

# Reductive Pentose Phosphate Cycle in *Nitrosocystis oceanus*<sup>1</sup>

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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 chemoautotrophic 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 MgCl<sub>2</sub> (30% at 0.01 M), MnCl<sub>2</sub> (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 C<sup>14</sup>O<sub>2</sub>, 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 CO<sub>2</sub> 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 chemoautotroph 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 C<sup>14</sup>O<sub>2</sub> for various time intervals, finding most of the label in the carboxyl group of 3-phosphoglyceric 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 C<sup>14</sup>O<sub>2</sub> in the presence of ribose-5-phosphate, Mg<sup>++</sup>, and adenosine triphosphate

(ATP), indicating the existence of ribosephosphate isomerase, phosphoribulokinase, and ribulosediphosphate (RuDP) carboxylase. Phosphoglycerate kinase, glyceraldehydephosphate dehydrogenase, fructosediphosphate 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 C<sup>14</sup>O<sub>2</sub> 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 CO<sub>2</sub> RuDP built up, whereas in the presence of CO<sub>2</sub> 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  $C^{14}$  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  $CO_2$  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  $C^{14}O_2$  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 (Aminco French pressure cell; 16,000 psi). The suspension was centrifuged in the cold for 20 min at  $20,000 \times g$ , and the supernatant fluid was used in the enzyme assays. The protein content of the extract was determined by the Folin-Ciocalteu 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 m $\mu$  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 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;  $MgSO_4$ , 3; glutathione (GSH), 2; ATP, 2; reduced nicotin-

amide adenine dinucleotide ( $NADH_2$ ), 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 ( $NADPH_2$ ) for  $NADH_2$ .

(ii) The triosephosphate isomerase system of Meyer-Arendt, Beisenherz, and Bücher (16) contained triethanolamine (pH 7.6), 50; EDTA, 5;  $NADH_2$ , 0.1; glyceraldehyde-3-phosphate, 2; glycerolphosphate dehydrogenase; and extract.

(iii) The aldolase system of Beisenherz et al. (8) contained triethanolamine (pH 7.6), 50; EDTA, 5;  $NADH_2$ , 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;  $MgCl_2$ , 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 glycylglycine (pH 7.5), 9; cysteine, 1.5;  $NADH_2$ , 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;  $NADH_2$ , 0.1; fructose-6-phosphate, 0.5; erythrose-4-phosphate, 0.6; triosephosphate isomerase; glycerolphosphate dehydrogenase; and extract.

(vii) The ribulosephosphate-3-epimerase system was the same as the transketolase system, with the omission of ribulosephosphate-3-epimerase.

(viii) The phosphoribulokinase system of Racker (19) contained Tris (pH 7.8), 50;  $MgCl_2$ , 10; KCl, 40; GSH, 5; ATP, 5;  $NADH_2$ , 0.1; phosphoenolpyruvic 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  $Na_2C^{14}O_3$  and RuDP, by a modification of the methods of Horecker, Hurwitz, and Weissbach (11). The reaction mixture contained 25  $\mu$ moles of Tris (pH 7.8), 0.05  $\mu$ mole of EDTA, 2.5  $\mu$ moles of  $MgCl_2$ , 2.5  $\mu$ moles of GSH, 2.5  $\mu$ moles of  $NaHCO_3$ , 1  $\mu$ c of  $NaC^{14}O_3$ , 1.5  $\mu$ moles of RuDP, and extract, in a final volume of 250  $\mu$ liters. After 10 min, the reaction was stopped with 25  $\mu$ liters of 1 N HCl. The mixture was aerated to drive off unreacted  $CO_2$ , a 25- $\mu$ liter 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- $\mu$ liter sample.

The reaction system used contained only a small amount of air above the reaction mixture. The gaseous  $CO_2$  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  $\text{CO}_2$  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  $\text{C}^{14}$  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  $\text{C}^{14}\text{O}_2$  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 sea-water 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  $(\text{NH}_4)_2\text{SO}_4$  (pH 7.8) was added as the ammonia source. After 1 min, 2 ml of 0.01 M  $\text{Na}_2\text{C}^{14}\text{O}_3$  (0.41 mc/ml) was added. The reaction was stopped by the addition of 50% trichloroacetic acid, to a final concentration of 6%. The mixture was aerated to remove unfixated  $\text{C}^{14}\text{O}_2$ , 5  $\mu$ moles 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  $20,000 \times g$  for 20 min. The trichloroacetic acid was extracted with ether, and the aqueous layer was adsorbed onto a Dowex 50 ( $\text{H}^+$ ) cation-exchange column. The sugar phosphates and organic acids were eluted with water, and the amino acids with 2 N  $\text{NH}_4\text{OH}$ . 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 cochromatographed 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  $\text{CO}_2$  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  $\mu$ ) by gentle filtration. As soon as the filter was dry, suction was stopped, and 200  $\mu$ liters of the culture medium was added. Then 50 to 100  $\mu$ liters of 0.04 M  $\text{Na}_2\text{C}^{14}\text{O}_3$  (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 cochromatographed 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 so 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 it to dryness on a planchet, and counting it with the Nuclear-Chicago gas-flow detector.

*Enzymes, substrates, and cofactors.* Fructose-6-phosphate, fructose-1,6-diphosphate, DL-glyceraldehyde-3-phosphate diethyl-acetal, D-erythrose-4-phosphate dimethylacetal, 3-phosphoglyceric acid, 2-phosphoenolpyruvic acid, glyceraldehydephosphate dehydrogenase, lactate dehydrogenase, triosephosphate isomerase, glucosephosphate isomerase, glycerolphosphate dehydrogenase, aldolase, phosphoglycerate kinase, and pyruvate kinase were purchased from Calbiochem; ribose-5-phosphate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; and NADH<sub>2</sub>, 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; ribulosephosphate-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  $\text{NaHCO}_3$  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  $K_m$  for  $\text{HCO}_3^-$  to be  $3 \times 10^{-3}$ . Similarly, by varying the RuDP concentration (Fig. 2), we found the  $K_m$  for RuDP to be  $3 \times 10^{-4}$  M. Concentrations of RuDP higher than  $10^{-3}$  M were inhibitory. At

TABLE 1. Activities of the enzymes of the reductive pentose phosphate cycle in cell-free extracts of *Nitrosocystis oceanus*\*

Enzyme	Activity (substrate per $\mu\text{g}$ of protein per min)
RuDP carboxylase.....	$9 \times 10^{-2} \mu\text{M CO}_2$
Phosphoglycerate kinase.....	$3 \times 10^{-1} \mu\text{M PGA}$
Glyceraldehydephosphate dehydrogenase NADH <sub>2</sub> .....	$1 \times 10^{-1} \mu\text{M GAP}$
Glyceraldehydephosphate dehydrogenase NADPH <sub>2</sub> .....	$3 \times 10^{-3} \mu\text{M GAP}$
Triosephosphate isomerase.....	$2 \times 10^{-2} \mu\text{M GAP}$
Aldolase.....	$2 \times 10^{-2} \mu\text{M FDP}$
Fructosediphosphatase.....	$1 \times 10^{-2} \mu\text{M FDP}$
Transaldolase.....	$8 \times 10^{-2} \mu\text{M F-6-P}$
Transketolase.....	$3 \times 10^{-2} \mu\text{M Ru-5-P}$
Ribosephosphate isomerase.....	$2 \times 10^{-1} \mu\text{M Ru-5-P}$
Ribulosephosphate-3-epimerase.....	$>3 \times 10^{-2} \mu\text{M Ru-5-P}$
Phosphoribulokinase.....	$1 \times 10^{-1} \mu\text{M Ru-5-P}$

\* Assay procedures were as described in Methods. Most of the assays were done several times on different extracts. Only the highest values are recorded here, as these represent optimal assay conditions. Values refer to the total protein concentration in the crude bacterial extract, as determined by the Folin-Ciocalteu method (14). RuDP = ribulosediphosphate; PGA = 3-phosphoglyceric acid; GAP = glyceraldehyde-3-phosphate; FDP = fructose-1,6-diphosphate; F-6-P = fructose-6-phosphate; Ru-5-P = ribulose-5-phosphate.

$4 \times 10^{-3}$  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  $144,000 \times g$  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<sup>++</sup> produced no effect at 0.001 M, 30% inhibition at 0.01 M, and 85% inhibition at 0.04 M. Mn<sup>++</sup> gave 70% inhibition at 0.01 M. NH<sub>4</sub><sup>+</sup> 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<sup>++</sup> 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<sup>++</sup>, Fe<sup>++</sup>, Co<sup>++</sup>, Cu<sup>++</sup>, GSH, EDTA, biotin, thiamine, or vitamin B<sub>12</sub>. 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<sup>14</sup>O<sub>2</sub> into whole cells.* The rate of CO<sub>2</sub> fixation in the early experiments was about  $10^{-5}$   $\mu\text{moles per mg (wet weight) per min}$ . 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<sub>2</sub> fixation of  $6 \times 10^{-3}$   $\mu\text{moles per mg (wet weight) per min}$  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- and 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 PGA or sugar phosphates. After 5 min of C<sup>14</sup>O<sub>2</sub> 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<sub>2</sub> as follows. Assuming that 5% of the wet weight is carbon, and using the observed generation time of 24 hr, the rate of CO<sub>2</sub> uptake is  $3 \times 10^{-3}$   $\mu\text{moles per mg (wet weight) per min}$  or  $3 \times 10^{-2}$   $\mu\text{moles per mg of protein per min}$ .

The activities in Table 1 compare favorably with this very rough figure. The lowest activity is  $10^{-2}$   $\mu\text{moles per mg of protein per min}$ , which

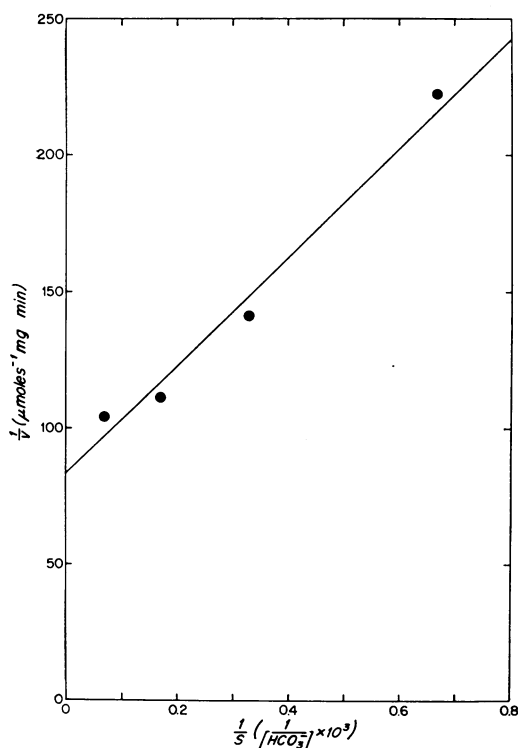


FIG. 1. Effect of  $\text{HCO}_3^-$  concentration on RuDP carboxylase activity. Assay conditions were as described in Materials and Methods, except for varying amount of  $\text{NaHCO}_3$ .

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  $K_m$  for RuDP and  $\text{HCO}_3^-$  were  $2.5 \times 10^{-4}$  M and  $1.1 \times 10^{-2}$  M, respectively. High concentrations of RuDP ( $3 \times 10^{-3}$  M) inhibited activity. RuDP carboxylase required  $\text{Mg}^{++}$  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  $\text{HCO}_3^-$  is an order of magnitude

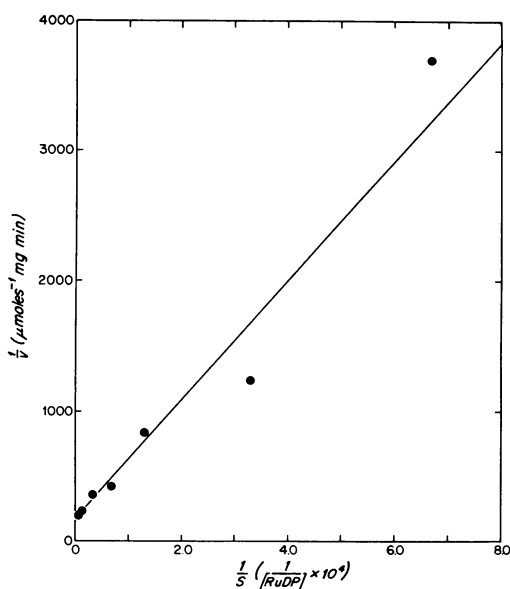


FIG. 2. Effect of RuDP concentration on RuDP carboxylase activity. Assay conditions were as described in Materials and Methods, except for varying amount of RuDP.

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  $\text{Mg}^{++}$  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  $\text{Mg}^{++}$  was somewhat less. This indicates that *N. oceanus* carboxylase may not need  $\text{Mg}^{++}$  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

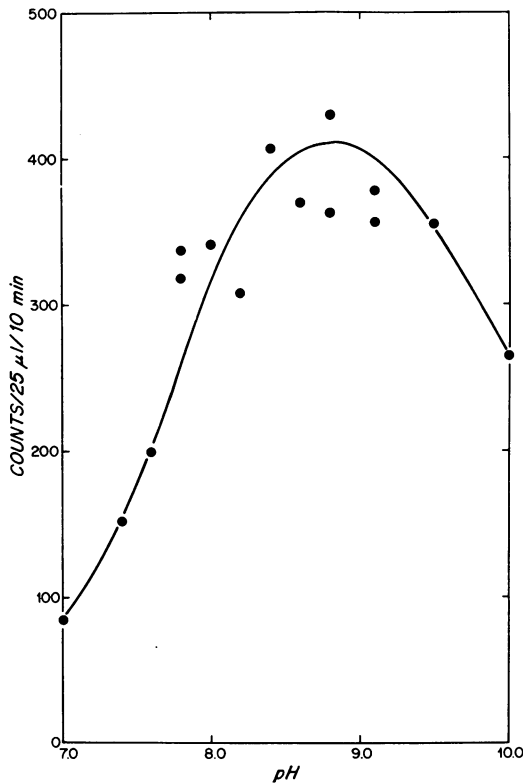
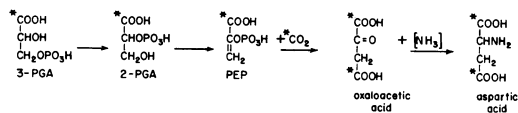


FIG. 3. Effect of pH on RuDP carboxylase activity. Assay conditions were as described in Materials and Methods, except for varying the pH of the Tris buffer.

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  $10^{-5}$   $\mu$ moles per mg (wet weight) per min, two orders of magnitude lower than the calculated rate. A large proportion 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,

TABLE 2. Distribution of radioactivity among intermediates of the pentose phosphate cycle and related pathways\*

Compound	1 min	5 min
3-Phosphoglyceric acid.....	+	+
Monophosphates.....	+ (?)	+ (?)
Diphosphates.....	+ (?)	+
Phosphoenolpyruvic acid.....	+	+
Pyruvic acid.....	++	+++
Malic acid.....	+	+
Oxaloacetic acid.....	+ (?)	+
Serine.....	+ (?)	+
Glycine.....	+ (?)	+
Aspartic acid.....	+++	++++
$\beta$ -Alanine.....	-	-
Glutamic acid.....	-	+
Alanine.....	-	-

\* Whole cells were exposed to  $\text{C}^{14}\text{O}_2$  for 1 and 5 min, as described in Materials and Methods (earlier experiments). The relative amount of activity in each compound was estimated from the intensity of the spot on the autoradiogram.

TABLE 3. Distribution of radioactivity on chromatograms corresponding with the locations of cochromatographed PGA, hexose mono- and diphosphates, and aspartic and glutamic acids\*

Compound	Radioactivity (count/min)			
	Expt 1		Expt 2	
	15 sec	30 sec	1 min	5 min
PGA.....	8	11	20	70
Hexose-monophosphate...	7	40	45	132
Hexose-diphosphate.....	3	6	20	44
Aspartic acid.....	0	0	0	20
Glutamic acid.....	0	0	0	12
Total on chromatogram...	48	100	195	860

\* The substances were eluted from the chromatograms, and their radioactivity was determined as described in Materials and Methods.

via the tricarboxylic acid cycle and  $\alpha$ -ketoglutaric acid. Serine and glycine are derived from PGA via phosphohydroxypyruvate and phosphoserine.

In later experiments, the rate of  $\text{CO}_2$  uptake was  $6 \times 10^{-3}$   $\mu$ 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  $\text{CO}_2$  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 CO<sub>2</sub> incorporation and growth.

In the later experiments, there was no detectable activity in aspartic and glutamic acids after 1 min of C<sup>14</sup>O<sub>2</sub> 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 CO<sub>2</sub> incorporation and that the pentose phosphate cycle was somehow inhibited. Under the more favorable conditions of the later experiments, the specific rate of CO<sub>2</sub> 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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