A simple procedure using ^{35}S incorporation for the measurement of microbial and undegraded food protein in ruminant digesta

BY J. C. MATHERS AND E. L. MILLER

Department of Applied Biology, University of Cambridge, Pembroke Street, Cambridge CB2 3DX

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1. A simple procedure using ^{35}S incorporation for the measurement of microbial protein in abomasal or duodenal digesta of ruminants was developed and tested.

2. Microbial protein synthesized in the rumen was labelled with ^{35}S by intraruminal infusion of $^{35}SO_4$ and a microbial fraction was isolated by differential centrifugation.

3. ^{35}S not bound by micro-organisms and present as inorganic ^{35}S in whole digesta and in microbial fractions was oxidized to $^{35}SO_4$ and the samples were brought into solution by acid-hydrolysis. $^{35}SO_4$ was precipitated as $Ba^{35}SO_4$.

4. The proportion of microbial non-ammonia-nitrogen (NAN) in digesta NAN was determined as ^{35}S:NAN (digesta) ÷ ^{35}S:NAN (microbial).

5. In sheep offered grass nuts at maintenance level of feeding, 48% of NAN flowing through the abomasum was of microbial origin. NAN flow to the small intestine was estimated using chromic oxide as a marker and it was calculated that 49% of the grass N was degraded in the rumen.

In ruminants consuming common foodstuffs, non-ammonia-nitrogen (NAN) flowing to the small intestine (SI) consists of microbial crude protein (N × 6·25) synthesized within the rumen, food protein which has escaped rumen fermentation and NAN from desquamated epithelial cells and from abomasal secretions (Miller, 1973). The quantitative determination of these components and how they vary with different foodstuffs and feeding regimens is central to recent proposals for new methods of evaluating the protein contribution of foodstuffs and calculating dietary protein requirements (Journet & Vérité, 1977; Roy *et al.* 1977; Satter & Roffler, 1977). Both food and microbial contributions can be assessed from a knowledge of NAN flow to the SI together with a determination of the proportion of microbial NAN in the total NAN present in abomasal or duodenal digesta. An assumption has to be made of the contribution of endogenous NAN.

Various procedures have been used to determine the proportion of microbial NAN in the mixture of nitrogenous materials flowing to the SI. All methods rely on the identification of microbial NAN from either a natural label, such as α, ε-diaminopimelic acid (DAP) or RNA occurring in microbial cells but presumed to be absent from undegraded foodstuffs, or an isotopic label, such as ^{35}S, ^{15}N, or ^{32}P which may be incorporated during microbial growth in the rumen. The advantages and disadvantages of current methods have been reviewed by Smith (1975) and Buttery & Cole (1977) and methods have been compared by Smith *et al.* (1978), Ling & Buttery (1978) and Mercer *et al.* (1980). In general, an isotopic method is preferred since both bacteria and protozoa become labelled; the use of ^{35}S has advantages of simplicity of measurement compared with ^{15}N and safety compared with ^{32}P.

With any marker, marker:microbial NAN is determined for an isolated microbial fraction that is assumed to be representative of the whole microbial mass and the proportion of microbial NAN is calculated from the dilution of the marker in the NAN of the whole digesta. When isotopic markers are used it is unnecessary to determine the specific activity (SA) of the isotope so long as the value for isotope:microbial NAN is the same for the isolated microbial fraction and the total microbial mass in the digesta. Using this principle

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Roberts & Miller (1969) first reported the use of ^{35}S:N to measure the microbial N in rumen digesta. Calculation of the flow of microbial N to the SI, however, depended on assumptions as to whether the microbial mass moved with the solid or liquid phases of digesta (Mercer *et al.* 1980). Direct measurement of the microbial NAN in abomasal or duodenal digesta would circumvent this problem. However, during passage through the abomasum, micro-organisms are subjected to hydrolysis by pepsin and hydrochloric acid which may disrupt and digest the cells with concomitant loss of soluble components so that isolated microbial fractions might contain unphysiologically high proportions of cell walls. Since S-containing amino acids are absent from the cell walls of Gram-positive bacteria (Salton, 1960), ^{35}S:NAN of a microbial fraction isolated from the abomasum or duodenum may be unrepresentative of the total microbial mass. This possibility was investigated in the present study.

In addition, the use of a radioactive marker such as ^{35}S assumes that the marker is associated only with the microbial fraction of digesta. However a portion of the ^{35}S in digesta is present as inorganic ^{35}S (Bird & Hume, 1971; Kennedy & Milligan, 1978), and an important aspect of this study was the development of a simple procedure for the removal of non-microbial inorganic ^{35}S from digesta samples.

We now report the development and testing of a simple procedure using ^{35}S incorporation for the identification and measurement of microbial NAN in abomasal or duodenal digesta. A brief account of part of this work has been published (Mathers & Miller, 1977*a*).

EXPERIMENTAL

Several in vitro and in vivo experiments were carried out to test various aspects of the proposed procedure.

Removal of inorganic ^{35}S from digesta fractions

Use of ion exchange resins. Following the procedure described by Beever *et al.* (1974), model solutions containing $^{35}SO_4$ with and without cysteic acid, methionine sulphone or performic acid oxidized, acid-hydrolysed digesta (OHD) were loaded on to columns of Dowex 2-X8 and Dowex 50-X8 ion-exchange resins (BDH Ltd, Poole). The columns were eluted with approximately 50 ml distilled water and the radioactivity in the eluates was determined.

Use of barium chloride. Portions (6 ml) of model solutions similar to those described previously were mixed with 1 ml saturated $BaCl_2$ and centrifuged at 3000 *g* for 1 min. Radioactivity and N remaining in solution were determined.

Estimation of food protein in mixtures of ^{35}S-labelled abomasal digesta and unlabelled feedingstuffs

After 4 d infusion of $^{35}SO_4$ to the rumen of a sheep offered dried grass, abomasal digesta was collected, freeze-dried and ^{35}S:N of the digesta was determined. Weighed portions of ground sunflower-seed meal (SFM) and of freeze-dried ^{35}S-labelled abomasal digesta were mixed and estimates of ^{35}S:N of the mixtures were obtained. The proportions of SFM-N in the mixtures were determined using the principle of isotope dilution and compared with the known proportions as calculated from the N contents of SEM and of digesta and the masses of each material in the mixtures.

Effects of $BaCl_2$ treatment on digesta ^{35}S:N values

^{35}S:N of various digesta samples from sheep offered conventional rations and intra-ruminally infused with $^{35}SO_4$ were determined with and without $BaCl_2$ treatment.

Comparison of the ^{35}S:NAN of rumen bacterial and duodenal microbial fractions

Sheep maintained on diets containing various proportions of chopped lucerne and rolled barley (Mathers & Miller, 1977*b*) were intraruminally infused with 200 μCi $^{35}SO_4$/d for 5 d. During days 3–5 of infusion, samples of rumen and of duodenal digesta were collected and bacterial and microbial fractions respectively were isolated immediately by the procedure detailed on p. 505 for digesta microbial fractions. ^{35}S:NAN of the fractions were determined.

The effects of acid-pepsin treatment on ^{35}S:NAN of rumen micro-organisms

Rumen micro-organisms were labelled with ^{35}S by in vitro incubation with $^{35}SO_4$. Rumen fluid was collected from sheep at pasture or consuming chopped lucerne, strained through two layers of muslin and a bacterial fraction was obtained as the supernatant fraction after centrifugation at 1000 *g* for 1 min. Each incubation vessel contained 150 ml strained rumen fluid or 150 ml bacterial fraction, 600 ml artificial saliva at pH 7·0 (McDougall, 1948), 3 g sucrose, 200 mg N (as ammonia), 20 mg S (as sulphate) and 10–12·5 μCi $^{35}SO_4$. Incubation proceeded anaerobically at 39° for 6 h. Approximately 350 ml of the incubation fluid were then taken for preparation of a microbial fraction by differential centrifugation as described on p. 505. Concentrated HCl was added to the remaining 400 ml incubation fluid to reduce the pH to 2·75 and pepsin (*EC* 3.4.4.1; Sigma Chemical Co., London) was added to produce a final concentration of approximately 500 Anson units/ml (Ben-Ghedalia *et al.* 1978). Incubation was continued at 39° for 2 h when a microbial fraction was isolated by differential centrifugation. All microbial fractions were freeze-dried before determination of ^{35}S:NAN values.

Analytical reproducibility of ^{35}S:N determinations

Freeze-dried duodenal digesta from a sheep given four different rations in turn (see Mathers & Miller, 1977*b*) and intraruminally infused with $^{35}SO_4$ were analysed in duplicate for ^{35}S:N on four separate occasions.

Preparation of ^{35}S-labelled microbial and abomasal or duodenal digesta fractions

Microbial protein synthesized in the rumen of sheep was labelled with ^{35}S by continuous intraruminal infusion of $Na_2{}^{35}SO_4$ (150–200 μCi and 500 mg Na_2SO_4/d) for 5 d. Digesta samples were collected during the final 3 d of infusion and a microbial fraction was isolated immediately. Approximately 75 ml digesta was centrifuged at 1000 *g* for 1 min at 4° to pellet food particles. The supernatant fraction was decanted into a second tube and centrifuged at 20000 *g* for 20 min at 4°. The supernatant fraction obtained was discarded and the microbial pellet resuspended in buffer (artificial saliva prepared according to McDougall (1948) at pH 3) and centrifuged once more at 20000 *g* for 20 min at 4° before being freeze-dried. Whole digesta collected at each sampling time was frozen, freeze-dried and ground before analysis.

Microscopical examination of microbial preparations

Freshly-prepared microbial isolates were examined by light microscopy, by scanning electron microscopy and by transmission electron microscopy (TEM). For scanning electron microscopy, microbial isolates harvested by centrifugation at 20000 *g* for 20 min were dehydrated by passage through a graded series of solutions containing 500, 700, 800, 850, 900, 950 and 1000 ml ethanol/l water and then critical-point dried in carbon dioxide (Echlin, 1971). Dried specimens were fixed on metal studs, coated with gold (scanning electron microscopy coating unit E5000; Polaron Equipment Ltd) and examined in a

Stereoscan S4 scanning electron microscope (Cambridge Scientific Instruments Ltd). For TEM, fresh digesta was centrifuged at 1000 *g* for 1 min at 4°. Portions (10 ml) of the supernatant fraction obtained were mixed with an equal volume of buffered glutaraldehyde-containing fixative and stored at 4°. Microbial fractions were then collected by centrifugation, embedded in Araldite and thin sections prepared for microscopical examination.

Determination of ^{35}S:NAN for microbial and digesta fractions and calculation of the proportion of microbial NAN in digesta NAN

Freeze-dried samples of the microbial fraction (150 mg) or of whole digesta (300 mg) were placed in 100 ml round-bottom Quickfit flasks and oxidized with performic acid (20 ml for 16 h at 4°) to convert inorganic ^{35}S to $^{35}SO_4$ and cystine and methionine to the more stable cysteic acid and methionine sulphone. Oxidation was stopped by the addition of 3 ml hydrogen bromide and the contents of the flasks were concentrated by rotary evaporation under reduced pressure at 40–50° to near dryness. Then 20 ml 6 M-HCl were added and the samples were hydrolysed under reflux for 22 h. The hydrolysate was filtered through a fluted paper (Whatman 541), concentrated by rotary evaporation under reduced pressure to near dryness and transferred using three 2 ml portions of distilled water to a centrifuge tube containing 1 ml saturated $BaCl_2$. The tube contents were thoroughly mixed and allowed to stand for 15 min before being centrifuged at 650 *g* for 15 min. Portions of the supernatant fraction were taken for determination of radioactivity and of N and radioactivity:N (disintegrations/min per mg N) was calculated. It was assumed that all the N originally present in the isolated freeze-dried microbial fraction was NAN i.e. any contaminating ammonia had been removed by washing during centrifugation. The total N and ammonia-N contents of the original freeze-dried whole digesta were determined and N:NAN was calculated. Multiplication of ^{35}S:N (digesta) by N:NAN (digesta) gave an estimate of ^{35}S:NAN (digesta). Finally, the proportion of microbial NAN in digesta NAN was calculated as:

$$^{35}S\text{:NAN (digesta)} \div {}^{35}S\text{:NAN (microbial)}.$$

In vivo determination of the synthesis of microbial protein and degradation of food protein in the rumen of sheep

Two mature ewes (40 and 56 kg body-weight) fitted with permanent cannulas to the rumen, abomasum and ileum were held in metabolism cages under continuous illumination. Each sheep was offered 960 g/d grass nuts at 2-hourly intervals by a mechanical feeder (A. Overhill, Cambridge) and water was available *ad lib.* Chromic oxide-impregnated paper (Corbett *et al.* 1960) (6 g/d) was given with the food as a flow marker. After 15 d, five to seven spot samples of abomasal digesta were collected from each sheep and analysed for Cr_2O_3, N and ammonia. Subsequently each animal was intraruminally infused with 150 μCi $^{35}SO_4$/d for 5 d and five spot samples of abomasal digesta were collected from each animal over the final 2–3 d. ^{35}S:NAN of whole digesta and of abomasal microbial fractions were determined.

Analytical procedures

N was determined by a micro-Kjeldahl procedure followed by colorimetric determination of ammonia using an autoanalyser (Fleck & Munro, 1965).

Residual ammonia in 200 mg samples of freeze-dried digesta was extracted into 5 ml 0·5 M-HCl and ammonia determined by a colorimetric procedure using the reactions of Fawcett & Scott (1960) as adapted for use with an autoanalyser (Allen, 1971).

Cr_2O_3 was estimated by the method of C. K. Milner (unpublished results) as described by Owen *et al.* (1967).

Table 1. *The elution of* $^{35}SO_4$ *from columns of Dowex 2-X8 and of Dowex 50-X8 ion-exchange resins with distilled water*

Resin	Test solution	Proportion of radioactivity eluted from column
2-X8	$^{35}SO_4$ alone	0·04*
2-X8	$^{35}SO_4$ in 0·65 M-chloride	0·25*
2-X8	$^{35}SO_4$ + OHD	0·47†
50-X8	$^{35}SO_4$ alone	1·00†
50-X8	$^{35}SO_4$ + OHD	0·96†

OHD, oxidized, hydrolysed digesta. * Single determination.
† Mean of duplicate determinations.

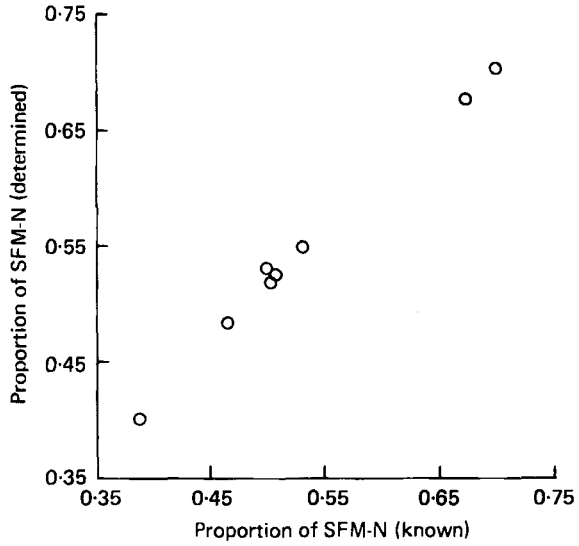

Fig. 1. Estimation of food protein in mixtures of ^{35}S-labelled abomasal digesta and unlabelled sunflower-seed-meal (SFM). ^{35}S-labelled abomasal digesta was obtained from a sheep consuming dried grass and intraruminally infused with $^{35}SO_4$. The ^{35}S:nitrogen of the freeze-dried digesta was determined as described on p. 504. Weighed portions of SFM and of freeze-dried abomasal digesta were mixed and ^{35}S:N determinations carried out on the mixtures. The proportions of sunflower-seed-meal N (SFM-N) in the mixtures were determined by isotope dilution and compared with the known proportions calculated from the N contents of SFM and of digesta and the masses of each material in the mixtures.

Radioactivity in hydrolysed digesta fractions was determined by liquid-scintillation counting after decolorization by the procedures of Jeffay *et al.* (1960) or of Mahin & Lofberg (1966); the latter procedure was preferred. Decolorized aqueous sample (0·8–1·0 ml) was mixed with 10 ml scintillation fluid (10 g 2-(4-tert-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (butyl PBD) dissolved in 1 l toluene and mixed with 500 g Triton X-100) and counted in a Corumetic 200 liquid-scintillation spectrometer (Tracerlab (GB) Ltd, Weybridge, Surrey).

RESULTS

Removal of inorganic ^{35}S *from digesta*

In the absence of competing anions, columns of Dowex 2-X8 removed almost all $^{35}SO_4$ from test solutions. In contrast, the presence of physiological concentrations of anions e.g.

Table 2. *Effects of barium chloride treatment on digesta ^{35}S:nitrogen*

Sample	Diet	Reduction in ^{35}S:N as proportion of control (no $BaCl_2$)
Duodenal digesta	Lucerne	0·32*
	Lucerne–barley (2:1, w/w)	0·25*
	Barley–lucerne (2:1, w/w)	0·24*
	Barley	0·15*
Rumen fluid	Straw+concentrates	0·43†

* Mean of duplicate determinations. † Single determination.

chloride or of oxidized, hydrolysed digesta (OHD) resulted in the elution from the columns of up to 47% of added radioactivity (Table 1). Whether loaded alone or with OHD, essentially all $^{35}SO_4$ was eluted from columns of Dowex 50–X8 (Table 1). Treatment with saturated $BaCl_2$ of model solutions or of OHD solutions containing $^{35}SO_4$ removed 98·6% (standard error of mean ±0·49%, r 11) of the added radioactivity. Such treatment had little effect on the recovery of the N in cysteic acid, methionine sulphone or in OHD with recoveries of 97, 98 and 96% respectively.

Estimation of food protein in mixtures of ^{35}S-labelled digesta and unlabelled feedingstuff

Fig. 1 shows a plot of the proportions of SFM-N in mixtures of SFM and ^{35}S-labelled abomasal digesta determined by the ^{35}S:N procedure *v.* the known proportions estimated from the N contents and the masses of each component in the mixtures. The linear correlation was highly significant ($P < 0.001$) but the slope of the line was significantly ($P < 0.05$) less than unity and the intercept greater than zero.

Effects of $BaCl_2$ treatment on digesta ^{35}S:N

For sheep offered a variety of diets, $BaCl_2$ treatment reduced ^{35}S:N of duodenal digesta by up to 32% (Table 2). For comparison, $BaCl_2$ treatment reduced ^{35}S:N of a single sample of rumen fluid by 43%.

Examination of microbial fractions

Microbial fractions isolated from the rumen by differential centrifugation were free of protozoa and have been termed bacterial fractions while those from the abomasum-duodenum have been termed microbial fractions (cf. Beever *et al.* 1974). Both light microscopy and scanning electron microscopy indicated that fractions from either section of the gut were largely free from contamination by food particles and contained a variety of morphological types of bacteria (Plate 1(*a*, *b*)). Microbial fractions from the duodenum appeared to be very similar to those from the rumen except that fewer large bacteria were apparent (Plate 1(*b*)). Partially-disrupted or digested cells were rare and protozoa were not observed in duodenal preparations or in fractions treated with pepsin and HCl (Plate 1(*c*)). However, TEM studies revealed that while bacteria isolated from the rumen consisted mainly of intact cells, those from duodenal digesta contained some cells from which the outer membranes had partially separated and other cells which had apparently lost most of their cytoplasm (Plate 2).

Table 3. *The synthesis of microbial protein and degradation of food protein in the rumen of sheep offered dried grass at maintenance level of feeding*

	Sheep A	Sheep B
SA abomasal microbial fractions (disintegrations/min per mg NAN)	4481	4619
SA abomasal digesta (disintegrations/min per mg NAN)	2054	2333
Microbial NAN as proportion of abomasal NAN*	0·458	0·505
Nitrogen intake (g/d)	20·7	20·7
NAN flow to SI (g/d)	23·6	25·0
Microbial NAN flow to SI (g/d)	10·8	12·6
Undegraded food NAN flow to SI (g/d)†	10·8	10·4
Rumen degradability of food NAN	0·478	0·498

SA, specific activity; NAN, non-ammonia N; SI, small intestine.
* Estimated as described on p. 506.
† Assuming 2 g/d endogenous NAN flow to SI (Phillipson, 1964; Harrop, 1974).

Comparison of ^{35}S:NAN of microbial fractions isolated from the rumen and from the duodenum, and the effect of treatment with pepsin and HCl on the ^{35}S:NAN of rumen bacteria

^{35}S:NAN determinations were made on microbial isolates from rumen and duodenal digesta collected during days 3–5 of intraruminal infusion of $^{35}SO_4$ when the SA of rumen micro-organisms has reached plateau (Beever *et al.* 1974). In twenty-four comparisons, there was no significant difference ($P > 0{\cdot}05$) between the mean ^{35}S:NAN of the rumen isolates and that of the duodenal isolates (5350 and 5021 disintegrations/min per mg N for rumen and duodenal samples respectively; pooled standard error of mean $\pm$ 155·1).

Similarly in in vitro experiments designed to simulate the actions of HCl and pepsin in the abomasum, there was no significant effect ($P > 0{\cdot}05$) of such treatment on ^{35}S:NAN of micro-organisms (mean ($\pm$ pooled standard error of mean, r 4) values for S:NAN were 10632 and 9472 $\pm$ 832·1 disintegrations/min per mg NAN for control and treated samples respectively).

Although the differences in ^{35}S:NAN between rumen and duodenal microbial isolates (6%) and between untreated and HCl+pepsin treated bacteria (11%) were not statistically significant it should be noted that the coefficients of variation for the two experiments, 14·7 and 16·6 respectively, were fairly large.

Analytical reproducibility of ^{35}S:N determination

Four samples of duodenal digesta from a sheep fed four different diets were analysed in duplicate on four occasions. The values obtained were examined by analysis of variance in which the 'samples of digesta' was considered to be a fixed source of variation while 'days of analysis' was random (Ridgman, 1975). There was no evidence that the day of analysis added to the variation associated with estimates of ^{35}S:N nor of any interaction between 'samples' and 'days'. The coefficient of analytical variation of a single determination on any one day was 3·6.

In vivo determination of the synthesis of microbial protein and degradation of food protein in the rumen of sheep offered grass nuts

In this study, the observations obtained with both sheep were similar (see Tables 3 and 4) and the mean values for the two sheep will be used in discussion. Comparison of the ^{35}S:NAN for whole abomasal digesta and for abomasal microbial fractions indicated that approximately 48% of the NAN flowing to the SI was of microbial origin (Table 3). This

Table 4. *Incorporation by micro-organisms of radioactivity infused as $^{35}SO_4$ to rumen*

	Sheep A	Sheep B
[^{35}S]sulphate infused ($\times 10^6$ disintegrations/min per d)	319·1	294·7
SA abomasal microbial fraction (disintegrations/min per mg NAN)	4481	4619
Microbial NAN flow to SI (g/d)*	10·8	12·6
Microbial ^{35}S flow to SI ($\times 10^6$ disintegrations/min per d)	48·4	58·2
Proportion of infused radioactivity incorporated by micro-organism	0·152	0·197

SA, specific activity; NAN, non-ammonia-nitrogen; SI small intestine.
* Estimated as described on p. 506.

corresponds to a daily flow of 11·7 g microbial NAN and, after allowing 2 g NAN/d as endogenous material (Phillipson, 1964; Harrop, 1974), indicates that 10·6 g food NAN/d escaped rumen degradation. The latter is equivalent to a rumen degradability of 0·49 for the N in grass nuts.

Knowledge of the quantity of $^{35}SO_4$ infused to the rumen, of ^{35}S:NAN of the microbial fraction and of the daily flow of microbial NAN to the SI permits calculation of the net incorporation of infused radioactivity by the rumen micro-organisms (Table 4). Approximately 17·5% of the infused radioactivity flowed to the SI as microbial organically-bound ^{35}S.

DISCUSSION

Development of a method for measuring rumen microbial protein synthesis

The use of ^{35}S is a convenient way of marking microbial protein. The proportion of microbial NAN in digesta NAN may then be determined from the dilution of the marker in digesta NAN. It is unnecessary to determine the SA of the isotope so long as the isotope: microbial NAN value is the same for the isolated microbial fraction and for the total microbial mass in the digesta. However, if the action of pepsin and HCl in the abomasum brings about disruption of microbial cells, an isolated microbial fraction may contain disproportionate amounts of cell walls and not be representative of the whole microbial mass. By determining the specific activities of cystine (Leibholz, 1972), methionine (Beever *et al.* 1974; Ling and Buttery, 1978) or organically-bound S (Hume, 1974; Walker & Nader, 1975), other workers have avoided this problem since S-containing amino acid SA are more likely to be constant in all fractions of the microbial cell. Such determinations give the proportion of S-containing amino acids in the digesta which is of microbial origin. To calculate the proportion of NAN which is of microbial origin, further determinations must be made of the cystine, methionine or organically-bound S content of the microbial mass and of the whole digesta (Ling & Buttery, 1978). Use of the S-containing amino acid content of a microbial fraction prepared from abomasal or duodenal digesta again makes the assumption that this is representative of the whole microbial mass and negates the reason for using SA values.

For example, it is readily demonstrated that the equation of Ling and Buttery (1978):

$$\text{microbial NAN flow to duodenum (g/d)} = \frac{\text{methionine SA of } D}{\text{methionine SA of } M} \times \frac{\text{methionine flow to duodenum (g/d)}}{\text{methionine:total NAN for } M},$$

where D is whole digesta and M is the microbial fraction isolated from duodenal digesta, reduces to:

$$\text{microbial NAN flow to duodenum (g/d)} = \frac{\text{radioactivity associated with methionine per g NAN in } D}{\text{radioactivity associated with methionine per g NAN in } M} \times \text{NAN flow to duodenum (g/d)}.$$

Therefore determination of the methionine contents of the M and D fractions is unnecessary. This relationship can be extended for use with other markers, e.g. organically-bound microbial ^{35}S and will give a valid estimate of microbial NAN in digesta NAN provided that the isolated microbial fraction is representative of the whole microbial mass.

Not all the ^{35}S in digesta will be present as organically-bound microbial ^{35}S. Bird & Hume (1971) and Kennedy & Milligan (1978) reported the flow of inorganic S compounds from the rumen. In addition, studies by Walker & Nader (1968) and by Allen (1971) showed that up to 20% of ^{35}S added to heat-sterilized incubations as $^{35}S^{2-}$ or as $^{35}SO_4$ was associated with trichloroacetic acid (TCA)-insoluble matter. The nature of this rapid non-enzymatic binding of ^{35}S to TCA-insoluble matter was not discovered but none of the radioactivity was present in amino acids (Walker & Nader, 1968). Consequently, an important aspect of the present study was the development of a simple procedure for the removal of non-microbial inorganic ^{35}S from digesta samples. This was achieved by performic acid oxidation, acid-hydrolysis and precipitation of SO_4 ions with $BaCl_2$.

The importance of barium precipitation of $^{35}SO_4$ was demonstrated by the reductions in ^{35}S:N values of digesta samples following this treatment (Table 2). The extent of reduction in ^{35}S:N observed was as expected since inorganic S may contribute 1–23% of the total S leaving the rumen (Bird & Hume, 1971; Kennedy & Milligan, 1978) and the SA of microbial organically-bound S may be lower than that of the sulphide pool (Gawthorne & Nader, 1976; McMeniman *et al.* 1976; Kennedy & Milligan, 1978). More complicated procedures of isolating a suitable marker such as chromatographic separation of cysteic acid (Leibholz, 1972), or methionine sulphone (Beever *et al.* 1974) or copious washing (Walker & Nader, 1975) do not appear to be necessary.

Beever *et al.* (1974) claimed to separate $^{35}SO_4$ from the S-containing amino acids on a column of Dowez 2 and to separate methionine sulphone from cysteic acid on a column of Dowex 50 before determination of the SA of methionine sulphone. Our studies indicated that the use of Dowex 2-X8 was not effective in retaining $^{35}SO_4$ in the presence of chloride or of the mixed anions of OHD and was not essential to their procedure since contaminating $^{35}SO_4$ would be effectively eluted, together with cysteic acid, from a column of Dowex 50-X8 which retained methionine sulphone.

In vitro experiments indicated that the analytical procedures used in determination of ^{35}S:NAN were associated with acceptably-low variability and could be used to predict the quantities of unlabelled food protein (SFM) in mixtures with ^{35}S-labelled digesta (Fig. 1). In this particular experiment the small deviations from equivalence in the estimated proportions of SFM in the mixtures could have been due to a small error in determination of the N content of either the SFM or the digesta.

For convenience of sampling, it was proposed that a microbial fraction might be isolated from abomasal or duodenal digesta (cf. Beever *et al.* 1974). Recent studies have suggested that in both sheep and cattle, a large proportion of protozoa are sequestered within the rumen (Hungate *et al.* 1971; Weller & Pilgrim, 1974; Bird *et al.* 1978; Harrison *et al.* 1979) so that the microbial matter flowing from the rumen may consist largely of bacterial cells.

During passage through the abomasum, bacterial cells may be lysed and some proteins partially hydrolysed by pepsin. It may be more difficult to isolate a microbial fraction from a mixture containing partially-digested food proteins. Therefore it was important to ascertain whether the ^{35}S:NAN value for the microbial fractions isolated from the abomasum–duodenum was representative of the whole microbial mass and similar to that of rumen bacteria.

The scanning electron microscopy studies indicated that there was little contamination of the microbial fractions isolated from the duodenum with non-microbial particles. However the TEM study suggested that some lysis and loss of cell contents had taken place. Both the in vitro investigation of the effect of pepsin + HCl treatment and the in vivo study where microbial preparations from the rumen and duodenum were compared showed no significant difference in ^{35}S:NAN values. However, in the former instance, the mean ^{35}S:NAN was reduced by 11% and in the latter by 6%. The 2 h-incubation in pepsin + HCl was probably excessive since estimates of mean retention times for flow markers in the abomasum include 0·5–1 h for polyethylene glycol (Hydén, 1961), 24 min for ^{51}Cr EDTA and 82 min for ^{144}Pr (Grovum & Williams, 1973). If the differences in ^{35}S:NAN between rumen and duodenal microbial preparations is a true difference, then the proportion of microbial NAN in digesta NAN will be over-estimated by 6–11% when the duodenal preparation is used.

However it is still uncertain whether a microbial fraction isolated by differential centrifugation from either the rumen or the duodenum is fully representative of the total microbial mass passing to the SI. Within the rumen, bacteria are closely attached to both food particles (Akin *et al.* 1974) and to the rumen epithelium (Bauchop *et al.* 1975) and neither of these populations is likely to be sampled adequately by the procedures normally used for collection of rumen fluid. Sampling digesta at the duodenum has the advantage of permitting collections of all material entering the SI, and it is possible that bacterial attachment to food particles may be reduced by passage through the abomasum. If bacteria attached to food particles have lower SA than those living in suspension in rumen fluid, this could account, at least partially, for the observed slightly lower ^{35}S:NAN of duodenal isolates compared with those of rumen isolates.

Although the use of a duodenal microbial preparation appears to be satisfactory when using ^{35}S-labelling for identification of microbial matter in duodenal digesta, such a preparation is unlikely to be satisfactory when DAP or nucleic acids are used as the microbial marker. These substances are found exclusively in the cell wall and cytoplasm respectively of bacterial cells and a microbial fraction enriched with cell walls is likely to give DAP:NAN and nucleic acid:NAN values which are unrepresentative of the whole microbial mass.

The procedure described in this paper for estimating microbial NAN in digesta NAN is technically simple and requires no sophisticated equipment apart from a liquid-scintillation counter. In addition, relatively few assumptions need to be made in interpretation of the results obtained.

Use of the ^{35}S:NAN procedure for measuring rumen microbial protein synthesis and food protein degradation in vivo

An experiment with two sheep offered grass nuts at maintenance was carried out to illustrate the use of the ^{35}S:NAN procedure for estimating rumen microbial protein synthesis and food protein degradation. Although the results for the two animals were in reasonable agreement (Tables 3 and 4), animal replication was small and undue emphasis should not be placed on the reported means for digestion of grass N in the rumen. A larger experiment has been carried out to examine sources of variations in in vivo estimation of microbial protein synthesis by the ^{35}S:N procedure (Mathers & Miller, 1977*b*).

Hogan & Weston (1970) indicated that for sheep offered forages with N:digestible organic matter (DOM) values less than approximately 4%, NAN flow to the SI exceeded N intake. The N:DOM of the grass nuts fed to two sheep in the present study was 3·9% and the observed gain in NAN (3·6 g/d) confirms the findings of Hogan & Weston (1970). Approximately 49% of the grass N was degraded in the rumen and microbial NAN contributed 48% of the total NAN flow to the SI (Table 3); both estimates are within the reported ranges for animals consuming pelleted dried grass (Proud, 1972; Coelho da Silva *et al.* 1972; Amos *et al.* 1976). Variation due to differences in species and maturity of grass, type and extent of processing, level of feeding and microbial marker used preclude more specific comparisons with values in the literature. In another experiment, this ^{35}S procedure gave degradabilities of 0·72 and 0·89 for lucerne and barley proteins respectively (Mathers & Miller, 1977*b*).

Approximately 17·5% of the radioactivity infused as $^{35}SO_4$ to the rumen flowed to the SI as microbial organically-bound ^{35}S (Table 4). Studies by Leibholz (1972), Bird (1973) and Kennedy *et al.* (1976) indicated that 4·4–40% of the sulphate entering the rumen left as microbial organically-bound S. In the rumen, SO_4 is rapidly reduced to S^{2-} (Lewis, 1954; Anderson, 1956) which is used for synthesis of S-containing amino acids, by the rumen micro-organisms (Block *et al.* 1951; Henderickx, 1961). If it is assumed that all SO_4 entering the rumen in the present experiment was reduced to S^{2-}, then approximately 17·5% of the S^{2-} pool was used for microbial protein synthesis. It is unlikely that availability of S limited microbial protein synthesis in this study.

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EXPLANATION OF PLATES

Plate 1. (*a*) Scanning electron micrograph of bacteria isolated from rumen fluid of sheep using the differential centrifugation procedure described on p. 505. Note the morphological diversity of organisms obtained and the absence of food particles. (*b*) Scanning electron micrograph of bacteria isolated from duodenal digesta of sheep by differential centrifugation. (*c*) Scanning electron micrograph of rumen bacteria following incubation with pepsin at pH 2·75 and 39° for 2 h. See p. 505 for experimental details.

Plate 2. (*a*, *b*). Transmission electron micrographs of thin sections of bacteria from rumen fluid of sheep. Note that cell membranes appear intact and that the cells are filled with electron dense matter (*c*, *d*). Transmission electron micrographs of thin sections of bacteria from duodenal digesta of sheep. Outer cell membranes have become detached and cytoplasmic matter may have been lost.

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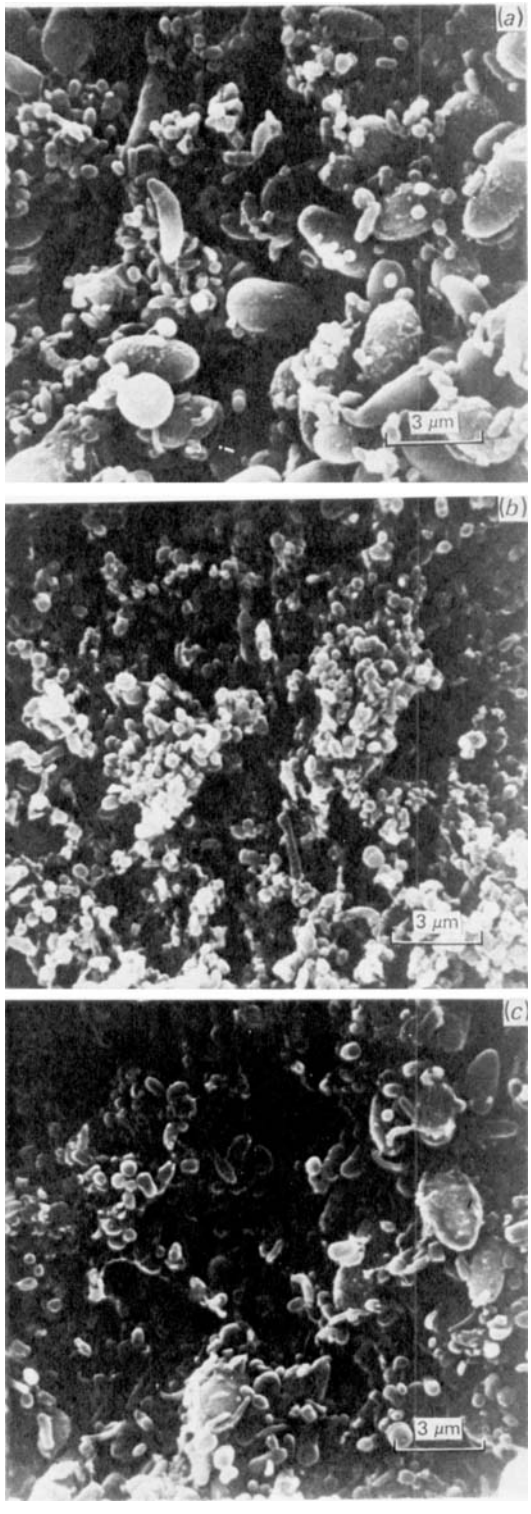

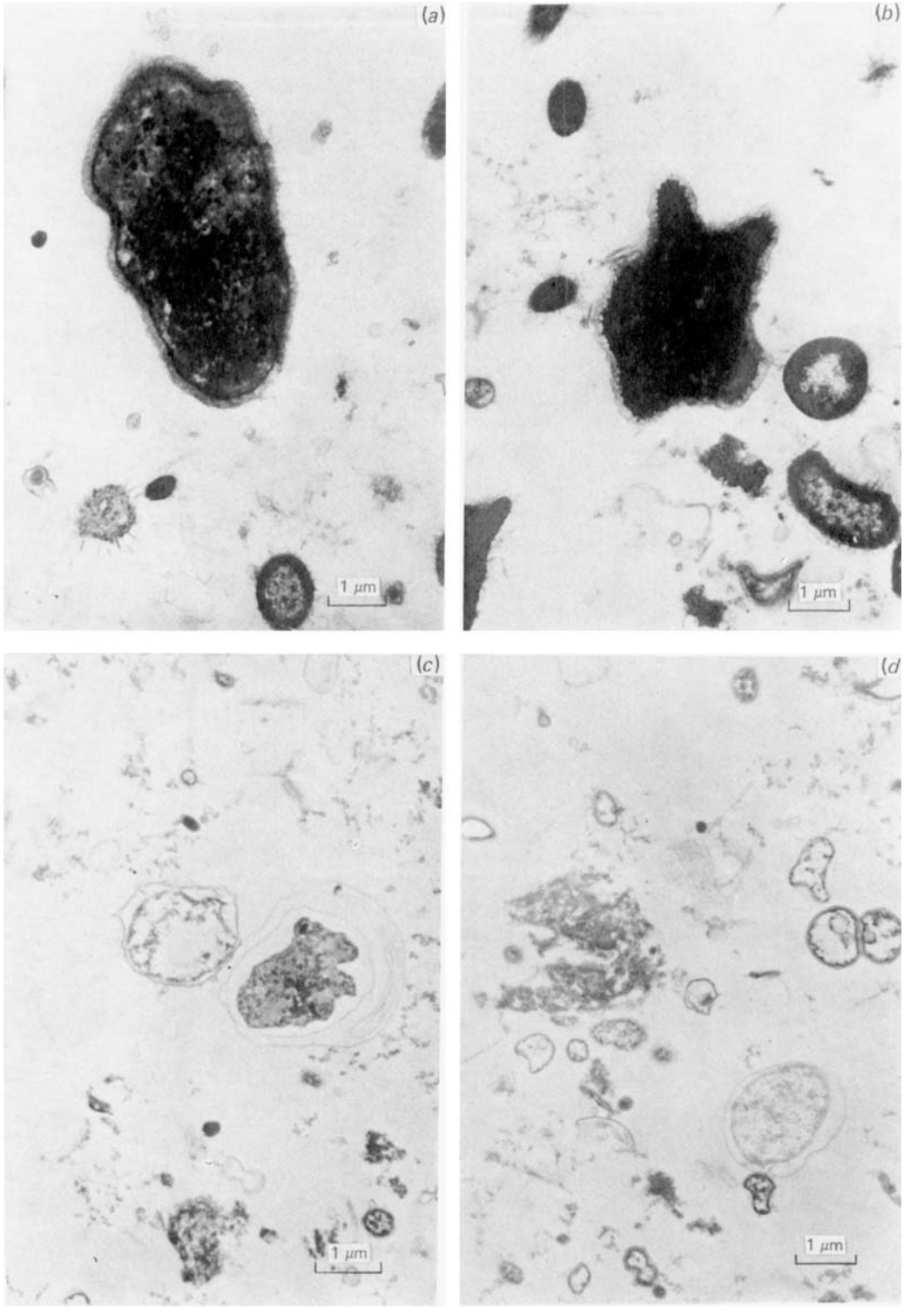

J. C. MATHERS AND E. L. MILLER