Enzymic Studies on the Biosynthesis of Amino Acids from Lactate by *Peptostreptococcus elsdenii*

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Cell-free extracts of *Peptostreptococcus elsdenii*, a strict anaerobe from the rumen, were examined for enzymes catalysing the steps in the biosynthesis from lactate of alanine, serine, aspartate and glutamate. Extracts contain the enzymes necessary for the formation of alanine from lactate via pyruvate. The presence of enzymes catalysing the interconversion of phosphoglycerate and phosphohydroxypyruvate, the transamination of the latter to phosphoserine and the cleavage of phosphoserine to serine and inorganic phosphate was demonstrated, suggesting that serine is formed via these intermediates. 'Malic' enzyme, malate dehydrogenase and glutamate-oxaloacetate transaminase are present in extracts and could account for aspartate formation. The extracts catalyse all of the steps of the tricarboxylic acid pathway leading from oxaloacetate plus acetate to glutamate. Together with substantive data from previous radioactive tracer studies the results provide strong evidence that these four amino acids are synthesized in this strict anaerobe by pathways closely similar to those operating in aerobic and facultatively aerobic organisms.

It has been reported (Somerville & Peel, 1964, 1967) that the strictly anaerobic rumen bacterium *Peptostreptococcus elsdenii* will incorporate radioactivity from ^{14}C -labelled sodium lactate into several amino acids when grown on a medium containing yeast extract. Alanine, serine, aspartate and glutamate were isolated and degraded to determine the distribution of radioactivity from both $[1-^{14}\text{C}]$ - and $[2-^{14}\text{C}]$ -lactate.

The results suggested that alanine and serine were synthesized directly from lactate by pathways that preserved the carbon chain of lactate. The distribution of radioactivity within aspartate indicated its formation by condensation of a C_3 unit with a C_1 unit derived from the lactate carboxyl group. The labelling pattern in glutamate was consistent with its formation by the tricarboxylic acid route involving the sequence pyruvate $\rightarrow$ oxaloacetate + acetate $\rightarrow$ citrate $\rightarrow$ isocitrate $\rightarrow$ 2-oxoglutarate $\rightarrow$ glutamate. An alternative pathway of glutamate formation was postulated, involving glutaconate as an intermediate and the reaction sequence pyruvate $\rightarrow$ acetyl-CoA $\rightarrow$ acetoacetyl-CoA $\rightarrow$ crotonyl-CoA $\rightarrow$ glutaconate $\rightarrow$ glutamate. The present paper reports enzymic investigations into the biosynthesis of these four amino acids in *P. elsdenii*. Some of the results have

been presented in preliminary form (Somerville, 1965).

METHODS AND MATERIALS

Maintenance and growth of micro-organisms. P. elsdenii was maintained as described by Somerville & Peel (1967). Two media were used for the growth of larger cultures (0.5–3 l.). Medium A contained: 1% (v/v) m-phosphate buffer ($\text{KH}_2\text{PO}_4\text{--KOH}$), pH 6.8, 0.4% Difco yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.), 0.05% NH_4Cl , 0.03% thioglycollic acid and 1.4% (w/v) sodium lactate (obtained as a 70% solution from British Drug Houses Ltd., Poole, Dorset). These were dissolved in tap water and the pH was adjusted to 6.8–7.0 with NaOH before autoclaving. Medium B contained: 1% (v/v) corn-steep liquor, 1.4% (w/v) sodium lactate and 0.02% $\text{Na}_2\text{S}_2\text{O}_4$. Individual components of this medium were sterilized separately, the corn-steep liquor being first neutralized with 10N-NaOH.

For larger cultures the organism was first subcultured into tubes of medium A and grown in tubes fitted with a sterile plug and an absorbent cotton-wool pad moistened with a few drops of 10% Na_2CO_3 solution, to which about 1 mg. of pyrogallol was added immediately before closing the tube with a rubber bung. Larger cultures were grown in conical flasks fitted with gassing attachments, which allowed gas ($\text{H}_2 + \text{CO}_2$, 95:5) to bubble through a tube immersed in the medium and excess of gas to escape through a side tube fitted with a Bunsen valve. The gas was passed through the medium, previously equilibrated to 37°, for 5 min. before and after inoculation. Despite these precautions the growth of the organism in large cultures was not reproducible and it

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was found best to harvest the cells when growth was nearly complete, as judged by the visual appearance of the culture, rather than by the time after inoculation.

Dried cells of the organism were kindly provided by Dr S. G. Mayhew. These cells were grown on medium B and the cell paste was prepared and dried under vacuum at room temperature, as described by Walker (1958).

Lactobacillus plantarum (*Lactobacillus arabinosus*) (N.C.I.B. 6376) was maintained and grown on the glucose-malate medium of Korkes (1957). Cells from 11. cultures were harvested by centrifuging after 30 hr. at 30°, washed once with 100 ml. of water and the resulting cell paste was freeze-dried. A suspension of dried cells of this organism was used for the decarboxylation of malate.

Clostridium welchii strain SR12 (N.C.I.B. 6784) was maintained and grown anaerobically at 37° on the complex medium of Krebs (1950). Cells from 11. of culture were harvested after 20 hr., washed twice with 100 ml. of 0.2 M-acetic acid-sodium acetate buffer, pH 5.0, and suspended in 20 ml. of the same buffer at 0°. Washed cells of this organism were used for the decarboxylation of glutamate.

Nocardia globerulea (N.C.I.B. 8852) was maintained on tubes of 2% peptone and larger cultures were grown in Roux bottles on 2% peptone-2% agar (Crawford, 1958). After incubation for 16 hr. at 30° in air, the cells were harvested by centrifuging, washed once and suspended in 0.9% NaCl (20 ml./g. wet wt. of cells). This organism was used for the decarboxylation of aspartate (Crawford, 1958).

Preparation of cell-free extracts of P. elsdonii. Cells were harvested by centrifuging at 5000g for 15 min. at 0° when growth appeared to be nearing completion, after 16-25 hr. After washing twice in 0.05 M-phosphate buffer (KH_2PO_4 -KOH), pH 7.4, containing 0.5 mM- Na_2S , and centrifuging as before, the cells were suspended in a suitable volume of the buffer-sulphide mixture (about 10-15 ml./g. dry wt. of cells). The suspension was subjected to ultrasonic treatment with a Mullard 1 kw ultrasonic generator operating at about 27 kcyc./sec.; the transducer and the stainless-steel flask were cooled in ice throughout. The disrupted suspension was then centrifuged at 22000g for 30 min. and the supernatant retained. A treatment of 2 min. was selected as the shortest exposure to ultrasonic disruption giving near-maximal protein release; the percentage of the dry weight released as protein showed little variation with the concentration of the cell suspension.

Extracts of dried cells of *P. elsdonii* were prepared by shaking under vacuum in Thunberg tubes for 1 hr. at 37° with 50 mM-phosphate buffer (KH_2PO_4 -KOH), pH 7.4, containing 0.5 mM- Na_2S (10 ml./g. of dried cells). The debris was removed by centrifuging at 22000g for 20 min.

To investigate the effect of phosphate, ultrasonic extracts were prepared as above except that the phosphate-sulphide mixture was replaced by 50 mM-tris-HCl buffer, pH 7.4, containing 0.5 mM- Na_2S . Such extracts are referred to below as 'phosphate-free' extracts.

To remove metal ions, some extracts (1 ml. containing about 40 mg. of protein) were passed down a column (120 cm. $\times$ 6 mm. diam.) of Sephadex G-25 (coarse grade) at 4°, and washed through with water, the main coloured band being collected. These are referred to below as 'metal-free' extracts. Dialysed extracts were prepared by dialysis against two changes of distilled water at 4°.

In some cases extracts were stored at -20° for about 16 hr. in Thunberg tubes that had been evacuated and filled

with CO. These are referred to below as 'CO-treated' extracts. This treatment decreased the oxidation of NADH by pyruvate.

Radioactive compound. The $\text{NaH}^{14}\text{CO}_3$ was obtained from The Radiochemical Centre, Amersham, Bucks.

Measurement of radioactivity. $^{14}\text{CO}_2$ was plated as BaCO_3 and counted in a Packard model 200A windowless gas-flow counter by the procedures described by Somerville & Peel (1967).

Paper chromatography and paper electrophoresis. Amino acid products of transamination reactions were identified by electrophoresis on Whatman 3MM paper in the pyridine-acetic acid-water (1:10:89, by vol.) system of Dixon, Kauffman & Neurath (1958), as described by Somerville & Peel (1967).

Glutamate was also identified by chromatography on washed Whatman no. 1 paper with the butan-2-ol-formic acid-water (15:3:2, by vol.) system of Haussman (1952).

The 2,4-dinitrophenylhydrazones of 2-oxo acids were prepared from deproteinized reaction mixtures and the spectra examined by using the procedure of Friedemann (1957). Samples of the extracted derivatives were also identified by paper chromatography on Whatman 3MM paper with the 2-methylbutan-2-ol-ethanol-water (5:1:4, by vol.) system of Altmann, Crook & Datta (1951).

Malate and pyruvate were separated as the free acids on Whatman no. 1 paper with water-saturated butan-1-ol-formic acid (Magasanik & Umbarger, 1950). The two-dimensional solvent system of Benson *et al.* (1950) was also used in the identification of malate.

Amino acids were detected as described by Somerville & Peel (1967) and organic acids were detected by spraying with 0.04% bromocresol green (Bryant & Overell, 1953).

Spectrophotometric methods. All measurements of enzymic activities were carried out at 37° in either the Unicam SP.500 or the Unicam SP.820 spectrophotometer. Other spectrophotometric measurements were carried out at room temperature in either the Unicam SP.500 or the Unicam SP.600 instrument. All measurements were made in cells of 1 cm. light-path.

All the enzymic activities determined spectrophotometrically involved the reduction of NAD^+ or NADP^+ or the oxidation of NADH or NADPH and were followed by observing the change in E_{340} . Cell-free extracts of *P. elsdonii* contained a powerful oxidase for NADH and NADPH and, to eliminate this cause of interference, assays were carried out under rigorously anaerobic conditions. 'O₂-free' N₂ (O.F.N.; British Oxygen Co. Ltd.) contained traces of O₂ sufficient to interfere with assays. The O₂ was removed by bubbling through a bead tower containing a solution of chromous sulphate, prepared by passing a saturated solution of chrome alum through a Jones reductor (Vogel, 1961). N₂ treated in this way is referred to below as 'purified N₂'. In some experiments CO was treated in the same way and this is referred to below as 'purified CO'.

Modified spectrophotometer cells were used with a ground-glass joint to which an extension with a side arm could be attached. The solution in the main compartment of this cell was gassed by inserting a stainless-steel needle through a self-sealing rubber cap (turnover type, size 17; William Freeman and Co. Ltd., Subaseal Works, Barnsley, Yorks.). After gassing had proceeded for 90 sec. with the extract in the side arm, the needle was withdrawn to a position just above the surface of the solution and the side

arm was stoppered. The needle was then withdrawn completely. This method was adapted for other anaerobic experiments by using ordinary test tubes with side arms.

Determination of protein. This was carried out by the biuret method of Gornall, Bardawill & David (1949).

Determination of inorganic phosphate. This was carried out by a phosphomolybdate method with ascorbic acid as reducing agent, as described by Chen, Toribara & Warner (1956).

Determination of citrate. This was carried out by the colorimetric method of Taylor (1953), the E_{450} value being corrected in the few cases required by measuring the E_{610} due to background opalescence (Stern, 1957). Some samples of oxaloacetate (mainly old preparations) appeared to interfere with the determination of citrate by giving rise to an absorption at 450 m μ . This was prevented by boiling at acid pH before starting the determination.

Chemical reagents. Unless otherwise stated all reagents used were A.R. grade and were obtained from either Hopkin and Williams Ltd., Chadwell Heath, Essex, or British Drug Houses Ltd., Poole, Dorset.

NAD⁺, NADP⁺, NADPH, ATP, isocitrate dehydrogenase and 3-phosphoglyceric acid were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

DL-Isocitric acid (trisodium salt), glyoxylic acid, 2-oxoglutaric acid, malic acid and all amino acids were obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

CoA and NADH were purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. Acetyl-CoA was prepared by treating CoA with acetic anhydride (Stadtman, 1957).

Glutaconic acid was obtained from the Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.

cis-Aconitic acid and the cyclohexylamine salt of the dimethylketal of phosphohydroxypyruvic acid were supplied by Calbiochem A.-G., Lucerne, Switzerland. Free phosphohydroxypyruvic acid was prepared from this derivative by the method of Ballou (1960).

Oxaloacetic acid was obtained from C. F. Boehringer und Soehne G.m.b.H. and from the Sigma Chemical Co., or was prepared from sodium diethylloxaloacetate (Eastman Organic Chemicals Department, Rochester, N.Y., U.S.A.) by the procedure of Heidelberger (1953).

Crystalline sodium pyruvate was a gift from Dr J. L. Peel and was prepared by neutralization of redistilled pyruvic acid as described by Robertson (1942). Lactic acid was obtained as a 70% solution of the sodium salt from British Drug Houses Ltd. Lithium hydroxypyruvate was a gift from Mr D. H. Williamson.

Solutions of free acids were neutralized where required by adding NaOH or KOH. Buffers were prepared by titration of the relevant solutions at a pH-meter.

Malate dehydrogenase and lactate dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H. Before use they were dialysed and diluted 1:1 with glycerol (Bergmeyer & Bernt, 1962).

RESULTS

Reactions interfering with enzymic assays

Several of the assays involved following the oxidation or reduction of NAD or NADP spectrophotometrically. NAD(P)H oxidase activity was

eliminated as described in the Methods and Materials section.

The assays of some enzymes were further complicated by secondary reactions in which pyruvate is a substrate. In this Laboratory, with extracts of *P. elsdonii*, pyruvate has been found to reduce NADP⁺ (J. L. Peel & R. J. Watkinson, unpublished work) and, to a smaller extent, to reduce NAD⁺. In *P. elsdonii*, the breakdown of pyruvate to acetyl-CoA, carbon dioxide and hydrogen involves the reduction of ferredoxin (Mayhew, 1966; Mortenson, Valentine & Carnahan, 1962). Reduced ferredoxin may be reoxidized either by the release of hydrogen or by the transfer of electrons to NAD⁺ and to NADP⁺ (Baldwin & Milligan, 1964).

On the other hand, pyruvate may also catalyse the oxidation of NAD(P)H. Acetyl-CoA produced in the initial cleavage of pyruvate by *P. elsdonii* may be converted into acetyl phosphate (Peel, 1960), or alternatively two molecules of acetyl-CoA may undergo a condensation reaction followed by reduction in two stages to give butyrate. Crotonyl-CoA is an intermediate in this process and can be reduced to butyryl-CoA by an acyl-CoA dehydrogenase that has been shown to be linked to NAD (Baldwin & Milligan, 1964). It is possible that the reductive formation of crotonyl-CoA could also utilize NADH or NADPH. Thus pyruvate, which gives rise to acetyl-CoA, might be expected to cause oxidation or reduction of the nicotinamide coenzymes depending on the exact balance of the above reactions in the particular preparation, and can therefore interfere with assays depending on the measurement of the oxidation or reduction of these coenzymes.

Enzyme activities were determined, unless otherwise indicated, at a pH close to the optimum and with the rate of the reaction proportional to the concentration of the extract in the assay mixture. The assay conditions were such that a change in E_{340} of 1 was equivalent to the oxidation or reduction of 0.5 μ mole of NAD(P). In general, progress curves were linear, sometimes with a preliminary lag, and the rates were measured over the linear phase; in other cases the rate was measured between 15 and 30 sec. after the addition of substrate.

Enzymic studies on alanine formation

Glutamate-pyruvate transaminase (L-alanine-2-oxoglutarate aminotransferase, EC 2.6.1.2). This reaction was demonstrated in both directions by incubating dialysed extracts with the appropriate amino acid plus oxo acid and examining the amino acid products by paper electrophoresis. Glutamate formation was also demonstrated by trapping the pyruvate formed with added authentic lactate

dehydrogenase (Fig. 1) by using a carbon monoxide-treated extract.

Enzymic studies on aspartate formation

Preliminary experiments showed that extracts of *P. elsdenii* catalysed the spectrophotometric reduction of both NAD^+ and NADP^+ by malate. The tentative conclusions were that both 'malic' enzyme and malate dehydrogenase were present in *P.*

Fig. 1. Glutamate-pyruvate transaminase in *P. elsdenii*. The complete reaction mixture contained in 3 ml. (μ moles): tris-HCl buffer, pH 7.4, 500; 2-oxoglutarate, pH 7.0, 25; L-alanine, 10; NADPH, 0.8; 5.4 mg. of protein of a CO-treated extract; 0.1 ml. of diluted authentic lactate dehydrogenase (see the Methods and Materials section). The gas phase was purified CO. Measurements of E_{340} were carried out in a Unicam SP.820 recording spectrophotometer. Curve 1, extract absent; curve 2, alanine or 2-oxoglutarate absent; curve 3, lactate dehydrogenase absent; curve 4, complete system.

elsdenii, and that these enzymes might be involved in aspartate formation.

'Malic' enzyme [L-malate-NADP oxidoreductase (decarboxylating), EC 1.1.1.40]. Enzymes other than the 'malic' enzyme may be involved in oxaloacetate synthesis from phosphoenolpyruvate or pyruvate. No net formation of oxaloacetate from carbon dioxide plus pyruvate or phosphoenolpyruvate was observed, with or without ADP or ATP, when an enzymic method was used to trap and detect oxaloacetate. Some of the extracts used in these attempts were treated with carbon monoxide in an attempt to prevent pyruvate breakdown.

On the other hand, evidence for the synthesis of malate from pyruvate and carbon dioxide was obtained by using $\text{NaH}^{14}\text{CO}_3$ and an extract treated with carbon monoxide in an attempt to prevent pyruvate breakdown (Table 1). Portions of the incubation mixture were treated, after the removal of protein, with a cell preparation of *L. plantarum* (see the Methods and Materials section). In the presence of semicarbazide, to prevent the decarboxylation of pyruvate by the preparation of *L. plantarum*, most radioactivity was evolved from the incubation mixture in which $\text{NaH}^{14}\text{CO}_3$, NADPH and pyruvate had been present. This indicates incorporation of $^{14}\text{CO}_2$ into the β -carboxyl group of malate. The failure of NADH to stimulate $^{14}\text{CO}_2$ incorporation in this way suggests that there is no NAD-linked 'malic' enzyme activity in the extracts. The $^{14}\text{CO}_2$ released from the reaction mixture when the decarboxylation was carried out in the absence of semicarbazide contained a much greater amount of radioactivity. This incorporation is presumably due to the exchange reaction between carbon dioxide and the carboxyl group of pyruvate, catalysed by cell-free preparations of *P. elsdenii*.

Table 1. Incorporation of $^{14}\text{CO}_2$ into pyruvate and malate by extracts of *P. elsdenii*

The initial incubations were carried out under purified N_2 in tubes similar to those described by Peel (1960). The complete reaction mixture contained in 1 ml. (μ moles): tris-maleate buffer, pH 7.35, 125; MnCl_2 , 4; NADPH or NADH, 2; sodium pyruvate, 10; $\text{NaH}^{14}\text{CO}_3$, 0.6 (2×10^7 counts/min.); 3 mg. of protein of an ultrasonic extract that had been stored under CO for 16 hr. at -20° . The tubes were incubated at 37° for 5 min. and then deproteinized by heating in a boiling-water bath for 10 min. Samples of each deproteinized incubation mixture were incubated with the *L. plantarum* preparation (see the Methods and Materials section) after the addition of 10 μ moles of carrier L-malate, with and without 50 μ moles of semicarbazide (Korkes, 1957). The CO_2 was collected, plated and counted as described in the Methods and Materials section.

Omission from reaction mixture	Coenzyme	$10^{-5} \times$ Total radioactivity in CO_2 evolved (counts/min.)	
		With semicarbazide	Without semicarbazide
None	NADPH	0.45	2.8
Pyruvate	NADPH	0	0
None	None	0.15	6.0
Pyruvate	NADH	0.04	0.11
None	NADH	0.16	4.2

Fig. 2. 'Malic' enzyme in *P. elsdonii*. (a) Effect of $MnCl_2$. The complete reaction mixture contained in 3 ml. (μ moles): potassium phosphate buffer, pH 7.4, 125; $MnCl_2$, 10; $NADP^+$, 0.3; potassium L-malate, 10; 3 mg. of protein of an ultrasonic extract. The gas phase was purified N_2 . Δ , Complete system; $\blacksquare$, $MnCl_2$ absent; $\blacktriangle$, malate absent. (b) Effects of cyanide and phosphate. The complete reaction mixture was as in (a) with phosphate buffer replaced by tris-HCl buffer, pH 7.4 (100 μ moles), and 3.1 mg. of phosphate-free extract was used; the complete mixture also contained (μ moles): KH_2PO_4 , pH 7, 2.5; KCN, 2. The gas phase was purified N_2 . Δ , Complete system, $\pm$ KCN; $\blacksquare$, phosphate absent; $\blacktriangle$, phosphate and KCN absent; $\square$, malate absent. (c) Oxaloacetate decarboxylation. The reaction was carried out in Warburg cups (total volume 10 ml.) at 37° in air. The complete reaction mixture contained in 1 ml. (μ moles): potassium phosphate buffer, pH 6.0, 100; $MnCl_2$, 2; $NADP^+$, 1; oxaloacetate pH 6.0, 20; in the side arm, 8.6 mg. of protein of an ultrasonic extract. $\bullet$, Complete system, $\pm NADP^+$; $\circ$, $MnCl_2$ absent; $\blacksquare$, extract absent, $\pm$ boiled extract.

Table 2. Decarboxylation of malate by extracts of *P. elsdonii*

The reaction was carried out in double-side-arm Warburg cups of total volume about 10 ml. The reaction mixture contained in 1.5 ml. (μ moles): potassium phosphate buffer, pH 7.2, 250; $MnCl_2$, 2; potassium L-malate, 5; other additions as indicated (main compartment); in side arm 1, 400 μ moles of H_2SO_4 ; in side arm 2, extract (20 mg. of protein); 100 μ moles of KOH were added to the centre well as CO_2 absorber where indicated. The gas phase was air in all cases.

Expt. no.	Incubation time (min.)	Addition to reaction mixture	Test system	Gas evolved (as CO_2) (μ moles)
1	60	NAD^+ (6 μ moles)	Complete	7.6
			No $MnCl_2$	6.2
			CO_2 absorbed	1.0
			No NAD^+	2.1
2	48	$NADP^+$ (6 μ moles)	Complete	3.1
			No $MnCl_2$	2.6
			Complete	6.3
			No NAD^+	0.7
3	60	Malate (5 μ moles) + $NADP^+$ (10 μ moles)	Complete	9.6
			Boiled extract	0.5
			No $NADP^+$	6.8

(Peel, 1962). On paper chromatography of the deproteinized reaction mixtures a radioactive spot corresponding approximately to the expected location of malate was obtained both in the system of Magasanik & Umbarger (1950) and in the two-dimensional system of Benson *et al.* (1950).

The $NADP^+$ -linked oxidation of malate was followed spectrophotometrically at $340m\mu$ (Fig. 2a). As with the 'malic' enzyme from other sources

Mn^{2+} stimulated the reaction, no stimulation being observed when manganous chloride was replaced by sodium chloride. Experiments with phosphate-free extracts showed an apparent stimulation by phosphate (Fig. 2b). This is attributed to the conversion of pyruvate into acetyl phosphate, thus preventing the possible reoxidation of NADPH during fatty acid formation, as proposed above. The rate of $NADP^+$ reduction in phosphate-free

Fig. 3. Malate dehydrogenase in *P. elsdenii*. (a) NAD⁺ reduction. The complete reaction mixture contained in 3 ml. (μmoles): tris-HCl buffer, pH 7.0, 500; MnCl₂, 2; potassium L-malate, 10; NAD⁺, 0.3; potassium phosphate buffer, pH 7.0, 5; 1.45 mg. of protein of an ultrasonic extract. The gas phase was purified N₂. Δ, Complete system; □, MnCl₂ absent; ■, phosphate absent; ▲, NAD⁺ or L-malate absent. (b) Oxidation of NADH. The complete reaction mixture contained in 3 ml. (μmoles): tris-maleate buffer, pH 7.35, 200; oxaloacetate, pH 7.0, 10; NADH, 0.8; 2.4 mg. of protein of CO-treated extract. The gas phase was purified CO. Measurements of E₃₄₀ were carried out in a Unicam SP.820 recording spectrophotometer. Curve 1, extract absent; curve 2, oxaloacetate absent or replaced by 10 μmoles of sodium pyruvate, or NADH replaced by NADPH; curve 3, complete system; curve 4, NADH absent.

extracts was also increased by cyanide (Fig. 2b), which was tested for comparison with the NAD-linked activity where no stimulation was observed. A possible explanation of this effect is that the oxidation of NADPH by acetyl-CoA may be inhibited by cyanide.

Carbon dioxide was released from malate by crude extracts of *P. elsdenii*, and required either NADP⁺ or NAD⁺ (Table 2). The effect of NAD⁺ is attributed to conversion of malate into oxaloacetate by malate dehydrogenase, followed by enzymic and spontaneous breakdown of oxaloacetate. Extracts of *P. elsdenii* catalysed the decarboxylation of oxaloacetate, the reaction being observed manometrically at pH 6.0 (Fig. 2c). The rate of gas output, as with malate, was stimulated by Mn²⁺. Some of the gas evolved was not carbon dioxide and was presumably hydrogen released by the further breakdown of the pyruvate formed.

Malate dehydrogenase (L-malate-NAD oxidoreductase, EC 1.1.1.37). The reactions of this enzyme are catalysed in both directions by crude extracts of *P. elsdenii* (Figs. 3a and 3b). Phosphate increased the rate of NAD⁺ reduction by malate and this effect is explained in terms of secondary reactions similar to those postulated for the effect of phosphate on 'malic' enzyme activity. Manganous chloride also stimulated this reaction. The pH optimum for NAD⁺ reduction was 6.6 in phosphate buffer and 7.2 in tris-maleate buffer. No activity

Table 3. *Glutamate-oxaloacetate transaminase* in *P. elsdenii*

The complete reaction mixture contained in 2.6 ml. (μmoles): tris-HCl buffer, pH 8.2, 500; pyridoxal phosphate, 0.1; L-glutamate, pH 8.0, 100; oxaloacetate, pH 8.0, 250; 24 mg. of extract protein. Incubation was for 30 min. before deproteinization by heating (100° for 10 min.) and determination of the aspartate in the supernatant.

Omission from reaction mixture	Total aspartate formed (μmoles)
None	6.2
Extract	0
Glutamate	2.1
Oxaloacetate	0.9
Pyridoxal phosphate	6.7

was found at pH 10 in glycine buffer. Malate dehydrogenase, in contrast with 'malic' enzyme, is not activated by Mn²⁺ and the rate of this reaction normally increases with pH to about pH 11; the equilibrium at neutral pH favours malate formation (Stern, Ochoa & Lynen, 1952). The observed pH optimum and the apparent stimulation by Mn²⁺ may be effects on the secondary reactions arising from oxaloacetate formation rather than on the malate dehydrogenase of *P. elsdenii*.

The oxidation of NADH by oxaloacetate was observed with carbon monoxide-treated extracts.

Fig. 4. Glutamate-oxaloacetate transaminase in *P. elsdennii*. The complete reaction mixture contained in 3 ml. (μ moles): potassium phosphate buffer, pH 7.6, 280; aspartate, pH 8.0, 250; NADH, 0.1; 2-oxoglutarate, pH 8.0, 250; 4.3 mg. of protein of a dialysed extract; 0.05 ml. of the diluted authentic malate dehydrogenase (see the Methods and Materials section). The gas phase was purified N_2 . Δ , Aspartate or 2-oxoglutarate absent; $\square$, extract absent; $\circ$, complete system, $\pm 1 \mu$ mole of pyridoxal phosphate; $\bullet$, malate dehydrogenase absent.

The blank rate (Fig. 3b) was measured with pyruvate replacing oxaloacetate in the reaction mixture. The pH optimum of this reaction was about pH 8.0 in tris-maleate buffer. Manganous chloride did not stimulate the reaction either in dialysed extracts or in metal-free extracts. Phosphate apparently completely inhibited the reaction in this case, and in the presence of phosphate and oxaloacetate NAD^+ was reduced. These results can be explained in terms of oxaloacetate breakdown as postulated above, and confirm that the effects on the forward reaction are due to secondary reactions.

Glutamate-oxaloacetate transaminase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1). This reaction was demonstrated qualitatively in both directions by incubating extracts with the appropriate amino acid plus oxo acid and testing for the expected amino acid product by paper electrophoresis. The formation of aspartate was also shown (Table 3) by using a decarboxylase method to determine aspartate. The rate of glutamate formation was measured by determining the oxaloacetate formed with an excess of added malate dehydrogenase (Fig. 4).

Enzymic studies on glutamate formation

Glutaconate is the immediate precursor of glutamate in one of the two pathways postulated to explain the labelling pattern found in glutamate (Somerville & Peel, 1967). Tests that use the

Table 4. Citrate formation by extract of *P. elsdennii*

In Expt. 1 the complete reaction mixture contained in 1 ml. (μ moles): tris-HCl buffer, pH 7.3, 125; cysteine hydrochloride, 1; CoA, 0.1; ATP, 10; $MgCl_2$, 10; fluoroacetate, 0.75; oxaloacetate, pH 7.0 (20 μ moles), and potassium acetate (10 μ moles) were added after preincubation of the above with 5.1 mg. of protein of an ultrasonic extract. The mixtures were incubated for 15 min. in air. In Expt. 2 the complete reaction mixture contained in 1 ml. (μ moles): tris-HCl buffer, pH 7.4, 100; ATP, 0.1; CoA, $MgCl_2$, oxaloacetate and potassium acetate as in Expt. 1; the mixtures were incubated after addition of 7.7 mg. of protein of an ultrasonic extract for 30 min. in air. In Expt. 3 the complete reaction mixture was as in Expt. 2, but with 7.4 mg. of protein of an ultrasonic extract dialysed against distilled water. The mixtures were incubated for 40 min. in air. In Expt. 4 the complete reaction mixture was as in Expt. 3, but with 4.8 mg. of a dialysed extract. The mixtures were incubated for 25 min. under purified N_2 . In each case the reaction was terminated by the addition of 20% trichloroacetic acid to 5% (final concentration). After centrifuging, citrate was determined in samples of the supernatant.

Omission from reaction mixture	Total citrate formed (μ moles)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
None	0.23	1.28	2.07	0.34
Extract	0	0	0	0
ATP	0.46	1.24	1.51	0.68
$MgCl_2$	0.13	1.16	—	—
Acetate	0.22	1.08	1.06	0.32
Oxaloacetate	0	0	0	0
CoA	0.24	1.08	—	0.14
Fluoroacetate	0.20	—	—	—

pronounced absorption peak of this compound at approx. 232 m μ showed it to be inert when incubated with extracts of *P. elsdennii*, with or without ammonium chloride. The enzymes of the alternative pathway via tricarboxylic acids were demonstrated in cell-free extracts as follows.

Citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7]. When oxaloacetate and acetate were incubated with extracts the amount of citrate formed was proportional to time for at least 20 min. Preincubation with monofluoroacetate, which was included to promote citrate accumulation, was omitted in later experiments (Table 4) as it was discovered that the aconitase activity was destroyed by the standard washing procedure with buffer containing 0.5 mM-sodium sulphide. Citrate formation was largely independent of the addition of acetate and was not increased either when acetate was replaced by pyruvate or when the reaction was carried out anaerobically. Similar effects have been observed with extracts of *Rhodospirillum rubrum* (Eisenberg, 1953) and of *Thiobacillus thioparus* (Cooper, 1964) and are consistent with the successive

Fig. 5. Isocitrate dehydrogenase in *P. elsdenii*. The complete reaction mixture contained in 3 ml. (μ moles): potassium phosphate buffer, pH 7.5, 100; NAD^+ , 0.2; MgCl_2 , 10; isocitrate (trisodium salt), 1; 2.6 mg. of an extract of dried cells. The gas phase was purified N_2 . O, Complete system; ●, MgCl_2 absent; Δ, isocitrate absent.

Fig. 6. Aconitase in *P. elsdenii*. The complete reaction mixture was as in Fig. 5 with 6.4 mg. of an ultrasonic extract and the following as substrate: O, 0.5 μ mole of isocitrate; ●, 1 μ mole of *cis*-aconitate; ▲, 1 μ mole of citrate; Δ, no substrate.

breakdown of oxaloacetate to pyruvate and acetyl-CoA. The observed rate of citrate formation with extracts of *P. elsdenii*, about 0.4 μ mole/hr./mg. of protein, is similar to the rate of non-enzymic decarboxylation of oxaloacetate observed under similar conditions, about 0.6 μ mole/hr.

Isocitrate dehydrogenase [threo-D₂-isocitrate- NAD oxidoreductase (decarboxylating), EC 1.1.1.41]. This enzyme was demonstrated spectrophotometrically by measuring the reduction of NAD^+ by isocitrate (Kornberg, 1955). NADP^+ was not reduced. The change in E_{340} was initially proportional to time, and was enhanced by magnesium chloride (Fig. 5). AMP, which was found by Kornberg & Pricer (1951) to be required for maximum activity of the NAD -linked isocitrate dehydrogenase of yeast, had no effect in the present system. The spectrum of the 2,4-dinitrophenylhydrazone of the reaction product was identical with that of the corresponding derivative of 2-oxoglutarate. The identity of the 2,4-dinitrophenylhydrazone was also confirmed by paper chromatography against authentic markers.

Aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3]. Aconitase activity in *P. elsdenii* was demonstrated by substitution of citrate and *cis*-aconitate for isocitrate in the isocitrate dehydrogenase assay. Aconitase in animal tissues has been found to be unstable and is inactivated by S^{2-} ions (Krebs & Eggleston, 1940). Accordingly, assays were carried out on extracts of cells that had been washed either with buffer containing 0.5 mM-thioglycolic acid or with buffer containing no reducing agent. With citrate a lag of about 3 min. and with *cis*-aconitate a lag of about 1 min. was observed before the rate became constant, the rate being greatest with isocitrate and least with citrate (Fig. 6). A similar effect was noted by Cooper (1964) with extracts of *T. thioparus*. Addition of commercial isocitrate dehydrogenase plus NADP^+ to the *P. elsdenii* reaction mixture had no effect on the rate, indicating that the activity of aconitase was limiting. Aconitase activity was not found in some of the extracts tested and this was attributed to the instability of the enzyme.

Glutamate dehydrogenase [L-glutamate- NAD oxidoreductase (deaminating), EC 1.4.1.2]. The reduction of NAD^+ by glutamate was readily observed with dialysed extracts (Fig. 7a). The 2,4-dinitrophenylhydrazone prepared from the products was identified as that of 2-oxoglutarate from its spectrum and its chromatographic behaviour. The reaction was also demonstrated in the reverse direction by following the oxidation of NADH in the presence of 2-oxoglutarate and ammonia (Fig. 7b). No activity was observed in either direction with NAD replaced by NADP .

Enzymic studies on serine formation

The results of the radioisotope experiments (Somerville & Peel, 1967) indicated that serine was formed from lactate via C_3 intermediates. Serine synthesis has been shown in *Escherichia coli* and *Salmonella typhimurium* (Umbarger, Umbarger &

Fig. 7. Glutamate dehydrogenase in *P. elsdonii*. (a) Reduction of NAD⁺. The complete reaction mixture contained in 3 ml. (μ moles): potassium phosphate buffer, pH 7.9, 240; phenylhydrazine hydrochloride, 12.5; L-glutamate, pH 8.0, 100; NAD⁺, 0.3; 3.4 mg. of a dialysed ultrasonic extract. The gas phase was purified N₂. ■, Complete system; □, phenylhydrazine absent; ▲, NAD⁺ or glutamate absent. (b) Oxidation of NADH. The complete reaction mixture contained in 3 ml. (μ moles): potassium phosphate buffer, pH 7.4, 150; 2-oxoglutarate, pH 7.0, 5; NH₄Cl, 5; NADH, 0.18; 2 mg. of protein of a dialysed ultrasonic extract. The gas phase was purified N₂. ○, Complete system; ■, 2-oxoglutarate or NH₄Cl absent; ●, extract absent.

Fig. 8. Phosphoglycerate dehydrogenase in *P. elsdonii*. The complete reaction mixture contained in 3 ml. (μ moles): tris-HCl buffer, pH 8.0, 500; MgCl₂, 10; NADH, 0.8; phosphohydroxypyruvate, pH 8.0, 1.8; 4.8 mg. of protein of an ultrasonic extract. The gas phase was purified N₂. Measurements of E_{340} were carried out in a Unicam SP. 820 recording spectrophotometer. Curve 1, phosphohydroxypyruvate absent; curve 2, extract absent; curve 3, complete system, $\pm$ MgCl₂; curve 4, NADH absent.

Phosphoglycerate dehydrogenase. This enzyme was demonstrated by following the oxidation of NADH by phosphohydroxypyruvate (Fig. 8). The rate was not affected by magnesium chloride, which has been shown to be required for maximum activity in extracts of *E. coli* (Umbarger *et al.* 1963). In a single experiment where hydroxypyruvate was tested, NADH was oxidized more rapidly than with an equimolar concentration of phosphohydroxypyruvate.

Activity of this enzyme was variable and was not found in all of the extracts tested. This enzyme has been postulated as a site of control of serine biosynthesis in other micro-organisms (Umbarger *et al.* 1963; Pizer & Potochny, 1964) and it may be that the variation in the observed activities in *P. elsdonii* reflects differences in the utilization of serine from the medium at the time of harvesting the cultures. Attempts to demonstrate the reaction in the direction of biosynthesis were unsuccessful with either NAD⁺ or NADP⁺ as the oxidant of phosphoglycerate, even in the presence of 30 mM-phenylhydrazine to trap the oxo acid.

Phosphohydroxypyruvate-glutamate transaminase. When extracts were incubated with phosphohydroxypyruvate and glutamate, the formation of phosphoserine was demonstrated by paper electrophoresis; with hydroxypyruvate replacing phosphohydroxypyruvate some serine was also formed. These transaminations were studied quantitatively in the reverse direction, when phosphoserine and free serine served equally as well as substrate for glutamate formation (Table 5).

Phosphoserine phosphatase (phosphoserine phosphohydrolase, EC 3.1.3.3). This enzyme was assayed

Siu, 1963) to proceed via phosphoglycerate, phosphohydroxypyruvate and phosphoserine. Extracts of *P. elsdonii* were assayed for enzymes catalysing the steps in this pathway.

Table 5. Formation of glutamate from 2-oxoglutarate and serine or phosphoserine in *P. elsdenii*

The complete reaction mixture contained in 3 ml. the following (μ moles): tris-HCl buffer, pH 8.2, 500; pyridoxal phosphate, 1; 2-oxoglutarate, pH 8.0, 250; DL-phosphoserine or DL-serine as indicated, 100; in Expt. 1 the incubation was for 20 min. with 38 mg. of protein and in Expt. 2 for 40 min. with 70 mg. of protein, and in both cases ultrasonic extracts were used. The gas phase was air. At the end of the reaction trichloroacetic acid was added to 5% final concentration, and after centrifugation the glutamate in the supernatant was determined by decarboxylation with a washed-cell suspension of *C. welchii*.

Amino acid	Omission from reaction mixture	Total glutamate formed (μ moles)	
		Expt. 1	Expt. 2
None	None	5.2	12.0
Phosphoserine	None	14.0	33.0
Phosphoserine	2-Oxoglutarate	7.1	3.3
Serine	None	20.0	35.0
Serine	2-Oxoglutarate	11.0	—
Phosphoserine	Extract	—	0
Phosphoserine + serine	Extract	0	—

Fig. 9. Phosphoserine phosphatase in *P. elsdenii*. The complete reaction mixture contained in 3 ml. (μ moles): tris-maleate buffer, pH 7.4, 400; substrate as indicated, 10; 12 mg. of protein of a phosphate-free, dialysed extract. The gas phase was air. ●, DL-Phosphoserine; □, DL-phosphoserine + 50 μ moles of L-serine; ■, phosphoglycerate; △, phosphohydroxypyruvate.

by determination of the inorganic phosphate released from the substrate with 'phosphate-free' extracts. A rather high phosphate blank was obtained from phosphoserine; recrystallization from aqueous ethanol decreased this but had no effect on the activity. A control with boiled extract showed that the non-enzymic breakdown of phosphoserine was negligible under these conditions. The activity was about 75% inhibited by

17 mM-L-serine (Fig. 9). Other investigators have found that the enzyme from other sources is sensitive to inhibition by L-serine. There was little or no phosphate release from 3-phosphoglycerate or phosphohydroxypyruvate at pH 5.0 or at pH 7.4.

DISCUSSION

The presence of lactate dehydrogenase in *P. elsdenii* has been established by Baldwin & Milligan (1964). The present results suggest that pyruvate formed in this way from lactate is transaminated to give alanine. This route of alanine formation is in agreement with the labelling pattern in alanine (Somerville & Peel, 1967).

The enzymic evidence suggests that, when *P. elsdenii* grows on lactate, oxaloacetate is the precursor of aspartate and that oxaloacetate is formed from pyruvate by the sequential action of 'malic' enzyme and malate dehydrogenase. This pathway is consistent with the previous tracer experiments (Somerville & Peel, 1967), but the possibility of other mechanisms being involved in the C₃ and C₁ condensation step cannot be precluded, particularly in view of the side reactions involving pyruvate. A C₃ and C₁ condensation has been implicated, by labelling data, in aspartate formation in other micro-organisms, including *Clostridium kluyveri* (Tomlinson & Barker, 1954) and anaerobically grown *R. rubrum* (Cutinelli, Ehrensward, Reio, Saluste & Stjernholm, 1951). 'Malic' enzyme has been demonstrated in cell-free extracts of the latter organism (Vernon & Kamen, 1953).

It is difficult to imagine a function other than a biosynthetic one for the 'malic' enzyme in *P. elsdenii*. On the other hand, a mutant of *E. coli* has been reported (Ashworth, Kornberg & Ward, 1965)

that lacks phosphoenolpyruvate carboxylase and requires C₄ acids for growth despite the presence of 'malic' enzyme.

P. elsdonii contains all of the enzymes of the tricarboxylic acid pathway leading from oxaloacetate and acetate to 2-oxoglutarate and glutamate. Together with the results of the tracer experiments, this provides very strong evidence for the formation of glutamate by this route. Burchall, Niederman & Wolin (1964) have presented evidence for the operation of the tricarboxylic acid pathway in *Streptococcus bovis*. The organism was grown anaerobically on a defined medium containing glucose; ¹⁴CO₂ was incorporated specifically into C-1 of glutamate in accordance with the tricarboxylic acid pathway. NAD-linked isocitrate dehydrogenase and an NADP-linked glutamate dehydrogenase were present in cell-free extracts, but no aconitase or citrate synthase activity could be found. Isocitrate dehydrogenase activity (NADP-linked) has also been found in extracts of *Clostridium pasteurianum*, *Diplococcus glycinophilus* and *Chlorobium thiosulphatophilum* (H. J. Somerville, unpublished work), all of which are strict anaerobes. These findings suggest that the tricarboxylic acid pathway to glutamate may be widespread among anaerobes.

Gottschalk & Barker (1966, 1967) have described glutamate formation via the tricarboxylic acid pathway in extracts of several strict anaerobes. In *C. kluyveri* and *Clostridium acidurici* they found that the stereospecificity of citrate synthase activity was opposite to that usually found in plant and animal cells. These observations account for the

results of Tomlinson (1954), who with *C. kluyveri* found that ¹⁴C from radioactive acetate was incorporated into glutamate to give a labelling pattern that could not then be explained. The observations of these workers indicate that glutamate formation from acetate in *C. kluyveri* proceeds via this atypical pathway. Other workers (Stern & Bambers, 1966; Stern, Hegre & Bambers, 1966) confirmed the presence of the necessary enzymes in *C. kluyveri*, but obtained conflicting results for the stereospecificity of the citrate synthase, which was attributed to mutation. The labelling data from *P. elsdonii* (Somerville & Peel, 1967) can only be explained by the normal tricarboxylic acid pathway.

In the present studies, the activity of glutamate dehydrogenase was much higher than that of the other enzymes (Table 6). This is consistent with this enzyme's acting as a major route of ammonia assimilation in *P. elsdonii*. The labelling data (Somerville & Peel, 1967) precluded the presence of aspartase; enzymic tests for this enzyme also proved negative.

The labelling pattern obtained in serine (Somerville & Peel, 1967) excludes the formation of serine from glycine and suggested that a pathway involving C₃ intermediates was involved. The present studies have demonstrated the enzymes catalysing the steps in serine formation from phosphoglycerate by the pathway phosphoglycerate → phosphohydroxypyruvate → phosphoserine, though the reaction catalysed by phosphoglycerate dehydrogenase has not been demonstrated in the biosynthetic direction. Studies by other

Table 6. *Enzymic activities in extracts of P. elsdonii*

The values quoted were obtained by using the assays described in the text and represent the average of at least two determinations corrected for controls without substrate. The forward reaction refers to the direction of implied amino acid synthesis. +, Reaction demonstrated qualitatively; —, demonstration of reaction attempted unsuccessfully.

Enzyme	Activity (μmoles/hr./mg. of protein)	
	Forward reaction	Reverse reaction
Glutamate-pyruvate transaminase	+	0.2
'Malic' enzyme	+	0.9*
Malate dehydrogenase	0.84*	2.9
Glutamate-oxaloacetate transaminase	+	0.64
Citrate synthase	0.35	
Aconitase	0.30	
Isocitrate dehydrogenase	0.33	
Glutamate dehydrogenase	2.2	0.33
Phosphoglycerate dehydrogenase	—	0.18
Glutamate-phosphohydroxypyruvate transaminase	+	+
Phosphoserine phosphatase	0.48	

* Approximate values determined in the presence of phosphate.

workers with *E. coli* and *Salm. typhimurium* (Umbarger *et al.* 1963; Pizer, 1963) have also failed to demonstrate this reaction spectrophotometrically. This failure has been attributed to the unfavourable equilibrium. The lack of phosphatase activity with phosphoglycerate suggests that the pathway involving the corresponding non-phosphorylated intermediates does not contribute significantly to serine formation in *P. elsdenii*. In *E. coli*, Smith, Shuster, Zimmerman & Gunsalus (1956) found that extracts contained an NAD-linked glycerate dehydrogenase, but found no activity with phosphohydroxypyruvate; this organism has since been shown to synthesize serine by the phosphorylated pathway (Umbarger *et al.* 1963). The conversion of pyruvate into phosphoenolpyruvate has not been demonstrated in *P. elsdenii*, but is known to occur in a number of other bacteria. Extracts of *P. elsdenii* catalyse the reverse of the triose phosphate dehydrogenase reaction, and phosphoenolpyruvate replaces 3-phosphoglycerate in this assay (R. J. Watkinson, personal communication), suggesting that enolase and phosphoglyceromutase are present in *P. elsdenii*. These results indicate that serine synthesis in *P. elsdenii* occurs by the same pathway shown to operate in *E. coli* and *Salm. typhimurium* (Umbarger & Umbarger, 1962; Umbarger *et al.* 1963). This pathway has not been previously demonstrated in a strict anaerobe, though the radioisotope-incorporation experiments of Tomlinson (1954) suggest that, in *C. kluyveri*, serine is formed from a C₃ precursor related to alanine.

The present investigation has demonstrated the presence in *P. elsdenii* of enzymes that can account for the biosynthesis of alanine, serine, aspartate and glutamate. Further, these enzymes are present in sufficient quantity. From the observed doubling time of an exponentially growing culture (about 2 hr. on medium A) it was calculated that the approximate rate of incorporation of each amino acid into cell material is 0.15 μ mole/hr./mg. of protein. In every case where an enzyme was assayed in the biosynthetic direction the activity was at least of this magnitude (Table 6). The present results, together with the evidence from the radioisotope-incorporation studies (Somerville & Peel, 1967), provide strong evidence that, in this strict anaerobe at least, the modes of biosynthesis of these four amino acids are similar to those discovered previously in aerobic and facultatively aerobic organisms.

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