Carbon Dioxide Fixation in Green Sulphur Bacteria

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1. About one-third of the CO₂ fixed during photosynthesis by washed suspensions of *Chlorobium thiosulfatophilum* strain 8346 gave rise to α-oxoglutarate and branched-chain oxo acids, mainly β-methyl-α-oxovalerate. Another one-third to one-half gave rise to a polyglucose. 2. The fixation of CO₂ was inhibited by fluoroacetate, increasing concentrations up to 1 mM stimulating the accumulation of α-oxoglutarate and causing a decrease in the formation of the branched-chain oxo acids and polyglucose. 3. Acetate was converted into the same products as was CO₂. 4. Fluoroacetate (1 mM) had a negligible effect on the formation of polyglucose from acetate and caused a slight inhibition of the formation of the branched-chain oxo acids and increased accumulation of α-oxoglutarate. 5. Iodoacetate (1 mM) strongly inhibited polyglucose formation from acetate and caused accumulation of pyruvate. The formation of the branched-chain oxo acids from acetate was only slightly affected by this inhibitor. 6. Pyruvate can be metabolized by this organism in the presence of a suitable electron donor whether CO₂ is present or not. In the absence of CO₂ pyruvate is converted into polyglucose. 7. The accumulation of oxo acids during CO₂ fixation is completely inhibited by NH₄⁺ ions. The formation of the branched-chain oxo acids is considerably decreased by the presence of isoleucine, leucine or valine, or a mixture of these. 8. CO₂ fixation in two other strains of *Chlorobium* appears to exhibit a similar pattern to that in *C. thiosulfatophilum* strain 8346. 9. It is concluded that in washed suspensions, CO₂ is fixed mainly by a mechanism involving the reductive carboxylic acid cycle. Acetate, the product of the cycle, is converted into polyglucose via pyruvate synthase and a reversal of glycolysis or into branched-chain oxo acids by an unknown mechanism.

Bacteria of the genus *Chlorobium* are photosynthetic anaerobes that use inorganic sulphur compounds or molecular H₂ as electron donors for the light-dependent reduction of CO₂ to cell material.

The green sulphur bacteria have traditionally been regarded as strict autotrophes (van Niel, 1941) and this was confirmed by Larsen (1953). However, Sadler & Stanier (1960) showed that *Chlorobium limicola* could use acetate as a source of cell carbon provided that bicarbonate and an electron donor such as sulphide were available. These observations were confirmed by Hoare & Gibson (1964) with *Chlorobium thiosulfatophilum* both in growing cultures and in washed suspensions.

It has been generally accepted that the mechanism of CO₂ fixation in autotrophic bacteria is similar to that in green plants, namely, the reductive pentose phosphate cycle (Elsden, 1962). Most of the enzymes of this cycle were indeed shown to be present in cell-free extracts of *C. thiosulfatophilum* by Smillie, Rigopoulos & Kelly (1962).

More recently two new, ferredoxin-dependent, CO₂-fixation reactions have been described in cell-free extracts of *C. thiosulfatophilum* (Evans & Buchanan, 1965; Buchanan & Evans, 1965):

The enzymes catalysing the reactions are pyruvate synthase and α-oxoglutarate synthase respectively. On the basis of these two reactions, Evans, Buchanan & Arnon (1966) proposed a new cyclic pathway for CO₂ assimilation, which they named the reductive carboxylic acid cycle. This mechanism is in principle a reversal of the well-known tricarboxylic acid cycle. It should be pointed out that van Niel (1949) envisaged operation of the tricarboxylic acid cycle in reverse as a mechanism of CO₂ fixation.

In support of the proposed pathway, Evans *et al.*

(1966) showed that all the enzymes of the cycle were present in cell-free extracts, although the activity of some of them was low compared with the rate of CO_2 fixation in whole cells. It was also shown that glutamate was among the first stable products labelled when ^{14}C bicarbonate was added to a growing culture. Although these results strongly suggest that the cycle operates in this organism, they give no indication of the extent of its activity. It is possible that both the reductive carboxylic acid cycle and the reductive pentose phosphate cycle operate simultaneously in *Chlorobium*. Indeed Evans *et al.* (1966) suggested that the reductive carboxylic acid cycle may be concerned mainly with synthesis of precursors of amino acids, lipids and porphyrins, and the reductive pentose phosphate cycle with carbohydrate synthesis.

We have used inhibitors in an attempt to differentiate between the two cyclic mechanisms. An earlier observation of inhibition of CO_2 fixation in *C. thiosulfatophilum* by fluoroacetate (Ormerod, 1957) prompted us to investigate further the action of this inhibitor.

A preliminary account of some of this work has already been published (Sirevåg & Ormerod, 1970).

MATERIALS AND METHODS

Organisms. *C. thiosulfatophilum* strains 8327 and 8346 were obtained from Dr Norbert Pfennig. *Chlorobium* strain R1 was isolated from Lake Polden, near Oslo, Norway. This organism is probably a strain of *C. limicola*.

Culture of organisms. Stock cultures were maintained in 65ml screw-capped bottles completely filled with Pfennig's (1961, 1962) medium modified as follows: the concentration of NH_4Cl was 0.1% and, for *C. thiosulfatophilum*, the $\text{Na}_2\text{S}_9\text{H}_2\text{O}$ concentration was decreased to 0.01% and $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ was added to a concentration of 0.1%. After inoculation with 1ml of a mature culture the bottles were incubated in a light-cabinet at 25–28°C at approx. 30 cm from a row of 25 W incandescent lamps for 24–48 h. The fully grown stock cultures were stored in the refrigerator and subcultured every 2–3 months. For use in experiments the organisms were grown in the same media in completely filled 320 ml screw-cap bottles. The inoculum was 1 or 10 ml of a mature culture and the incubation time 40–48 or 20–24 h respectively. Both these procedures gave similar cell densities at the time of harvesting.

Preparation of washed suspensions. Growth was determined in cultures immediately before harvesting by measuring extinction values in an EEL colorimeter with an ND-3 filter and referring to a standard graph relating extinction to dry weight. Elementary S produced during growth sedimented in the culture bottles. Therefore, to obtain bacterial suspensions of low sulphur content (as confirmed by microscopic observation), cultures were carefully decanted before being centrifuged at 40000g for 1 min at 5°C. The cells were washed once with a boiled and cooled mineral solution of composition similar to that of the thiosulphate growth medium, but without vitamin B_{12} , NH_4Cl , NaHCO_3 and $\text{Na}_2\text{S}_2\text{O}_3$, and then suspended

in the same solution. Na_2S was added after cooling. The volume of the washed suspensions was adjusted to give 4.4 mg dry wt./ml.

Chemicals. Sodium fluoroacetate was a gift from Professor H. Larsen. Sodium malonate and PtO_2 (Adam's catalyst) were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. L-Amino acid oxidase, catalase, isocitrate dehydrogenase, amino acids and sodium pyruvate, α -oxoglutarate, iodoacetate and D-isocitrate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. β -Methyl- α -oxovalerate (3-methyl 2-oxo-pentanoate), α -oxoisovalerate (3,3-dimethyl-2-oxopropionate) and α -oxoisocaproate (4,4-dimethyl-2-oxobutyrate) were prepared from L-isoleucine, L-valine and L-leucine respectively by oxidative deamination with L-amino acid oxidase as described by Meister (1951, 1952). Sodium succinate (anhydrous, pure) was obtained from Fluka A.-G., Buchs S.G., Switzerland; glucose oxidase (Blood Sugar Colorimetric Test) was from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany; light petroleum (A.R. grade) was from BDH Chemicals Ltd., Poole, Dorset, U.K. The diethyl ether used was sodium-distilled, commercial grade. Anthrone (synthesis grade), ammonium vanadate (pure) and all other chemicals were of A.R. grade, and obtained from E. Merck A.G., Darmstadt, Germany. $\text{Na}_2\text{S}_2\text{O}_3$ was recrystallized twice before use. Solutions of $\text{Na}_2\text{S}_2\text{O}_3$ and Na_2S were prepared immediately before use.

Experimental procedure. CO_2 fixation was measured in volumetric respirometers (Scholander & Iversen, 1958) with 20–25 ml flasks. The respirometers were shaken in a water bath at 30°C and illuminated by a row of six 60 W incandescent lamps 20 cm away. The respirometer flasks contained, in a final volume of 2.0 ml, 4.4 mg (dry wt.) of cells and mineral solution of the same composition as that used to wash the cells, together with $15 \mu\text{mol}$ of NaHCO_3 . Each flask was gassed by means of a hypodermic needle inserted through the rubber injection stopper. The cell suspension was added with a 1 ml syringe through the stopper and the flasks were regassed. Unless otherwise stated, the gas phase was $\text{Ar} + \text{CO}_2$ (99:1). When fluoroacetate was used it was added to the cells approx. 15 min before the substrate. Substrates (0.05 ml) were added by volume-compensated injection with micro-syringes (Scholander & Iversen, 1958) through the rubber stopper, and the reaction was started by turning on the lights. The cells were inactivated at the desired time by adding, through the stopper, 0.05 ml of 50% (w/v) citric acid, or, when analysis for citrate or isocitrate was to be carried out, 2 M- H_2SO_4 . The acid served both to inactivate the cells and to liberate bound CO_2 , which could then be measured. During the process of photosynthesis, H_2SO_4 and other acids produced by the cells liberate CO_2 from the bicarbonate in the suspension. The gaseous exchange measured during the experiment thus represents the difference between CO_2 assimilated and that liberated from bicarbonate by the acid produced. In each experiment one respirometer was used to measure initial bound CO_2 . CO_2 fixation was then calculated as the sum of the observed gas uptake and the change in bicarbonate concentration in each case. A control flask without substrate was also run in each experiment. In general the endogenous gas exchange was negligible. At the end of each experiment the acidified cell suspensions were centrifuged at 3000

rev./min for 5 min and the supernatants and sediments were used for analysis.

The values obtained by the respirometric method for CO_2 fixed/ μmol of thiosulphate added (Table 1), were generally considerably lower than those obtained by Larsen (1953). The method used was therefore checked by absorbing the CO_2 remaining after a fixation experiment. 10M-NaOH (0.05 ml) was added by syringe to a filter-paper disc placed on an absorption plate in the respirometer flask (Scholander, Claff, Andrews & Wallach, 1952), and the subsequent decrease in volume was recorded. The initial value for the CO_2 content of the same respirometer was determined separately.

A further check was made by measuring CO_2 fixation in Warburg manometers with Dixon-Keilin vessels (Dixon, 1943) to determine total CO_2 after fixation by the cells. Initial CO_2 was determined in a control manometer. Essentially identical results were obtained with all of these methods.

Analysis showed that all the thiosulphate added had disappeared when CO_2 fixation had ceased, and that 80–90% had been converted into sulphate. The reason for the difference between these results and those of Larsen (1953) is not known, but the discrepancy is not considered to have any bearing on the results to be described.

Analytical methods. Thiosulphate was determined by conventional iodometry and sulphate turbidimetrically as described by van Gernerden (1968a). Citrate was determined as described by Taylor (1953). Isocitrate was determined enzymically (Ochoa, 1955) with isocitrate dehydrogenase and NADP^+ by following the increase in E_{340} in a Bausch and Lomb Spectronic 20 colorimeter.

The 2,4-dinitrophenylhydrazones derivatives of oxo acids in the experimental supernatants were prepared and extracted for chromatography by the method of Cavallini, Frontali & Toschi (1949) as modified by Kogut & Podoski (1953). The 2,4-dinitrophenylhydrazones of pyruvate, α -oxoglutarate, α -oxoisovalerate, β -methyl- α -oxovalerate and α -oxoisocaproate were prepared and treated by the same procedure. T.l.c. was carried out on Woelm TLC aluminium sheets precoated with silica gel, by using light petroleum (b.p. 100–120°C)–toluene–acetic acid–water (10:10:17:3, by vol.) (Bush & Hockaday, 1962). The same solvent or aq. 5% (w/v) NaHCO_3 (Smith, 1967) was used for descending paper chromatography on Whatman no. 1 paper. Developed chromatograms were sprayed with or dipped in 1.0M-NaOH in aq. 80% (v/v) ethanol (Bush & Hockaday, 1962).

As described below (see the Results section) a mixture of oxo acids was present in the experimental supernatants, the main components being α -oxoglutarate and β -methyl- α -oxovalerate with smaller amounts of α -oxoisocaproate and α -oxoisovalerate and sometimes traces of pyruvate. In one case (Table 2) it was shown that a considerable amount of pyruvate accumulated. For determination of oxo acids in experimental supernatants, the procedure of Friedemann & Haugen (1943) was modified as follows: the first extraction of the 2,4-dinitrophenylhydrazone mixture was carried out with light petroleum (b.p. 100–120°C), and the second with ethyl acetate. Extinctions were measured in an EEL colorimeter with an OB 10 (blue) filter. A test with the authentic oxo acid derivatives showed that the light-petroleum extraction procedure gave a colour recovery of 63% with the derivatives of β -methyl- α -oxo-

valerate and α -oxoisovalerate. The corresponding values for α -oxoglutarate and pyruvate were 4 and 8% respectively. For the subsequent ethyl acetate extraction procedure the values with α -oxoglutarate and pyruvate were 72 and 54% respectively, and the β -methyl- α -oxovalerate and α -oxoisovalerate derivatives gave a recovery of less than 0.5%.

Thus the oxo acids measured by the light-petroleum extraction procedure are mostly the branched-chain compounds (as represented by β -methyl- α -oxovalerate) and will be referred to as branched-chain oxo acids. The ethyl acetate extraction procedure gives values for α -oxoglutarate and pyruvate. These two oxo acids were not determined individually. The preponderance of one or the other was assessed by t.l.c. or paper chromatography. When pyruvate was present in more than trace amounts (as judged from chromatograms) the oxo acids determined by the ethyl acetate procedure are referred to as polar oxo acids.

Mass-spectrometric analysis was carried out by using an AEI MS 902 mass spectrometer with a resolving power of 20 000.

N.m.r. spectra were obtained with a Varian A 60A instrument with deuteriochloroform as solvent and tetramethylsilane as internal standard.

Catalytic hydrogenation of the 2,4-dinitrophenylhydrazones of oxo acids to the corresponding amino acids was carried out as described by Meister & Abendschein (1956) except that H_2 at atmospheric pressure was used. Ascending paper chromatography of the amino acids was carried out on Whatman no. 1 paper with pyridine–3-methylbutan-1-ol–water (7:6:6, by vol.) (Heyns & Anders, 1951), or with water-saturated phenol–conc. NH_3 (200:1, v/v) (Consden, 1968). The same solvents were used for ascending t.l.c. on Woelm TLC aluminium sheets precoated with silica gel.

DL-Isoleucine, L-leucine, DL-valine, DL-alanine and L-glutamic acid were used as standards and amino acids were detected with ninhydrin.

Unless otherwise stated, total carbohydrate was determined by the anthrone method (Trevelyan & Harrison, 1952). Interfering pigments were removed from the cell material by washing twice with 5 ml of ethanol and centrifuging. For enzymic determination of polyglucose, cell material was hydrolysed by heating at 100°C in 1M- H_2SO_4 for 1 h. Glucose in the neutralized hydrolysate was determined colorimetrically by use of glucose oxidase.

For paper chromatography of the sugars in hydrolysed cell material a sample of the acid hydrolysate was centrifuged and saturated $\text{Ba}(\text{OH})_2$ solution was added to slight excess (pH 10–11). CO_2 was bubbled through to precipitate the excess of $\text{Ba}(\text{OH})_2$ and the suspension was centrifuged. The supernatant was used for descending paper chromatography with butan-2-one–acetone–water, (15:5:3, by vol.) (Høgstrøm, 1957) on Whatman no. 1 paper and sugars were detected with aniline hydrogen phthalate (Consden, 1968).

RESULTS

Products of carbon dioxide fixation in washed suspensions of C. thiosulfatophilum strain 8346. Most (60–90%) of the CO_2 fixed could be accounted

Fig. 1. Relationship between thiosulphate metabolized and products formed by photosynthesis in *C. thiosulfatophilum*. Cells were grown, harvested and washed as described in the text. A 500 ml Erlenmeyer flask was fitted with a rubber stopper equipped with a glass tube (6 mm external diam.) and the upper portion of a screw-capped test tube (16 mm external diam.). This arrangement facilitated gassing, addition of cells and substrate and removal of samples. The flask was mounted on a shaker and illuminated from below by a 150 W incandescent lamp, 10 cm distant and cooled by a fan. The flask contained in a final volume of 24 ml/52.8 mg dry wt. of washed cells, mineral solution of the same composition as that used to wash the cells and 180 μmol of NaHCO₃. The flask was gassed with argon+CO₂ (99:1) before and after addition of the cells. The reaction was started by adding 120 μmol of thiosulphate and turning on the light. At zero time and at 15 min intervals, a 5 ml sample was removed from the suspension. The flask was gassed during sample taking. Citric acid (0.2 ml, 50% w/v) was added to the sample and the suspension was centrifuged for 5 min at 3000 rev./min. The supernatant fluid was analysed for thiosulphate, sulphate and oxo acids, and the cell material for polysaccharide. ○, Polysaccharide (as glucose); △, α-oxoglutarate; □, branched-chain oxo acids; ●, sulphate; ▲, thiosulphate.

for as insoluble polysaccharide in the cell material and oxo acids in the supernatant. The relationship between thiosulphate metabolized and products formed by photosynthesis as a function of time is shown in Fig. 1. The increase in cell carbohydrate

as measured by the anthrone method in unhydrolysed cell material was the same as that measured by glucose oxidase in acid-hydrolysed cell material. This shows that the carbohydrate formed from CO₂ is a polyglucose as in other photosynthetic bacteria (Stanier, Doudoroff, Kunisawa & Contopoulou, 1959; van Gernerden, 1968b). The presence of glucose in the cell hydrolysate was further confirmed by paper chromatography. Only two spots appeared, corresponding in *R_F* value and colour reaction to glucose and ribose.

Preliminary examination by paper chromatography and t.l.c. of the 2,4-dinitrophenylhydrazones of the oxo acids in supernatants from CO₂-fixation experiments showed the presence of substances corresponding in *R_F* value and alkaline colour reaction to the derivatives of α-oxoglutarate and either β-methyl-α-oxovalerate or α-oxoisocaproate.

The identification of the oxo acids was further substantiated by conversion of the 2,4-dinitrophenylhydrazones into the corresponding amino acids followed by paper chromatography or t.l.c. Material for this was obtained in a large-scale experiment in a 500 ml Erlenmeyer flask under conditions similar to those described for respirometer experiments but with 12 times the amount of cells and 24 times the amount of substrate (thiosulphate). The flask was closed by a rubber stopper fitted with glass tubes to facilitate gassing. After 3 h of incubation with shaking in the light, the cells were inactivated by adding 0.25 ml of 2M-H₂SO₄ and the suspension was centrifuged. The supernatant was clarified by filtration through a membrane filter (Millipore, 0.45 μm pore diam.). The 2,4-dinitrophenylhydrazones were prepared, extracted and reduced to amino acids by the procedures referred to in the Materials and Methods section. Paper chromatography and t.l.c. showed that the major components were isoleucine and glutamic acid. Also present, though in smaller amounts, were amino acids with *R_F* values corresponding to leucine and valine, and several other very faint unidentified spots were also present.

The 2,4-dinitrophenylhydrazones were also isolated in the crystalline state from the supernatant of an experiment carried out in a manner similar to that for the large-scale experiment described above, except that the gas phase was H₂+CO₂ (99:1), since this had been shown by experiment to increase the yield of oxo acids. A sample of the crystalline precipitate was recrystallized from warm aq. 80% (v/v) ethanol and dried *in vacuo* over P₂O₅. T.l.c. showed that only branched-chain oxo acid hydrazones were present in this material.

Mass-spectrometric analysis revealed a component with molecular weight 310.092 (C₁₂H₁₄N₄O₆), thus agreeing with the results from paper chromatography, which indicated the presence of the deriva-

Fig. 2. Effect of fluoroacetate on CO_2 fixation and formation of oxo acids and polysaccharide in *C. thiosulfatophilum* strain 8346. Respirometers contained 4.4 mg (dry wt.) of washed cells, mineral solution of the same composition as that used to wash the cells and $15 \mu\text{mol}$ of NaHCO_3 in a final volume of 2.0 ml. Fluoroacetate to give the concentrations indicated was added 15 min before the substrate. Gas phase was $\text{Ar} + \text{CO}_2$ (99:1) and the temperature 30°C . Thiosulphate ($10 \mu\text{mol}$) was added with a microsyringe and the reaction was started by turning on the lights. The reaction was stopped in all flasks by adding 0.05 ml of 50% (w/v) citric acid when CO_2 uptake had ceased in the flask with thiosulphate alone (90 min). The cells were removed by centrifugation and supernatants were analysed for oxo acids, and cell material for polysaccharide. ○, Polysaccharide (as glucose); △, α -oxoglutarate; □, branched-chain oxo acids; ■, CO_2 fixed.

tive of β -methyl- α -oxovalerate or of α -oxoisocaproate or a mixture of both.

The n.m.r. spectrum of the same material had a very complex coupling pattern in the region corresponding to the protons of the aliphatic carbon chain, indicating the presence of an asymmetrically branched molecule, namely β -methyl- α -oxovalerate.

The presence of the α -oxoisocaproate derivative is, however, not excluded by this evidence.

Effect of inhibitors on carbon dioxide fixation.
Fluoroacetate. The inhibitory action of fluoroacetate in aerobic cells is due to its conversion product, fluorocitrate, which inhibits aconitate hydratase, thus blocking the tricarboxylic acid cycle at that point (Morrison & Peters, 1954). Fluoroacetate has a profound effect on the metabolism of *C. thiosulfatophilum*, 10 mM concentrations completely inhibiting photosynthesis. The effect of various concentrations of fluoroacetate on CO_2 fixation and formation of polysaccharide and oxo acids is shown in Fig. 2. Inhibition of CO_2 fixation is accompanied by a decrease in the formation of polysaccharide and branched-chain oxo acids. Since addition of fluoroacetate to cells having tricarboxylic acid-cycle activity generally leads to accumulation of citrate, one would expect *a priori* that inhibition of the reversed tricarboxylic acid cycle at the same point would cause accumulation of isocitrate. No accumulation of either of these two compounds could be detected in experimental supernatants after CO_2 fixation in the presence of thiosulphate and various concentrations of fluoroacetate. On the other hand, increasing concentrations of fluoroacetate up to 1 mM augmented the accumulation of α -oxoglutarate. (Fig. 2).

Arsenite. CO_2 uptake was completely inhibited by 0.1 mM-arsenite and no oxo acids accumulated, indicating that the oxo acid synthase reactions of Evans *et al.* (1966) involve lipoic acid as cofactor, just as do the oxo acid oxidase reactions.

Malonate. Malonate, the classic inhibitor of the tricarboxylic acid cycle, had no effect on oxo acid accumulation but caused a slight increase in CO_2 fixation and a larger increase in polysaccharide formation. These results indicate that malonate is assimilated by this organism.

Metabolism of organic compounds by washed suspensions.
Acetate. The addition of acetate to washed suspensions of *C. thiosulfatophilum* in the presence of CO_2 and thiosulphate or molecular H_2 results in a large increase in accumulation of α -oxoglutarate, branched-chain oxo acids and polysaccharide (Sirevåg & Ormerod, 1970). T.l.c. of the 2,4-dinitrophenylhydrazones also showed that traces of pyruvate were present. In comparison with suspensions without added acetate (Fig. 2), 1 mM-fluoroacetate in the presence of acetate causes only a slight inhibition of CO_2 fixation and of branched-chain oxo acid formation, whereas accumulation of α -oxoglutarate is increased (Sirevåg & Ormerod, 1970).

In contrast with fluoroacetate, iodoacetate caused a large decrease in the amount of polysaccharide formed in the presence of acetate. At the same time, the formation of the branched-chain

Table 1. *Effect of iodoacetate on acetate metabolism in C. thiosulfatophilum strain 8346*

Experimental conditions and procedure were the same as for Fig. 2, except that acetate was added as indicated, together with the thiosulphate, and iodoacetate (0.1 mM) replaced fluoroacetate. The duration of the experiment was 60 min.

Additions	CO ₂ fixed (μ mol)	Polar oxo acids (μ mol)	Branched-chain oxo acids (μ mol)	Polysaccharide (as glucose) (μ mol)
None (initial control)	—	0.08	0.0	0.94
Thiosulphate	11.5	0.12*	0.39	1.68
Thiosulphate, acetate	9.4	0.25*	0.86	2.26
Thiosulphate, acetate, iodoacetate	10.3	0.58	0.70	1.62

* α -oxoglutarate

Table 2. *Metabolism of acetate and pyruvate in the absence of carbon dioxide in C. thiosulfatophilum strain 8346*

The experimental conditions were the same as for Fig. 2, except that NaHCO₃ was omitted and the gas phase was H₂. In addition a CO₂-absorption plate containing 0.1 ml of 10 M-NaOH and a folded filter paper were included in each flask. Acetate (5 μ mol) and pyruvate (5 μ mol) were added by injection as indicated. The duration of the experiment was 120 min. Supernatants were analysed for oxo acids, and cell material for polysaccharide.

Additions	H ₂ uptake (μ mol)	Polar oxo acids (μ mol)	Branched-chain oxo acids (μ mol)	Polysaccharide (as glucose) (μ mol)
None (initial control)	—	0.06	0.00	0.49
None	1.5	0.05	0.01	0.55
Acetate	1.8	0.06	0.00	0.54
Pyruvate	2.8	0.14	0.01	0.75

oxo acids was inhibited considerably less, and the formation of the more polar oxo acids was increased (Table 1). T.l.c. and paper chromatography showed that a considerable amount of pyruvate was present in addition to α -oxoglutarate.

Pyruvate. Like acetate, pyruvate is metabolized by the organism in the presence of a suitable electron donor and CO₂. A washed suspension metabolized 5 μ mol of pyruvate in the presence of CO₂ and 10 μ mol of thiosulphate with the production of approx. 1.2 μ mol of oxo acids, about half of which consisted of branched-chain oxo acids. This result indicates that at least 80% of the pyruvate added had been metabolized; there was an increase in polysaccharide (as glucose) of 0.83 μ mol over and above the amount formed with thiosulphate alone.

Pyruvate is converted into polysaccharide in the complete absence of CO₂ provided that molecular H₂ is present (Table 2). The process is accompanied by a slow H₂ uptake, slightly greater than that occurring in the control without pyruvate. Acetate assimilation, on the other hand, is completely dependent on the presence of CO₂.

Succinate. In some experiments succinate was found to stimulate CO₂ uptake in the presence of thiosulphate and to increase approximately three-fold the accumulation of α -oxoglutarate. However,

the results with succinate varied from suspension to suspension.

Effect of nitrogen and nitrogen compounds on carbon dioxide fixation and oxo acid accumulation. Since oxo acids are the immediate precursors of amino acids, the relationship between oxo acid accumulation and the presence of nitrogen sources was investigated. The substitution of N₂ for argon in the gas phase had no effect on oxo acid accumulation or CO₂ fixation. However, ammonium chloride completely inhibited the accumulation of oxo acids without appreciably affecting CO₂ uptake (Table 3).

The accumulation of oxo acids in washed suspensions may be due to relief of allosteric feedback inhibition on removal of the nitrogen source. To test this idea, the effect of the branched-chain amino acids on photosynthesis was investigated. The addition of isoleucine, leucine or valine or a mixture of the three decreased the accumulation of branched-chain oxo acids by up to 40% (Table 4). The presence of an amino acid not belonging to this biosynthetically related group (phenylalanine), or of calcium pantothenate, did not have this effect.

Carbon dioxide fixation in washed suspensions of other Chlorobium strains. CO₂ fixation in *C. thiosulfatophilum* strain 8327 resulted in a pattern

Table 3. *Effect of ammonium chloride on carbon dioxide fixation and oxo acid formation in C. thiosulfatophilum strain 8346*

Experimental conditions and procedure were the same as for Fig. 2. NH_4Cl was added to the cells as indicated to give a final concentration of 0.05%. The duration of the experiment was 180 min. Supernatants were analysed for oxo acids.

Additions	CO_2 fixed (μmol)	α -Oxo- glutarate (μmol)	Branched-chain oxo acids (μmol)
None	0.45	0.02	0.00
Thiosulphate	10.7	0.16	0.67
Thiosulphate, NH_4Cl	11.9	0.01	0.00

Table 4. *Effect of amino acids on carbon dioxide fixation and oxo acid formation in C. thiosulfatophilum strain 8346*

Experimental conditions were similar to those for Fig. 2. L-Amino acids ($1 \mu\text{mol}$ of each) and calcium pantothenate ($0.1 \mu\text{mol}$) were added as indicated approx. 15 min before addition of thiosulphate. The duration of the experiment was 105 min. Supernatants were analysed for oxo acids.

Additions	CO_2 fixed (μmol)	α -Oxo- glutarate (μmol)	Branched-chain oxo acids (μmol)
None	—	0.08	0.03
Thiosulphate	13.0	0.17	0.53
Thiosulphate, isoleucine	13.8	0.13	0.29
Thiosulphate, leucine	14.6	0.13	0.39
Thiosulphate, valine	14.0	0.13	0.28
Thiosulphate, pantothenate	13.0	0.19	0.52
Thiosulphate, isoleucine, leucine, valine, pantothenate	13.7	0.13	0.31
Thiosulphate, phenylalanine	12.9	0.19	0.48

Table 5. *Products of carbon dioxide fixation in C. thiosulfatophilum strain 8327: effect of fluoroacetate*

Experimental conditions were similar to those for Fig. 2. Fluoroacetate concentrations were as indicated. The duration of the experiment was 105 min. Supernatants were analysed for oxo acids, and cell material for polysaccharide.

Concn. of fluoroacetate	CO_2 fixed (μmol)	α -Oxo- glutarate (μmol)	Branched-chain oxo acids (μmol)	Polysaccharide (as glucose) (μmol)
0 (initial control)	—	0.05	0.03	0.89
0 (no thiosulphate)	3.0	0.07	0.10	0.97
0	12.7	0.19	0.39	1.80
$2 \mu\text{M}$	13.4	0.11	0.38	1.86
$10 \mu\text{M}$	13.6	0.13	0.32	1.83
0.2 mM	3.2	0.21	0.07	0.74
1 mM	3.0	0.21	0.06	0.84

of assimilation similar to that occurring in strain 8346, as shown in Table 5. Further, fluoroacetate caused an increase in the accumulation of α -oxo-glutarate and a pronounced decrease in the formation of branched-chain oxo acids and polysaccharide.

Chlorobium R1 cannot utilize thiosulphate; molecular H_2 was therefore used as electron donor

for CO_2 fixation in respirometer experiments with washed suspensions. In one experiment, the cells assimilated $29 \mu\text{mol}$ of gas ($\text{H}_2 + \text{CO}_2$) over a period of 60 min. At the end of the experiment, analysis of the supernatant liquid showed the presence of $0.42 \mu\text{mol}$ of branched-chain oxo acids and $0.24 \mu\text{mol}$ of α -oxoglutarate.

DISCUSSION

The fact that α -oxoglutarate accumulates during photosynthesis in washed suspensions of *C. thiosulfatophilum* is in agreement with the suggestion of Evans *et al.* (1966) that a reversed tricarboxylic acid cycle is involved in CO₂ fixation. Proof for the operation of a cyclic biochemical mechanism should be based on several lines of evidence. Among these may be mentioned: (i) demonstration of activity of the necessary enzymes in cell-free extracts in amounts consistent with the rate of corresponding metabolism in intact cells; (ii) the appearance of radioactivity in intermediate compounds in a manner consistent with operation of the cycle when radioactive substrate is added to the cells; (iii) inhibitors specific for enzymes of the cycle should have the expected effect in terms of lowered overall rates and accumulation of intermediates.

With regard to the reductive carboxylic acid cycle, the first two of the above criteria were partially fulfilled by the work of Evans *et al.* (1966). Our work with inhibitors, particularly fluoroacetate, provides further support for the operation of the reductive carboxylic acid cycle.

The inhibition of CO₂ fixation by fluoroacetate was not accompanied by accumulation of citrate, thus showing that the tricarboxylic acid cycle is not operating in the forward direction. If the tricarboxylic acid cycle were blocked at the aconitate hydratase stage, one would expect operation of the cycle in reverse to result in accumulation of intermediates on the isocitrate side of the inhibited enzyme. The fact that α -oxoglutarate and not isocitrate accumulates may be due to the equilibrium of the isocitrate dehydrogenase reaction, which is in favour of decarboxylation. Another possibility is that isocitrate dehydrogenase is the enzyme inhibited.

When α -oxoglutarate accumulates as a result of inhibition by fluoroacetate, there is a large decrease in the formation of branched-chain oxo acids and polysaccharide (Fig. 2). This indicates that α -oxoglutarate is an intermediate in the biosynthesis of these products from CO₂. Traditionally, the synthesis of polysaccharide from CO₂ in autotrophic organisms is considered to proceed via the reductive pentose phosphate cycle, the enzymes of which are not, as far as is known, affected by fluoroacetate. Yet, as can be seen from Fig. 2, polysaccharide formation is inhibited to the same extent as CO₂ fixation in the presence of various concentrations of fluoroacetate. It follows then that polysaccharide is synthesized by a mechanism that is sensitive to fluoroacetate, i.e. the reductive carboxylic acid cycle.

However, with high concentrations of fluoro-

acetate (1 mM), polysaccharide formation is less inhibited than the formation of branched-chain oxo acids (Fig. 2). This suggests that a fraction of the polysaccharide formed during CO₂ fixation may be due to a mechanism other than the reductive carboxylic acid cycle, i.e. the reductive pentose phosphate cycle.

Evans *et al.* (1966) state that the primary product of the reductive carboxylic acid cycle is acetate. It is not difficult to visualize the conversion of acetate into hexose by a mechanism involving pyruvate synthase and a reversal of the Embden-Meyerhof pathway; the stimulation of polysaccharide formation by acetate (Sirevåg & Ormerod, 1970) is in keeping with this. Similarly, the formation of polysaccharide from acetate and CO₂ is practically unaffected by fluoroacetate, suggesting that the reductive carboxylic acid cycle need not be involved in this conversion. It might be argued that added acetate in some way (e.g. competitively) overcomes inhibition of the cycle by fluoroacetate. However, α -oxoglutarate still accumulates in the presence of the inhibitor even though acetate is also present, indicating that the inhibition of the cycle is effective.

The formation of polysaccharide from acetate is inhibited by iodoacetate, and pyruvate accumulates as a result of this inhibition (Table 1). These results are in keeping with the well-known effect of iodoacetate on triose phosphate dehydrogenase in glycolysis. It also follows that the organism should be able to convert exogenous pyruvate into polysaccharide even in the absence of CO₂, provided that a suitable electron donor is present; this was readily demonstrated (Table 2).

Washed suspensions of *C. thiosulfatophilum* showed variation in their ability to assimilate succinate. The reason for this is unknown, but the increased accumulation of α -oxoglutarate in cases where succinate was metabolized may be regarded as strong evidence for the operation of the α -oxoglutarate synthase reaction *in vivo*.

Our experiments on CO₂ fixation can be explained by Scheme 1. The results show that acetate, the primary product of the reductive carboxylic acid cycle, can be converted into polyglucose, or branched chain oxo acids, or it can re-enter the cycle after carboxylation to oxaloacetate via pyruvate. The metabolic sequences outlined in Scheme 1 can provide precursors not only for amino acids, lipids and porphyrins (Evans *et al.* 1966) but also for carbohydrates. If the reductive pentose phosphate cycle operates in this organism, its function may be connected with the supply of pentose units for nucleic acid synthesis.

The manner of CO₂ assimilation characteristic of *C. thiosulfatophilum* strain 8346 seems to hold also for strain 8327 (Table 5), and at least as far as oxo acid formation is concerned, for *Chlorobium*

Scheme 1. Suggested mechanism of CO_2 fixation in *Chlorobium* (based on Evans *et al.* 1966).

strain R1. It is reasonable to conclude, then, that the green sulphur bacteria as a group share the same CO_2 -fixing mechanism.

Bacteria subjected to nitrogen starvation usually respond by converting the carbon substrate into insoluble intracellular reserve material such as polyglucose or poly- β -hydroxybutyrate (Holme & Palmstierna, 1956; Stanier *et al.* 1959). This is also true for *Chlorobium*, but in addition a substantial part of the CO_2 fixed is excreted as soluble oxo acids into the supernatant. A similar phenomenon was observed by Iwasaki, Iwasaki & Gest (1962) in cultures of *Rhodospirillum rubrum*. Aromatic oxo acids appeared in the medium on exhaustion of the nitrogen source. As far as we are aware, the production of oxo acids by washed suspensions of *R. rubrum* has not been reported.

During growth the biosynthesis of amino acids and other monomers is regulated partly by feedback mechanisms in which the end products play a decisive role. On exhaustion of the nitrogen source these end products are no longer produced. As a result, feedback regulation cannot take place, and precursors of the end products, such as oxo acids, would be expected to accumulate. In *Chlorobium*, the rates of formation of both polyglucose and

branched-chain oxo acids can be increased by adding acetate (Sirevåg & Ormerod, 1970), indicating that neither of these rates limits CO_2 fixation in the absence of acetate. It is possible, therefore, that during nitrogen starvation, carbon metabolism proceeds unregulated in these bacteria.

The biosynthetic pathways of β -methyl- α -oxovalerate, α -oxoisocaproate and α -oxoisovalerate are well established in *Escherichia coli* and are closely related, but little is known about the synthesis of these substances in *Chlorobium*. In *E. coli* the biosynthesis of isoleucine involves α -oxobutyrate, which is produced by oxidative deamination of threonine. Since in *Chlorobium* β -methyl- α -oxovalerate accumulates in the complete absence of a nitrogen source, it seems unlikely that threonine would be involved in its biosynthesis. If α -oxobutyrate is an intermediate in the biosynthesis of isoleucine in *Chlorobium*, it is conceivable that it may arise by the reductive carboxylation of propionyl-CoA (Buchanan, 1969). Hoare & Gibson (1964) observed that the carboxyl carbon of [$1\text{-}^{14}\text{C}$]acetate was incorporated into the carboxyl group of isoleucine, thus suggesting a novel mechanism of biosynthesis for this amino acid. The present experimental system in which large amounts of β -methyl- α -oxovalerate accumulate offers a good opportunity for investigation of this biosynthetic pathway.

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