STUDIES ON THE METABOLIC FUNCTION OF BRANCHED-CHAIN VOLATILE FATTY ACIDS, GROWTH FACTORS FOR RUMINOCOCCI

I. INCORPORATION OF ISOVALERATE INTO LEUCINE

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ABSTRACT

ALLISON, MILTON J. (Dairy Cattle Research Branch, U. S. Department of Agriculture, Beltsville, Md.), M. P. Bryant, and R. N. Doetsch. Studies on the metabolic function of branched-chain volatile fatty acids, growth factors for ruminococci. I. Incorporation of isovalerate into leucine. J. Bacteriol. 83:523-532. 1962.—Ruminococcus flavefaciens strain C94, a cellulolytic rumen bacterium, requires either isobutyrate or isovalerate for growth. The organism was grown in the presence of C14-labeled isovalerate, and the metabolic fate of the labeled carbon was studied to obtain information on the functions of this growth factor. Radioactivity from isovalerate-1-C14 and isovalerate-3-C14 was found mainly in the protein and lipid fractions of the cells. The C14 in protein was all in leucine, indicating that a function of isovalerate was to serve as a carbon skeleton for leucine synthesis.

As C14 in leucine synthesized from isovalerate-1-C14 was entirely in carbon 2, the intact isovalerate molecule was apparently incorporated into leucine. This is evidence that leucine was synthesized by a mechanism different from that previously demonstrated in other microorganisms.

R. flavefaciens has a definite but limited ability to incorporate exogenous amino acids, including leucine. It incorporated 2% of the C14 during growth in uniformly labeled (UL) C14-Chlorella protein hydrolyzate; Escherichia coli incorporated 37% of the label under similar conditions. In another experiment, a limited amount of exogenous leucine-2-C14 was incorporated into protein of R. flavefaciens. The requirement for isovalerate was not replaced by DL-leucine or 2-ketoisocaproate. It is suggested that isovalerate or isobutyrate is required because R. flavefaciens has a limited ability to incorporate exogenous branched-chain amino acids and a limited ability to synthesize the isopropyl group found in these amino acids and in other components of the cell.

A number of strains of several genera of rumen bacteria have an obligate nutritional requirement for certain volatile fatty acids. A strain of the cellulolytic anaerobe, Bacteroides succinogenes, studied by Bryant and Doetsch (1955), required a branched-chain acid (isobutyrate, isovalerate, or 2-methyl-3-butryrate), in combination with any five- to eight-carbon straight-chain saturated monocarboxylic acid. Stearic or palmitic acid partially replaced the requirement for the straight-chain acid. Allison, Bryant, and Doetsch (1958) found that three cellulolytic strains of Ruminococcus flavefaciens and two cellulolytic strains of R. albus required volatile fatty acids. Bryant and Robinson (1961a) extended this finding to four additional strains of R. albus, one of which was noncellulolytic. However, five strains of Ruminococcus which required other growth factors were noted. Ayers (1958) studied a strain of R. flavefaciens and Fletcher (1956), a strain of R. albus that also required other, as yet unidentified, growth factors.

Other noncellulolytic rumen bacteria with volatile fatty acid requirements have recently been noted. Wegner and Foster (1960) isolated a Borrelia species and an unnamed gram-positive rod which require both a branched- and a straight-chain acid; Bryant and Robinson (unpublished data) found that all of several strains.

of *Eubacterium ruminantium* (Bryant, 1959) require volatile fatty acids and that a number of other noncellulolytic bacteria are greatly stimulated by these acids.

The requirement for volatile fatty acids may not be a unique characteristic of rumen bacteria. Hall (1952) isolated from the rabbit cecum cellulytic cocci which required either rumen fluid or cecal extract. It is possible that volatile fatty acids were the growth factors in these substances and that other bacteria which require volatile fatty acids may be found in similar environments.

Since information on the physiological function of such acids was lacking, we studied the fate of C\(^{14}\) after culture of a cellulytic strain of *R. flavefaciens* in isovalerate-1-C\(^{14}\) and isovalerate-3-C\(^{14}\). We obtained evidence which indicates that a function of isovalerate is to provide part of the carbon skeleton for the synthesis of leucine. A preliminary report of this work has been published (Allison, Bryant, and Doetsch, 1959).

**MATERIALS AND METHODS**

Organisms. Strain C94 of *R. flavefaciens*, described by Bryant et al. (1958), which requires either isovalerate or isobutyrate for growth (Allison et al., 1958) was selected for study as a representative of organisms requiring volatile fatty acids. *Escherichia coli*, strain B, was obtained from D. B. Cowie, Carnegie Institution of Washington.

Organisms were cultured anaerobically, using the methods of Hungate (1950) as modified by Bryant and Burkey (1953), except that gases were freed of oxygen by passing them through a column of hot reduced copper filings.

**Nutrition experiments.** Table 1 gives the composition of the basal medium used. The inoculum was prepared as outlined by Allison et al. (1958). Growth was measured as optical density (OD) with a Bausch and Lomb Spectronic 20 colorimeter at 600 nm in 13 by 100 mm Pyrex rubber-stoppered tubes.

Clariﬁed rumen fluid (CRF) was prepared from rumen contents obtained by a stomach tube from a cow on an alfalfa hay-grain ration 4 hr after feeding. The rumen material was filtered through cheesecloth and centrifuged at 25,000 × g for 30 min. The supernatant was decanted, adjusted to pH 6.7, and sterilized in the autoclave at 121 C for 20 min under an atmosphere of CO\(_2\).

CRF was fractionated into acid volatile and acid nonvolatile fractions by steam distillation, after adjustment to pH 1.5 with concentrated H\(_2\)SO\(_4\). The distillate was titrated with standardized NaOH and found to contain 80 μmoles of volatile acid/ml. It was then evaporated to a small volume on a steam bath at pH 11.5, and

**TABLE 1. Composition of the basal medium**

<table>
<thead>
<tr>
<th>Ingredient*</th>
<th>mg/100 ml</th>
<th>Ingredient</th>
<th>mg 100/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine-HCl</td>
<td>0.2</td>
<td>Casein hydrolyzate(^b)</td>
<td>200</td>
</tr>
<tr>
<td>Ca-pantothenate</td>
<td>0.2</td>
<td>Cellobiase</td>
<td>300</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.2</td>
<td>Ressurin</td>
<td>0.1</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0.2</td>
<td>KH(_2)PO(_4)</td>
<td>90</td>
</tr>
<tr>
<td>Pyridoxamine-2-HCl</td>
<td>0.1</td>
<td>NaCl</td>
<td>90</td>
</tr>
<tr>
<td>Pyridoxal-HCl</td>
<td>0.1</td>
<td>(NH(_4))(_2)SO(_4)</td>
<td>90</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
<td>0.1</td>
<td>CaCl(_2)</td>
<td>1</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>0.01</td>
<td>MgSO(_4)-7H(_2)O</td>
<td>1</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.005</td>
<td>MnSO(_4)-H(_2)O</td>
<td>1</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.005</td>
<td>CoCl(_2)-6H(_2)O</td>
<td>0.4</td>
</tr>
<tr>
<td>Cobalamin</td>
<td>0.0005</td>
<td>Na(_2)CO(_3)*</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na(_2)S-9H(_2)O(^d)</td>
<td>50</td>
</tr>
</tbody>
</table>

* Solution of materials, other than Na\(_2\)CO\(_3\) and Na\(_2\)S-9H\(_2\)O, adjusted to pH 6.7 and autoclaved for 15 min at 15 lb under CO\(_2\).  
\(^b\) "Vitamin-free" acid-hydrolyzed casein (Nutritional Biochemicals Corp., Cleveland, Ohio).  
\(^c\) Autoclaved separately under N\(_2\), after which it was cooled and equilibrated with CO\(_2\) before adding to the medium.  
\(^d\) Autoclaved separately under N\(_2\), and added to the tubed medium just prior to inoculation.
the pH of both nonvolatile and volatile fractions was adjusted to 6.7 with NaOH and H₂SO₄. Solutions were made to the original volume of the CRF and sterilized by autoclaving under oxygen-free N₂. Solutions of salts of fatty acids were prepared by titrating a known volume of standardized NaOH to pH 8.0 with a dilution of the acid. Solutions were made to a known volume and were autoclaved and tubed under N₂. Solutions of amino acids were also sterilized by autoclaving. Solutions of 2-ketoisovalerate, 2-ketoisocaproate, and DL-2-hydroxyisovalerate (California Corp. for Biochemical Research, Los Angeles) were sterilized by passing them through a Millipore filter (0.45 μ), and oxygen was displaced by bubbling with CO₂ before these were added to the culture medium.

Radioactive chemicals. The following compounds were employed: sodium isovalerate-1-C¹⁴, uniformly labeled *Chlorella* protein C¹⁴ hydrolysate, and acetone-2-C¹⁴ (Volk Radiochemical Co.); and DL-leucine-2-C¹⁴ (California Corp. for Biochemical Research). Isovalerate-3-C¹⁴ was synthesized as follows: 2-cyanoethyl isovalerate was prepared by a condensation-reduction reaction between acetone-2-C¹⁴ and ethyl cyanocetate (Alexander and Cope, 1944) in the hydrogenation apparatus described by Siggia (1949). The reaction mixture contained 10 mg of palladium black, 106 mg of ethyl cyanacetic acid, 14 mg of ammonium acetate, 0.02 ml of acetic acid, and 200 μmoles of acetone-2-C¹⁴ in 2.7 ml of ethanol. Uptake of hydrogen by the catalyst, while stirring at atmospheric pressure, was followed manometrically. Hydrogen uptake ceased after 6 hr, at which time the acetone solution was injected with a hypodermic syringe through a rubber serum-bottle closure. After hydrogenation for 165 min, the reaction mixture was filtered to remove the catalyst, and isopropyl malonate-2-C¹⁴ was produced from 2-cyanoethyl isovalerate in the filtrate by hydrolysis with 1.5 ml of 15% NaOH under reflux at 110°C for 20 hr. Ethanol was removed by distillation, the mixture was acidified, and isopropyl malonic acid was removed by continuous extraction with ethyl ether. To decarboxylate the extract, it was evaporated to dryness and heated under reflux in an oil bath at 200°C for 30 min. Isovaleric acid-3-C¹⁴ was recovered by steam distillation and purified by chromatography, using a modification of the method of Wiseman and Irwin (1957). Approximately 25% of the radioactivity from acetone-2-C¹⁴ was recovered as a five-carbon volatile acid.

Both isovalerate-1-C¹⁴ and isovalerate-3-C¹⁴ gave single spots corresponding to a five-carbon fatty acid on radioautographs of paper chromatograms developed by the method of Elsden and Lewis (1953).

Measurement of radioactivity. Radioactivity was measured with a Nuclear Chicago D-47 windowless gas-flow counter operating in the Geiger-Müller region. Samples, other than the BaCO₃ samples, were evaporated to dryness on stainless steel planchets and contained material weighing less than 0.5 mg/cm², so that no correction for self-absorption was required. Samples containing volatile acids were kept alkaline to phenolphthalein while drying, and a drop of dilute detergent was added to aqueous samples to obtain uniform spreading. Samples of BaC⁴O₃ were prepared by the method of Sakami (1955), except that a Millipore filter was used to collect the BaCO₃ and the filter was fixed to the planchet with a few drops of acetone. Counts of BaCO₃ samples were corrected for self-absorption.

Methods for determining incorporation and distribution of labeled metabolites. Growth during incorporation experiments was estimated as OD in tubes 18 mm in diameter. Incorporation of isovalerate-1-C¹⁴ by *R. flavefaciens* was measured after exponential growth from OD 0.18 to 0.72 (6.75 hr) in the basal medium plus 10% CRF and isovalerate-1-C¹⁴ (0.2 μmole/ml). Cells were harvested by centrifugation, washed three times by centrifugation from a 0.85% NaCl solution, and then fractionated by the method of Abelson, Bolton, and Aldous (1952).

The protein fraction was hydrolyzed and amino acids were separated by paper chromatography, using the methods of Roberts et al. (1957) and Work (1949). Amino acids were located on the chromatograms by spraying them with 0.25% ninhydrin in n-butanol and by holding them in the open doorway of a drying oven for 5 to 10 min. Radioautographs were prepared to detect radioactive areas on the chromatograms.

The diffusion vessel of Katz, Abraham, and Baker (1954) was employed to test for the presence of C⁴O₂ in the lipid fraction of the cells and in the culture supernatant. Radioautographs of paper chromatograms prepared by the method of Elsden and Lewis (1953) were used to detect C¹⁴ in volatile fatty acids in the lipid fraction of the cell.

Isovalerate-3-C¹⁴ incorporation was deter-
mined after growth from OD 0 to 0.64 (70 hr) in basal medium plus sodium acetate (20 μmoles/ml) and sodium isovalerate-3-C\(^14\) (2 μmoles/ml). A small inoculum was used in this experiment to obtain maximal uptake of the isovalerate-3-C\(^14\), as the supply of it was limited.

Incorporation of C\(^14\) from Chlorella protein-C\(^14\) hydrolyzate by \textit{R. flavefaciens} was determined after growth in the basal medium (with the casein hydrolyzate replaced by a trace quantity of the labeled hydrolyzate). Growth factor was supplied by adding both isovalerate (2 μmoles/ml) and CRF (10% v/v). Cells were harvested and fractionated after exponential growth from OD 0.19 to 0.76 in 6.25 hr. \textit{E. coli} was cultured under the same conditions, except glucose replaced the cellulose and incorporation was measured after growth from OD 0.175 to 0.70 in 4.75 hr. The protein fractions of cells grown in media containing labeled amino acids were washed successively with ethanol plus 1% (v/v) HCl, ethyl ether, acid-hydrolyzed casein (2 mg/ml), acidic alcohol, and finally with ethyl ether. The wash with casein hydrolyzate was performed to remove traces of nonpeptide labeled amino acids which might have been adsorbed to the protein.

Incorporation of leucine-2-C\(^14\) was measured after exponential growth of \textit{R. flavefaciens} from OD 0.18 to 0.72 in 12 hr. Isovalerate (2.5 μmoles/ml) and sodium acetate (20 μmoles/ml) were added to the basal medium, and the casein hydrolyzate was replaced by a mixture of amino acids. The amino acids were the \(\ell\) isomers of alanine, arginine, aspartic acid, glutamic acid, glycine, histidine-HCl, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and valine (all at 1 mg/ml), plus a trace amount of \(\text{dL}\)-leucine-2-C\(^14\). The protein fraction of these cells was washed as above to remove any adsorbed nonpeptide leucine-2-C\(^14\).

\textbf{Degradation of leucine.} The position of C\(^14\) in leucine was determined, using methods similar to those of Sakami (1955). Radioactivity in carbon 1 was determined as BaCO\(_3\) after decarboxylation with ninhydrin. Isovaleraldehyde from that reaction was oxidized to isovaleric acid with alkaline KMnO\(_4\), and isovaleric acid was recovered from the reaction mixture by steam distillation at pH 1.5. After distillation, the material was evaporated to near dryness at pH 11, and a paper chromatogram (Elsden and Lewis, 1953) of a sample was prepared. The \(R_p\) of the radioactive area coincided with the \(R_p\) of known isovalerate. The isovalerate was decarboxylated by the Schmidt reaction, and CO\(_2\) was trapped in NaOH and counted as BaCO\(_3\). The reaction mixture was adjusted to the alkaline color of thymol blue, and isobutylamine was steam-

![FIG. 1. Growth of Ruminococcus flavefaciens in response to CRF, fractions of CRF, and a mixture of salts of volatile fatty acids.](http://jb.asm.org/)

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FIG. 2. Growth response of Ruminococcus flavefaciens to levels of isovalerate, isobutyrate, and a combination of isovalerate and isobutyrate.

distilled into HCl and was counted as the hydrochloride.

RESULTS

Growth response to volatile fatty acids. The unsupplemented basal medium did not support growth of R. flavefaciens. The acid-volatile fraction of CRF supported growth similar to that obtained with whole CRF, but the nonvolatile fraction did not support significant growth (Fig. 1). A mixture of salts of commercially obtained fatty acids in proportions similar to those found in rumen fluid supported growth similar to that obtained with CRF. When sodium salts of the fatty acids were added individually to the basal medium, only isovalerate and isobutyrate supported growth. Other sodium salts, which did not support growth when tested singly or in various combinations, included: acetate, propionate, n-butyrate, n-valerate, n-caproate, DL-2-methyl-n-butyrate, and isocaproate. When acetate was added to medium containing isovalerate, the lag phase of growth was shortened but the final turbidity of the culture was not changed.

The growth response to isovalerate and isobutyrate in the basal medium containing 20 μmoles acetate/ml is shown in Fig. 2. The combination of isobutyrate and isovalerate supported better growth than did equivalent levels of single acids. The branched-chain acids used in this experiment were commercial samples supplied by I. R. Hunter, Field Crops Laboratory, Western Utilization and Development Division, U. S. Dept. of Agriculture, Albany, Calif.; these acids yielded single peaks when tested by gas chromatography.

With casein hydrolyzate deleted from the basal medium, and with acetate and isovalerate added (20 and 1 μmoles/ml, respectively), strain C94 grew to OD 0.13. In this medium plus a mixture of salts of volatile fatty acids similar to those found in rumen fluid (Annison, 1954) growth to OD 0.50 was obtained. This demonstrated that the organism could grow with ammonia as the sole source of nitrogen (other than the small amount present in B vitamins). Bryant and Robinson (1961b) have also found that this strain and seven other strains of Ruminococcus can grow with ammonia as the sole source of nitrogen.

Growth of strain C94 was not obtained when 2-ketoisovalerate or 2-ketoisocaprate (1 or 0.1 μmole/ml) or 2-hydroxyisovalerate (1 μmole/ml) were added to the basal medium. Racemic mixtures of leucine, isoleucine, and valine, added to the basal medium singly or together at 0.1 mg/ml, were also incapable of supporting growth.

Distribution of C\textsuperscript{14} in cells grown in labeled isovalerate. Most of the C\textsuperscript{14} from isovalerate-1-C\textsuperscript{14} and isovalerate-3-C\textsuperscript{14} was located in the protein...
and lipid fractions of the cells (Table 2). Similar distribution of C\textsuperscript{14} was obtained in other experiments with both 1 and 3 labeled isovalerate.

A radioautograph of a two-dimensional chromatogram of the hydrolyzed protein from cells grown in isovalerate-1-C\textsuperscript{14} was prepared by the method of Roberts et al. (1957). Radioactivity was noted only in the area corresponding to leucine, isoleucine, and phenylalanine. These amino acids were resolved, using the method of Work (1949), and the only labeled amino acid was then found to be leucine (Fig. 3). Leucine was also the only labeled amino acid detected in hydrolyzates of the protein of cells grown in isovalerate-3-C\textsuperscript{14}.

Radioactive CO\textsubscript{2} could not be detected in the lipid fraction of cells grown in isovalerate-1-C\textsuperscript{14}, nor was there appreciable C\textsuperscript{14}O\textsubscript{2} in the culture supernatant. The labeled material in the lipid fraction had an $R_f$ of 0.88 on chromatograms prepared by the method of Elsden and Lewis (1953); the $R_f$ of isovalerate was 0.59 and that of isocaproate was 0.70. This demonstrated that there was no appreciable C\textsuperscript{14} in the cells in CO\textsubscript{2}, acetate, or isovalerate. A preliminary report of studies on the nature of labeled materials in the lipid fraction of cells has been published (Allison et al., 1961). Most of the radioactivity in lipid from cells grown in the presence of isovalerate-1-C\textsuperscript{14} was present as a 15-carbon branched-chain fatty acid; some of the C\textsuperscript{14} was detected as a long-chain fatty aldehyde. Studies are in progress to identify further the labeled components of the lipid fraction.

To determine whether isovalerate functioned as a leucine precursor in another bacterium, E. coli was cultured in a medium containing mineral salts, glucose, and isovalerate-1-C\textsuperscript{14}, and incorporation of C\textsuperscript{14} was determined. After growth from OD 0.13 to 0.52 in medium containing $12.5 \times 10^6$ count/min, only 4,700 count/min were found in the washed cells. This would appear to be insignificant incorporation compared with that by R. flavefaciens. The labeled material in the cells was not identified.

**Incorporation of exogenous amino acids.** It seemed likely that the requirement for isovalerate by *R. flavefaciens* was due to an inability or limited ability to assimilate exogenous leucine or to synthesize this amino acid in the absence of isovalerate. *R. flavefaciens* does have the ability to incorporate exogenous amino acids, including leucine, into cellular protein, but the ability to do so, as compared with *E. coli*, is greatly limited (Table 3). *E. coli* incorporated 37% of the C\textsuperscript{14} from the labeled hydrolyzate; *R. flavefaciens* incorporated about 2%.

### Table 2. Distribution of radioactivity after culture of Ruminococcus flavefaciens in medium containing isovalerate-1-C\textsuperscript{14} and isovalerate-3-C\textsuperscript{14}

<table>
<thead>
<tr>
<th>Distribution</th>
<th>Isovalerate-1-C\textsuperscript{14}</th>
<th>Isovalerate-3-C\textsuperscript{14}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole culture</td>
<td>199,000\textsuperscript{a}</td>
<td>441,000\textsuperscript{a}</td>
</tr>
<tr>
<td>Cells</td>
<td>47,500</td>
<td>22,400</td>
</tr>
<tr>
<td>Saline wash of cells</td>
<td>1,850</td>
<td></td>
</tr>
<tr>
<td><strong>Cellular fractions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid</td>
<td>20,250</td>
<td>5,700</td>
</tr>
<tr>
<td>Protein</td>
<td>22,350</td>
<td>10,400</td>
</tr>
<tr>
<td>Nucleic acid</td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td>Wash of protein</td>
<td>600</td>
<td>1,000</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Culture volume was 100 ml.  
\textsuperscript{b} Culture volume was 10 ml.  
\textsuperscript{c} Count/min per ml of culture.

![Fig. 3. A tracing of ninhydrin-positive areas of a chromatogram superimposed on a radioautograph of the chromatogram. Spotted, left to right, (1) mixture of five amino acids, (2) leucine, (3) hydrolyzate of protein of C94 cells cultured in presence of isovalerate-1-C\textsuperscript{14}, (4) phenylalanine, (5) mixture of five amino acids. Spots from mixture were identified from top to bottom as: valine, methionine, isoleucine, leucine, and phenylalanine.](http://jb.asm.org/)
Radioautographs of paper chromatograms of cellular protein hydrolyzate demonstrated that leucine was the only labeled amino acid in cells grown in leucine-2-C\textsuperscript{14}. Valine, leucine, isoleucine, phenylalanine, tyrosine, arginine, lysine, and glycine or serine or both were the labeled amino acids detected in the protein hydrolyzate of \textit{R. flavefaciens} cells cultured in the \textit{Chlorella} protein-C\textsuperscript{14} hydrolyzate. Other labeled amino acids may have been present but were not detected because of the low specific activity of the amino acids.

\textbf{Position of label in leucine synthesized from isovalerate-1-C\textsuperscript{14}}. Leucine synthesized by \textit{R. flavefaciens} in the presence of isovalerate-1-C\textsuperscript{14} was obtained by elution from the radioactive area of a paper chromatogram (Work, 1949) of the protein hydrolyzate. No radioactivity was detected in CO\textsubscript{2} when leucine containing 120,000 count/min was decarboxylated. The isovaleraldehyde was oxidized to isovaleric acid, and a sample containing 15,300 count/min was decarboxylated. All of the radioactivity (16,600 count/min) was recovered as BaCO\textsubscript{3}, and thus was present as carbon 2 of leucine. No C\textsuperscript{14} could be detected in isobutyramine.

\section*{DISCUSSION}

\textit{R. flavefaciens}, strain C94, requires for growth a factor present in the volatile fraction of rumen fluid. This factor can be supplied by either isobutyrate or isovalerate. Since these acids are homologues, it seems likely that they would have a common function. However, since growth is better with both acids than with an equivalent level of a single acid, it is suggested that they have individual functions as well. Since single branched-chain acids will support growth of certain strains of \textit{R. flavefaciens} and \textit{R. albus}, their requirement is different from that of \textit{B. succinogenes}, which requires for growth both a branched and a straight-chain fatty acid.

There is evidence that branched-chain volatile fatty acids in the rumen are formed by Stickland-type reactions from corresponding amino acids (el Shazy, 1952; Dehority et al., 1958). Bladen, Bryant, and Doetsch (1961) found that an amino acid-catabolizing strain of \textit{B. ruminicola} produced a labeled five-carbon acid, presumably isovaleric acid-1-C\textsuperscript{14}, from leucine-2-C\textsuperscript{14}. It is also significant that in vitro digestion of cellulose by mixed cultures of rumen microorganisms is stimulated by leucine, valine, proline, and isoleucine, as well as by corresponding volatile fatty acids produced by anaerobic catabolism of these amino acids (Dehority et al., 1958).

Sijpesteijn (1948), who first described and named \textit{R. flavefaciens}, found that \textit{Clostridium sporogenes} produced a diffusible factor necessary for growth of \textit{R. flavefaciens}. \textit{C. sporogenes} produces volatile fatty acids, including isobutyrate and isovalerate, from corresponding amino acids by means of the Stickland reaction; it is possible that these acids were responsible for the growth stimulation observed.

Incorporation by \textit{R. flavefaciens} of radioactivity from both isovalerate-1-C\textsuperscript{14} and isovalerate-3-C\textsuperscript{14} into leucine is evidence that a function of isovalerate is to serve as part of the carbon skeleton for synthesis of leucine. Since 2-ketoisocaproate is considered to be the immediate precursor of leucine (Strassman et al., 1955), its inability to substitute nutritionally for isovalerate in strain C94 suggests that the fatty acid has an essential function other than its participation in leucine biosynthesis, or that a permeability barrier prevents the exogenous keto acid from reaching the site of leucine biosynthesis.

Radioactivity from isovalerate was incorporated into leucine even when the medium contained 2 mg/ml of acid-hydrolyzed casein. This means that appreciable leucine was being synthesized, even though the medium contained more leucine than would be required by a bacterium unable to synthesize this amino acid (Snell, 1951).
This fact, and the findings of Bryant and Robinson (1961b), indicate that biosynthesis of amino acids, in this strain and in certain other rumen bacteria, is not greatly inhibited by an exogenous supply of amino acids. These organisms differ in this respect from several other microorganisms in which exogenous amino acids repress synthesis of amino acids by repressing formation of biosynthetic enzymes or by inhibition of the activity of these biosynthetic enzymes (Pardee, 1959).

*R. flavefaciens* has a limited ability to incorporate exogenous amino acids into cellular protein (Table 2). This is in contrast with results we obtained with *E. coli*, and with the results of Roberts et al. (1957) with *E. coli*, *Torulopsis utilis*, and Neurospora crassa; these organisms incorporate exogenous amino acids in preference to synthesizing them. It seems probable that the failure to note inhibition of amino acid biosynthesis and the inability to demonstrate incorporation of appreciable exogenous amino acids into cellular protein may be due to the absence of an efficient amino acid transport mechanism.

Data of several workers suggest that many rumen bacteria are quite unique in that they, like the strain of *R. flavefaciens* studied here, synthesize much of their cellular amino acids in preference to utilization of amino acids in the medium. Warner (1955) found that mixed cultures of rumen bacteria differed from both *E. coli* and Leuconostoc mesenteroides; considerable lysine was synthesized by the rumen microflora from $^{14}$H and nonradioactive carbon, even though exogenous lysine-C$^{14}$ was in the medium. Wright (1960) found that a strain of Streptococcus bovis from the rumen had a limited ability to utilize exogenous aspartic acid, and suggested that protein synthesis was limited by the intracellular supply of this amino acid, even though an adequate level was present in the medium. Bryant and Robinson (1961b) found that ammonia could serve as sole source of nitrogen for strain C94 and for all of seven other strains of *Ruminococcus* studied. They found that *R. flavefaciens*, *R. albus*, and *B. succinogenes* require and fix ammonia approximately equal to the amount of cellular nitrogen produced, even when grown in a medium rich in organic nitrogen.

The experiments of Roberts et al. (1957), Strassman et al. (1955), Reiss and Bloch (1955), and Rafelson (1957) have shown that in *E. coli*, *T. utilis*, *N. crassa*, Saccharomyces cerevisiae, and *Aerobacter aerogenes* leucine is synthesized by a mechanism which involves attachment of a two-carbon fragment to the alpha carbon of 2-ketoisovalerate. The carboxyl carbon from the 2-ketoisovalerate is decarboxylated, and 2-ketoisocaproate, the immediate precursor of leucine, is formed. Our results indicate that in *R. flavefaciens* the intact isovalerate molecule is incorporated into leucine, since both carbons 1 and 3 of isovalerate were incorporated into cellular leucine and since the radioactivity in leucine synthesized from isovalerate-1-C$^{14}$ was present in carbon 2 of leucine. This is evidence that a biosynthetic mechanism different from those previously described is operative. The data do not preclude the possibility that the previously recognized mechanism also functions. Since *R. flavefaciens* requires CO$_2$ for growth, we might speculate that a function of CO$_2$ might be to provide the carboxyl carbon of leucine.

Wegner (personal communication) found that this same strain of *R. flavefaciens* incorporated radioactivity from isobutyrate-1-C$^{14}$ into cellular valine and into the lipid fraction of the cells. This substantiates the conclusion that a function of these branched-chain fatty acids is to provide the carbon skeleton for biosynthesis of branched-chain amino acids, and suggests that similar carboxylation reactions occur during synthesis of both valine and leucine from fatty acids.

A consideration of the ruminal environment suggests that organisms capable of using the carbon skeleton of branched-chain fatty acids for synthesis of branched-chain amino acids might be expected to outcompete organisms wholly dependent upon an exogenous supply of the amino acids. Annison (1956) found that the level of free $\alpha$-amino nitrogen in rumen contents of sheep on various rations varied between 0.3 and 1.5 mg N/100 ml, except for a short time during and immediately after the intake of food. If we assume that about 5% of the free $\alpha$-amino nitrogen is in leucine (Loosli et al., 1949), we can calculate that the level of free leucine in the rumen ranges between 0.011 and 0.054 $\mu$mol/ml. These values may in fact be high, because the method used by Annison to prepare rumen fluid for analysis may have freed amino acids from bacterial cells. The isovalerate concentration is usually near 1 $\mu$mol/ml (Annison, 1954), and thus its concentration is at least 20 to 100 times the concentration of leucine. Also, the ammonia
level in rumen fluid is almost always high in relation to the amount needed for bacterial growth (Lewis, 1961). It would be of interest to determine the extent of utilization of isovalerate and other volatile fatty acids for protein synthesis by the mixed rumen microflora.

It now seems likely that branched-chain fatty acids are required, because \textit{R. flavefaciens} C94 has a limited ability to incorporate exogenous amino acids and has a limited ability to synthesize the isopropyl moiety found in valine, leucine, and in the lipid fraction of the cell.

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