The Biosynthesis of Valine from Isobutyrate by *Peptostreptococcus elsdenii* and *Bacteroides ruminicola*

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1. Growing cultures of *Peptostreptococcus elsdenii* and *Bacteroides ruminicola* incorporate ^{14}C from $[1-^{14}\text{C}]$ isobutyrate into the valine of cell protein. With *P. elsdenii* some of the ^{14}C is also incorporated into leucine. 2. Crude cell-free extracts of both organisms in the presence of glutamine, carbon dioxide and suitable sources of energy and electrons incorporate ^{14}C from $[1-^{14}\text{C}]$ isobutyrate into valine but not into leucine. 3. With extracts of *P. elsdenii* treated with DEAE-cellulose the reaction is dependent on ATP, CoA, thiamin pyrophosphate, molecular hydrogen and a low-potential electron carrier (ferredoxin, flavodoxin or benzyl viologen). 4. The same extracts incorporate ^{14}C from $\text{NaH}^{14}\text{CO}_3$ into valine in the presence of isobutyrate plus ATP, CoA, glutamine and ferredoxin; isobutyryl-CoA or isobutyryl phosphate plus CoA will replace the isobutyrate plus CoA and ATP. With acetyl phosphate in place of isobutyryl phosphate, ^{14}C is incorporated into alanine. With isovalerate or 2-methylbutyrate in place of isobutyrate, ^{14}C is incorporated into leucine and isoleucine respectively. 5. When carrier 2-oxoisovalerate is added to the carboxylating system ^{14}C from $[1-^{14}\text{C}]$ isobutyrate passes into the oxo acid fraction. 6. It is concluded that these two organisms form valine from isobutyrate by the sequence isobutyrate $\rightarrow$ isobutyryl-CoA $\rightarrow$ 2-oxoisovalerate $\rightarrow$ valine and that the reductive carboxylation of isobutyrate is catalysed by a system similar to the pyruvate synthetase of clostridia and photosynthetic bacteria.

The microflora of the rumen is capable of both producing and utilizing branched-chain fatty acids. Thus mixed ruminal micro-organisms degrade branched-chain amino acids to produce branched-chain fatty acids with one less carbon atom (el Shazly, 1952). These fatty acids are growth factors for important ruminal bacteria of several genera (Bryant & Doetsch, 1955; Allison, Bryant & Doetsch, 1962a; Bryant & Robinson, 1962) and their metabolic fate has been studied in both pure and mixed cultures by using ^{14}C as tracer. Carbon skeletons of branched-chain fatty acids were incorporated mainly into the lipid and protein fractions of the cells. Higher branched-chain fatty acids and fatty aldehydes were synthesized from isovalerate and isobutyrate (Allison, Bryant, Katz & Keeney, 1962b; Wegner & Foster, 1963). In the protein fraction leucine was synthesized from isovalerate (Allison *et al.* 1962a), valine from isobutyrate (Allison & Bryant, 1963) and isoleucine from 2-methylbutyrate (Robinson & Allison, 1969). The tracer evidence also indicated that the carboxyl groups of these amino acids were formed by car-

boxylation. Thus *Ruminococcus flavefaciens* incorporated ^{14}C from $^{14}\text{CO}_2$, but not from $[^{14}\text{C}]$ -formate, into all three of the branched-chain amino acids and in each case the ^{14}C was confined to the carboxyl group; in addition, ^{14}C from $[1-^{14}\text{C}]$ isovalerate was incorporated exclusively into C-2 of leucine. The present investigation was conducted to obtain more information about the carboxylation process with *Peptostreptococcus elsdenii* and *Bacteroides ruminicola* as experimental organisms. Part of the work has been reported in brief (Allison & Peel, 1968).

MATERIALS AND METHODS

Micro-organisms. *P. elsdenii* strain B-159 and *B. ruminicola* strains 23 and GA-33 were obtained from the culture collection of Dr M. P. Bryant. *P. elsdenii* strain LC-1 (N.C.I.B. 8927) was the organism described by Elsden, Volcani, Gilchrist & Lewis (1956).

Growth of bacteria and preparation of extracts. Cultures of *P. elsdenii* were maintained and grown on the 'complete' medium of Bryant & Robinson (1961) with the following modifications: (a) an amino acid mixture (L-serine,

L-glutamic acid, L-proline, L-lysine hydrochloride, L-histidine, L-arginine, L-methionine, DL-aspartic acid, DL-threonine, DL-alanine and glycine, each at a final concentration of 0.1 mg/ml) was used instead of casein hydrolysate, (b) glucose (3 mg/ml) was substituted for cellobiose, (c) Na_2S was omitted and (d) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (10 μg /ml) was added. After autoclaving, CO_2 filtered through sterile cotton was bubbled through the medium while it was cooled under running water and the medium was then supplemented with the Na_2CO_3 , cysteine hydrochloride and FeSO_4 that had been sterilized separately under N_2 . The organism was transferred serially (0.1 ml/5 ml) in this medium by using the anaerobic technique described by Hungate (1966). Cultures in rubber-stoppered tubes (18 mm $\times$ 150 mm) were incubated at 37°C until good growth was obtained (usually about 20 h) and then were held at room temperature for 1 or 2 days before the next transfer was made.

Growth was followed by measuring the turbidity with a colorimeter (Bausch and Lomb Spectronic 20) at 600 nm in 18 mm-diameter tubes. Cells used for enzymic studies were obtained from batch cultures at the end of the exponential phase of growth [20 litres, 5% inoculum grown for 20–40 h at 38°C, harvested with a continuous-flow centrifuge (Sorvall model R-C2) and stored as cell paste at –40°C under N_2]. All subsequent treatment of cells and extracts was at 0–4°C and under CO_2 or N_2 where possible. During column operations the collecting tubes and the space above the column were flushed with N_2 .

For the preparation of ultrasonic extracts the cell paste was suspended in 5 ml of buffered thiol solution/g of cell paste. The buffered thiol solution was prepared by boiling 0.02 M-phosphate buffer, pH 7.0 (KH_2PO_4 – Na_2HPO_4 mixture), cooling under N_2 and then adding dithiothreitol to a final concentration of 0.25 mg/ml. The suspension was treated for 4 min in a 20 kHz ultrasonic oscillator (MSE) while flushing with N_2 and then centrifuged at 24 000 g for 15 min. The supernatant is referred to as 'crude extract'.

DEAE-cellulose-treated extracts were obtained by passing up to 20 ml of crude extract through a column (1.2 cm diam. $\times$ 5.5 cm) of DEAE-cellulose (BioRad Laboratories, Richmond, Calif., U.S.A.) previously equilibrated with the buffered thiol solution under N_2 . The column was eluted with buffered thiol solution and a volume of eluate equal to that of the original extract was collected, starting when the amber material began to emerge from the column.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with dry twice-crystallized bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) as the standard.

B. ruminicola was grown and extracted in the same way as *P. elsdenii* except that L-methionine (45 μg /ml) replaced the amino acid mixture in the growth medium and haemin (0.5 μg /ml) was added.

Incorporation of isobutyrate by growing cultures. The organisms were grown in rubber-stoppered tubes (10 ml) as described above except that the mixture of branched-chain fatty acids was replaced by [$1\text{-}^{14}\text{C}$]isobutyrate (final concentration 100 μM with *P. elsdenii*, 60 μM with *B. ruminicola*; 2.8 $\mu\text{Ci}/\mu\text{mol}$). The cells were harvested at or before the end of the exponential growth stage, washed and fractionated by the method of Roberts, Cowie, Abelson,

Bolton & Britten (1957). Samples were dissolved in the XDC counting solution of Bruno & Christian (1961) and radioactivity was measured in a model 314EX scintillation spectrometer (Packard Instrument Co.). Efficiency was measured after the addition of an internal standard ([^{14}C]toluene), and results are given as d.p.m.

The amino acids of the protein hydrolysates (Roberts *et al.* 1957) were examined by chromatography as described below.

Fixation of isobutyrate and carbon dioxide by cell-free extracts. Reaction mixtures, with either [$1\text{-}^{14}\text{C}$]isobutyrate or $\text{H}^{14}\text{CO}_3^-$ and other components as indicated in individual experiments, were contained in tubes (11 mm diam. $\times$ 100 mm) and flushed with the appropriate gas phase for 45 s before addition of the microbial extract. The tube was then closed with a rubber bung as the needle admitting the gas was withdrawn. After incubation the reaction was stopped by adding 0.3 ml of 12 M-HCl. The precipitate was removed by centrifuging at 10 000 g for 15 min and suitable portions of the supernatant were taken for the measurement of non-acid-volatile radioactivity. Each sample was dried in a glass scintillation vial under an i.r. lamp, redissolved in 0.5 ml of 0.2 M-HCl, dried again and redissolved in 0.5 ml of water. The XDC counting solution (15 ml) of Bruno & Christian (1961) was added and the radioactivity of the sample counted in the scintillation spectrophotometer.

Where required, the 2,4-dinitrophenylhydrazones of oxo acids were isolated from the reaction mixture as described by Rabinowitz (1960), except that when 2-oxo-isovalerate was present the precipitated hydrazone was dissolved in chloroform-methanol (2:1, v/v) since it was difficult to dissolve in Na_2CO_3 . The solvent was then evaporated off, the residue dissolved in 1.5 M-NaOH and the hydrazone reprecipitated by the addition of 10 M-HCl. The precipitated hydrazones were plated by filtration; they were dried and the radioactivity was measured with an end-window counter (model D47; Nuclear-Chicago Corp.) or with the Panax equipment described by Somerville & Peel (1967). The weight of hydrazones was reasonably constant and no correction was applied for self-absorption.

Separation of amino acids. Amino acids in protein hydrolysates or reaction mixtures were separated by paper chromatography of suitable portions. The solvent systems used were: 1, butan-1-ol-acetone-water (2:2:1, by vol.) as used by Hardy, Holland & Nayler (1955); 2, butan-1-ol-acetic acid-water (12:3:5, by vol.), Smith (1960); 3, the buffered benzyl alcohol-butan-1-ol system at pH 8.4 of McFarren (1951). Samples that contained excessive amounts of salt were treated with Dowex 50 (Roberts *et al.* 1957) before chromatography. The identity of isotopically labelled valine, alanine and leucine was established by chromatography in at least two of the solvent systems and by co-chromatography with authentic markers. System 3 was used when the separation of leucine from isoleucine was required.

Radioactive areas on chromatograms were located by radioautography (Knight, 1962) and with a chromatogram scanner (model 7200; Packard Instrument Co. Inc.).

Materials. Unless otherwise stated reagents were of analytical-reagent grade. Amino acids, coenzymes, ATP, ADP, 2-phosphoenolpyruvate, 2-oxoisovalerate, sodium pyruvate, 2-mercaptoethanol and dithiothreitol were

from Calbiochem (Los Angeles, Calif., U.S.A.); acetyl phosphate (dilithium salt) was from Sigma Chemical Co. (St Louis, Mo., U.S.A.); avidin (3300 units/mg) was from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.); benzyl viologen was from Mann Research Laboratories (New York, N.Y., U.S.A.). Ferredoxin from *Clostridium pasteurianum* was obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) and ferredoxin from *P. elsdenii* LC-1 was prepared as described by Mayhew (1966); crystalline flavodoxin from *P. elsdenii* LC-1 was a gift from Dr S. G. Mayhew.

Gases were freed of trace amounts of O₂ by passing them through reduced copper turnings heated at 320–340°C (Hungate, 1966).

Isobutyryl-CoA was synthesized from isobutyric anhydride (City Chemicals Corp., New York, N.Y., U.S.A.), and CoA as described by Simon & Shemin (1953). Isobutyryl phosphate was synthesized from K₂HPO₄ and isobutyric anhydride by the method of Avison (1955) as given by Stadtman (1957); the concentration was determined by the procedure of Lipmann & Tuttle (1945).

Sodium [1-¹⁴C]isobutyrate was obtained from Mallinckrodt Nuclear (Orlando, Fla., U.S.A.). It was separated from trace amounts of radioactive material not volatile in acid by descending paper chromatography on Whatman no. 3 MM paper. The solvent was butan-1-ol saturated with 1.5M-NH₃ (Brown, 1950). The NaH¹⁴CO₃ was obtained from Volk Radiochemical Co. (Burbank, Calif., U.S.A.).

RESULTS

Incorporation of isobutyrate by growing cells. When *Peptostreptococcus elsdenii* strain B-159 was grown with [1-¹⁴C]isobutyrate from a small inoculum (0.1ml/10ml) to a final extinction of 0.5 at 600nm in 21 h, 14% of the ¹⁴C was incorporated into the cells. On fractionation of the cells 90% of the incorporated ¹⁴C was recovered in the protein fraction, and when a chromatogram of the protein hydrolysate was scanned radioactivity was confined to two areas corresponding to valine and leucine (70 and 30% of the ¹⁴C in the protein respectively). The identity of these radioactive products was confirmed by chromatography of the eluted radioactive material from these two areas.

The defined medium used for this experiment did not contain valine; when the organism was grown on a complex medium containing corn-steep liquor as a growth-factor supplement (Walker, 1958) the amount of ¹⁴C incorporated from [1-¹⁴C]isobutyrate was diminished to about one-fortieth. Another strain of *P. elsdenii* (LC-1) that had been maintained in artificial culture for several years incorporated less than 0.01% of the ¹⁴C from [1-¹⁴C]isobutyrate during growth in the defined medium. All subsequent experiments were done with strain B-159.

B. ruminicola strain 23 incorporated 4% of the ¹⁴C from [1-¹⁴C]isobutyrate during growth. Of the incorporated ¹⁴C only 48% was recovered in the protein fraction and 38% was in the lipid fraction.

Table 1. *Incorporation of ¹⁴C from [1-¹⁴C]isobutyrate into valine by crude cell-free extract of P. elsdenii*

The complete reaction mixture contained, in a final volume of 2ml, crude cell-free extract equivalent to 2.6 (Expt. 1) or 2.0 (Expt. 2) mg of protein, 5μmol of ATP, 0.2μmol of CoA, 10μmol of NaHCO₃, 10μmol of L-glutamine, 100μmol of phosphate buffer, pH 7.0 (KH₂PO₄-Na₂HPO₄ mixture), 2μmol of MnCl₂, 1μmol of MgSO₄, 1μmol of CoCl₂, 10μmol of sodium [1-¹⁴C]isobutyrate (9×10⁵ d.p.m.) and, where indicated, 10μmol of sodium pyruvate, 10μmol of phosphoenolpyruvate and 5μmol of ADP. Tubes were incubated at 38°C for 60 min under H₂. For incubations without H₂ the gas phase was N₂. Boiled enzyme refers to a control with crude extract previously heated at 100°C for 10 min. Isobutyrate fixed was measured as the ¹⁴C recovered in the non-acid-volatile fraction.

System	Isobutyrate fixed (d.p.m.)	
	Expt. 1	Expt. 2
Complete	24 000	12 000
Pyruvate added	41 000	28 000
H ₂ omitted	2 700	3 300
H ₂ omitted, pyruvate added	7 100	—
ATP omitted	960	—
ATP omitted, phosphoenolpyruvate added	4 600	—
ATP omitted, phosphoenolpyruvate and ADP added	52 000	—
Boiled enzyme	—	130

A single radioactive peak that corresponded to the position of valine was detected when chromatograms of hydrolysed protein were scanned. With strain GA-33 of *B. ruminicola* grown in [1-¹⁴C]isobutyrate the relative recoveries of ¹⁴C in protein and lipid fractions of the cells were different (71 and 18% respectively), but again the only radioactive area detected on paper chromatograms of protein hydrolysates corresponded to that of valine.

Incorporation of isobutyrate by cell-free extracts. Crude cell-free extracts of *P. elsdenii* B-159 fixed ¹⁴C into the non-acid-volatile fraction when incubated with [1-¹⁴C]isobutyrate under conditions similar to those of Buchanan, Bachofen & Arnon (1964) (Table 1). Radioactive valine was identified as a product by chromatography and was the sole radioactive product detected on the chromatograms. With some preparations of the bacterial extract pyruvate stimulated the reaction. Since extracts of the organism are known to catalyse the phosphoclastic reaction (Peel, 1960), pyruvate could be serving as a source of CO₂, electrons or energy. Isobutyrate fixation was only observed under strictly anaerobic conditions. In the absence of H₂ (under N₂) the reaction was greatly diminished but could be restored in part by pyruvate, indicating that a source of low-potential electrons is essential.

Table 2. *Requirements for incorporation of ^{14}C from $[1\text{-}^{14}\text{C}]\text{isobutyrate}$ by extracts of *P. elsdenii* and *B. ruminicola**

In the experiments with *P. elsdenii* the complete system was as in Table 1 except that (i) DEAE-cellulose-treated extract was used containing 1.4, 1.6 or 2.4 mg of protein in Expts. 1, 2 and 3 respectively, (ii) the $10\text{ }\mu\text{mol}$ of $[1\text{-}^{14}\text{C}]\text{isobutyrate}$ contained 9.4×10^5 d.p.m. (Expt. 1), 7.1×10^5 d.p.m. (Expt. 2) or 3.3×10^5 d.p.m. (Expt. 3) and (iii) $10\text{ }\mu\text{mol}$ of sodium pyruvate, $130\text{ }\mu\text{g}$ of ferredoxin prepared from *P. elsdenii* and $0.5\text{ }\mu\text{mol}$ of thiamin pyrophosphate were added to the mixture. Where indicated $64\text{ }\mu\text{g}$ of flavodoxin or $1.5\text{ }\mu\text{mol}$ of benzyl viologen was added. The gas phase was $\text{H}_2 + \text{CO}_2$ (50:50). In the experiments with *B. ruminicola* the details were as with *P. elsdenii* except that crude extract containing 2.2 mg of protein was used, no ferredoxin was added, the isobutyrate contained 9.6×10^5 d.p.m., MgSO_4 and CoCl_2 were omitted and the gas phase was N_2 . Other details were as described in Table 1 or the Materials and Methods section.

System	Isobutyrate fixed (d.p.m.)			
	With <i>P. elsdenii</i> (DEAE-cellulose-treated extract)			With <i>B. ruminicola</i> (crude extract)
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Complete	8700	21200	24500	8400
Glutamine omitted	8000	—	—	5600
Pyruvate omitted	8000	7400	—	1650
ATP omitted	4600	—	—	1000
CoA omitted	1600	—	—	3100
Thiamin pyrophosphate omitted	3700	—	—	—
Ferredoxin omitted	1900	3400	4800	—
Ferredoxin omitted, flavodoxin added	—	—	26400	—
Ferredoxin omitted, benzyl viologen added	—	13600	—	—
Metal ion(s) omitted	8200	—	—	5100
Boiled enzyme	—	220	—	100

The reaction was also dependent on ATP; this requirement could be replaced by phosphoenolpyruvate plus ADP and this combination was in fact more effective.

The requirement for other components of the reaction mixture was examined with extracts treated with DEAE-cellulose. An almost complete dependence on CoA and ferredoxin and a stimulation by thiamin pyrophosphate were observed (Expt. 1, Table 2). Further examination of the electron-carrier requirement showed that ferredoxin could be replaced by flavodoxin or benzyl viologen (Expts. 2 and 3, Table 2).

Under the conditions of these experiments ^{14}C incorporation was approximately linear over the first 30 min and the rate was proportional to the concentration of the enzyme preparation up to the maximum tested (2.5 mg of extract protein/ml of incubation mixture).

Extracts of *B. ruminicola* strain 23 were examined in less detail as they were much less stable than those of *P. elsdenii* and active extracts were not obtained consistently. The *B. ruminicola* extracts contained no hydrogenase as tested by the ability to reduce benzyl viologen in the presence of H_2 (under conditions similar to those used for isobutyrate fixation) and were unable to use H_2 as reductant. Isobutyrate fixation was, however, observed with crude extracts

under N_2 when pyruvate was added. This system was also dependent on ATP and CoA. Partial stimulations were also observed with Mn^{2+} and glutamine (Expt. 4, Table 2). The lack of effect of glutamine and metals with the *P. elsdenii* preparations may be due to the presence of available metal ions and a source of amino groups in these extracts.

Incorporation of carbon dioxide by cell-free extracts. When extracts of *P. elsdenii* were incubated with unlabelled isobutyrate plus $\text{H}^{14}\text{CO}_3^-$ under conditions similar to the previous experiments, ^{14}C was again incorporated into the non-acid-volatile fraction (Table 3). A paper chromatogram of this fraction was prepared and all of the radioactivity detected by radioautography migrated with valine. Much less ^{14}C was fixed in the absence of isobutyrate. When acetate, isovalerate or 2-methylbutyrate replaced isobutyrate, most of the radioactivity fixed from $^{14}\text{CO}_2$ migrated with alanine, leucine and isoleucine respectively. Like $[^{14}\text{C}]\text{isobutyrate}$ fixation, $\text{H}^{14}\text{CO}_3^-$ fixation required ATP, CoA and ferredoxin. Again ferredoxin could be replaced by flavodoxin or benzyl viologen (Table 3). Isobutyrate, CoA and ATP could be replaced by isobutyryl-CoA or isobutyryl phosphate and CoA. With acetyl phosphate instead of isobutyryl phosphate most of the radioactivity fixed from $\text{H}^{14}\text{CO}_3^-$ was in alanine.

Table 3. *Incorporation of ^{14}C from $^{14}\text{CO}_2$ by cell-free extracts of *P. elsdenii**

Details were as for the complete system of Table 1 except that (i) DEAE-cellulose-treated extract from *P. elsdenii* was used containing 1.3, 3.0 or 1.2 mg of protein in Expts. 1, 2 and 3 respectively, (ii) the isobutyrate was unlabelled, (iii) $10\ \mu\text{mol}$ of $\text{NaH}^{14}\text{CO}_3$, containing 4.4×10^6 d.p.m. (Expts. 1 and 2) or 9.5×10^5 d.p.m. (Expt. 3), and $100\ \mu\text{g}$ of ferredoxin from *C. pasteurianum* were added and (iv), where indicated, flavodoxin or benzyl viologen as for Table 2, $2\ \mu\text{mol}$ of isobutyryl-CoA, $10\ \mu\text{mol}$ of isobutyryl phosphate or 4 units of avidin were present. The gas phase was $\text{H}_2 + \text{CO}_2$ (50:50) in Expt. 1 and H_2 in Expts. 2 and 3. Other details were as described in Table 1 or the Materials and Methods section. CO_2 fixed was measured as the ^{14}C recovered in the non-acid-volatile fraction.

System	CO_2 fixed (d.p.m.)		
	Expt. 1	Expt. 2	Expt. 3
Complete	3500	26 000	30 500
Isobutyrate omitted	1250	1600	7100
CoA omitted	780	—	—
Ferredoxin omitted	140	—	4000
Ferredoxin omitted, flavodoxin added	—	—	38 700
Ferredoxin omitted, benzyl viologen added	—	—	39 400
ATP omitted	—	2400	—
Isobutyrate + CoA + ATP replaced by isobutyryl-CoA	5600	—	—
Isobutyrate + ATP replaced by isobutyryl phosphate	—	66 000	—
Avidin added	4400	—	—
Boiled enzyme	—	200	100

Table 4. *Effect of 2-oxoisovalerate on the incorporation of ^{14}C from $[1-^{14}\text{C}]$ isobutyrate by extracts of *P. elsdenii**

The incubation mixture was as for the complete system of Table 1 except that crude extract containing 3.4 mg of protein was used, the $10\ \mu\text{mol}$ of $[1-^{14}\text{C}]$ isobutyrate contained 3.3×10^5 d.p.m. and $10\ \mu\text{mol}$ of sodium pyruvate was present. When indicated, $50\ \mu\text{mol}$ of sodium 2-oxoisovalerate was added and subsequently isolated as the 2,4-dinitrophenylhydrazone. Other details are given in Table 1 and the Materials and Methods section. Isobutyrate fixed was measured as the ^{14}C recovered in the fraction indicated.

System	Isobutyrate fixed	
	In non-acid-volatile fraction (d.p.m.)	In oxo acid hydrazone (c.p.m.)
(a) Oxo acid added after incubation	62 000	190
(b) Oxo acid added before incubation	6200	1300
(c) As (a) but boiled enzyme	240	5

The reaction was not inhibited by avidin, indicating that biotin-containing enzymes are not involved.

Oxo acid formation from isobutyrate. When carrier 2-oxoisovalerate was added at the end of an incubation with $[1-^{14}\text{C}]$ isobutyrate and was isolated from the mixture as the 2,4-dinitrophenylhydrazone, a small amount of ^{14}C was recovered (Table 4). When the 2-oxoisovalerate was present during the incubation the ^{14}C recovered with the oxo acid was increased sevenfold, suggesting that the oxo acid is an intermediate in the formation of valine (or in close metabolic connexion with one). In the same experiment the ^{14}C passing into the non-acid-volatile (valine) fraction was decreased to about one-tenth. This is attributed to the lowered specific radioactivity of the oxo acid pool together, possibly, with a mass-action effect on the carboxyl-

ation reaction, and is consistent with the role of 2-oxoisovalerate as intermediate in the conversion of isobutyrate into valine.

DISCUSSION

The present investigation shows that cell-free extracts of *P. elsdenii* and of *B. ruminicola* catalyse a carboxylation of isobutyrate to produce valine; 2-oxoisovalerate is probably the immediate carboxylation product and a reaction sequence based on a reversal of the reactions involved in the anaerobic oxidation of pyruvate may be proposed as follows:

The individual steps have not yet been demonstrated directly nor the reactants and products fully characterized, but the present observations are all consistent with this reaction sequence. An alternative to reactions (1) and (2) is that isobutyrate may be activated to isobutyryl-CoA by an acyl-CoA synthetase (EC 6.2.1.-) with the elimination of pyrophosphate or by a CoA transferase (EC 2.8.3.-) at the expense of acetyl-CoA.

The key reaction in this sequence is the reductive carboxylation of isobutyrate to 2-oxoisovalerate, and the present results support the suggestion of Elsdén (1966) that this reaction is similar to that of the 'pyruvate synthetase' found in *C. pasteurianum* (Bachofen, Buchanan & Arnon, 1964), *Clostridium kluyveri* (Andrew & Morris, 1965) and certain anaerobic photosynthetic bacteria (Buchanan *et al.* 1964; Evans & Buchanan, 1965). Some evidence for pyruvate synthetase activity in *P. elsdenii* extracts was also obtained in the present work, since labelled alanine was synthesized instead of valine when acetate replaced isobutyrate in a system containing $\text{H}^{14}\text{CO}_3^-$. Extracts of both *P. elsdenii* and *B. ruminicola* catalyse a slow exchange between carbon dioxide and 2-oxoisovalerate and a more rapid exchange between carbon dioxide and pyruvate (M. J. Allison & J. L. Peel, unpublished work). The reductive carboxylation of succinate to give 2-oxoglutarate has been demonstrated in *Chlorobium thiosulfatophilum* (Buchanan & Evans, 1965) and in *B. ruminicola* (Allison & Robinson, 1970). It seems likely that analogous systems are involved in the synthesis by ruminal micro-organisms of leucine from isovalerate, isoleucine from 2-methylbutyrate, phenylalanine from phenylacetate and tryptophan from indolyl-acetate (e.g. Allison, 1969). Whether one single or several enzyme systems are responsible for these various carboxylations is not yet clear.

The reductive carboxylation step requires a low potential source of electrons and a suitable electron carrier. With the preparations from *P. elsdenii* hydrogen or pyruvate can act as the electron source; pyruvate is presumably the natural source in the growing cell since the organism oxidizes pyruvate anaerobically and produces H_2 as an end product (Elsden & Lewis, 1953; Peel, 1960). Either ferredoxin or flavodoxin can act as electron carrier for the carboxylation of isobutyrate, and extracts of *P. elsdenii* are known to reduce these carriers at the expense of pyruvate (Mayhew, 1966).

Ferredoxin is present in appreciable quantities in *P. elsdenii* grown in a medium containing adequate iron, but when the iron concentration is low flavodoxin is synthesized instead (Mayhew & Massey, 1969). Ferredoxin is probably the operative electron carrier when the organism is grown in its natural environment.

With *B. ruminicola* pyruvate could act as the electron donor, but molecular hydrogen did not and no hydrogenase activity was found in the extracts. When cells of *B. ruminicola* were examined for ferredoxin and flavodoxin by isolation procedures, based on fractionation with DEAE-cellulose (Mayhew, 1966; Knight & Hardy, 1966), neither electron carrier was found. It is possible that *B. ruminicola* contains such proteins but that they are not revealed by these procedures; alternatively this organism may contain some other low-potential electron carrier.

Growing cells of *P. elsdenii* incorporated ^{14}C from [$1\text{-}^{14}\text{C}$]isobutyrate into leucine as well as valine, whereas the cell-free extracts only incorporated the ^{14}C into valine. It is probable that in the growing cells leucine is synthesized via 2-oxoisovalerate as proposed by Abelson & Vogel (1955) and Strassman, Locke, Thomas & Weinhouse (1955), but that conditions with the cell-free extracts are not conducive for this longer sequence of reactions. Growing cells of *P. elsdenii* are also able to synthesize leucine by carboxylation of isovalerate and isoleucine by carboxylation of 2-methylbutyrate (Allison, 1969); these reactions were observed with cell-free extracts in the present study.

The contribution of these carboxylation reactions to the synthesis of cellular amino acids of *P. elsdenii* during growth in the rumen is uncertain and probably varies with the conditions. Rumen fluid contains the necessary branched-chain fatty acids (el Shazly, 1952; Annison, 1954) but may also contain low concentrations of valine and leucine (Wright & Hungate, 1967). These amino acids may affect the incorporation of branched-chain acids into *P. elsdenii* since growth in complex medium diminished the uptake of isobutyrate. *P. elsdenii* does not require the branched-chain fatty acids as growth factors, though a number of other rumen bacteria do (Bryant & Robinson, 1962).

Ruminal bacteria found to incorporate ^{14}C from [$1\text{-}^{14}\text{C}$]isobutyrate into valine during growth under conditions similar to those in this study include *Methanobacterium ruminantium* strain M-1 and *Ruminicoccus flavefaciens* strain C94 (Allison, 1969, and unpublished work). The possible selective advantage of the ability to synthesize valine by the reductive carboxylation pathway rather than by the longer route from less complex carbon skeletons has been pointed out elsewhere (Allison *et al.* 1962a).

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REFERENCES

- Abelson, P. H. & Vogel, H. J. (1955). *J. biol. Chem.* **213**, 355.
- Allison, M. J. (1969). *J. Anim. Sci.* **29**, 797.
- Allison, M. J. & Bryant, M. P. (1963). *Archs Biochem. Biophys.* **101**, 269.
- Allison, M. J., Bryant, M. P. & Doetsch, R. N. (1962a). *J. Bact.* **83**, 523.
- Allison, M. J., Bryant, M. P., Katz, I. & Keeney, M. (1962b). *J. Bact.* **83**, 1084.
- Allison, M. J. & Peel, J. L. (1968). *Bact. Proc.* p. 180.
- Allison, M. J. & Robinson, I. M. (1970). *J. Bact.* **104**, 50.
- Andrew, I. G. & Morris, J. G. (1965). *Biochim. biophys. Acta*, **97**, 176.
- Annison, E. F. (1954). *Biochem. J.* **57**, 400.
- Avison, A. W. D. (1955). *J. chem. Soc.* p. 732.
- Bachofen, R., Buchanan, B. B. & Arnon, D. I. (1964). *Proc. natn. Acad. Sci. U.S.A.* **51**, 690.
- Brown, F. (1950). *Biochem. J.* **47**, 598.
- Bruno, G. A. & Christian, J. E. (1961). *Analyt. Chem.* **33**, 1216.
- Bryant, M. P. & Doetsch, R. N. (1955). *J. Dairy Sci.* **38**, 340.
- Bryant, M. P. & Robinson, I. M. (1961). *Appl. Microbiol.* **9**, 91.
- Bryant, M. P. & Robinson, I. M. (1962). *J. Bact.* **84**, 605.
- Buchanan, B. B., Bachofen, R. & Arnon, D. I. (1964). *Proc. natn. Acad. Sci. U.S.A.* **52**, 839.
- Buchanan, B. B. & Evans, M. C. W. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 1212.
- Elsden, S. R. (1966). *Pathologia microbiol.* **29**, 602.
- Elsden, S. R. & Lewis, D. (1953). *Biochem. J.* **55**, 183.
- Elsden, S. R., Volcani, B. E., Gilchrist, F. M. C. & Lewis, D. (1956). *J. Bact.* **72**, 681.
- el Shazly, K. (1952). *Biochem. J.* **51**, 647.
- Evans, M. C. W. & Buchanan, B. B. (1965). *Proc. natn. Acad. Sci. U.S.A.* **53**, 1420.
- Hardy, T. L., Holland, D. O. & Nayler, J. H. C. (1955). *Analyt. Chem.* **27**, 971.
- Hungate, R. E. (1966). *The Rumen and its Microbes*, pp. 26-30. New York: Academic Press Inc.
- Knight, E., jun. & Hardy, R. W. F. (1966). *J. biol. Chem.* **241**, 2752.
- Knight, M. (1962). *Biochem. J.* **84**, 170.
- Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **159**, 21.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McFarren, E. F. (1951). *Analyt. Chem.* **23**, 168.
- Mayhew, S. G. (1966). Ph.D. Thesis: University of Sheffield.
- Mayhew, S. G. & Massey, V. (1969). *J. biol. Chem.* **244**, 794.
- Peel, J. L. (1960). *Biochem. J.* **74**, 525.
- Rabinowitz, J. C. (1960). *J. biol. Chem.* **235**, 50.
- Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T. & Britten, R. J. (1957). *Publ. Carnegie Instn Wash.* no. 607.
- Robinson, I. M. & Allison, M. J. (1969). *J. Bact.* **97**, 1220.
- Simon, E. J. & Shemin, D. (1953). *J. Am. chem. Soc.* **75**, 2520.
- Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 84. New York: Interscience Publishers Inc.
- Somerville, H. J. & Peel, J. L. (1967). *Biochem. J.* **105**, 299.
- Stadtman, E. R. (1957). In *Methods in Enzymology*, vol. 3, pp. 228-231. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Strassman, M., Locke, L. A., Thomas, A. J. & Weinhouse, S. (1955). *Science, N.Y.*, **121**, 303.
- Walker, D. J. (1958). *Biochem. J.* **69**, 524.
- Wegner, G. H. & Foster, E. M. (1963). *J. Bact.* **85**, 53.
- Wright, D. E. & Hungate, R. E. (1967). *Appl. Microbiol.* **15**, 148.