A Direct Pathway for the Metabolism of Propionate in Cell Extracts from *Moraxella lwoffii*

By B. HODGSON AND J. D. MCGARRY*

Department of Biochemistry, University of Manchester Institute of Science and Technology

(Received 21 August 1967)

1. Extracts from *Moraxella lwoffii* oxidize propionate, but at a low rate when compared with whole cells. 2. This oxidative activity requires the formation of propionyl-CoA. 3. Enzymes catalysing the formation of propionyl phosphate and propionyl-CoA are present. The presence of a propionyl-CoA hydrolase is considered to be an artifact, but partly responsible for the low rates of oxidation. 4. Enzymes catalysing the reduction of NAD⁺ and the formation of pyruvate with propionyl-CoA as substrate are also present. 5. That the only pathway for the metabolism of propionate in extracts is a direct one to acetate via pyruvate was confirmed by the use of ¹⁴C-labelled materials. 6. A possible sequence of enzyme-catalysed reactions that will account for the experimental observations is described.

In the preceding paper (Hodgson & McGarry, 1968) on the pathway of propionate metabolism in *Moraxella lwoffii*, with whole cells, it was shown that only a pathway that allowed a direct formation of pyruvate with C₍₁₎, C₍₂₎ and C₍₃₎ of propionate yielding C₍₁₎, C₍₂₎ and C₍₃₎ of pyruvate respectively would account for all the experimental observations. Of the previously described pathways for the conversion of propionate into tricarboxylic acid-cycle intermediates, the only one that would suffice was the so-called 'acrylate pathway' that Mahler & Huennekens (1953) originally suggested occurred in rabbit liver, but with the slight modification that perhaps CoA derivatives were the actual intermediates. A study of the pathway of propionate oxidation as it occurs in cell-free extracts has been made in an attempt to determine the precise nature of the individual reactions concerned.

METHODS

Organism, cultural conditions and preparation of cell extracts. *Moraxella lwoffii* N.C.I.B. 8250 was maintained and cultured, and extracts were prepared from it, as described in the preceding paper (Hodgson & McGarry, 1968).

Enzyme assays. ATP-monocarboxylate phosphotransferase, e.g. EC 2.7.2.1, was measured with Dowex 2-treated extracts. The incubation mixture (10 ml.) at 30° contained 0.25 m-mole of tris buffer, pH 7.6, 0.2 m-mole of MgCl₂, 0.2 m-mole of ATP, 2.0 m-moles of neutralized hydroxylamine hydrochloride (equal volumes of 4.0 M-hydroxylamine

hydrochloride and 3.5 N-NaOH), 0.1 m-mole of substrate and about 20 mg. of protein. At intervals samples (1 ml.) were mixed with 3.0 ml. of FeCl₃ reagent (see below), protein was sedimented and *E*₅₄₀ of the supernatants was measured with a Unicam SP.600 spectrophotometer. Specific activities, expressed as μmoles of hydroxamate/mg. of protein/hr., were then calculated.

Acyl-CoA-orthophosphate acyltransferase was assayed by the arsenolysis method of Stadtman (1955) for acetyl-CoA-orthophosphate acetyltransferase (EC 2.3.1.8) and the method of Stadtman (1957b) to determine the concentration of acyl hydroxamate.

Acyl-CoA hydrolase with acyl-CoA-orthophosphate acyltransferase was measured with Dowex 2-treated extracts. Incubation mixture (10 ml.) at 30° contained 300 μmoles of tris buffer, pH 8, 100 μmoles of neutralized cysteine hydrochloride, 100 μmoles of acyl phosphate as substrate, 1 μmole of CoA where indicated and about 10 mg. of protein. At intervals over 30 min., samples (0.5 ml.) were mixed with 1.5 ml. of neutralized 1.3 M-hydroxylamine hydrochloride and the concentration of hydroxamate was subsequently determined. The activity of the enzyme tended to fall during the 30 min. incubation period, so specific activities, expressed as μmoles of hydroxamate utilized/mg. of protein/hr., were calculated from the initial rates.

Reduction of NAD⁺ by propionyl-CoA. Because of the presence of an active NADH oxidase in the extracts, it was necessary to carry out the reactions under strictly anaerobic conditions. Each silica cuvette contained, in a volume of 2.6 ml., tris buffer, NAD⁺ and substrate as described in the legend to Fig. 2. A stream of N₂ was bubbled through the solution for 2 min. with the aid of a fine Pasteur pipette. The pipette was then raised to a point just above the surface of the liquid, and the extract (0.4 ml.), previously gassed with a stream of N₂ for about 5 min., was run in. The lid of the cuvette was then placed in position, the contents were mixed and the cuvettes were placed in the Unicam

* Present address: Department of Internal Medicine, University of Texas Southwestern Medical School, Dallas, Tex. 75235, U.S.A.

SP.800 spectrophotometer. E_{340} was then measured continuously at room temperature.

Determination of hydroxamic acid concentration. This was measured essentially by the method described by Stadtman (1957b). Samples were mixed with 3 ml. of FeCl_3 reagent [equal volumes of 12% (w/v) trichloroacetic acid, 3N-HCl and 5% (w/v) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 0.1N-HCl]. Protein was sedimented and E_{540} of the supernatants was determined with a Unicam SP.600 spectrophotometer. Amounts of hydroxamate formed were calculated by referring to E_{540} with succinic anhydride as standard.

Identification of monocarboxylic acid hydroxamates. A 1 ml. portion of reaction mixture from the ATP-mono-carboxylate phosphotransferase assay system was mixed with 25 ml. of 95% (v/v) ethanol. Protein was sedimented and the supernatant was evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in 2.0 ml. of ethanol and applied to sheets of chromatography paper together with authentic samples of hydroxamates.

Spectrophotometric procedure for following keto acid production in extracts. This was essentially as described in the legend to Fig. 3. When lactate, propionate or succinate was substrate, the propionyl-CoA in cuvette (1) was replaced with 5 μ moles of the appropriate neutralized acid, 5 μ moles of ATP and 0.1 μ mole of CoA. When acrylyl-CoA, lactyl-CoA or succinyl-CoA was substrate, 0.5–1.5 μ moles were used in place of the propionyl-CoA. E_{324} was measured continuously with a Unicam SP.800 spectrophotometer.

Determination and identification of the keto acid produced during the oxidation of propionate by extracts. The determination was based on the method described by Olson (1959). An incubation mixture (3 ml.) at 30° contained 50 μ moles of tris buffer, pH 7.4, 10 μ moles of ATP, 0.6 μ mole of CoA, 1.2 mg. of NAD^+ , 10 μ moles of MgCl_2 , 20 μ moles of neutralized semicarbazide hydrochloride, 10 μ moles of substrate and 1.0 ml. of extract containing about 20 mg. of protein. Control incubations lacked substrate. After 70 min., 1.0 ml. of reaction mixture was added to 5.0 ml. of 10% (w/v) trichloroacetic acid solution at 0°. Protein was sedimented and 3.0 ml. samples of the supernatant were treated with 1.0 ml. of 2,4-DNP-hydrazine reagent (0.1% 2,4-DNP-hydrazine in 2N-HCl, filtered) for 20 min. The concentration of 2,4-DNP-hydrazone was determined by the method of Friedemann & Haugen (1943) with ethyl acetate as solvent.

For the identification of the keto acid the remainder of the assay solution above (2 ml.) was incubated for a further 120 min. and the protein was precipitated with 4 ml. of ice-cold 10% (w/v) trichloroacetic acid solution. Protein was then sedimented and 2 ml. of 2,4-DNP-hydrazine reagent was added. The 2,4-DNP-hydrazine derivatives were then prepared as described in the preceding paper (Hodgson & McGarry, 1968) and characterized by paper chromatography and by their absorption spectra in alkali (equal volumes of 10% Na_2CO_3 and 1.5N-NaOH) over the range 350–700 m μ .

Conversion of [^{14}C]propionate into [^{14}C]acetate and [^{14}C]succinate. Reactions were carried out at 30° in a total volume of 4.8 ml. containing 150 μ moles of tris buffer, pH 7.4, 15 μ moles of ATP, 2 μ moles of CoA, 1.5 mg. of NAD^+ , 15 μ moles of [^{14}C]propionate (15 μC) and 40 mg. of protein. After the mixture had been shaken for 60 min. in air, 1.5 ml. portions were withdrawn and mixed with either 3 ml. of ethanol standing at 65° or 1 ml. of 20% (v/v) H_2SO_4 and 1 ml. of 3.5% (w/v) sodium tungstate solution. In each case

protein was sedimented at 14000g for 10 min. and the sediment was washed once with either 1 ml. of 20% (v/v) ethanol or 2 ml. of water. The combined ethanol supernatants were then analysed directly by paper chromatography or by column chromatography with Celite. The other supernatants were brought to pH 2 with 10N-KOH and continuously extracted with ether for 24 hr. in a Quickfit liquid-liquid extractor. The ether was then evaporated under reduced pressure at room temperature. Residues were dissolved in either water for analysis by Celite chromatography or 50% (v/v) ethanol for analysis by paper chromatography.

Chromatography. Column chromatography with Celite for separating organic acids was carried out by the method of Swim & Krampitz (1954) as described by Hodgson & McGarry (1968). Fractions (10 ml.) were collected and acids were determined by titration with CO_2 -free 10mN-NaOH to the first permanent pink colour with phenol red indicator, while being continuously agitated with a stream of CO_2 -free N_2 . It was not possible to separate succinate from lactate.

Paper chromatography. Non-volatile organic acids were separated on Whatman no. 1 paper by using a descending technique with any of the following solvent systems: (i) the upper phase of pentan-1-ol-formic acid-water (40:25:1, by vol.) after it had been well mixed and kept for 36 hr.; (ii) propan-1-ol-aq. NH_3 (sp.gr. 0.88) (7:3, v/v); (iii) butan-1-ol-acetic acid-water (4:1:5, by vol.) After the papers had been dried at room temperature, acids were detected by dipping the papers in a solution of 0.2% bromocresol purple dissolved in a mixture of 40% (w/v) formalin (1 vol.) and ethanol (4 vol.), and then exposing the paper to NH_3 fumes. Acids appeared as yellow spots on a purple background.

Acid hydroxamates were separated on Whatman no. 4 paper by using a descending technique. The solvent was butan-1-ol-acetic acid-water (4:1:5, by vol.). After the solvent had been mixed and kept, the aqueous phase was used in the tank for pre-equilibration of the paper for 2 hr. before the organic phase was added to the trough. After the paper had been dried, acid hydroxamates were detected by spraying with a FeCl_3 reagent [50 g. of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 1 l. of 95% (v/v) ethanol in 0.1N-HCl]. Hydroxamates appeared as purple spots on a yellow background.

2,4-DNP-hydrazine derivatives of keto acids were separated on Whatman no. 1 paper with the solvent of Cavallini, Frontali & Toschi (1949), as modified by Dagley, Fewster & Happold (1952). 2,4-DNP-hydrazones appeared as yellow spots on a white background, but could be further characterized by the colour change observed when sheets were sprayed with 2% (w/v) KOH in aq. 90% (v/v) ethanol.

Miscellaneous methods. Consumption of O_2 was measured by conventional methods (Umbreit, Burris & Stauffer, 1957) in constant-volume respirometers at 30°. Flasks had a final volume of 3.2 ml., 0.2 ml. of which was 20% (w/v) KOH in the centre well. Substrates (25 μ moles unless otherwise stated) were initially present in the side arm and each flask had 10–25 mg. of protein dissolved in tris buffer, pH 7.4. Oxidation rates were calculated from maximum linear rates. Protein concentration was estimated by either the method of Stickland (1951) or the method of Gornall, Bardawill & David (1949) with crystallized bovine plasma albumin, dried over P_2O_5 , as standard.

Assay of ^{14}C -labelled compounds. ^{14}C -labelled compounds

were assayed as described by Hodgson & McGarry (1968). Radioautographs of chromatographs were prepared by standard techniques with Kodak Blue Brand X-ray film.

MATERIALS

Chemicals and buffer solutions. These were generally as described in the preceding paper (Hodgson & McGarry, 1968).

Miscellaneous materials. Authentic propionhydroxamate and acetoxyhydroxamate were prepared from ethyl propionate and ethyl acetate respectively by the method of Thompson (1951). Dilithium propionyl phosphate was synthesized from propionic anhydride by the method of Stadtman (1957a). Propionyl-CoA, acrylyl-CoA and succinyl-CoA were synthesized from the corresponding anhydrides by the method of Simon & Shemin (1953). Lactyl-CoA was prepared from lactylthiophenol by the method of Trams & Brady (1960). Sodium lactate was prepared from zinc lactate, which was dissolved in 2N-H₂SO₄ and continuously extracted with ether for 24 hr. in a Quickfit liquid-liquid extractor. The ether was removed by evaporation and the remaining acid was titrated with standard NaOH to determine its concentration.

Dilithium acetyl phosphate was purchased from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). The purity of this compound was estimated to be 71%.

RESULTS

Oxidation of organic acids by cell extracts. Extracts from cells growing exponentially with propionate were tested for their ability to oxidize various substrates. With no substrate, the oxygen uptake (μ l. of oxygen/mg. of protein/hr.) was 7.5, and this was not altered when either acrylate or 3-hydroxypropionate was present. With acetate, the value was 9; with 2-oxoglutarate (5 μ moles), propionate and lactate, 14; with 2-hydroxyglutarate (8 μ moles), 16.5; with succinate, 19; with pyruvate, 25. Although slightly higher rates of oxidation sometimes occurred with propionate as substrate this was always accompanied by a higher rate of endogenous respiration, e.g. in one experiment oxygen uptake with propionate as substrate was 23, but without substrate it was 14. Extracts from cells growing exponentially with acetate were also tested. With no substrate, oxygen uptake was 8; with propionate and acetate, 12.5; with succinate, 16; with lactate, 20. Rates of oxidation for those substrates tested were therefore comparable.

Although it might be expected that the initial reactions concerned with the oxidation of both propionate and acetate would involve the formation of CoA derivatives, the addition of CoA, ATP or NAD⁺ singly or together had no effect on the rates of oxidation observed with these extracts. A similar situation had previously been found with this organism when extracts from octanoate-grown cells were tested for their ability to oxidize octanoate

(Callely, Dagley & Hodgson, 1958). In this case a requirement for these cofactors was established by treatment of the extract with Dowex 2. Therefore an extract from propionate-grown cells was divided into two parts; one part was treated with Dowex 2 (Cl⁻ form) (Chantrenne & Lipmann, 1950), and both extracts were then adjusted with buffer to the same protein content (20 mg. of protein/ml.). The oxidation of propionate was then tested with and without the addition of cofactors (Fig. 1). Treatment with Dowex 2 considerably decreased the endogenous respiration from a value of 7.5 before treatment to a value of 4.5 μ l. of oxygen/mg. of protein/hr. after treatment, and also abolished the increase in the oxidation rate caused by the presence of propionate. Although ATP itself had no effect on the rate of propionate oxidation by Dowex 2-treated extracts, in the presence of both CoA and NAD⁺ an oxygen uptake of 14 μ l./mg. of protein/hr. was obtained, which was only slightly less than the initial rate for propionate oxidation by the normal extract (15 μ l. of oxygen/mg. of protein/hr.).

Formation of CoA derivatives of acetate and propionate. The activities of ATP-monocarboxylate phosphotransferase in extracts from both acetate- and propionate-grown cells were determined as described in the Methods section with acetate and propionate as substrates. The results are shown in Table 1. Although extracts from propionate-grown cells in each case had more activity than those from acetate-grown cells, with both extracts hydroxamate was produced more readily with acetate than with propionate as substrate. The products of the reaction with propionate and acetate were identified, by paper chromatography, as propionhydroxamate and acetoxyhydroxamate respectively, with authentic samples of the two hydroxamates as standards. As the formation of these hydroxamates occurred in the absence of added CoA and was dependent on the presence of ATP, it was assumed that the products of the reaction were propionyl phosphate and acetyl phosphate. It was to be expected then that there would be present an enzyme system for the transfer of the acyl group from the acyl phosphate to CoA to form the acyl-CoA. Dowex 2-treated extracts were therefore tested for the presence of acyl-CoA-orthophosphate acyltransferase as described in the Methods section (Table 2). Each extract catalysed the arsenolysis of acetyl phosphate and propionyl phosphate at the same rate, but the extracts from propionate-grown cells were about twice as active as those from acetate-grown cells. In each case the specific activity was very similar to that of the ATP-monocarboxylate phosphotransferase. This was surprising, for Hodgson (1959) had found that, in extracts from *M. lwoffi*, hydrolysis of acetyl phosphate occurred without arsenate and this

Fig. 1. Oxidation of propionate by normal or Dowex 2-treated extracts of *M. lwoffi* with or without cofactors. Each incubation mixture contained 20 mg. of protein in 3 ml. of phosphate buffer, pH 7, with, where indicated, propionate (40 μ moles), ATP (5 μ moles), CoA (0.4 μ mole) and NAD⁺ (0.8 mg.). ●, Normal extract + propionate; ○, Dowex 2-treated extract + propionate + ATP + CoA + NAD⁺; ▲, Dowex 2-treated extract + propionate + ATP + CoA; Δ, normal extract; ■, Dowex 2-treated extract; □, Dowex 2-treated extract + propionate + ATP and Dowex 2-treated extract + propionate.

deacylase activity might have been expected to contribute to the observed activities of acyl-CoA-orthophosphate acyltransferase. Accordingly extracts were assayed for acyl-CoA hydrolase as described in the Methods section. These results are shown in Table 3. Hydrolysis of the acyl phosphates was again dependent on the presence of CoA and in fact the rate of hydrolysis was dependent on the concentration of CoA. Neither in this assay nor in that for the acyl-CoA-orthophosphate acyltransferase were conditions chosen so that enzymes were saturated with respect to CoA. Hence in each case maximum activity may have been higher than the recorded values. With each extract, the rate of hydrolysis of propionyl phosphate was about twice that of acetyl phosphate, but the two extracts differed very little in their ability to hydrolyse acetyl phosphate. The rates of hydrolysis were not significantly altered by anaerobic conditions, nor

Table 1. *ATP-monocarboxylate phosphotransferase activities in extracts from M. lwoffi grown with either acetate or propionate*

Incubation mixtures (10 ml.) at 30° contained 0.1 m-mole of substrate, 0.25 m-mole of tris buffer, pH 7.6, 0.2 m-mole of MgCl₂, 0.2 m-mole of ATP, 2.0 m-moles of neutralized hydroxylamine hydrochloride and about 20 mg. of protein from Dowex 2-treated cell extract. Portions (1 ml.) were removed at various times and *E*₅₄₀ was determined with FeCl₃ reagent as described in the Methods section.

Assay substrate	Growth substrate	Enzyme activity (μ moles of hydroxamate/mg. of protein/hr.)
Acetate + ATP	Propionate	4.0
Acetate + ATP	Acetate	3.2
Propionate + ATP	Propionate	2.3
Propionate + ATP	Acetate	1.3
Acetate	Propionate	0
Propionate	Acetate	0

Table 2. *Acyl-CoA-orthophosphate acyltransferase activities in extracts from M. lwoffi grown with either acetate or propionate*

Incubation mixtures (1 ml.) at 30° contained 10 μ moles of tris buffer, pH 8.0, 10 μ moles of neutralized cysteine hydrochloride, 6 μ moles of substrate (either dilithium propionyl phosphate or dilithium acetyl phosphate), 0.2 μ mole of CoA where indicated and about 1 mg. of protein from Dowex 2-treated extract. After 13 min. 50 μ moles (0.1 ml.) of potassium arsenate, pH 8, were added and 15 min. later 0.5 ml. of neutralized 2 M-hydroxylamine hydrochloride was added. After a further 10 min. the amount of hydroxamate present was determined as described in the Methods section.

Assay substrate	Growth substrate	Cofactor	Enzyme activity (μ moles of hydroxamate lost/mg. of protein/hr.)	
			Acetyl phosphate	Propionyl phosphate
Propionate		CoA	4.6	4.7
Propionate		None	0.4	0.4
Acetate		CoA	2.4	2.9
Acetate		None	0.3	0.4

when extracts had been previously dialysed against water for 18 hr. at 4°.

Effect of glyoxylate on the rate of loss of acyl phosphates from extracts. The rate of loss of acyl phosphate was determined from incubation mixtures (5 ml.) at 30° with 150 μ moles of tris buffer, pH 8.0, 50 μ moles of acyl phosphate, 0.3 μ mole of CoA, 5 μ moles of GSH, 50 μ moles of either gly-

Table 3. *Acyl-CoA hydrolase with acyl-CoA-orthophosphate acyltransferase activities in extracts from M. lwoffi grown with acetate or propionate*

Incubation mixtures (10 ml.) at 30° contained 0.3 m-mole of tris buffer, pH 8.0, 0.1 m-mole of neutralized cysteine hydrochloride, 0.1 m-mole of acyl phosphate, 1 μ mole of CoA where indicated and about 10 mg. of protein from Dowex 2-treated extract. Portions (0.5 ml.) were removed at intervals and treated with neutralized hydroxylamine hydrochloride and FeCl₃ reagent as described in the Methods section.

Assay substrate		Enzyme activity (μ moles of hydroxamate lost/mg. of protein/hr.)	
Growth substrate	Cofactor	Acetyl phosphate	Propionyl phosphate
Propionate	CoA	12.5	23.0
Propionate	None	0	—
Acetate	CoA	11.0	22.0
Acetate	None	—	0

oxylate or DL-isocitrate and about 9 mg. of protein from untreated extract from propionate-grown cells. Assay of samples (0.5 ml.) by the hydroxamic acid method showed that, although both glyoxylate and DL-isocitrate stimulated the removal of acetyl phosphate, they both had a slight inhibitory effect on the rate of removal of propionyl phosphate.

Reduction of NAD⁺ with propionyl-CoA. A stimulation of oxygen uptake by NAD⁺ was observed in Dowex 2-treated extracts with propionate as substrate (Fig. 1). As these extracts contained a very active NADH oxidase it was thought that, under anaerobic conditions, they might reduce NAD⁺ with propionate as substrate. Such a reaction was shown to take place when propionyl-CoA (or propionate + ATP + CoA) was added to a mixture of the extract plus NAD⁺ held under an atmosphere of nitrogen. Under similar conditions NADP⁺ was not reduced and lactate, succinate, lactyl-CoA, acrylyl-CoA and succinyl-CoA would not reduce NAD⁺. Although from ϵ_{340} for NADH it was obvious that all the NAD⁺ in the cuvette was not being reduced, further additions of propionyl-CoA or NAD⁺ failed to increase the amount of NADH produced (Fig. 2). However, when the cuvette was opened to the air, stirred and then returned to anaerobic conditions, reduction of NAD⁺ occurred again, but at a slightly decreased rate compared with that found at the outset, owing perhaps to the presence of dissolved oxygen. Probably an inhibitor of NAD⁺ reduction accumulated under the conditions of the assay and was removed in the presence of air. Accordingly the reduction of NAD⁺ by propionyl-CoA was allowed

Fig. 2. Effect of further additions of propionyl-CoA, NAD⁺ and air on the reduction of NAD⁺. Initially the cuvette in an atmosphere of N₂ gas contained, in a volume of 3 ml., 100 μ moles of tris buffer, pH 7.4, 2 mg. of NAD⁺, 1.5 μ moles of propionyl-CoA and 0.4 ml. of extract (8 mg. of protein). E_{340} was measured continuously, 0.1 ml. (0.5 μ mole) of propionyl-CoA was added at the time indicated by the arrow marked *a* and 0.1 ml. (0.5 mg.) of NAD⁺ at the arrow marked *b*. At the arrow marked *c* the cuvette was opened, quickly stirred and then returned to anaerobic conditions.

to proceed to completion in a cuvette under nitrogen. At this point it was calculated from ϵ_{340} for NADH that about 0.5 μ mole of NADH was present. The lid of the cuvette was removed and, with a stream of nitrogen blowing over the surface of the liquid, 0.03 ml. of oxaloacetate (0.45 μ mole) was added. NADH was immediately almost completely oxidized, presumably because of the presence of a very active malate dehydrogenase (EC 1.1.1.37) previously shown to be present in the extract; but then NAD⁺ was again reduced.

After dialysis against water for 15 hr. at 4°, extracts that had actively reduced NAD⁺ in the presence of propionyl-CoA now failed to do so. Extracts prepared from cells harvested in the post-exponential phase of growth with propionate did not catalyse the reduction of NAD⁺ with propionyl-CoA.

Conversion of propionate into pyruvate. In the presence of a suitable concentration of arsenite, *M. lwoffi* converted propionate quantitatively into pyruvate (Hodgson & McGarry, 1968). Under normal conditions the oxidation rate for extracts with pyruvate as substrate was almost twice that with propionate as substrate. Should pyruvate be produced during the oxidation of propionate it would not accumulate unless its further metabolism was selectively blocked. Arsenite at 1 mM inhibited the oxygen uptake by extracts with propionate as substrate by 70%, and with pyruvate as substrate by 85%. It was unlikely therefore that arsenite would be a sufficiently selective inhibitor.

If pyruvate was the first keto acid intermediate of

propionate oxidation it should be possible to follow its production continuously by using phenylhydrazine in the same way that it is used to measure the formation of glyoxylate from isocitrate in the isocitrate lyase assay (Dixon & Kornberg, 1959). An increase in E_{324} was observed when extracts were incubated as described in the legend to Fig. 3, with phenylhydrazine and NAD^+ . The rate of increase was significantly greater when, in addition, $0.5 \mu\text{mole}$ of propionyl-CoA was present, and with $1.5 \mu\text{moles}$ this rate increased further (Fig. 3). Although in this assay, propionate + ATP + CoA was an effective substitute for propionyl-CoA, lactate, succinate, acrylyl-CoA, lactyl-CoA and succinyl-CoA were not.

Determination of the keto acid produced with propionate + ATP + CoA as substrate and semicarbazide as trapping agent instead of phenylhydrazine showed that $1.24 \mu\text{moles}$ were produced in 1 hr. from $10 \mu\text{moles}$ of propionate. Under the same conditions, lactate did not give rise to keto acid. The 2,4-DNP-hydrazine derivatives were then prepared from an incubation with propionate and semicarbazide. Paper chromatography of these derivatives showed the presence of two 2,4-DNP-hydrazones that had R_F values identical with those of the two 2,4-DNP-hydrazine derivatives of genuine pyruvate. A comparison of the spectrum of the prepared derivatives in alkali with that of genuine pyruvate 2,4-DNP-hydrazone showed them to be identical over the range $350\text{--}700 \text{ m}\mu$.

Conversion of [^{14}C]propionate into ^{14}C -labelled intermediates associated with the tricarboxylic acid cycle. When *M. lwoffi* metabolized [^{14}C]propionate it was found that the concentrations of the metabolic intermediates within the cells were very low (Hodgson & McGarry, 1968). With extracts, intermediary metabolites might accumulate as a result of the dilution of the individual enzymes. With ^{14}C -labelled substrates it should be possible to trap ^{14}C -labelled metabolic intermediates by adding a pool of unlabelled compound to the incubation mixtures. Extracts from propionate-grown cells were incubated with [$3\text{-}^{14}\text{C}$]propionate, with and without either $15 \mu\text{moles}$ of DL-lactate or $30 \mu\text{moles}$ of DL-2-hydroxyglutarate. Reactions were stopped after 1 hr. by pouring the mixtures into ethanol at 65° , and, after the protein had been sedimented, the supernatants were prepared for column chromatography in the presence of $10 \mu\text{moles}$ of propionate, acetate, pyruvate and lactate. The percentage of the initial radioactivity that was recovered in each fraction was then determined. Details are given in the Methods section. With [$3\text{-}^{14}\text{C}$]propionate alone 33% of the initial radioactivity was recovered in the fraction that contained propionate, 36% in the fraction that contained acetate and 1.5% in the fraction that con-

Fig. 3. Formation of a keto acid during the incubation of an extract from propionate-grown cells with propionyl-CoA. All cuvettes contained, in a final volume of 3.0 ml. , $100 \mu\text{moles}$ of tris buffer, pH 7.4, $10 \mu\text{moles}$ of neutralized phenylhydrazine hydrochloride and 0.2 ml. of extract (4 mg. of protein). In addition cuvette (1) contained $1.5 \mu\text{moles}$ of propionyl CoA and 2 mg. of NAD^+ ; cuvette (2) contained $0.5 \mu\text{mole}$ of propionyl-CoA and 2 mg. of NAD^+ ; cuvette (3) contained $1 \mu\text{mole}$ of propionyl-CoA; cuvette (4) contained 2 mg. of NAD^+ . E_{324} was measured continuously.

tained lactate (Fig. 4). In the presence of a pool of unlabelled lactate, 5% of the initial radioactivity in propionate was recovered in the fraction containing lactate, and with unlabelled 2-hydroxyglutarate 33% in this fraction. With this type of column, lactate and succinate are eluted in the same fractions. Portions of the ethanol supernatants were therefore subjected to paper chromatography in three solvent systems (see the Methods section), with succinate and lactate as standards. When the positions of the ^{14}C -labelled compounds were revealed by radioautography and these were compared with the positions of acids revealed on the chromatograms by spraying with indicator, it was established that the major ^{14}C -labelled material was in each case identical with succinate. Although other ^{14}C -labelled compounds were present, these contained much less ^{14}C and none had an R_F value that corresponded in all solvents to that of either lactate or 2-hydroxyglutarate.

Fig. 4. Separation on a Celite column of ^{14}C -labelled acids produced during the metabolism of $[3\text{-}^{14}\text{C}]\text{propionate}$ by cell extracts of *M. lwoffi*. The reaction was carried out at 30° in a total volume of 4.8 ml. containing $15\text{ }\mu\text{moles}$ of this buffer, pH 7.4, $15\text{ }\mu\text{moles}$ of ATP, $2\text{ }\mu\text{moles}$ of CoA, 1.5 mg. of NAD^+ , $15\text{ }\mu\text{moles}$ of $[3\text{-}^{14}\text{C}]\text{propionate}$ ($15\text{ }\mu\text{C}$) and 40 mg. of protein. After 60 min. 1.5 ml. was mixed with 3 ml. of ethanol standing at 65° . The protein was sedimented and washed with 1 ml. of 20% (v/v) ethanol. A portion of the combined supernatant fluid was separated on a 7 g. Celite column with $10\text{ }\mu\text{moles}$ each of acetate, lactate, propionate and pyruvate as standards. Fractions (10 ml.) were titrated with CO_2 -free 0.01 N-NaOH, and after the aqueous phase had been adjusted to a volume of 2 ml. with 0.01 N-NaOH the ^{14}C content was determined. Acetate was eluted in fractions 6, 7, 8, 9 and 10; lactate in fractions 18, 19, 20 and 21; propionate in fractions 3, 4 and 5; pyruvate in fractions 10, 11, 12, 13 and 14. Δ , % of radioactivity of the original propionate recovered in each fraction; O, vol. of 10 mN-NaOH required for neutralizing the fraction.

With $[3\text{-}^{14}\text{C}]\text{propionate}$, then, the major ^{14}C -labelled products of metabolism by extracts were acetate and succinate. When extracts were incubated under similar conditions but with $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]\text{-propionate}$ as substrate, paper chromatography of ethereal extracts of deproteinized reaction fluids with the butan-1-ol-acetic acid-water solvent followed by radioautography showed that $[^{14}\text{C}]\text{succinate}$ was produced only from $[2\text{-}^{14}\text{C}]\text{propionate}$. Neither $[^{14}\text{C}]\text{succinate}$ nor any other ^{14}C -labelled compound was detected with the $[1\text{-}^{14}\text{C}]\text{propionate}$ sample.

It seemed probable that $[^{14}\text{C}]\text{propionate}$ gave

rise to $[^{14}\text{C}]\text{succinate}$ after first being oxidized to $[^{14}\text{C}]\text{acetate}$ by a pathway that involved the loss of $\text{C}_{(1)}$. To test this suggestion more fully extracts were incubated with equal quantities of $[3\text{-}^{14}\text{C}]\text{-propionate}$ or $[2\text{-}^{14}\text{C}]\text{acetate}$, either alone or together with either oxaloacetate or pyruvate, under both aerobic and anaerobic conditions for 90 min. Reactions were terminated with acid tungstate, non-volatile carboxylic acids were extracted with ether, the radioactivities were determined and then the acids were separated on Celite columns. The percentages of the initial radioactivity of propionate that were recovered in the ethereal solution and in each fraction from the column were then calculated. The results in Table 4 show the percentages of initial radioactivity recovered in the ethereal solution and in the fractions that contained succinate. $[^{14}\text{C}]\text{-Malate}$ was also present in each sample, accounting for 7% of the initial radioactivity in the sample incubated with oxaloacetate and propionate under anaerobic conditions, but considerably less than this in all other samples. Fractions from the column that contained citramalate, citrate, 2-hydroxyglutarate, methylmalonate, 2-oxoglutarate or pyruvate did not contain ^{14}C -labelled material.

DISCUSSION

The oxidative capacity of extracts was found to be very low compared with that of whole cells (see Hodgson & McGarry, 1968). Extracts from propionate-grown cells oxidized propionate at a rate of $6.5\text{ }\mu\text{l.}$ of oxygen/mg. of protein/hr. (corrected for endogenous respiration). This is approximately equivalent to an uptake of $0.25\text{ }\mu\text{mole}$ of oxygen/mg. of protein/hr. The rate of formation of propionyl phosphate in the same extracts was $2.3\text{ }\mu\text{moles/mg.}$ of protein/hr. and the rate of formation of propionyl-CoA from propionyl phosphate was even higher. It would seem, therefore, that in extracts the rate-limiting reaction of propionate oxidation occurs after the formation of propionyl-CoA. It could be that the presence of the acyl-CoA hydrolase limits the supply of propionyl-CoA to the oxidative enzymes. In fact the rate of the acyl-CoA hydrolase with acyl-CoA-orthophosphate acyltransferase (Table 3) appears to be greater than the rate of acyl-CoA-orthophosphate acyltransferase (Table 2) measured independently. This is obviously not possible for the rate of the latter must be at least equal to that of the former. Presumably the different conditions must account for this discrepancy and we must assume that the assay method of Stadtman (1955) does not necessarily measure the maximum activity of the acyl-CoA-orthophosphate acyltransferase. The presence of this acyl-CoA hydrolase *in vivo* would be nonsensical in terms of energy conservation by the cell, so we must

Table 4. Conversion of [3-¹⁴C]propionate into succinate and other non-volatile organic acids

Each incubation mixture (1.5 ml.) at 30° in 25 ml. conical flasks contained 25 μ moles of tris buffer, pH 7.4, 5 μ moles of ATP, 0.25 μ mole of CoA, 0.6 mg. of NAD⁺ and 10 mg. of protein. Flasks 1, 2 and 3 contained [3-¹⁴C]propionate (5 μ C, 5 μ moles), and flasks 4, 5 and 6 [2-¹⁴C]acetate (5 μ C, 5 μ moles). Sodium oxaloacetate (10 μ moles) was added to flasks 2 and 5 and sodium pyruvate (10 μ moles) to flasks 3 and 6. In each case the reaction was started by adding the extract. One duplicate set of flasks was gassed with N₂ before the addition of extract and then they were tightly stoppered during the 90 min. incubation. Reactions were terminated by adding H₂SO₄-sodium tungstate and the deproteinized reaction fluids were analysed for ¹⁴C-labelled organic acids by ether extraction. The distribution of ¹⁴C between the various organic acids was determined by Celite chromatography. P, [3-¹⁴C]Propionate; A, [2-¹⁴C]acetate; OA, sodium oxaloacetate; Py, sodium pyruvate; —, no addition.

Incubation						Air						N ₂					
Flask no.	...	...	...	...	...	1	2	3	4	5	6	1	2	3	4	5	6
Substrate	...	...	...	...	...	P	P	P	A	A	A	P	P	P	A	A	A
Addition	...	...	...	...	...	—	OA	Py	—	OA	Py	—	OA	Py	—	OA	Py
% of initial ¹⁴ C in ether extract						7	30	11	38	54	38	16	27	17	34	48	25
% of initial ¹⁴ C in succinate						4	19	5	20	30	19	1	10	1	15	20	7

assume that its occurrence *in vitro* is an artifact created by the extraction procedure.

Propionyl-CoA, as well as being a substrate for the deacylase, is also a substrate for a reaction that results in the reduction of NAD⁺ and also the formation of pyruvate. Although no attempt was made to measure the stoichiometry of NADH and pyruvate formation, it is reasonable to assume that at least 1 mole of NADH will be produced during the conversion of 1 mole of propionyl-CoA into pyruvate. A reversal of the pathway, such as is postulated to occur from lactate to propionate in *Peptostreptococcus elsdenii* (Ladd, 1959), together with an NAD-linked lactate dehydrogenase, could account for some of the observations. However, acrylyl-CoA, lactate and lactyl-CoA do not act as substrates for NADH production nor do they result in the formation of pyruvate under conditions where propionyl-CoA does. We have assumed that perhaps acyl-enzyme derivatives are the true substrates for the reactions. Such derivatives are known to be involved in the biosynthesis of fatty acids (Vagelos, 1964) and, even though Baldwin, Wood & Emery (1965) managed to purify most of the enzymes of the *Peptostreptococcus elsdenii* system, they failed to demonstrate a net conversion of lactyl-CoA into propionate in a synthetic system consisting of the isolated enzymes.

One noteworthy finding was the failure of the propionyl-CoA to reduce the NAD⁺ completely, even after the addition of more propionyl-CoA. This inhibition was removed by stirring the incubation mixture in air. The first step in the oxidation of acyl-CoA derivatives in the fatty acid spiral is an FAD-linked acyl-CoA dehydrogenase. The conversion of succinate into fumarate is also catalysed by an FAD-linked oxidoreductase, so, if acrylyl-enzyme was the first oxidized intermediate, then

we might expect an FAD-linked oxidoreductase to be involved. Under anaerobic conditions it is possible that conversion of propionate into pyruvate was limited by the availability of FAD, and that when the incubation mixture was stirred in air more FAD was made available as well as the NADH being oxidized. To test this possibility, the concentration of NADH was selectively decreased by adding oxaloacetate under anaerobic conditions. As NAD⁺ was then reduced again, the concentration of FAD is unlikely to be a rate-limiting factor. It is also possible that both oxidation-reduction reactions involved in the conversion of propionate into pyruvate are NAD-linked. Either the first of these or both are inhibited by the presence of NADH at a concentration of about 0.2 mM with 8 mg. of protein in the crude extract. Baldwin *et al.* (1965) found that the acyl-CoA dehydrogenase present in extracts of *Peptostreptococcus elsdenii* was stimulated by NAD⁺.

However these results are interpreted, this work does establish that a pathway from propionate to pyruvate does exist in extracts and that at least one NAD-linked reaction is involved. It was still possible that other pathways existed, and it was hoped that these might be revealed with ¹⁴C-labelled materials. The finding that glyoxylate did not stimulate the removal of propionyl phosphate from extracts, while at the same time it did stimulate the removal of acetyl phosphate, ruled out any significant formation of 2-hydroxyglutarate, and this was adequately confirmed by adding a pool of unlabelled 2-hydroxyglutarate to extracts metabolizing [¹⁴C]-propionate. No radioactive 2-hydroxyglutarate was isolated. However, there was a considerable accumulation of [¹⁴C]succinate; in fact, 33% of the initial radioactivity was recovered in this fraction. Initially this finding seemed difficult to explain in

terms of the direct pathway of propionate oxidation. 2-Hydroxyglutarate and 2-oxoglutarate were oxidized by extracts at an initial rate that was higher than the final rate. With 2-oxoglutarate the rate seemed to change when a sufficient quantity of oxygen to convert all the 2-oxoglutarate into succinate had been utilized. It seemed that the rate of succinate oxidation in extracts was rate-limiting, so it was not improbable that succinate did accumulate. However, why did [^{14}C]succinate accumulate, when we had started with [^{14}C]propionate?

With the 'direct' pathway, propionate would be first converted into pyruvate and then into acetate, before producing succinate via citrate and isocitrate. Oxaloacetate would be required for the formation of citrate. With the 'succinate' pathway, the formation of [^{14}C]succinate would simply require a source of active carboxyl groups such as found in oxaloacetate. There would be no oxidation reactions involved and all three carbon atoms of pyruvate would give rise to the carbon atoms of succinate. Under both anaerobic and aerobic conditions, very little of the ^{14}C of propionate appeared in succinate unless oxaloacetate was present and, although both [2- ^{14}C]- and [3- ^{14}C]-propionate gave rise to [^{14}C]succinate, [1- ^{14}C]-propionate did not. A considerable proportion of ^{14}C from acetate was incorporated into succinate under all conditions tested, but the stimulation of incorporation resulting from the presence of oxaloacetate was very similar to that observed with propionate, i.e. about a 10% increase in the incorporation of initial radioactivity (Table 4). In addition, in one experiment without oxaloacetate with [3- ^{14}C]propionate, 44% of the initial radioactivity was recovered in acetate.

Under neither aerobic nor anaerobic conditions did pyruvate have any effect on the incorporation of ^{14}C into the ether-soluble materials. A stimulation of incorporation was not expected, but on the other hand the oxidation of pyruvate to acetate might have been expected to decrease the incorporation of [^{14}C]propionate and acetate. Presumably, then, the added pyruvate was not metabolized.

The stimulation of incorporation of [^{14}C]propionate into succinate by oxaloacetate under anaerobic conditions can be readily explained by the oxaloacetate acting as an acceptor of electrons from NADH produced during the conversion of propionate into acetate. In fact, when the samples from the anaerobic incubations were analysed by column chromatography, as well as a fair proportion of the ^{14}C of propionate being incorporated into malate a reasonable titre with sodium hydroxide was obtained, in spite of the fact that no carrier malate was added. This result supports the contention that only NAD-linked oxidation-

reduction reactions are involved in the conversion of propionate into acetate by extracts.

As a consequence of these results and those obtained previously, we postulate that the pathway of propionate metabolism in *M. lwoffi* proceeds by the following reactions:

Enzymes for the reactions (1), (2), (8), (9), (10), (11), (12), (13) and (14) have been demonstrated in extracts of *M. lwoffi*.

Reactions (1) to (7) inclusive account for the findings that propionate + ATP + CoA will reduce

NAD⁺ and give rise to pyruvate whereas compounds such as acrylate, lactate, succinate, acrylyl-CoA, lactyl-CoA and succinyl-CoA will not. The oxidation of pyruvate by reaction (8) explains why pyruvate will not accumulate in extracts metabolizing propionate unless a trapping agent such as semicarbazide or phenylhydrazine is present. At the same time reaction (8) in conjunction with reactions (1) to (7) will account for the conversion of propionate into acetate. It is possible to explain how, in an atmosphere of either air or nitrogen, propionate will give rise to both acetate and succinate. For example, if we assume that the rate of conversion of propionate into pyruvate is three times the rate of the conversion of acetyl-CoA into citrate (reaction 9) and also that acetate can be readily produced from acetyl-CoA by a reversal of the reactions (2) and (1), then a combination of reactions (1) to (13) could result in the overall reaction: 3 propionate + 2 ATP + 8 H₂O + 10 NAD⁺ → 1 acetate + 1 succinate + 3 CO₂ + 2 ADP + 2 P_i + 10 NADH. Under aerobic conditions the NADH would be rapidly oxidized by reaction (14), whereas under anaerobic conditions it would be necessary to add oxaloacetate to reoxidize the NADH by a reversal of reaction (13). Under anaerobic conditions with oxaloacetate, the pool of unlabelled malate might be expected to trap some of the ¹⁴C derived from either [2-¹⁴C]- or [3-¹⁴C]-propionate. The presence of the acyl-CoA hydrolase would slightly complicate the picture and lead to a more extensive conversion of propionate into acetate than into succinate, and again this is what was found in the extracts. However, *in vivo* the activity of this enzyme must be carefully controlled or not present, so it has not been included in the overall mechanism.

The results in this paper were presented to the University of Manchester by J.D.McG. as part of a Thesis for the degree of Ph.D. (McGarry, 1965).

REFERENCES

- Baldwin, R. L., Wood, W. A. & Emery, R. S. (1965). *Biochim. biophys. Acta*, **97**, 202.
- Callely, A. G., Dagley, S. & Hodgson, B. (1958). *Biochem. J.* **69**, 173.
- Cavallini, D., Frontali, N. & Toschi, G. (1949). *Nature, Lond.*, **163**, 568.
- Chantrenne, H. & Lipmann, F. (1950). *J. biol. Chem.* **187**, 757.
- Dagley, S., Fewster, M. E. & Happold, F. C. (1952). *J. gen. Microbiol.* **8**, 1.
- Dixon, G. H. & Kornberg, H. L. (1959). *Biochem. J.* **72**, 3 p.
- Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Hodgson, B. (1959). Ph.D. Thesis: University of Leeds.
- Hodgson, B. & McGarry, J. D. (1968). *Biochem. J.* **107**, 7.
- Ladd, J. N. (1959). *Biochem. J.* **71**, 16.
- McGarry, J. D. (1965). Ph.D. Thesis: University of Manchester.
- Mahler, H. R. & Huennekens, F. M. (1953). *Biochim. biophys. Acta*, **11**, 575.
- Olson, J. A. (1959). *J. biol. Chem.* **234**, 5.
- Simon, E. J. & Shemin, D. (1953). *J. Amer. chem. Soc.* **75**, 2520.
- Stadtman, E. R. (1955). In *Methods in Enzymology*, vol. 1, p. 596. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Stadtman, E. R. (1957a). In *Methods in Enzymology*, vol. 3, p. 228. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Stadtman, E. R. (1957b). In *Methods in Enzymology*, vol. 3, p. 231. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Stickland, L. H. (1951). *J. gen. Microbiol.* **5**, 698.
- Swim, H. E. & Krampitz, L. O. (1954). *J. Bact.* **67**, 419.
- Thompson, A. R. (1951). *Aust. J. sci. Res. B*, **4**, 180.
- Trams, E. G. & Brady, R. O. (1960). *J. Amer. chem. Soc.* **82**, 2972.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques*, 3rd ed., pp. 1-17. Minneapolis: Burgess Publishing Co.
- Vagelos, P. R. (1964). *Annu. Rev. Biochem.* **33**, 139.