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ROLE OF THE REDUCTIVE CARBOXYLIC ACID CYCLE IN A PHOTOSYNTHETIC BACTERIUM LACKING RIBULOSE 1,5-DIPHOSPHATE CARBOXYLASE

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SUMMARY

Evidence is presented that a green photosynthetic bacterium (*Chlorobium thiosulfatophilum*, Tassajara) lacks ribulose 1,5-diphosphate carboxylase, the key enzyme of the reductive pentose phosphate cycle—the photosynthetic carbon reduction mechanism characteristic of green plants. The bacterium appears to use exclusively the reductive carboxylic acid cycle (and its associated reactions) not only, as previously recognized, in the photosynthetic conversion of CO_2 to amino acids and organic acids but also to carbohydrates. This conclusion is based on: (i) the absence in cell-free preparations of ribulose 1,5-diphosphate carboxylase; (ii) the assimilation by cell suspensions of $^{14}CO_2$, [^{14}C]acetate, and [^{14}C]succinate to give the products expected from the operation of the reductive carboxylic acid cycle; (iii) the demonstration in cell-free preparations of all enzymes needed for the conversion of CO_2 and the primary product of the reductive carboxylic acid cycle, acetyl-CoA, to carbohydrate.

The results also show that CO_2 concentration influences the products formed by the reductive carboxylic acid cycle, with a low level of CO_2 favoring the synthesis of carbohydrates.

INTRODUCTION

The reductive carboxylic acid cycle (see below) was proposed[1,2] as a new cyclic pathway for the assimilation of CO_2 by photosynthetic bacteria. The reductive carboxylic acid cycle is, in effect, a reversal of the oxidative citric acid cycle of Krebs and yields one molecule of acetyl-CoA from two molecules of CO_2. The reductive carboxylic acid cycle depends on the strong reducing potential of ferredoxin [−420 mV at pH 7 (ref. 3)] to drive acetyl-CoA synthesis. Reduced ferredoxin is needed to form (by reversal of α-decarboxylation) α-ketoglutarate, a key intermediate of the cycle, and also pyruvate, a product made from acetyl-CoA and CO_2. The initial proposal[1,2] was that the reductive carboxylic acid cycle functions chiefly in the synthesis of amino acids—the main products of bacterial photosynthesis[4-6,1].

Subsequently, two conflicting views have emerged to account for the role of the reductive carboxylic acid cycle in CO_2 assimilation by photosynthetic bacteria:

(i) bacterial photosynthetic CO_2 assimilation occurs *via* the reductive pentose phosphate cycle as in green plants[7] and the reactions of the reductive carboxylic acid cycle are basically nonessential and ancillary[8]; (ii) the two carbon cycles coexist in photosynthetic bacteria—the reductive pentose phosphate cycle being mainly concerned with carbohydrate synthesis whereas the reductive carboxylic acid cycle functions in the synthesis of amino acids and precursors of lipids and porphyrins[1,2].

The importance of the reductive carboxylic acid cycle in bacterial photosynthesis was recently emphasized by the finding of Sirevåg and Ormerod[9,10] that in photosynthetic green bacteria the reductive carboxylic acid cycle accounts for most of the CO_2 assimilated and leads to the synthesis not only of amino and organic acids but also of carbohydrates. The synthesis of carbohydrates by reactions leading from the reductive carboxylic acid cycle raises the question whether that cycle functions in the total absence of the reductive pentose phosphate cycle. To test this possibility we have reinvestigated (with both whole cells and cell-free extracts) the path of carbon assimilation in the photosynthetic green bacterium *Chlorobium thiosulfatophilum*, Tassajara, an organism known to contain all enzymes of the reductive carboxylic acid cycle[1]. We now report new evidence (i) that photosynthetic green bacteria lack ribulose 1,5-diphosphate carboxylase—the key enzyme of the reductive pentose phosphate cycle—and use exclusively the reductive carboxylic acid cycle for the photosynthetic assimilation of carbon and (ii) that the ambient CO_2 concentration regulates the type of cellular products formed by the reductive carboxylic acid cycle. A preliminary report of some of these findings has been published[11].

METHODS

C. thiosulfatophilum, Tassajara, cells were grown with Na_2CO_3 (40 mM)* as sole carbon source in a modified *Chromatium* thiosulfate medium[12] (final pH 6.7). For ^{14}C-labeling experiments, two media were used: a complete medium (A) and a "*minus* CO_2" culture medium (B) in which Na_2CO_3 had been omitted and the pH adjusted to 6.7 with NaOH. Freshly harvested cells were suspended in Medium B in a ratio of 1 g packed cells to 2 ml medium, and 1 ml was placed in a "lollipop"[7] containing 20 ml of either Medium A or B under argon. For 40 mM CO_2 treatments, Medium A was used. For 0.8 mM CO_2 treatments, Medium B was used with the CO_2 added as either $NaH^{14}CO_3$ (for $^{14}CO_2$ uptake) or unlabeled $NaHCO_3$ (for [^{14}C]acetate uptake).

The cell suspension was gassed (5 min, argon, dark), preilluminated 3 min, and then one of the following was injected: $NaH^{14}CO_3$ (17.5 μmoles, 800 μCi); [1,2-$^{14}C_2$]-acetic acid (0.9 μmole, 50 μCi); [2,3-$^{14}C_2$]succinic acid (2.2 μmoles, 50 μCi). *Chlorobium* chlorophyll concentration determined according to Stanier and Smith[13] was 0.2 mg/ml. Light intensity, 10000 lux (white light); temperature, 25 °C. After the indicated times of photosynthesis, 1-ml samples were withdrawn with a syringe and immediately added to 2 ml of boiling methanol. Labeled products were analyzed by thin-layer electrophoresis–chromatography and counted as described previously[14,15].

Cell-free extracts of *C. thiosulfatophilum* were prepared (at 4 °C) form 6 g of frozen cell paste suspended in 12 ml of one of the indicated solutions: (i) 0.02 M potas-

* The total concentration of Na_2CO_3 + $NaHCO_3$ + CO_2 (added to culture medium as either Na_2CO_3 or $NaHCO_3$) is expressed as "CO_2 concentration".

sium phosphate buffer, pH 6.5 (for pyruvate synthase); (ii) 0.1 M potassium phosphate buffer, pH 7.4 (for phosphoenolpyruvate synthase and glycolytic enzymes); or (iii) 0.025 M Tris–HCl buffer, pH 7.3, containing 0.1 mM neutralized ethylene diamine tetraacetic acid and 5 mM 2-mercaptoethanol (for sedimentation velocity ultracentrifugation). For (i) and (ii) the suspension was sonicated for 6 min in a Raytheon 10 kcycles sonic generator cooled with ice water; for (iii) the suspended cells of *C. thiosulfatophilum* (and *Chlorella*, grown in a modified Myers medium[16]) were broken by passage through a French pressure cell (10000 lb/inch^2). In all cases the cell residue was centrifuged off (10 min, 10000 × *g*) and the supernatant solution clarified in a preparative ultracentrifuge (1 h, 105000 × *g*). The supernatant solution obtained at this step represented the soluble protein fraction used in the enzyme assays. Prior to sedimentation velocity ultracentrifugation, soluble fractions of the *C. thiosulfatophilum* and *Chlorella* were dialyzed overnight (4 °C) against a solution of 0.1 M potassium phosphate buffer, pH 7.3, and 0.1 M NaCl.

Phosphoenolpyruvate synthase and the glycolytic enzymes were assayed in 0.05 M Tris–HCl buffer, pH 7.4; other assays were described elsewhere: phosphoenolpyruvate synthase[17]; enolase *plus* phosphoglyceromutase (assayed together[18]); phosphoglycerokinase *plus* triose phosphate dehydrogenase (assayed together[18]); triose phosphate isomerase[19]; and aldolase[20]. Pyruvate synthase was assayed as described by Buchanan and Arnon[21].

Protein estimation and analytical ultracentrifugation were as described previously[22].

RESULTS AND DISCUSSION

Ribulose 1,5-diphosphate carboxylase, the enzyme that catalyzes the carboxylation of ribulose 1,5-diphosphate to yield two molecules of 3-phosphoglyceric acid, is essential for (and peculiar to) the reductive pentose phosphate cycle[7]. The carboxylase is present at high levels in green plants and in certain photosynthetic bacteria and is easily detected both by its enzymic activity and by analytical ultracentrifugation[23]. These properties make the carboxylase particularly useful as a diagnostic probe for the presence of the reductive pentose phosphate cycle in organisms such as *C. thiosulfatophilum*.

Preparative and assay methods (based on those of Paulsen and Lane[24] and Sugiyama *et al.*[25]) were found in the present study to be effective for demonstrating carboxylase activity in a related photosynthetic bacterium (*Chromatium*) and in green algae, blue-green algae, and leaves but gave negative results with *C. thiosulfatophilum*, Tassajara. A procedure reported[26] to yield low levels of carboxylase activity from another *Chlorobium* strain (*C. thiosulfatophilum*, L strain) also gave no activity with the Tassajara strain in recent attempts and when we first assayed cell-free preparations for the carboxylase 6 years ago.

Attempts were also made to demonstrate in *C. thiosulfatophilum*, Tassajara, a 16- to 19-S protein component typical of ribulose 1,5-diphosphate carboxylase from other organisms[23]. The enzyme [migrating in the ultracentrifuge as a 15-S component (value uncorrected for protein concentration and solvent)] was readily observed in identically prepared cell-free preparations of the green alga *Chlorella* but not in *Chlorobium* (Fig. 1). The 15-S component of *Chlorella* was identified as the carboxylase

by enzymic assay[24] after isolation by sucrose density gradient centrifugation[27]. The corresponding fractions from different *C. thiosulfatophilum* preparations contained only traces of protein with sedimentation coefficients around 15 S and, like the parent extract, showed no carboxylase activity. The *C. thiosulfatophilum* fractions representing the 4- to 7-S components, which could possibly contain an atypical ribulose 1,5-diphosphate carboxylase, such as the 5-S enzyme described for the purple photosynthetic bacterium, *Rhodospirillum rubrum*[28], also showed no carboxylase activity.

The absence of the carboxylase prompted the conclusion that the reductive pentose phosphate cycle cannot function in *C. thiosulfatophilum*, Tassajara. Such a conclusion led us to abandon the view[1] that the pentose cycle is the mechanism of

Fig. 1. Sedimentation velocity pattern of cell-free preparations of a photosynthetic bacterium, *Chlorobium thiosulfatophilum*, and an alga, *Chlorella*. Patterns were determined iwth 0.8-ml samples, each containing 12 mg protein per ml. Measurements were made at a rotor speed of 59780 rev./min; average temperature, 20 °C. Photograph taken 26 min after full speed was reached. 15-S component = ribulose 1,5-diphosphate carboxylase.

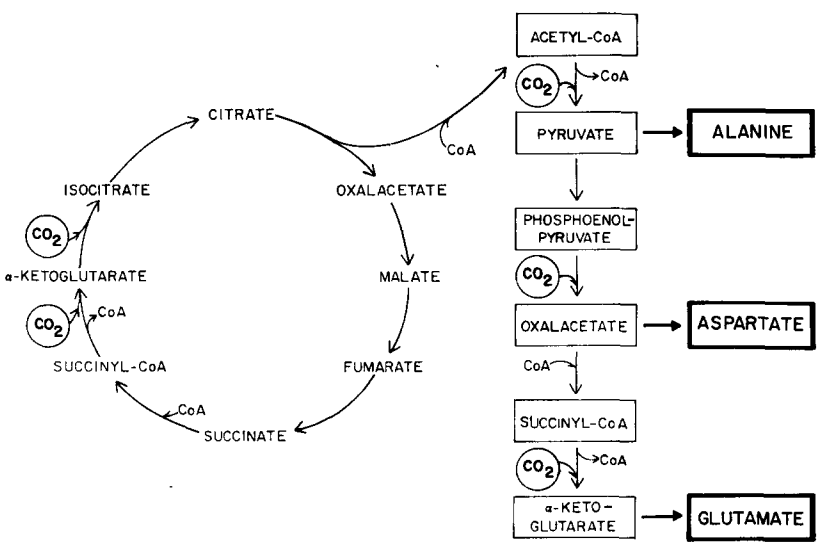

Fig. 2. Role of the reductive carboxylic acid cycle in the synthesis of amino acids.

carbohydrate synthesis in photosynthetic green bacteria and to test the proposal[9,10] that carbohydrates, like organic and amino acids (Fig. 2), are formed by reactions leading from the reductive carboxylic acid cycle. The proposed route is based on a conversion to sugar phosphates of CO_2 and the primary product of the reductive carboxylic acid cycle, acetyl-CoA (Fig. 3). Such a conversion requires certain enzymes of glycolysis which, like pyruvate synthase and phosphoenolpyruvate synthase, were found in *C. thiosulfatophilum* (Table I).

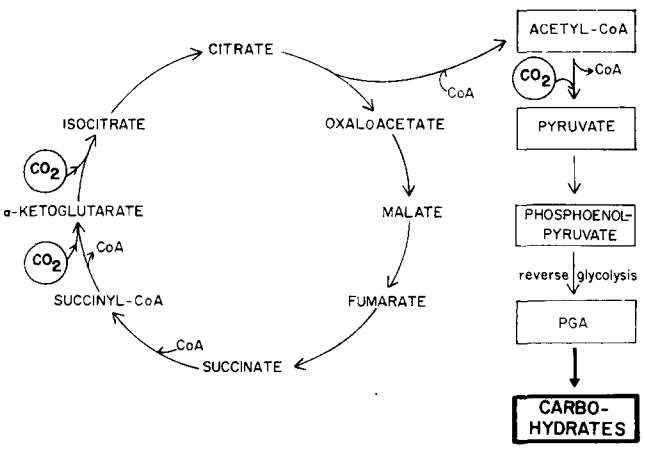

Fig. 3. Role of the reductive carboxylic acid cycle in the synthesis of carbohydrates. PGA = 3-phosphoglycerate.

TABLE I

PRESENCE OF ENZYMES NEEDED FOR THE CONVERSION OF ACETYL-CoA *plus* CO_2 TO CARBOHYDRATE IN THE SOLUBLE PROTEIN FRACTION OF *C. thiosulfatophilum* *

	Activity (*μmoles/mg protein per h*)
Pyruvate synthase	0.8
Phosphoenolpyruvate synthase	2.3
Enolase *plus* phosphoglyceromutase	21.8**
Phosphoglycerokinase *plus* triose phosphate dehydrogenase	8.5**
Triose phosphate isomerase	662.0
Aldolase	2.5

* Data of B. B. Buchanan and M. C. W. Evans.
** Represents activity of the two enzymes assayed simultaneously.

Since acetate assimilation by *Chlorobium* requires CO_2[29,30], the proposed route of carbohydrate formation (Fig. 3) could be tested in whole cells supplied either (i) unlabeled CO_2 and [^{14}C]acetate (converted intracellularly to [^{14}C]acetyl-CoA[1]) or (ii) $^{14}CO_2$ and endogenous acetyl-CoA. The path in Fig. 3 predicts that a similar labeling pattern of 3-phosphoglyceric acid and sugar phosphates would be observed under both conditions.

Carbohydrate formation from [^{14}C]acetate was examined in cell suspensions incubated at two different CO_2 concentrations (Fig. 4). At 40 mM CO_2 (the level present in culture medium), 80% of the [^{14}C]acetate assimilated after 30 s was recovered in organic acids and amino acids and only 20% was recovered in sugar phosphates *plus* 3-phosphoglyceric acid. Lowering the CO_2 concentration to 0.8 mM effected a sharp increase (to 60%) in the relative amount of carbohydrates formed and a corresponding drop (to 40%) in the organic and amino acids. In agreement with the proposed route of carbohydrate synthesis (Fig. 3), the labeled acids formed

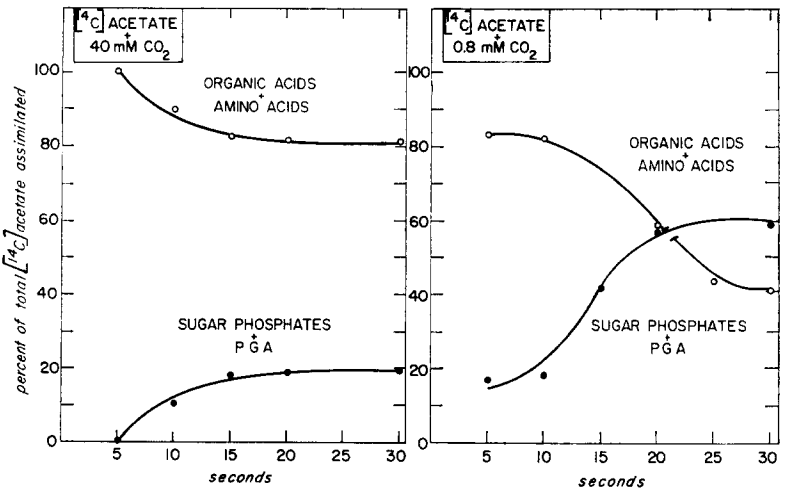

Fig. 4. Effect of [CO_2] on the labeled products of [^{14}C]acetate assimilation in *C. thiosulfatophilum* PGA = 3-phosphoglycerate.

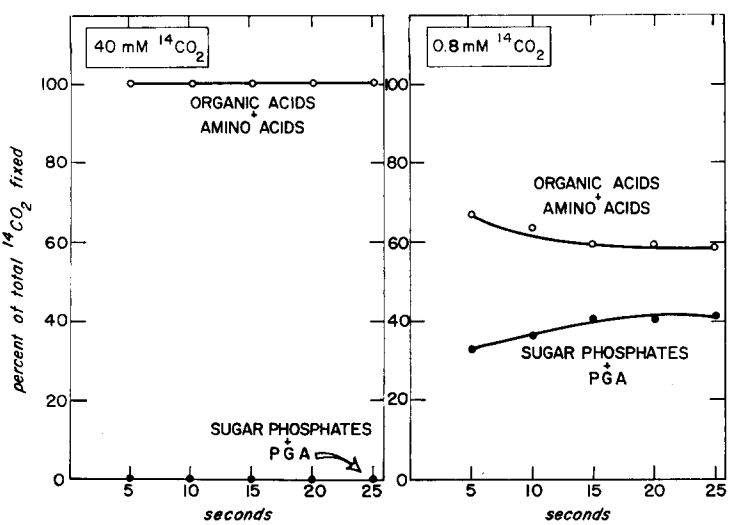

Fig. 5. Effect of [CO_2] on the labeled products of $^{14}CO_2$ assimilation in *C. thiosulfatophilum*. The rate of CO_2 assimilation at each of the two levels of CO_2 was (in μmoles/h per mg chlorophyll): 2.1 at 40 mM $^{14}CO_2$ and 0.4 at 0.8 mM $^{14}CO_2$. PGA = 3-phosphoglycerate.

from [^{14}C]acetate, at both levels of CO_2, were identified as intermediates of the reductive carboxylic acid cycle and its associated amino acids.

The low level of CO_2 was found also to enhance the amount of carbohydrate formed from $^{14}CO_2$ (Fig. 5). At 0.8 mM (but not at 40 mM), $^{14}CO_2$ was fixed rapidly into sugar phosphates *plus* 3-phosphoglyceric acid. Both levels of $^{14}CO_2$ supported the formation of large amounts of organic and amino acids.

The profiles of the individual products formed at the two levels of CO_2 constitute further evidence for the reductive carboxylic acid cycle and its role in carbohydrate

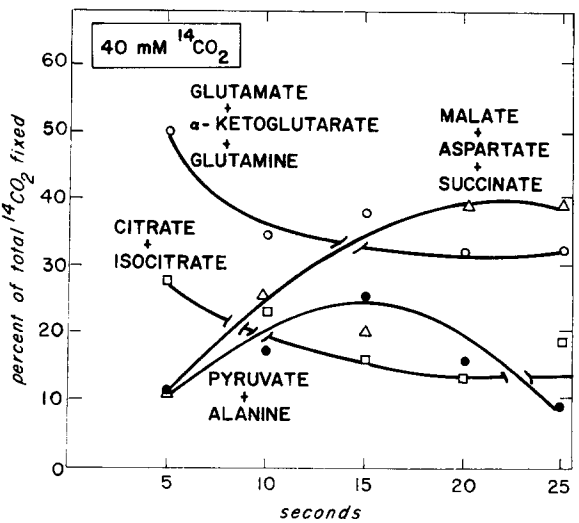

Fig. 6. Product profile of photosynthesis by *C. thiosulfatophilum* (40 mM $^{14}CO_2$). The rate of CO_2 assimilation was 2.1 μmoles/h per mg chlorophyll.

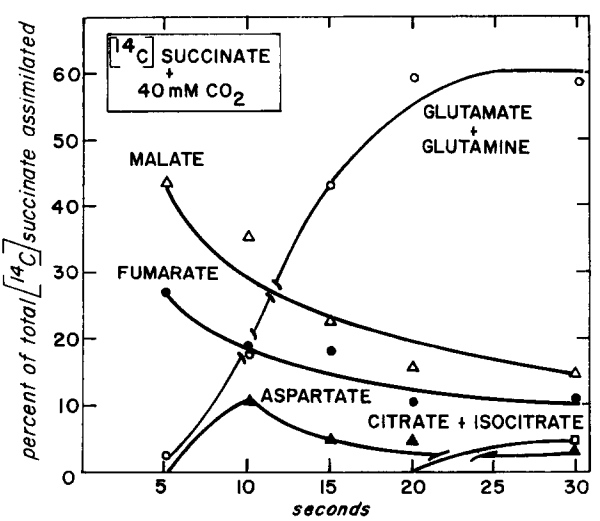

Fig. 7. Labeled products formed in the assimilation of [^{14}C]succinate and unlabeled CO_2 by *C. thiosulfatophilum*.

synthesis. At 40 mM $^{14}CO_2$ (Fig. 6), C_5-compounds (α-ketoglutarate, glutamate, glutamine) and C_6-acids (isocitrate, citrate) represented early major products, in agreement with CO_2 fixation by the two carboxylation steps of the reductive carboxylic acid cycle, the α-ketoglutarate synthase reaction and reversal of the isocitrate dehydrogenase reaction. The labeling observed in C_3-compounds (pyruvate, alanine) and in C_4-compounds (malate, aspartate, succinate) is accounted for by the two carboxylations associated with the cycle, pyruvate synthase and phosphoenolpyruvate carboxylase (Fig. 2). The *in vivo* activity of the α-ketoglutarate synthase system was confirmed in a parallel treatment by a pronounced formation of glutamate (and glutamine) from [^{14}C]succinate *plus* unlabeled CO_2 (Fig. 7).

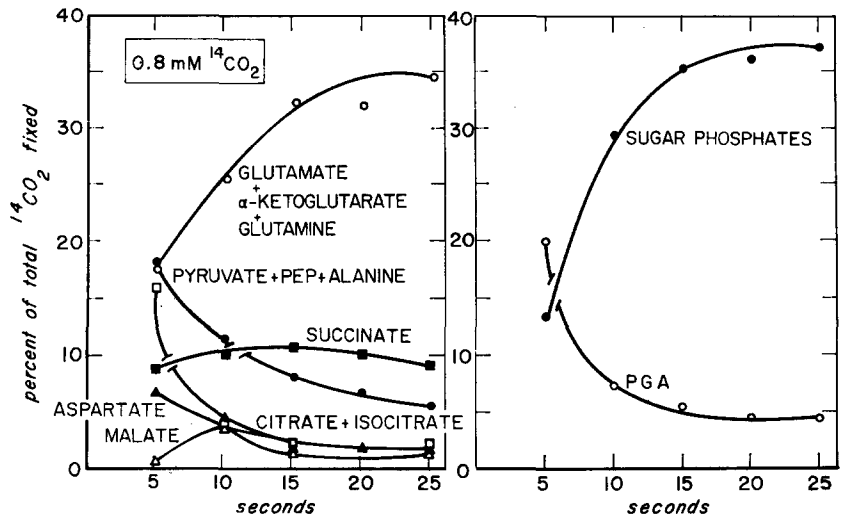

Fig. 8. Product profile of photosynthesis by *C. thiosulfatophilum* (0.8 mM $^{14}CO_2$). The rate of CO_2 assimilation was 0.4 μmoles/h per mg chlorophyll. PGA = 3-phosphoglycerate; PEP = phosphoenolpyruvate.

Evidence for sugar phosphate synthesis by reactions leading from the reductive carboxylic acid cycle to pyruvate, phosphoenolpyruvate, and 3-phosphoglyceric acid is shown in Fig. 8 (0.8 mM $^{14}CO_2$). An identification of the sugar phosphate fraction revealed the expected intermediates of reverse glycolysis (triose phosphates, fructose 1,6-diphosphate, fructose 6-phosphate, glucose 6-phosphate) and of ribose 5-phosphate. The latter is probably an intermediate of an oxidative pentose phosphate cycle[31] needed for pentose synthesis by *C. thiosulfatophilum*. Ribulose 1,5-diphosphate, an intermediate peculiar to the reductive pentose phosphate cycle, was not detected.

CONCLUDING REMARKS

The above results constitute the first evidence that a photosynthetic (autotrophic) organism grows without ribulose 1,5-diphosphate carboxylase and hence without the mechanism heretofore considered universal in photosynthetic cells, the reductive pentose phosphate cycle. *Chloropseudomonas ethylicum*, another photo-

synthetic green bacterium, has also been reported to lack the carboxylase[8] but that organism, unlike *C. thiosulfatophilum*, requires a source of organic carbon and cannot grow autotrophically.

The present findings therefore suggest that the reductive carboxylic acid cycle can serve as an independent carbon reduction mechanism for synthesis of carbohydrates as well as amino acids and organic acids. The dependence of products formed by the reductive carboxylic acid cycle on the ambient CO_2 concentration raises the possibility that the prevailing level of CO_2 may govern the products of bacterial photosynthesis.

NOTE ADDED IN PROOF (Received September 5th, 1972)

According to a recent report[32], the organism referred to above as *Chloropseudomonas ethylicum* is a mixed culture comprised of a green photosynthetic bacterium (*Chlorobium limicola*) and an unidentified nonphotosynthetic bacterium. It will be of interest to know whether *C. limicola* grown autotrophically in pure culture will, as reported previously for the mixed culture *Chloropseudomonas ethylicum*[8], resemble *C. thiosulfatophilum* in lacking the enzyme ribulose 1,5-diphosphate carboxylase.

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