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Photosynthesis in Rhodospirillum rubrum
I. Autotrophic Carbon Dioxide Fixation[1]

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Summary. The incorporation and distribution of activity from $^{14}CO_2$ was investigated under autotrophic conditions in the facultative photoautotroph, *Rhodospirillum rubrum*, with cells cultured on hydrogen, carbon dioxide, and ammonium sulfate. In 1 second $^{14}CO_2$ fixation experiments essentially all of the activity was found in 3-phosphoglyceric acid; plotted against time percent incorporation into phosphate esters has a strikingly negative slope. These results suggest that under autotrophic conditions the reductive pentose phosphate cycle or the key reactions of the cycle play a major role in carbon metabolism in this photosynthetic bacterium. Incorporation into amino acids and into intermediates of the tricarboxylic acid cycle was quite low.

The photosynthetic bacterium, *Rhodospirillum rubrum*, one of the Athiorhodaciae, can be grown under a number of dissimilar conditions: photoheterotrophically, when provided with a suitable carbon source; heterotrophically in the absence of light, and aerobically, on the same medium; or photoautotrophically on molecular hydrogen, CO_2, and nitrogen gas or another inorganic nitrogen source.[3] This organism is a truly facultative phototroph, as distinct from those photosynthetic bacteria, such as *Chromatium* and *Chlorobium*, which are able to grow photoheterotrophically or photoautotrophically, but are not able to grow in the absence of light. It is, therefore, ideally suited for an investigation of the variability in, and control of, carbon metabolism accompanying heterotrophic and autotrophic growth.

Photosynthetic CO_2 fixation has been shown to occur through the reductive pentose phosphate cycle in plants, algae, and chemoautotrophic bacteria. Since the rate of photosynthetic CO_2 fixation is much lower in the photosynthetic bacteria (9) than in green plants or algae (11), strictly analogous experiments using short term kinetic analysis to show the operation of the reductive pentose phosphate cycle under either photoautotrophic or photoheterotrophic conditions in the bacteria have not been performed previously. After 30 seconds both 3-phosphoglyceric acid (3-PGA) and aspartic acid appeared to be primary products of CO_2 fixation in autotrophically cultured *Chromatium* (7). The key enzymes of the reductive pentose phosphate cycle are present in this organism (7). Recently a ferredoxin-dependent CO_2 fixation cycle distinct from the reductive pentose phosphate cycle has been proposed to account for autotrophic growth and bacterial photosynthesis in *Chlorobium* (6). The purpose of the present experiments, with *Rhodospirillum rubrum*, was to investigate, using chromatographic techniques, the kinetics of $^{14}CO_2$ incorporation under strictly autotrophic conditions in short term experiments. The results obtained are consistent with the operation of the reductive pentose phosphate cycle (or of the key reactions of the cycle) in such cells under strictly photoautotrophic conditions.

Materials and Methods

Growth of Bacterium. *Rhodospirillum rubrum*, strain S-1, was obtained from Dr. Howard Gest. The medium was 2.5 mM in $(NH_4)_2SO_4$ and contained the same levels of minerals, trace elements, and biotin, as that employed by Ormerod, et al., (10), except that the phosphate concentration was 10 mM. The inoculum was cultured and transferred as described by Ormerod, et al.; a 10 % inoculum was used for autotrophic growth. Cells were grown in Roux bottles in a water bath at 30° and were bubbled continuously with 1 % CO_2 in H_2. The light source consisted of 3 lumiline 60

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[3] For simplicity the term "autotrophic" is used here to describe photolithotrophic growth; "Photoheterotrophic" to describe photoorganotrophic growth; "Heterotrophic" to describe dark, aerobic, organotrophic growth. Biotin is required for growth in all cases.

watt lights approximately 15 cm from the culture yielding approximately 1000 foot candles of incident light. Growth was measured by turbidity at 650 mμ. One OD_{650} unit equals 0.353 mg dry weight per ml.

Experimental Procedure. Cells were harvested by centrifugation and re-suspended in a pH 7 buffer solution consisting of the minerals, trace elements, and phosphate used in the original media, diluted 10-fold. For experiments over 10 seconds in duration Warburg flasks, gassed with hydrogen (except as indicated), were used. For shorter term experiments 125 ml Erlenmeyer flasks with a small side opening closed with a rubber cap which permitted the injection of radioactive bicarbonate from a syringe were employed. The depth of the cell suspension was the same in either case. Cells were preincubated for 30 minutes in the light with $NaH^{12}CO_3$ prior to the addition of radioactive $NaH^{14}CO_3$. Temperature was controlled at 30°. The light source consisted of 10 photoenlarger bulbs, General Electric number 212, 150 watt, 115 to 125-volt. Experiments were terminated by the addition of sufficient bioling absolute ethanol to bring the final alcohol concentration to 80 %. Extracts were analyzed chromatographically by the methods of Benson, Bassham, and Calvin (5).

Radioactive spots on duplicate chromatograms were counted using a thin-window Geiger tube. The total activity used in computation of the percent of incorporation into any specific compound was the sum of the radioactivity measured in the spots on the paper and not the activity of the origin before development of the chromatogram, since the origin was self absorbent with respect to counting. Occasionally a few counts remained on the origin after development. These were not included in the tabulation of counts.

Reagents. High specific activity (39 to 46 mc/mM) $Ba^{14}CO_3$ was obtained from New England Nuclear Corporation; $NaH^{14}CO_3$ was formed by trapping, in NaOH, $^{14}CO_2$ which had been generated by the addition of concentrated H_2SO_4 to the barium salt. Phenol, used in the development of chromatograms, was freshly redistilled. All other chemicals were analytical reagent grade.

Results

The highest level of carbon dioxide fixation was found with young cells in the log phase of growth. There was a sharp decline in fixation as the stationary phase was approached. In preliminary experiments the relationship of culture age to rates of CO_2 fixation was a critical factor in obtaining optimal and reproducible results (fig 1). Under the conditions used in these experiments fixation was linear for 10 minutes indicating that steady state conditions prevailed.

The distribution of activity in the photosynthate

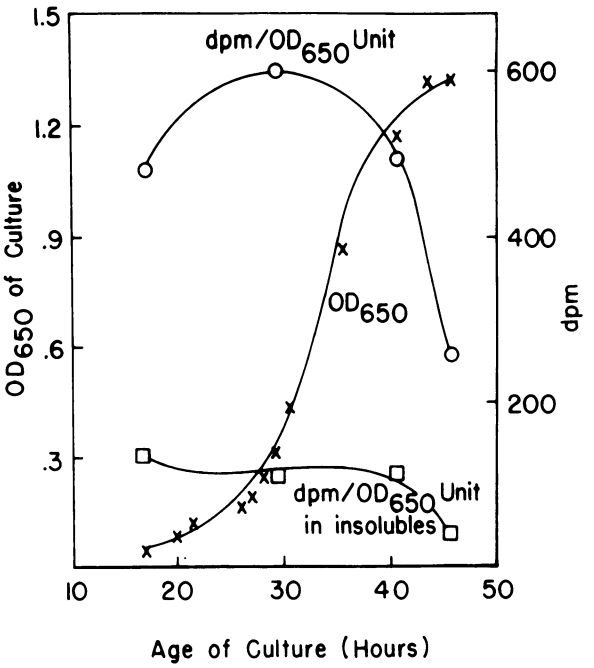

FIG. 1. One minute incorporation of $^{14}CO_2$ in the light versus culture age. Cells used in the experiment (strain R_1 grown on 2.5 mM $(NH_4)_2SO_4$, 25 mM DL-malate under 4 % CO_2, He from a 1 % inoculum) were harvested at times indicated, and resuspended in buffer solution. Each manometer flask contained 100 μmoles L-malate, 10 μmoles $NaHCO_3$, cells equal to approximately 0.4 mg dry weight, and in the side arm, 2 μc $NaH^{14}CO_3$. Gas phase was 4 % CO_2 in He. Experiment was terminated 1 minute after introduction of $NaH^{14}CO_3$. Upper curve is total dpm incorporated/OD_{650} unit. Lower curve is dpm/OD_{650} unit in ethanol and water insoluble precipitate. Similar results were obtained with cells grown autotrophically and photoheterotrophically with acetate as carbon source.

of autotrophically cultured *R. rubrum* fixing $^{14}CO_2$ under an atmosphere of H_2 is presented graphically in figures 2a and b.

When the percent incorporation of isotope into phosphate esters is plotted against time the slope of the curve is strongly negative (fig 2a). The first products noted on the chromatograms were 3-PGA and sugar diphosphate. 75 % Of the activity at 1 second was in 3-PGA. After 1 second several phosphate esters became labelled. In 11 seconds there were 8 spots in the phosphate ester portion of the chromatogram containing 129 counts (70 % of the activity fixed into the soluble portion): 1 spot corresponding to sugar diphosphate, 5 to monophosphates, and 2 to P-enolpyruvate and 3-PGA. Percent incorporation into 3-PGA versus time was negative between 1 and 30 seconds (75 %–20 %); percent incorporation into the diphosphate area was positive.

The initial increase in labelling in alanine and glutamate (fig 2b) corresponds to incorporation

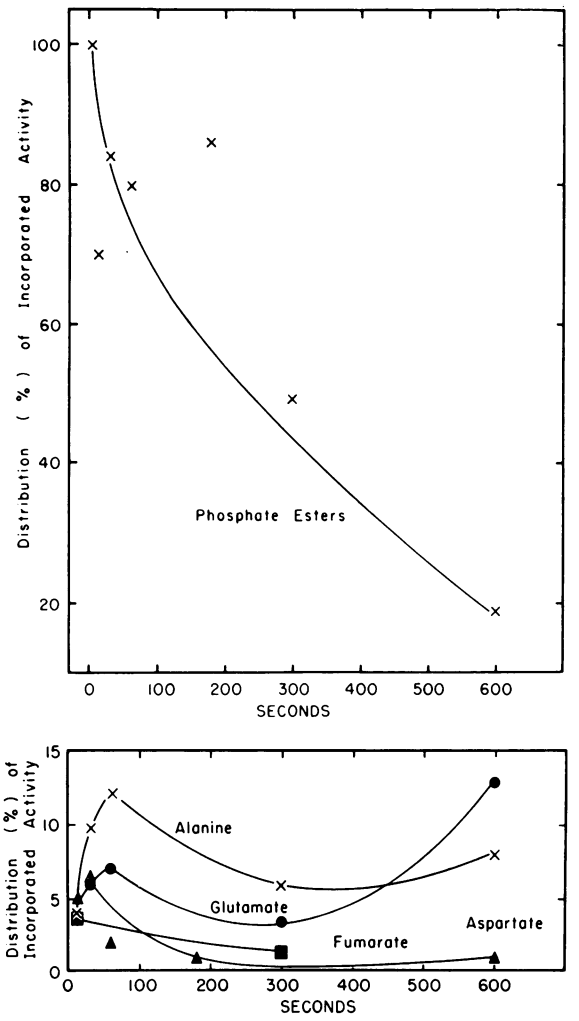

FIG. 2. (a) (upper) Distribution of activity incorporated into phosphate esters versus time of exposure to $^{14}CO_2$. Each manometer flask contained cells, equal to 8.2 mg dry weight, suspended in buffer solution, 11 μmoles $NaHCO_3$ and 50 μc $NaH^{14}CO_3$ in a total volume of 2 ml. All the components except $NaH^{14}CO_3$ were quadrupled in the 1 second experiment. 100 μc $NaH^{14}CO_3$ were used in the 1 second and 11 second experiments. The OD_{650} of the cells when harvested was 0.73. After 1 minute 154,200 dpm (18,800 dpm/min/mg dry wt cells) had been incorporated. Of the activity 10 % was incorporated into the water insoluble precipitate after 10 minutes; 7 % after 5 minutes, none after 1 second. The 180 second point deviates more than 3 times the standard error (10 %) from the curve and may therefore not be significant. The first products noted on the chromatogram appeared by location to be 3-PGA and a diphosphate, most of the counts being in 3-PGA. (b) (lower) Distribution of activity incorporated into amino acids and fumarate versus time of exposure to $^{14}CO_2$. Very low levels of activity were observed in malate and α-ketoglutarate after 1 and 3 minutes. Data from the experiment illustrated in figure 2a.

into the carboxyl carbon since most (85 % of activity fixed into glutamate after 30 seconds; 70 % of activity in alanine) of the activity was lost with ninhydrin treatment. Most of the radioactivity found in these compounds after 10 minutes was stable to ninhydrin (65 % of activity in glutamate; 75 % of activity in alanine), indicating that during longer periods of exposure the internal carbons in these compounds had become labelled. Very low levels of activity were found in malate and in α-ketoglutarate after 1 minute and in citrate and succinate after 3 minutes.

Discussion

The reductive pentose phosphate cycle is clearly implicated in the pattern of photosynthetic CO_2 fixation observed in this organism. The negative slope of incorporation into phosphate esters, the observation that 3-PGA is labelled prior to other compounds, the secondary labelling of the diphosphate ester area of the chromatogram are all consistent with the operation of the key steps of the cycle. In addition recently we have observed extremely high levels of ribulose-diphosphate carboxylase activity in extracts of autotrophically grown *R. rubrum* and have demonstrated the presence of several of the enzymes of the reductive pentose phosphate cycle. These experiments are reported in the third paper in this series (4).

Since the incorporation of radioactivity into tricarboxylic acid cycle intermediates is low, an anaerobic tricarboxylic acid cycle as proposed by Gest et al. (8) for photoheterotrophic metabolism in *R. rubrum* probably does not operate under strictly photoautotrophic conditions.

In addition to the above observation, the positive slopes of incorporation, and the low activity in alanine and glutamate rule out any important participation in this organism of the ferredoxin-dependent cycle proposed recently by Evans, Buchanan, and Arnon (6) to account for photosynthetic CO_2 fixation of *Chlorobium thiosulfatophilum*. The only known or postulated pathway for net CO_2 fixation consistent with these data is the reductive pentose phosphate cycle.

In *R. rubrum* cultured on L-malate, glycolic acid appears to be the first stable product of carbon dioxide fixation under photoheterotrophic conditions (2, 3). Apparently 2 pathways for the uptake of CO_2 occur in *R. rubrum*, one, involving 3-PGA, which accounts for the major part of CO_2 fixation in autotrophically grown cells, and the other, involving glycolic acid, in photoheterotrophically grown cells.

Clearly the nature of the carbon source during growth controls and influences both the kinetic pattern of fixation and the photometabolism of CO_2 in this organism.

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