A Direct Pathway for the Conversion of Propionate into Pyruvate in *Moraxella lwoffii*

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(Received 21 August 1967)

1. The identity of the organism previously known as *Vibrio* O1 (N.C.I.B. 8250) with a species of *Moraxella* is established. 2. The ability of cells to oxidize propionate is present only in cells with an endogenous respiration and this ability is increased 80-fold when the organism is grown with propionate. 3. Isocitrate lyase activity in extracts from propionate-grown cells is the same as that in extracts from lactate-grown cells, about tenfold greater than that in extracts from succinate-grown cells and slightly greater than half the activity in extracts from acetate-grown cells. 4. With arsenite as an inhibitor conditions were found in which the organism would catalyse the quantitative oxidation of propionate to pyruvate. When propionate was completely utilized pyruvate was metabolized further to 2-oxoglutarate. 5. The oxidation of propionate by cells was incomplete both in a 'closed system' with alkali to trap respiratory carbon dioxide and in an 'open system' with an atmosphere of oxygen + carbon dioxide (95:5). Acetate accumulated. Under these conditions [2-¹⁴C]- and [3-¹⁴C]-propionate gave rise to [¹⁴C]acetate. The rate of conversion of [2-¹⁴C]propionate into ¹⁴CO₂, although much less than the rate of conversion of [1-¹⁴C]propionate into ¹⁴CO₂, was slightly greater than the rate of conversion of [3-¹⁴C]propionate into ¹⁴CO₂. 6. The oxidation of propionate by cells was complete in an 'open system' with an atmosphere of either oxygen or air. Under these conditions very little [1-¹⁴C]propionate was converted into ¹⁴C-labelled cell material. The conversion of [2-¹⁴C]- and [3-¹⁴C]-propionate into ¹⁴C-labelled cell material occurred at an appreciable rate, the rate for the incorporation of [3-¹⁴C]propionate being slightly more rapid. In the absence of a utilizable nitrogen source part of the [¹⁴C]propionate was incorporated into some reserve material, which was oxidized when added substrate had been completely utilized. 7. [¹⁴C]-Pyruvate produced from [¹⁴C]propionate was chemically degraded. The C₍₁₎ of propionate was found only in C₍₁₎ of pyruvate. At least 86% of C₍₂₎ of pyruvate was derived from C₍₂₎ of propionate and at least 92% of C₍₃₎ of pyruvate from C₍₃₎ of propionate. 8. These results are incompatible with the operation of any of the previously described pathways for propionate metabolism except the direct one, perhaps via an activated acrylate.

Previous studies (Callely, Dagley & Hodgson, 1958) on the mechanism of breakdown of fatty acids with four to ten carbon atoms by *Moraxella lwoffii* have shown that those with an even number of carbon atoms are oxidized to acetate and those with an odd number of carbon atoms to a mixture of acetate and propionate. Acetate is further oxidized by the tricarboxylic acid cycle. Studies with suspensions of washed cells grown with different substrates indicated that the ability to oxidize propionate is rapidly lost during starvation unless

the organism has been previously grown with a substrate that gives rise to propionate.

The specific activity of isocitrate lyase (EC 4.1.3.1) in extracts from cells grown with propionate, or other fatty acids with an odd number of carbon atoms, was similar in extracts from cells grown with fatty acids with an even number of carbon atoms (Hodgson, 1959). This activity was ten times that found in extracts from cells grown with succinate or compounds that by metabolism gave rise to succinate (Callely *et al.* 1958). This suggested that succinate was not readily produced from propionate.

When suspensions of washed propionate-grown cells metabolized propionate in the presence of

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arsenite, pyruvate was produced during the initial stages of metabolism in amounts that were equivalent to the propionate utilized (Hodgson, 1959; Hodgson & McGarry, 1964).

These findings prompted a more detailed investigation into the pathway of propionate metabolism by *Moraxella lwoffii*.

METHODS

Organism. The organism was the *Vibrio* used in earlier work (Callely *et al.* 1958). This is deposited with the National Collection of Industrial Bacteria where it is strain number 8250. During an investigation of the properties of a large number of *Vibrio* species Sébald & Veron (1963) identified this strain with *Moraxella lwoffii* IP 55112. We have confirmed this and have also found that the structure of the organism, as revealed by both light and electron microscopy, is very similar to that found for other species of *Moraxella* (Ryter & Piechaud, 1963). Plate 1 shows the presence of filaments protruding radially from the edges of the organism. Ryter & Piechaud (1963) suggested that these were responsible for the bizarre movements that they had observed. The halo around the organism in Plate 1(b) also suggests the presence of a capsular-like material. Sections of the organism (Plate 2) show the presence of mesosomes at the region of the cell where division is taking place. Septa were not observed, division taking place by a process of strangulation. The plasmalemma appears as a conventional 'unit membrane', but the cell wall appears as a multilayered structure that is not like that found in *Escherichia coli* (De Petris, 1965). An electron-transparent region external to the plasmalemma is bounded by a rigid electron-dense layer approximately 50 Å thick. This is bounded by another electron-transparent region of varying thickness, which is again bounded by an undulating structure that superficially resembles the plasmalemma. This is again similar to the structure found in other species of *Moraxella* (Ryter & Piechaud, 1963). We therefore refer to the organism as *Moraxella lwoffii* N.C.I.B. 8250.

Growth of the organism. Stock cultures were freeze-dried in horse serum from 24 hr.-old nutrient-broth (Oxoid) cultures and stored *in vacuo*. Working cultures were maintained at 4° on slopes of synthetic nutrient agar containing (g./100 ml.): agar, 2; peptone (Evans), 1; yeast extract (Difco), 0.5; NaCl, 0.5. The basal mineral salts medium contained (g./l. of water): KH_2PO_4 , 2; $(\text{NH}_4)_2\text{SO}_4$, 1; NaCl, 1. The pH was adjusted to 7.1 with 5N-NaOH and, after sterilization, sterile $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (10%, w/v) was added to give a final concentration of 0.04% (w/v). The carbon source (0.02 M unless otherwise stated) was dissolved in the basal medium before neutralization and sterilization. The organism is a strict aerobe, so liquid medium was dispensed, 50 ml. in 250 ml. conical flasks, 250 ml. in 1 l. conical flasks and 6 l. in 10 l. flat-bottomed flasks. The small volumes were agitated at 30° on a reciprocating shaker and the 6 l. lots were agitated with a vigorous stream of sterile compressed air. Growth was started by inoculation of the 50 ml. lots with a loopful of culture from a slope; volumes of 25 ml. and 250 ml. of actively growing culture were used to inoculate 250 ml. and 6 l. lots respectively. Unless otherwise stated cells were harvested in the exponential phase of growth. Growth was measured turbidimetrically

with a Hilger Spekker photoelectric absorptiometer with a neutral filter (Ilford H 508) and the weight of bacteria was estimated with a calibration graph. Volumes of cell suspensions not greater than 1 l. were harvested by centrifuging at 14 000 g for 10 min. at 5°. Larger volumes were harvested in a rotor-driven Sharples super-centrifuge.

Cell extracts. Cells were harvested and washed with cold (5°) tris buffer, pH 7.4. Each 10 g. wet wt. of cells was placed in a Hughes (1951) bacterial press, frozen at -20° for 3-15 hr., crushed and mixed with an equal weight of polishing alumina (grade 3/50; Griffin and George Ltd., Manchester). The mixture was homogenized in an MSE homogenizer, the cup of which was surrounded by ice, while 25 ml. of ice-cold tris buffer, pH 7.4, was slowly added. Alumina, cell debris and large particulate matter was removed by centrifuging at 14 000 g for 40 min. at 5°. The clear viscous supernatant was carefully decanted and 0.1 ml. of M-MgSO₄ was added for every 10 ml. of extract obtained. Unless the extract was to be used immediately it was dispensed into several Pyrex tubes and stored at -20°. The enzyme activities assayed in these extracts did not change during storage for 4-6 weeks. Measurements were made on extracts that had been frozen and thawed only once. Crystallized bovine plasma albumin from Armour Laboratories (Eastbourne, Sussex), dried over P₂O₅, was used as standard for protein determinations by the method of Stickland (1951).

Manometric methods. Cells were harvested, washed twice and resuspended (about 4 mg. dry wt. of cells/ml.) in phosphate buffer. Consumption of O₂ was measured by conventional methods (Umbreit, Burris & Stauffer, 1957) in constant-volume respirometers at 30°. Flasks contained 1.0 ml. of cell suspension and 1.5 ml. of phosphate buffer in the main compartment, 0.2 ml. of 20% (w/v) KOH in the centre well and 0.5 ml. (25 μmoles unless otherwise stated) of substrate, pH 7, or water in the side arm. Substrates were tipped in after equilibration and oxidation rates expressed as Q_o values (μl. of O₂/mg. bacterial dry wt. or mg. of protein/hr.) were calculated from maximum linear rates. Solutions of inhibitors adjusted to the appropriate pH value were added to the main compartment and the volume of buffer was decreased accordingly.

Preparation and extraction of 2,4-DNP-hydrazine derivatives of keto acids. A solution containing not more than 5 μmoles of keto acid/ml. was mixed with 0.5 or 1.0 vol. of 0.1% (w/v) 2,4-DNP-hydrazine hydrochloride in 2N-HCl (filtered before use) and left for up to 30 min. The 2,4-DNP-hydrazine derivatives were extracted into ethyl acetate and then re-extracted into 10% (w/v) Na₂CO₃ solution. This was cooled in ice and slowly brought to pH 2 by the addition of conc. HCl. The 2,4-DNP-hydrazones were then re-extracted into ethyl acetate and this solution was concentrated with a stream of N₂ gas and dried by mixing with anhydrous Na₂SO₄. For chromatography, this solution was applied directly to the paper, whereas for experiments involving the degradation of ¹⁴C-labelled derivatives the solution was filtered, carefully evaporated to dryness and stored *in vacuo* over KOH pellets before use.

Separation of propionate, acetate and other organic acids. Organic acids were separated by partition chromatography on ether-washed air-dried Celite by the method of Swim & Krampitz (1954) with 7 g. of Celite packed in columns of 11 mm. internal diam. and with 70 ml. of each solvent previously equilibrated with 0.2N-H₂SO₄ for elution. It

EXPLANATION OF PLATE I

(a) *M. lwoffi* shadowed with carbon-platinum at an angle of 60°, showing the presence of filaments (f) protruding radially from the edge of the organism. Magnification $\times 70\,000$. (b) *M. lwoffi* negatively stained with phosphotungstate, showing the presence of filaments, and a halo that suggests the presence of a capsular-like material. Magnification $\times 70\,000$.

EXPLANATION OF PLATE 2

Sections through *M. lwoffi* fixed and stained by the method of Kellenberger *et al.* (1958). The structure of the cell wall (cw) can be clearly seen, with a single rigid layer overlain by an undulating plasmalemma-type membrane. Mesosomes (m) can be seen at the point of strangulation (dp). pm, plasmalemma. Magnification $\times 150\,000$.

was not possible to separate either propionate from acrylate or lactate from succinate.

Determination of pyruvate and 2-oxoglutarate. Invariably these acids had to be determined in the presence of each other, so either the differential-extraction method, as outlined by Friedemann (1957), or specific enzyme methods were used.

(a) *Differential-extraction method.* Two identical 1 ml. portions of the sample in phosphate buffer, containing not more than a total of 3 μ moles of keto acid or 1.5 μ moles of each keto acid separately, were mixed with 2.0 ml. of 10% (w/v) trichloroacetic acid solution and 1.0 ml. of 2,4-DNP-hydrazine reagent. After exactly 5 min. 3.0 ml. of ethylbenzene was added to one solution and it was then mixed by agitation with a stream of air for 2 min. The other solution was left for 25 min., 8 ml. of benzyl alcohol was added and the mixture was agitated as was the first sample. In both cases separation of the two phases was facilitated by a short centrifugation at about 1000g. The organic phases were separated and extracted as before with 6 ml. of 10% (w/v) Na_2CO_3 with the whole of the ethylbenzene phase, but only 7 ml. of the benzyl alcohol phase. The phases were separated by centrifuging and 5 ml. samples of the aqueous phase were mixed with 5 ml. of 1.5 N-NaOH. After 15 min. the extinction was determined with a Hilger Spekker photoelectric absorptiometer with a 601 filter (max. transmission at 435 μ m). Since with both organic solvents there was a linear relationship between the concentration of each keto acid (up to 1.5 μ moles) and the extinction, it was assumed that with a mixture of keto acids the total extinction measured was the sum of the individual extinctions. E was then determined with 1 μ mole of each keto acid in each solvent system. With ethylbenzene as solvent, E with pyruvate was 0.895 and with 2-oxoglutarate was 0.055. With benzyl alcohol as solvent, E with pyruvate was 0.090 and with 2-oxoglutarate was 0.685. Hence for any mixture of these two keto acids the amount of pyruvate in μ moles present in the sample was $1.13 E$ (ethylbenzene) — $0.11 E$ (benzyl alcohol) and the amount of 2-oxoglutarate present in the sample was $1.47 E$ (benzyl alcohol) — $0.15 E$ (ethylbenzene). The validity of these two equations was checked with known quantities of mixed acids.

(b) *Enzyme methods for determining the concentration of pyruvate and 2-oxoglutarate in mixtures.* Pyruvate and 2-oxoglutarate were determined spectrophotometrically with lactate dehydrogenase (EC 1.1.1.27) and glutamate dehydrogenase (EC 1.4.1.2) respectively, by measuring the fall in E_{340} after the addition of solutions, pH 7, of unknown keto acid concentration to phosphate buffer, appropriate enzyme, NADH (0.3 mg.) and, in the glutamate dehydrogenase assay, 0.15 M-mole of NH_4Cl , in a total volume of 3 ml. in a spectrophotometer cell with a light-path of 1 cm. The methods were calibrated with solutions of known concentration of either lithium pyruvate or 2-oxoglutarate. Propionate, acetate, arsenite and the other keto acid at the concentration present in the assay solution did not interfere with either assay.

Determination of propionate and acetate. A modification of the steam-distillation method of McClendon (1944) with the apparatus described by Markham (1942) was devised that gave quantitative recoveries of steam-volatile acids and abolished significant interference by pyruvate. The solution (2 ml.) to be analysed, containing not more than 20 μ moles of steam-volatile acids, was treated with 1.0 ml. of 30% (w/v)

metaphosphoric acid solution. Precipitated protein was removed by centrifuging and the pellet was re-extracted with a further 1.0 ml. of metaphosphoric acid solution. The combined supernatants were then treated for 15–30 min. with 1.0 ml. of 10% (w/v) phenylhydrazine hydrochloride solution (Weil-Malherbe, 1937) and then added to the still. The flask was rinsed with 4.0 ml. of water in which 8 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ had been dissolved by warming (Pennington, 1952). Silicon MS antifoam emulsion (0.5 ml.) (prepared by ultrasonic treatment of the silicone in water) and 0.5 ml. of syrupy phosphoric acid were added to the flask and the mixture was quickly added to the still. The first 50 ml. of distillate was collected and titrated with approx. 10 mM-NaOH to the first permanent pink colour with phenol red as indicator while the distillate was gassed with a continuous stream of CO_2 -free N_2 . To avoid the need for standardized NaOH, known quantities of propionate or acetate were distilled at the end of each series of determinations. So long as the quantities of steam-volatile acids did not exceed 20 μ moles quantitative recoveries were obtained. When acetate and propionate were present together their total concentration was determined as described above and then individual concentrations were determined by concentrating the distillate under alkaline conditions and separating the acetate and propionate on Celite columns.

Assay for isocitrate lyase (EC 4.1.3.1). The method was based on that of Dixon & Kornberg (1959). The assay system contained, in 3 ml., 200 μ moles of tris buffer, pH 8.0, 20 μ moles of MgCl_2 , 5 μ moles of GSH, 20 μ moles of phenylhydrazine hydrochloride (brought to pH 8 with NaOH) and 0.2 ml. of extract diluted with buffer to contain approx. 1 mg. of protein. The reaction was started by adding 10 μ moles of DL-isocitrate (trisodium salt). After a lag of 1–2 min. the rate of change of E_{324} was linear for 10–12 min. This rate was proportional to enzyme concentration so that the specific activity of the enzyme in terms of μ moles of glyoxylate produced/mg. of protein/hr. was calculated from $\epsilon_{324} \cdot 1.7 \times 10^4$ for glyoxylate phenylhydrazone (Dixon & Kornberg, 1959). GSH was used in preference to cysteine because glyoxylate rapidly reacted with cysteine. The rate of reaction of GSH with glyoxylate was much lower.

Quantitative conversion of propionate into pyruvate. Propionate-grown cells harvested in exponential growth were suspended (4.6 mg. dry wt. of cells/ml.) in phosphate buffer. The cell suspension (250 ml.) and phosphate buffer (500 ml.) containing 7.5 M-moles of propionate and 0.75 M-mole of sodium arsenite were aerated at 30°. At intervals during a 4 hr. incubation, 20 ml. samples were removed and mixed with 5.0 ml. of 3 N- H_2SO_4 standing in ice. Cells were sedimented and the supernatants assayed for steam-volatile acids, pyruvate and 2-oxoglutarate.

Electron microscopy. Suspensions of growing bacteria were diluted with water and a drop was placed on a carbon-coated Formvar-covered Athens grid. The preparations were dried and shadowed at an angle of 60° with carbon-platinum. For negative staining the method of Zwillenberg (1964) was followed except that the organism was grown in liquid media and sedimented, and then the pellet was resuspended in the ammonium acetate-sucrose solution. For sections, suspensions of growing bacteria were fixed by the method of Kellenberger, Ryter & Séchaud (1958), embedded in Vestopal W and sectioned with glass knives on an LKB Ultratome Mk 1. Specimens were examined in either a

Philips EM75B or an Associated Electrical Industries EM 6B instrument.

Experiments with radioactive materials

Rapid-sampling technique. The methods for carrying out the rapid-sampling experiments for separating the products of metabolism and for the purification of [^{14}C]acetate and [^{14}C]propionate were essentially those described by Kornberg (1958) with acetate as substrate.

Conversion of [^{1-14}C], [^{2-14}C] or [^{3-14}C]propionate into carbon dioxide: 'closed system'. Experiments were performed in double-side-armed Warburg flasks at 30°. The main compartment contained 2.0 ml. of a suspension of propionate-grown cells (3 mg. dry wt./ml.) in phosphate buffer and 0.3 ml. of water. The centre well contained 0.2 ml. of *m*-Hyamine hydroxide in methanol and a filter-paper wick. One side arm contained 10 μmoles of [^{14}C]propionate (0.2 μC) and the other 0.3 ml. of freshly prepared 25% (w/v) sodium dodecyl sulphate solution in water. Flasks were gassed with O_2 , stoppered and equilibrated for 10 min. Substrate was tipped in and the reaction stopped at various times up to 60 min. by tipping the sodium dodecyl sulphate into the main compartment. (Sodium dodecyl sulphate solution is a bacteriolytic substance even at neutral pH, and under the above conditions immediately blocks metabolism.) Flasks were shaken for a further 45 min. to ensure complete absorption of carbon dioxide, and the contents of the centre wells were transferred to a tube. The wells were washed with methanol (3 $\times$ 0.3 ml.), the washings were combined with the contents and the mixture was vigorously shaken to break up the filter paper. The paper was sedimented, washed with methanol (4 ml.) and resedimented, and the combined supernatants were made up to 10 ml. with methanol. Portions (generally 0.5 ml.) were used for liquid-scintillation counting. This procedure is referred to in the text as the 'closed system'.

Conversion of [^{1-14}C], [^{2-14}C] or [^{3-14}C]propionate into cell material: 'open system'. Reactions were carried out in 100 ml. conical flasks each with propionate-grown washed cells suspended (4 mg. dry wt. of cells/ml.) in 10 ml. of phosphate buffer containing 50 μmoles of sodium propionate and 0.5 μC of [^{14}C]propionate. Flasks were shaken at 30° and 1 ml. samples were removed at intervals and mixed into 2.0 ml. of water standing in an ice bath. Cells were immediately sedimented and the pellets were washed with 2.0 ml. of ice-cold water. Portions of the combined supernatants were counted by liquid-scintillation counting. Pellets were suspended in 2.0 ml. of water, boiled for 15 min., resedimented and resuspended in 2.0 ml. of water. Portions (1 ml.) were plated and counted to give the radioactivity incorporated into cellular material.

Determination of the distribution of ^{14}C in pyruvate produced during the metabolism of [^{14}C]propionate. A suspension of bacteria (8 mg. dry wt.) in phosphate buffer (1 ml.) was incubated with 60 μmoles (30 μC) of [^{1-14}C], [^{2-14}C] or [^{3-14}C]propionate and 6.0 μmoles of sodium arsenite in a total volume of 6 ml., in 50 ml. flasks stoppered with rubber bungs containing glass capillary inlet and outlet tubes. CO_2 -free air was drawn over the incubation liquid by suction and the CO_2 produced was trapped in 2*N*-NaOH. After 90 min. bacteria were sedimented, the supernatant was separated and a portion (2 ml.) was frozen for subsequent determination of propionate, acetate, pyruvate and 2-oxoglutarate. The remaining supernatant

(4 ml.) was combined with 3 ml. of water used for washing the bacterial pellet and the solution was incubated with 1 ml. of 2,4-DNP-hydrazine reagent for 4 hr. The 2,4-DNP-hydrazones were then prepared. A sample of the pyruvate 2,4-DNP-hydrazone from each incubation was degraded to acetate and CO_2 by a modification of the method of Martin & Batt (1957): 5.0 ml. of 0.1*N*- KMnO_4 , 0.5 ml. of 5*N*- H_2SO_4 and 0.5 ml. of catalyst solution in an atmosphere of O_2 were used; CO_2 was collected in 0.4 ml. of 2*N*-NaOH, which was contained in a small glass tube suspended in the flask, which was itself sealed with a rubber Suba-Seal cap. The sample (1.5 ml. of 0.1*N*-NaOH) was injected through the rubber cap and the CO_2 trap was removed after 90 min. The manganese oxides were removed by centrifuging and the excess of KMnO_4 was decomposed by the addition of *m*-oxalic acid. Solutions were then made slightly alkaline and, after evaporation to a small volume, added to a Celite column. Acids were eluted and extracted from each 10 ml. of eluate with 2 ml. of 10*MN*-NaOH. A portion of this was plated and counted and the percentage of the radioactivity in the pyruvate 2,4-DNP-hydrazones that appeared in each fraction was calculated. The remainder of the aqueous fractions, which contained the acetate, together with 200 μmoles of carrier, was then used directly in a Schmidt degradation as described by Martin & Batt (1957) but in the apparatus of Greenberg & Rothstein (1957). The CO_2 from the carboxyl group was trapped and the methylamine from the methyl group was distilled into 2 ml. of *N*- H_2SO_4 after adjustment of the pH. The distillate was concentrated to a small volume *in vacuo* at 45°. Methylamine sulphate was oxidized to CO_2 with the Van Slyke & Folch (1940) chromic acid solution and the method described by Sakami (1955), and the CO_2 was trapped. At each stage CO_2 was trapped with 5 ml. of 2*N*-NaOH in a Geissler-Mohr absorption bulb (A. Gallenkamp and Co. Ltd., Widnes, Lanes.). The degradation procedures for acetate were checked with both [^{1-14}C] and [^{2-14}C]acetate.

Assay of ^{14}C -labelled compounds. The $^{14}\text{CO}_2$ that was trapped in 2*N*-NaOH was converted into $\text{Ba}^{14}\text{CO}_3$ by precipitation with 5% (w/v) BaCl_2 solution, collected on filter-paper disks and dried with an infrared lamp. These samples, samples from Celite columns, [^{14}C]pyruvate derivatives of 2,4-DNP-hydrazine and bacterial suspensions were dried on to aluminium planchets and counted with a windowless scintillation counter (type SC-A9; Panax Ltd., Redhill, Surrey) and conventional scaling equipment. The counting efficiency was about 20%. Counts for $\text{Ba}^{14}\text{CO}_3$ (corrected for background and coincidence) were corrected to infinite thinness by using a self-absorption correction curve prepared under conditions identical with those described above by the method described by Wang & Willis (1965). For the other materials weights on planchets were invariably considerably less than 1 mg. and no corrections for self-absorption were required.

Liquid-scintillation counting. The $^{14}\text{CO}_2$ collected in methanolic Hyamine hydroxide and supernatants from bacterial suspensions incubated with [^{14}C]propionate were counted by mixing 0.5 ml. samples with 8.0 ml. of either NE213 liquid scintillator [Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh] or DEM liquid phosphor (Panax Ltd.) respectively, in glass sample jars. These were cooled for 30 min. at 0° and counted in a Panax assembly type SC-LP (cooled by continuously circulating tap water) and connected to a scaler type D657 (Panax Ltd.).

Efficiency was approx. 70% for NE213 and 60% for DEM phosphors.

MATERIALS

Chemicals. NAD⁺ (free acid), NADPH (sodium salt), NADH (disodium salt), phosphoenolpyruvate (tricyclohexylammonium salt) and GSH were from Boehringer Corp. (London) Ltd. (London, W. 5). Glyoxylic acid monohydrate and 2-hydroxyglutarate (zinc salt) were from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). The 2-hydroxyglutarate was dissolved in dil. H₂SO₄, extracted with ether, re-extracted with a small volume of 2N-NaOH run through a Zeo-Karb 225 (H⁺ form) column and the aqueous eluate titrated with standard NaOH to determine the concentration of the acid. Silicone MS antifoam A was from Hopkin and Williams Ltd. (Chadwell Heath, Essex). Hyamine hydroxide (1.0M in methanol) was from Nuclear Enterprises (G.B.) Ltd. Celite 535 was from Johns-Manville and Co. Ltd. (London, S.E. 1). Other chemicals were of A.R. grade wherever possible, from Hopkin and Williams Ltd. or British Drug Houses Ltd. (Poole, Dorset).

Radioactive compounds. Na¹⁴CO₃, sodium [1-¹⁴C]- and [2-¹⁴C]-acetate and sodium [1-¹⁴C]- and [2-¹⁴C]-propionate were from The Radiochemical Centre (Amersham, Bucks.) and sodium [3-¹⁴C]propionate was from NV Philips-Duphar (Amsterdam, The Netherlands).

Enzymes. Lactate dehydrogenase (EC 1.1.1.27) and glutamate dehydrogenase (EC 1.4.1.2) were obtained from Boehringer Corp. (London) Ltd.

Miscellaneous. Suba-Seal stoppers were from A. Gallenkamp and Co. Ltd., London, E.C. 2. Athene Electron microscope grids, new type 200, were from Smethurst High-Light Ltd., Bolton, Lancs.

Buffers. The phosphate buffer contained 2g. of KH₂PO₄/l., neutralized with NaOH to pH 7. The tris buffer was 0.05M, prepared as described by Gomori (1955).

RESULTS

Oxidation of propionate and various other substrates. In an attempt to discover which of the possible alternative pathways of metabolism

operated, the rates of oxidation of possible intermediates were examined. Cells grown with acetate, propionate or succinate and harvested in exponential growth oxidized acetate, lactate, malate, oxaloacetate, propionate and pyruvate at rates that varied slightly depending on the growth substrate (Table 1). Acrylate, glyoxylate, citramalate, 2-hydroxyglutarate, methylmalonate and 3-hydroxypropionate were not oxidized by cells grown with any of the three substrates. Citrate and 2-oxoglutarate were oxidized only by cells grown with succinate, and isocitrate was occasionally oxidized by cells grown with acetate. Extracts from all these cells oxidized citrate and 2-oxoglutarate with an initial rate of uptake of about 40 μ l. of oxygen/hr./mg. of protein. This high initial rate of oxidation quickly fell to about 15 μ l./hr./mg. of protein, which was very similar to that at which succinate, fumarate and malate were oxidized. Although there were differences in the rates at which citrate and 2-oxoglutarate were oxidized by cell extracts, these were not of the same magnitude nor did they mirror the variations found with whole cells. Extracts from propionate-grown cells, which did oxidize propionate, also oxidized 2-hydroxyglutarate but did not oxidize 3-hydroxypropionate, acrylate or methylmalonate.

When cells were harvested after they had reached the stationary phase of growth it was found that washed suspensions did not oxidize propionate even though their ability to oxidize other substrates was not impaired. For example, in Table 1 compare the cells grown with acetate and harvested in exponential growth, which had Q_{O_2} 33 with propionate, with those harvested after growth had ceased, which had Q_{O_2} 0 with propionate. This effect was not dependent on the growth substrate but was found with all cells that had a very low endogenous respiration.

Table 1. Rates of oxidation of organic acids by *M. lwoffi* grown with different substrates

The organism was harvested either during exponential growth or when growth had ceased. Washed-cell suspension (1 ml.; 4 mg. dry wt. of cells/ml. of phosphate buffer), 1.5 ml. of phosphate buffer, 0.5 ml. of substrate (25 μ moles), pH 7, and 0.2 ml. of 20% (w/v) KOH in the centre well were incubated in Warburg flasks at 30°. Q_{O_2} values were calculated from maximum linear rates.

Growth substrate ... Period of growth when harvested ...	Q_{O_2} (μ l. of O ₂ /mg. dry wt./hr.)					
	Acetate		Propionate		Succinate	
	Exponential	Stationary	Early exponential	Late exponential	Exponential	Stationary
Substrate						
Nil	9	0	8	24	17	0
Acetate	262	238	294	265	127	115
Isocitrate	10	43	9	24	20	0
Lactate	75	87	70	69	142	175
Propionate	33	0	268	189	28	0
Succinate	157	172	104	163	306	356

Inhibition of oxidation reactions by arsenite. Arsenite is a particularly effective inhibitor of keto acid oxidations (Krebs, 1933) and, when it is used as an inhibitor of so-called catabolic reactions, keto acid intermediary metabolites are often found to accumulate. Oxygen uptake by suspensions of washed propionate-grown cells (4mg.) in the presence of propionate (25 μ moles) was inhibited by arsenite at concentrations greater than 10 μ M. After about 30 min. oxidation, analysis of the contents of the Warburg flasks showed that an appreciable quantity of keto acid was present when arsenite (1mM) had decreased the rate of oxidation to about 80% of that of the control without arsenite. Very little keto acid was found when the concentration of arsenite was such that the rate of oxygen uptake was decreased by less than 10% (0.1mM-arsenite) or more than 50% (10mM-arsenite). 2,4-DNP-hydrazine derivatives of the accumulated keto acids were then separated by paper chromatography. After oxidation in the presence of 1mM-arsenite for 15 min. only two derivatives, with similar R_F values to the two derivatives of genuine pyruvate, were detected, whereas after a further 15 min. a third derivative that had an R_F value corresponding to the derivative of genuine 2-oxoglutarate was also present.

Arsenite also inhibited oxidation with acetate as substrate to about the same extent as it did that with propionate; however, at all times only very small quantities of pyruvate could be detected, whereas appreciable quantities of 2-oxoglutarate were always present. With succinate as substrate arsenite had no effect on the rate of oxidation but both pyruvate and 2-oxoglutarate accumulated in about equal quantities.

Cells grown with succinate did not produce keto acids in the presence of arsenite from either propionate or acetate, although with both substrates the rates of oxidation were decreased compared with controls. With succinate as substrate behaviour was the same as when the cells had been grown with propionate.

Identification of pyruvate as product of propionate metabolism. Determination of pyruvate produced from propionate in the presence of arsenite gave identical values with either the differential-extraction method or the lactate dehydrogenase assay. The 2,4-DNP-hydrazone derivatives of the product formed during a 30 min. oxidation were prepared and purified by paper chromatography. The pyruvate 2,4-DNP-hydrazone derivatives were eluted and their properties compared with those of genuine pyruvate 2,4-DNP-hydrazone derivatives. This showed that: (1) in alkali they had identical absorption spectra in the range 350–600 m μ ; (2) after paper chromatography in the solvent systems of Dagley, Fewster & Happold (1952) they

Fig. 1. Conversion of propionate into keto acids by *M. lwoffi* in the presence of arsenite. The organism was harvested in exponential growth from propionate medium. Washed cells (1.15 g. dry wt.) in 750 ml. of phosphate buffer containing 10mM-propionate and 1mM-arsenite were vigorously aerated at 30°. Samples (20 ml.) were removed at various times and mixed with 5.0 ml. of 3N-H₂SO₄ at 0°, the cells were sedimented and the supernatants analysed. ●, Propionate utilized; △, pyruvate formed; ○, 2-oxoglutarate formed.

had identical R_F values, and each corresponding spot gave the same colour change when sprayed with 2% (w/v) potassium hydroxide in 90% (v/v) ethanol; (3) after electrolytic reduction by the method described by Smith (1958), followed by paper chromatography in three different solvent systems, each gave one ninhydrin-positive spot that had an R_F value identical with that of alanine.

Quantitative conversion of propionate into pyruvate. The accumulation of the keto acids and the disappearance of propionate were determined quantitatively in a scaled-up incubation as described in the Methods section. The results (Fig. 1) show that under these conditions there was a 1:1 conversion of propionate into pyruvate during the first 90 min. When the propionate had been completely utilized the pyruvate disappeared and there was a rapid accumulation of 2-oxoglutarate. The amounts of acetate determined were in all cases extremely low, and their values have not been recorded.

Isocitrate lyase activities in extracts from cells grown with various substrates. Cell extracts were prepared from cells harvested in exponential growth from medium with acetate, propionate, lactate or succinate as sole carbon source. The specific

Table 2. *Isocitrate lyase activities in extracts from M. lwoffii grown with various substrates*

Activities were determined at pH 8 by following the rate of glyoxylate production in 3 ml. of assay solution containing about 1 mg. of protein, 10 μ moles of DL-isocitrate, under other conditions described in the Methods section. Inhibition of activity by succinate was determined by including 10 μ moles of succinate in the normal assay solution.

Growth substrate	Glyoxylate produced (μ moles/mg. of protein/hr.)	Inhibition of activity by succinate (%)
Acetate	8.0	40
Propionate	5.0	50
Lactate	5.0	45
Succinate	0.6	50

activities of isocitrate lyase found are shown in Table 2 together with the percentage inhibition of activity found when 10 μ moles of succinate were included in the assays. Phosphoenolpyruvate, which Ashworth & Kornberg (1963) claim inhibits the activity of isocitrate lyase in *E. coli* Bm, had no effect on the rate of glyoxylate production in *M. lwoffii* when 10 μ moles were included in the assay system for isocitrate lyase. In this latter respect the isocitrate lyase of *M. lwoffii* resembles that of *E. coli* E26 (Rabin, Reeves, Wegener, Megraw & Aji, 1965).

Metabolism of [14 C]propionate

Rapid-sampling technique. The initial products of acetate metabolism have been identified in *Pseudomonas* KB1 (Kornberg, 1958) and yeast (Barnett & Kornberg, 1960) by using a rapid-sampling technique after the addition of [14 C]acetate to cells metabolizing unlabelled acetate at a constant rate. When this method was tried with *M. lwoffii* with [14 C]propionate or [14 C]acetate, the amounts of radioactivity found in the ethanol-soluble components of the cells were extremely small and radioautographs of these components separated by two-dimensional paper chromatography revealed no difference between the products of propionate metabolism and those of acetate metabolism.

Conversion of [14 C]propionate into carbon dioxide. Experiments carried out as described in the Methods section with [14 C]-, [14 C]- or [14 C]-propionate gave the results shown in Fig. 2. After 60 min., 42%, 19% and 10% respectively of the radioactivity initially present in the samples were recovered as carbon dioxide. To locate the remainder of the radioactivity a companion experiment was done in which cells were incubated with each compound and after 60 min. 0.3 ml. of water, instead of sodium dodecyl sulphate solution, was tipped into the main compartment. A portion

(1.3 ml.) of the cell suspension was removed and mixed into 2.0 ml. of water at 0° and the cells were quickly sedimented at 14000g for 10 min. at 4°. The supernatants were decanted and the pellets each washed with 2.0 ml. of water, and the amount of radioactivity was determined by liquid-scintillation counting on portions of the combined supernatants. About 40%, 67% and 89% of the initial radioactivity were found in the supernatants from [14 C]-, [14 C]- and [14 C]-propionate respectively. As experiments with the 'open system' revealed that the rates of metabolism of the differently labelled propionates were identical, the different percentages of the initial radioactivity found in the supernatants from the different substrates could not have been due to the presence of inhibitors in the original [14 C]- and [14 C]-propionate. Samples of the supernatants from the 'closed system' (Fig. 2) were therefore concentrated, acidified and run through Celite columns with acetate and propionate as carriers. In each case about 90% of the radioactivity remaining in the supernatants was recovered in the fractions shown by titration to contain acetate and propionate. In the example shown in Fig. 3, which is for the [14 C]propionate sample, about 35% of the radioactivity remaining in the supernatant was recovered in the acetate fraction. From the [14 C]-propionate sample about 40% and from the [14 C]propionate sample only about 5% of the radioactivity were recovered in acetate. As no corrections were made for self-absorption these values can only be approximate.

Conversion of [14 C]propionate into cell material. By using the 'open system' described in the Methods section it was first established that the rates of loss of 14 C from the supernatants with [14 C]-, [14 C]- and [14 C]-propionate were identical. After 40 min. incubation, less than 20% of the initial radioactivity was recovered in the cell-free supernatants, and as the rate of loss of 14 C over the next 80 min. period was low it was assumed that after 40 min. incubation most of the propionate had been utilized. After 40 min., 2%, 12% and 16% of the initial radioactivity of [14 C]-, [14 C]- and [14 C]-propionate respectively were recovered in the cellular material (Fig. 4). However, during the next 80 min., when very little further loss of 14 C from the supernatants occurred, there was a fall in the radioactivity present in the cells to 1.5%, 6.5% and 10.5% of the initial values respectively. The presence of 12 mg. of ammonium sulphate in the incubation mixture prevented this loss of 14 C from the cells that had utilized [14 C]-propionate and also slightly increased the initial rate at which propionate was converted into cell material. When the supernatant from the incubation with [14 C]propionate was run through a

Fig. 2. Rate of conversion of [¹⁴C]propionate into CO₂ by *M. lwoffi* in a 'closed system'. Propionate-grown cells (6 mg. dry wt.) were incubated at pH 7.0 and 30° in phosphate buffer with 10 μmoles of [¹⁴C]propionate (0.2 μC). The liberated CO₂ was trapped in 0.2 ml. of 1.0 M-Hyamline hydroxide in methanol. Metabolism was stopped at various times with 0.3 ml. of 25% (w/v) sodium dodecyl sulphate solution. Radioactivity was determined by liquid-scintillation counting and other conditions were as described in the Methods section. ●, [1-¹⁴C]Propionate; △, [2-¹⁴C]propionate; ○, [3-¹⁴C]propionate.

Celite column, radioactivity was not detected in the fractions that contained acetate. However, it was later found that, when cells were incubated with [3-¹⁴C]propionate in an 'open system' that was continuously gassed with a stream of oxygen + carbon dioxide (95:5), only 27% of the initial radioactivity had disappeared from the cell-free supernatants after 60 min., compared with 89% of the radioactivity in a companion incubation gassed with a stream of air. When the supernatant from the incubation gassed with oxygen + carbon dioxide (95:5) for 60 min. was chromatographed on a Celite column, 26% of the remaining radioactivity was recovered in the fraction that contained acetate.

Distribution of ¹⁴C in pyruvate produced during the metabolism of [¹⁴C]propionate. Chemical analysis of the supernatant fluids from the sedimented incubation mixtures showed that in each case only

Fig. 3. Separation of acetate and propionate on Celite columns to show the distribution of the radioactivity in the supernatants prepared from the 'closed-system' experiment described in the text and Fig. 2 with [3-¹⁴C]propionate as substrate. Acetate (10 μmoles) and propionate (10 μmoles) were added to the supernatants before they were concentrated. Acetate was eluted in fractions 9, 10 and 11 and propionate in fractions 5, 6 and 7. △, % of radioactivity in the supernatant recovered in each fraction; ○, volume of 10 M-NaOH required for neutralizing each fraction.

20 μmoles of propionate had been utilized and that only about 2 μmoles of pyruvate were present, together with a small quantity (less than 0.2 μmole) of 2-oxoglutarate. Celite chromatography of the acids that were present in the supernatants at the end of the incubation revealed that 66% (40 μmoles) of the original radioactivity was present as propionate and 20% (12 μmoles) as acetate from the [2-¹⁴C]- and [3-¹⁴C]-propionate samples but only 0.4% from the [1-¹⁴C]propionate sample, and that in each sample there was a trace of [¹⁴C]succinate and 2-oxo[1-¹⁴C]glutarate. Apparently the conditions of this particular experiment favoured the decarboxylation of the accumulated keto acids.

The degradation of the pyruvate 2,4-DNP-hydrazones to acetate and carbon dioxide (see the Methods section) was not quantitative. However, 25.5% of the radioactivity in the pyruvate 2,4-DNP-hydrazone isolated from the incubation with [1-¹⁴C]propionate was recovered as carbon dioxide

Fig. 4. Rate of conversion of $[^{14}\text{C}]$ propionate into cellular material by *M. lwoffii* in an 'open system'. Propionate-grown washed cells (40 mg. dry wt.) were incubated at pH 7 and 30° in 10 ml. of phosphate buffer with 50 μmoles of $[^{14}\text{C}]$ propionate (0.5 μC). Samples (1 ml.) were removed at various times, cells were sedimented and their radioactivity was determined. Other conditions are described in the Methods section. ○, $[3\text{-}^{14}\text{C}]$ Propionate substrate; ▲, $[2\text{-}^{14}\text{C}]$ propionate substrate; △, $[1\text{-}^{14}\text{C}]$ propionate substrate; ●, $[3\text{-}^{14}\text{C}]$ propionate + $(\text{NH}_4)_2\text{SO}_4$ (12 mg.) substrate.

and no radioactivity was detected in acetate. About 22% and 23% of the radioactivity in the pyruvate 2,4-DNP-hydrazone isolated from the incubations with $[2\text{-}^{14}\text{C}]$ - and $[3\text{-}^{14}\text{C}]$ -propionate respectively were recovered in acetate, and only 0.6% and 0.5% in carbon dioxide. Together these results indicate that only about 25% of the pyruvate 2,4-DNP-hydrazone from the three incubations was degraded to carbon dioxide and acetate.

When this acetate was isolated and degraded most of the radioactivity in the acetate sample obtained from the incubation with $[2\text{-}^{14}\text{C}]$ -propionate was found in the carboxyl carbon and in the acetate sample from the $[3\text{-}^{14}\text{C}]$ propionate in the methyl carbon. These results are summarized in Table 3.

DISCUSSION

A number of different pathways for the conversion of propionate into intermediates that can be directly oxidized by the tricarboxylic acid cycle have been described (for summary see Calley & Lloyd, 1964). The so-called succinate pathway, which involves the carboxylation of propionyl-CoA to methylmalonyl-CoA, the conversion of this into succinyl-CoA and the removal of the CoA to yield succinate, is the pathway most frequently found in animals and bacteria.

There are several lines of evidence against the operation of this pathway in *M. lwoffii*. First, the activity of isocitrate lyase in extracts from propionate- and lactate-grown cells was closer to that in extracts from acetate- than from succinate-grown cells. One of the characteristic properties of *M. lwoffii* is that, although it will grow with a large number of aromatic compounds and organic acids as sole carbon source, it is unable to utilize carbohydrates. It is also likely that in terms of anaplerotic sequences for the tricarboxylic acid cycle *M. lwoffii* is equivalent to the PN-1/IR mutants of *E. coli* B described by Kornberg (1966), i.e. the only pathway for the formation of phosphoenolpyruvate is via the C_4 acids.

Further evidence against the operation of the succinate pathway came from studies with labelled propionate. According to this pathway it might be expected that $\text{C}_{(1)}$ of propionate would quickly give rise to carbon dioxide and, owing to the equivalence of pyruvate with acetate, there should be very little, if any, incorporation of $\text{C}_{(1)}$ into cell material. This is exactly what was found (Figs. 2 and 4). However, the operation of this hypothetical route would result in the formation of a symmetrical intermediate, succinic acid. This should cause both $\text{C}_{(2)}$ and $\text{C}_{(3)}$ of propionate to be incorporated into both $\text{C}_{(2)}$ and $\text{C}_{(3)}$ of pyruvate, i.e. complete randomization of $\text{C}_{(2)}$ and $\text{C}_{(3)}$ should occur. It should not therefore be possible to distinguish between $\text{C}_{(2)}$ and $\text{C}_{(3)}$ of propionate. This is obviously not so, either under conditions where there is a quantitative conversion of propionate into pyruvate, or under more normal conditions (Figs. 2 and 4). Hence, unless the organism can in some way distinguish between the two methylene groups of succinate, we must assume that the succinate pathway does not operate in *M. lwoffii*.

The operation of either the malonic semialdehyde pathway or the malonyl semialdehyde-CoA pathway is precluded in *M. lwoffii* under conditions when propionate is quantitatively converted into pyruvate. Under more normal conditions the malonyl semialdehyde-CoA pathway does not operate, for $\text{C}_{(3)}$ of propionate should be more rapidly converted into carbon dioxide than is $\text{C}_{(1)}$, and this does not

Table 3. *Distribution of ^{14}C in pyruvate produced by *M. lwoffi* from [^{14}C]propionate*

Propionate-grown washed cells (8 mg. dry wt.) were incubated at pH 7 and 30° in 6 ml. of phosphate buffer, with $60\mu\text{moles}$ of [^{14}C]propionate ($30\mu\text{C}$) and $6\mu\text{moles}$ of arsenite. After 90 min., pyruvate was isolated as the 2,4-DNP-hydrazine derivative. This was degraded by acid KMnO_4 to acetate and CO_2 and the percentages of the radioactivity appearing in these products were determined. Acetate was isolated by Celite chromatography and further degraded to CO_2 , after the addition of unlabelled carrier acetate, by the Schmidt degradation followed by the isolation and oxidation of the resulting methylamine sulphate. The percentages of the radioactivity in the acetate recovered as CO_2 at the end of each of these two stages were determined. The details are given in the Methods section. The samples were derived initially from incubation with: [$1\text{-}^{14}\text{C}$]propionate (a); [$2\text{-}^{14}\text{C}$]propionate (b); [$3\text{-}^{14}\text{C}$]propionate (c).

Sample	Method of degradation	% of radioactivity of sample recovered as CO_2		
		(a)	(b)	(c)
Pyruvate 2,4-DNP-hydrazone	Acid KMnO_4 oxidation	25.5	0.6	0.5
Acetate	Schmidt degradation	—	86	9
Acetate	Methylamine sulphate oxidation	—	3	92
		% of radioactivity of sample recovered as acetate		
		(a)	(b)	(c)
Pyruvate 2,4-DNP-hydrazone	Acid KMnO_4 oxidation	0	22	23

occur. The operation of the malonic semialdehyde pathway would allow a rapid conversion of $\text{C}_{(1)}$ of propionate into carbon dioxide, but then $\text{C}_{(2)}$ and $\text{C}_{(3)}$ of propionate should give rise to $\text{C}_{(1)}$ and $\text{C}_{(2)}$ of acetate respectively, so that the further metabolism of acetate would result in $\text{C}_{(3)}$ of propionate being converted more rapidly into carbon dioxide, and more slowly into cell material, than is $\text{C}_{(2)}$. Again this is not the result found, so neither of these two pathways operates in *M. lwoffi*.

The condensation of propionyl-CoA with glyoxylate to yield 2-hydroxyglutarate was first described in extracts from propionate-grown *E. coli* by Reeves & Ajl (1962). Later Reeves & Ajl (1963) described the breakdown of this to lactate and acetate and postulated that these reactions played a role in the growth of *E. coli* adapted to use propionate as sole source of carbon. Rabin *et al.* (1965) extended the possible intermediates in this hypothetical pathway and it was then possible to devise a pathway leading to the net conversion of propionate into pyruvate when the further metabolism of pyruvate was blocked (Scheme 1). Initially only $\text{C}_{(3)}$ of propionate would appear in the pyruvate, but later after further turns of the cycle $\text{C}_{(2)}$ and $\text{C}_{(1)}$ would appear, perhaps slightly diluted with ^{12}C but still in the same sequence as in the original propionate. There would be no randomization of $\text{C}_{(2)}$ and $\text{C}_{(3)}$ of propionate.

Under more normal conditions pyruvate would be oxidized to acetate either as a result of pyruvate oxidase activity or even by the more complicated pathway via glycerate suggested by Rabin *et al.*

(1965). However, it is unlikely that the only pathway for the formation of acetate would be via pyruvate. The formation of acetate during the degradation of citramalate would result in $\text{C}_{(1)}$ and $\text{C}_{(2)}$ of propionate being converted into $\text{C}_{(1)}$ and $\text{C}_{(2)}$ of acetate respectively. This might be expected to result in a slight lag in the conversion of $\text{C}_{(1)}$ of propionate into carbon dioxide and its more rapid incorporation into cell material when compared with what would happen if the sole pathway for acetate formation was via pyruvate, either through the 2-hydroxyglutarate pathway or perhaps by a more direct pathway such as via acrylate. However, it could be argued that the methods described here are not sufficiently sensitive to distinguish between these alternatives. Fortunately, however, it was found that when cells were incubated with propionate in the 'closed system' acetate accumulated in the suspension. If this was produced by the reactions described in Scheme 1 then a significant proportion of $\text{C}_{(1)}$ of propionate should have been found in the acetate. With [$1\text{-}^{14}\text{C}$]propionate only 5% of the radioactivity that remained in the supernatant was recovered in acetate whereas with [$3\text{-}^{14}\text{C}$]propionate 35% and with [$2\text{-}^{14}\text{C}$]propionate 40% of the remaining radioactivity was recovered in acetate. This behaviour was confirmed in the experiment in which [$1\text{-}^{14}\text{C}$]pyruvate was isolated. Hence, unless the organism has some means for distinguishing between acetate produced from pyruvate and acetate produced by the breakdown of citramalate, then the evidence from experiments with ^{14}C -labelled materials does

Scheme 1. Hypothetical pathway for the conversion of propionate into pyruvate via 2-hydroxyglutarate and a modified tricarboxylic acid cycle. The behaviour of the individual carbon atoms, C₁, C₂ and C₃, of propionate under conditions when the further metabolism of pyruvate is blocked is indicated.

not support the existence of a pathway for propionate metabolism in *M. lwoffi* that includes 2-hydroxyglutarate.

The evidence is entirely consistent with a more direct pathway for propionate metabolism, perhaps via acrylate. Cell-free extracts that oxidized propionate did not oxidize acrylate, so it is unlikely that free acrylate is a normal metabolic intermediate. However, metabolism of most fatty acids is preceded by some activating mechanism, generally for formation of a CoA derivative. The rapid loss of the ability to oxidize propionate during starvation and the inability of whole cells with negligible endogenous respiration to oxidize propionate certainly suggests that there is some unusually labile feature in the pathway for propionate oxidation in *M. lwoffi*. The inhibition of propionate utilization in an atmosphere containing appreciable quantities of carbon dioxide might also be caused by inactivation of this labile feature, perhaps as a result of the accumulation of acetate.

B. H. is indebted to Mrs Jacqueline R. Bennett for skilful technical assistance and to Miss Beryl Murphy for the electron microscopy.

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