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

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Peptostreptococcus elsdenii, a strict anaerobe from the rumen, was grown on a medium containing yeast extract and [1-^{14}C]- or [2-^{14}C]-lactate. Radioisotope from lactate was found in all cell fractions, but mainly in the protein. The label in the protein fraction was largely confined to a few amino acids: alanine, serine, aspartic acid, glutamic acid and diaminopimelic acid. The alanine, serine, aspartic acid and glutamic acid were separated, purified and degraded to establish the distribution of ^{14}C from lactate within the amino acid molecules. The labelling patterns in alanine and serine suggested their formation from lactate without cleavage of the carbon chain. The pattern in aspartic acid suggested formation by condensation of a C_3 unit derived directly from lactate with a C_1 unit, probably carbon dioxide. The distribution in glutamic acid was consistent with two possible pathways of formation: (*a*) by the reactions of the tricarboxylic acid cycle leading from oxaloacetate to 2-oxoglutarate, followed by transamination; (*b*) by a pathway involving the reaction sequence 2 acetyl-CoA→crotonyl-CoA→glutaconate→glutamate.

Over the past 20 years, the pathways of formation of the amino acids have been established. A great deal of the pertinent evidence has come from experiments with micro-organisms, notably *Neurospora* and *Escherichia coli*. These organisms were selected because they are convenient to grow and because considerable knowledge of their genetic and biochemical properties was available. The results obtained indicate that amino acids are synthesized by closely similar pathways in these organisms. However, the existence of alternative biosynthetic pathways has been shown for several amino acids in other micro-organisms. For example, there is evidence that the carbon skeleton of glutamic acid, which is synthesized by the reactions of the tricarboxylic acid cycle in *E. coli* and *Neurospora*, may be formed by a number of different routes in other micro-organisms (Hoare, 1963*a*).

With anaerobic micro-organisms, the relatively sparse data suggest that unusual pathways may operate. Studies with radioisotopically labelled precursors have indicated novel pathways of glutamate formation in *Clostridium kluyveri* (Tomlinson, 1954*a*,*b*), anaerobically grown *Rhodospirillum rubrum* (Hoare, 1963*b*) and *Chlorobium thiosulphatophilum* (Hoare & Gibson, 1964).

Peptostreptococcus elsdenii is a strict anaerobe, originally isolated from the sheep rumen (Elsden, Volcani, Gilchrist & Lewis, 1956). Little is known about biosynthesis in *P. elsdenii* and in other rumen bacteria. Bryant & Robinson (1962) showed that *P. elsdenii* requires growth factors present in casein hydrolysate; the organism has not yet been grown on a defined medium. The experiments of Bryant, Robinson & Chu (1959) and Bryant & Robinson (1961) have shown that *Bacteroides succinogenes* and several *Ruminococcus* species require ammonia for growth even in the presence of a complex mixture of amino acids. This suggests that these rumen bacteria may not respond to the presence of exogenous amino acids by repression of the relevant biosynthetic pathways (Umbarger, 1964).

The present investigation was undertaken to discover whether lactate carbon was incorporated into amino acids by *P. elsdenii* and, if so, to determine the pathways involved. A preliminary account of some of the results has been reported (Somerville & Peel, 1964).

MATERIALS AND METHODS

Micro-organisms

Peptostreptococcus elsdenii (*N.C.I.B.* 8927). Cultures of this organism were maintained by using the medium and

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method of Walker (1958). For the radioactive cultures the following medium was used: 1% (w/v) of M-KH_2PO_4, pH 6·8 (adjusted with KOH); 0·4% of Difco yeast extract (Difco Laboratories, Detroit 1, Mich., U.S.A.); 0·05% of NH_4Cl; 0·03% (v/v) of thioglycollic acid; 1·4% of sodium lactate. The solution was made up in tap water and the pH was adjusted to 6·8–7·0 before autoclaving. The organism was first subcultured into tubes containing 10 ml. of this medium and fitted with a sterile plug and an absorbent cotton-wool pad moistened with a few drops of 10% (w/v) Na_2CO_3 solution, to which about 1 mg. of pyrogallol was added immediately before the tube was closed with a rubber bung. All cultures were grown at 37°. The radioactive cultures were grown in 100 ml. flasks fitted with gassing attachments and containing 45 ml. of the above medium but without the lactate. The sodium lactate was sterilized separately with the radioactive lactate in a volume of 5·0 ml. A sample was removed for the determination of specific activity and the remainder added aseptically to the medium before adding the 5% inoculum. The flasks were gassed with H_2+CO_2 (95:5) for 5 min. before and after inoculation.

Veillonella alcalescens (*formerly* Micrococcus lactilyticus). The strain used was isolated in this Department by Dr J. N. Ladd. It was maintained by using the same medium and method as for *P. elsdenii*. For the decarboxylation of succinic acid, in the degradation of glutamic acid, washed-cell suspensions of the organism were prepared from cells grown on the lactate–yeast extract–salts medium of Johns (1951). The 1 l. culture was incubated for 18 hr. at 37° under H_2+CO_2 (95:5) and the cells were harvested by centrifuging (3000*g* for 15 min. at 0°) and washed twice with 100 ml. of 5 mM-Na_2S, pH 7·4, before suspension in 1–2% of the culture volume of 0·1 M-phosphate buffer (KH_2PO_4–Na_2HPO_4), pH 5·8, containing 5 mM-Na_2S.

Nocardia globerula (*N.C.I.B.* 8852). This organism was used for the decarboxylation of aspartic acid (Crawford, 1958). It was maintained on tubes of 2% peptone (Oxoid Division, Oxo Ltd., London, S.E. 1). Larger cultures were grown in Roux bottles on 2% peptone in 2% agar (Crawford, 1958). After incubation for 16 hr. at 30° in air, the cells were harvested and washed and suspended in 0·9% NaCl.

Clostridium welchii (*N.C.I.B.* 6784). Washed cells of this organism were used for the decarboxylation of glutamic acid. The organism was maintained and grown anaerobically at 37° on the complex medium of Krebs (1950). Cells from 1 l. of culture were harvested after 20 hr., washed twice with 0·2 M-sodium acetate–acetic acid buffer, pH 5·0, and suspended in 20 ml. of the same buffer. The suspension was stored at 0°.

Radioactive compounds

The sodium salts of [1-^{14}C]- and [2-^{14}C]-lactic acid were obtained from The Radiochemical Centre, Amersham, Bucks. Chromatography of a sample of the [2-^{14}C]lactate in the two-dimensional solvent system of Benson *et al.* (1950), followed by radioautography, revealed six different radioactive areas on the chromatogram. The largest of these contained some 90% of the total radioactivity, as determined by elution and counting at infinite thinness. On repeating the chromatography and radioautography, only one radioactive area was detected. The radioactive sodium lactate used for growth experiments B, C and D (see the Results and Discussion section) was partially purified by electrophoresis in 0·5 M-$(NH_4)_2CO_3$, pH 8·9 (see under 'Chromatography and electrophoresis'). The $(NH_4)_2CO_3$ was removed by drying overnight at room temperature. Radioautography showed several bands, the most dense corresponding to marker sodium lactate. This band was eluted and examined by two-dimensional chromatography and radioautography. Several faint areas of radioactivity were present, but one major spot accounted for 99·5% of the radioactivity detected after elution and counting as before.

Separation and fractionation of cells

Cultures were harvested at the end of growth, i.e. when gassing ceased and most cells had settled out, by centrifuging (3400*g* for 15 min. at 0°). The supernatant was retained and the cells were washed three times with 100 ml. of water at 4°. The washings were discarded.

Cells were fractionated by using a modification of the procedure of Roberts, Cowie, Abelson, Bolton & Britten (1955).

(1) The washed cells were suspended in 50 ml. of 5% (w/v) trichloroacetic acid at 4° and transferred to a glass centrifuge tube. After 30 min. at 4° the suspension was centrifuged (2000*g* for 15 min. at 0°). The supernatant was decanted and is referred to as the 'cold-trichloroacetic acid fraction'.

(2) The pellet was suspended in 40 ml. of aq. 75% (v/v) ethanol. After 30 min. at 48° the suspension was centrifuged as in (1). The supernatant is referred to as the 'ethanol fraction'.

(3) The pellet from (2) was suspended in 40 ml. of an ethanol–ether mixture prepared by mixing equal volumes of aq. 75% (v/v) ethanol and diethyl ether. The suspension was kept at 48° for 15 min. and then centrifuged as before. The supernatant is referred to as the 'ethanol–ether fraction'.

(4) The pellet from (3) was suspended in 40 ml. of 5% (w/v) trichloroacetic acid at 100° and heated at 100° for 30 min. The suspension was then centrifuged (2500*g* for 30 min. at 0°); the prolonged centrifugation was found necessary to form a clean pellet. The supernatant from this step is referred to as the 'hot-trichloroacetic acid fraction'.

(5) The residue was washed with 40 ml. of 50 mN-HCl in ethanol and finally in 40 ml. of ether. The supernatants were discarded after centrifuging as for (4).

(6) The remaining material, containing most of the cell protein, was suspended in about 3 ml. of 6 N-HCl and transferred to a 50 ml. flask, with washings of 6 N-HCl. The protein was hydrolysed by refluxing for 18 hr. The hydrolysate was evaporated to dryness under reduced pressure at about 60°; 1 ml. of distilled water was added and the evaporation repeated three times to remove HCl.

Measurement of radioactivity

The following three types of counting equipment were used. (*a*) Panax D 657 scaler fitted with G.E.C. type 2B2 large-diameter end-window tube. This counter was used for counting radioactive areas on chromatograms. (*b*) Packard model 200A windowless gas-flow counter with a Baird–Atomic model 1035 scaler and a gas phase of '98% minimum stench propane' (Shell-Mex and B.P. Gases Ltd., London, W.C. 2). This counter was used for counting plates of $BaCO_3$ and for counting other substances at infinite

thinness. (*c*) Frieseke–Hoepfner FH407 flow counter fitted with a thin end window (0·8 mg./cm.^{2}) coupled to an FH90 scaler, FH449 printing timer and FH448 automatic sample-changer. The samples were supported on blocks of the same dimensions as those used for counting in the Packard counter. The carrier gas was methane. This counter was used for counting at infinite thinness and for $BaCO_3$ plates; when the latter were counted, the radioactivities were converted into equivalent counts in the Packard counter by using a conversion factor obtained by counting several samples in each counter.

Samples of $BaCO_3$ were prepared by the method of Knight (1962). All radioactivities obtained from $BaCO_3$ plates were corrected for self-absorption to radioactivity at infinite thinness. Measurements of radioactivity at infinite thinness were made on disposable steel planchets, which were kept in ethanol and flamed before use. This allowed solutions to spread evenly before evaporating to dryness under an infrared lamp. Formaldehyde, produced in the degradation of serine, was counted as the dimedone derivative. The plates, containing approx. 40 μmoles of the derivative, were prepared and dried as for $BaCO_3$.

Radioactive areas on chromatograms were detected by radioautography as described by Knight (1962). Radioautographs were exposed for a time determined approximately by the following relationship between the number of radioactive spots expected (*A*) and the radioactivity applied (*B*), measured as counts/min. in the Panax counter:

$$\text{Exposure time in days} = \frac{A \times 10000}{B}$$

Chromatography and electrophoresis

Paper chromatography. Unless otherwise stated, paper for chromatography was not treated before use. For a few experiments, washed papers were prepared by the method of Eggleston & Hems (1952). All chromatograms were developed in the descending direction; the temperature of the chromatography room was maintained at 22°.

Samples of protein hydrolysates were examined by chromatography on washed Whatman no. 4 paper in the two-dimensional system of Benson *et al.* (1950). This system is referred to below as CS 1. Glutamic acid and aspartic acid were separated on washed Whatman no. 1 paper with the butan-2-ol–formic acid–water (15:3:2, by vol.) solvent of Haussman (1952) (system CS 2). Neutral amino acids were separated on Whatman 3MM paper with the propan-1-ol–water (4:1, v/v) system of Kemble & Macpherson (1954) (system CS 3). Alanine was separated from threonine on Whatman no. 1 paper with the acetone–butan-1-ol–diethylamine–water (10:10:5:2, by vol.) system of Hardy, Holland & Naylor (1955) (system CS 4). Radioactive diaminopimelic acid isolated from cells was identified as the LL-isomer with the methanol–water–pyridine (77:20:10, by vol.) system of Rhuland, Work, Denman & Hoare (1955) (system CS 5).

Column chromatography. Individual volatile fatty acids were purified on an Amberlite CG-50 resin column by the method of Seki (1958). Before application to the column, samples were made neutral to phenol red with HCl to avoid heating of the resin by reaction with excess of alkali.

Paper electrophoresis. Three types of apparatus were used. (1) A glass tank with the Whatman 3MM paper strip (66 cm. × 12 cm.) supported on a glass stand between two troughs containing buffer (Ryle, Sanger, Smith & Kitai, 1955). The stand was wholly immersed in light petroleum, which was cooled by a coil through which cold water was circulated. This apparatus was used in most separations. (2) The apparatus used by Peel (1958). Whatman 3MM paper strips (67 cm. × 12 cm.) were used in this apparatus, which was used only with system ES 1 (see below). (3) A horizontal double-surface apparatus similar to that described by Gross (1961). The Whatman 3MM paper strip (67 cm. × 12 cm.) was enclosed between two aluminium blocks through which tap water circulated, and was insulated from the metal by thin sheets (0·3 mm.) of polyvinyl chloride. This apparatus was used in the purification of radioactive sodium lactate.

The following buffer systems were used in the separation of amino acids by paper electrophoresis: 0·033 M-ammonium acetate–acetic acid, pH 6·0, for 80 min. at 15 v/cm. (system ES 1); pyridine–acetic acid–water (25:1:225, by vol.), pH 6·5 (Ryle *et al.* 1955), for 100 min. at 25 v/cm. (system ES 2); pyridine–acetic acid–water (1:10:89, by vol.), pH 3·6 (Dixon, Kauffman & Neurath, 1958), for 100 min. at 25 v/cm. (system ES 3); pyridine–formic acid–water (3:40:960, by vol.), pH 2·2 (Richmond & Hartley, 1959), at 25 v/cm. for 100 min. (system ES 4).

Sodium [^{14}C]lactate was partially purified by electrophoresis in 0·5 M-$(NH_4)_2CO_3$, pH 8·9, for 60 min. at 20 v/cm.

Table 1 shows the successive electrophoretic and chromatographic procedures used in the isolation of amino acids from protein hydrolysates.

Detection and recovery of amino acids and lactate. Amino acids and lactate, after electrophoresis in $(NH_4)_2CO_3$ solution, were detected by dipping the paper in 0·1% ninhydrin in acetone and heating at 100° for 5 min. The areas in which radioactivity was detected by radioautography were cut out and eluted with distilled water in an air-tight cabinet. The volume collected was about 0·5 ml.; little or no radioactivity could be detected on counting the extracted paper after drying.

Degradation of ^{14}C-labelled compounds

Collection of carbon dioxide. The CO_2 evolved in degradations was collected by flushing the apparatus concerned with a stream of CO_2-free air for at least 20 min. after the reaction had ceased. The alkali and washings with CO_2-free distilled water were made up to 10 ml. and, where necessary, the CO_2 was estimated manometrically by displacement with acid. The solution was plated and counted as described above, after the addition of carrier Na_2CO_3 to give a final concentration of 60 mM. The CO_2 from manometric decarboxylations was collected by the method of Ormerod (1956).

Total combustion, by the method of Knight (1962), was used in the determination of the specific activities of amino acids and in the final stages of degradations.

Alanine. This was degraded via acetic acid by the method of Hoare & Gibson (1964).

Aspartic acid. Two procedures were used. (1) Aspartic acid was degraded completely by the method of Hoare & Gibson (1964), washed-cell suspensions of *Nocardia globerula* being used for the initial decarboxylation (Krebs & Bellamy, 1960). The alanine was recovered from the reaction products by electrophoresis (system ES 3) and

Table 1. *Separation and isolation of amino acids*

Expt.	Amino acid	Purification procedure Step (1)	Step (2)	Step (3)
A	Glutamic acid	ES 1	ES 2	CS 2
A	Aspartic acid	ES 1	ES 2	CS 2
A	Alanine	ES 1	CS 3	CS 4
A	Diaminopimelic acid	ES 1	CS 3	
B, C, D	Glutamic acid	ES 3	CS 2	
B, C	Aspartic acid	ES 3	CS 2	
B, C, D	Alanine	ES 3	CS 3	CS 4
B, C, D	Serine	ES 3	CS 3	ES 4

chromatography (system CS 4), with radioautography after each step. An alanine marker was used to check the behaviour of the radioactive alanine in both cases. After determination of the specific activity on a sample, the remainder was degraded by the method mentioned above. (2) The C-1 and C-4 carboxyl groups of aspartic acid were simultaneously converted into CO_2 manometrically by the method of Kemble & Macpherson (1954).

Glutamic acid. Several procedures were used for the complete and partial degradation of glutamic acid. (1) Degradation of glutamic acid via succinic acid was carried out by using the method of Hoare (1963*b*). This method does not distinguish C-2 of glutamic acid from C-5, or C-3 from C-4. (2) Complete degradation of glutamic acid was accomplished by a method similar to that of Hoare & Gibson (1964). The initial reaction was carried out by adding an equimolar amount of chloramine-T slowly to a solution of approx. 300 μmoles of glutamic acid through which a stream of CO_2-free air was passing slowly. When all the chloramine-T had been added, the temperature of the reaction mixture was raised slowly to 50° and maintained at 50° for 1 hr. The CO_2 absorber was then removed and the reaction mixture cooled to 0° to precipitate the toluene-*p*-sulphonamide, which was filtered off and washed once with ice-cold water. The reduction of succinic semialdehyde to butyric acid and the subsequent steps were carried out as described by Hoare & Gibson (1964). (3) Decarboxylation of glutamic acid C-1 was carried out with a washed-cell suspension of *Cl. welchii* by the manometric method described by Gale (1945). (4) Removal of C-5 of glutamate with NaN_3, by direct Schmidt reaction, was carried out by the method of Cutinelli, Ehrensvärd, Reio, Saluste & Stjernholm (1951).

Serine. A modification of the method of Sakami (1955) was used. CO_2-free air was passed through a 0·5 M-NaH_2PO_4 solution, containing approx. 300 μmoles of serine, for 10–15 min. After the addition of 3 ml. of 0·5 M-sodium metaperiodate solution, the CO_2 was collected at room temperature for 45 min. The reaction mixture was then made up to 50 ml. with water and a 20 ml. portion of this solution was taken for the oxidation of formic acid to CO_2. After the addition of 5 ml. of M-$BaCl_2$, pH 6·8, and 5 ml. of N-KOH, the precipitate was removed by centrifuging and the pellet washed twice with 10 ml. of water at 0°. The combined supernatants were adjusted to about pH 3 with acetic acid. The reaction mixture was flushed with CO_2-free air for 10 min. before adding 25 ml. of a solution containing 8% (w/v) of $HgCl_2$, 2% (w/v) of sodium acetate and 2% (w/v) of acetic acid (Pirie, 1946). The CO_2 was collected while the solution was boiled under reflux for 40 min., and then for a further 30 min. without heating.

Two 10 ml. portions of the original reaction mixture were taken for the estimation of formaldehyde as the dimedone derivative. Excess of periodate in each portion was removed by the addition of 2 ml. of N-H_2SO_4 and 3 ml. of 1·2 M-$NaAsO_2$. After a few minutes 6 ml. of M-sodium acetate, followed by 1 ml. of 8% (w/v) dimedone in ethanol, was added and the flask heated for 10 min. at 100°. After cooling, the crystalline derivative was filtered on a tared paper and washed with water.

Amino acids. These were estimated by the ninhydrin method of Yemm & Cocking (1955).

Volatile fatty acids. These were estimated by steam-distillation with the Markham (1942) still, or in the lactic acid apparatus of Elsden & Gibson (1954), followed by titration in a stream of CO_2-free air with CO_2-free alkali.

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

Chemical reagents. Unless otherwise stated all reagents were A.R. grade. All amino acids except diaminopimelic acid were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Butyric acid, propionic acid and acetic acid were redistilled A.R. grade. Lactic acid was obtained as a 70% solution of the sodium salt from British Drug Houses Ltd., Poole, Dorset, and was diluted to 35% sodium lactate with water before use. Samples of isomers of diaminopimelic acid were a gift from Dr D. S. Hoare.

RESULTS AND DISCUSSION

Incorporation of ^{14}C *from lactate into growing cells of* P. elsdenii

One initial growth experiment was carried out with [2-^{14}C]lactate to determine the extent to which lactate carbon was incorporated into amino acids. Radioactive amino acids from this and similar experiments were then degraded to determine the distribution of radioisotope within the amino acid molecules. In all, four growth experiments (Expts. A, B and C with sodium [2-^{14}C]lactate and Expt. D with sodium [1-^{14}C]lactate) were carried out; in Expts. B, C and D the lactate was purified by electrophoresis as described in the Materials and Methods section. These experiments used cultures growing on a complex medium. Washed cells of the organism were not used as results from this type of experiment cannot be assumed to apply to growing cells.

When the organism was grown on a medium containing [1-^{14}C]- or [2-^{14}C]-lactate, about 1% of the radioactivity was recovered in the protein hydrolysate in each case. With one culture on [2-^{14}C]lactate as substrate (Table 2), the radioactivity was estimated in each of the cell fractions; the protein fraction was more heavily labelled than any other fraction. The bulk of the radioactivity was in the medium, presumably as fermentation products, since lactate itself was absent from the supernatant of similar cultures with unlabelled

Table 2. *Incorporation of* [2-^{14}C]*lactate into cell fractions of* P. elsdenii

Cells from Expt. C were separated, washed and fractionated as described in the Materials and Methods section. Total radioactivity was determined as $BaCO_3$ after combustion of samples; the total carbon in each fraction was also determined by combustion except with the protein fraction where it was calculated from the amino nitrogen content.

Fraction	Contents	μequiv. of carbon	10^{-3} × Radioactivity (counts/min.)	% of radioactivity relative to lactate
Lactate		14000	45000	100
Supernatant	Fermentation products	14000	36000	80
Supernatant	CO_2	1800	0	0
'Cold-trichloroacetic acid'	Intermediates	—	50	0·1
'Ethanol'	Lipids and ethanol-soluble protein	500	140	0·3
'Ethanol–ether'	Lipids and ethanol-soluble protein	250	3	0·01
'Hot-trichloroacetic acid'	Nucleic acid	—	150	0·3
Hydrolysate	Protein	500	500	1·1

Table 3. *Specific activities of isolated amino acids*

Radioactivity was determined by combustion to CO_2 and plating as $BaCO_3$. Amino acids were determined by the ninhydrin method of Yemm & Cocking (1955).

^{14}C source and amino acid	Amount (μmoles)	10^{-3} × Radioactivity (counts/min.)	Sp. activity (counts/min./μmole)	Sp. activity relative to lactate
[2-^{14}C]Lactate	7400	40000	5400	1
Supernatant CO_2	3200	0	0	0
Alanine	9·4	9·9	1050	0·19
Aspartic acid	7·0	12·5	1800	0·33
Glutamic acid	6·0	13·7	2300	0·43
Serine	2·0	2·3	1150	0·21
[1-^{14}C]Lactate	7500	65000	8700	1
Supernatant CO_2	2100	13000	6200	0·71
Alanine	9·4	33·8	3600	0·41
Aspartic acid	11·6	120·0	10300	1·21
Glutamic acid	13·0	27·0	2100	0·24
Serine	5·0	13·0	2600	0·30

substrate. The low overall recovery (82%) is attributed to discarding the washings during harvesting and possibly to the loss of volatile fermentation products. The carbon dioxide recovered from the supernatant of the culture grown with [1-^{14}C]lactate contained 20% of the total radioactivity used and had a specific activity (counts/min./μmole) about 0·7 times that of the lactate used for growth (Table 3).

Radioautographs of two-dimensional chromatograms of samples of the protein hydrolysates showed that the radioactivity was concentrated in a few amino acids, which were identified as alanine, serine, aspartic acid and glutamic acid. The same amino acids were labelled from cultures with both [1-^{14}C]- and [2-^{14}C]-lactate. The amino acids in the bulk of the protein hydrolysates were separated and the most heavily labelled amino acids were rigorously purified and identified as the above, by using the procedures detailed in the Materials and Methods section (Table 1). The identity of the alanine serine, aspartic acid and glutamic acid was confirmed after purification by co-chromatography with authentic amino acid with a solvent system that had not been previously used in the isolation of the particular amino acid. In each case the radioactive area on the chromatogram, detected by radioautography, coincided with the sole ninhydrin-positive area on the paper. Other radioactive amino acids were identified as diaminopimelic acid from all the cultures, and threonine from Expts. C and D. Diaminopimelic acid from Expt. A was identified as the LL-isomer by chromatography in system CS 5 against markers of the LL-, DD- and DL-isomers of the compound.

The specific activities of the purified alanine, serine, aspartic acid and glutamic acid (Table 3) are, with the exception of the aspartic acid from [1-^{14}C]-lactate, less than the specific activity of the lactate used in each experiment. These values indicate that

these compounds are not synthesized entirely from lactate, though lactate carbon is incorporated to an appreciable extent; the specific activity of the aspartate from [1-^{14}C]lactate shows that more than one lactate C-1 has been incorporated. Traces of radioactivity were observed in other amino acids, which were not identified. This suggests that *P. elsdenii* may be able to synthesize most amino acids from lactate but that the organism uses preferentially the amino acids in the medium. Bryant & Robinson (1963) have shown that *P. elsdenii* utilizes exogenous amino acids in preference to synthesizing them from ammonia and glucose carbon. The results of the present experiments agree with these observations. They do not show whether lactate is used for the formation of any individual amino acid only when the supply of the latter in the medium is exhausted, or whether a diminished synthesis from lactate occurs in the presence of the amino acid concerned.

Degradation of radioactive amino acids

Samples of the alanine, aspartic acid, glutamic acid and serine were degraded, by the procedures described in the Materials and Methods section, to determine the distribution of radioactivity incorporated from [1-^{14}C]- and [2-^{14}C]-lactate within each amino acid molecule.

Before each degradation a known amount of pure carrier amino acid was added to a measured portion of the radioactive amino acid. A quantitative method was used in each case for the estimation of the recovery of intermediates and, where carrier was added, the amount was measured accurately, allowing subsequent specific activities to be related to that of the amino acid starting material. Only with alanine, formed in the degradation of aspartate, and succinic acid, formed in the degradation of glutamate, were the specific activities of intermediates independently determined.

Patterns of ^{14}C incorporation from lactate are presented by using the symbols L-1 and L-2 for lactate C-1 and C-2 respectively and L-3 where incorporation of lactate C-3 is assumed from the absence of label from either [1-^{14}C]- or [2-^{14}C]-lactate. The possibility that carbon atoms designated L-3 may be derived wholly from media constituents other than lactate is not excluded, but is considered remote; such carbon atoms always occurred adjacent to L-2 carbon atoms and no reaction is known in *P. elsdenii* that splits the lactate 'skeleton' between C-2 and C-3. Incomplete recoveries of radioactivity are attributed to limitations in the degradation procedure or to manipulative errors. In the overall labelling patterns, amounts of radioisotope less than 10% of that found in the most radioactive carbon atoms have not been taken into account in interpreting the results.

Alanine. Samples of alanine from all growth experiments were degraded. The details of illustrative degradations of alanine from both a [2-^{14}C]-lactate and a [1-^{14}C]lactate culture are summarized in Table 4. All the results were clear-cut in that label from both [1-^{14}C]- and [2-^{14}C]-lactate was confined to a single carbon atom. Thus C-2 of lactate appeared only in C-2 of alanine, and C-1 of lactate in C-1 of alanine. There was close agreement between the results of the degradations of alanine from the three different growth experiments (Expts. A, B and C).

The results indicate that the carbon atoms of alanine have the following origins:

$$\underset{\text{L-1}}{CO_2H}\cdot\underset{\text{L-2}}{CH(NH_2)}\cdot\underset{\text{(L-3)}}{CH_3}$$

This labelling pattern suggests that alanine is formed directly from lactate without rearrangement of the carbon chain. This conversion would be readily explained by the following known reactions:

$$\text{Lactate} \rightarrow \text{pyruvate} \rightarrow \text{alanine}$$

P. elsdenii contains a lactate dehydrogenase (Baldwin, Emery & Wood, 1963) and reactions catalysing the conversion of pyruvic acid into alanine have been widely demonstrated in bacteria (see Umbarger & Davis, 1962). *P. elsdenii* also catalyses the carbon dioxide–pyruvate exchange reaction, and it is possible that a small amount of carbon dioxide from the medium is incorporated into alanine C-1 in this way.

Serine. Label from [2-^{14}C]lactate was specifically incorporated into serine C-2, and from [1-^{14}C]lactate into serine C-1 (Table 5). This distribution of radioactivity gives a labelling pattern similar to that obtained with alanine:

$$\underset{\text{L-1}}{CO_2H}\cdot\underset{\text{L-2}}{CH(NH_2)}\cdot\underset{\text{(L-3)}}{CH_2\cdot OH}$$

This suggests that serine is synthesized by a pathway that does not involve breakage of the lactate carbon chain.

Serine synthesis is known to occur in *E. coli* and *Salmonella typhimurium* from 3-phosphoglyceric acid via a series of C_3 intermediates (Umbarger & Umbarger, 1962; Umbarger, Umbarger & Siu, 1963). Phosphoglyceric acid is a known intermediate in carbohydrate metabolism and mechanisms are known for its formation from lactate or pyruvate without disrupting the C_3 chain of these compounds (Wood & Stjernholm, 1962). A similar pathway probably operates in *Cl. kluyveri*; Tomlinson (1954*a*) found that serine was formed from a C_3 unit related to alanine. An alternative known route of serine formation would involve

Table 4. *Degradation of alanine from cells grown with* [1-^{14}C]- *and* [2-^{14}C]-*lactate*

Samples of the alanine isolated from cells grown with [1-^{14}C]- and [2-^{14}C]-lactate were degraded. Additional carrier was added where indicated. Reaction steps: (1) decarboxylation with ninhydrin; (2) decarboxylation by Schmidt reaction; (3) combustion of methylamine.

Expt.	^{14}C source	Reaction step	Reactant (μmoles)	Carrier added (μmoles)	Total radioactivity (counts/min.)	Sp. activity (counts/min./ μmole)	Product (alanine carbon atom)	Amount (μmoles)	Total radioactivity (counts/min.)	Sp. activity (counts/min./ μmole)	Sp. activity relative to alanine
D	[1-^{14}C]Lactate	(1)	Alanine (1·0)	107	3400	31·4	CO_2 (C-1)	107	3210	30·0	0·96
							Acetate	62	—	—	
		(2)	Acetate (62)	0	—	—	CO_2 (C-2)	52	0	0	0
		(3)					Methylamine (C-3)	71	0	0	0
A	[2-^{14}C]Lactate	(1)	Alanine (2·0)	107	1845	16·9	CO_2 (C-1)	106	0	0	0
							Acetate	85	—	16·9*	
		(2)	Acetate (85)	387	—	3·1*	CO_2 (C-2)	441	1365	3·1	1·00
		(3)					Methylamine (C-3)	456	25	0·05	0·02

* These values were calculated assuming the acetic acid recovered had the same specific activity as the alanine.

Table 5. *Degradation of serine from cells grown with* [1-^{14}C]- *and* [2-^{14}C]-*lactate*

Samples of the serine isolated from cells grown with [1-^{14}C]- and [2-^{14}C]-lactate were degraded. Reaction steps: (1) degradation with sodium metaperiodate; (2) oxidation of formate, in two-fifths of reaction products, to CO_2; (3) conversion of formaldehyde into the dimedone derivative.

Expt.	^{14}C source	Reaction step	Reactant (μmoles)	Carrier added (μmoles)	Total radioactivity (counts/min.)	Sp. activity (counts/min./ μmole)	Product (serine carbon atom)	Amount (μmoles)	Total radioactivity (counts/min.)	Sp. activity (counts/min./ μmole)	Sp. activity relative to serine
D	[1-^{14}C]Lactate	(1)	Serine (2·5)	284	6600	23·0	CO_2 (C-1)	230	5700	24·8	1·08
		(2)					Formate (C-2)	100	50	0·5	0·02
		(3)					Formaldehyde (C-3)	48	0	0	0
C	[2-^{14}C]Lactate	(1)	Serine (1·0)	305	1470	4·80	CO_2 (C-1)	272	0	0	0
		(2)					Formate (C-2)	92	480	5·2	1·08
		(3)					Formaldehyde (C-3)	49	0	0	0

condensation of a C_2 unit (glycine) with a C_1 unit (Lascelles & Woods, 1954). This can be excluded for the following reasons: (1) if such a pathway used unlabelled glycine from the medium, then no isotope from lactate would enter C-1 and C-2 of serine; (2) if radioactive glycine were formed from lactate, this would be detected in the purification procedure used for serine (Table 1), in which glycine was separated at the last step. With both [1-^{14}C]- and [2-^{14}C]-lactate (Expts. B, C and D) the radioautograph showed only one radioactive band corresponding to serine, whereas the glycine band, which was readily detected with ninhydrin, contained no radioactivity.

Aspartic acid. Samples of aspartate from both [2-^{14}C]lactate (Expts. A and B) and [1-^{14}C]lactate were degraded completely (Table 6), the degradation being repeated in the latter case. Almost all the radioactivity from lactate C-2 was found in C-2 of aspartate. Partial degradation of the aspartic acid from Expt. A, by decarboxylation of both carboxyl groups with chloramine-T (Kemble & Macpherson, 1954), confirmed the absence of significant amounts of label from either carboxyl carbon.

The label in aspartic acid from [1-^{14}C]lactate was distributed between the carboxyl carbon atoms, with C-4 containing approx. 70% of the radioactivity in C-1. The results may be summarized in the labelling pattern:

$$\underset{\text{L-1}}{CO_2H}\cdot\underset{\text{L-2}}{CH(NH_2)}\cdot\underset{\text{(L-3)}}{CH_2}\cdot\underset{\text{L-1}}{CO_2H}$$

This labelling pattern indicates that the carbon 'skeleton' of aspartic acid is formed by condensation of a C_3 unit derived directly from lactate with a C_1 unit, probably carbon dioxide, derived partially from lactate C-1. The fact that the specific activity of aspartic acid C-4 was less than that of C-1 is in agreement with this route of aspartic acid formation, as the carbon dioxide in the [1-^{14}C]lactate culture supernatant was shown to be of lower specific activity than the [1-^{14}C]lactate (Table 3).

Isotope studies with other organisms, both aerobic and anaerobic, have implied a similar route of aspartate formation (Hoare, 1963*a*), and several enzyme systems are known that can account for this pattern (Wood & Stjernholm, 1962).

The present results enable certain pathways to be definitely eliminated. First, the oxidation of lactate to acetate with subsequent formation of the aspartate 'skeleton' via the glyoxylate cycle would place isotope from [2-^{14}C]lactate into both carboxyl groups of aspartate and is thus excluded. Secondly, any pathway with the aspartase reaction as the terminal step would involve fumarate, a symmetric intermediate, and lead to randomization of label between C-1 and C-4 and between C-2 and C-3 of aspartate; this was not observed.

Table 6. *Degradation of aspartic acid from cells grown with* [1-^{14}C]- *and* [2-^{14}C]-*lactate*

Samples of the aspartic acid isolated from cells grown with [1-^{14}C]- and [2-^{14}C]-lactate were degraded. Additional carrier was added where indicated. Reaction steps: (1) decarboxylation with a washed-cell suspension of *Nocardia globerula*; (2) decarboxylation with ninhydrin; (3) decarboxylation by Schmidt reaction; (4) combustion of methylamine.

Expt.	^{14}C source	Reaction step	Reactant (μmoles)	Carrier added (μmoles)	Total radioactivity (counts/min.)	Sp. activity (counts/min./μmole)	Product (aspartate carbon atom)	Amount (μmoles)	Total radioactivity (counts/min.)	Sp. activity (counts/min./μmole)	Sp. activity relative to aspartate
D	[1-^{14}C]Lactate	(1)	Aspartate (3·8)	9·4	37800	2860	CO_2 (C-4)	13·0	17600	1350	0·47
							Alanine	9·2	13300	1450	
		(2)	Alanine (5·0)	105	7700	70	CO_2 (C-1)	99	7800	79	0·57
							Acetate	80	—	—	
		(3)	Acetate (80)	0	—	—	CO_2 (C-2)	55	0	0	0
		(4)					Methylamine (C-3)	65	0	0	0
A	[2-^{14}C]Lactate	(1)	Aspartate (1·5)	7·5	1260	140	CO_2 (C-4)	8·6	60	7	0·05
							Alanine	8·3	1010	122	
		(2)	Alanine (5·0)	107	580	5·2	CO_2 (C-1)	109	0	0	0
							Acetate	93	—	—	
		(3)	Acetate (93)	387	—	—	CO_2 (C-2)	421	390	0·93	0·94
		(4)					Methylamine (C-3)	80	0	0	0

Table 7. *Degradation of glutamic acid from cells grown with* [1-^{14}C]*lactate* (*Expt. D*)

Glutamic acid was degraded: I, by decarboxylation with chloramine-T; II, by direct Schmidt reaction; III, by decarboxylation with a washed-cell suspension of *Cl. welchii*.

Degradation procedure	Reactant (μmoles)	Carrier added (μmoles)	Total radio-activity (counts/min.)	Sp. activity (counts/min./μmole)	Product (glutamate carbon atom)	Amount (μmoles)	Total radio-activity (counts/min.)	Sp. activity (counts/min./μmole)	Sp. activity relative to glutamate
I	Glutamate (0·6)	6·6	1350	188	CO_2 (C-1)	6·9	1332	193	1·03
II	Glutamate (0·2)	360	340	0·95	CO_2 (C-5)	240	10	0·04	0·04
III	Glutamate (0·6)	8·0	1350	157	CO_2 (C-1)	8·4	1170	139	0·89

Glutamic acid. A sample of the glutamic acid from [1-^{14}C]lactate was decarboxylated with chloramine-T. All the radioactivity was located in C-1 (Table 7). This degradation was repeated with similar results, and the absence of label from other carbon atoms of glutamic acid was confirmed by completing the degradation via succinic acid. Decarboxylation of C-1 with a washed-cell suspension of *Cl. welchii* (Table 7) also showed that most of the radioactivity was located in this carbon atom, relatively little label being present in the residue. The decarboxylation of C-5 by the Schmidt reaction (Table 7) confirmed the absence from this carbon atom of label from [1-^{14}C]lactate.

Glutamate from both Expt. A (Table 8) and Expt. B with [2-^{14}C]lactate was degraded via succinate. Since succinate is a symmetrical molecule it is not possible to distinguish between glutamate C-2 and C-5, or between C-3 and C-4. The results show that about half of the label was located in C-2, C-5 or both, and the remainder in C-3, C-4 or both. To determine which of these carbon atoms were labelled, a sample of the glutamate from Expt. C was degraded completely via butyric acid. The results (Table 8) show that label was confined to C-3 and C-5, and suggest that, of the two, C-3 contained slightly more radioactivity. The results from Schmidt decarboxylation of glutamate from [2-^{14}C]lactate (Table 8) confirmed that this was the case, rather less than half of the radioactivity being released in the carbon dioxide from C-5 of glutamate.

The distribution of label found in glutamic acid may be expressed in the following labelling pattern:

$$\underset{\text{L-1}}{CO_2H}\cdot\underset{\text{(L-3)}}{CH(NH_2)}\cdot\underset{\text{L-2}}{CH_2}\cdot\underset{\text{(L-3)}}{CH_2}\cdot\underset{\text{L-2}}{CO_2H}$$

This pattern cannot be explained by any simple condensation involving C_2 and C_3 units. Assuming the labelling of aspartic acid to reflect that in oxaloacetate, this pattern would be produced by the reactions of the tricarboxylic acid pathway (Scheme 1). Other studies have shown this pathway to operate widely in micro-organisms under aerobic conditions (Hoare, 1963*a*). 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 and ammonia as nitrogen source; it was found that $^{14}CO_2$ was incorporated specifically into glutamate C-1, in accordance with the tricarboxylic acid pathway, and two of the necessary enzymes, isocitrate dehydrogenase and glutamate dehydrogenase, were demonstrated in cell-free extracts. However, $^{14}CO_2$ can be incorporated into C-1 of glutamate by routes other than the tricarboxylic acid pathway as in *Rhodospirillum rubrum* (Cutinelli *et al.* 1951; Hoare, 1963*b*).

An alternative, though perhaps less probable, pathway that could account for the present tracer studies and those of Burchall *et al.* (1964) involves crotonyl-CoA and glutaconate as intermediates (Scheme 1). There is no complete biochemical precedent for this pathway, but most of the reactions are known. There is strong evidence that crotonyl-CoA is an intermediate in the formation of butyrate from lactate by *P. elsdenii* (Baldwin & Milligan, 1964), and reactions similar to the remaining steps of the pathway have been demonstrated in mammalian tissues (Tustanoff & Stern, 1963).

Isotopic studies of the type described above should ideally be carried out with cells growing on a defined medium and with a radioactive substrate of absolute purity. The possibility of impurities being incorporated specifically into the amino acids in the present work can be largely discounted, on the following grounds. Radioactive amino acids present in the lactate would be separated during the electrophoretic purification of the lactate. The amino acids were separated first as protein, then as

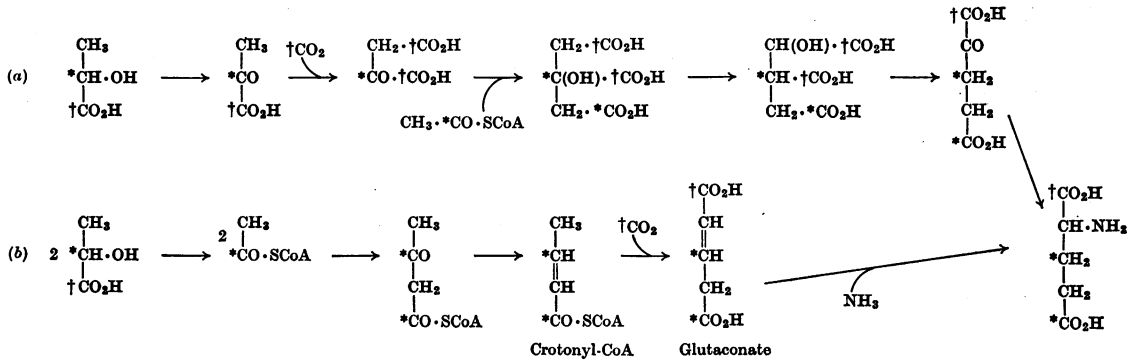
Table 8. *Degradation of glutamic acid from cells grown with* [2-^{14}C]*lactate*

Glutamic acid was degraded: I, via succinate (Hoare, 1963*b*); II, by a modification of the procedure of Hoare & Gibson (1964); III, by direct Schmidt reaction.

Degradation procedure	Expt.	Reactant (μmoles)	Carrier added (μmoles)	Total radioactivity (counts/min.)	Sp. activity (counts/min./μmole)	Product (glutamate carbon atom)	Amount (μmoles)	Total radioactivity (counts/min.)	Sp. activity (counts/min./μmole)	Sp. activity relative to glutamate
I	A	Glutamate (1·6)	13·5	2000	132	CO_2 (C-1)	15·0	0	0	0
						Succinate	12·1*	1600	132	
		Succinate (9·7)*	0	1280	132	$CO_2\left(\frac{C\text{-}2+C\text{-}5}{2}\right)$	9·7	280	29	0·22 (mean C-2+C-5)
						Propionate	10·0	—	—	
		Propionate (8·3)	799	—	1·36†	$CO_2\left(\frac{C\text{-}2+C\text{-}5}{2}\right)$	660	220	0·33	0·24 (mean C-2+C-5)
						Ethylamine (C-3+C-4)	1150	435	0·38	0·28 (mean C-3+C-4)
II	C	Glutamate (1·8)	300	7850	26·0	CO_2 (C-1)	165	0	0	0
						Butyrate	54	—	—	
		Butyrate (54)	86	—	10·1†	CO_2 (C-5)	89	409	4·6	0·46
						Propionate	65	—	—	
		Propionate	65	—	10·1†	CO_2 (C-4)	72	0	0	0
						Acetate	34	—	—	
		Acetate (34)	546	—	0·59†	CO_2 (C-3)	396	158	0·40	0·68
						Methylamine (C-2)	488	0	0	0
III	B	Glutamate (0·45)	115	1030	9·0	CO_2 (C-5)	109	432	4·0	0·44

* The amount of succinic acid was calculated from the yield in the subsequent decarboxylation.

† These specific activities were calculated assuming no loss of radioactivity in the formation of the intermediates from glutamate.



Scheme 1. Possible pathways of glutamate formation: (*a*) via tricarboxylic acids; (*b*) via glutaconate. † and * denote carbon from lactate C-1 and lactate C-2 respectively. Route (*b*) involves a shifting of the double bond and the thio ester of vinylacetate (but-3-enoate) is an alternative intermediate to crotonyl-CoA. In route (*a*) it is assumed that the conversion of oxaloacetate into isocitrate proceeds with the same stereospecificity as in animal tissues (see e.g. Lorber, Utter, Rudney & Cook, 1950).

amino acids. This would seem to eliminate the danger of unrelated radioactive impurities being present in the purified amino acids. Loeb & Lichtenberger (1950) have shown that multiple spots arise during chromatography of lactic acid as a result of polymerization by esterification. If these were utilized by *P. elsdenii*, they would presumably be hydrolysed and then metabolized in the same way as lactate. Finally, the results of degradation of amino acids isolated from cells grown on untreated [2-^{14}C]lactate, or on partially purified [2-^{14}C]lactate, were closely similar.

These tracer experiments show that *P. elsdenii* can utilize lactate carbon for the formation of at least some amino acids and, where the amino acids were degraded, they suggest, though they do not prove, the operation of known pathways. They further eliminate a number of alternative pathways and thus provide a basis for a more detailed enzymic investigation.

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