the cytochrome system when organic acids of the Krebs cycle are the substrates, and by a flavoprotein oxidase with glycolate. Certain inhibitory effects of glycolate and glyoxylate on the operation of the Krebs cycle have been described and their possible physiological importance considered.

Acknowledgements

Grateful acknowledgement is made to Dr. Hubert B. Vickery for helpful discussion and to the National Science Foundation which provided a part of the financial support for this investigation.

Literature Cited