Method for Measuring Microbial Growth in Rumen Content

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Radioactive sodium sulfide was used to label the sulfide pool of rumen contents in vitro. Microbial protein synthesis was calculated from the size and rate of dilution of label in the sulfide pool, and from the radioactivity incorporated into protein together with a conversion factor specifying the nitrogen-sulfur ratio determined for microbial protein. The microbial cell yield, calculated on the basis of the adenosine triphosphate (ATP) available from fermentation, was 13 to 14 g (dry weight) per mole of ATP, which is in good agreement with the values obtained for pure cultures of bacteria. Calculation of microbial protein yield per kilogram of ration agreed fairly well with previous estimates for similar rations.

Quantitative estimation of the rate of microbial cell synthesis in rumen content has long been a difficult problem. Essentially, the difficulties are associated with the necessity of differentiating plant material from microbial cell substances, and to overcome these difficulties a number of techniques have been developed.

McDonald (15) investigated the breakdown of the protein zein, which, since it does not contain lysine, is distinguishable from microbial protein. Blackburn and Hobson (3) took advantage of solubility differences between microbial protein and casein to study problems of dietary protein degradation. Both of these techniques suffer from the disadvantage that purified protein must be supplied in the diet; thus, they cannot be applied under normal ruminant feeding conditions.

Weller, Gray, and Pilgrim (20) and El-Shazly and Hungate (8) have used methods based upon the fact that bacterial cells contain diamino-pimelic acid (DAP), whereas plant cells do not. These methods, however, are tedious and do not directly measure the growth or concentration of protozoal cell material.

To assess protein synthesis, Henderickx (9) employed radioactive sulfite and measured incorporation of $^{35}$S into microbial cells. Again, however, this method is only applicable to a rumen fluid system to which known protein and energy sources are added.

In this paper, we report a method in which the sulfide pool of rumen content is labeled with $^{35}$S; this method allows the estimation of microbial protein synthesis in untreated rumen ingesta from animals under normal feeding conditions.

Materials and Methods

**Rumen content.** A rumen fistulated Merino ewe maintained on a diet of 500 g of wheat hay and plus 500 g of lucerne hay chaff daily, was used as a source of rumen content. The rumen content was collected, as described by Walker and Forrest (18), into a warmed Dewar flask.

**Bacterial and protozoal suspension.** For determination of the nitrogen-sulfur ratio of the rumen microflora, rumen fluid was expressed from whole ingesta by squeezing through four layers of surgical gauze. Repeated centrifugation at about $10 \times g$ was used to remove protozoa and yield a bacteria-rich supernatant fluid. The deposited protozoal fraction was repeatedly washed with 0.9% NaCl solution and was centrifuged at about $100 \times g$ until microscopically free from plant material and essentially free from bacteria. The bacterial fraction was centrifuged at 22,000 $\times g$ for 10 min, and the deposit was washed three times with 0.9% NaCl. Finally, both fractions were centrifuged and resuspended in distilled water, and samples were removed for dry weight determination. Samples of each fraction, containing about 30 mg (dry weight) of cells, were made 0.4 N with respect to perchloric acid in order to release soluble materials, and the acid-precipitable residue was washed once with 0.4 N perchloric acid. Nitrogen was determined by a micro-Kjeldahl technique, and sulfur was determined by the method of Johnson and Nishita (10), after wet ashing in perchloric-nitric acid.

**Preparation of Na$^{35}$S.** A 0.5-ml amount of an H$_2$$^{35}$SO$_4$ solution (Radiochemical Centre, Amersham, England) containing about 200 $\mu$C of radioactivity and 0.5 ml of K$_2$SO$_4$ solution equivalent to 5 mg of sulfur were converted to H$_2$S by the method of Johnson and Nishita (10); the liberated H$_2$S was absorbed in 20 ml of 0.02 N NaOH. This solution was freshly prepared for each experiment.
Chemical estimations. Sulfide in rumen content was estimated by acidifying with 6 N HCl (10 ml to 10 g of rumen content), flushing the liberated H2S into zinc acetate-sodium acetate trapping solution, and estimating the methylene blue formed in the reaction described by Johnson and Nishita (10). The flushing gas in this operation was nitrogen which had been bubbled through a solution of 10% HgCl2 plus 2% KMnO4 to remove traces of sulfur gases. A flushing time of 30 min at about 200 ml/min was used for each sample, and a temperature of about 70 C was maintained.

Steam volatile fatty acids (VFA) were estimated on samples of fluid expressed from a portion of the rumen content incubated separately for this purpose. VFA concentrations were expressed as micromoles per gram (dry weight) of the rumen content used.

Radiochemical estimations. For estimation of radioactivity in sulfide, H2S was removed from the incubation mixture as described above. However, the effluent gas stream was passed into a Dreschel bottle containing 20 ml of 1.5% NH4OH in 3% H2O2, which oxidizes the sulfide to sulfate. It has been shown that no significant amount of sulfide escaped oxidation in this system. After disconnecting the H2S trap, the pH of the H2O2-NH4OH solution was adjusted to about 3 with HCl, with methyl orange as the internal indicator. A 1-ml amount of 1 N H2SO4 was added as the carrier and the solution was heated to almost boiling; then 10 ml of hot 10% BaCl2 solution was added, and the mixture was allowed to stand overnight. The BaSO4 was then quantitatively collected by centrifugation and was washed three times. Small amounts of redistilled ethyl alcohol were then used to transfer the precipitate to weighed scintillation-counter glass vials, which were then dried overnight at 90 to 100 C prior to reweighing. For counting, 400 mg of finely divided silica (Cab-o-Sil, Packard Instrument Co., Inc., Downers Grove, Ill.) was added to each vial, followed by 10 ml of Dioxan phosphor (16). The BaSO4 was then evenly suspended by manual shaking. Counting was done in a Packard Model 3375 liquid scintillation counter at 30% amplifier gain and a discriminator gate width of 50 to 1,000. We corrected for minor differences in quenching by means of the automatic external standardization provided on the instrument.

Radioactivity incorporated into protein was estimated after conversion of the nonvolatile insoluble sulfur compounds to sulfate. The residues after removal of radioactive sulfide were quantitatively transferred to Whatman no. 1 filter paper (covered with a thin layer of cellulose powder to aid filtration) in a Buchner funnel. The solids were washed with 5 lots of 20 ml of distilled water; the first 20 ml of filtrate was slightly cloudy and was filtered again. The washed solids plus filter paper were transferred to a 500-ml Kjeldahl flask and were digested with a mixture of 20 ml of glass-distilled HNO3 and 10 ml of HClO4; the heating was continued for at least 1 hr after the first appearance of white HClO4 fumes. Residual nitric acid was removed by further heating after the addition of 5 ml of 6 N HCl. The digest was filtered into a 250-ml Erlenmeyer flask with copious washings, 1 ml of 1 N H2SO4 was added as carrier, and BaSO4 was precipitated as described above. The counting procedure was as previously described.

When the radioactivity of nonvolatile, acid-soluble materials was measured, the filtrate plus washings collected from the treatment of the residues of rumen content after removal of H2S were reduced to dryness with a rotary evaporator. The residues were digested in HNO3-HClO4 as described above, and the radioactivity was measured as BaSO4 after the addition of carrier.

RESULTS

Measurement of radioactivity. Initially, attempts were made to measure radioactivity by direct solubility liquid scintillation counting in Bray's (6) phosphor. However, when neutralized acid digests of the acid-insoluble residues from rumen content incubated with Na235S were counted in this manner, peculiar results were obtained. Zero-time samples had an apparently high radioactivity and samples which had been incubated with Na235S for up to 2 hr did not show any increase in counting rate. Similarly, rumen content which had been heated at 100 C for 10 min and cooled prior to addition of Na235S also showed an apparently high incorporation of label into acid-insoluble material. However, when the neutralized digests from samples incubated with Na235S for 2 hr were dried on filter paper strips and counted by strip-scanning, considerable radioactivity was detected, whereas heat-treated controls processed in the same manner revealed low radioactivity. This artifact of direct scintillation counting led to the development of the suspension counting technique for Ba234SO4 described above.

Suspension counting as BaSO4 has been shown to be free of the artifact described above. By comparing standardized samples of pure H234SO4 counted by direct liquid scintillation techniques with the same samples precipitated and counted as BaSO4, the suspension counting technique was shown to give a counting efficiency of about 55%.

Source of 35S. Although the measurement of 35S incorporated into protein from the H2S pool is the basis of our method, for convenience of handling, initial studies were done with 35SO4. The rationale for this was the known coversion of sulfate to sulfide in the rumen (1, 9, 11). However, we observed that the conversion of added sulfate to sulfide was relatively slow, resulting in an increase in the labeling of H2S for about 80 min, followed by a decrease thereafter, due presumably to dilution from nonradioactive sources and utilization for synthetic processes (Fig. 1). This situation complicated the kinetic basis upon which the calculations of protein synthesis were to be made, and the use of sulfate was therefore abandoned.
In all further studies, freshly prepared Na$_{2}^{35}$S solution was used as source of radioactive sulfur.

**Sulfide binding phenomenon.** In all experiments in which $^{35}$S-sulfide was added to rumen content, an initial very rapid incorporation of radioactivity into acid-insoluble material occurred. Thereafter, in the normal incubation mixtures, label was incorporated with first-order kinetics. This phenomenon was further investigated by use of rumen content samples which had been heated in sealed vessels at 100 C for 10 min. When Na$_{2}^{35}$S was added to such material, about 20% of the added tracer was bound within 20 min, but no further binding occurred over a period of 2 hr (Table 1). The nature of the compound formed is still unknown, but hydrolysis of the protein present, followed by purification of the amino acids on ion exchange resins and paper chromatography, failed to reveal any radioactivity in the amino acids present. However, with unheated rumen contents, incubated with Na$_{2}^{35}$S for 2 hr, both cystine and methionine were found to be heavily labeled. Thus, it was reasonable to assume that, after the rapid nonenzymatic binding had occurred, any further incorporation was due to the synthesis of new protein.

**Reversibility of sulfide incorporation.** To ensure that the label incorporated into acid-insoluble material was not being recycled, microorganisms labeled with $^{35}$S by incubation of rumen content for 2 hr with Na$_{2}^{35}$S were separated from the rumen solids by squeezing through surgical gauze and were centrifuged. The cells were then washed with fresh cell-free rumen liquor to remove extracellular $^{35}$S and were added to fresh rumen content containing a large excess of unlabeled Na$_{2}S$. This mixture was then incubated under the standard conditions for 2 hr, and samples for H$_{2}^{35}$S determination were withdrawn at various times. Table 2 shows that there was no exchange of incorporated $^{35}$S with the sulfide pool.

**Stability of Na$_{2}S$ solutions.** In standardizing Na$_{2}S$ solutions during early studies, it was observed that the sulfide recovery by distillation from acid solution fell rapidly. Table 3 shows that at 0 C 60% of the sulfide solution, which had been stored at a concentration of 1 mg of Na$_{2}S$ per ml, was not recoverable after 96 hr. Paper electrophoresis of a solution of Na$_{2}S$ (100 mg of sulfur per ml), which had been stored at 0 C for 8 weeks, showed that about 30% of the sulfur was present as sulfate and that sulfite was also present. This rapid oxidation of dilute sulfide solutions made it imperative that such solutions be freshly prepared for experimental work.

**Incubation technique.** For some time, our studies were plagued by an inability to recover all of the radioactivity added to rumen contents as Na$_{2}^{35}$S. Ultimately, we realized that by opening the incubation vessels, even for a few seconds, a con-
considerable quantity of sulfide was allowed to escape. Therefore, a completely closed system for incubation and subsequent removal of sulfide was devised, and this system is an essential part of the successful use of the proposed method.

Clin Britic (Britton-Malcolm & Co., Ltd., London, England) vaccine bottles (50-ml capacity) were modified by drilling a hole (10 mm in diameter) in the Bakelite cap. Carefully mixed rumen content (10 g) was placed in the preweighed bottle and the gas space was briefly flushed with nitrogen-40% CO₂ before replacing the thick rubber diaphragm-type seal and cap. Addition of the rumen content was facilitated by use of a funnel made from a polyethylene bottle cut in two; the neck of this bottle fitted snugly over the neck of the incubation vessel. For each experiment, 12 such vessels were set up. The vessels were then placed in a water bath at 39 C for 30 min to allow regeneration of H₂S in the rumen content (see below), and 200 μl of Na₂³⁵S solution (about 2 μc) was injected into each of eight reaction vessels with a Hamilton microsyringe fitted with a Chaney adaptor. After thorough mixing, an additional 15 min of incubation was allowed for the nonenzymatic binding phenomenon, already mentioned, to occur.

Three incubation vessels were then withdrawn. Into one, we injected 100 μl of 10 n NaOH to stop further biological activity. This vessel was then stored at 0 C until required for VFA analysis. A second vessel was connected to a nitrogen gas stream by means of an all-glass tube (1 to 2 mm in diameter), which was sharpened to pierce the rubber seal and which was pushed into the incubation mixture. A second glass tube, connected to an H₂S trap containing peroxide-ammonia, was inserted through the rubber seal so that its tip was well above the incubation mixture. After starting the nitrogen gas flow, the reaction mixture was acidified by injection through the rubber seal, and H₂³⁵S was collected as described previously. The third incubation vessel was treated in exactly the same way, except that the H₂S trap was sodium acetate-zinc acetate.

After removal of H₂S, protein-bound ³⁵S was measured as previously described. Further sets of three incubation vessels were withdrawn at predetermined time intervals and were treated similarly.

Recovery of sulfide. Table 4 illustrates the excellent recoveries of added ³⁵S when the closed-bottle system was used. With this system, we obtained recoveries of 96 to 99%, as compared with 55 to 70% recoveries prior to the use of this technique.

A second finding which illustrates the extreme ease with which H₂S is lost is that the collection of this rumen content and the subsequent mixing prior to placing in the incubation vessels result in a low concentration of H₂S, which slowly rises to a higher constant level upon incubation in a closed system (Fig. 2). To attain a constant H₂S pool size quickly, carrier sulfide, about 2.5 μg of sulfur per g of rumen content, was added along with the radioactive sulfide. Table 5 shows that

<table>
<thead>
<tr>
<th>Fraction assayed</th>
<th>Radioactivity measured (counts/min)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>H₂S pool.........</td>
<td>221,000</td>
</tr>
<tr>
<td>Acid-washed solids</td>
<td>85,000</td>
</tr>
<tr>
<td>Acid-soluble materials</td>
<td>24,000</td>
</tr>
<tr>
<td>Recovery (%)....</td>
<td>96</td>
</tr>
</tbody>
</table>

a Initial radioactivity added as Na₂³⁵S = 344,500 counts/min.

FIG. 2. Regeneration of sulfide pool during incubation in a closed system.

<table>
<thead>
<tr>
<th>Time</th>
<th>Amt of H₂S sulfur per g of rumen content</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>μg</td>
</tr>
<tr>
<td>15</td>
<td>4.35</td>
</tr>
<tr>
<td>50</td>
<td>4.42</td>
</tr>
<tr>
<td>90</td>
<td>4.80</td>
</tr>
<tr>
<td>125</td>
<td>4.30</td>
</tr>
</tbody>
</table>
protein, \( RtP \)

For incorporation of radioactivity into protein

\[
dR_t^P = \sigma \ dt \cdot R_t^s / S
\]

where \( R_t^P \) is the radioactivity in protein at time \( t \), \( \sigma \) is the rate of incorporation of sulfur into protein, and \( S \) is the sulfide sulfur pool size.

\[R_t^p = \frac{\sigma}{S} \int_0^t R_t^s \ dt\]

\[= \frac{\sigma}{Sk} R_0^s (1 - e^{-kt})\]

\[k \text{ being the rate constant for dilution of the sulfide pool.}\]

\[= \frac{\sigma}{Sk} (R_0^s - R_t^s)\]

Hence, total sulfur incorporated into protein at time \( t \)

\[= \sigma t \cdot \frac{R_t^p \cdot Sk}{R_0^s - R_t^s}\]

This treatment assumes that steady-state conditions apply during the experiment, but makes no assumption regarding the proportion of the sulfide sulfur pool incorporated into protein.

Analyses of the nitrogen to sulfur ratios of ruminal bacterial and protozoal fractions have shown them to be virtually identical, 10.7 and 11.0, respectively. Therefore, if one knows the amount of sulfur incorporated into microbial protein, the protein nitrogen synthesized may be calculated, and further multiplication by the factor 6.25 results in an estimate of the amount of protein synthesized. Table 7 lists results from three experiments on samples of rumen contents taken at different times after feeding.

In all experiments, the rate of VFA productivity was measured, since this parameter must represent an assessment of the energy available for microbial growth (17). Table 7 shows that the protein synthesized per unit of VFA produced is comparable at 11 and 20 hr after feeding, but that the ratio is lower 3 hr after feeding.

The ration fed in these experiments contained about 130 g of crude protein per kg, and Weller, Pilgrim, and Gray (22) have estimated an intraruminal conversion of about 80% of plant nitrogen to microbial nitrogen with a roughage ration. With similar rations, Weller et al. (21) have shown the production of about 5 moles of VFA per kg of ration. Using the average ratio of protein to VFA for the 11- and 20-hr samples, we obtained a microbial protein synthesis of about 92 g per kg of ration, i.e., 71% conversion of the dietary protein. The protein to VFA ratio for the 3-hr sample has been ignored for reasons given in the Discussion.

To further check if the protein synthesis figures were reasonable, a further conversion factor was used to translate protein synthesis into terms of

\[R_t^p = \frac{\sigma}{S} \int_0^t R_t^s \ dt\]

\[= \frac{\sigma}{Sk} R_0^s (1 - e^{-kt})\]

\[k \text{ being the rate constant for dilution of the sulfide pool.}\]

\[= \frac{\sigma}{Sk} (R_0^s - R_t^s)\]
TABLE 7. Protein synthesis in rumen content at various times after feeding

<table>
<thead>
<tr>
<th>Time after feeding of rumen content (hr)</th>
<th>Radioactivity (counts per min per g)</th>
<th>Rate of sulfur incorporation (μg per g per hr)</th>
<th>Rate of protein synthesis (μg per g per hr)</th>
<th>Rate of VFA production (μg per g per hr)</th>
<th>Protein/VFA (μg per μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k(min⁻¹)</td>
<td>S² μg</td>
<td>t (min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.0073</td>
<td>8,450 4,080 2,500</td>
<td>100</td>
<td>1.17</td>
<td>80.3</td>
</tr>
<tr>
<td>11</td>
<td>0.0061</td>
<td>14,700 7,800 5,600</td>
<td>110</td>
<td>1.31</td>
<td>90</td>
</tr>
<tr>
<td>20</td>
<td>0.0085</td>
<td>5,580 3,340 1,340</td>
<td>60</td>
<td>1.37</td>
<td>94</td>
</tr>
</tbody>
</table>

*Sulfide sulfur pool size.

total microbial cell material synthesized. Luria and Bauchop and Elsden (2) and others have shown that about 10.5 g (dry weight) of cell material is synthesized for each mole of adenine triphosphate (ATP) available, and Walker (17) has calculated that about 2 moles of ATP become available for each mole of VFA produced during rumen fermentation. Taking these factors into consideration, in the experiments reported in Table 7, we found that 8.5, 14.4, and 12.8 μg of cells per μmole of ATP were formed in the 3-, 11-, and 20-hr samples.

**DISCUSSION**

The basic premise upon which the method described is based is that all of the sulfur incorporated into microbial protein is first passed through the free H₂S pool. In the case of inorganic sulfur sources, there is abundant evidence for this (1, 9, 11). Similarly, there is evidence which supports the concept that sulfur amino acids are first degraded to H₂S prior to resynthesis by the microflora. Lewis (11) reported the production of H₂S from cysteine by washed cells of mixed rumen organisms, a finding supported by Anderson (1) and Lewis and Emery (13). In vivo, Lewis (12) further demonstrated the breakdown of methionine, and Bosman (5) reported high ruminal H₂S concentrations when highly digestible crude protein was combined with a low starch equivalent in the diet of cattle.

Synthesis of rumen microbial protein sulfur from H₂S has been amply demonstrated prior to the studies reported here (1, 4, 9). No studies on direct assimilation of S-amino acids by rumen organisms have been reported, but Portugal and Sutherland (16) have shown that little glutamate or aspartate is directly incorporated into microbial protein, and Wright and Hungate (23) have shown the same for glycine. There is also a great deal of information suggesting that, by and large, the rumen bacteria synthesize most or all of their amino acids de novo. Thus, it is reasonable to assume that this is the case for S-amino acids.

Again, there are no published values for S-amino acid concentration in cell-free rumen fluid, but Wright and Hungate (24) have published figures for other amino acids. Assuming that amino acids become available in roughly the proportions ingested as forage proteins, the amount of amino acid sulfur which might be expected, from the data of Weller (19) for analysis of herbage and those of Wright and Hungate (24) for free amino acid concentrations in rumen fluid, would be 0.3 to 0.5 μg/ml of rumen fluid. This is far below the figure of 5 to 15 μg of sulfur per g of rumen content in the H₂S pool, suggesting that H₂S is by far the more readily available source of sulfur for protein synthesis. Therefore, considering current knowledge, it is very reasonable to assume that sulfide is the precursor for the bulk of the microbial protein sulfur.

In developing the method described, a number of important aspects of the use of radioactive sulfide have become obvious. First, it is of paramount importance that a completely closed incubation system is adopted because of the ease with which sulfide escapes into the atmosphere. For the same reason, it is necessary to restore the sulfide pool size after setting up the incubation vessels, since the removal of rumen content from the animal and further handling result in a substantial loss of H₂S. This can be achieved by a short preincubation period and the addition of carrier Na₂S along with the Na₂S³⁵⁰. Lastly, in view of the rapid oxidation of dilute solutions of sulfide, it is essential that only freshly prepared solutions of Na₂S³⁵⁰ be used.

The rapid, nonenzymatic binding of some of the added sulfide is not yet understood; however, since it is not associated with the labeling of S-amino acids and is not reversible, our method is not affected by this phenomenon.

For convenience of handling, it would be advantageous if labeled sulfate could be used instead of sulfide. However, the rate of sulfate reduction, although fairly rapid, would require a fairly lengthy preincubation period before it could be assumed that the sulfide pool was not being significantly enriched by label from Na₂S³⁵⁰.
In relation to other methods previously devised for the estimation of conversion of dietary nitrogen to microbial protein, the method described here has certain advantages; it can be applied to rumen content which has not been altered, save that it has been transferred to an in vitro environment. This contrasts with the technique of Henderickx (9), which uses rumen fluid and added sources of nitrogen and energy. The method described by Weller, Gray, and Pilgrim (20), based upon the DAP content of the bacteria, involves slaughter of the animals and tedious separation of bacteria and protozoa from plant particles, followed by chromatography and estimation of the amino acids obtained after hydrolysis of the microbial proteins. Similarly, the method proposed by el-Shazly and Hungate (8), also based upon the DAP content of bacteria, involves protein hydrolysis and separation of the amino acids; and, if increase in microbial growth is to be measured, one must be able to measure a small difference between two large values unless the incubation period is to be unduly long.

The sensitivity of the method described herein is good, since radioactivity incorporated into nonradioactive material is the measurement used and the estimations are relatively simple and rapid. In addition, whereas the methods based upon DAP are limited to bacterial growth, our method, based upon incorporation of $^{35}$S, should include the bulk of the protozoa, since Coleman (7) has shown protozoal utilization of bacterial amino acids.

At 11 and 20 hr after feeding, the values obtained for microbial protein synthesis showed reasonable agreement with the estimate of Weller et al. (21) of about 80% conversion of plant to microbial nitrogen. The somewhat lower value of 70% that we obtained could be due to the fact that the ration we used contained 13% crude protein, which in turn contained more digestible nitrogen than the organisms could use, resulting in some wastage as ammonia.

When protein synthesis was translated to microbial cell synthesis and the yield of the latter was divided by the anticipated ATP production, the values of 14.4 and 12.8 g of cells per mole of ATP for the 11- and 20-hr samples were somewhat higher than that of 10.5 observed in pure culture (2). This may reflect an underestimation of ATP yield during ruminal fermentation. Part of this underestimation can be accounted for in methane synthesis, a reaction sequence of which the energy yield is still in doubt.

The lower yield of protein in relation to ATP and VFA observed in the 3-hr sample of rumen content may be explained by the fact that, at this stage of the fermentation, the more readily degradable carbohydrates were being fermented at a rate such that energy became available faster than it could be used for growth, and part of it was probably channelled into reserve carbohydrate rather than protein synthesis. Undoubtedly, some of this carbohydrate could be used as an energy source for protein synthesis at a later stage of the fermentation, when the energy supply becomes limiting.

Work is in progress to investigate the diurnal variations in protein synthesis in relation to energy supply.

ACKNOWLEDGMENTS

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LITERATURE CITED