The Effect of Monofluoroacetate on the Metabolism of *Rhodospirillum rubrum*

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The Athiorhodaceae are photosynthetic bacteria which grow anaerobically on media containing any one of a number of organic compounds, plus carbon dioxide and a source of growth factors, provided that the cultures are illuminated. In contrast to the green plants, no oxygen is produced during photosynthesis. A number of strains will grow in the dark provided that oxygen is present.

The most generally accepted concept of the photo-metabolism of these organisms is that developed by van Niel (1941, 1944, 1949). He has proposed that, in the light, water is broken down to an oxidized and a reduced component. The oxidized component so produced acts as electron acceptor for the oxidation of part of the substrate, and the remainder of the substrate is converted into cell material. The reduced component is thought to act as electron donor in the reduction of carbon dioxide to cell material. This hypothesis is illustrated by the following equations, in which H_2A is the substrate and (CH_2O) is a convenient but not precise expression of cell material:

In the Thiorhodaceae and Chlorobacteriaceae, H_2S can act as electron donor and, according to conditions, either elementary sulphur or sulphate is produced. In these circumstances H_2A acts solely as an electron donor.

When organic substrates are used by the Athiorhodaceae and the Thiorhodaceae, considerable amounts of the substrate are assimilated by the cells, as was first shown by Müller (1933) and later by van Niel (1944).

van Niel (1941) found that the rate of metabolism of acetate anaerobically in the light was the same as its rate of oxidation aerobically in the dark, and he concluded that acetate was oxidized by the same mechanism in both the light and the dark, and that the oxidation of electron donors during photosynthesis was a 'dark reaction'. Some evidence as

to the mechanism of acetate oxidation in the dark was also obtained. van Niel found that suspensions of *Rhodospirillum rubrum* deprived of endogenous reserves by prolonged shaking in the dark in the presence of air do not oxidize acetate in the absence of carbon dioxide. Introduction of carbon dioxide, or the addition of catalytic amounts of succinate, fumarate, malate or oxaloacetate (but not citrate, isocitrate, or α -oxoglutarate), stimulated the oxidation of acetate. These observations suggest that, at least in the dark, acetate is oxidized by a pathway involving the tricarboxylic acid cycle. Further evidence for the operation of such a mechanism was obtained by van Niel & Barker (see van Niel, 1949), who showed that labelled carbon dioxide is fixed into succinic acid under aerobic conditions in the dark, and that oxidation of labelled acetate in the presence of unlabelled succinate gives rise to labelled succinate.

More recently, Vernon & Kamen (1953) have shown that succinic dehydrogenase, malic dehydrogenase and Ochoa's 'malic enzyme' are present in 'sonic extracts' of *Rsp. rubrum*, and Eisenberg (1953) has shown that all the enzymes of the tricarboxylic acid cycle are present in dried-cell preparations of the organism. The results of Eisenberg (1953) have been in part confirmed by the work of Scardovi (1955), who found that dried preparations of *Rsp. rubrum* oxidized citrate, α -oxoglutarate, succinate, fumarate, malate and pyruvate aerobically in the dark, but not acetate. The inability to oxidize acetate was explained by the absence of phosphotransacetylase in the preparation. Similar preparations of *Rhodopseudomonas vannielii* oxidized all of these compounds except fumarate and acetate. In the presence of arsenite, citrate gave rise quantitatively to α -oxoglutarate. Malonate in the same preparations had the effect of reducing the oxygen uptake with α -oxoglutarate to that required for oxidative decarboxylation to succinate.

Kamen, Ajl, Ransom & Siegel (1951) found that the carbon dioxide produced during the oxidation of labelled acetate by *Rhodospirillum rubrum* under aerobic conditions in the dark arises from the methyl and carboxyl groups to an equal extent. This is what would be expected if the tricarboxylic acid cycle were operating. In the light, no such

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equivalence of the methyl and carboxyl groups of acetate was found. The small amount of carbon dioxide produced was derived solely from the carboxyl group, and the bulk of the acetate was assimilated. This also can be explained in terms of the tricarboxylic acid cycle as was shown by Elsdén (1954).

The metabolism of acetate by *Rsp. rubrum* in light was further studied by Glover, Kamen & van Genderen (1952), who used [*meth*yl-¹⁴C]acetate. The washed suspension of cells was allowed to reach a steady state, metabolizing non-isotopic acetate in the light, and then the labelled acetate was added and the reaction was stopped after a suitable period of time by rapidly transferring the suspension to boiling ethanol. Extracts were then examined chromatographically, on paper.

Glover *et al.* found that, when the cells were exposed to the labelled substrate for short periods (24 sec.), there was labelling in succinic, malic and α -oxoglutaric acids, but no isotope was present in citrate or isocitrate. Longer exposures to the labelled acetate produced labelling in all the acids of the tricarboxylic acid cycle. These results suggest that the tricarboxylic acid cycle does not play a significant part in the light-metabolism of acetate by *Rsp. rubrum*.

Monofluoroacetate was shown by Liébecq & Peters (1949) to inhibit the oxygen uptake of kidney homogenates with fumarate as substrate. Under these conditions citrate accumulated and fluoroacetate was considered to inhibit the tricarboxylic acid cycle in such a way as to cause accumulation of citrate. Subsequently it was found that the inhibitor was not fluoroacetate, but rather a compound produced from it. This compound has now been isolated (Peters, Wakelin & Buffa, 1953) and shown by Peters, Wakelin, Rivett & Thomas (1953) to be fluorocitrate. Fluoroacetate is therefore a useful alternative to malonate in the study of the tricarboxylic acid cycle, and we decided to investigate its effect on the metabolism of *Rsp. rubrum* both in the light and dark to see whether we could obtain evidence for the participation of the tricarboxylic acid cycle in these reactions.

The work described below has been published in part in an abridged form (Elsden & Ormerod, 1953; Elsdén, 1954).

MATERIALS AND METHODS

Cultural procedure

Organism. The organism used throughout the work was *Rhodospirillum rubrum*, strain S1, obtained from the collection of Dr C. B. van Niel, Hopkins Marine Station, Stanford University, Pacific Grove, California, U.S.A.

Culture of organism. The organism was maintained as stab cultures in a medium containing either 0.5% (w/v) Oxoid yeast extract (Oxo Ltd., London, E.C. 4), or 0.3%

(w/v) Difco yeast extract (Difco Laboratories Inc., Detroit, Michigan, U.S.A.), together with 1.5% (w/v) Difco Bacteriological Agar. This medium was dispensed into test tubes and sterilized by autoclaving at 15 lb./in.² for 15 min.

Inoculated tubes were incubated for 6–7 days in a cabinet illuminated by eight 60 w incandescent lamps. The cabinet was ventilated by an electric fan and the temperature was 25–30°. Stock cultures were kept at 4°, at which temperature they remained viable for at least 3 months.

Inocula were grown in tubes containing 15 ml. of the following medium made up in tap water: yeast autolysate (Barker & Beck, 1942) 10% (v/v), or, alternatively, 0.5% (w/v) Oxoid yeast extract and 0.1% (w/v) sodium citrate dihydrate. The pH was adjusted to 7.0 with N-NaOH. Sodium citrate was added to prevent the formation of inorganic precipitates (Hutner, 1946). The medium was tubed and autoclaved at 15 lb./in.² for 15 min. A tube of this medium was inoculated with a loopful of the stab culture and incubated in the light. When good growth had occurred (4–5 days), 1–2 ml. was transferred to a new tube of medium which was incubated in the light for 72 hr., when it was ready for use. Inoculation tubes were prepared daily, but to reduce the risk of contamination a return was made to stock agar stabs every 6–8 weeks.

For use in experiments, the organisms were grown in the following liquid medium: 3.15 g. of DL-malic acid; 1.3 g. of sodium citrate dihydrate; 1.0 g. of (NH₄)₂SO₄; 0.2 g. of MgSO₄·7H₂O; 0.1 g. of CaCl₂; 0.25 g. of Difco yeast extract (or 0.5 g. of Oxoid yeast extract) in 1 l. of tap water. (Citrate was again included to prevent the formation of a precipitate on autoclaving.) The pH was adjusted to 7.0 with 20% (w/v) NaOH, and N-NaOH, and 5 ml. of phosphate buffer, pH 7.0, composed of K₂HPO₄ (Hopkin and Williams Pure Grade) and KH₂PO₄ (A.R.), was added. This medium was dispensed in 400 ml. amounts in 16 oz. screw-cap medical flats, and sterilized by autoclaving at 15 lb./in.² for 25 min.

Each bottle of medium was inoculated with 15 ml. of a 72 hr. culture prepared as described above, and incubated in the light for 40–45 hr. The yield of cells from 400 ml. of medium was 300–400 mg. dry wt. As a precaution against contamination, wet mounts of these cultures were examined microscopically before harvesting.

Cells grown in this manner were found to metabolize propionate and DL-lactate very slowly. Consequently for use in experiments with these substrates the organisms were grown either on a medium similar to the above but with the DL-malic acid replaced by 0.4% (w/v) sodium DL-lactate and 0.3% (w/v) NaHCO₃, as carbon sources (the NaHCO₃ was sterilized by filtration through a Seitz filter and added separately after autoclaving), or on 5% (v/v) yeast autolysate. The last medium was not altogether satisfactory, as different batches of yeast autolysate differed somewhat in composition.

Preparation of washed suspensions. Cells were harvested by centrifuging, washed twice with, and suspended in, 0.01M potassium phosphate buffer, pH 7.0 (K₂HPO₄ and KH₂PO₄ mixture). Dry weight was determined by measuring optical density in the Hilger Spekker absorptiometer (Hilger and Watts, Ltd., London, N.W. 1), with a neutral-grey filter, Ilford No. H508 (Ilford, Ltd., London) and referring to a standard graph relating optical density to dry wt. For the photosynthesis experiments the master suspension was adjusted to 5 mg. dry wt./ml. in 0.025M-NaHCO₃, save in those cases where the bound CO₂ was to be

estimated, when the concentration of NaHCO_3 was 0.005 M. The cells were suspended in 0.01 M potassium phosphate buffer, pH 7.0, for the respiration experiments.

Substrates

All organic acids were used as solutions of their sodium salts. Crystalline sodium pyruvate was prepared from crystalline pyruvic acid by the method of Robertson (1942). Oxaloacetic acid was the gift of Professor H. A. Krebs, and a solution of the sodium salt was prepared immediately before use by dissolving a weighed amount of the free acid in a small volume of water, adjusting to pH 7 with N-NaOH , and making up to volume. The oxaloacetic content of the solution was measured by the aniline citrate method as described below. Sodium fluoroacetate was the gift of Dr B. C. Saunders, University Chemical Laboratory, Cambridge. The succinic acid and DL-lactic acid used were A.R. grade. Propionic and butyric acids were purified by distillation, b.p.'s 141° and 164° , respectively. Sodium acetate solutions were prepared from A.R. $\text{CH}_3\text{CO}_2\text{Na}\cdot 3\text{H}_2\text{O}$. The fumaric acid used was a commercial product obtained from Light and Co., Colnbrook, Bucks., and the DL-malic acid was a product of B.D.H. Ltd., Poole, Dorset.

Analytical methods

Pyruvic acid. This was estimated by the method of Westerkamp (1933). The carboxylase preparation used was stored at -20° , and under these conditions it retained its activity for at least three months.

Lactic acid. This was estimated by the method of Elsdon & Gibson (1954).

Volatile fatty acids. Acetic, propionic and butyric acids were estimated by steam distillation of the acidified solutions in the apparatus of Markham (1942) and titration of the distillate with CO_2 -free 0.01 N-NaOH in a stream of CO_2 -free air with phenol red as indicator. The volume of solution distilled was generally 2-4 ml., and this was acidified in the apparatus by the addition of 1 ml. of 10 N- H_2SO_4 . The volume of distillate collected was 80 ml., in two portions of 40 ml. Fluoroacetate also distils under these conditions, 43% coming over in the first 40 ml. and a further 21% in the second 40 ml.

Succinate. This was estimated by the method of Krebs (1937).

Malate and fumarate. These compounds were estimated by the method of Nossal (1952) with suspensions of *Lactobacillus arabinosus*, strain 17-5. L-Malate is quantitatively decarboxylated by this organism, and this stereochemical specificity was of great use to us because, although we were using DL-malate as substrate, only the L-isomer was metabolized. To determine fumarate, a preparation of fumarase made by Nossal's procedure from baker's yeast was added. Stock solutions of fumarate were assayed manometrically by catalytic hydrogenation with colloidal palladium (Harrison 1939). The catalyst was placed in the main compartment and the fumarate solution in the side bulb; this ensured the rapid saturation of the catalyst with H_2 .

Oxaloacetic acid. This was estimated by the aniline citrate method of Greville (1939). The experiments with oxaloacetate as substrate were carried out in double-side-bulb cups and the reaction was stopped and the bicarbonate concentration measured by tipping in 0.2 ml. of 2 N-HCl from the side bulb, at the required time. The manometers

were then removed from the bath, and the cups were placed in ice to minimize the spontaneous decomposition of the residual oxaloacetic acid. Citric acid [0.5 ml. of 50% (w/v) solution] was added to the acidified suspension in the main compartment and 0.5 ml. of aniline citrate was placed in the side bulb which had contained the 2 N-HCl. The stoppers were replaced and the manometers allowed to equilibrate in the bath at 30° . The aniline citrate was then tipped into the suspension and the volume of CO_2 evolved was determined. In addition, a control cup was run without cells present, to determine the rate of the spontaneous decomposition of oxaloacetate under the conditions of the experiment.

Citric acid. The method of Weil-Malherbe & Bone (1949) as modified by Taylor (1953) was used. The optical density was measured with micro cells (capacity 0.5 ml.) in the Hilger Spekker absorptiometer, with Ilford spectrum-violet filters no. 601 (peak transmission 430 m μ) and heat filters no. H503. Two standards of 0.31 and 0.75 μmole of citric acid respectively were run with each set of 'unknowns'. The volume of sulphide solution used for the extraction was 2.0 ml., and under these conditions the range covered by the method was 0.05-0.83 μmole . For larger amounts of citric acid the sulphide extract was diluted with the appropriate volume of 2% (w/v) $\text{Na}_2\text{S}_9\text{H}_2\text{O}$ solution. Some difficulty was experienced with amounts of citric acid less than 0.1 μmole , since the colour tended to fade if the sodium sulphide extract was left for any length of time, especially in sunlight (cf. Weil-Malherbe & Bone, 1949). This was overcome by doing only two sulphide extractions at a time, thus minimizing the delay between extraction and the colorimetric measurement. With this precaution the method was satisfactory.

Experimental procedure

Gaseous exchange was measured with Warburg manometers and cups of capacity 20-25 ml. fitted with centre wells. Substrates were added from the side bulb and, when bicarbonate was to be estimated, cups with two side bulbs were used, one of which contained 0.2 ml. of either 2 N- H_2SO_4 or 2 N-HCl. The volume of bacterial suspension plus substrate was 3.0 ml. and the temperature 30° . Where large amounts of material were required for analysis the experiments were carried out in vessels made from 100 ml. Pyrex conical flasks as follows: The neck of the flask was a B14 socket into which fitted a B14 cone fused on to a stopcock. The flask had a centre well, approximately 5 cm. high, and a side bulb of about 2 ml. capacity fitted with a B10 socket and stopper. This arrangement facilitated the addition of substrate at the desired moment, and made it possible to fill the vessels with the appropriate gas mixture. The vessels were attached to the shaking mechanism of the Warburg bath. When necessary, gaseous exchange was followed in parallel experiments in manometers.

Photosynthesis experiments. The Warburg bath used was similar to that described by Larsen (1953). Each side of the bath was illuminated by a bank of seven 100 w incandescent lamps, providing light of an intensity of 500 f.t.c.

An electric blower of the centrifugal type was attached at the end of each row of lamps to keep them cool. A sheet of glass ($\frac{1}{4}$ in. thick) was inserted between the lamps and the glass panels of the bath, leaving an air space of 1 cm. Between the metal sides of the bath and the lamps a sheet of asbestos board ($\frac{1}{4}$ in. thick) was fixed, leaving an air space of 2 cm. These precautions ensured that the heat generated by

the lamps did not appreciably affect the temperature of the bath.

Buffering system used in photosynthesis experiments. The system used was $\text{NaHCO}_3\text{-CO}_2$, and the manometers were gassed with $\text{N}_2 + \text{CO}_2$ (95:5, v/v). The fact that the organisms were grown in a medium containing $(\text{NH}_4)_2\text{SO}_4$ as the nitrogen source and that the gas phase of the manometers contained N_2 ensured that no photoproduction of H_2 occurred (Gest & Kamen, 1949). The final NaHCO_3 concentration in 3 ml. was 0.0166 M except when bound CO_2 was to be estimated, when it was 0.0033 M. Rates of photosynthesis are expressed as Q_{CO_2} (light) = $\mu\text{l. of CO}_2$ at s.t.p./mg. dry wt./hr.

The pressure changes observed when the sodium salt of an organic acid is photometabolized by *Rsp. rubrum* is the result of three processes: (a) the binding of CO_2 by the buffer due to removal of anions; (b) the fixation of CO_2 by the cells; (c) the production of CO_2 by oxidation of the substrate. The amount of CO_2 bound by the buffer is obtained by measuring the initial and final bicarbonate content of the system. The actual amounts of CO_2 involved in each of the other two processes cannot be determined by manometric methods alone, but it is possible to calculate the net CO_2 uptake or output if the total CO_2 exchange (calculated from the observed pressure changes) and the increase in bicarbonate are known. The net CO_2 is either positive or negative, according to the nature of the substrate. Thus with substrates more oxidized than cell material, more CO_2 is produced from the substrate than is fixed by the cell, and the net figure is positive; with substrates such as pyruvate and oxaloacetate, the net CO_2 is positive and is of about the same magnitude as the amount of CO_2 bound by the buffer; thus with these two substrates the observed pressure changes are very small and cannot be used as an index of the rate of metabolism. Acetate is slightly more oxidized than cell substance and the net figure is positive but far smaller than the amount of CO_2 bound by the buffer; consequently, the observed pressure change is negative. Propionate and butyrate are more reduced than cell substance; the net figure is negative and the pressure change observed is also negative.

Effect of yellow P as an O_2 absorbent. Whilst van Niel (1941) had shown that illumination of suspensions of *Rsp. rubrum* completely suppressed O_2 uptake, some trials were carried out to ensure that under the conditions of our experiments the traces of O_2 found in commercial gas mixtures had no effect on the light-metabolism of this organism. In the course of these experiments it was found that the photometabolism of acetate was markedly inhibited when freshly scraped sticks of yellow P were used as an O_2 absorbent (Fig. 1). On the other hand, yellow P had no apparent effect on the photometabolism of either butyrate or succinate. We have no explanation to offer for these results, but they indicate that caution is necessary in the use of yellow P as an O_2 absorbent. It was also found that the rate of photosynthesis was the same whether the gas mixture was used direct or whether it was freed from O_2 by passage over heated copper turnings.

Aerobic experiments. Oxygen consumption was measured in Warburg manometers in the photosynthesis bath which, for this purpose, was covered with a black cloth supported on a frame of aluminium strip to exclude the light; in addition, the glass panels were covered in by strips of hard board. The gas phase was air and the centre wells of the manometers

Fig. 1. Effect of yellow phosphorus on photometabolism of acetate. Manometer cups contained, in the main compartment, 2.0 ml. of washed suspension (5 mg. dry wt. of cells/ml.) in 0.025 M- NaHCO_3 , and water to make 2.8 ml. A freshly scraped stick of yellow phosphorus was placed in the centre well in B. Acetate (0.2 ml., 0.10 M) was added from the side bulb and the lights were switched on. Gas phase, $\text{N}_2 + \text{CO}_2$ (95:5, v/v). Temp., 30°. A, 20 μmoles of acetate; B, acetate + yellow phosphorus.

contained 0.2 ml. of 20% (w/v) KOH, and a folded strip of filter paper, to absorb CO_2 . The substrate solution (0.2 ml.) was added from the side bulb and the total volume in which the cells were suspended after addition of substrate was 3.0 ml. The concentration of phosphate buffer in the cups was 0.066 M. The rate of O_2 uptake is expressed as Q_{O_2} (dark) = $\mu\text{l. of O}_2$ at s.t.p./mg. dry wt./hr.

RESULTS

Conditions for maximum inhibition by fluoroacetate. Kalnitsky & Barron (1947) showed that inhibition of acetate oxidation in yeast by fluoroacetate developed only slowly, and that in order to obtain maximum inhibition it was necessary to incubate the suspension with the inhibitor before the addition of substrate. A similar effect was observed in our system, as can be seen from Fig. 2.

The effect of two different concentrations of fluoroacetate is shown in Fig. 3. It was decided to use a concentration of 8.3×10^{-4} M fluoroacetate throughout the work, and to add the inhibitor to the cells about 30 min. before the addition of substrate.

Effect of fluoroacetate on the Q_{CO_2} (light). Table 1 gives the results obtained with a range of substrates under standard conditions. It will be seen that a strong inhibition occurs only with acetate and butyrate.

Photometabolism of pyruvate and oxaloacetate. There was only a small pressure change when these substrates were metabolized. These compounds are much more oxidized than cell material, and in consequence the amount of CO_2 evolved as a result of oxidation is approximately equal to the sum of CO_2 fixed by photosynthesis and that bound by the buffer due to the removal of anions. It was therefore necessary to follow the metabolism of these compounds either by determining the increase in HCO_3^- ions or by measuring directly the amount of

Fig. 2. Effect of pre-incubation of cells with fluoroacetate on photometabolism of acetate. Experiment carried out in the light in double-side-bulb manometer cups containing 0.2 ml. of 0.05 M acetate in one side bulb and 0.1 ml. of 0.025 M fluoroacetate in the other, except in the control cup. The main compartment contained 2.0 ml. of washed suspension containing 5 mg. dry wt. of cells/ml. in 0.025 M $NaHCO_3$ and water to make the total volume 3.0 ml. A, Acetate alone; B, acetate and fluoroacetate added together; C, acetate added 10 min. after fluoroacetate; D, acetate added 30 min. after fluoroacetate.

Fig. 3. Effect of concentration of fluoroacetate on photometabolism of acetate. Manometer cups contained, in the main compartment, 2.0 ml. of washed suspension (5 mg. dry wt./ml.) in 0.025 M $NaHCO_3$, with or without fluoroacetate in the concentrations indicated. Water was added to make 2.8 ml. and 0.2 ml. of 0.05 M acetate was added from the side bulb, and the lights were switched on. Gas phase, $N_2 + CO_2$ (95:5, v/v). Temp., 30°. A, Acetate alone; B, acetate plus 8.3×10^{-6} M fluoroacetate; C, acetate plus 8.3×10^{-4} M fluoroacetate.

Table 1. *Effect of fluoroacetate on the Q_{CO_2} (light)*

Manometer cups contained, in the main compartment, 2.0 ml. of washed suspension of bacteria (5 mg. dry wt./ml.) in 0.025 M $NaHCO_3$, with or without 0.1 ml. of 0.025 M fluoroacetate, and distilled water to 2.8 ml. Substrate solution (0.2 ml.) (0.05 M, except DL-malate and acetate, which were 0.1 M), or water, was added from the side bulb at zero time and the lights were switched on. Gas phase, $N_2 + CO_2$ (95:5, v/v). Temp., 30°. Figures in parentheses refer to the number of experiments performed. Q_{CO_2} values were calculated from the linear parts of the progress curves.

Substrate	Q_{CO_2}	Q_{CO_2} with fluoroacetate
None (7)	0.9 ± 0.1	0.5 ± 0.1
Acetate (10)	18.7 ± 5.1	2.7 ± 0.1
Propionate (5)	13.1 ± 3.4	10.0 ± 3.2
Butyrate (8)	9.7 ± 3.1	3.1 ± 1.3
Succinate (10)	13.3 ± 3.0	10.3 ± 2.7
Fumarate (10)	9.0 ± 1.8	6.6 ± 1.8
DL-Malate (10)	8.9 ± 1.5	5.9 ± 1.1

substrate disappearing. Fig. 4 shows the effect of fluoroacetate on the photometabolism of pyruvate and oxaloacetate. It will be seen that fluoroacetate inhibits the utilization of both compounds.

*Effect of fluoroacetate on the H_2 - CO_2 reaction. *Resp. rubrum* can reduce CO_2 in the light with molecular*

Fig. 4. Effect of fluoroacetate on photometabolism of pyruvate and oxaloacetate. Experiment carried out in double-side-bulb manometer cups containing, in the main compartment, 2.0 ml. of washed suspension (5 mg. dry wt. of cells/ml.) in 0.005M- $NaHCO_3$ with or without 0.1 ml. of 0.025M fluoroacetate, and water to 2.8 ml. Substrate solution (0.2 ml. 0.05M) was placed in one side bulb and 0.2 ml. of 2N- H_2SO_4 in the other. Gas phase, $N_2 + CO_2$ (95:5, v/v). Temp., 30°. Substrate was added at zero time and light switched on. Acid was added at appropriate times and residual substrate estimated. A, Pyruvate alone; B, pyruvate plus fluoroacetate; C, oxaloacetate alone; D, oxaloacetate plus fluoroacetate.

H_2 as electron donor. The ratio of H_2 to CO_2 was found by van Niel (1941) to be approximately 2:1. It was impossible with the equipment available to measure H_2 and CO_2 separately, so in these experiments Warburg cups of approximately the same size (25 ml.) were used and the results expressed as mm. of manometric fluid. The effect of fluoroacetate on the pressure changes occurring during this reaction is shown in Fig. 5; the inhibition was more variable than that observed with other substrates.

Fig. 5. Effect of fluoroacetate on photoreduction of CO_2 by hydrogen. Manometer cups had 2.0 ml. of washed suspension containing 5 mg. dry wt. of cells/ml. in 0.025M- $NaHCO_3$ with (A) or without (B) 0.1 ml. of 0.025M fluoroacetate and with water to make 3.0 ml. Gas phase, $H_2 + CO_2$ (95:5, v/v). Temp., 30°. Volume of manometers, 25 ml.

Table 2. *Effect of fluoroacetate on the Q_{O_2} (dark)*

Manometer cups contained, in the main compartment, 2.0 ml. of washed suspension having 5 mg. dry wt. of cells/ml. in 0.01M phosphate buffer, pH 7.0, with or without 0.1 ml. of 0.025M fluoroacetate, and with distilled water to make 2.8 ml. Substrate solution (0.2 ml.) (0.05M, except DL-malate and acetate, which were 0.1M), or water, was added from the side bulb at zero time. 0.2 ml. of 20% (w/v) KOH and a filter-paper absorber were placed in the centre well; gas phase, air; temp., 30°. Q_{O_2} values were calculated from the linear parts of the progress curves.

Substrate	Q_{O_2} endogenous (no fluoroacetate)	Q_{O_2} endogenous (+ fluoroacetate)	Q_{O_2} + substrate (no fluoroacetate)	Q_{O_2} + substrate (+ fluoroacetate)
Acetate	4.8	1.6	11.2	1.6
Propionate	4.8	1.6	10.9	1.6
Butyrate	4.2	1.7	16.6	3.4
Pyruvate	4.8	1.5	17.8	3.0
Succinate	4.8	1.6	14.0	2.9
Fumarate	4.8	1.6	13.0	2.3
DL-Malate	3.4	1.7	15.0	3.4
Oxaloacetate	4.8	1.4	8.6	2.0

Effect of fluoroacetate on the Q_{O_2} (dark). Table 2 shows results of experiments in the dark, obtained with the same substrates as were used in the experiments in the light. The oxidation of all substrates

is strongly inhibited by fluoroacetate. This is in marked contrast to the results of experiments in the light.

Accumulation of citrate in the presence of fluoroacetate. Addition of fluoroacetate to suspensions of *Rsp. rubrum* incubated in the light under $N_2 + CO_2$ (95:5, v/v) but in the absence of substrate showed that a material accumulated which reacted as citrate in the test used. Fig. 6 shows the course of citrate accumulation under these conditions. Table 3 shows the amount of citrate which accumulates when suspensions of the organism are incubated in the light with various substrates in the presence and absence of fluoroacetate. Substrates, the photometabolism of which is but slightly affected by fluoroacetate, gave rise to negligible amounts of citrate. Of the substrates whose metabolism is strongly inhibited by fluoroacetate, acetate, pyruvate and oxaloacetate gave rise to citrate, whereas butyrate gave no increase over and above that found in the control. The results of experiments on the accumulation of citrate during the dark oxidation of substrates (Table 4) present a very different picture. The disappearance of all substrates was inhibited by fluoroacetate; and, whereas acetate, butyrate, propionate and pyruvate gave little or no citrate, the C_4 dicarboxylic acids and lactate caused a significant accumulation.

Effect of CO_2 on citrate formation. It seemed possible that the small amount of citrate formed from pyruvate in the aerobic experiments was due to the low tension of CO_2 in the experimental vessels. The effect of introducing CO_2 with pyruvate as substrate can be seen in Table 5. Although CO_2 increased the amount of citrate formed by the cells

Fig. 6. Endogenous formation of citrate in the presence of fluoroacetate in the light. Experiment carried out in five 100 ml. flasks containing 10 ml. of washed suspension (50 mg. dry wt. of cells) in 0.025 M- $NaHCO_3$ and 0.125 ml. of 0.10 M fluoroacetate. Water was added to make 15 ml. and incubated in the light at temp. 30° under a gas phase of $N_2 + CO_2$ (95:5, v/v). Reaction was stopped in different flasks at appropriate time by adding 1 ml. of 2 N- H_2SO_4 .

Table 3. Accumulation of citrate in the presence of fluoroacetate anaerobically in the light

All experiments were performed in large (100 ml.) flasks containing 10.0 ml. of washed suspension of organisms (5 mg. dry wt./ml.) in 0.025 M- $NaHCO_3$, with or without 0.125 ml. of 0.10 M fluoroacetate, and with distilled water to make 14.0 ml. Substrate solution (1.0 ml.) (0.05 M, except for DL-malate and acetate, which were 0.1 M), or water, was added from the side bulb at zero time and the lights were switched on. Gas phase, $N_2 + CO_2$ (95:5, v/v). In the experiment with $H_2 + CO_2$ (95:5, v/v) the control vessels were gassed with $N_2 + CO_2$ (95:5, v/v); temp., 30° .

Substrate	Citrate (μmoles)				Substrate used (μmoles)		Duration (min.)
	Endogenous	Endogenous + fluoroacetate	With substrate	With substrate + fluoroacetate	No fluoroacetate	With fluoroacetate	
Acetate	0.22	0.90	0.22	2.16	91	22	195
Propionate	0.13	1.34	0.20	1.51	45	33	134
Butyrate	0.13	1.46	0.17	1.53	17	4	130
Pyruvate	0.28	1.44	0.45	3.70	43	13	120
Succinate	0.06	0.67	0.30	0.86	46	43	160
Fumarate	0.16	1.74	0.15	1.89	29	21	194
DL-Malate	0.18	1.91	0.18	2.02	45*	44*	145
Oxaloacetate	0.30	1.03	0.78	4.22	39	26	180
H ₂ + CO ₂ (95:5, v/v)	0.14	0.37	0.14	0.37	272 mm.† (manometric fluid)	78 mm.† (manometric fluid)	100

* These figures refer to the amount of L-malate used.

† These figures refer to pressure changes occurring in parallel manometers run at the same time; volume of flasks, 25 ml.

Table 4. *Accumulation of citrate in the presence of fluoroacetate aerobically in the dark*

All experiments were carried out in 100 ml. flasks with 10.0 ml. of washed suspension containing 5 mg. dry wt. cells/ml. in 0.01M phosphate buffer, pH 7.0, with or without 0.12 ml. of 0.1M fluoroacetate, and made up to 14 ml. with distilled water. Substrate solution (1.0 ml.) (0.05M, except DL-malate and acetate, which were 0.1M), or water, was added from the side bulb at zero time. The centre wells contained 0.5 ml. of 20% (w/v) KOH and a folded strip of filter paper. Gas phase, air; temp., 30°.

Substrate	Citrate (μ moles)				Substrate used (μ moles)		Duration (min.)
	Endogenous	Endogenous + fluoro-acetate	With substrate	With substrate + fluoro-acetate	No fluoro-acetate	With fluoro-acetate	
Acetate	0.15	1.60	0.16	1.61	52	13	120
Propionate	0.15	1.63	0.20	1.91	50	19	135
Butyrate	0.13	1.66	0.13	1.78	37	2	120
Pyruvate	0.36	1.12	0.73	1.48	22	10	160
Succinate	0.07	2.80	0.07	4.90	48	20	250
Fumarate	0.16	1.82	0.13	2.99	49	9	120
DL-Malate	0.03	1.83	0.10	3.72	35*	23*	120
Oxaloacetate	0.32	2.30	0.77	4.45	21	5	100

* These figures refer to the amount of L-malate used.

Table 5. *Effect of CO₂ on formation of citrate from pyruvate and acetate in the presence of fluoroacetate aerobically in the dark*

Vessels (100 ml.) contained in a total reaction volume of 9.0 ml. (Expt. 1) or 12.0 ml. (Expt. 2) these final concentrations: 3.33 mg. dry wt. of washed cells of the organism/ml.; 0.0067M phosphate buffer, pH 7.0; 0.00083M fluoroacetate; and in the high-CO₂-tension vessels only, 0.0167M-NaHCO₃. Where indicated, 0.6 ml. of 0.05M sodium pyruvate (Expt. 1) or 1.0 ml. of 0.1M sodium acetate (Expt. 2) was initially present in the side bulb. In addition, in vessels with low-CO₂-tension the centre well contained 0.5 ml. of 20% (w/v) KOH and a folded strip of filter paper. In the high-CO₂-tension vessels of Expt. 2, the gas phase was O₂ + CO₂ (93:7, v/v); in others the gas phase was air. Duration of experiments, 90 min. (Expt. 1) or 73 min. (Expt. 2).

Substrate	Citrate formed (μ moles)			
	Endogenous		With substrate	
	Low CO ₂	High CO ₂	Low CO ₂	High CO ₂
Expt. 1.				
Pyruvate	1.57	2.52	2.12	2.59
Expt. 2.				
Acetate	0.71	4.15	0.82	4.49

alone, addition of pyruvate caused practically no increase over and above that formed endogenously. A similar result was obtained with acetate as substrate.

Catalysis of acetate photometabolism. From these results it would appear that the tricarboxylic acid cycle, or part of it, is involved in both the light- and the dark-metabolism of acetate. van Niel's (1949) experiment, in which it was found that the dark-oxidation of acetate was stimulated either by CO₂ or by small amounts of succinate, fumarate, malate or oxaloacetate, had already given a strong indication

that the tricarboxylic acid cycle might be operating under these conditions. If the mechanism of acetate oxidation is the same in the light as in the dark, then under suitable conditions these compounds should also stimulate the light-metabolism of acetate. A washed suspension, from a culture of *Rsp. rubrum* grown in 5% (v/v) yeast autolysate, was left overnight in the dark in the refrigerator. Next day the cells were centrifuged, washed in phosphate buffer and suspended in dilute NaHCO₃. Manometers were set up containing the suspension, and in the side bulb was the substrate, acetate, with or without 1 μ mole of succinate, fumarate or L-malate. The gas phase was N₂ + CO₂ (95:5, v/v) and the reaction was carried out in the light. The result is shown in Fig. 7.

It will be seen that the rate of the photometabolism of acetate alone is low, and that it is increased by the addition of succinate, fumarate or malate.

Effect of CO₂ on succinate metabolism. The effect of fluoroacetate on the H₂-CO₂ reaction is difficult to explain in terms of an inhibition of the tricarboxylic acid cycle, for it seems unlikely that the cycle is involved in the oxidation of molecular H₂. Almost complete inhibition of CO₂ fixation by fluoroacetate has been observed with the green sulphur bacterium *Chlorobium thiosulphatophilum* with thiosulphate as electron donor (unpublished results of Larsen & Ormerod, 1954), and an alternative site for inhibition might be the CO₂-fixation mechanism. However, the inhibitor has no observable effect on the amount of CO₂ fixed when propionate is the electron donor in *Rsp. rubrum*. With substrates such as succinate, it is impossible at present to say whether or not any CO₂ is fixed during the reaction, since there is a net output of CO₂. Table 6 shows that, in the absence of CO₂, the rate of succinate metabolism

Fig. 7. Stimulatory action of C_4 dicarboxylic acids on photometabolism of acetate. Experiment carried out in the light in manometer cups containing, in the main compartment, 2 ml. of washed suspension (10 mg. dry wt. of cells) in 0.025 M NaHCO_3 and water to make a final volume of 3.0 ml. Where indicated below, 0.2 ml. of 0.1 M acetate and/or 0.1 ml. of 0.01 M succinate, fumarate or 0.1 ml. of 0.02 M DL-malate was placed in the side bulb. Gas phase, $\text{N}_2 + \text{CO}_2$ (95:5, v/v). Temp., 30°. A, 20 μmoles of acetate + 1 μmole of succinate; B, 20 μmoles of acetate + 1 μmole of fumarate; C, 20 μmoles of acetate + 1 μmole of malate; D, 20 μmoles of acetate; E, 1 μmole of DL-malate; F, 1 μmole of succinate + 1 μmole of fumarate.

Table 6. Effect of CO_2 on the photometabolism of succinate

Experiment carried out in vessels of 100 ml. capacity containing 10.0 ml. of washed suspension of organisms (5 mg. dry wt./ml.) in 0.01 M phosphate buffer, pH 7.0. Sodium succinate (1.0 ml., 0.05 M) was placed in the side bulb. The vessel with the low tension of CO_2 contained, in the centre well, 0.5 ml. of 20% (w/v) KOH and a filter-paper absorber, and was evacuated and filled with N_2 (O_2 -free) three times, and finally filled with O_2 -free N_2 . The vessel with a high tension of CO_2 contained NaHCO_3 in a final concentration of 0.0166 M and was gassed with $\text{N}_2 + \text{CO}_2$ (95:5, v/v). Total volume of liquid/vessel excluding KOH, 15.0 ml. Substrate was added at zero time, and the light was switched on. Temp., 30°; time, 140 min.

	No CO_2	With CO_2
Succinate used (μmoles)	11.1	33.2

in the light is about one-third of that in the presence of CO_2 ; this suggests that CO_2 , and hence CO_2 fixation, play an essential part in the light-metabolism of succinate.

DISCUSSION

As far as is known fluoroacetate as such does not inhibit enzyme reactions. The inhibition of respiration in animal tissues and the accumulation of citrate are thought to be due to an inhibition of aconitase by fluorocitrate, which is produced from fluoroacetate, presumably in the form of fluoroacetyl coenzyme A and oxaloacetate (Peters, Wakelin, Rivett & Thomas, 1953). It would then seem likely that the inhibition of a metabolic process accompanied by the accumulation of citrate can be taken as evidence for the participation of part at least of the tricarboxylic acid cycle in that process. Fluoroacetate is believed to behave, metabolically, like acetate, and if this supposition is correct other fluorinated metabolites will be formed from fluoroacetate. So far only fluorocitrate has been described, and the discovery of this compound was facilitated by its inhibitory action on aconitase. It appears to us quite possible that other fluorinated metabolites formed from fluoroacetate will be specific inhibitors of particular metabolic processes and, in view of this, inhibition of a complex series of metabolic reactions by fluoroacetate must be interpreted with caution, particularly if the inhibition is not accompanied by an accumulation of citrate. When the effect of fluoroacetate on the metabolism of *Rsp. rubrum* in the light was examined it was found that the results obtained varied according to the substrate being tested. The substrates can be divided into three groups: (a) those whose metabolism is inhibited only slightly and from which little or no citrate is formed, e.g. succinate, fumarate, DL-malate and propionate; (b) those whose metabolism is inhibited without the accumulation of citrate over and above that formed in the absence of substrate (included in this group are *n*-butyrate, and hydrogen plus carbon dioxide); (c) those whose metabolism is inhibited and from which a citrate-like compound is produced (these include acetate, pyruvate and oxaloacetate). In every experiment fluoroacetate caused an increase in the amount of citrate produced by cells alone. The evidence that the material accumulating in the presence of fluoroacetate is citrate is as follows: The material reacts as citrate in the Weil-Malherbe & Bone (1949) colorimetric method for citrate estimation; it cannot be separated from citrate by paper-partition chromatography (Buch, Montgomery & Porter, 1952) with the *n*-pentanol-formic acid-water solvent of these authors; it runs as citrate on the Celite column of Swim & Krampitz (1954), and the

amount present in the citrate fraction estimated by titration agrees with the value obtained by the colorimetric method.

The results of the experiments on the effect of fluoroacetate on the dark-oxidation of substrates were in marked contrast to those described above. In each case substrate oxidation was inhibited, and with all substrates except acetate and butyrate an accumulation of citrate resulted.

From the aerobic experiments in the dark, it seems reasonable to conclude that the tricarboxylic acid cycle is playing an important part in terminal respiration in *Rsp. rubrum*. That no citrate over and above that found in the control is produced from acetate and butyrate is probably due to the limited amount of C_4 dicarboxylic acids present in the cell suspension, assuming, of course, that, aerobically in

beyond the stage of acetyl-coenzyme A is an essential part of their photometabolism.

On the other hand, the photometabolism of succinate, fumarate, malate and propionate is scarcely affected by fluoroacetate, and the amounts of citrate formed are small. We conclude from these results that the tricarboxylic acid cycle does not play a vital part in the photometabolism of succinate, fumarate, malate and propionate. The most reasonable explanation would appear to be that in the photometabolism of these compounds, the amount of hydrogen made available in the process of converting them into a compound which can be assimilated is also adequate for the reduction of the oxidized component of the light reaction. Thus the photometabolism of succinate can be envisaged as shown in the diagram below:

the dark, C_4 dicarboxylic acids are produced only slowly, if at all, from these substrates. Under these conditions the C_4 dicarboxylic acids present in the cells become immobilized as citrate, with consequent inhibition of respiration. These conclusions are in keeping with those reached by van Niel (1949) and Eisenberg (1953).

The amount of citrate formed from propionate and pyruvate under these conditions was less than the amount formed from any of the C_4 dicarboxylic acids. Further experiments showed that this effect was not due to the low tension of carbon dioxide in the experimental vessels. On the other hand, it was observed that carbon dioxide increased the amount of endogenous citrate formed by cell suspensions treated with fluoroacetate aerobically in the dark.

The results of the experiments in the light require more detailed consideration. According to the hypothesis of van Niel (1941) radiant energy is used to split water molecules into an oxidized and a reduced component. The oxidized component is reduced back to water by the substrate in the presence of the appropriate enzymes, and the reduced component is used for the reduction of carbon dioxide or a product or products derived from carbon dioxide. A very large portion of the substrate is assimilated by the cell, and the experiments of Cutinelli, Ehrensward, Reid, Saluste & Stjernholm (1951) with acetate make it seem unlikely that assimilation of this substrate involves a preliminary conversion into carbon dioxide. The inhibition of the photometabolism of both pyruvate and oxaloacetate by fluoroacetate, coupled with the accumulation of citrate, suggests that the tricarboxylic acid cycle is participating in the process. This implies that the oxidation of these compounds

Phosphoenolpyruvate is suggested as an intermediate in view of the work of Utter & Kurahashi (1954) and Bartley & Avi-Dor (1955).

The photometabolism of acetate and butyrate is strongly inhibited by fluoroacetate, but citrate accumulates only in the presence of acetate, suggesting that under these conditions C_4 dicarboxylic acids can be synthesized from acetate, but not from butyrate. On the other hand, little or no citrate is formed from acetate in the dark, even when the CO_2 tension in the experimental vessels is high (Table 6). The results of Cutinelli *et al.* (1951) are interesting in this respect. They grew *Rsp. rubrum* on $^{13}CH_3$, $^{14}CO_2H$ and $NaHCO_3$, and on $NaH^{14}CO_3$ and unlabelled acetate, and examined the distribution of the isotopes in alanine, serine, glutamic and aspartic acids isolated from the cell protein. Their results indicated that carbon dioxide, or a derivative of it, combines with a C_2 compound derived from acetate to give a C_4 compound which is either converted into alanine or is further carboxylated to a C_4 dicarboxylic acid. Furthermore, the isotopes were distributed in glutamic acid in a way which could be explained only if the cycle were operative. The stimulation of the photometabolism of acetate by catalytic amounts of the C_4 dicarboxylic acids also supports the idea that acetate is metabolized in the light via the tricarboxylic acid cycle.

In contrast to all these results is the work of Glover *et al.* (1952), who in very short-term (24 sec.) experiments with $[2-^{14}C]$ acetate found that, whereas succinate and α -oxoglutarate were labelled, the tricarboxylic acid areas of their paper chromatograms were unlabelled. The tricarboxylic acids became equilibrated with the tracer only after a relatively long time. Maximum radioactivity was found in an

area of the paper which could not be identified with any known compound, and it was considered that this represented a new intermediary which Glover *et al.* (1952) referred to as 'compound X'. In this work, total counts, but not specific activities, were measured. The fact that succinate and α -oxoglutarate were labelled before the tricarboxylic acids is not easy to explain in terms of established reactions. Nor is it possible at the moment to equate their results with ours. It seems to us important for the experiments of Glover *et al.* (1952) to be repeated and confirmed as a preliminary to the clearing up of this discrepancy.

As was pointed out above, fluoroacetate may not be a specific inhibitor of the tricarboxylic acid cycle; the fact that the hydrogen-carbon dioxide reaction is inhibited by this compound supports this view, for it is not easy to understand how the cycle participates in this reaction, the more so since Glover *et al.* (1952) have shown that carbon dioxide fixation in *Rsp. rubrum* involves phosphoglyceric acid just as in green-plant photosynthesis.

SUMMARY

1. *Rhodospirillum rubrum* S1 metabolizes acetate, propionate, butyrate, pyruvate, oxaloacetate, succinate, fumarate and L-malate anaerobically in the light or aerobically in the dark.

2. Monofluoroacetate ($8.3 \times 10^{-4} M$) strongly inhibits (70–80 %) the photometabolism of acetate, butyrate, pyruvate and oxaloacetate. The effect of the inhibition on the photometabolism of the remaining substrates is much smaller (20–30 %).

3. A material which behaved like citrate accumulated when acetate, pyruvate and oxaloacetate were metabolized anaerobically in the light in the presence of fluoroacetate. No significant amounts of citrate accumulated, over and above that formed in the control without substrate in the presence of fluoroacetate, when propionate, butyrate, succinate, fumarate and L-malate were metabolized under these conditions.

4. Anaerobically in the dark the oxidation of all substrates was strongly inhibited by $8.3 \times 10^{-4} M$ fluoroacetate.

5. Under these conditions citrate was produced from succinate, L-malate, fumarate and oxaloacetate; the amount of citrate produced from propionate and pyruvate was small; the amount accumulating in the presence of acetate and butyrate was no greater than that formed by the cells alone.

6. Succinate, fumarate and L-malate catalysed the photometabolism of acetate.

7. Carbon dioxide stimulated the photometabolism of succinate.

8. Aerobically in the dark carbon dioxide increased the endogenous citrate formed by suspensions of *Rsp. rubrum* in the presence of fluoroacetate.

9. Carbon dioxide had little effect on the amount of citrate formed aerobically in the dark from acetate and pyruvate in the presence of fluoroacetate.

10. It is concluded that the tricarboxylic acid cycle plays an essential part in the light-metabolism of acetate, butyrate, pyruvate and oxaloacetate by washed suspensions of *Rsp. rubrum*, but not of propionate, L-malate, fumarate and succinate; that aerobically in the dark the oxidation of all substrates involves the tricarboxylic acid cycle.

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