SULPHUR METABOLISM AND EXCRETION STUDIES IN RUMINANTS

VI.* THE DIGESTIBILITY AND UTILIZATION BY SHEEP OF ^{35}S FROM ^{35}S-LABELLED RUMINAL MICROORGANISMS

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Abstract

Ruminal microorganisms were labelled using [^{35}S]sulphate during growth on a sulphur-deficient medium. More than 99% of the ^{35}S incorporated by the cells was in the organic sulphur fraction. The labelled microorganisms were then infused into the abomasum of six sheep which were each fed 775 g of a ration containing $2 \cdot 1$% nitrogen and $0 \cdot 17$% sulphur.

In a 6-day collection period the mean apparent digestibility of the incorporated ^{35}S ($\pm$ S.E. of the mean) was $71 \cdot 3$ ($\pm 0 \cdot 94$)%; $51 \cdot 7$ ($\pm 1 \cdot 38$)% of the dose was retained and $19 \cdot 7$ ($\pm 1 \cdot 59$)% was excreted in the urine. Reducible ^{35}S (i.e. [^{35}S]sulphate) accounted for $8 \cdot 0$% of the ^{35}S activity in the faeces and $55 \cdot 3$% of the urinary ^{35}S activity. The proportion of the ^{35}S activity residing in the non-cell wall fraction of the infused microorganisms was $35 \cdot 7$%, but was $51 \cdot 4$ ($\pm 2 \cdot 32$)% in the faeces. Over a 21-day period, representing about 14 days post-infusion wool growth, $20 \cdot 2$ ($\pm 2 \cdot 20$)% of the infused ^{35}S, or 28% of the absorbed ^{35}S, was recovered in the wool.

The estimated digestibility of ruminal microorganisms in sheep closely corresponded with estimates obtained from experiments using rats, but the estimated utilization of the absorbed protein was a little lower. However, the efficiency of utilization of ^{35}S from ^{35}S-labelled bacteria was comparable to that obtained by other workers where radioactive casein and sulphur containing amino acids were infused.

I. INTRODUCTION

Despite the major contribution that microbial protein makes to the ruminant's amino acid requirements, estimates of the digestibility and utilization of that protein have been based largely upon data obtained from systems unrepresentative of the ruminant. Such information has been obtained from the feeding of ruminal micro-organisms to rats, or from *in vitro* systems, which use a pepsin–pancreatin digestion. While these results (see Table 1) may indicate the potential utilization by ruminants of microbial protein, they must be treated with caution, as Purser (1970) has indicated.

An alternative direct approach is to infuse tracer-labelled microorganisms into the abomasum of sheep and then determine the absorption and retention of the label. A basic requirement is that the behaviour of the labelled atoms should indicate the behaviour of the protein.

Estimates of endogenous nitrogen excretions are essential, but uncertain, quantities in bioassay calculations. The difficulty will also occur with the isotope

TABLE 1

ESTIMATES OF THE TRUE DIGESTIBILITY (TD), BIOLOGICAL VALUE (BV), AND NET PROTEIN UTILIZATION (NPU) OF RUMINAL MICROORGANISMS

Method A, values obtained using rats as test animals; method B, values obtained from *in vitro* enzymic methods; method C, a calculation made by Mason using estimates of bacterial nitrogen assimilated and excreted by sheep

Method	Reference	TD (%)	BV (%)	NPU (%)
	Protein source—bacteria			
A	Johnson *et al.* (1944)	55	66	36
	Reed, Moir, and Underwood (1949)	62, 65	80, 78	50
	McNaught *et al.* (1950)	73	88	64
	McNaught *et al.* (1954)	74	81	60
B	Bergen, Purser, and Cline (1968a)	75	85	63
	Bergen, Purser, and Cline (1967)	74(44–93)	80(53–111)	58(37–80)
	Bergen, Purser, and Cline (1968b)	65–70	—	71–77
	Protein source—protozoa			
A	Johnson *et al.* (1944)	86	68	58
	McNaught *et al.* (1954)	91	80	73
	Bergen, Purser, and Cline (1968b)	87	82	71
B	Bergen, Purser, and Cline (1968b)	75–85	—	71–78
	Protein source—mixed (bacteria and protozoa)			
B	Bergen, Purser, and Cline (1968b)	67–82	—	70–79
C	Mason (1969)	69	—	—

technique where errors may arise due to recycling of the absorbed isotope back into the gut. The isotope used must be incorporated into the cell structure and not be readily exchangeable. Further, organisms representative of the ruminal biota should be used. All ruminal bacteria have a similar amino acid composition (Purser and Buechler 1966) which alter little with dietary changes (Bergen, Purser, and Cline 1968b), but there is considerable variation in digestibility and in "protein quality" (biological value) between strains (Bergen, Purser, and Cline 1967). This

variation may account, in part, for the high apparent digestibility of ^{14}C in ^{14}C-labelled *Bacillus subtilis* (85%) and *E. coli* (91%) in sheep (Hoogenraad *et al.* 1970). Although the isotope-labelling method may be used to advantage in evaluating the nutritive value of single species of microorganisms, generalization from such results may be misleading.

In the present experiment mixed ruminal microorganisms, labelled using [^{35}S]sulphate during growth *in vitro* on a sulphur-deficient media, were infused into the abomasum of sheep. The excretion of ^{35}S and the incorporation of ^{35}S into wool was then followed, in an endeavour to determine the digestibility and utilization of microbial protein.

II. METHODS

Rumen fluid was collected from six rumen- and omasal-fistulated sheep, 24 hr after feeding 775 g of a ration comprising 57% oaten chaff, 29% of a proprietary concentrate ("Wesfeeds" Sheep Cubes), 13% lucerne chaff, and 1·3% minerals. The ration contained 2·1% nitrogen and 0·17% sulphur. The rumen fluid was strained through two layers of fine bolting silk. 500 ml of a solution containing 10 g cellobiose, 5 g glucose, 2 g urea, 2 g $NaHCO_3$, 0·175 g KH_2PO_4, and 200 μCi of [^{35}S]Na_2SO_4 was added to each of six flasks containing 250 ml of the strained rumen liquor; the system was sparged and continuously flushed with CO_2, and incubated for 24 hr at 38°C, with occasional agitation.

The labelled microorganisms were collected by centrifugation at 18,000 g for 20 min, suspended in isotonic saline, and stored for several hours at 5°C prior to infusion into the sheep. Approximately 64% of the added [^{35}S]sulphate was recovered in the microbial fraction and 2% in the supernatant fraction. About 34% was not accounted for, presumably lost to the air as H_2S during incubation. Only 0·1% of the ^{35}S in the microbial suspension was in a reducible form. The specific activity of ^{35}S in the microbial suspension was 5·15 μCi/mg S.

The labelled cells were infused into the abomasum by way of a vinyl tube (2 mm int. diam.) extending from the omasal cannula through the omaso-abomasal orifice. The method of omasal cannulation has been described by Hume, Moir, and Somers (1970). A tared 50-ml capacity polythene syringe (Atlas) was used to deliver the radioactive cell suspension. Three portions, each approximately 50 g, were given by single infusion to each of the six sheep at 2-hourly intervals, starting 3 hr after feeding. The total amount of radioactivity given to each sheep was 124·1 $\pm$0·17 μCi (mean $\pm$ S.E.). Each sheep received 20 mg of microbial sulphur, or on the basis of an N/S ratio of 11/1 (Walker and Nader 1968) about 1·38 g microbial protein.

Urine and faeces were collected over a 6-day period. Collections for day 1–2, 3–4, and 5–6, respectively, were combined, frozen, and stored.

The adoption of a 6-day period was based on an estimated mean retention time of a solid in the post-omasal tract of 16–21 hr (Weston 1968). In 6 days less than 1% of the infused dose would be retained in the gut.

Samples of wool were taken from a midside patch 21 days after the infusion and therefore represent about 14 days wool growth after the infusion (e.g. see Downes and Sharry 1971). Total wool growth was estimated from the wool grown per unit area in this period and a ratio of patch wool weight per unit area to fleece weight grown over a 5-month period prior to this experiment.

An ultrasonic probe (Dawes Soniprobe, 20 kHz, operating at 6–7 A) was used to disrupt cells in the infusate and in the faecal samples for days 1–2. Duplicate 5 g aliquots of the microbial infusate or 3 g aliquots of the faeces were each suspended in 35 ml water and subjected to six 45-sec periods of ultrasonic disintegration. The method used was similar to that of Hoogenraad and Hird (1970), and it was assumed that the majority of the cells were broken by this treatment. These samples were then diluted to 180 ml with 0·1M phosphate buffer and centrifuged for 20 min at 20,000 g. The supernatants were decanted, centrifuged again, and the cell-free liquid collected in separate flasks. Any precipitate remaining in the supernatant bottles was rinsed back into the

original bottles with 100 ml of phosphate buffer. Glass beads were added, the bottles were sealed, and the precipitates were dispersed by shaking prior to centrifuging at 20,000 g. The supernatants resulting were collected as previously described and the washing procedure repeated. Finally, the volumes of the cell-free solutions containing the cell-content ^{35}S were measured.

Analysis of sulphur and of ^{35}S in faeces, urine, wool, and the microbial preparation were made by the methods described by Bird and Fountain (1970). The total [^{35}S]sulphate content of faeces and of the infusate was, however, determined by direct reduction of the sample without the extraction procedure.

Diaminopimelic acid (DAP) and total nitrogen analyses were made on hydrolysates of the infusate and of rumen bacteria. The method of hydrolysis and DAP analyses were as described by Mason (1969); bacteria were isolated from rumen digesta as described by Hutton, Bailey, and Annison (1971). The ratio of N/DAP in rumen bacteria compared with the infusate was used to estimate the proportion of non-bacterial protein in the microbial infusate.

III. RESULTS

The mean excretion of ^{35}S (as % of dose given) in the urine and faeces of six sheep after infusing ^{35}S-labelled ruminal microorganisms is shown in Table 2. Of the

TABLE 2

EXCRETION OF ^{35}S BY SIX SHEEP FOLLOWING THE INFUSION OF ^{35}S-LABELLED
RUMINAL MICROORGANISMS

Values given are means $\pm$ S.E.

Sulphur fraction	Interval (days)	Excretion (as % of dose)	
		Faeces	Urine
Total sulphur	1–2	23·4 ($\pm$0·73)	14·4 ($\pm$1·33)
	3–4	3·5 ($\pm$0·18)	3·1 ($\pm$0·29)
	5–6	1·8 ($\pm$0·05)	2·2 ($\pm$0·30)
	Σ1–6	28·7 ($\pm$0·90)	19·7 ($\pm$1·59)
Reducible sulphur*	1–2	1·3 ($\pm$0·05)	7·5 ($\pm$0·73)
	3–4	0·6 ($\pm$0·03)	2·0 ($\pm$0·21)
	5–6	0·4 ($\pm$0·03)	1·3 ($\pm$0·07)
	Σ1–6	2·3 ($\pm$0·07)	10·9 ($\pm$0·91)
Organic sulphur†	Σ1–6	26·4 ($\pm$0·91)	8·8 ($\pm$0·78)

* i.e. sulphate sulphur.   † i.e. neutral sulphur.

totals for the 6-day collection period (Σ1–6), 82% of the faecal ^{35}S activity and 73% of the urinary ^{35}S activity recovered was accounted for in the first 2 days. The range of values for non-absorbed ^{35}S was 26–32% of the ^{35}S dose, and for ^{35}S absorbed but not retained 16–26% of the ^{35}S dose. Reducible ^{35}S (i.e. [^{35}S]sulphate) accounted for 8·0% of the faecal ^{35}S and 55·3% of the urinary ^{35}S activity. The contributions made by organic sulphur was estimated by difference between total ^{35}S and reducible ^{35}S. The organic ^{35}S component of faeces and urine was equivalent to 26·4 and 8·8%, respectively, of the total organic ^{35}S infused.

The incorporation of ^{35}S into wool varied from 12 to 26% of the infused ^{35}S (Table 3). The specific activity of ^{35}S in wool ranged from 0·165 to 0·201 μCi/g. The between-animal variation in ^{35}S incorporated was due mainly to differences in estimated wool growth rates, which varied from 4·1 to 8·4 g/day.

The mean apparent digestibility of ^{35}S was 71·3% (68–74%) (Table 3). An adjusted value for the digestibility of the microorganisms was obtained by subtracting the [^{35}S]sulphate component from the total ^{35}S activity in the faeces. This estimate was 73·6% (70–76%).

The estimated portion of the total cell ^{35}S activity residing in the cell-content fraction of the microbial infusate was 35·7%; that for the faeces from sheep A–F was 58·0, 56·0, 53·7, 39·3, 47·5, and 53·7%, respectively (mean 51·4%).

The N/DAP ratios in the sample of rumen bacteria and the infusate were similar, 21 and 23 respectively. This indicates that most, if not all, of the protein in the microbial infusate was bacterial. Protozoa may have been removed from the rumen digesta used for the ^{35}S-incubation by the initial straining process or have failed to survive the incubation treatment or both may have occurred. Microscopic examination of the microbial infusate was intended, but the sample was inadvertently frozen before this objective could be achieved.

TABLE 3

DIGESTIBILITY AND UTILIZATION OF ABSORBED ^{35}S FOLLOWING THE INFUSION OF ^{35}S-LABELLED RUMINAL MICROORGANISMS

	Sheep						Mean
	A	B	C	D	E	F	
Apparent digestibility (% of dose)	71·9	69·2	68·1	72·2	72·4	74·0	71·3
Adjusted digestibility (% of dose)*	74·4	71·4	70·3	74·4	74·9	76·1	73·6
Retention of ^{35}S (% of dose)	55·4	47·3	52·6	54·8	51·8	48·2	51·7
^{35}S in wool (% of dose)	23·3	25·9	17·4	24·3	11·8	18·4	20·2

* Estimate derived from faecal organic ^{35}S data.

IV. DISCUSSION

The microbial cells were effectively labelled by the incubation procedure as 99·9% of the cellular ^{35}S was found in the organic sulphur fraction. It is probable that most species of ruminal organisms were labelled during growth on this sulphur-deficient medium containing [^{35}S]sulphate. Although some bacterial strains are presumed to be unable to reduce sulphate (see Emery, Smith, and Fai To 1957; Prescott 1961) they could incorporate the sulphide arising from sulphate reduced by other bacteria. Protozoa require an organic source of sulphur but could have been labelled during the incubation following the ingestion of ^{35}S-labelled bacteria. However, the results indicate that few, if any, protozoa were finally present in the microbial infusate.

^{14}C is incorporated into a diversity of cellular and extracellular compounds, but ^{35}S is restricted to a smaller, less labile group of compounds and in this regard is a better marker. The cystine plus methionine sulphur content of bacteria, calculated from Purser and Buechler's (1966) data, is about 0·8 g/100 g amino acids. In *E. coli* more than 95% of the sulphur is in the sulphur-containing amino acid fraction (Roberts *et al.* 1955). All of the methionine and half the cystine is found in cellular protein, while the remaining cystine is in the glutathione tripeptide. When the cells

are grown in a sulphur-deficient media the glutathione reserve diminishes and at cessation of growth all of this sulphur is located in the residual protein (Roberts *et al.* 1955).

In attempting to estimate the true digestibility of ruminal microorganisms several factors must be considered:

(1) Recycling of ^{35}S to the gut, following digestion and absorption of the [^{35}S]protein;

(2) Incorporation of recycled ^{35}S into microbial protein within the gut;

(3) Degradation of [^{35}S]protein in the hindgut;

(4) Incomplete recovery of undigested [^{35}S]protein from the gut tract.

It is clear that if (1) and (2) occur then an underestimate of digestibility may result. If (3) or (4) occurs then the reverse may result. Bray (1969), and others, have shown that a portion of an intravenous dose of [^{35}S]sulphate is excreted in the faeces. This may be due to the entry of ^{35}S into the rumen and caecum and synthesis into microbial protein; the intestinal secretion of mucous ester sulphate or inorganic sulphate; the secretion of pancreatic and biliary organic sulphur compounds; or from the shedding of mucosal cells. The relative contribution of each of these outputs is not known. Endogenous protein secreted into the gut might account for some faecal [^{35}S]protein. Mason (1969) has estimated that for sheep fed on high fibre diets the faecal nitrogen of animal origin varied from 0 to $15 \cdot 5\%$ of the bacterial plus endogenous debris nitrogen in the non-dietary nitrogen fraction.

Both [^{35}S]taurine and [^{35}S]sulphate, when given intravenously to sheep, are secreted in the bile as sulphates or as tauro-conjugated bile acids (Bird, unpublished data). However, bacterial hydrolysis of bile acid conjugates occurs within the large intestine, consequently little bile taurocholic acid is excreted in the faeces by rats (Gustafsson *et al.* 1957) or by sheep (Bird, unpublished data). The sulphide resulting from taurine degradation in the rat is absorbed and excreted in the urine as sulphate (Boquet and Fromageot 1967). However, the sulphide could presumably also meet the bacterial sulphur requirement in the hindgut and thereby permit a portion of the ^{35}S from recycled bile acids to be incorporated into new bacterial protein.

The greater proportion of cell-content ^{35}S in the faecal samples, compared with the infusate, could suggest substantial reincorporation of ^{35}S by bacteria in the hindgut. From the proportions of ^{35}S in the cell-content fraction of faeces and infusate, and from the amount of infused ^{35}S excreted in the organic sulphur fraction in the faeces, about $13 \cdot 6\%$ of the dose was found in the faeces cell-content fraction; $9 \cdot 4\%$ is expected if digestion of cell walls and cell contents proceeded at the same rate. Reincorporation of the ^{35}S arising from the digestion of the infused ^{35}S-labelled bacteria during fermentation in the caecum, or from the ^{35}S recycled in bile acids or mucin, could occur in the hindgut. These species may be different from the species of ruminal microorganisms labelled *in vitro*. Since Gram-positive bacteria have no sulphur-containing amino acids in their cell walls (Salton 1960), an increased proportion of these species would produce a greater proportion of cell-content ^{35}S/cell-wall ^{35}S in the faeces than originally infused. A less credible alternative is that the bacterial cell-wall ^{35}S material is more readily digestible than the cell-content ^{35}S.

It has been suggested that differential digestion of microbial cell components may occur in the intestines with the cell-wall component being more resistant than

the remaining protein (see Allison 1969). The data of Hoogenraad et al. (1970) could, however, suggest a differentially greater digestion of cell-wall ^{14}C than of cell-content [14]C. In the present experiment the apparent change in the ratio of cell-content ^{35}S/cell-wall ^{35}S in faeces compared with the infusate does not necessarily support an argument counter to that stated by Allison (1969). Apart from the possibility of a resynthesis of ^{35}S-containing cells in the hindgut, if organic ^{35}S in forms such as tauro-conjugated bile acids, pancreatic trypsin, or mucin was not completely degraded in the sheep's intestine, then these secretions could contribute to the "cell-content" fraction, as determined here. This would also result in underestimating the true digestibility of the infused microorganisms.

The possibility that backflow of labelled microorganisms from the omasum to the rumen could occur and thus invalidate the conclusions drawn from this experiment, must be considered. A preliminary experiment had shown that the infusion of labelled (and presumably viable) microorganisms into the rumen of each of four sheep resulted in $31 \cdot 9$ ($\pm 0 \cdot 35$)% and $22 \cdot 3$ ($\pm 1 \cdot 31$)% of the dose appearing in the faeces and urine, respectively, over a 10-day collection period. There was $97 \cdot 7$% organic ^{35}S in the infusate. The similarity of those and the present results may indicate that any ^{35}S arising from degradation of labelled cells in the rumen re-entered new cells which subsequently passed to the abomasum. The methods used for the labelling and infusion of the ruminal microorganisms were the same in both cases. However, it is unlikely that backflow of labelled cells from the omasum could occur, and, in fact, in another experiment, continuous infusion of Cr-EDTA solution into the omasum of each of four sheep, at a rate of 290 mg Cr/day over 2 days, did not elevate the concentration of chromium in the rumen fluid (Bird, unpublished data).

If one assumes that all of the [^{35}S]sulphate in the faeces originates from recycled [^{35}S]sulphate and therefore must be discounted, then the adjusted estimate of the digestibility of microorganisms is a little greater ($73 \cdot 6$% compared with $71 \cdot 3$%). Hogan and Weston (1968) estimated by regression analysis that over a wide range of diets the mean true digestibility of protein in the abomasal digesta was 78%, a value not substantially different from that obtained here, or by others, for ruminal microbes. Thus, from Table 1, the rat bioassay method gives a mean value for digestibility of bacteria of 67%, and 88% for protozoa. The in vitro enzymic method gives comparable results, and the estimate by Bergen, Purser, and Cline (1968b) of the digestibility of mixed ruminal organisms obtained from sheep fed four different rations was 67–82%. It is concluded from these comparisons that the digestibility of ruminal microorganisms in rats and sheep is similar.

A mean value of 20% of the infused ^{35}S, or 28% of the absorbed ^{35}S, was incorporated into wool. From 30–40% of the ^{35}S from intravenous doses of L-[^{35}S]cystine (Downes 1961; Downes et al. 1970a) and approximately 20% of [^{35}S]methionine given per abomasum (Downes et al. 1970b) may be incorporated into wool. Data from Weller (1957) and Purser and Buechler (1966) show that the methionine sulphur/cystine sulphur ratio in ruminal bacteria is about $2 \cdot 11$, i.e. approximately two-thirds of the sulphur is in methionine (see also Roberts et al. 1955). It is evident, therefore, that the microbial sulphur amino acids were relatively efficiently absorbed and utilized in the current experiment. Approximately $31 \cdot 5$% of the dose (or 44% of the absorbed ^{35}S) was retained in the body in tissues other than wool. Since both methionine and cystine stimulate wool growth (Reis 1967) and body

weight gains of sheep fed a variety of diets (Reis and Schinckel 1963; Hume and Bird 1970; Bird, unpublished data), the sulphur-containing amino acids in the digesta entering the duodenum are probably the primary limiting amino acids. This suggestion is supported by the finding of Nimrick et al. (1970) that methionine was the first limiting amino acid in the nutrition of the growing lamb. Cystine was also indicated as the most limiting amino acid of bacteria and second limiting amino acid of protozoa when fed to rats (Bergen, Purser, and Cline 1968a, 1968b). It is, therefore, expected that catabolism of absorbed ^{35}S-labelled amino acids would not be great and that the efficiency of usage of absorbed ^{35}S would reflect the "net protein utilization" of the ruminal microorganisms. Only 20% of the infused ^{35}S (or 28% of absorbed ^{35}S) was excreted in the urine, and inorganic sulphate accounted for approximately half of this indicating that the absorbed sulphur-containing amino acids were reasonably efficiently used under these conditions. Downes et al. (1970a, 1970b) have found that about 20% of the ^{35}S from either intravenous or intraperitoneal infusions of L-[^{35}S]cystine and approximately 12% of the ^{35}S from [^{35}S]methionine or [^{35}S]casein infused per abomasum was excreted in the urine over 14- and 7-day periods, respectively. It should be noted that the urinary ^{35}S loss measured in the current experiment may be underestimated, due to the restricted collection interval. If this is so, then a slight overestimation of protein utilization will result.

The estimated net protein utilization obtained in this experiment, 52%, is lower than the mean values of 55% for bacteria and 67% for protozoa obtained with rats (Table 1). It is not clear whether this is due to differences in experimental approach or indicates a species difference in the ability to utilize absorbed microbial protein.

V. Acknowledgments

VI. References

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