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# Amino Acid Biosynthesis in Mixed Rumen Cultures

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Mixed rumen micro-organisms, maintained in continuous culture readily incorporated labelled $HCO_3^-$ and acetate into amino acids. Labelled propionate, in contrast, was utilized only for isoleucine biosynthesis, but failed to label other amino acids to any significant extent. Evidence was obtained showing that in these mixed, i.e. symbiotic, cultures forward tricarboxylic acid-cycle reactions only proceed to 2-oxoglutarate. $^{14}C$ distribution in amino acids clearly shows that 2-oxoglutarate is not oxidized further by tricarboxylic acid-cycle enzymes. Instead, acetate is carboxylated to pyruvate which is then carboxylated to oxaloacetate. Oxaloacetate equilibrates with fumarate and thereby carbon atoms 1 and 4 as well as carbon atoms 2 and 3 are randomized. Evidence was also obtained for the carboxylation of propionate to 2-oxobutyrate, isovalerate to 4-methyl-2-oxopentanoate, phenylacetate and hydroxyphenylacetate to the corresponding phenyl- and hydroxyphenyl-pyruvic acids and succinate to 2-oxoglutarate. Of the amino acid precursors investigated, only 3-hydroxypyruvate, the precursor of serine, appeared to be synthesized via an oxidative step, i.e. 3-phosphoglyceric acid to 3-phosphohydroxypyruvic acid. Most 2-oxo precursors of amino acids in these organisms appear to be formed via reductive carboxylation of the precursor acid.

Most strains of rumen microbes utilize ammonia as their principal source of nitrogen. In contrast not all species of rumen bacteria can utilize exogenous amino acids, and even with those that can, better growth is obtained when ammonia is added to the medium (Allison, 1969). This would suggest that the normal rumen microbial population depends largely on the synthesis *de novo* of amino acids from ammonia and carbon precursors derived from products of cellulose fermentation.

To gain an insight into the relative rates of amino acid synthesis in the rumen from simple carbon precursors, the normal mixed rumen flora were grown in continuous culture under simulated rumen conditions. The present investigation deals with the time-dependent incorporation of $^{14}C$ from $H^{14}CO_3^-$, [1-$^{14}$C]acetate or [1-$^{14}$C]propionate into bacterial amino acids.

The importance of ferredoxin-dependent carboxylation reactions in the synthesis of 2-keto acids, originally described by Buchanan (Evans *et al.*, 1966) in a photosynthetic bacterium, has now been shown to include some strains of rumen bacteria (Allison & Peel, 1971). The experiments reported here were conducted to determine the role of reductive carboxylation in the formation of the 2-oxo precursors of amino acids under the experimental conditions. The results of these studies indicate that the rumen flora synthesize a large proportion of amino acids *de novo* and that reductive carboxylation reactions probably play a major role in providing precursors of several of these amino acids. It is becoming increasingly apparent that pathways of amino acid biosynthesis described in aerobic bacteria may not necessarily be operative to any significant extent in rumen bacteria and that in these organisms the biosynthetic pathways of some amino acids should be reinvestigated.

## Experimental

### *Materials*

The following radioactive substrates were purchased from New England Nuclear Corp. (Canada Ltd., Dorval, Que., Canada): [1-$^{14}$C]acetate (59mCi/mmol); [1-$^{14}$C]propionate (22mCi/mmol); L-[U-$^{14}$C]phenylalanine (380mCi/mmol); L-[U-$^{14}$C]-serine (135mCi/mmol); L-[U-$^{14}$C]tyrosine (380mCi/mmol); L-[U-$^{14}$C]aspartate (167mCi/mmol); L-[U-$^{14}$C]alanine (156mCi/mmol); L-[U-$^{14}$C]glutamate (269mCi/mmol); L-[U-$^{14}$C]leucine (327mCi/mmol); L-[U-$^{14}$C]isoleucine (250mCi/mmol); L-[U-$^{14}$C]-lysine (258mCi/mmol). Radioactive amino acids were diluted with unlabelled amino acids as required before use. $Na_2{}^{14}CO_3$ (96.8% $^{14}C$ to total C present) was purchased from Atomic Energy (Ottawa, Ont., Canada) and carefully neutralized before use.

All reagents and chemicals were of the highest purity commercially available.

### *Methods*

*Continuous rumen culture technique.* The rumen microbial population was maintained in the con-

tinuous culture fermentor described by Slyter *et al.* (1964). The apparatus was modified by the use of a 500ml water-jacketed spinner flask (O. H. Johns, Toronto, Ont., Canada) fitted with a stirrer seated in a gas-tight Teflon bearing and coupled by a flexible shaft to a gear-driven variable-stirrer (Cole–Parmer, Chicago, Ill., U.S.A.).

Rumen fluid was collected by stomach tube with vacuum from a lactating Holstein cow on a diet of ground corn meal (82%) and soya-bean meal (15%) concentrate, containing dicalcium phosphate (2%) and cobalt iodized salt (1%). In addition, a forage mixture of corn silage and chopped hay was fed *ad libitum*. The fluid was transported to the laboratory in a Thermos flask under $CO_2$ and 500ml filtered through a single-layer cheese cloth into the fermentor vessel. The microbial population was maintained by adding 7.5g of a ground mixture of corn silage, hay and concentrate (82:2:2, by weight) twice daily to the fermentors.

Under the conditions of these experiments total protozoal counts (direct microscopic) decreased to less than $2\times10^3$/ml after 48h. The microbial population, assayed as DNA concentration/ml of culture fluid (Ceriotti, 1952), however, remained constant, i.e. $72.6\pm4.06\mu$g/ml during the 3-week test period.

To detect any unusual quantitative or qualitative drifts in the resident microbial population, the volatile fatty acid concentration in the fermentors was continually monitored. Volatile fatty acids were measured by gas chromatography with a Hewlett–Packard model 5710A gas chromatograph on 1.83m glass columns packed with 20% (w/w) Tween 80 and 2% (w/w) phosphoric acid on Chromosorb W (60–80mesh). Although the total concentration of combined volatile fatty acids (acetate, propionate, butyrate, isovalerate and valerate) varied from 45 to 197mM with different cultures and different time of sampling, the molar percentage ratio of the acids remained remarkably constant with time, i.e. (mean±S.E.M. for eight determinations) acetate $54.98\pm0.61$, propionate $26.1\pm0.92$, butyrate $12.0\pm0.82$, isovalerate $3.6\pm0.26$ and valerate $3.4\pm0.18$. These did not change unless different feed mixtures were added.

*$^{14}C$ experiments and amino acid isolation.* Tracer experiments were done 48h after the fermentor was charged with rumen fluid. Radioactive tracer (2mCi), dissolved in minimal volume of distilled water and adjusted to pH7.5, was added to the culture through a fermentor port at time zero. Initial specific radioactivity values of $NaH^{14}CO_3$, [1-$^{14}$C]acetate and [1-$^{14}$C]propionate in the fermentor were 116.6, 114.3 and $273.5\mu$Ci/mmol respectively. At indicated time-intervals, 8ml of culture fluid was withdrawn through a fermentor port and filtered through 53-mesh nylon under $N_2$ pressure; 5ml of filtered fluid was centrifuged at 15000*g* for 10min. The bacterial pellet was hydrolysed with 2ml of 6M-HCl at 105°C for 48h. The protein hydrolysate was dried and taken up in 5ml of water.

Amino acids were separated on an automatic Jeol model 5 AH amino acid analyser, and peaks quantified with a Systems AA computing integrator (Spectra Physics, Palo Alto, Calif., U.S.A.). Fractions (5min) were collected after splitting the effluent stream (1:1), and portions of each amino acid were mixed with Aquasol (New England Nuclear Corp.) and the radioactivity was counted in a Packard liquid-scintillation counter. Corrections for quenching were made by use of the external-standard technique.

The collected fractions for each radioactive amino acid were combined (30–420min), diluted threefold with distilled water and put on a column (1cm×5cm) of Dowex 50 ($H^+$ form). This was necessary to remove the citrate buffer from the amino acid analyser fractions. The resin was washed with 50ml of water and the amino acid was eluted with 150ml of 1M-HCl and freeze-dried.

When large samples of radioactive amino acid were required, protein hydrolysates were separated by combined electrophoresis and paper chromatography. For the isolation of lysine, the hydrolysate was subjected to high-voltage paper electrophoresis (3500V, 70mA; 2M-acetic acid adjusted to pH2.6 with pyridine) for 1.5h. Lysine was eluted with water and further purified by ascending paper chromatography with butan-1-ol – acetone – diethylamine – water (10:10:2:5, by vol.). Purity of the final product was checked on a Beckman 121 M amino acid analyser.

*Amino acid degradation.* All amino acid-degradative procedures were first tested with uniformly labelled standard amino acids before the chemical degradation of bacterial amino acids.

(i) Alanine. To radioactive alanine was added 0.5mmol of carrier and this was transferred in 2.0ml of water to a steam-distillation tube (Quickfit 19MU). The tube was chilled in ice and 0.1ml of sodium acetate buffer (4M, pH5.5) added followed by 1.0ml of $CO_2$-free ninhydrin solution (7.5g of ninhydrin; 50mg of $SnCl_2,2H_2O$; 37.5ml of Cellosolve; 12.5ml of sodium acetate buffer, 4M, pH5.5). The unit was assembled with the exit tube from the Liebig condenser submerged in 3ml of ice-cold 1.2M-NaOH. Steam was generated for 20min and the resulting $CO_2$ and acetaldehyde were both trapped in the alkali. $^{14}CO_2$ was removed by centrifugation as $BaCO_3$, washed twice, dried, weighed and counted for radioactivity. The supernatant was brought to approx. 30ml with distilled water, 10ml of 5% (w/v) KI was added and followed by 5% (w/v) NaOCl until iodoform precipitation was complete. Iodoform was washed, dried, taken up

in ethanol, quantified spectrophotometrically at 340nm ($\varepsilon = 2.22 \times 10^3$ litre·mol$^{-1}$·cm$^{-1}$) and counted for radioactivity. The supernatant was reduced to approx. 4ml, acidified with 0.5M-$H_2SO_4$, and $Na_2S_2O_4$ was added dropwise until the solution was clear. The residual formic acid was steam-distilled and titrated, and a sample removed for radioactive counting.

(ii) Glutamic acid. The macro-scale procedure of Hoare (1963) for removal of C-1 was followed. After removal of toluene-*p*-sulphonamide the supernatant which contained succinate was dried, taken up in 4ml of 0.25M-$H_2SO_4$, and mixed with 14g of silicic acid until a free-flowing powder was obtained. Succinate was eluted from the resulting column with diethyl ether. Succinate was degraded by the Schmidt reaction (Phares & Long, 1955). C-5 of glutamic acid was measured separately by decarboxylation with sodium azide, as described by Finlayson (1966). C-3 and C-4 of glutamate were not separated.

(iii) Lysine. The degradative procedure of Strassman & Weinhouse (1953) was followed, modified only in that dicarboxylic acids (succinic acid and glutaric acid) were recovered by elution with diethyl ether from silicic acid rather than by continuous liquid–liquid ether extraction (Sauer *et al.*, 1970).

(iv) Aspartic acid. Aspartic acid (0.5mmol) was dissolved in 10ml of distilled water (50°C) followed by the addition of 1ml of sodium acetate buffer (4M, pH5.5). This was placed in a three-neck flask and connected to the $CO_2$-absorbing train. Ninhydrin solution (10ml) was added through a dropping funnel. The solution was brought to boiling which was continued for 10min to decarboxylate both C-1 and C-4 of aspartic acid. Recovery experiments with [U-$^{14}$C]aspartate gave yields of C-1 plus C-4 equal to 70% of theoretical. C-2 and C-3 were measured as described by Sakami (1955*d*). The resulting acetaldehyde bisulphite was oxidized to acetic acid (Sakami, 1955*a*) which was degraded by the Schmidt reaction (Sakami, 1955*b*).

(v) Isoleucine. The degradative procedure of Strassman *et al.* (1956) was followed.

(vi) Phenylalanine. Phenylalanine (1mmol) was dissolved in 10ml of water–0.5ml of sodium acetate buffer (4M, pH5.5) and decarboxylated with 5ml of ninhydrin solution. The α-carbon, β-carbon and aromatic ring of phenylalanine were determined as described by Hoare & Gibson (1964).

(vii) Tyrosine. Tyrosine was decarboxylated with ninhydrin as described above except that volumes were adjusted for 0.5mmol of tyrosine, 25ml of ninhydrin solution and 2.5ml of acetate buffer. The specific radioactivity of the aromatic ring and β-carbon were measured as recrystallized 4-hydroxybenzoic acid derived from tyrosine by alkaline fusion (Baddiley *et al.*, 1950). The α-carbon radioactivity was calculated by the difference.

(viii) Serine. Serine was decarboxylated as described by Sakami (1955*c*).

## Results and Discussion

Table 1 shows the molar percentage ratios of the amino acids in rumen bacteria. These results agree with values obtained by Purser & Buechler (1966) for the amino acid composition of 22 strains of rumen bacteria isolated in pure culture.

Figs. 1, 2, 3 and 4 show the kinetics of tracer incorporation into microbial amino acids with [1-$^{14}$C]acetate, [1-$^{14}$C]propionate and $H^{14}CO_3^-$ as labelled precursors. Of the basic amino acids, histidine showed the highest specific radioactivity with $H^{14}CO_3^-$ added and the lowest specific radioactivity with [1-$^{14}$C]acetate added (Figs. 1*a* and 2*a*). Lysine had the highest specific radioactivity with [1-$^{14}$C]acetate as precursor (Fig. 2*a*). [1-$^{14}$C]-Propionate was incorporated into histidine and arginine at higher rates than into lysine (Fig. 3*a*); however, all amino acids (except isoleucine; Fig. 4) had much lower specific radioactivities with [1-$^{14}$C]-propionate than with the other $^{14}$C sources. (Note the tenfold ordinate-scale expansion in Fig. 3.)

Glutamate specific radioactivity exceeded that of aspartate at all time-intervals when [1-$^{14}$C]acetate or [1-$^{14}$C]propionate were precursors (Figs. 2*b* and 3*b*), but when $H^{14}CO_3^-$ was added aspartate

Table 1. *Amino acid composition of rumen bacteria isolated from continuous culture fermentors*

Analyses based on hydrolysates of pellets of whole bacteria; mean values are given for 22 estimations±S.E.M. Amounts (mol) are relative to glutamate assigned an arbitrary value of 1000.

| Amino acid | Present work | Purser & Buechler (1966) |
|---|---|---|
| Lysine | 501.1±26.7 | 669 |
| Histidine | 132.5± 6.3 | 155 |
| Arginine | 251.0±10.1 | 326 |
| Aspartic acid | 895.5±24.1 | 883 |
| Threonine | 467.6±12.4 | 485 |
| Serine | 424.7±12.0 | 380 |
| Glutamic acid | 1000 ±21.1 | 1000 |
| Proline | 498.8±36.4 | 375 |
| Glycine | 726.9±16.3 | 854 |
| Alanine | 814.8±19.5 | 767 |
| Valine | 505.3±13.4 | 592 |
| Methionine | 215.0± 8.9 | 184 |
| Isoleucine | 432.9±10.5 | 514 |
| Leucine | 674.8±29.9 | 585 |
| Tyrosine | 287.9± 8.2 | 243 |
| Phenylalanine | 311.0± 6.5 | 325 |

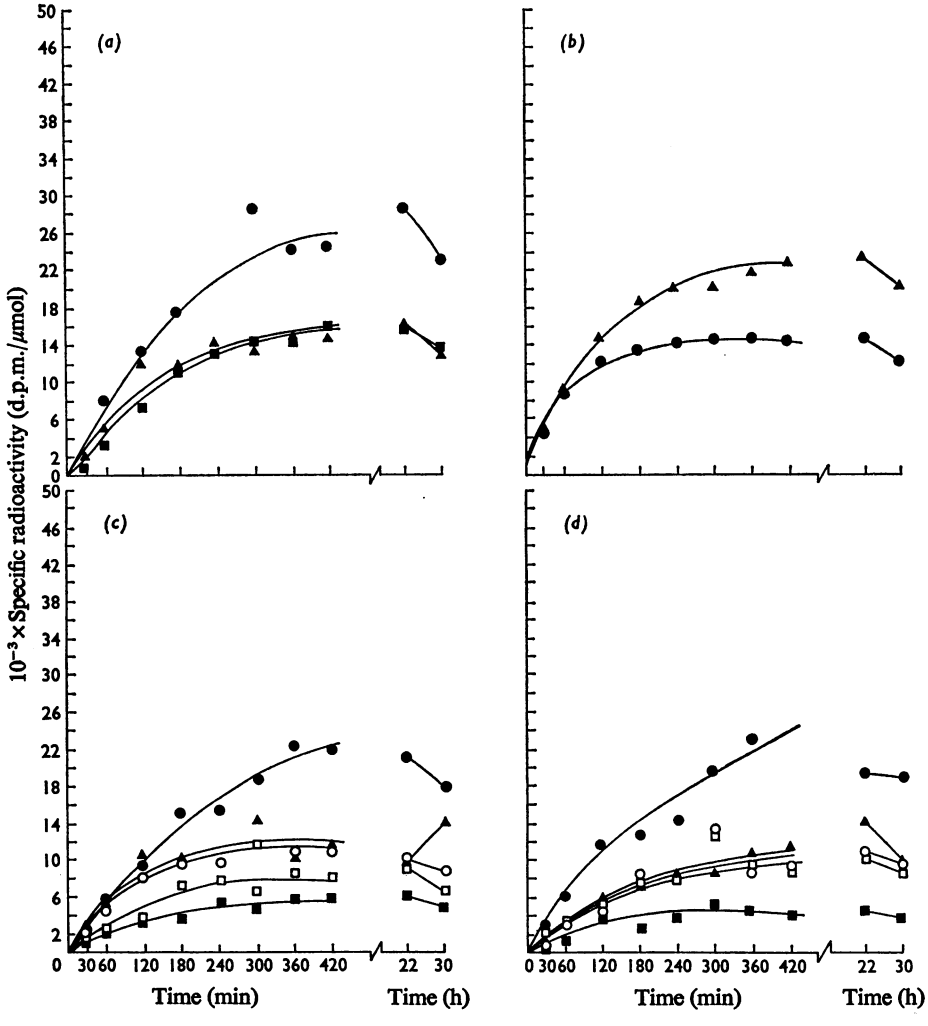



Fig. 1. *Kinetics of $H^{14}CO_3^-$ incorporation into rumen microbial amino acids*

Bacterial samples were removed from continuous fermentors at the indicated time-intervals. Bacterial pellets were hydrolysed and specific radioactivity of each amino acid was determined as described in the text. The fixation of $H^{14}CO_3^-$ into (*a*) ●, histidine, ▲, lysine and ■, arginine, (*b*) ▲, aspartic acid and ●, glutamic acid, (*c*) ●, serine, ▲, threonine, ○, alanine, □, valine and ■, glycine, (*d*) ●, methionine, ▲, isoleucine, ○, tyrosine, □, phenylalanine and ■, leucine is indicated.

specific radioactivity was greater than that of glutamate (Fig. 1*b*).

A comparison of Figs. 1(*b*) and 2(*b*) shows a distinct lag in the labelling of aspartate and glutamate from [1-$^{14}$C]acetate which was not so when $H^{14}CO_3^-$ was the precursor.

Serine synthesized from $H^{14}CO_3^-$ (Fig. 1*c*) had a higher specific radioactivity than that synthesized from [1-$^{14}$C]acetate (Fig. 2*c*). Glycine synthesized either from $H^{14}CO_3^-$ or [1-$^{14}$C]acetate had a low specific radioactivity at all time-periods. This finding was expected as serine, the precursor of glycine, probably has a smaller intracellular pool size than the latter (Table 1) and would therefore undergo dilution of radioactivity in the conversion into glycine.

Threonine reflected the specific radioactivity of its precursor, aspartate. The specific radioactivity of threonine was consistently higher with $H^{14}CO_3^-$ than with [1-$^{14}$C]acetate as the radioactive source (Figs. 1*c* and 2*c*). The specific radioactivity of alanine was the same with either source, but valine showed more radioactivity from [1-$^{14}$C]acetate than from $H^{14}CO_3^-$. Similarly leucine, which is on the same biosynthetic pathway as valine, had a threefold higher

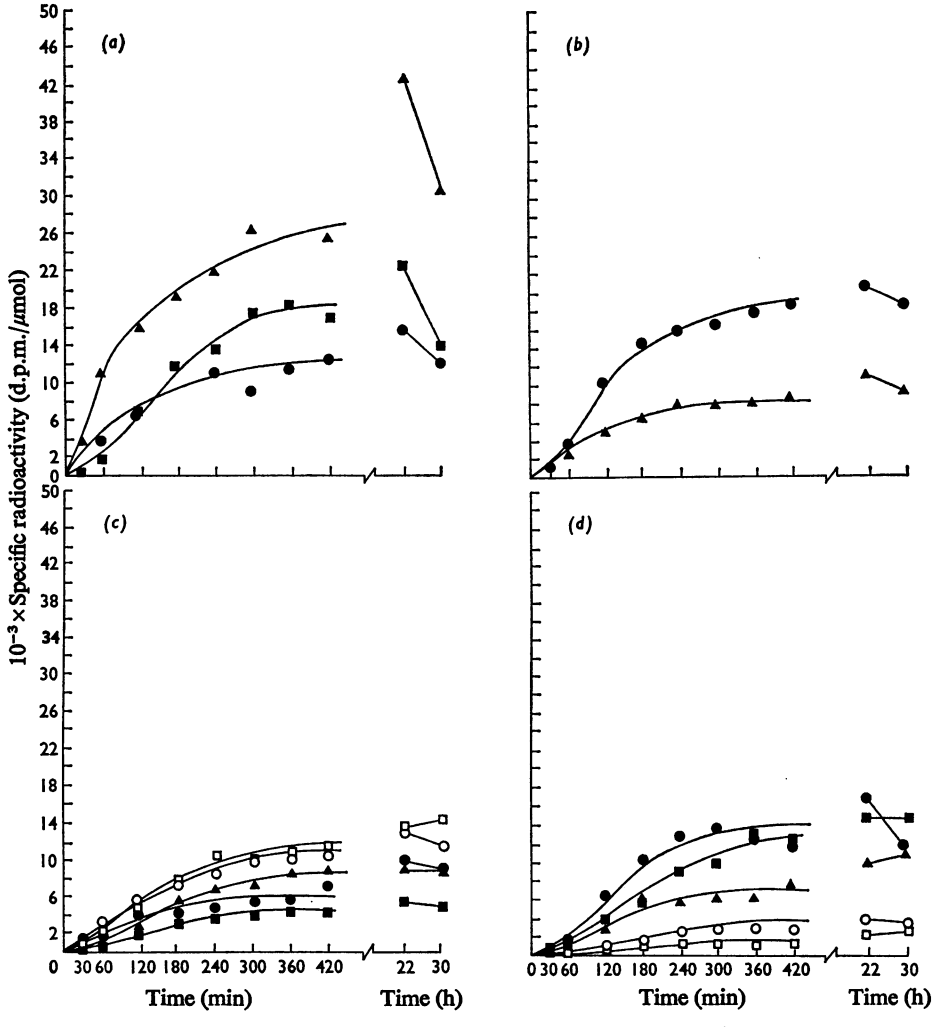



Fig. 2. *Kinetics of* [1-$^{14}$C]*acetate incorporation into rumen microbial amino acids*

Bacterial samples were removed from continuous fermentors at indicated time-intervals. Bacterial pellets were hydrolysed and the specific radioactivity of each amino acid was determined as described in the text. The symbols representing amino acids are the same as in Fig. 1.

specific radioactivity when [1-$^{14}$C]acetate instead of $H^{14}CO_3^-$ was the labelled precursor. [1-$^{14}$C[Propionate incorporation into these amino acids was negligible (Figs. 3*c* and 3*d*).

Methionine labelling was greater with $H^{14}CO_3^-$ than with the other substrates (Figs. 1*d*, 2*d* and 3*d*). As expected, its specific radioactivity was approximately the same as that of its precursor, aspartic acid (Figs. 1*b* and 1*d*). However, when [1-$^{14}$C]acetate was the precursor, the specific radioactivity of methionine exceeded that of aspartic acid (Figs. 2*b* and 2*d*).

The aromatic amino acids tyrosine and phenylalanine were also more highly labelled from $H^{14}CO_3^-$ than from [1-$^{14}$C]acetate (Figs. 1*d* and 2*d*). With all three radioactive precursors the specific radioactivity of phenylalanine was slightly less than that of tyrosine.

Fig. 4 shows the specific radioactivity of isoleucine in relationship to all other amino acids when [1-$^{14}$C]propionate was added to the culture system. The specific radioactivity of isoleucine was fourfold

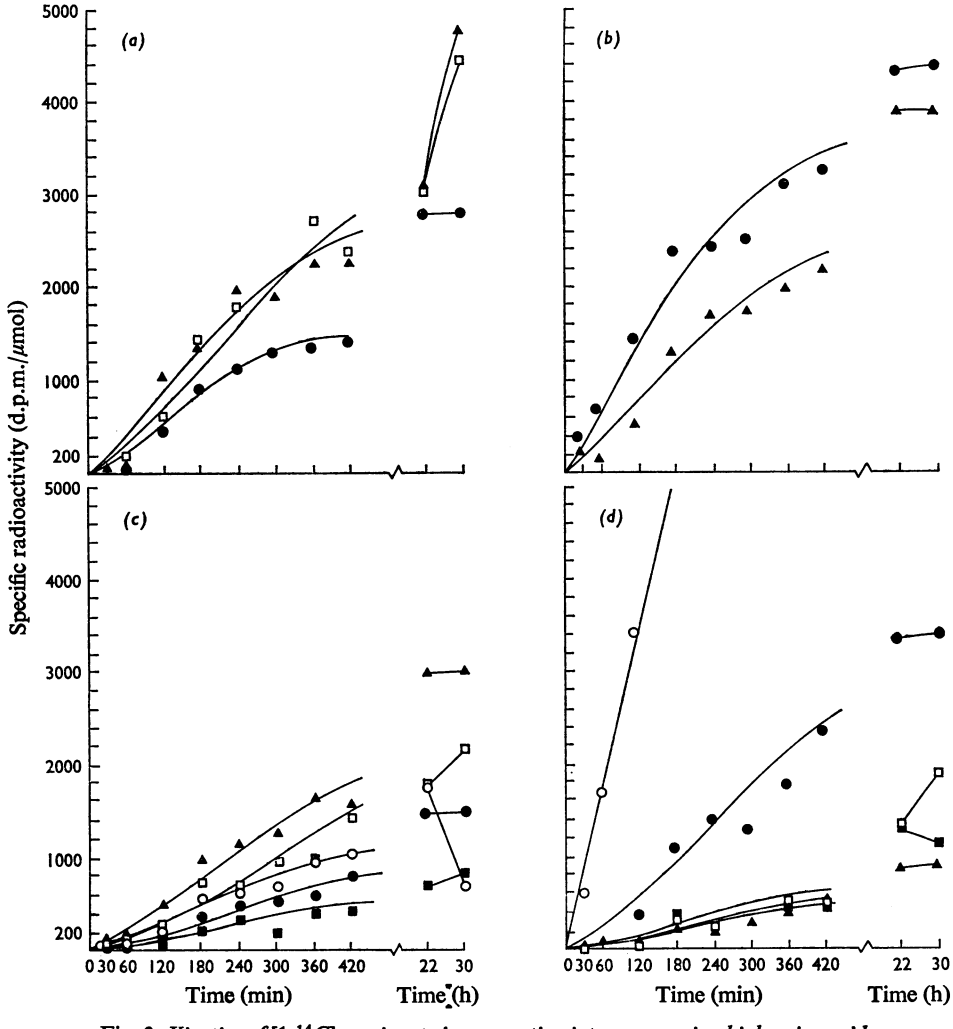



Fig. 3. *Kinetics of* [1-$^{14}$C]*propionate incorporation into rumen microbial amino acids*

Bacterial samples were removed from continuous fermentors at indicated time-intervals. Bacterial pellets were hydrolysed and the specific radioactivity of each amino acid was determined as described in the text. Incorporation of [1-$^{14}$C]propionate into amino acids is represented by the following symbols: (*a*) ●, lysine, ▲, arginine and □, histidine; (*b*) ●, glutamic acid and ▲, aspartic acid; (*c*) ▲, threonine, □, serine, ○, alanine, ●, valine and ■, glycine; (*d*) ○, isoleucine, ●, methionine, □, tyrosine, ▲, leucine and ■, phenylalanine. (Note the tenfold ordinate-scale expansion compared with Figs. 1 and 2.)

higher than that of the next most highly labelled amino acid, glutamate.

If one ranks the specific radioactivity of each amino acid in order of decreasing radioactivity for each of the three radioactively labelled precursors, a general pattern emerges. Amino acids which are synthesized by methyl group transfer, carboxylation or reductive carboxylation show a trend to higher specific radioactivity with $H^{14}CO_3^-$ as precursor. Amino acids which are primarily dependent on 2-carbon-unit incorporation or citrate-cycle reactions generally have greater specific radioactivity with [1-$^{14}$C]-acetate as precursor. To gain some insight into the principal pathway of amino acid biosynthesis from $CO_2$ and acetate by rumen micro-organisms, key amino acids were chemically degraded to determine the position of the $^{14}$C label (Tables 2–8). The details and significance of these results are discussed below.

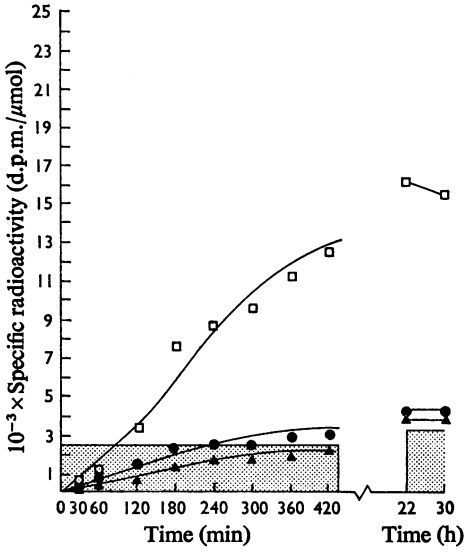



Fig. 4. *Comparison of isoleucine specific radioactivity with that of other amino acids with [1-$^{14}$C]propionate as precursor*

Shaded block represents specific radioactivity of amino acids shown in Fig. 3. Specific radioactivity of glutamic acid (●), aspartic acid (▲) and isoleucine (□) are plotted for comparison.

Table 2. *Percentage $^{14}$C distribution in alanine from [1-$^{14}$C]acetate and $H^{14}CO_3^-$*

Values are calculated on the basis of initial radioactivity equals 100% and corrected for losses incurred at each degradative step.

| | Percentage $^{14}$C distribution | | |
|---|---|---|---|
| Alanine carbon number | ... 3 | 2 | 1 |
| Labelled compound | $CH_3$ | $CHNH_2$ | $CO_2H$ |
| [1-$^{14}$C]Acetate | 3.2 | 94.5 | 2.3 |
| $H^{14}CO_3^-$ | <2.0 | <2.0 | 96.5 |

*Alanine*

Pyruvate synthase (EC 1.2.7.1), an enzyme first discovered in *Clostridium pasteurianum* (Bachofen *et al.*, 1964), catalyses the reaction:

$$\text{Acetyl-CoA} + CO_2 + Fd_{red.} \rightarrow \text{pyruvate} + \text{CoA} + Fd_{ox.}$$

where $Fd_{red.}$ and $Fd_{ox.}$ are reduced and oxidized ferredoxin respectively. Evidence that the reductive carboxylation of acetate to pyruvate is a fundamental reaction of primary importance in rumen micro-organisms was obtained from experiments wherein labelled alanine was chemically degraded (Table 2). These showed that alanine synthesized from [1-$^{14}$C]-acetate was labelled exclusively in C-2 whereas alanine labelled from $H^{14}CO_3^-$ was labelled exclusively in C-1. The failure of $H^{14}CO_3^-$ to label either C-2 or C-3 of alanine also shows that the synthesis *de novo* of acetate from $CO_2$ (Ljungdahl & Wood, 1969) cannot be quantitatively significant in the mixed microbial culture system.

*Lysine*

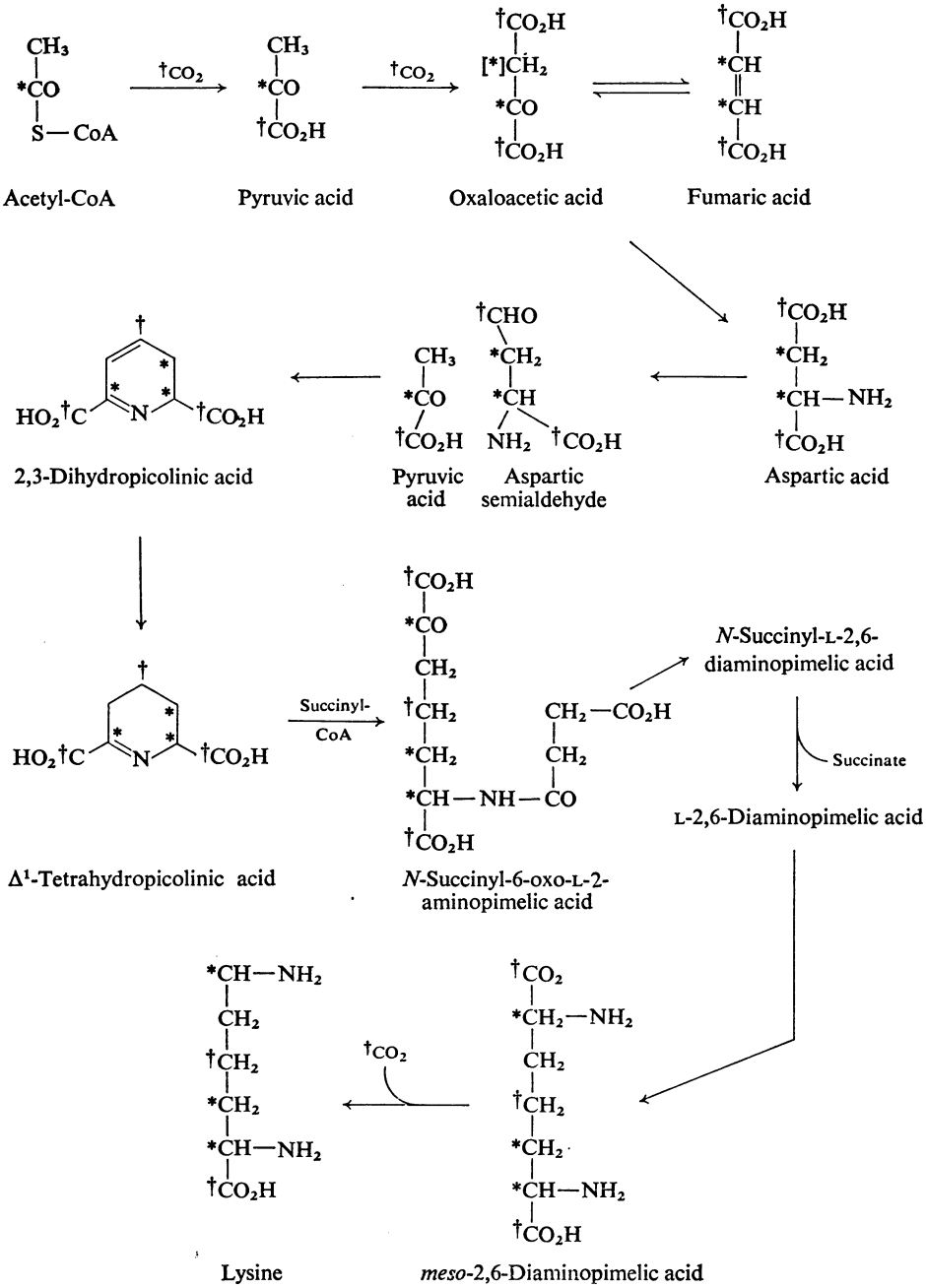
The labelling pattern of lysine (Table 3) synthesized from [1-$^{14}$C]acetate is clearly consistent with the sequence of reactions shown in Scheme 1, in which lysine is synthesized with the $^{14}$C label in C-2, C-3 and C-6. The carboxylation of pyruvate to oxaloacetate (Scheme 1) by enzymes of rumen micro-organisms was described previously (Atwal & Sauer, 1974).

In the carboxylation of acetate to pyruvate and pyruvate to oxaloacetate $^{14}CO_2$ appears in C-1 and C-4. After condensation with pyruvate (labelled in C-1) and ring opening, *N*-succinyl-6-oxo-2-amino-pimelic acid is labelled in positions 1, 4 and 7. In subsequent reactions which convert this compound into lysine, C-7 is lost and lysine is labelled in C-1 and C-4. As shown in Table 3, experimental values for lysine synthesized from $H^{14}CO_3^-$ and chemically

Table 3. *Percentage $^{14}$C distribution in lysine from [1-$^{14}$C]acetate and $H^{14}CO_3^-$*

Values are calculated on the basis of initial radioactivity equals 100% and corrected for losses incurred at each degradative step.

| | Percentage $^{14}$C distribution | | | | | |
|---|---|---|---|---|---|---|
| Lysine carbon number | ... 6 | 5 | 4 | 3 | 2 | 1 |
| Labelled compound | $H_2NCH_2$ | $CH_2$ | $CH_2$ | $CH_2$ | $CHNH_2$ | $CO_2H$ |
| [1-$^{14}$C]Acetate | 36.3 | 0 | 0 | 25.5 | 30.6 | 7.6 |
| $H^{14}CO_3^-$ | 1.0 | 57.7 | | <1.0 | 1.0 | 40.0 |




Scheme 1. *Labelling pattern of lysine synthesized from* [1-$^{14}$*C*]*acetate* (*) *and* $H^{14}CO_3^-$ (†)

Label [*] in C-3 of oxaloacetate is indicated as the result of randomization in the equilibration with fumarate.

degraded, indicate that the $^{14}$C label is distributed principally in C-1, C-4 and C-5, as expected. Carbon atoms 4 and 5 of lysine are, of course, indistinguishable by chemical degradation.

*Aspartic acid, threonine and methionine*

The data on the degradation of labelled aspartate (Table 4) agree with the pathway outlined in Scheme

Table 4. *Percentage $^{14}C$ distribution in aspartic acid from [1-$^{14}C$]acetate and $H^{14}CO_3^-$*

Values are calculated on the basis of initial radioactivity equals 100% and corrected for losses at each degradative step.

| | Percentage $^{14}C$ distribution | | |
|---|---|---|---|
| Aspartic acid carbon number ... | 1+4* | 3 | 2 |
| Labelled compound | $2(HO_2C)$ | $CH_2$ | $CHNH_2$ |
| [1-$^{14}C$]Acetate | 5.0 | 28.5 | 66.4 |
| $H^{14}CO_3^-$ | 96.9 | 1.5 | 1.5 |

* Both aspartic acid carboxyl groups were released by ninhydrin reaction.

1. From [1-$^{14}C$]acetate the $^{14}C$ label should appear in both positions 2 and 3 of aspartate; however, more radioactivity appeared in C-2 than in C-3. This shows that complete equilibration of C-2 and C-3 of fumarate was not reached. The moiety of lysine derived from aspartic semialdehyde reflects this general labelling pattern. Lysine derived from [1-$^{14}C$]acetate was labelled in C-2 and C-3 (C-2 more than C-3) with C-1 and C-4 essentially devoid of radioactivity (Table 3), exactly as would be predicted from the results in Table 4.

[1-$^{14}C$]Acetate metabolized via a forward tricarboxylic acid cycle labels oxaloacetate and aspartate in positions 1 and 4. Since no label was detected in these positions the complete tricarboxylic acid cycle was not operative in these mixed culture organisms. However, equilibration of the label in positions 2 and 3 suggests that the reaction sequence

Aspartate $\rightleftharpoons$ oxaloacetate $\rightleftharpoons$ malate $\rightleftharpoons$ fumarate

was functioning.

As one would predict from Scheme 1, $H^{14}CO_3^-$ was incorporated into aspartate solely into positions 1 and 4 (Table 4).

Aspartic acid, after reduction to its semialdehyde, is also the precursor of threonine and methionine. As expected, threonine specific radioactivity was always less than that of aspartic acid irrespective of the radioactive precursor tested (Figs. 1*b* and 1*c*; 2*b* and 2*c*; 3*b* and 3*c*).

The specific radioactivity of methionine was either equal to or higher than that of aspartate and in all cases exceeded that of threonine. Again, this result is expected since methionine can derive its label from (*a*) aspartic semialdehyde:

Aspartic semialdehyde $\xrightarrow{NAD(P)H}$ homoserine $\xrightarrow{succinyl\text{-}CoA}$ *O*-Succinylhomoserine $\xrightarrow{cysteine}$ cystathionine $\longrightarrow$ homocysteine

and (*b*) from $^{14}CO_2$, if rumen micro-organisms can convert $CO_2$ into the methyl group of methionine as has been suggested for other anaerobic micro-organisms (Jungermann *et al.*, 1968). For this conversion to proceed, a key reaction is a ferredoxin-dependent reduction of $CO_2$ to formate (Ljungdahl & Wood, 1969). It is not known if rumen micro-organisms have the required enzyme, formate dehydrogenase.

*Isoleucine*

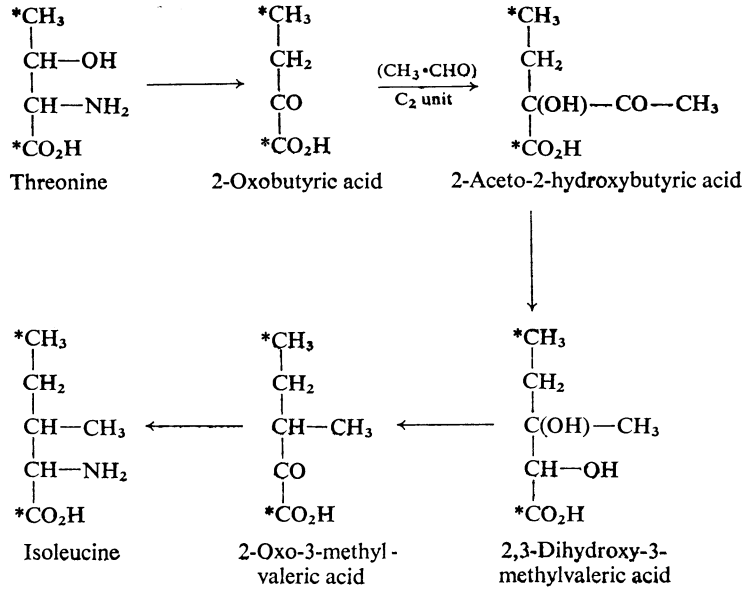
The specific radioactivity of isoleucine synthesized from [1-$^{14}C$]propionate was at least fourfold greater than that of the other amino acids synthesized from this precursor (Fig. 4).

2-Oxobutyrate is an intermediate in the biosynthesis of isoleucine in micro-organisms (Meister, 1965). In most organisms 2-oxobutyrate is synthesized from threonine by threonine dehydratase (EC 4.2.1.16), an allosteric enzyme subject to regulation by isoleucine concentrations (Barman, 1969). By this conventional pathway or the *Escherichia coli* pathway, [1-$^{14}C$]propionate via forward tricarboxylic acid cycle, forms [1,4-$^{14}C$]succinate, which after conversion into [1,4-$^{14}C$]oxaloacetate, [1,4-$^{14}C$]-aspartate and aspartic semialdehyde forms [1,4-$^{14}C$]-threonine. As indicated in Scheme 2, by this pathway [1-$^{14}C$]propionate would yield isoleucine labelled in positions 1 and 5. Similarly, $H^{14}CO_3^-$ would label C-4 of threonine and subsequently C-5 of isoleucine. [1-$^{14}C$]Acetate, as indicated in Scheme 1, would yield [2-$^{14}C$]aspartate, [2-$^{14}C$]threonine and [2-$^{14}C$]isoleucine, and the introduction of a 2-carbon unit (Scheme 2) derived from acetate and labelled in C-1 would place an additional label in C-3 of isoleucine.

However, as shown by the results in Table 5, [1-$^{14}C$]propionate did not label isoleucine in C-1 and C-5 to any significant degree, but instead labelled it predominantly in position 2. This shows that propionate could not have formed 2-oxobutyrate by the conventional pathway but instead must have been directly carboxylated to 2-oxobutyrate with the label in C-2. 2-[2-$^{14}C$]Oxobutyrate forms [2-$^{14}C$]isoleucine by reactions shown in Scheme 2. Buchanan (1969) has isolated an enzyme, 2-oxobutyrate synthase, from a photosynthetic bacterium, *Chromatium*, that catalyses a reduced ferredoxin-dependent reaction:

$$\text{Propionyl-CoA} + Fd_{red.} + CO_2 \rightarrow \text{2-oxobutyrate} + Fd_{ox.} + \text{CoA}$$

Since abundant quantities of propionate are always present in rumen fluid, this pathway of isoleucine biosynthesis may well be of major quantitiative significance in rumen bacteria.




Scheme 2. *Labelling pattern of isoleucine synthesized from [1-$^{14}$C]propionate (*) via the threonine dehydratase pathway*

*Glutamic acid*

Glutamate derived from [1-$^{14}$C]acetate can be labelled by a number of pathways.

(i) Via forward tricarboxylic acid cycle:

(*a*) Oxaloacetate+[1-$^{14}$C]acetate → [1-$^{14}$C]citrate
[1-$^{14}$C]Citrate → 2-[5-$^{14}$C]oxoglutarate
2-[5-$^{14}$C]Oxoglutarate → [5-$^{14}$C]glutamate

(*b*) [1-$^{14}$C]Acetate+$CO_2$ → [2-$^{14}$C]pyruvate
[2-$^{14}$C]Pyruvate+$CO_2$ → [2-$^{14}$C]oxaloacetate
[2-$^{14}$C]Oxaloacetate → [3-$^{14}$C]citrate
[3-$^{14}$C]Citrate → 2-[3-$^{14}$C]oxoglutarate → [3-$^{14}$C]glutamate

(ii) Via reverse tricarboxylic acid cycle:

(*c*) [1-$^{14}$C]Acetate+$CO_2$ → [2-$^{14}$C]pyruvate+$CO_2$ → [2-$^{14}$C]oxaloacetate → [2-$^{14}$C]malate → [2,3-$^{14}$C]fumarate
[2,3-$^{14}$C]Fumarate → [2,3-$^{14}$C]succinate
[2,3-$^{14}$C]Succinate+$CO_2$ → 2-[3,4-$^{14}$C]oxoglutarate
2-[3,4-$^{14}$C]Oxoglutarate → [3,4-$^{14}$C]glutamate

The data in Table 6 show that 57% of the [1-$^{14}$C]-acetate label is recovered in position 5 of glutamate. Therefore in mixed rumen microflora, pathway (*a*) via forward tricarboxylic acid cycle is of principal importance in the biosynthesis of glutamate from acetate; 30% of the [1-$^{14}$C]acetate label was recovered in C-3 and C-4 of glutamate. Since in the chemical degradation of glutamate, C-3 and C-4 are not distinguishable, it is not possible to state whether these positions are labelled via pathway (*b*) (forward tricarboxylic acid cycle) or pathway (*c*) (reverse tricarboxylic acid cycle).

Table 5. *Percentage $^{14}$C distribution in isoleucine from [1-$^{14}$C]propionate*

Values are calculated on the basis of initial radioactivity equals 100% and corrected for losses incurred at each degradative step.

| Isoleucine carbon number | ... 5 | 4 | 3+6 | 2 | 1 |
|---|---|---|---|---|---|
| | Percentage $^{14}$C distribution | | | | |
| | $CH_3$ | $CH_2$ | $CH(CH_3)$ | $CHNH_2$ | $CO_2H$ |
| | <1.0 | <1.0 | <1.0 | 88.4 | 11.6 |

Table 6. *Percentage $^{14}$C distribution in glutamic acid from [1-$^{14}$C]acetate and $H^{14}CO_3^-$*

Values are calculated on the basis of initial radioactivity equals 100% and corrected for losses incurred at each degradative step.

| Glutamic acid carbon number | ... 5 | 4+3 | 2 | 1 |
|---|---|---|---|---|
| | Percentage $^{14}$C distribution | | | |
| Labelled compound | $HO_2C$ | $CH_2$–$CH_2$ | $CHNH_2$ | $CO_2H$ |
| [1-$^{14}$C]Acetate | 56.9 | 29.7 | 9.8 | 3.6 |
| $H^{14}CO_3^-$ | 16.9 | 9.9 | 3.1 | 70.1 |

C-2 of glutamate most likely derives its label from [1-$^{14}$C]acetate via the following reactions:

(*d*) [2,3-$^{14}$C]Succinate (from pathway *c*) →
[2,3-$^{14}$C]oxaloacetate
[2,3-$^{14}$C]Oxaloacetate+acetate →
[3,4-$^{14}$C]citrate
[3,4-$^{14}$C]Citrate → 2-[2,3-$^{14}$C]oxoglutarate
2-[2,3-$^{14}$C]Oxoglutarate → [2,3-$^{14}$C]glutamate

Failure of [1-$^{14}$C]acetate to label C-1 of glutamate significantly, again emphasizes that these micro-organisms do not carry out the reactions of a complete forward tricarboxylic acid cycle, since this C-1 would be labelled from [1,4-$^{14}$C]oxaloacetate formed from [1-$^{14}$C]acetate on the second turn of the tricarboxylic acid cycle.

Milligan (1970) found $^{14}$C distribution in glutamate to be 70.4% in C-1, 10.9% in C-2, 0.6% in C-3 and C-4 and 10.0% in C-5 when rumen micro-organisms were incubated with $H^{14}CO_3^-$. These results are in general agreement with those reported in Table 6, where $H^{14}CO_3^-$ labelled glutamate almost exclusively in position 1, with some label (16.9%) in position 5. C-1 becomes labelled by either (*a*) the carboxylation of pyruvate to [4-$^{14}$C]oxaloacetate, which, via forward tricarboxylic acid cycle, gives [1-$^{14}$C]glutamate, or by (*b*) the reductive carboxylation of succinate with $^{14}CO_2$ to give 2-[1-$^{14}$C]oxoglutarate, which, as above, is transaminated to [1-$^{14}$C]glutamate. From these results it is not possible to assess the relative contribution of either pathway.

The pathway of $H^{14}CO_3^-$ incorporation into C-5 of glutamate (Table 6) is not clear. Gottschalk & Barker (1967) reported on an atypical citrate synthase in *Clostridium kluyveri* which forms the isotope antipode of [$^{14}$C]citrate formed by the usual synthase. The presence of an atypical citrate synthase in rumen micro-organisms could explain the presence of label in C-5 of glutamate and indeed Milligan (1970) offered this explanation. However, this enzyme would also label C-1 of glutamate with [1-$^{14}$C]acetate as precursor and as seen from the results in Table 6 this did not occur. Therefore the pathway of incorporation of $H^{14}CO_3^-$ into C-5 of glutamate still needs clarification.

From these data it is apparent that in rumen micro-organisms, forward tricarboxylic acid-cycle reactions proceed to 2-oxoglutarate only. Since the reversible reaction sequence of oxaloacetate to succinate is apparently functioning (see the section on lysine) the block is most likely in the reaction of 2-oxoglutarate to succinyl-CoA catalysed by 2-oxoglutarate dehydrogenase (EC 1.2.4.2). Possibly this enzyme is lacking in the microbial systems. Alternatively, any 2-oxoglutarate that is synthesized can be metabolized to glutamate by transamination or to 2-hydroxyglutarate by reduction with 2-hydroxyglutarate dehydrogenase (EC 1.1.99.2). These reactions, combined, could possibly lower the 2-oxoglutarate concentration sufficiently so that the 2-oxoglutarate dehydrogenase reaction is effectively blocked.

*Leucine and valine*

Leucine and valine biosynthesis proceed through condensation of pyruvate and a 2-carbon unit (acetaldehyde) as shown in Scheme 3.

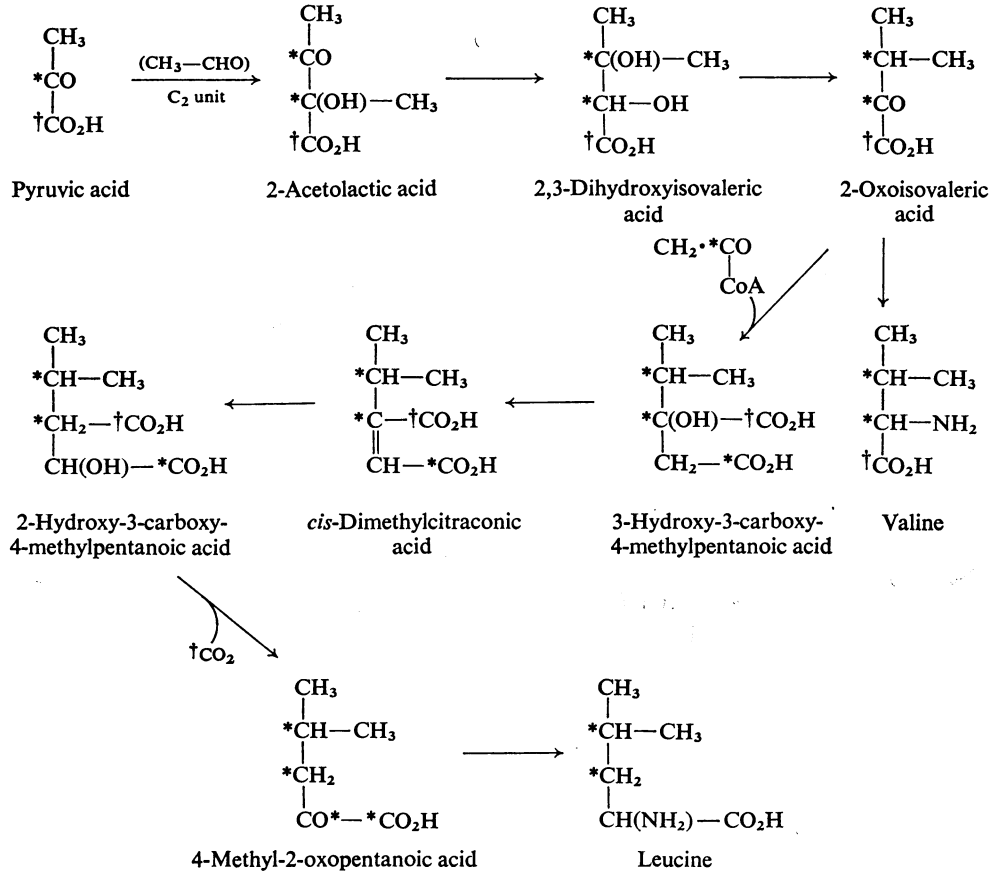
In the present experiments valine specific radioactivity was greater with [1-$^{14}$C]acetate than with $H^{14}CO_3^-$ as substrate (Figs. 1*c* and 2*c*). This is expected since in the thiamine pyrophosphate-catalysed condensation, one of the two pyruvate molecules is decarboxylated, thus one-half of the fixed $^{14}CO_2$ is lost, whereas all the radioactive carbon in the pyruvate formed by carboxylation of [1-$^{14}$C]acetate is retained in 2-acetolactate.

The specific radioactivity of leucine synthesized from [1-$^{14}$C]acetate is greater than that synthesized from $H^{14}CO_3^-$ (Figs. 1*d* and 2*d*). This is not surprising since, as indicated in Scheme 3, [1-$^{14}$C]acetate can label C-1, C-3 and C-4 of leucine. On the other hand, no $H^{14}CO_3^-$ should be retained in leucine since this $^{14}CO_2$ is lost in the decarboxylation of 2-hydroxy-3-carboxy-4-methylpentanoic acid. The results in Fig. 1*d*, however, clearly show that leucine does become labelled from $^{14}CO_2$. This undoubtedly represents reductive carboxylation of isovalerate with $^{14}CO_2$. This pathway has been demonstrated for *Ruminococcus flavefaciens*, *Bacteroides ruminicola* and *Peptostreptococcus elsdenii* although no quantitative assessment was made (Allison, 1969; Allison & Peel, 1971). These organisms also showed evidence for the reductive carboxylation of isobutyrate. The present results, showing that valine synthesized from [1-$^{14}$C]acetate has greater specific radioactivity than valine synthesized from $H^{14}CO_3^-$, assign a quantitatively minor role to the isobutyrate carboxylation pathway.

*Serine*

Serine was effectively labelled with either [1-$^{14}$C]-acetate or $H^{14}CO_3^-$ as precursor (Figs. 1*c* and 2*c*); however, the specific radioactivity of serine was greater with $H^{14}CO_3^-$ than with [1-$^{14}$C]acetate. The labelling pattern of serine (Table 7) is again consistent with the reductive carboxylation of acetate to pyruvate (Scheme 4).

In recent years attention has focused on the phosphorylation of pyruvate to phosphoenolpyruvate. Cooper & Kornberg (1965) first demonstrated the synthesis of phosphoenolpyruvate from pyruvate and ATP by an enzyme, phosphoenolpyruvate synthase, present in *E. coli*. Since then another enzyme, pyruvate,orthophosphate dikinase (EC 2.7.9.1),




Scheme 3. *Labelling pattern of valine and leucine synthesized from* [1-$^{14}$*C*]*acetate* (*) *and* $H^{14}CO_3^-$ (†)

Table 7. *Percentage* $^{14}$*C distribution in serine from* [1-$^{14}$*C*]*acetate and* $H^{14}CO_3^-$

Values are calculated on the basis of initial radioactivity equals 100% and corrected for losses incurred at each degradative step.

| | Percentage $^{14}$C distribution | | |
|---|---|---|---|
| Serine carbon ... | 3 | 2 | |
| Labelled compound | $CH_2OH$ | $CHNH_2$ | $CO_2H$ |
| [1-$^{14}$C]Acetate | 4.5 | 85.3 | 10.2 |
| $H^{14}CO_3^-$ | 9.9 | 5.0 | 85.1 |

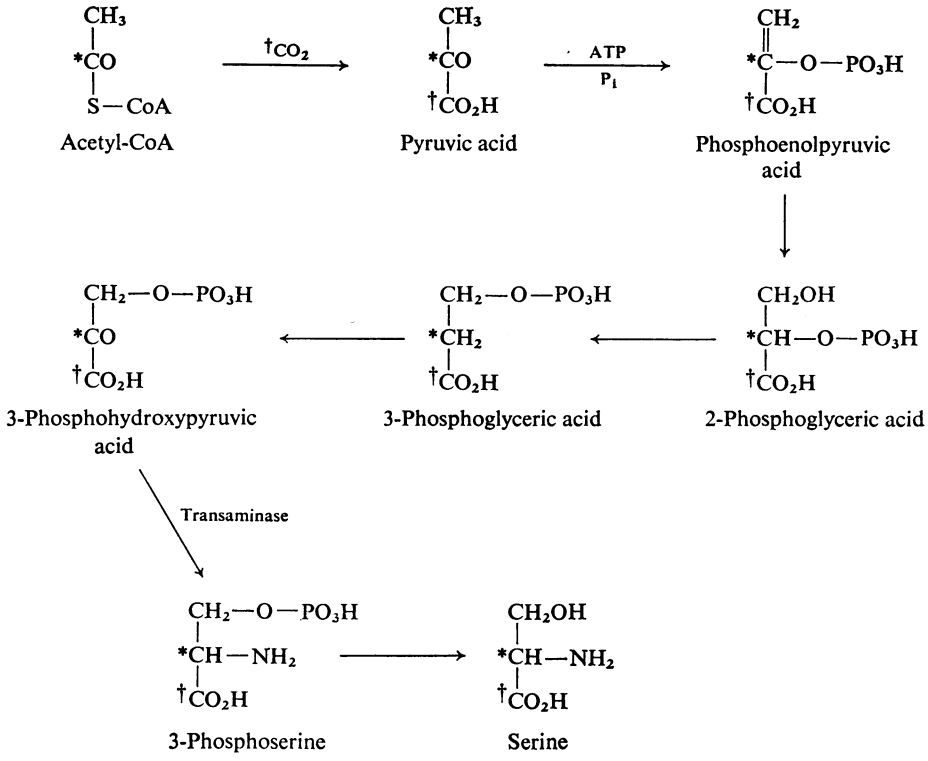
which catalyses this reaction has been found in photosynthetic (Buchanan, 1974) and non-photosynthetic bacteria (Evans & Wood, 1971). It is therefore probable that the reaction can proceed in rumen micro-organisms.

In rumen micro-organisms many of the 2-oxo acid precursors of amino acids appear to be synthesized via reductive carboxylation of carboxylic acids. Serine may be one of the exceptions to this in that the 2-oxo precursor, 3-phosphohydroxypyruvic acid, is usually formed by an $NAD^+$-catalysed oxidation of 3-phosphoglyceric acid. This oxidative step apparently proceeds in spite of the high reducing potential of the anaerobic rumen bacteria.

With the operation of the sequence of reactions in Scheme 4, serine synthesized from either $H^{14}CO_3^-$ or [1-$^{14}$C]acetate should have equal specific radioactivity. As seen in Figs. 1(*c*) and 2(*c*) this was not so, and serine synthesized from $H^{14}CO_3^-$ had a two- to three-fold greater specific radioactivity than serine formed from [1-$^{14}$C]acetate. This raises the question of whether some serine is synthesized by reductive carboxylation of glycollate to form 3-hydroxypyruvate which could then be transaminated to give serine.

*Tyrosine and phenylalanine*

Phosphoenolpyruvate, synthesized from $H^{14}CO_3^-$ and acetate, labels shikimic acid in C-7, which is

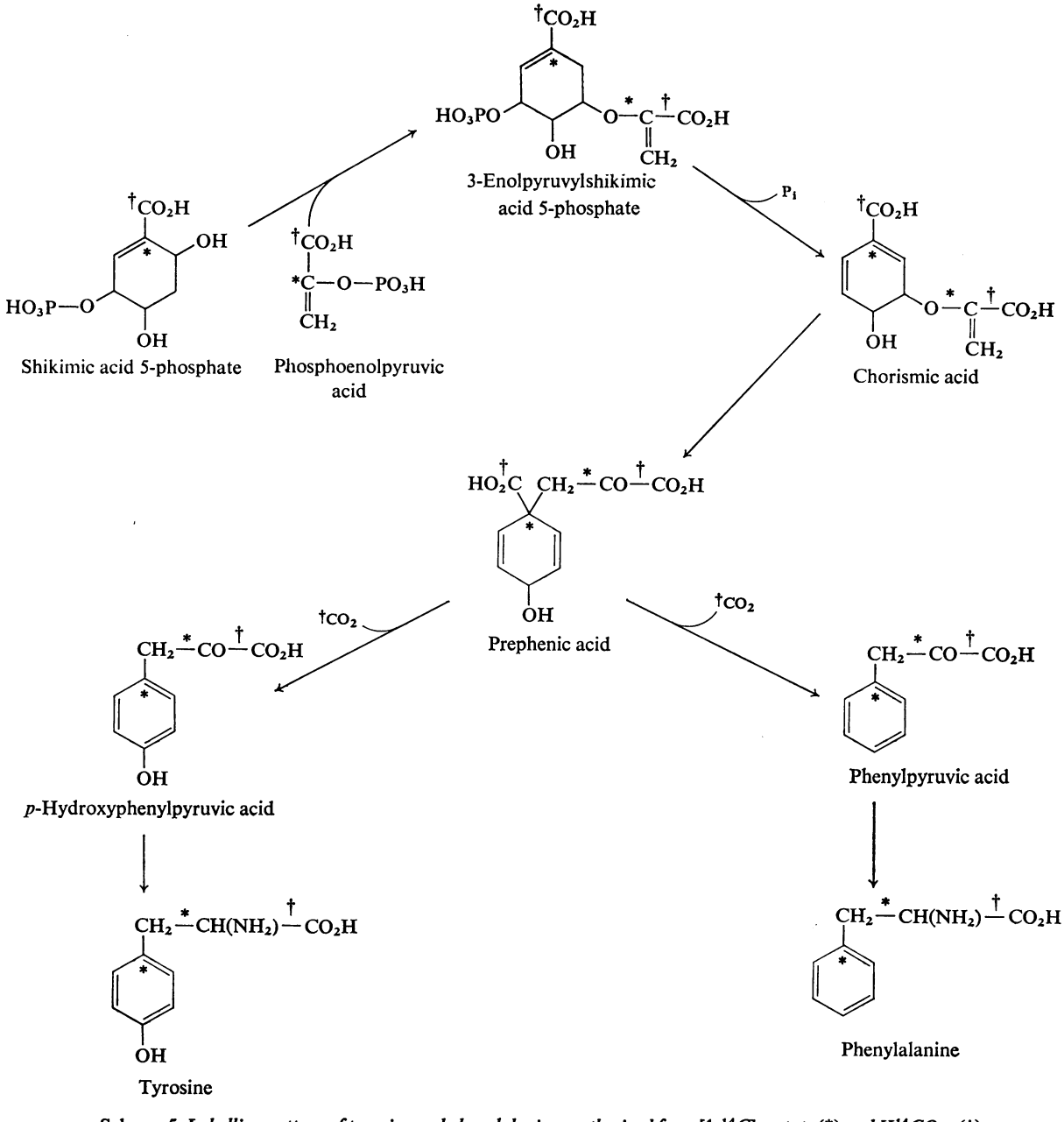



Scheme 4. *Labelling pattern of serine synthesized from* [1-$^{14}$C]*acetate* (*) *and* $H^{14}CO_3^-$ (†)

subsequently lost in the decarboxylation of prephenic acid (Scheme 5). A second molecule of phosphoenolpyruvate also labelled in C-1, is condensed with shikimic acid 5-phosphate to yield the 3-enolpyruvyl derivative. This $H^{14}CO_3^-$ is retained in the carboxyl carbon of tyrosine and phenylalanine. [1-$^{14}$C]Acetate, which after reductive carboxylation yields phosphoenol[2-$^{14}$C]pyruvate, labels both ring and side chain of tyrosine and phenylalanine with no loss of $^{14}$C. From this, one would expect the aromatic amino acids to have greater specific radioactivity with [1-$^{14}$C]acetate as precursor than with $H^{14}CO_3^-$ as precursor. In fact, as the Results in Figs. 1(*d*) and 2(*d*) show, the opposite is true. The specific radioactivity of phenylalanine is approximately tenfold higher with $H^{14}CO_3^-$ as precursor than with [1-$^{14}$C]acetate as precursor. A similar five- to seven-fold increase in specific radioactivity was observed with tyrosine synthesized from $H^{14}CO_3^-$.

The phenylalanine- and tyrosine-degradation results (Table 8) indicate that rumen microbes synthesize aromatic amino acids by the well-known shikimic acid phosphate pathway. With $H^{14}CO_3^-$ as precursor the ring of either tyrosine or phenylalanine was practically devoid of label. In contrast, appreciable ring labelling occurred in both tyrosine and phenylalanine when [1-$^{14}$C]acetate was the radioactive precursor. Hoare & Gibson (1964) also observed that acetate was incorporated into the aromatic ring of phenylalanine. Scheme 5, however, does not account for the higher specific radioactivity observed with the aromatic acids when $H^{14}CO_3^-$ instead of [1-$^{14}$C]acetate was used as radioactive precursor.

The reductive carboxylation of the phenylalanine precursor, phenylacetate has been qualitatively, but not quantitatively, demonstrated by Allison (1969) in six species of rumen micro-organisms. The present data fully support his observations and further indicate that the reductive carboxylation of phenylacetate and hydroxyphenylacetate may constitute a quantitatively important pathway for phenylalanine and tyrosine biosynthesis in rumen micro-organisms. In addition to the carboxyl carbon, which was formed from $HCO_3^-$, the present results (Table 8) show that the α-carbon of the aromatic amino acid side chain was also derived from $HCO_3^-$. In contrast, the β-carbon and ring were almost devoid of radioactivity when $H^{14}CO_3^-$ was added to the culture.




Scheme 5. *Labelling pattern of tyrosine and phenylalanine synthesized from* [1-$^{14}$C]*acetate* (*) *and* $H^{14}CO_3^-$ (†)

Rumen micro-organisms apparently reutilize the substituted benzene ring in the biosynthesis of phenylalanine and tyrosine. This obviates the necessity of synthesizing these amino acids *de novo* from erythrose 4-phosphate and phosphoenolpyruvate, or at least minimizes the quantitative significance of the pathway *de novo*.

*Histidine*

The specific radioactivity of histidine labelled with $H^{14}CO_3^-$ was greater than that of histidine labelled with [1-$^{14}$C]acetate (Figs. 1*a* and 2*a*). This is not unexpected in view of the known biosynthetic pathways of histidine formation. $CO_2$,

Table 8. *Percentage $^{14}C$ distribution in phenylalanine and tyrosine from [1-$^{14}C$]acetate and $H^{14}CO_3^-$*

Values are calculated on the basis of initial radioactivity equals 100% and corrected for losses incurred at each degradative step.

| | Percentage $^{14}C$ distribution | | | |
|---|---|---|---|---|
| Phenylalanine carbon ... | | β | α | |
| Labelled compound | Ring | $CH_2$ | $CHNH_2$ | $CO_2H$ |
| [1-$^{14}C$]Acetate | 27.5 | | 72.5 | |
| $H^{14}CO_3^-$ | 2.1 | 4.8 | 72.1 | 21.0 |
| | | β | α | |
| Tyrosine carbon ... | HO–ring | $CH_2$ | $CHNH_2$ | $CO_2H$ |
| [1-$^{14}C$]Acetate | 26.3 | | 65.5 | 8.2 |
| $H^{14}CO_3^-$ | 4.1 | | 40.2 | 55.7 |

after reduction to formate, is incorporated into the purine ring as C-2 which becomes C-2 of the imidazole ring of histidine. Alternatively, $CO_2$ (and acetate) could be incorporated into histidine through incorporation into pyruvate, which through reversal of glycolysis and formation of phosphoribosyl pyrophosphate, goes to constitute the remainder of the histidine carbon chain.

*General comments*

The results of this investigation show that acetate and $CO_2$ form an active precursor pool of carbon for amino acid synthesis by rumen bacteria. Although acetate was incorporated directly into amino acid carbon the possibility that some acetate dismutation to $CO_2$ and methane occurred before amino acid biosynthesis cannot be excluded.

Propionate carbon, in contrast with acetate or $CO_2$, was only poorly utilized for amino acid biosynthesis. Propionate apparently only serves as $CO_2$ acceptor for 2-oxobutyrate in isoleucine biosynthesis.

The present data indicate that succinate carboxylation to 2-oxoglutarate may proceed in mixed rumen culture. Since propionate was poorly incorporated into glutamic acid it follows that propionate carboxylation to methylmalonyl-CoA, and conversion into succinyl-CoA and succinate are slow reactions in this system. This is not surprising since in rumen bacteria propionate is primarily an end product of carbohydrate fermentation via the following pathway (Lewis & Elsden, 1955):

Pyruvate → lactate → acryloyl-CoA → propionyl-CoA → propionate

Buchanan and collaborators (Bachofen *et al.*, 1964; Buchanan *et al.*, 1964; Buchanan & Evans, 1965; Evans *et al.*, 1966; Buchanan, 1969) have described three reductive carboxylation reactions:

$$\text{Acetyl-CoA} + CO_2 + Fd_{red.} \rightarrow \text{pyruvate} + Fd_{ox.} + \text{CoA} \quad (1)$$

$$\text{Propionyl-CoA} + Fd_{red.} + CO_2 \rightarrow \text{2-oxobutyrate} + Fd_{ox.} + \text{CoA} \quad (2)$$

$$\text{Succinyl-CoA} + CO_2 + Fd_{red.} \rightarrow \text{2-oxoglutarate} + Fd_{ox.} + \text{CoA} \quad (3)$$

Reactions (1) and (2) were present in a non-photosynthetic bacterium, *Cl. pasteurianum*. Reaction (3) has been described in photosynthetic bacteria. Reactions (1) and (3) appear to be catalysed by different enzymes (Buchanan, 1969). Characteristic of these reactions is the direct involvement of ferredoxin without the mediation of nicotinamide–adenine dinucleotides. Ferredoxin, the most negative electron carrier known, i.e. $\simeq -420$mV for $NAD^+$ or $NADP^+$ at pH7, has been successfully isolated from mixed rumen micro-organisms (Emmanuel & Milligan, 1972).

The results of this investigation are fully consonant with reactions (1), (2) and (3) proceeding actively in rumen micro-organisms. The evidence also supports the presence of reductive carboxylations for the biosynthesis of 2-oxoisovaleric acid and 4-methyl-2-oxopentanoic acid, phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid. A further reductive carboxylation, i.e. that of ribulose 1,5-diphosphate to 3-phosphoglycerate should also be considered in future investigations. In addition, the results of the present investigations show that one tricarboxylic acid-cycle reaction, the oxidation of 2-oxoglutarate to succinate, is effectively blocked in rumen bacteria. Studies are currently in progress to determine the nature of the block imposed on this reaction.

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