Photosynthesis in Rhodospirillum rubrum
II. Photoheterotrophic Carbon Dioxide Fixation

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Summary. The contribution of the reductive pentose phosphate cycle to the photometabolism of carbon dioxide and to carbon metabolism in Rhodospirillum rubrum grown photoheterotrophically with L-malate as the carbon source is nil, unlike autotrophically grown R. rubrum. Glycolic acid appears to be the first stable product of CO₂ fixation in R. rubrum cultured photoheterotrophically on L-malate. The results obtained in ¹⁴CO₂ fixation experiments suggest that the photometabolism of CO₂ through glycolate into malate is a major pathway of CO₂ fixation in such cells. However, L-malate was a much more efficient precursor of phosphate esters, and of glutamic acid, than was carbon dioxide: L-malate is therefore, in this case, a far more important source of cell carbon than is carbon dioxide.

The products of the light-dependent incorporation of CO₂ and of acetate were investigated in R. rubrum grown photoheterotrophically on acetate. Carboxylation reactions and the reductive pentose phosphate cycle are apparently of greater significance in the photometabolism of acetate heterotrophs than in malate heterotrophs: the photometabolism of the acetate photoheterotrophs seems to be intermediate between the photoheterotrophy of malate heterotrophs and strict autotrophy.

Organic carbon compounds photometabolized during growth by the facultative phototroph, Rhodospirillum rubrum, are utilized directly as a source of reduced organic carbon, and not, as was originally proposed by van Niel (19) solely as a source of reducing power for the assimilation of CO₂ (6, 8, 9, 14, 17). The relative contributions of photoheterotrophic and of photoautotrophic carbon metabolism in photoheterotrophically cultured cells have not, however, been previously investigated in short term kinetic experiments. The purpose of the present study was, therefore, to investigate the metabolism of CO₂ in cells cultured with L-malate, or with acetate, as the major carbon source for growth, and to assess the relative importance of the growth substrate and of carbon dioxide utilization in the metabolism of such cells.

Materials and Methods

Growth of Bacterium. Rhodospirillum rubrum, strain S-1, was cultured on 50 mM L-malate, 2.5 mM (NH₄)₂SO₄, or 50 mM acetate, 5 mM (NH₄)₂SO₄, biotin, and mineral salts, under an atmosphere of 4% CO₂ in He as previously described (3). A 1% inoculum was used to initiate growth in malate media; a 5% inoculum was used to initiate growth in acetate media.

Experimental Procedure. The experimental procedure was essentially the same as that used previously (3). Cells were preincubated 30 minutes in the light in the presence of 10 µmoles L-malate or acetate and 10 µmoles NaHCO₃ under He, unless otherwise noted. Dark control flasks were draped in aluminum foil during preincubation. Foil was removed and NaH¹⁴CO₃ was added in dim light.

Reagents. L-Malate used for culturing cells was C-grade obtained from Calbiochem. Sodium acetate used both for growth and experimentally was neutralized, steam distilled, J. T. Baker Chemical Company reagent grade acetic acid. Uniformly labelled L-malic acid was obtained from New England Nuclear Corporation: acetic acid-2,¹⁴C, from Nuclear Chicago. Other reagents were as described in the preceding paper.

Results

The results of a ¹⁴CO₂ fixation experiment with cells cultured on L-malate, ammonium sulfate media were similar to those reported previously for L-malate grown photoheterotrophs (2). There

1 This investigation was supported in part by Public Health Research Grant GM-10705-03 from the National Institute of General Medical Sciences. A preliminary report of these findings has been published (1).

2 National Institute of Health Trainee, Training Grant 5T1-GM-961-02.
is a strongly negative slope for percent incorporation into glycolic acid (Fig 1). After 3 seconds only 1 phosphate ester had activity; this spot corresponded to P-enolpyruvate. Malate was detect-

able on the radioautogram after 3 seconds. The slope for percent incorporation into malate was initially positive but became negative. Likewise plots of percent incorporation into alanine, succinic, and fumaric acids versus time gave slopes which were initially positive.

The total incorporation of $^{14}$C in the 5 minute dark control, in this experiment, was the same as that in the light. Of the incorporated counts 69% were found in succinic acid and 7% in phosphate esters. The remainder of the activity was spread among the compounds which were labelled in the light experiment. The pattern of anaerobic dark $^{14}$CO$_2$ fixation was completely dissimilar to $^{14}$CO$_2$ fixation in the light.

The results of an experiment with labelled l-malate and $^{14}$CO$_2$ are shown in Table I. In this experiment all of the conditions, save the location of the radioactive carbon atoms, were duplicated in the 2 sets of flasks. The relative pool sizes of the compounds labelled must be, within the limits of experimental error, the same in both samples. Hence the activity incorporated into any 1 compound from l-malate-$^{14}$CO$_2$ can be directly compared with the activity incorporated into the same compound from $^{14}$CO$_2$ in the corresponding sample. After 30 seconds the relative incorporation into phosphate esters from l-malate was 210-fold higher than that from CO$_2$. Clearly l-malate is a much more important precursor of phosphate ester carbon than is CO$_2$ under these conditions.

The results of an experiment in which $^{14}$CO$_2$ was administered to cells cultured on acetate, ammonium sulfate are shown in figures 2a, b, and c. After 3 seconds activity was found in several compounds, including aspartic, fumaric, citric, glutamic, and malic acids, alanine, and 1 phosphate ester, which appeared to be 3-phosphoglyceric acid (3-PGA) by location on chromatogram. The percent incorporation into phosphate esters plotted against time had a positive slope; negative slopes were obtained for percent incorporation into aspartic and succinic acids. The relative percentage of

![Image of Figure 1: Distribution of activity incorporated from $^{14}$CO$_2$ into phosphate esters, glycolic acid, and malic acid with time in cells cultured on l-malate, ammonium sulfate media. Each manometer flask contained, in 2 ml, 10 μmoles l-malic acid, 10 μmoles NaHCO$_3$, and cells equivalent to 30 mg dry weight suspended in dilute buffer solution. After 30 minutes preincubation under He in the light, 2 μmoles, 100 μc NaH$^{14}$CO$_3$ was added to the side arm and tipped into the flask. Volumes were quadrupled in the 3 and 12 second runs. Optical density of the culture when harvested was 2.12. After 1 minute a total of 445,000 dpm (15,000 dpm/min/mg dry wt cells) had been incorporated, 2% being in the alcohol and water insoluble precipitate. Are results from an experiment with glutamate, malate photoheterotrophs (2).

Table I. Relative Incorporation of $^{14}$C from Malate and from CO$_2$ Into Phosphate Esters and Glutamate in Malate Photoheterotrophs

<table>
<thead>
<tr>
<th>Administered compound</th>
<th>Cpm incorporated into phosphate esters</th>
<th>Cpm incorporated into glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 sec</td>
<td>5 min</td>
</tr>
<tr>
<td>l-Malate-$^\mu$-$^{14}$C</td>
<td>43,300</td>
<td>586,000</td>
</tr>
<tr>
<td>$^{14}$CO$_2$</td>
<td>880</td>
<td>10,350</td>
</tr>
<tr>
<td>Relative incorporation malate carbon/CO$_2$ carbon</td>
<td>210</td>
<td>250</td>
</tr>
</tbody>
</table>
activity incorporated into glutamic acid is much higher in acetate cells than in either malate photoheterotrophs or in autotrophically cultured cells. The initial incorporation of activity in both malate and aspartate was relatively high (19 and 16 % respectively after 3 seconds). There was no detectable activity in glycolic acid.

When the relative incorporation of activity from acetate-2-14C and from 14CO₂ under essentially identical conditions was measured, the results given in Table II were obtained. The incorporation of activity into tricarboxylic acid cycle acids or derivative amino acids, excluding glutamate and succinate, was 2 to 4-fold higher from acetate-2-14C than from 14CO₂. This is indicative of the operation of the light-dependent anaerobic tricarboxylic acid cycle (10). The relative incorporation of activity into glutamate and succinate from acetate-2-14C was disproportionately high. The relative labelling of α-ketoglutarate, however, is similar to that of the remaining tricarboxylic acid cycle acids. These data indicate that glutamate is derived neither from α-ketoglutarate, in agreement with the glutamate degradation data of Hoare (12), nor from succinate, and that succinate is derived, in part, from glutamate.

H₂ gas and a variety of carbon compounds were tested for possible effects on the products of 1

Fig. 2a (upper). Distribution of activity incorporated from 14CO₂ into phosphate esters and malic acid with time in cells cultured on acetate, ammonium sulfate media. Each manometer flask contained, in 2 ml, 10 μmoles sodium acetate, 10 μmoles NaHCO₃, and cells equivalent to 26 mg dry weight suspended in dilute buffer solution. Volumes were quadrupled in the 3 and 8 second runs. After 30 minutes preincubation under He in the light 50 μc, 1 μmole NaH¹⁴CO₃ was added to the side arm and tipped. After 1 minute 506,000 dpm (19,500 dpm/min/mg dry wt cells) had been incorporated, 15 % of the activity being found in the precipitate. The OD₆₅₀ of the cells when harvested was 1.88.

Fig. 2b (lower). Distribution of activity incorporated into acids of the tricarboxylic acid cycle in cells cultured on acetate, ammonium sulfate media. Results from the experiment described in Fig. 2a were analyzed in this manner.

Fig. 2c. Distribution of activity incorporated into amino acids in cells cultured on acetate, ammonium sulfate media. Results from the experiment described in Fig. 2a were analyzed in this manner.
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Table II. Distribution of $^{14}$C from $^{14}$CO$_2$ and from Acetate-2-$^{14}$C after 3 Minutes

Each flask contained, in 2 ml, 10 $\mu$moles NaHCO$_3$ and cells equivalent to 33 mg dry weight in dilute buffer solution. After 30 minutes preincubation in the light 10 $\mu$moles sodium acetate, 2 $\mu$moles, 100 $\mu$C NaH$^{14}$CO$_3$ were added to 1 flask and 3.3 $\mu$moles, 33 $\mu$C sodium acetate-2-$^{14}$C to the other. The experiment, which was performed in the light, was terminated after 3 minutes. Cells were cultured on 50 mM sodium acetate, 5 mM (NH$_4$)$_2$SO$_4$. The OD$_{660}$ when harvested was 1.56.

<table>
<thead>
<tr>
<th>Isolated product</th>
<th>cpm From acetate-2-$^{14}$C*</th>
<th>cpm From $^{14}$CO$_2$*</th>
<th>Relative incorporation acetate-2/$^{14}$CO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>1400</td>
<td>570</td>
<td>2</td>
</tr>
<tr>
<td>$\alpha$-Ketoglutarate</td>
<td>8170</td>
<td>1440</td>
<td>4.5</td>
</tr>
<tr>
<td>Glutamate**</td>
<td>239,000</td>
<td>13,320</td>
<td>14</td>
</tr>
<tr>
<td>Succinate</td>
<td>30,800</td>
<td>1520</td>
<td>16</td>
</tr>
<tr>
<td>Fumarate</td>
<td>2610</td>
<td>540</td>
<td>3.8</td>
</tr>
<tr>
<td>Malate</td>
<td>5230</td>
<td>1410</td>
<td>3</td>
</tr>
<tr>
<td>Aspartate</td>
<td>4450</td>
<td>1560</td>
<td>2.3</td>
</tr>
<tr>
<td>Phosphate esters</td>
<td>108,000</td>
<td>34,000</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* cpm On chromatogram corrected to give total cpm in compound.

** Approximately 80% of the activity incorporated from $^{14}$CO$_2$ was located in the carboxyl carbon of glutamic acid (only 20% of the activity remained after ninhydrin treatment). Only 4% of the activity derived from acetate-2-$^{14}$C was located in the carboxyl carbon of glutamate. If one compares the activity remaining on the paper after ninhydrin treatment the relative incorporation into carbons 2 through 5 is 63.

Table III. Effect of Exogenous Substrates on One Minute $^{14}$CO$_2$ Fixation

Each manometer flask contained, in 2.0 ml, 10 $\mu$moles of the indicated substrate, 10 $\mu$moles NaHCO$_3$, and cells suspended in dilute buffer solution. 1 $\mu$ mole, 50 $\mu$C NaH$^{14}$CO$_3$ was added from the side arm. Gas phase: He. OD$_{660}$ of the malate culture when harvested was 1.32; 28 mg dry weight cells were used per vessel. OD$_{660}$ of the acetate culture was 1.9; 45 mg dry weight cells were used per vessel. Preincubation was 30 minutes in the light.

<table>
<thead>
<tr>
<th>Growth carbon source</th>
<th>Acetate</th>
<th>t-Malate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exogenous substrate</td>
<td>Total Incorporation Phosphate esters</td>
<td>Total Incorporation Phosphate esters</td>
</tr>
<tr>
<td></td>
<td>dpm/$\mu$C added mg$^{-1}$ dry wt of cells</td>
<td>dpm/$\mu$C added mg$^{-1}$ dry wt of cells</td>
</tr>
<tr>
<td>L-Malate</td>
<td>160</td>
<td>91</td>
</tr>
<tr>
<td>Acetate</td>
<td>170</td>
<td>81</td>
</tr>
<tr>
<td>None</td>
<td>130</td>
<td>64</td>
</tr>
</tbody>
</table>

minute CO$_2$ fixation in acetate and malate grown cells (table III). Previously Ormerod (15) and Glover and Kamen (11) have observed that acetate inhibits fixation of $^{14}$CO$_2$. Acetate quantitatively lowered incorporation into phosphate esters in t-malate phototrophs but not in acetate phototrophs. Clearly the effect of exogenous compounds on CO$_2$ fixation by resting cell suspensions is mediated by the nature of the carbon source during growth.

Discussion

The pattern of CO$_2$ fixation in malate phototrophs is distinctly different from that of autotrophs. These experiments provide no evidence for the operation of the reductive pentose phosphate cycle in R. rubrum cultured on t-malate in the light. The positive slope for percent incorporation of activity from $^{14}$CO$_2$ into phosphate esters, the lower total percent of activity found in phosphate esters, and the observation that the first phosphate ester labelled was P-enolpyruvate indicate that fixation of CO$_2$ by ribulose-diphosphate carboxylase can be only of minor importance in the metabolism of CO$_2$ by this organism when cultured photoheterotrophically on t-malate.

The percent incorporation of activity into glycolate plotted against time had a strikingly negative slope. A preliminary report of the kinetics of incorporation of $^{14}$CO$_2$ into this compound in cells cultured on t-malate, glutamate media has been published (2). Clearly in cells cultured on t-malate, glycolate is a primary product of incorporation of activity from $^{14}$CO$_2$. The kinetics of $^{14}$CO$_2$ incorporation suggest that glycolate gives rise to malate (fig 1). Reactions are known for the conversion of glycolic acid to t-malate. Malate synthetase has been shown in this organism (18) and glycolic acid oxidase has been shown in other photosynthetic organisms (16). Apparently one of the major pathways of incorporation of label from
\(^{14}\)CO\(_2\) in this case is through glycolic acid into \(\alpha\)-malate and hence into intermediates of the tri-carboxylic acid cycle and into phosphate esters. \(\alpha\)-malate is apparently converted to an early intermediate of the Embden-Meyerhof pathway and gluconeogenesis, with malate as the ultimate carbon source, ensues. Similar conclusions were reached by Stanier, et al., in experiments with isotopically labelled succinate (17).

Although in *R. rubrum* cultured photoheterotrophically on \(\alpha\)-malate one of the major pathways of photometabolism of CO\(_2\) is through glycolic acid, the fixation of CO\(_2\) by this pathway, as compared to direct utilization of malate, is of little overall significance in photoheterotrophic carbon metabolism. It is evident that the reductive pentose phosphate cycle plays essentially no part in the carbon metabolism of this organism under these conditions.

The distribution of activity when \(^{14}\)CO\(_2\) was administered to acetate photoheterotrophs was quantitatively and qualitatively dissimilar to that obtained either with autotrophs or with \(\alpha\)-malate photoheterotrophs. Apparently several carboxylation reactions occur when acetate is the sole carbon source. Although the first phosphate ester to become labelled was 3-PGA the positive slope of percent incorporation into phosphate esters indicates that the contribution of the reductive pentose phosphate cycle to the overall metabolism of CO\(_2\) is limited. Negative slopes of percent incorporation were obtained for aspartate and succinate. Carboxylation of pyruvate, mediated by pyruvic carboxylase, an enzyme which apparently occurs in *Chromatium* (7), to give oxaloacetate, and transamination (13), or a similar series of reactions, would account for the labelling of aspartic acid. Succinate could arise through carboxylation of propionyl-CoA catalyzed by propionyl-CoA carboxylase, an enzyme which has been shown in this organism (14), or from oxaloacetate via reactions of the tricarboxylic acid cycle. Incorporation into alanine is low and the slope for percent incorporation is positive, which eliminates pyruvic synthetase (5) as an important enzyme for CO\(_2\) fixation in acetate grown cells.

The ability of this organism, which lacks isocitritase, to grow photoheterotrophically with acetate as the sole organic carbon source has not been previously explained. The only known mechanisms for the conversion of acetyl units to intermediates of the Embden-Meyerhof pathway are the glyoxylate cycle and conversion of acetate to pyruvate through the action of pyruvic synthetase (5). The results of the present experiment suggest that fixation of CO\(_2\), either through the pentose phosphate reduction cycle or possibly through glycolate, makes growth on acetate possible. We have been unable to obtain growth of this organism on acetate in the dark when the nitrogen source was ammonium sulfate in the completely synthetic media of Ormerod, et al. (unpublished observation). Further evidence for the participation of the reductive pentose phosphate cycle in the photometabolism of the acetate heterotroph is presented elsewhere (4).

It is apparent from these experiments that in this photosynthetic bacterium the photosynthetic carbon metabolism is entirely altered by, and dependent upon, environmental metabolic variables.

**Acknowledgment**

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**Literature Cited**


