Metabolic Fate of Cysteine and Methionine in Rumen Digesta

C. J. NADER and D. J. WALKER
C. S. I. R. O., Division of Nutritional Biochemistry, Adelaide, South Australia

Received for publication 8 June 1970

Estimates were obtained of the extent to which cysteine and methionine were incorporated into the protein of the microbes of rumen digesta without prior degradation and resynthesis. By using the amino acids labeled with both $^{35}$S and $^{14}$C, it was observed that a large proportion of the $^{35}$S appeared in the sulfide pool and of the $^{14}$C appeared in volatile fatty acids. By isolating the appropriate amino acid, obtaining the $^{14}$C to $^{35}$S ratio, and comparing this with the ratio in the added amino acid, the degree of direct incorporation was calculated. For cysteine it was estimated that at most 1% and for methionine, at most 11% of the amino acid in the free pool was incorporated unchanged into microbial protein. As a consequence of these findings, it is considered that the method for measuring microbial protein synthesis in rumen digesta based upon incorporation of $^{35}$S from the free sulfide pool is not seriously affected by direct utilization of sulfur amino acids arising from dietary sources.

Several reports have appeared (2-5, 9, 11, 15) indicating that inorganic sulfur compounds in the rumen are used in the synthesis of sulfur amino acids and that the pathway involves reduction to sulfide prior to upgrading into the protein of rumen microorganisms. Evidence cited by Allison (1) supports the idea that microorganisms of the rumen obtain most of their amino acid requirement by de novo synthesis. There seems good reason (2, 11) to apply the same concept to the sulfur amino acids, and this has been made the basis of a method for measurement of protein-synthesis rates of rumen microbes (22).

The possibility remains that some direct incorporation of sulfamino acid may occur and there is a good deal of qualitative work (6, 7, 12, 20, 24) dealing with this. It seems, however with one exception (14), that no attempt has been made to determine quantitatively the extent to which this may occur under normal conditions in rumen contents. We report here such a quantitative appraisal based upon change of the $^{14}$C to $^{35}$S ratio of added, doubly labeled methionine and cysteine.

MATERIALS AND METHODS

Labeled sulfamino acids. $^{35}$S- and $^{14}$C-labeled cysteine and methionine were obtained from the Radiocimical Centre, Amersham, England. Cysteine-$U^{14}$C of specific activity 18.3 mCi/mmole, containing a total of 12 $\mu$Ci of $^{14}$C, was made to a volume of 1 ml (solution A). $^{35}$S-cysteine of specific activity 26 mCi/mmole, containing a total of 500 $\mu$Ci of $^{35}$S, was made to a volume of 16 ml (solution B). A 1-ml amount of solution A was mixed with 0.4 ml of solution B. Methionine-1,2,3,4-$^{14}$C of specific activity 39 mCi/mmole, containing a total of 100 $\mu$Ci, was made to a volume of 5 ml (solution C). $^{35}$S-methionine of specific activity 270 mCi/mmole, containing a total of 500 $\mu$Ci, was made to a volume of 16 ml (solution D). A 1.2-ml amount of solution C was mixed with 0.8 ml of solution D. To maintain concentrations of sulfur amino acids in rumen contents as close to normal as possible during the experiments, no carrier amino acid was added to either mixture.

Incubation of labeled amino acids with rumen content. Rumen-content samples were obtained via a rumen fistula from a merino ewe which had been fed daily a diet of 500 g of wheaten hay chaff and 500 g of lucerne hay chaff. On the day of each experiment, approximately 200 g of rumen content was taken as described by Walker and Forrest (21). A 10- $g$ amount of this material was introduced into each of two preweighed Clin-Britvic vaccine bottles (Britton-Malcolm and Co. Ltd., London, England), and the space above the rumen contents was filled with a gas mixture of 60% $N_2$-40% CO$_2$. To each vessel was added 350 $\mu$liters of the prepared mixture of cysteine $U^{14}$C: $^{35}$S by injection through the rubber seal. For the methionine experiment, the design was identical except that 250 $\mu$liters of the prepared mixture of methionine-1,2,3,4-$^{14}$C:$^{35}$S was added. The unused portion of both radiotracer solutions was deep frozen and kept for measurement of the precise amount of each radioactive species present.

Treatment of reaction mixtures containing labeled sulfamino acids. After incubation at 39 C in a shaking bath for 105 min, the rumen contents were acidified with 10 ml of 6 N HCl, the evolved H$_2$S was collected into 10% H$_2$O$_2$-1.5% NH$_4$ mixture, and the $^{35}$S present
in this solution was measured as BaSO₄ (22). The solids after washing and extraction with 0.05 M Na₂S were hydrolyzed in 6 n HCl for 16 hr after the addition of 10 µmoles of the appropriate unlabeled amino acid as carrier. When methionine was the amino acid studied, 2-mercaptoethanol (1:2,000, v/v; reference 13) was added to the samples to minimize oxidative losses during the hydrolysis. HCl was removed from hydrolysates by rotary evaporation with two additions of distilled H₂O. Humin was removed from the samples by treatment with activated carbon and filtration.

**Isolation and purity testing of cystine and methionine.**

Cystine was purified on the basis of its insolubility at neutral pH. The hydrolysate from the rumen-content sample incubated with labeled cysteine was made to a volume of 20 ml, 2 ml was absorbed to a column of cation exchange resin (H⁺ form), and the amino acids were eluted from the washed column with 2 n NH₄OH. Cystine was precipitated from the eluate, after concentration, by adjustment of the pH to neutrality in a centrifuge tube. The precipitated cystine, after repeated water washing to remove traces of other amino acids, was redissolved in 3 ml of 0.1 n HCl. The sample was tested for purity by electrophoresis in 0.01 M HCl, paper chromatography in n-butanol-acetic acid-water (25:6:25, v/v; reference 18), and thin layer chromatography in 2-methyl propanol-2-butanol-propanol-methanol-water-(0.88) ammonia (40:20:20:1:14.5, v/v) and 2-propanol-butanol·n HCl (60:15:25, v/v; reference 10).

The hydrolysate from the methionine experiment was made to a volume of 20 ml, and samples of this were used for methionine preparation by two independent methods. First, 2 ml was absorbed to a cation exchange resin and the amino acids were eluted with 2 n NH₄OH. The eluate was dried and the redissolved amino acids (10% ethanol-water), after streaking on several 3-mm Whatman papers, separated into neutral, basic, and acidic fractions by electrophoresis in pyridine-acetic acid-water (2.5:1:5.96) buffer (pH 5.8) at 20 v/cm for 75 min. The neutral bands were eluted with 10% aqueous ethanol; the eluates were pooled, dried in a rotary evaporator, and redissolved in 1 ml of the same solvent. The neutral fraction was chromatographed in n-butanol-acetic acid-water (25:6:25, v/v; reference 18). A band corresponding in position to a marker methionine spot was eluted and rechromatographed in n-butanol-pyridine-water (1:1:1, v/v; reference 18). The band eluted from the first solvent system was found to contain valine as well as methionine which were sufficiently well separated in the second solvent system to allow elution of a pure sample of methionine. This material was further tested for purity by electrophoresis in 1.5 M formic acid solution (8).

In the second method, 200 µlitters of hydrolysate was separated into component amino acids by chromatography in a Beckman amino acid analyzer (model 120C) on a 150-cm column eluting with 0.2 M citrate buffer (pH 3.25 initially) and changing to 0.2 M citrate buffer of pH 4.30 (16, 17). Buffer change occurred just prior to the position of the methionine peak which was determined from a reference mixture of amino acids. The five fractions comprising the methionine peak were pooled, evaporated to dryness, and redissolved in 300 µlitters of water. Purity of the fraction was established by using 60 µlitters for electrophoresis in 1.5 M formic acid on Whatman no. 4 paper at 21 v/cm for 90 min, by using a reference solution of methionine containing the same final concentration of citrate as the unknown.

**Radioactivity in amino acids.** Total ³⁵S plus ¹⁴C was determined in added and purified samples of cysteine and methionine by counting small portions of sample dissolved in 2.5 ml of ethyl cellulose with the addition of 5 ml of diphenylazo-toluene (0.3%, w/v) phosphor. A Packard 3375 scintillation counter was used throughout. Settings used were those optimal for ¹⁴C-window 50-500, amplifier gain 8%. To count ³⁵S, the same volume of sample as for ¹⁴C counting was first wet-ashed in HNO₃-HClO₄ mixture after the addition of 1 ml of n H₂SO₄ as carrier. Total sulfate was precipitated as BaSO₄ and counted as a suspension (22) at conditions optimal for ³⁵S (window 50-1000, amplifier gain 20%) as well as at ¹⁴C settings. Corrections for minor quenching differences measured by using automatic external standardization were applied, and the ¹⁴C to ³⁵S ratio was calculated from:

- counts per minute of undigested sample at ¹⁴C settings
- counts per minute of wet-ashed sample at ¹⁴C settings

The elimination was due to isotope decay, ³⁵S activity in the purified amino acid was determined at the same time as the ³⁵S activity of the appropriate added amino acid. For convenience of presentation of results, however, ³⁵S activities were adjusted back to the day of each experiment.

**¹⁴C in volatile fatty acids.** The volumes of the aqueous washings obtained from acid-treated rumen contents before Na₂S washings were measured, 2 ml samples were steam-distilled, and pH of the distillate was adjusted to 10 with NaOH. After concentration over a microburner to a volume of about 20 ml, 500 ulitters was counted in 10 ml of Bray's phosphor.

**RESULTS AND DISCUSSION**

From analysis of ³⁵S present in the sulfide pool and of ¹⁴C present in the VFA pool after incubation of ¹⁴C:³⁵S amino acid with rumen contents, it is clear that both cysteine and methionine were extensively degraded (Table 1). The fact that a greater proportion of the added ³⁵S than the ¹⁴C was recovered in these pools is undoubtedly due to formation of ¹⁴CO₂ and ¹⁴CH₃ which were not recovered for assay. The size of the H₂S pool during the incubations (about 10 µg of S per ml) was such that the amount of sulfur added as amino acid was low by comparison, being about 3% in the cysteine experiment and 0.3% in the methionine experiment. Consequently, there was a large trap for sulfide arising by degradation of the added amino acid. Thus, the finding of almost 90% of the added cysteine ³⁵S- and over 50% of the added ³⁵S-methionine in the sulfide pool gives a rough indication of the extent of degradation.
Table 1. Labeling of volatile fatty acids and \( \text{H}_2\text{S} \) during incubation of \( ^{14}\text{C}:^{35}\text{S} \)-sulfamino acids with rumen content

| Reaction mixture       | Radioactivity (counts per min per 10 g of rumen content) | Per cent recovered |
|------------------------|----------------------------------------------------------|--------------------|
|                        | Recovered       | Added             |                     |
|                        | \( ^{14}\text{C} \) in VFA | \( ^{35}\text{S} \) in VFA | \( ^{14}\text{C} \) in VFA | \( ^{35}\text{S} \) in VFA | %            |
| Cysteine \( ^{14}\text{C}:^{35}\text{S} \) | 2.75 \( \times 10^6 \) | 3.48 \( \times 10^6 \) | 4.02 \( \times 10^6 \) | 3.95 \( \times 10^6 \) | 68.4 | 88 |
| Methionine \( ^{14}\text{C}:^{35}\text{S} \) | 2.82 \( \times 10^6 \) | 3.37 \( \times 10^6 \) | 4.02 \( \times 10^6 \) | 3.95 \( \times 10^6 \) | 70.2 | 85.3 |
|                        | 2.02 \( \times 10^6 \) | 2.09 \( \times 10^6 \) | 4.1 \( \times 10^6 \) | 4.05 \( \times 10^6 \) | 49.3 | 51.6 |
|                        | 1.88 \( \times 10^6 \) | 2.06 \( \times 10^6 \) | 4.1 \( \times 10^6 \) | 4.05 \( \times 10^6 \) | 45.9 | 50.8 |

Table 2. Change of the ratio \( ^{14}\text{C} \) to \( ^{35}\text{S} \) in sulfamino acids

| Amino acid sample* | \( ^{14}\text{C} \) counts/min | \( ^{35}\text{S} \) counts/min | \( ^{14}\text{C}:^{35}\text{S} \) | Maximum direct incorporation |
|--------------------|-----------------|-----------------|-----------------|-----------------|
| Added cysteine \( ^{14}\text{C}:^{35}\text{S} \) | 128.6 \( \times 10^3 \) | 127 \( \times 10^3 \) | 1:1 | 6 |
| Cystine from protein | 0.11 \( \times 10^4 \) | 1.95 \( \times 10^4 \) | 1:1.75 | 44 |
| Added methionine \( ^{14}\text{C}:^{35}\text{S} \) | 874 \( \times 10^3 \) | 873 \( \times 10^3 \) | 1:1 | 6 |
| Methionine from protein* | 1.03 \( \times 10^4 \) | 2.38 \( \times 10^4 \) | 1:2.3 | 44 |
| Methionine from protein* | 0.34 \( \times 10^4 \) | 0.77 \( \times 10^4 \) | 1:2.3 | 44 |

* Approximately 3% of total added dose of labeled cysteine and 20% of methionine were used for estimation of each isotopic species.

\[ \text{radioactive} = \text{volatile} \]
\[ \text{cysteine} = \text{methionine} \]
\[ \text{incubation} = \text{proteinsynthesis} \]

Table 2 shows the \( ^{14}\text{C} \) to \( ^{35}\text{S} \) ratios in the amino acids as added to rumen contents and as isolated from microbial protein. The dramatic change in the ratio for microbial cysteine indicates an enrichment of 17.5-fold of \( ^{35}\text{S} \) over \( ^{14}\text{C} \). Consequently, it can be stated that over the incubation period a maximum of about 6% of the added radioactive cysteine was directly incorporated into microbial protein. In the case of methionine, the dilution of \( ^{14}\text{C} \) compared to \( ^{35}\text{S} \) in the amino acid isolated from microbial protein is substantially lower than the dilution obtained with cysteine. As a result, the calculated maximum direct incorporation for added radioactive methionine is 44%.

In both experiments, the incubation period (105 min) was long enough to allow substantial total incorporation of radioactivity. This, together with the fact that very small quantities of amino acid were added as labeled species, allows us to assume that the behavior of the labeled amino acids represented that of the corresponding pool, and that virtually all of the labeled amino acid would have been metabolized.

From Table 1 it is seen that 86% of the added \( ^{85}\text{S} \) of cysteine was present in the \( \text{H}_2\text{S} \) pool after 105 min, leaving 14% of the label incorporated into protein. Of the 14%, it is evident from the change in \( ^{85}\text{S} \) to \( ^{14}\text{C} \) ratio (Table 2) that only 0.06 arose directly from added cysteine. If all of the incorporated \( ^{85}\text{S} \) was present in the cysteine of protein then \( 14 \times 0.06 = 0.8\% \) of the cysteine pool was directly used for protein synthesis and 99% was metabolized via \( \text{H}_2\text{S} \). Since some \( ^{85}\text{S} \) must have been incorporated into methionine in protein, the extent of direct incorporation of free cysteine into protein is overestimated.

In the case of methionine, the proportion of the added \( ^{85}\text{S} \) finally recovered in the \( \text{H}_2\text{S} \) pool was 51%, leaving 49% incorporated into protein. From the change in \( ^{85}\text{S} \) to \( ^{14}\text{C} \) ratio, 0.44 of the label incorporated into methionine arose directly from the methionine pool. Thus, if all of the \( ^{85}\text{S} \) present in the protein finally were in methionine, \( 49 \times 0.44 = 22\% \) of the methionine pool would be directed unchanged into protein. However, as we have just seen, virtually all of the cysteine sulfur entering the free amino acid pool is metabolized via \( \text{H}_2\text{S} \), requiring de novo synthesis of cysteine for protein synthesis. Since there are approximately equal amounts of cysteine- and methionine-S in rumen microbial protein (23) and since the larger proportion of methionine-S is obviously metabolized via \( \text{H}_2\text{S} \), roughly half of the label incorporated in the methionine experiment would have been in
cysteine. Consequently, only about 11% of the methionine would be directly incorporated into protein and 89% would be degraded and the sulfur metabolized via H2S.

Extensive degradation of added cysteine is compatible with results published by Landis (14), in that he found that a large proportion of 35S label from cysteine appeared in methionine of the rumen microbes of the goat. However, his conclusion that 75 to 83% of methionine is directly incorporated into microbial protein is at variance with our work. Since Landis did not isolate and purify the amino acids from the microbes, we consider that he was estimating "bound-but-not incorporated" 35S-sulfide (22) as well as 35S-methionine, thus obtaining falsely high results.

Emery, Smith, and Huffman (6, 7) in studies with rumen liquor incubated with 35SO4- found that the label was incorporated into cystine, methionine, and glutathione of the microorganism protein, but, by using the relatively insensitive technique of microradioautography, suggested that the majority of the organisms did not take up the label. They also reported on the ability of 10 different strains of ruminal organisms, in pure culture, to utilize SO4--S and found that only three were able to do so. This is not surprising since it is most unlikely that all strains of organisms in the rumen have the ability to use SO4- as a terminal oxidant, converting it to sulfide which is required for de novo sulfamino acid synthesis (2, 11). Emery et al. (6, 7) also found that Lachnospira multiformis uses cysteine-sulfur in preference to inorganic SO4- in pure culture. Such a finding tells nothing of the pathway of utilization of cysteine, that is whether it was first desulfhydrated to H2S, pyruvate, and NH3 prior to cysteine synthesis de novo. Consequently, the findings of these workers do not have direct bearing on sulfamino acid metabolism by the mixed rumen microflora.

Wright (24) has demonstrated the incorporation into microbial protein of 14C from radioactive peptides incubated with rumen contents, the peptide apparently being utilized slightly faster than the corresponding amino acids. However, from the figures given, the concentration of peptide amino acid added was about 2 μmoles/g of rumen content which is about 30 times the concentration of total amino acids generally found by Wright and Hungate (25). Since there are no reports of the concentration of peptides as such in rumen fluid, it is not possible to assess the direct incorporation of sulfamino acids into microbial protein by peptide utilization.

The finding that a small proportion of the methionine entering the free amino acid pool is reutilized without prior degradation means that the method for determining microbial protein synthesis based upon incorporation of 35S from the sulfide pool (22) yields a slight underestimate. From the amino acid analyses given for plant and microbial protein by Weller (23), it can be calculated that 0.58 g of cyst(e)ine-sulfur and 0.55 g of methionine-sulfur are present in 100 g of crude plant and microbial protein, so that approximately equal amounts of sulfur are presented to and incorporated by the organisms as methionine and cyst(e)ine. But since calculations presented here show that 89% of methionine-sulfur turnover and 99% of cysteine-sulfur turnover is directed through H2S, the method for measuring microbial protein synthesis based upon 35S incorporation from the H2S pool (22) would account for (89 + 99)/2 or 94% of total sulfur incorporation, an underestimate of 6%, under conditions where all of the sulfur available for protein synthesis arises from dietary amino acids. Since normally other sources such as inorganic salts would contribute to the sulfur available, the above underestimate must be considered maximal.

**LITERATURE CITED**

1. Allison, M. J. 1965. Nutrition of rumen bacteria, p. 369-378. In Physiology of digestion in the ruminant. Butterworth Inc., Washington, D.C.

2. Anderson, C. M. 1956. The metabolism of sulphur in the rumen of sheep. N.Z. J. Sci. Technol. 37:579-594.

3. Block, R. J., and J. A. Stekol. 1950. Synthesis of sulfur amino acids from inorganic sulfate by ruminants. Proc. Soc. Exp. Biol. Med. 73:391-394.

4. Block, R. J., J. A. Stekol, and J. K. Loosli. 1951. Synthesis of cysteine and methionine from sodium sulfate by the goat and by microorganisms from the rumen of the ewe. Arch. Biochem. Biophys. 33:353-363.

5. Duncan, C. P., I. P. Agrawala, C. F. Huffman, and R. W. Luecke. 1953. A quantitative study of rumen synthesis in the bovine on natural and purified rations. J. Nutr. 49: 41-48.

6. Emery, R. S., C. K. Smith, and C. F. Huffman. 1937. Utilization of inorganic sulfate by rumen microorganisms. I. Incorporation of inorganic sulfate into amino acids. Appl. Microbiol. 5:360-362.

7. Emery, R. S., C. K. Smith, and C. F. Huffman. 1957. Utilization of inorganic sulfate by rumen microorganisms. II. The ability of single strains of rumen bacteria to utilize inorganic sulfate. Appl. Microbiol. 5:363-366.

8. Gross, D. 1955. Paper electrophoresis of amino acids and oligopeptides at very high potential gradients. Nature (London) 176:72-73.

9. Hale, W. H., and U. S. Garrigue. 1953. Synthesis of cystine in wool from elemental sulphur and sulphate sulphur. J. Anim. Sci. 12:492-496.

10. Haworth, C. and J. G. Heathcote. 1969. An improved technique for the analysis of amino acids and related compounds on thin layers of cellulose. J. Chromatogr. 41:380-385.

11. Hendericks, H. 1962. Utilisation des traces. C. R. Rech. Inst. Ecoucour. Rech. Sci. Ind. Agr. 28:65-81.

12. Hift, H., and G. I. Wallace. 1949. A study of the synthesis of
cystine by some lactic acid bacteria. J. Biol. Chem. 177: 927-931.
13. Keutman, H. T., and J. T. Potts. 1968. Improved recovery of methionine after acid hydrolysis using mercaptoethanol. Anal. Biochem. 29:175–185.
14. Landis, J. 1963. Studies of the sulphur metabolism of the ruminant. Z. Tierphysiol. Tierernähr. Futtermittelk. 18:357–370.
15. Lewis, D. 1954. The reduction of sulphate in the rumen of the sheep. Biochem. J. 56:391–399.
16. Moore, S., D. H. Spackman, and W. H. Stein. 1958. Chromatography of amino acids on sulphonated polystyrene resins. Anal. Chem. 30:1185–1190.
17. Moore, S., D. H. Spackman, and W. H. Stein. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30:1190–1206.
18. Peterson, P. J., and G. W. Butler. 1962. Paper chromatographic and electrophoretic systems for the identification of sulfur and selenium amino acids. J. Chromatogr. 8:70–74.
19. Pimnan, K. A., and M. P. Bryant. 1964. Peptides and other nitrogen sources for growth of Bacteroides ruminocola. J. Bacteriol. 88:401–410.
20. Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten. 1955. Studies of biosynthesis in Escherichia coli. Carnegie Inst. Wash. Publ. 607.
21. Walker, D. J., and W. W. Forrest. 1964. The application of calorimetry to the study of the rumen fermentation in vitro. Aust. J. Agr. Res. 15:299–315.
22. Walker, D. J., and C. J. Nader. 1968. Method for measuring microbial growth in rumen content. Appl. Microbiol. 16:1124–1131.
23. Weller, R. A. 1957. The amino acid composition of hydrolysates of microbial preparations from the rumen of sheep. Aust. J. Biol. Sci. 10:384–389.
24. Wright, D. E. 1967. Metabolism of peptides by rumen microorganisms. Appl. Microbiol. 15:547–550.
25. Wright, D. E., and R. E. Hungate. 1967. Amino acid concentrations in rumen fluid. Appl. Microbiol. 15:148–151.