Reductive Pentose Phosphate Cycle in *Nitrosocystis oceanus*¹

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ABSTRACT

CAMPBELL, ANN E. (Woods Hole Oceanographic Institution, Woods Hole, Mass.), JOHAN A. HELLEBUST, AND STANLEY W. WATSON. Reductive pentose phosphate cycle in *Nitrosocystis oceanus*. J. Bacteriol. 91:1178-1185. 1966.—Assays in cell-free extracts of *Nitrosocystis oceanus*, a marine chemosynthetic bacterium, have demonstrated the presence of all of the enzymes of the reductive pentose phosphate cycle, with activities high enough to account for the normal growth rate of the cells. Studies on ribulosediphosphate carboxylase activity in these extracts showed that it is inhibited by MgCl₂ (30% at 0.01 M), MnCl₂ (70% at 0.01 M), NaCl and KCl (100% at 0.5 M, 63% at 0.2 M), and by sulfate (35% at 0.01 M); phosphate, glutathione, and ethylenediaminetetraacetic acid had no effect. The bacterial enzyme differs from the spinach enzyme with respect to its affinity for bicarbonate and its pH optimum. Whole cells were incubated with C⁴O₂, and the acid-soluble fraction was analyzed by paper chromatography and autoradiography. Phosphoglyceric acid and the sugar phosphates were the earliest labeled compounds; several amino acids and organic acids were also labeled. It is concluded that *N. oceanus* incorporates CO₂ primarily via the reductive pentose phosphate cycle.

*Nitrosocystis oceanus* is a nitrifying bacterium isolated by Watson (27) from the open ocean. It is an obligate chemosynthetic organism using carbon dioxide and ammonia as sole sources of carbon and energy.

The reductive pentose phosphate cycle has been established as the path of carbon dioxide fixation in photosynthetic organisms (7). Studies on some autotrophic bacteria have indicated that they too incorporate carbon dioxide via this pathway.

Suzuki and Werkman (22) incubated *Thiobacillus thiooxidans* with C⁴O₂ for various time intervals, finding most of the label in the carboxyl group of 3-phosphoglycerate acid (PGA) after 2 sec; at the end of 10 sec, 80% of the activity was in PGA, glucose phosphates, and aspartic acid. The distribution of label in these compounds conformed to that predicted by the cycle. Suzuki and Werkman found that cell-free extracts could fix C⁴O₂ in the presence of ribose-5-phosphate, Mg²⁺, and adenosine triphosphate (ATP), indicating the existence of ribosephosphate isomerase, phosphoribulokinase, and ribulosediphosphate (RuDP) carboxylase. Phosphoglycerate kinase, glyceraldehydephosphate dehydrogenase, fructosephosphate aldolase, and triosephosphate isomerase activities were also found in the extracts, with indications of the presence of transketolase, transaldolase, and ribulosephosphate-3-epimerase.

In another strain of sulfur bacteria, *Thiobacillus denitrificans*, Aubert, Milhaud, and Millet (2) identified labeled compounds in whole cells exposed to C⁴O₂ for various times. An analysis of the kinetics of incorporation showed that the time variation in the amount of label in each intermediate could be predicted by the reductive pentose phosphate cycle. In addition, they found that in the absence of CO₂ RuDP built up, whereas in the presence of CO₂ and absence of thiosulfate RuDP was consumed and PGA accumulated.

Similar experiments on *Thiobacillus thioparus* (21, 26), *Hydrogenomonas facilis* (10), *Nitrobacter agilis* (15), and *Nitrosomonas europaea* (17) have established that the reductive pentose phosphate cycle is operative in these organisms and is the major pathway of carbon dioxide incorporation.

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The above investigations utilized three approaches: (i) the kinetics of appearance of label in the intermediates, (ii) the distribution of C¹⁴ in selected intermediates, and (iii) enzyme assays. The most complete enzyme work has been done on the thiobacilli, but even this was not quantitative. Hence, we undertook a quantitative study of the enzymes of the reductive pentose phosphate cycle in _N. oceanus_, with special emphasis on RuDP carboxylase, the enzyme catalyzing the incorporation of carbon dioxide into the cell. Our objectives were: (i) to demonstrate the presence of these enzymes in cell-free extracts and to correlate their activities with the observed rate of CO₂ fixation in whole cells and with the growth rate of the cells; (ii) to investigate the properties of RuDP carboxylase, specifically its substrate affinities and cofactor requirements, and to compare these with the properties of RuDP carboxylase in higher plants; and (iii) to show that these enzymes operate in the intact organism by demonstrating that whole cells incorporate C¹⁴O₂ into PGA and other intermediates of the reductive pentose phosphate cycle.

**Materials and Methods**

*_N. oceanus_ was cultured in 30-liter pH-stat fermentors as described by Watson (27).

**Preparation of the cell-free extract.** The bacteria were harvested by continuous-flow centrifugation, and were washed in filtered seawater. About 2 g of cells (wet weight, equivalent to approximately 400 mg of cell protein) were suspended in 10 ml of tris-(hydroxymethyl)aminomethane (Tris) buffer (0.05 M, pH 7.8), and disrupted by pressure (Amino French pressure cell; 16,000 psi). The suspension was centrifuged in the cold for 20 min at 20,000 × g, and the supernatant fluid was used in the enzyme assays. The protein content of the extract was determined by the Folin-Ciocalteau method (14) and varied from 20 to 40 mg of protein per ml of extract.

**Enzyme assays.** Most of the assays involved directly, or were coupled to, the oxidation or reduction of the nicotinamide adenine dinucleotides. These assays were carried out in a total volume of 1 ml in 1-cm cuvettes. The change in absorbance at 340 mμ was recorded with a Bausch & Lomb Spectronic-505 ratio-recording spectrophotometer against a distilled water blank. (The amount of extract was adjusted to give rates of change of absorbance of 0.1 to 0.5 optical density units per min. The total change recorded was about one optical density unit. Controls from which the substrate was omitted were always run, and the assays were conducted at two extract concentrations.) The following systems were used (amounts are given in micromoles per milliliter).

(i) The phosphoglycerate kinase and glyceraldehydephosphate dehydrogenase system of Beisenherz et al. (8) contained: triethanolamine (pH 7.6), 50; ethylenediaminetetraacetic acid (EDTA), 5; MgSO₄, 3; glutathione (GSH), 2; ATP, 2; reduced nicotinamide adenine dinucleotide (NADH₂), 0.1; PGA, 8; phosphoglycerate kinase or glyceraldehydephosphate dehydrogenase; and extract. The nucleotide specificity of glyceraldehydephosphate dehydrogenase was examined by substituting reduced nicotinamide adenine dinucleotide phosphate (NADPH₂) for NADH₂.

(ii) The triosephosphate isomerase system of Meyer-Arendt, Beisenherz, and Bücher (16) contained triethanolamine (pH 7.6), 50; EDTA, 5; NADH₂, 0.1; glyceraldehyde-3-phosphate, 2; glyceraldehyde-3-phosphate dehydrogenase; and extract.

(iii) The aldolase system of Beisenherz et al. (8) contained triethanolamine (pH 7.6), 50; EDTA, 5; NADH₂, 0.1; fructose-1,6-diphosphate, 5; triosephosphate isomerase; glycerolphosphate dehydrogenase; and extract.

(iv) The fructosediphosphatase system of Racker and Schroeder (20) contained Tris (pH 8.8), 100; MgCl₂, 5; EDTA, 0.12; nicotinamide adenine dinucleotide phosphate (NADP), 0.1; fructose-1,6-diphosphate, 5; glucosephosphate isomerase; glucose-6-phosphate dehydrogenase; and extract.

(v) The transketolase system contained glyoxylate (pH 7.5), 9; cysteine, 1.5; NADH₂, 0.1; ribose-5-phosphate, 10; triosephosphate isomerase; glycerolphosphate dehydrogenase; ribosephosphate isomerase; ribulosephosphate-3-epimerase; and extract.

(vi) The transaldolase system of Venkataraman and Racker (25) contained Tris (pH 7.8), 50; MgCl₂, 10; KCl, 40; GSH, 5; ATP, 5; NADH₂, 0.1; phosphonooxypurinic acid, 5; ribose-5-phosphate, 5; ribosephosphate isomerase; pyruvate kinase; lactate dehydrogenase; and extract.

**Nonspectrophotometric assays.** RuDP carboxylase activity was determined from the amount of radioactivity incorporated when the extract was incubated with Na₂CΟ₃ and RuDP, by a modification of the methods of Horecker, Hurwitz, and Weissbach (11). The reaction mixture contained 25 μmoles of Tris (pH 7.8), 0.05 μmole of EDTA, 2.5 μmoles of MgCl₂, 2.5 μmoles of GSH, 2.5 μmoles of NaHCO₃, 1 μ of NaC₅O₄, 1.5 μmoles of RuDP, and extract, in a final volume of 250 μlitters. After 10 min, the reaction was stopped with 25 μliters of 1 N HCl. The mixture was aerated to drive off unreacted CO₂, a 25-μliter sample was plated and dried, and its radioactivity was counted with a Nuclear-Chicago model D47 gas-flow detector. The amount of extract was adjusted to give an incorporation of several hundred counts per minute per 10-μliter sample.

The reaction system used contained only a small amount of air above the reaction mixture. The gaseous CO₂ in this air phase did not under any condition amount to more than 10% of the amount of bicarbonate in the reaction mixture. Possible errors in the
determination of RuDP carboxylase rates due to the dilution of the specific activity of the labeled bicarbonate by unlabeled CO₂ in the air phase must therefore be relatively small.

In the control assay (no RuDP), incorporation was less than 10% of the incorporation in samples with RuDP. Furthermore, the amount of C⁴ incorporated was proportional to the amount of extract used. Thus we concluded that we were measuring the carboxylation of RuDP by RuDP carboxylase.

Ribosephosphate isomerase was assayed by the method of Axelrod and Jang (3).

Incorporation of C⁴O₂ into whole cells. Two methods were used to expose whole cells to labeled carbon dioxide for short periods of time. The earlier involved a relatively large reaction volume (7 ml). Cells were harvested by centrifugation, washed in filtered seawater or in phosphate buffer, and 1 g (wet weight) was suspended in 5 ml of 3% NaCl. A 0.1-mL amount of 0.5 M (NH₄)₂SO₄ (pH 7.8) was added as the ammonia source. After 1 min, 2 ml of 0.01 M NaC⁴O₃ (0.002 μM) were added. The reaction mixture was quenched by the addition of 50% trichloroacetic acid, to a final concentration of 6%. The mixture was aerated to remove unixed C⁴O₂, 5 μmoles each of PGA, ribose-5-phosphate, and fructose-1,6-diphosphate were added as carrier, and the mixture was homogenized in a glass homogenizer and centrifuged in the cold at 20,000 × g for 20 min. The trichloroacetic acid was extracted with ether, and the aqueous layer was adsorbed onto a Dowex 50 (H⁺) cation-exchange column. The sugar phosphates and organic acids were eluted with water, and the amino acids with 2 N NH₄OH. The sugar phosphate fraction was lyophilized, and the residue was taken up in a small volume of water. Samples were spotted on acid-washed Whatman 1 MM paper and chromatographed one-dimensionally, descending, in butanol-propionic acid-water (10:5:7; 9). The amino acid fraction was evaporated to dryness, and the residue was taken up in a small volume of 50% ethyl alcohol. Samples were spotted on unwashed Whatman 1 MM paper and chromatographed two-dimensionally, ascending, in phenol-water and in propanol-water-ethyl acetate (7:2:1).

Radioactive compounds were located by autoradiography, phosphate esters with ammonium molybdate-perchloric acid spray (6), amino acids with ninhydrin (18), and organic acids by the quenching of fluorescence of 8-hydroxyquinoline when the latter compound was added to the solvent and the chromatogram was viewed under ultraviolet light. Radioactive compounds were eluted and chromatographed with known carriers to confirm their identity.

Aspartic acid from the 1-min sample was isolated by paper chromatography and degraded with ninhydrin (24). The carbon dioxide from both carboxyl groups was collected in an ion chamber, and its radioactivity was determined with a Nuclear-Chicago model 6000 Dynacon Electrometer.

The specific rate of CO₂ fixation was much lower than the calculated rate (see Results and Discussion). In an effort to increase this rate, fewer cells were used in later experiments. The bacteria (about 2 mg, wet weight) were collected on a Millipore filter (pore size, 1.2 μ) by gentle filtration. As soon as the filter was dry, suction was stopped, and 200 μl of the culture medium was added. Then 50 to 100 μlitters of 0.04 M NaC⁴O₃ (1 mc/ml) was added, with enough 0.001 N HCl to bring the pH of the system to 7.8. At the end of the incubation period, suction was again applied, and the filter was soaked in 10% trichloroacetic acid. The solution was decanted, and trichloroacetic acid was extracted with ether. The aqueous layer was lyophilized, and the residue was taken up in a small volume of water. Samples were spotted on acid-washed Whatman 3 MM paper and chromatographed with PGA, fructose-6-phosphate, and fructose-1,6-diphosphate, and glutamic and aspartic acids in butanol-propionic acid-water. Phosphate esters and amino acids were located as above. Since few cells were used, the total amount of radioactivity was too small to permit autoradiography. The radioactivity in each compound was determined by eluting it from the paper with 50% ethyl alcohol, evaporating the radioactivity, and counting it with the Nuclear-Chicago gas-flow detector.

Enzymes, substrates, and cofactors. Fructose-6-phosphate, fructose-1,6-diphosphate, α-glyceraldehyde-3-phosphate diethyl-acid, α-erythrose-4-phosphate dimethyl-acetal, 3-phosphoglyceric acid, 2-phospho-enol-pyruvic acid, glyceraldehydephosphate dehydrogenase, lactate dehydrogenase, triosephosphate isomerase, glycerophosphate dehydrogenase, aldolase, phosphoglycerate kinase, and pyruvate kinase were purchased from Calbiochem; ribose-5-phosphate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; and NADH, NADP, and ATP were purchased from Sigma Chemical Co., St. Louis, Mo.

Glyceraldehydephosphate and erythrose-4-phosphate were converted to the sodium salts by the methods of Ballou and Fischer (4) and of Ballou, Fischer, and MacDonald (5), respectively.

RuDP carboxylase (28) and ribosephosphate isomerase (12) were prepared from spinach; ribulose-phosphate-3-epimerase was prepared from rabbit muscle (23). Ribulose-1,5-diphosphate was prepared by the method of Horecker, Hurwitz, and Weissbach (11). The RuDP used in the carboxylase enzyme assays was found to be chromatographically pure when chromatographed one-dimensionally in butanol-propionic acid-water (9).

RESULTS

Enzyme assays. The results of the enzyme assays are shown in Table 1. The carboxylation enzyme was studied in some detail. By varying the amount of NaHCO₃ in the assay for carboxylase and making a Lineweaver-Burk plot from the observed velocities as a function of bicarbonate concentration (Fig. 1), we found the Kₘ for HCO₃⁻ to be 3 × 10⁻². Similarly, by varying the RuDP concentration (Fig. 2), we found the Kₘ for RuDP to be 3 × 10⁻⁴ M. Concentrations of RuDP higher than 10⁻³ M were inhibitory.

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4 × 10^{-3} \text{ M}, the activity was 40\% of the observed maximal rate. The pH profile (Fig. 3) obtained by varying the pH of the Tris, shows that the pH optimum is around 8.6. The enzyme is soluble, or is solubilized in making the extract. Centrifuging the preparation at 144,000 × g for 1 hr and removal of the precipitated material did not decrease the activity. After 3 days of storage at 4 °C, the preparation retained 80\% of its original carboxylase activity. After 4 days, the activity had dropped to 30\%. The enzyme was sensitive to salt. NaCl or KCl at 0.5 M inhibited the activity 100\%; at 0.2 M, the inhibition was 63\%. Other cations and anions tested for inhibition were as follows. Mg^{2+} produced no effect at 0.001 M, 30\% inhibition at 0.01 M, and 85\% inhibition at 0.04 M. Mn^{2+} gave 70\% inhibition at 0.01 M. NH_{4}^{+} had no effect at 0.02 M, phosphate had no effect at 0.01 M, and sulfate produced 35\% inhibition at 0.01 M. GSH and EDTA had little or no effect.

To investigate the Mg^{2+} requirement, we dialyzed the crude bacterial extract against 0.05 M Tris (pH 7.8) and 0.001 M EDTA for 15 hr in the cold. The enzyme was inactivated by the procedure and could not be reactivated with 0.001 M Mg^{2+}, Fe^{3+}, Co^{2+}, Cu^{2+}, GSH, EDTA, biotin, thiamine, or vitamin B_{12}. The dialyzed extract showed no synergistic effect in combination with the undialyzed preparation, so we concluded that the dialysis had irreversibly inactivated the enzyme.

**Incorporation of C^{14}O_{2} into whole cells.** The rate of CO_{2} fixation in the early experiments was about 10^{-5} \text{ mol per mg (wet weight)} per min. Incubation times were 1 and 5 min. Estimates of relative amounts of radioactivity appearing in various compounds are given in Table 2. The degradation of aspartic acid after elution from the chromatograms showed that all of the activity was in the two carboxyl groups.

In the later experiments, a maximal rate of CO_{2} fixation of 6 × 10^{-4} \text{ mol per mg (wet weight)} per min was achieved. The incubation times in experiment 1 were 15 and 30 sec; in experiment 2, they were 1 and 5 min. The results from these experiments are given in Table 3. Only the radioactivity in compounds coincident with carrier hexose mono- diphosphates, PGA, aspartic, and glutamic acids, and the total recovered radioactivity on the chromatograms are presented. Most of the radioactivity (>75\%) at 15 and 30 sec was found to be either coincident with or close to the added carrier PEP or sugar diphosphates. After 5 min of C^{14}O_{2} assimilation, about 30\% of the recovered radioactivity on the chromatogram was found in positions of the chromatogram roughly corresponding to those of pyruvic and malic acids. Less than 5\% of the label was in aspartic and glutamic acids after 5 min.

**Discussion**

We estimated the rate at which healthy cells in the log phase of growth fix CO_{2} as follows. Assuming that 5\% of the wet weight is carbon, and using the observed generation time of 24 hr, the rate of CO_{2} uptake is 3 × 10^{-3} \text{ mol per mg (wet weight)} per min or 3 × 10^{-4} \text{ mol per mg of protein per min}.

The activities in Table 1 compare favorably with this very rough figure. The lowest activity is 10^{-2} \text{ mol per mg of protein per min, which}
is of the same order of magnitude. Of course, in vitro activities cannot be interpreted as actual in vivo rates, but we may say that *N. oceanus* has all of the enzymes needed to produce PGA by carboxylating RuDP and to regenerate the acceptor via the pentose phosphate cycle. We did not assay for the specific sedoheptulose diphosphatase, but the formation and dephosphorylation of sedoheptulose diphosphate is not essential to the cycle.

Weissbach, Horecker, and Hurwitz (28) purified and characterized RuDP carboxylase from spinach. They found that the *K*<sub>m</sub> for RuDP and HCO<sub>3</sub> were 2.5 × 10<sup>-4</sup> M and 1.1 × 10<sup>-2</sup> M, respectively. High concentrations of RuDP (3 × 10<sup>-3</sup> M) inhibited activity. RuDP carboxylase required Mg<sup>2+</sup> and a sulfhydryl compound or a sequestering agent. Its pH optimum was 7.8; there was little or no activity above pH 9.2.

Phosphate at 0.01 M inhibited the activity by 70%.

A comparison of these properties with those of RuDP carboxylase in *N. oceanus* extracts reveals striking differences. The affinity of the bacterial enzyme for HCO<sub>3</sub> is an order of magnitude greater than that of the spinach enzyme. It is interesting that the concentration of bicarbonate required by *N. oceanus* carboxylase for half-maximal velocity is the same as the concentration of bicarbonate in seawater.

The inhibition of carboxylase activity by moderate concentrations of salt is difficult to rationalize. Seawater has a salt concentration of about 0.5 M, and probably the salt concentration within the cell is of the same magnitude (for discussion, see 13). We can only presume that the activity of these ions inside the cell is decreased by binding to proteins and other materials.

Akoyunoglou and Calvin (1) investigated the effect of Mg<sup>2+</sup> concentration on the activity of purified spinach enzyme and found no inactivation at concentrations as high as 0.1 M, but they found that at 0.01 M the activity was about 70% of the maximum. In contrast, we found 30% inhibition at 0.01 M and 85% at 0.04 M. We also observed no inhibition of activity by EDTA, although in the presence of EDTA the inhibition by Mg<sup>2+</sup> was somewhat less. This indicates that *N. oceanus* carboxylase may not need Mg<sup>2+</sup> as a cofactor, or that it is needed at a very low concentrations.

This is the first characterization of the properties of RuDP carboxylase from a marine microorganism. The enzyme was not purified, and we expect that the properties of the purified enzyme...
may differ somewhat from the data we obtained. We believe, however, that the bacterial enzyme is significantly different from the carboxylase of freshwater algae or of higher plants.

The specific rate of carbon dioxide fixation in the early experiments was $10^{-5}$ moles per mg (wet weight) per min, two orders of magnitude lower than the calculated rate. A large portion of the activity was located in the two carboxyl groups of aspartic acid. This is consistent with a pathway of synthesis from PGA via phosphoenolpyruvic acid:

The first three enzymes of this pathway have been found in extracts of *N. oceanus* (Williams and Watson, unpublished data). This scheme also explains the labeling of pyruvic and malic acids, which can be derived from phosphoenolpyruvic acid and oxaloacetic acid, and of glutamic acid, via the tricarboxylic acid cycle and $\alpha$-ketoglutaric acid. Serine and glycine are derived from PGA via phosphohydroxypropionate and phosphoserine.

In later experiments, the rate of $CO_2$ uptake was $6 \times 10^{-5}$ moles per mg (wet weight) per min, in good agreement with the calculated rate. We think that this increase in rate was due to two factors. First, the density of cells in the medium was much greater in the earlier set (140 mg/ml compared with 10 mg/ml). Ketchum and Watson (unpublished data) observed that the ratio of the rate of $CO_2$ fixation to the rate of nitrite production decreased as the density of the...
culture increased. Second, in the earlier experiments, the cells were subject to repeated centrifugation in the processes of harvesting and washing, and were then suspended in 3% NaCl. This procedure was much harsher on the cells than filtration, and would have removed from the environment any cofactors in the culture medium necessary for maximal CO₂ incorporation and growth.

In the later experiments, there was no detectable activity in aspartic and glutamic acids after 1 min of CO₂ incorporation. Most of the radioactivity was found in compounds coinciding with, or near, carrier sugar phosphates. This evidence indicates that, in the earlier experiments, the carboxylation of phosphoenolpyruvate was the primary route of CO₂ incorporation and that the pentose phosphate cycle was somehow inhibited. Under the more favorable conditions of the later experiments, the specific rate of CO₂ fixation was increased, becoming comparable to the normal steady-state rate, and the pentose phosphate cycle became the predominant pathway of carbon dioxide incorporation.

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