THE TRICARBOXYLIC ACID CYCLE IN RHODOSPIRILLUM RUBRUM

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The oxidation of acetic acid in the dark by the non-sulfur purple bacterium, Rhodospirillum rubrum, was shown by van Niel (1, 2) to require the presence of either carbon dioxide or trace amounts of the C4-dicarboxylic acids of the Krebs cycle. These results could readily be interpreted to mean that the oxidation of acetic acid proceeds by way of the Krebs cycle, the presence of CO2 being required to maintain a sufficient concentration of the C4 acids. However, α-ketoglutaric, citric, and isocitric acids were unable to spark acetic acid oxidation in this organism. In addition, citric acid could not be utilized as the sole carbon source for growth. The latter findings would imply either that the Krebs cycle is inoperative in R. rubrum for the terminal oxidation of acetic acid or that the cell membrane is impermeable to α-ketoglutaric, citric, and isocitric acids.

The inability of yeast and Escherichia coli to utilize citric acid was interpreted by Krebs and Johnson (3, 4) as evidence against the functioning of a tricarboxylic acid cycle. Karlsson and Barker (5) were unable to demonstrate a tricarboxylic acid cycle in Azotobacter agilis for the terminal oxidation of acetic acid. More recently Ajl and coworkers (6-9) claimed to have demonstrated a C4-dicarboxylic acid cycle in E. coli for the terminal oxidation of acetic acid which enters the cycle by a Wieland-Thunberg condensation. In Aerobacter aerogenes, on the other hand, they found that the tricarboxylic acid and the C4-dicarboxylic acid cycles are co-existent, whereas in Micrococcus lysodeikticus only the tricarboxylic acid cycle operates.

While the evidence mentioned above would indicate that the Krebs cycle is not the major pathway for the oxidation of acetic acid by microorganisms, evidence to the contrary has also accumulated. Thus it was found by Lynen and Necuillah (10), Campbell and Stokes (11), and Lara and Stokes (12) that the inability of yeast, Pseudomonas aeruginosa, and E. coli, respectively, to utilize citric acid may be due to the impermeability of the cell membrane to this substance. A comparable explanation of
Karlsson and Barker’s results with A. agilis is indicated by the recent studies of Stone and Wilson (13, 14) with cell-free extracts of Azotobacter vinelandii. Similarly, Krampitz (15) obtained evidence for the operation of a tricarboxylic acid cycle in cell-free extracts of E. coli.

The present investigation is a study of the mechanism for the terminal oxidation of acetic acid in R. rubrum.

Materials and Methods

R. rubrum was grown under conditions of continuous illumination at 25° in a medium consisting of 0.1 per cent ammonium sulfate, 0.05 per cent magnesium chloride, 0.3 per cent sodium dl-malate, 0.5 per cent yeast autolysate, 0.6 per cent phosphate buffer, pH 7.0, and 0.01 per cent calcium chloride in distilled water. The phosphate buffer and calcium chloride solutions were sterilized separately and added aseptically to the remainder of the medium. Strictly anaerobic conditions were not maintained in growing the organism; usually a quantity of 400 ml. of medium in a 500 ml. cotton-stoppered bottle was inoculated with about 40 ml. of a 24 hour culture of the organism and the new culture incubated in a light cabinet.

To obtain a dried cell preparation, the cells from a 24 hour culture were harvested by centrifugation, resuspended in M/30 phosphate buffer, pH 7.0, and placed overnight on a rotary shaker in darkness at 30°. This treatment was included to reduce the endogenous respiration of the final preparations to a conveniently low value. The cells were centrifuged off, washed once with tap water, spread in a thin layer along the sides of the centrifuge tube, and dried over concentrated sulfuric acid in vacuo. After 18 to 24 hours of drying, the flaky material was ground in a mortar and the resulting powder stored at either 4° or -10°.

The cell-free extracts were prepared from living cells according to the method of McIlwain (16). The cells were treated in a manner identical to that indicated for the dried cell preparations prior to the drying process. The washed cells were ground with twice their weight of alumina¹ and extracted with 2 to 5 volumes of M/15 phosphate buffer, pH 7.0. The suspension was centrifuged in the cold in a Servall centrifuge at 14,000 X g.

Phosphotransacetylase was prepared according to the method of Ochoa et al. (17). The preparation contained trace amounts of condensing enzyme.

Citric acid was determined by the method of Natelson et al. (18), modified to cover the concentration range of 10 to 100 γ of citric acid. The sample, prepared in 3 N sulfuric acid, was autoclaved at 15 pounds pressure for 30 minutes to destroy oxalacetic acid, which also gives a pentabromacetone reaction.

¹ 1557 AB levigated alumina powder, Buehler, Ltd., Chicago, Illinois.
Oxalacetic acid was prepared according to the method of Krampitz and Werkman (19). The preparation was 96 per cent pure, as determined by the aniline citrate method. Acetyl phosphate was prepared by the method of Stadtman and Lipmann (20). d-Isocitric acid was prepared by alkaline hydrolysis of dimethyl-d-isocitric lactone (21). The methanol produced by this treatment was not eliminated since it is not oxidized by either the dried cells or the cell-free extracts.

DPN § "90," TPN "10," ATP, and cytochrome c were obtained from the Sigma Chemical Company, TPN (25 per cent) was kindly furnished by Dr. P. Stumpf, and coenzyme A (170 units per mg.) by Dr. Fritz Lipmann or obtained from the Pabst Laboratories. Cocarboxylase was obtained from Merck and Company, Inc.

Results

Dried Cell Preparations

Oxidation of Citric and Isocitric Acids—In preliminary experiments with dried cells, citric acid was not oxidized. The addition of DPN, TPN, cytochrome c, methylene blue, Mg++, and Mn++, either singly or in combinations, failed to cause oxidation. However, when isocitric acid was added to the same preparations, a rapid uptake of oxygen occurred, as shown in Fig. 1. These results indicated that the dried cells were devoid of aconitase activity. The extremely labile nature of this enzyme has been demonstrated by Krebs (21) with animal tissue extracts, and it seemed reasonable to expect that the enzyme could have been inactivated either during the drying process or during storage of the dried cells.

Therefore, a fresh batch of dried cells was prepared and checked at intervals of time for "citric acid oxidase" activity. The results shown in Fig. 2, corrected for endogenous respiration, indicate clearly that freshly prepared dried cells are capable of oxidizing citric acid and at a rate comparable to that of isocitric acid oxidation. As the preparation aged, the rate of citric acid oxidation fell off at an almost constant rate and was negligible after 12 to 14 days. There is a lag period in the oxidation of citric acid which increases with the age of the preparation. In the older preparations (7 to 10 days), 30 to 40 minutes may elapse before oxygen uptake occurs. This lag is probably due to the low rate of conversion of citric acid to isocitric acid as a result of the decreased aconitase activity. It was with the older preparations that many of the preliminary experiments were performed, which explains the negative results obtained initially.

The addition of either DPN or TPN increased the rate of isocitric oxida-

§ The following abbreviations are used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ATP, adenosinetriphosphate; OAA, oxalacetic acid; CoA, coenzyme A.
The addition of methylene blue also increased the rate of oxidation in the presence of TPN, but exerted no appreciable effect in the presence of DPN. The pH optimum for this system was found to be about 7.5.

The ability of both DPN and TPN to stimulate isocitric dehydrogenase activity would seem to indicate that both a DPN and a TPN isocitric dehydrogenase are present in this organism. This possibility was examined by preparing a cell-free extract and fractionating the latter according to the procedure described by Kornberg and Pricer for yeast (22).

![Graph](http://www.jbc.org/)

**Fig. 1.** Oxidation of citric and isocitric acids by dried cells of *R. rubrum*. The reaction mixture contained 20 mg. of dried cells, 125 μM of phosphate buffer, pH 7.5, and 5 μM of substrate; total volume, 2 ml. 0.2 ml. of 10 per cent KOH in the center well; temperature 30°. Δ, endogenous, ○, citric acid, •, isocitric acid.

Each of the various fractions was tested for its ability to reduce TPN and DPN, respectively, in the presence of isocitric acid. None of the fractions caused a reduction of DPN, even when the test solution was fortified with the cofactors found to be necessary for the yeast enzyme. Reduction of TPN occurred under the influence of the precipitate obtained at 0.7 saturation.

These results suggest that a DPN-dependent isocitric dehydrogenase does not exist in *R. rubrum*, in which case the stimulation of oxidation by DPN must be accounted for in another manner. Since the dried cells do possess an α-ketoglutaric acid oxidase, the observed increase in oxygen uptake might be due to an oxidation of isocitric acid beyond the α-ketoglutaric acid stage, with DPN playing a rôle in the latter oxidation. DPN has
been shown recently to be a cofactor in the α-ketoglutaric acid oxidase system (23, 24). This hypothesis was tested in an experiment in which the oxidation of isocitric acid by the dried cells was carried out in the presence of arsenite. As can be seen from Fig. 3, the oxidation of α-ketoglutaric acid is almost completely suppressed in the presence of arsenite and DPN, but the rate of isocitric acid oxidation is still markedly increased by the addition of DPN.

The favorable effect of DPN could also be accounted for by assuming the presence, in R. rubrum, of a pyridine nucleotide transhydrogenase enzyme system similar to that recently described by Colowick et al. (25) for Pseudomonas fluorescens. In favor of this explanation is the fact that the rate of isocitric acid oxidation does not increase with increasing DPN concentrations, which would be expected if the concentration of TPN is limiting the rate of reaction. The absence of methylene blue stimulation in the presence of DPN would also fit into this explanation.

Oxidation of α-Ketoglutaric Acid—The oxidation of α-ketoglutaric acid, like that of citric acid, can be demonstrated with dried, but not with intact, cells. It is evident from Fig. 4 that the rate of oxidation is not constant, but falls off gradually and assumes a value which is identical with that of malic acid oxidation. Up to the time of the break in the curve, the molar ratio of oxygen taken up to substrate oxidized approaches 1.0. This ratio is consistent with the occurrence of a two-step oxidation of α-ketoglutaric acid to fumaric and malic acids. The fact that the rate of oxygen con-

![Graph](http://www.jbc.org/)

**Fig. 2.** Effect of age of dried cell preparations on the oxidation of citric acid. The reaction mixture contained 20 mg. of dried cells, 135 μM of phosphate buffer, pH 7.0, 5 μM of methylene blue, and 5 μM of substrate; total volume, 2.2 ml. 0.2 ml. of 10 per cent KOH in the center well; temperature 30°. O, 17 hours; ●, 65 hours; ●, 137 hours; ▲, 233 hours.
sumption does not immediately return to the endogenous level after the uptake of 1.0 mole of O₂ per mole of added α-ketoglutaric acid can be taken as evidence for the oxidation of malic acid.

The pH optimum for this system is about 7.0. The addition of either coenzyme A, diphosphothiamine, or DPN to the preparations resulted in an increase in the initial rate of oxidation (Fig. 5). The three cofactors, when added together, produced a 4-fold increase in this rate. These three cofactors have been shown recently by Green and Beinert (23) and Kaufman (24) to be specific for the oxidation of α-ketoglutaric acid in a soluble enzyme system from heart muscle. The requirements for Mg²⁺, Mn²⁺, and phosphate could not be demonstrated, as all attempts to remove these ions by dialysis resulted in the complete inactivation of the enzyme system.

It was found that the addition of methylene blue to the dried cell preparations resulted in the inhibition of α-ketoglutaric acid oxidation, as in-
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When potassium ferricyanide was substituted as an electron acceptor, it also proved to be inhibitory, $2.5 \times 10^{-3}$ M causing 95 per cent inhibition. This latter finding is difficult to interpret at present in view of its action in the assay system for $\alpha$-ketoglutaric acid oxidase from heart muscle (26).

**Oxidation of Succinic Acid**—The dried cell preparations, without additional cofactors, oxidize succinic acid more rapidly than they oxidize $\alpha$-ketoglutaric or malic acid (Fig. 4). The rate of oxidation is high, and constant for only a very short interval of time; thereafter the rate changes rapidly and soon becomes identical with that of malic acid oxidation. At the time of the break in the curve, approximately 0.5 mole of oxygen has been taken up per mole of substrate oxidized. This ratio is consistent with the occurrence of a one-step oxidation to fumaric and malic acids. Also, the rate of oxidation falls off with the age of the dried cells; approximately 50 per cent of the activity is lost after 8 days. Nevertheless, some of the preparations have been found to retain about 20 per cent of the original activity even after 7 months.

![Graph showing oxidation of α-ketoglutaric, succinic, and malic acids by dried cell preparations.](http://www.jbc.org/)

**Fig. 4.** Oxidation of $\alpha$-ketoglutaric, succinic, and malic acids by dried cell preparations. The reaction mixture contained 20 mg. of dried cells, 135 $\mu$M of phosphate buffer, pH 7.0, and 5 $\mu$M of substrate; total volume, 2.1 ml. 0.2 ml. of 10 per cent KOH in the center well; temperature 30°. $\Delta$, endogenous, O, malic acid, ▲, succinic acid, ●, $\alpha$-ketoglutaric acid.
In some of the older preparations it was observed that, in addition to the decreased rate of succinic acid oxidation, there was also less than the theoretical 0.5 mole of oxygen taken up per mole of substrate oxidized. The addition of cytochrome c produced a 50 per cent increase in the initial rate of oxidation and increased the $O_2$-substrate ratio from 0.18 to 0.25 (Fig. 7). Doubling the cytochrome c concentration produced no further increase. The addition of either ATP, coenzyme A, DPN, or methylene blue was without effect.

The ability of cytochrome c to raise the ratio to only 0.25 would seem to suggest that an inhibitory reaction may also be involved. Pardee and Potter (27) have shown by kinetic measurements that oxalacetic acid in extremely low concentrations inhibits succinic dehydrogenase activity of animal tissue by forming a dissociable complex with the enzyme such as that formed by succinic and malonic acids. It appeared possible to account for the results obtained with older preparations by assuming that oxalacetic acid accumulates. This was tested by carrying out the oxidation of succinic acid in the presence of glutamic acid, which removes the oxalacetic acid by converting it into aspartic acid via transamination.
Glutamic acid was not oxidized by this preparation, as is shown in Fig. 7. However, the rate of succinic acid oxidation was increased 3-fold by the simultaneous addition of glutamic acid and cytochrome c. The amount of oxygen taken up during the linear portion of the curve increased from 0.25 to 0.40 mole per mole of substrate oxidized.

Oxidation of Malic and Fumaric Acids—From Fig. 4 it may be concluded that, at pH 7.0, malic acid is oxidized by unfortified dried cells, although at a much lower rate than those at which either α-ketoglutaric or succinic acid is oxidized. Furthermore, the rate decreases with increasing age of the preparation. The inhibitory action of oxalacetic acid on malic dehydrogenase was shown by Green (28) to account for the low rate of malic acid oxidation in animal tissues.

The necessary components and the optimal conditions for this system were therefore determined. From the results shown in Fig. 8, it can be seen that the oxidation of malic acid is extremely slow. The addition of cyanide for trapping oxalacetic acid caused a small increase in the rate of oxygen uptake. A greater effect was produced by the addition of DPN, although the increase in rate is only 2-fold. However, the simultaneous addition of both cyanide and DPN resulted in a 5-fold greater rate. Methylene blue was included in all these experiments to permit a valid com-
parison between the effectiveness of cyanide and DPN in influencing the rate of malic acid oxidation. In the absence of any cofactors and cyanide, it was found that methylene blue increased the rate by 30 to 50 per cent.

On the other hand, the addition of nicotinamide, a treatment which was meant to prevent the breakdown of DPN by DPNase, caused an inhibition of the malic acid oxidation, the degree of inhibition increasing with increasing nicotinamide concentrations. Similar results have been ob-

![Graph](http://www.jbc.org/)  
Fig. 7. Effect of cytochrome c and glutamic acid on succinic acid oxidation in old dried cell preparations. The reaction mixture contained 20 mg. of dried cells, 125 $\mu$M of phosphate buffer, pH 7.0, and 5 $\mu$M of substrate; final volume, 2.0 ml. 0.2 ml. of 10 per cent KOH in the center well; temperature 30°. ▲, endogenous; ●, 10 $\mu$M of glutamic acid, ◇, succinic acid, △, succinic acid + 1.4 X $10^{-4}$ $\mu$M of cytochrome c, ○, succinic acid + 10 $\mu$M of glutamic acid + 1.4 X $10^{-4}$ $\mu$M of cytochrome c.

tained by Feigelson et al. (29) with purified malic dehydrogenase enzyme from animal tissue.

The rate of malate oxidation increased with increasing pH. In Fig. 8 the rates of malate oxidation at pH 7 and 8.5 are compared under otherwise identical conditions. There is a 5-fold increase in the rate of oxidation. The pH optimum for this system is about 9.0. In view of the high pH optimum for malic dehydrogenase activity of dried cells of *R. rubrum*, it is evident why, in unfortified preparations at pH 7.0, the rates of oxidation of $\alpha$-ketoglutaric, succinic, citric, and isocitric acids fall off to that of malic acid.
A buffer mixture consisting of 0.2 M glycine-M/15 KH₂PO₄ was used over the pH range of 8.5 to 10.0. At pH 9.0 the rate of malate oxidation in a fortified system was the same in the presence as in the absence of cyanide. This appeared to be in direct contradiction to the stimulating effect of cyanide obtained at pH 7.0 and 8.5. However, since α-keto acids are readily decarboxylated by amines (30), it appeared possible that the glycine in the buffer was exerting a similar effect. This was tested by adding 10 μM of oxalacetic acid to the buffer solution and measuring the decarboxylation manometrically. It was found that within the first 20 minutes the CO₂ evolution accounted for 70 to 80 per cent of the oxalacetic acid added. The rate then decreased very rapidly, indicating that the decarboxylation of oxalacetic acid by glycine followed first order kinetics. It would thus appear that glycine, under the conditions used, can replace cyanide as a means of removing oxalacetic acid. Additional evidence for the action of glycine is illustrated in Fig. 9. The oxygen consumption of dried cells suspended in a phosphate and in a phosphate-glycine buffer at pH 9.0 was determined following the addition of 5 μM of malic acid. The markedly higher rate as well as the greater extent of malic acid oxidation in the phosphate-glycine buffer is evident. The rapid decrease in the rate of malate oxidation in the phosphate buffer can be ascribed to an in-
creasing inhibition by accumulating oxalacetic acid. The fact that after 30 minutes the rate of oxygen consumption had become constant suggests that a progressive accumulation of oxalacetic acid did not occur and that a stationary concentration was maintained as a result of the occurrence of secondary reactions by which oxalacetic acid is removed as rapidly as it is produced. When oxalacetic acid was added to the two suspensions, the inhibition of malic acid oxidation in the system containing glycine lasted for only 10 minutes, after which the rate rapidly increased to a value comparable to that of the control suspension. In the absence of glycine, the inhibition was extended over a longer period of time, 80 to 90 minutes; later, the rate increased slowly.

The total oxygen uptake for malic acid oxidation in the glycine buffer at pH 9.0 exceeds the amount required for a one-step oxidation of malic to oxalacetic acid. At the point where the curve in Fig. 9 assumes the slope of the endogenous respiration, the amount of oxygen utilized per mole of substrate oxidized is 0.75; the theoretical ratio for a one-step oxidation is 0.5. This ratio of 0.75 has been obtained consistently with preparations which are unable to oxidize citric acid. The additional O\textsubscript{2} consumption is

![Graph showing the inhibitory action of oxalacetic acid on malic acid oxidation in phosphate and phosphate-glycine buffers at pH 9.0.](http://www.jbc.org/)

Fig. 9. Inhibitory action of oxalacetic acid on malic acid oxidation in phosphate and phosphate-glycine buffers at pH 9.0. The reaction mixture contained 20 mg. of dried cells, 2.0 ml. of 0.25 M phosphate buffer, pH 9.0, or 2 ml. of 0.25 M K\textsubscript{2}HPO\textsubscript{4} + 0.2 M glycine buffer, pH 9.0, 5 \mu M of methylene blue, 0.64 \mu M of DPN, 5 \mu M of substrate, and 10 \mu M of oxalacetic acid where indicated; final volume, 2.5 ml. 0.2 ml. of 10 per cent KOH in the center well; temperature 30°. Glycine-phosphate buffer at pH 9.0. ○, endogenous, ●, malic acid, ○, malic acid + oxalacetic acid. (Phosphate buffer at pH 9.0.) ▲, endogenous, ■, malic acid, △, malic acid + oxalacetic acid.
exactly equivalent to that needed for a secondary transformation of oxalacetate to citric acid. That this conversion can be accomplished by dried cell preparations will be shown in the section on acetic acid oxidation.

It seemed justifiable to assume that the oxidation of fumaric acid by the dried cell preparations would be accomplished via primary conversion of fumaric to malic acid by the enzyme fumarase. A suspension of dried cells in phosphate-glycine buffer at pH 9.0 consumes oxygen at a low rate in the presence of fumaric acid, as measured manometrically. The pH optimum for fumarase activity covers the pH range of 6.5 to 7.5, while at pH 9.0 its activity is only about a tenth as high (31). Therefore, the reaction mixture in phosphate buffer was incubated for 1 hour at a pH of 7.0; following this treatment, a concentrated glycine solution at pH 9.7 was added from the side arm of the reaction flask, raising the pH to about 9.0. Malic acid formed from fumaric acid during the first part of the experiment will be rapidly oxidized under the new conditions.

The results of such an experiment are presented in Fig. 10. Neither fumaric nor malic acid was oxidized during the 1st hour at pH 7.0, but as soon as the pH of the reaction mixture was raised to 9.0, a rapid oxygen uptake occurred. The rate of oxidation of fumaric acid was identical with that of malic acid.

Oxidation of Acetic Acid—Manometric experiments showed that dried
cells of *R. rubrum* are unable to oxidize acetic acid, even in the presence of CO₂ and C₄-dicarboxylic acids which sparked acetate oxidation in the living cells. Also, the addition of coenzymes, cofactors, and hydrogen carriers failed to influence the results. Since the previously discussed experiments had revealed that, under appropriate conditions, the various components of the cycle can be oxidized by dried cells, it seemed logical to infer that the inactivity of the dried cells toward acetic acid was due to the inactivation of an enzyme system concerned with bringing acetic acid into the cycle.

On the basis of all information available (32–35), a search was made for the presence in the cells of *R. rubrum* of the various enzymes responsible for the stepwise reactions involved in citric acid formation from acetic acid. For this purpose, aged preparations of dried cells were used. These are no longer able to oxidize citric acid, so the determination of citrate formation could serve as a quantitative measure of the occurrence of the individual processes.

The addition of oxalacetic acid to dried cell preparations resulted in the formation of considerable quantities of citric acid, as shown in Table I. Acetic acid alone did not yield citric acid, but when both acetic and oxalacetic acids were added, the amount of citrate formed was 15 to 30 per cent above that obtained with oxalacetic acid alone. The addition of ATP in low concentrations increased the amount of citrate formed, but with increasing concentrations of ATP the yield decreased. Acetyl phosphate did not yield citric acid when added alone, nor did it increase citric acid production above that obtained with oxalacetic acid alone. The amount of citric acid formed varied from one preparation to another, and also with the age of the preparations.

Citrate formation by the dried cell preparations from oxalacetic acid alone can readily be accounted for as the result of a decarboxylation of the substrate to pyruvic acid and the conversion of the latter compound to acetyl CoA which condenses with the residual oxalacetic acid to form citric acid. In line with this hypothesis is the extra oxygen uptake in the oxidation of malic acid, and also the fact that pyruvic acid is not oxidized by the dried cells unless malic acid is simultaneously supplied.

The conversion of oxalacetic acid to citric acid can be largely eliminated by dialyzing a dried cell suspension for 18 to 24 hours at 4°C against 0.02 M phosphate buffer, pH 7.0. This treatment reduces the extent of citric acid formation by as much as 75 per cent of that observed with a freshly prepared suspension; simple storage of a suspension at 4°C for the same length of time causes a reduction of only 15 per cent (Table II). This drastic reduction in citric acid formation can be explained by the removal of the coenzyme necessary for the transformation of pyruvic acid (36).
As the data of Table II demonstrate, the dialyzed dried cell preparations of *R. rubrum* respond to the addition of these substances. Addition of DPN increases the citric acid formation by 300 per cent; coenzyme alone exerts but a minor influence, but the two coenzymes together cause an increase in yield of 500 per cent. Additional evidence for the involvement of DPN is the fact that nicotinamide completely suppresses citrate formation from oxalacetate in freshly prepared suspensions.

The dialyzed preparations were well suited for a study of the occurrence of condensation reactions between oxalacetic acid, acetic acid, and ATP or acetyl phosphate. Experiments listed in Table III show that the amount

**Table I**

*Formation of Citric Acid by Dried Cells of R. rubrum*

The system contained 10 mg. of dried cells, 25 µM of phosphate buffer, pH 7.0, 5 µM of MgCl₂, and 10 µM of cysteine per ml. Additions: 20 µM of acetic acid, 20 µM of acetyl phosphate, 20 µM of oxalacetic acid, and ATP as indicated. The results are expressed as micromoles of citric acid per ml. of reaction mixture. Incubated for 2 hours at 30°.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Preparation 1 (aged 10 days)</th>
<th>Preparation 2 (aged 10 days)</th>
<th>Preparation 3 (aged 10 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>0.12</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>OAA</td>
<td>2.18</td>
<td>2.68</td>
<td>2.00</td>
</tr>
<tr>
<td>Acetic acid + OAA</td>
<td>2.89</td>
<td>3.10</td>
<td>1.28</td>
</tr>
<tr>
<td>Acetyl phosphate + OAA</td>
<td>1.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid + OAA + 0.05 mg. ATP</td>
<td>1.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid + OAA + 1.00 &quot; + 1.00 &quot;</td>
<td>1.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid + OAA + 1.50 &quot; + 1.50 &quot;</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

of citric acid formed from acetic plus oxalacetic acid is approximately twice as great as that produced with oxalacetic acid alone. The effect of the addition of acetyl phosphate is no greater than that of acetic acid. But a further notable increase results from the simultaneous addition of acetic acid and ATP. As in the case of the undialyzed suspensions, high concentrations of ATP reduce the extent of citric acid formation. The optimum appeared to be about 5 × 10⁻⁸ M ATP, which agrees well with the results reported by Ochoa et al. for pigeon liver preparations (17). Coenzyme A causes an increase of approximately 50 per cent in the amount of citric acid formed (Table IV). Nevertheless, as demonstrated by the data in Table IV, citric acid production from acetic acid, ATP, and oxalacetic acid, even under the best conditions, is small compared to the citric acid formation from oxalacetic acid alone via the pyruvic oxidase system.
The inability of acetyl phosphate to replace acetic acid and ATP in the condensation reaction with oxalacetic acid indicated that the formation of acetyl CoA was ineffective through the lack of phosphotransacetylase activity. Further evidence was obtained from arsenolysis tests (35). With

**Table II**

*Effect of Dialysis and Addition of Coenzymes on Citric Acid Formation*

Composition of reaction mixture as in Table I, phosphate buffer, pH 8.0. Additions: 0.64 μM of DPN, 0.1 μM of cocarboxylase, 800 μM of nicotinamide. The results are expressed as micromoles of citric acid per ml. of reaction mixture. Incubated for 2 hours at 30°.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Freshly prepared</th>
<th>Stored 18 hrs. at 4°</th>
<th>Dialyzed 18 hrs. at 4°</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.08</td>
<td>1.68</td>
<td>0.47</td>
</tr>
<tr>
<td>DPN</td>
<td></td>
<td>1.80</td>
<td></td>
</tr>
<tr>
<td>Cocarboxylase</td>
<td>0.57</td>
<td>2.51</td>
<td></td>
</tr>
<tr>
<td>DPN + cocarboxylase</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table III**

*Citric Acid Formation by Dialyzed Dried Cells from Acetic Acid, Acetyl Phosphate, and Oxalacetic Acid; Effect of ATP*

Composition as in Table II. Additions: 20 μM of oxalacetic acid, 20 μM of acetic acid, and ATP as indicated. The results are expressed as micromoles of citric acid per ml. of reaction mixture. Incubated for 2 hours at 30°.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Acetyl phosphate</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>OAA</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>OAA</td>
<td>0.30</td>
</tr>
<tr>
<td>Acetyl phosphate</td>
<td>OAA</td>
<td>0.30</td>
</tr>
<tr>
<td>Acetic acid + OAA + 2.3 μM ATP</td>
<td>0.53</td>
<td>0.55</td>
</tr>
<tr>
<td>&quot; &quot; + &quot; + 4.8 &quot; &quot;</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; + &quot; + 6.9 &quot; &quot;</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; + &quot; + 9.2 &quot; &quot;</td>
<td>0.45</td>
<td></td>
</tr>
</tbody>
</table>

Dried cell preparations, the results were strictly negative, and with a cell-free extract prepared from freshly harvested bacteria the outcome was no different. On the other hand, the addition of a phosphotransacetylase preparation, obtained in the form of a cell-free extract of *E. coli*, to dialyzed dried cells of *R. rubrum* resulted in the abundant formation of citric acid from oxalacetic acid and acetyl phosphate (Table V).
Cell-Free Extracts

All members of the Krebs cycle tested are oxidized by cell-free extracts of \( R. \) rubrum. The results of a typical experiment, performed at pH 7.0 with an unfortified extract, are illustrated in Fig. 11. The rates of oxid-

**Table IV**

Citric Acid Formation by Dialyzed Suspensions of Dried Cells; Effect of Coenzyme A

Composition as in Table II. Additions: 20 \( \mu \text{M} \) of acetic acid, 20 \( \mu \text{M} \) of oxalacetic acid, CoA\( ^* \) as indicated, 1.9 \( \mu \text{M} \) of ATP, 0.32 \( \mu \text{M} \) of DPN, and 0.1 \( \mu \text{M} \) of cocarboxylase. The results are expressed as micromoles of citric acid per ml. of reaction mixture. Incubated for 2 hours at 30°.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Citrate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAA</td>
<td>0.25</td>
</tr>
<tr>
<td>&quot; + acetic acid</td>
<td>0.30</td>
</tr>
<tr>
<td>&quot; + &quot; + &quot; + ATP</td>
<td>0.39</td>
</tr>
<tr>
<td>&quot; + &quot; + &quot; + &quot; + 3.5 units CoA</td>
<td>0.55</td>
</tr>
<tr>
<td>&quot; + &quot; + &quot; + &quot; + 7.0 &quot; &quot;</td>
<td>0.64</td>
</tr>
<tr>
<td>&quot; + ATP + 3.5 units CoA</td>
<td>0.24</td>
</tr>
<tr>
<td>&quot; + DPN + cocarboxylase</td>
<td>2.49</td>
</tr>
<tr>
<td>&quot; + &quot; + &quot; + 3.5 units CoA</td>
<td>3.13</td>
</tr>
</tbody>
</table>

* I am indebted to Mr. R. G. Bartsch for the coenzyme A determination.

**Table V**

Effect of Transacetylase on Citrate Formation by Dialyzed Dried Cell Preparations of \( R. \) rubrum

Composition as in Table II. Additions: \( E. \) coli extract (1.6 mg. of protein), 20 \( \mu \text{M} \) of acetyl phosphate, 20 \( \mu \text{M} \) of oxalacetic acid. The results are expressed in micromoles of citric acid per ml. of reaction mixture. Incubated for 2 hours at 30°.

<table>
<thead>
<tr>
<th>Additions</th>
<th>With dried cells</th>
<th>Without dried cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAA</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>&quot; + acetyl phosphate</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>( E. ) coli extract + OAA</td>
<td>0.40</td>
<td>0.00</td>
</tr>
<tr>
<td>&quot; + &quot; + &quot; + acetyl phosphate</td>
<td>1.45</td>
<td>0.13</td>
</tr>
</tbody>
</table>

OAA and isocitric acids were found to be identical, as were the rates of oxidation of malic and fumaric acids. The rates of oxidation of citric and \( \alpha \)-ketoglutaric acids, while high initially, gradually decrease and become identical with the rate of succinic acid oxidation. The fact that the initial rates are high would indicate that the necessary cofactors and coenzymes are present in the extracts in satisfactory concentrations. The oxygen uptake per mole of substrate oxidized during the linear portion
of the curves approaches the ratio of 1.0 and 0.5 for citrate and α-ketoglutarate, respectively. These are the values required for a two-step and a one step oxidation to succinic acid.

While in dried cell preparations the initial rate of oxidation was higher for succinic acid than for any other member of the cycle, in the cell-free extracts succinic acid oxidation occurs at the lowest rate of all, but it is more steady than in the young dried cell preparations. The curve for succinic acid oxidation resembles those in the older dried cell preparations, suggesting that the low rate of oxidation in the cell-free extracts may also be due to oxalacetic acid inhibition. Similar results have been obtained with cell-free extracts of A. vinelandii (13, 14).

The rate of malic acid oxidation varied somewhat from one preparation to another, but in each preparation the initial rate of oxidation of malic acid was higher than that of succinic acid. In experiments with unfortified extracts (Fig. 11) the difference was very small, but when the preparation was fortified with DPN and methylene blue, the rate could be as much as twice as high for malic as for succinic acid oxidation. The pH optimum for this system, as for the dried cell preparations, was found to be about 9.0. The rate of malic acid oxidation, even under the best conditions at

![Fig. 11. Oxidation of the Krebs cycle members by cell-free extracts of R. rubrum.](http://www.jbc.org/)

The reaction mixture contained 1.8 ml. of enzyme extract (equivalent to 5.2 mg. of N), 1.8 ml. of 1/15 phosphate buffer, pH 7.0, 10 μM of MgCl₂, and 5 μM of substrate; total volume, 2.0 ml. 0.2 ml. of 10 per cent KOH in center well; temperature 30°. ▲, endogenous, ●, citric and isocitric acids, ○, α-ketoglutaric acid, ●, succinic acid, Δ, malic and fumaric acids.
pH 7.0, is rather low compared to that shown by cell-free extracts of *A. vinelandii*. Similarly, the extent of oxidation by *R. rubrum* extracts appears to be that of a one-step oxidation to oxalacetic acid, while in *A. vinelandii* extracts the oxidation proceeds via citric acid to succinic acid. This would indicate either a block in citric acid formation in *R. rubrum* or a difference in mechanism for citric acid formation in the two organisms.

Acetic acid was not oxidized by the cell-free extracts. The addition of C_4_-dicarboxylic acids was without effect on acetic acid oxidation, as in the dried cells. These preliminary results are in marked contrast to those obtained with *A. vinelandii* extracts, where acetate is oxidized, though at a low rate, and all the Krebs cycle members can "spark" the reaction.

**DISCUSSION**

The oxidation of citric, isocitric, and α-ketoglutaric acids by dried cells and cell-free extracts of *R. rubrum* suggests that the failure of the living cells to oxidize these compounds is not due to the lack of the appropriate enzymes in the organism. It is, therefore, reasonable to ascribe this discrepancy to the existence in the living cell of permeability barriers, a situation that has already been encountered in a variety of bacteria, yeast, algae, and fungi. The present experiments emphasize again the need for caution in interpreting data obtained with living cells, especially in experiments on simultaneous adaptation and with tracers, intended to determine whether a particular substance can be considered as an intermediate product in the degradation of a given substrate.

The oxidation of citric, isocitric, and α-ketoglutaric acids occurs at a fairly high rate in both dried cells and cell-free extracts. The oxidation of malic and succinic acids, however, shows a different pattern in the two preparations. In young dried cell preparations, at neutrality, succinic acid is rapidly oxidized and malic acid accumulates, the further oxidation becoming the rate-limiting reaction of the over-all process. Cell-free extracts, on the other hand, oxidize malic acid more rapidly than succinic acid; here the oxidation of succinic acid appears as the rate-limiting step. A possible explanation may be found in the nature of the malic dehydrogenase enzyme which may occur in a "conjugated" or "dissociated" form (37).

While the dried cells and cell-free extracts are unable to oxidize acetic and pyruvic acids by themselves in manometric experiments, these acids are capable of entering the tricarboxylic acid cycle as shown by their condensation with oxalacetic acid to form citric acid in the dried cell preparations. The formation of citric acid from acetic acid and ATP, but not from acetyl phosphate, and the absence of the enzyme phosphotransacetylase are evidence for acetic acid entering the cycle via a mechanism which is probably identical with that described for animal tissue and yeast (32).
According to this mechanism, the rate of the over-all reaction in the presence of catalytic amounts of coenzyme A can be maintained only if the CoA is continuously regenerated by the condensation of acetyl CoA and oxalacetic acid to form citric acid. This would explain the sparking effect of the C\textsubscript{1}-dicarboxylic acids in the oxidation of acetic acid by living cells. The sparking action of CO\textsubscript{2} could be attributed to the formation of oxalacetic acid as the result of a fixation reaction. The oxidation of pyruvic acid by the pyruvic oxidase system would also require a mechanism for the regeneration of CoA, and therefore one would expect that this reaction would be sparked by members of the Krebs cycle. This is supported by the fact that dried cells do not oxidize pyruvic acid unless malic acid is also supplied. In the living cells, the oxidation of pyruvic acid does not require sparking, presumably because of the ability of the cells to form oxalacetic acid from pyruvic acid through CO\textsubscript{2} fixation.

The experimental evidence obtained with refortified suspension of dialyzed dried cells indicates that citric acid formation from acetic acid, ATP, and oxalacetic acid is extremely small compared to that from oxalacetic acid alone via the pyruvic acid oxidase system. The low rate of citric acid formation in the former system can be attributed to the lower rate of acetyl CoA formation. This would account for the failure to observe acetic acid oxidation in the dried cells by manometric techniques. Assuming that dried cell suspensions and cell-free extracts in the presence of acetic acid consume O\textsubscript{2} only for the oxidation of primarily formed citric acid, the data in Table V show that the rate of O\textsubscript{2} utilization is of the order of magnitude of only 0.5 \( \mu \)l. per 10 minutes; this is so low as to escape manometric detection. The failure to observe a sparking effect by members of the Krebs cycle on acetic acid oxidation in dried cells and cell-free extracts can be explained on the same basis.

Consequently, the results of the present investigation are entirely compatible with the hypothesis that the oxidation of acetic acid by \textit{R. rubrum} is accomplished via the tricarboxylic acid cycle.

**SUMMARY**

1. Dried cell preparations and cell-free extracts of \textit{Rhodospirillum rubrum} can oxidize all members of the tricarboxylic acid cycle tested. The failure of living cells to oxidize citric, isocitric, and \( \alpha \)-ketoglutaric acids is attributed to the impermeability of the living cell to these compounds.

2. The cofactor and coenzyme requirements and the pH optima of the various enzyme systems have been determined and found to be identical with those of the corresponding enzymes in animal tissues.

3. Manometric experiments failed to show acetic acid oxidation by both dried cells and cell-free extracts. However, the dried cell preparations
are capable of forming citric acid from acetic acid, ATP, and oxalacetic acid, though at a low rate. The absence of the enzyme phosphotransacetylase and the inability of acetyl phosphate to substitute for acetic acid and ATP in this reaction indicate that the mechanism for citric acid formation from acetic acid and ATP is probably identical with that found in animal tissue and yeast.

4. A pyruvic acid oxidase system is also present in the dried cell preparations; it can effect the production of citric acid at a much greater rate than can the acetic acid-ATP system.

5. The presence of all the enzymes required for the oxidation of the Krebs cycle members and the ability of the dried cells to form citric acid from acetic and pyruvic acids indicate that the tricarboxylic acid cycle is probably the pathway for the terminal oxidation of these acids in R. rubrum.

The author wishes to thank Professor C. B. van Niel for his encouragement and advice throughout the course of this work.

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Max A. Eisenberg


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