A NEW FERREDOXIN-DEPENDENT CARBON REDUCTION CYCLE IN A PHOTOSYNTHETIC BACTERIUM

By M. C. W. Evans, Bob B. Buchanan, and Daniel I. Arnon*

DEPARTMENT OF CELL PHYSIOLOGY, UNIVERSITY OF CALIFORNIA, BERKELEY

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The view is widely held that carbon assimilation by photosynthetic and autotrophic cells must be a cyclic process that continuously regenerates an acceptor for carbon dioxide. Until now, the only known cyclic pathway for the assimilation of carbon dioxide in photosynthetic cells[1] and in autotrophic bacteria (see review, ref. 2) has been the reductive pentose phosphate cycle, which uses and regenerates ribulose 1,5-diphosphate as the sole acceptor for carbon dioxide and employs as the reductive step the reduction of 1,3-diphosphoglyceric acid—a reaction which is a reversal of a key step in glycolysis.[3] One complete turn of this cycle incorporates one molecule of carbon dioxide. Thus, beginning with 1 molecule of ribulose 1,5-diphosphate and 1 molecule of CO_2, one complete turn of the cycle gives, on balance, a net synthesis of $^1/_3$ molecule of triose phosphate.

This communication describes a new cyclic process for the reductive assimilation of carbon dioxide by the photosynthetic bacterium, *Chlorobium thiosulfatophilum.* One complete turn of the new cycle, which we will call the reductive carboxylic acid cycle (Fig. 1), incorporates four molecules of CO_2 and results in the net synthesis of oxalacetate, a four-carbon dicarboxylic acid, which is itself an intermediate in the

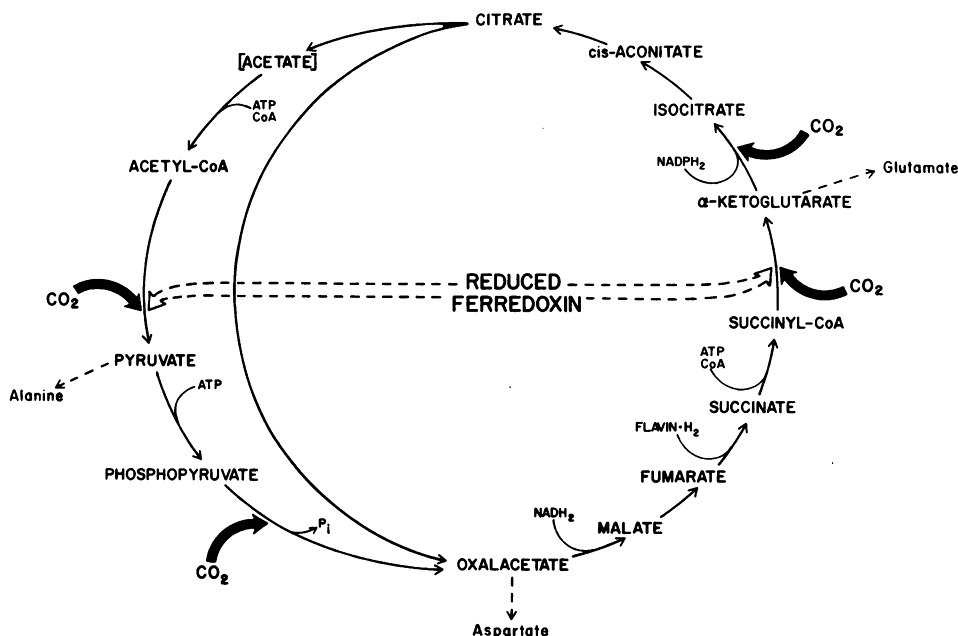


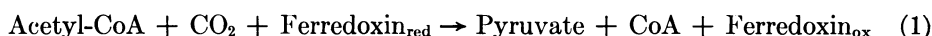
Fig. 1.—Reductive carboxylic acid cycle in *C. thiosulfatophilum.* One turn of the complete cycle (represented by the one-sided ellipse) results in the incorporation of four molecules of CO_2. One turn of the short cycle (represented by the circle) results in the incorporation of two molecules of CO_2. Further details are given in the text.

cycle. Thus, beginning with one molecule of oxalacetate, one complete turn of the reductive carboxylic acid cycle will regenerate it and yield, in addition, a second molecule of oxalacetate formed by the reductive fixation of four molecules of CO_2.

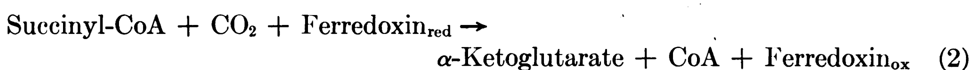
A variant of the complete reductive carboxylic acid cycle (one-sided ellipse in Fig. 1) is the "short" reductive carboxylic acid cycle (*circle* in Fig. 1) which, in one turn, incorporates two molecules of CO_2 and yields one molecule of acetate. As shown in Figure 1, the complete cycle and the short cycle have the same sequence of reactions from oxalacetate to citrate. Thus, beginning again with oxalacetate, a complete turn of the short reductive carboxylic acid cycle would result in the regeneration of the oxalacetate and the synthesis of acetate from CO_2.

In its over-all effect, the short reductive carboxylic acid cycle (*circle* in Fig. 1) which generates acetyl-CoA from two molecules of carbon dioxide is a reversal of the Krebs[4] citric acid cycle which degrades acetyl-CoA to two molecules of carbon dioxide. A fundamental distinction between the two cycles is that the reductive carboxylic acid cycle is endergonic in nature and hence must be linked with energy-yielding reactions, which, in this instance, are the photoreduction of ferredoxin and photophosphorylation. Moreover, although several reversible enzyme reactions of the citric acid cycle function also in the reductive carboxylic acid cycle, the reductive cycle as a whole cannot function without several key enzymes peculiar to it, such as the pyruvate and α-ketoglutarate synthases.

The four carboxylation steps in the complete reductive carboxylic acid cycle include two recently discovered primary reactions for CO_2 fixation that are dependent on reduced ferredoxin: the pyruvate synthase[5-7] [eq. (1)] and the α-ketoglutarate synthase[8] [eq. (2)].



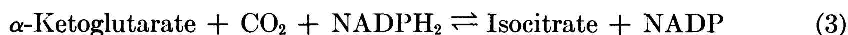
$$\text{Acetyl-CoA} + CO_2 + \text{Ferredoxin}_{red} \rightarrow \text{Pyruvate} + \text{CoA} + \text{Ferredoxin}_{ox} \quad (1)$$



$$\text{Succinyl-CoA} + CO_2 + \text{Ferredoxin}_{red} \rightarrow \alpha\text{-Ketoglutarate} + \text{CoA} + \text{Ferredoxin}_{ox} \quad (2)$$

These two new ferredoxin-dependent carboxylations reverse two reactions of the citric acid cycle of Krebs[4] that in aerobic cells are irreversible: (*i*) the decarboxylation of pyruvate to acetyl-CoA and CO_2, and (*ii*) the decarboxylation of α-ketoglutarate to succinyl-CoA and CO_2. In *C. thiosulfatophilum*, these two reactions can be reversed because this photosynthetic bacterium is able to generate photochemically reduced ferredoxin[7, 8] and use its strong reducing power to overcome the large energy barrier in these two carboxylations. Ferredoxin is the most electronegative known electron carrier in cellular metabolism. Its oxidation-reduction potential is about 100 mv more electronegative than that of the nicotinamide adenine dinucleotides.[9]

The other two carboxylation reactions in the reductive carboxylic acid cycle pose no thermodynamic difficulties: isocitrate dehydrogenase,[10] an NADP-specific enzyme which catalyzes reversibly the carboxylation of α-ketoglutarate to isocitrate [eq. (3)] and phosphoenolpyruvate carboxylase,[11] which catalyzes the carboxylation of phosphoenolpyruvate to oxalacetate [eq. (4)].



$$\alpha\text{-Ketoglutarate} + CO_2 + NADPH_2 \rightleftharpoons \text{Isocitrate} + \text{NADP} \quad (3)$$

$$\text{Phosphoenolpyruvate} + CO_2 \rightarrow \text{Oxalacetate} + P_i \quad (4)$$

The energy requirements for reactions (3) and (4) are accounted for by the ability of *C. thiosulfatophilum* to generate reduced ferredoxin and ATP at the expense of radiant energy. Reduced ferredoxin is known to reduce nicotinamide adenine dinucleotides [9, 12–14] and hence the supply of these reductants would be assured. The ATP required in the reductive carboxylic acid cycle would come from photophosphorylation. Evidence for an enzyme, phosphoenolpyruvate synthase, that catalyzes the synthesis of phosphoenolpyruvate from ATP and pyruvate in *E. coli* has recently been reported by Cooper and Kornberg.[15] The presence of phosphoenolpyruvate synthase in *C. thiosulfatophilum* was demonstrated by Buchanan and Evans.[16] The equilibrium of the phosphoenolpyruvate synthase reaction lies far on the side of phosphoenolpyruvate formation[15] and would thus favor the operation of the reductive carboxylic acid cycle. A similar effect would also result from the irreversibility of the phosphoenolpyruvate carboxylase[17] [eq. (4)].

Apart from the four carboxylation reactions, the evidence which we now present for the operation of the reductive carboxylic acid cycle rests on (*i*) the identification in extracts of *C. thiosulfatophilum* of the other enzymes that are required to catalyze the sequence of reactions shown in Figure 1, (*ii*) measurements of their rates, and (*iii*) the identification of the C^{14}-labeled products that are formed from $C^{14}O_2$ in short-term experiments.

The reductive carboxylic acid cycle appears to function as a biosynthetic pathway that is particularly suited to provide the carbon skeletons for the main products of bacterial photosynthesis, which are predominantly amino acids.[18–20] Thus, the reactions of the cycle supply α-ketoglutarate for the synthesis of glutamate, oxalacetate for aspartate, and pyruvate for alanine. The application of the reductive carboxylic acid cycle to these and to other biosynthetic reactions in photosynthetic bacteria and in other organisms is being investigated.

Results and Discussion.—Figure 2 shows the results of an experiment in which an illuminated suspension of *C. thiosulfatophilum* cells was exposed to $C^{14}O_2$. Samples were collected at short intervals, killed in ethanol, and centrifuged. Independent experiments showed that practically all of the $C^{14}O_2$ fixed in short-term experiments was in the 80 per cent ethanol soluble fraction. The labeled compounds in the soluble fraction were identified by two-dimensional paper chromatography and radioautography, and their radioactivity was counted directly on the chromatogram.

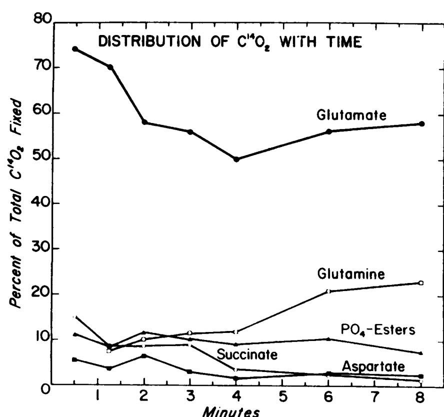


FIG. 2.—Distribution of $C^{14}O_2$ with time in *C. thiosulfatophilum*. Ten ml of a suspension of unwashed fresh cells (300 mg/ml wet weight) and 30 ml growth medium[6] (minus bicarbonate) were mixed in a "lollipop" filled with argon. After gassing with argon for 5 min, the lollipop was illuminated (10,000 lux) for 4 min and then 110 μmoles (240 μc) $Na_2C^{14}O_3$ were injected. With a syringe, 5-ml samples were removed at the times indicated and were injected into ethanol to give a final concentration of 80% ethanol. The cell suspension was stirred with a magnetic stirrer. A positive pressure of argon was maintained in the lollipop throughout the experiment. The ethanol-soluble fractions were analyzed by two-dimensional paper chromatography with two different solvent systems[27, 30] and radioautography.

Glutamate was the earliest stable product identified; after 30 sec it accounted for 75 per cent of the total $C^{14}O_2$ fixed. The negative slope of the glutamate curve with time (Fig. 2) is consistent with the interpretation that glutamate was formed from α-ketoglutarate, which in turn was synthesized by α-ketoglutarate synthase.

Small amounts of labeled succinate, aspartate, and compounds with R_f values corresponding to phosphate esters were also identified after 30 sec exposure to $C^{14}O_2$. The early appearance of succinate and the slightly negative slope of its curve (Fig. 2) are consistent with its role as an important intermediate in the cycle. The proportion of $C^{14}O_2$ fixed into phosphate esters remained relatively constant with time and did not exceed 10 per cent of the total fixation. The rate of total $C^{14}O_2$ incorporation was linear with time for at least 30 min.

The results shown in Figure 2, which, in general, are similar to those obtained for other photosynthetic bacteria,[18-20] are consistent with the operation of the reductive carboxylic acid cycle as a metabolic mechanism that is specially adapted for the synthesis *de novo* of amino acids.

The presence of the enzymes of the reductive carboxylic acid cycle was first established by incubating extracts of *C. thiosulfatophilum* with C^{14}-labeled substrates and identifying the labeled products by paper chromatography and radioautography. In all cases except the fumarate hydratase reaction, which is known to be reversible,[21] the activity of the enzymes was measured in the direction appropriate for their respective positions in the reductive carboxylic acid cycle. Table 1 summarizes the results of these experiments and shows that all enzymes necessary for the reductive carboxylic acid cycle were present in extracts of *C. thiosulfatophilum*. These include two enzymes, the lack of which, in animal cells, constitutes another barrier to the reversibility of the citric acid cycle: citrate lyase and fumarate reductase. (We did not attempt here to separate fumarate reductase from succinate dehydrogenase.)

TABLE 1

ENZYMES OF, AND RELATED TO, THE REDUCTIVE CARBOXYLIC ACID CYCLE FOUND IN EXTRACTS OF *C. thiosulfatophilum*

Enzyme	Additions	Identified C^{14}-labeled products
Aceto-CoA synthetase and pyruvate synthase	$C^{14}O_2$, acetate, ATP, CoA, reduced ferredoxin, glutamate, and heart muscle alanine amino transferase	Alanine
Phosphoenolpyruvate synthase	C^{14}-pyruvate, ATP	Phosphoenolpyruvate
Phosphoenolpyruvate carboxylase and aspartate amino transferase	$C^{14}O_2$, phosphoenolpyruvate, glutamate	Aspartate
Malate dehydrogenase and phosphoenolpyruvate carboxylase	$C^{14}O_2$, phosphoenolpyruvate, $NADH_2$	Malate
Fumarate hydratase and fumarate reductase	C^{14}-fumarate, $NADH_2$	Succinate, malate
Succinyl CoA synthetase and α-ketoglutarate synthase	$C^{14}O_2$, succinate, ATP, CoA, reduced ferredoxin	α-Ketoglutarate, glutamate
Isocitrate dehydrogenase and aconitate hydratase	$C^{14}O_2$, α-ketoglutarate, $NADPH_2$	Isocitrate, citrate
Aconitate hydratase	C^{14}-isocitrate	Citrate
Citrate lyase, aconitate hydratase, and aspartate amino transferase	C^{14}-isocitrate, ATP, CoA, glutamate	Aspartate

Cell-free extracts of *C. thiosulfatophilum* were incubated with the indicated C^{14}-labeled substrates and other additions in the presence of necessary cofactors. After incubation, an 80% ethanol-soluble fraction was prepared and analyzed for radioactive products by two-dimensional paper chromatography and radioautography.[27,31] Detailed procedures for the identification of enzymes will be published elsewhere.

Measurements of the rate of activity of the enzymes of the reductive carboxylic acid cycle in extracts of *C. thiosulfatophilum* are given in Table 2. With the exception of succinate dehydrogenase, which was bound to the chlorophyll-containing particles,[22] all of the enzymes were soluble. The activity of the enzymes ranged from 0.15 to 118 μmoles substrate converted per hour per mg soluble protein at 25°. Except for isocitrate dehydrogenase, fumarate hydratase, and succinate dehydrogenase, the activity of each enzyme was measured in the direction of the cycle (Fig. 1). The lowest activity found for any enzyme in the extract was about one tenth of the rate of CO_2 fixation by whole cells. The rate calculated with whole cells is based on the amount of soluble protein released from the cells by sonic oscillation and, therefore, probably represents a maximum value.

In similar experiments, Peterkofsky and Racker[23] compared the activity of the individual enzymes of the reductive pentose phosphate cycle in leaf and algal extracts with the rate of $C^{14}O_2$ fixation by whole cells. In their experiments, the lowest rate for a single enzyme of the reductive pentose phosphate cycle was only one thirtieth of the rate of CO_2 fixation by whole cells.

More detailed evidence for the presence of phosphoenolpyruvate synthase in *C. thiosulfatophilum,* as well as some other photosynthetic bacteria, has been presented elsewhere.[16] Certain enzymes shown in Table 2 (malate dehydrogenase, fumarate hydratase, succinate dehydrogenase, isocitrate dehydrogenase, aconitate hydratase) were previously assayed in extracts of *C. thiosulfatophilum* by Smillie and Evans[22] with essentially comparable results, although they were unable to demonstrate aconitate hydratase.

In a brief communication, Smillie *et al.*[24] reported that enzymes of the reductive pentose phosphate cycle were present in *C. thiosulfatophilum* and suggested that this cycle was operative in the assimilation of CO_2 by this organism. The assessment of the relative contribution of the reductive carboxylic acid cycle and the reductive pentose phosphate cycle in the assimilation of CO_2 in *C. thiosulfatophilum* and in other photosynthetic bacteria must await future experiments.

TABLE 2

ACTIVITIES OF ENZYMES OF THE REDUCTIVE CARBOXYLIC ACID CYCLE* IN EXTRACTS OF *C. thiosulfatophilum*

Enzyme	Activity (μmoles/mg prot/hr)
Aceto-CoA synthetase	0.8
Pyruvate synthase	0.2
Phosphoenolpyruvate synthase	2.3
Phosphoenolpyruvate carboxylase	4.8
Malate dehydrogenase	37
Fumarate hydratase	118
Succinate dehydrogenase	0.85
Succinyl-CoA synthetase	1.6
α-Ketoglutarate synthase	0.4
Isocitrate dehydrogenase	102
Aconitate hydratase	3.1
Citrate lyase	0.15

Assays for pyruvate synthase,[4] α-ketoglutarate synthase,[7] and phosphoenolpyruvate synthase[15] are described elsewhere. Other enzymes were assayed by standard procedures[28, 29] and will be described in detail subsequently. The rate of photosynthetic $C^{14}O_2$ fixation by whole cells was measured anaerobically with unwashed fresh cells suspended in growth medium.[6]

* Corresponding rates were also determined for the following: alanine amino transferase, 2.2; aspartate amino transferase, 7.4; and total $C^{14}O_2$ fixation by whole cells, 1.8. The following enzymes were not detected under the conditions used: pyruvate kinase, malate dehydrogenase (decarboxylating), isocitrate lyase, glutamate dehydrogenase, and lactate dehydrogenase.

Concluding Remarks.—Evidence has been presented for the occurrence of a reductive carboxylic acid cycle in *Chlorobium thiosulfatophilum*, a green sulfur photosynthetic bacterium. In one complete turn of the cycle, four molecules of CO_2 are fixed in sequence to give a *de novo* synthesis of a C_4-acid. The C_4-acid can be further metabolized through the cycle to provide 2, 3, 4, 5, or 6 carbon compounds for the synthesis of amino acids, lipids, porphyrins, or other cellular constituents. The cycle depends for its operation on an input of energy, supplied by photochemical reactions which generate reduced ferredoxin and ATP.

It is premature to assess the importance of the new cycle in relation to the reductive pentose phosphate cycle which hitherto has been regarded as the sole cyclic mechanism for CO_2 assimilation and which has been reported[24] in *C. thiosulfatophilum*. It is possible that the reductive pentose phosphate cycle is mainly concerned with sugar synthesis, whereas the reductive carboxylic acid cycle functions mainly in the synthesis of amino acids and precursors of lipids and porphyrins.

Ferredoxin-dependent CO_2 fixation has been demonstrated only in anaerobic bacteria; therefore, the reductive carboxylic acid cycle cannot, on present evidence, be extended to green plants. As far as other phosynthetic bacteria are concerned, it is significant that short-exposure experiments with *Chromatium*[19] and *R. rubrum*[20] show amino acids as the main early products of $C^{14}O_2$ fixation. It is likely, therefore, that the reductive carboxylic acid cycle, or a variant of it, functions in these organisms. It is also possible that the new cycle operates in anaerobic, nonphotosynthetic bacteria, such as *Clostridium thermoaceticum*, which contains ferredoxin[25] and reduces CO_2 to acetic acid[26] in its fermentation.

Abbreviations: P_i, inorganic phosphate; ox, oxidized; red, reduced; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CoA, coenzyme A; NAD and $NADH_2$, oxidized and reduced nicotinamide adenine dinucleotide; NADP and $NADPH_2$, oxidized and reduced nicotinamide adenine dinucleotide phosphate; acetyl-CoA and succinyl-CoA, thiolesters of coenzyme A and acetate or succinate; flavin·H_2, reduced flavin nucleotide.

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*THE PARTICIPATION OF SRNA IN THE ENZYMATIC SYNTHESIS OF O-L-LYSYL PHOSPHATIDYLGYLCEROL IN STAPHYLOCOCCUS AUREUS**

BY W. J. LENNARZ,† J. A. NESBITT, III, AND J. REISS

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE, BALTIMORE, MARYLAND

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"Lipoamino acids," containing amino acids esterified to phosphatidylglycerol, have been found in a number of bacteria. Macfarlane first reported the occurrence of the *O*-L-lysyl and alanyl esters of phosphatidylglycerol in *Clostridium welchii* and the *O*-L-lysyl ester in *Staphylococcus aureus* (Fig. 1).[1] These findings have been confirmed by van Deenen and his co-workers,[2, 3] who have also found *O*-L-lysyl phosphatidylglycerol in *Streptococcus faecalis*[3] and in *Bacillus megaterium*,[4] as well as ornithinyl phosphatidylglycerol in *Bacillus cereus*.[5] Amino acids (lysine and alanine) bound to lipids have also been reported in the lactobacilli.[6] No information has been available on the mechanism of biosynthesis of this class of compounds.

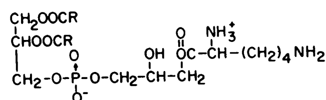

FIG. 1.—Structure of *O*-L-lysyl phosphatidylglycerol. The position of linkage of the lysyl group to the glyceryl moiety (2′ or 3′) is not known.

In this communication we report that cell-free extracts of *S. aureus* catalyze the incorporation of C^{14}-L-lysine into lysyl phosphatidylglycerol. The over-all reaction appears to take place in at least two enzymatic steps. In the first, free lysine is activated with the formation of lysyl-sRNA. The latter then serves as the lysyl group donor in the enzymatic formation of lysyl phosphatidylglycerol.

Methods.—Staphylococcus aureus, a penicillin-resistant 81/80 strain obtained from the Department of Microbiology, the Johns Hopkins University School of Medicine, was grown to the late log phase (450 Klett units at 660 mμ) in 1-liter cultures at 37° with shaking. Details on the syn-