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The effect of increasing methionine supply on the methionine conversion to cyst(e)ine in sheep

BY P. M. PISULEWSKI* AND P. J. BUTTERY†

Department of Applied Biochemistry & Food Science, University of Nottingham, School of Agriculture, Sutton Bonington, Loughborough, Leicestershire LE12 5RD

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1. The conversion of methionine to cyst(e)ine was determined in sheep infused with different amounts of methionine (0–5 g/d) into the duodenum by assaying the incorporation of ^{35}S from intravenously-infused L-[^{35}S]methionine into cyst(e)ine in wool, plasma albumin and the free plasma pool.

2. The percentage of cystine-S in the plasma originating from methionine increased linearly from 4·5 to 18 with increasing supplemental methionine supply.

3. The percentage of cysteine-S in albumin increased from 15 to 50; methionine supply increased to 3 g/d but then remained constant, indicating that the transsulphuration pathway of the liver was exceeded.

4. The percentage of wool cysteine-S originating from methionine was high (~ 70) at all methionine supplementation rates.

There is considerable evidence that the supply to the duodenum of the sulphur-containing amino acids methionine and cystine can limit ruminant productivity. The two-phase dose–response relation between post-ruminal methionine supply and plasma free methionine concentration has frequently identified methionine as the first-limiting amino acid in both sheep (Chalupa & Chandler, 1972; Reis *et al.* 1973; Strath & Shelford, 1978) and cattle (Williams & Smith, 1974; Fenderson & Bergen, 1975; Mathers *et al.* 1979). These results, however, are appropriate only to the conditions used in each experiment and cannot be readily applied to other situations (Bergen, 1979; Mathers & Miller, 1979).

It is recognized that knowledge of the whole-body amino acid metabolism can aid attempts to define amino acid allowances for farm animals (Mathers & Miller, 1979; MacRae & Reeds, 1980). For example, Gill & Ulyatt (1979) have used studies of plasma amino acid kinetics to investigate whole-body methionine metabolism in sheep and similar studies have been conducted in cattle (Mathers & Miller, 1983). However, no attempt was made in these studies to estimate the extent of conversion of methionine to cystine quantitatively.

The objective of the present experiment was to estimate quantitatively the conversion of methionine to cystine in sheep. The transsulphuration pathway of methionine catabolism produces cystine (Findelstein, 1970) and probably constitutes a major drain on methionine in sheep. Cystine is a main component amino acid of wool (Hogan, 1975). In the present studies, sheep were infused intravenously with L-[^{35}S]methionine and the plasma plateau specific activities of both methionine and cystine were measured. From this the proportion of cystine originating from methionine was calculated. Subsequently cystine flux was determined following intravenous infusion of L-[^{35}S]cystine. The estimated proportion of cystine originating from methionine and the cystine flux were then used to calculate the extent of the conversion of methionine to cyst(e)ine.

The predominant sites of the transsulphuration pathway in sheep are the liver (Radcliffe & Egan, 1978) and the skin (Downes *et al.* 1964). In an attempt to assess the contribution

* Present address: Department of Animal Nutrition, Institute of Animal Production, 32–083 Balice, Poland.
† For reprints.

Table 1. *The composition of the experimental diet (g/kg)*

Ingredient*	
Barley	225
Oats	450
Grass meal	250
Vitamins and minerals†	25
Nutramol‡	100

* Fresh weight basis.
† Wrightmin-Sheep (Frank Wright Ltd).
‡ Molassed peat (Rumenco Ltd).

of these sites to whole-body methionine metabolism, the proportion of cystine originating from methionine was estimated in plasma albumin (synthesis is in the liver) and in wool samples.

The importance of methionine supply in limiting ruminant production is illustrated by the number of compounds investigated as potential derivatives (see, for example, Buttery *et al.* 1977). Some of these derivatives have been shown directly to increase methionine supply to the duodenum (Langar *et al.* 1978), but it is not known how much this extra methionine is being used to satisfy the cystine requirement of the animal. The possibility that the conversion of methionine to cysteine can be saturated under physiological or near physiological conditions was examined by studying the extent of the methionine conversion to cysteine as the supply of methionine to the duodenum was increased by infusion of L-methionine into the proximal duodenum.

MATERIALS AND METHODS

Animals, diet and feeding

Cross-bred wethers weighing approximately 30 kg were used. The sheep were fitted with single cannulas in the rumen and in the proximal duodenum 6 weeks before the start of the experiment. Throughout the experimental periods the animals were kept in metabolism crates at ambient temperatures and with continuous lighting. The composition of the experimental diet is shown in Table 1. The diet was offered in equal portions at 2-h intervals by an automatic feeder at a daily rate of 800 g. Water was constantly available. The animals were adapted to the diet and the feeding regimen for 14 d before the start of the experimental period.

Experimental design

Six wethers were randomly allocated to six intraduodenal L-methionine infusion levels (0, 1, 2, 3, 4 and 5 g/24 h). Each infusion lasted the entire 10 d of the experimental period. L-Methionine (Sigma Chemical Co. Ltd, Poole, Dorset), dissolved in physiological saline (9 g sodium chloride/l), was infused at a rate of 500 ml/24 h.

Collection and preparation of samples

During the 10 d experimental period the samples of blood plasma, plasma albumin and wool were collected as indicated later (p. 123). On the 3rd day of the experimental period both jugular veins of each sheep were catheterized, one with a short (150 mm) and the other with a long (250 mm) catheter. The catheters were filled with sterile saline containing 250 units heparin/ml. On day 4 a continuous infusion of L-[^{35}S]methionine (Amersham International

plc, Amersham, Bucks) was made via the long catheter while blood was sampled from the short one. The infusion solutions contained approximately 1 mCi L-[^{35}S]methionine and 12·5 μmol L-methionine in 250 ml sterile saline and were infused at a rate of 20 ml/h. Blood samples (30 ml) were withdrawn into heparinized tubes at 0 h and then at 1, 2, 3, 4, 5, 6, 7 and 8 h after the beginning of the infusion. The catheters were flushed with heparinized saline after each sample. The whole-blood samples were immediately centrifuged at 2000 ***g*** for 15 min and the plasma obtained and stored in liquid nitrogen. The catheters were removed at the end of the methionine infusion. Both jugular veins were catheterized again on day 9 of the experimental period and on the following day the sheep were infused with L-[^{35}S]cystine (Amersham International plc). The infusion solutions contained approximately 1 mCi L-[^{35}S]cystine and 12·5 μmol L-cystine (Sigma Chemical Co. Ltd) in 250 ml sterile saline and were infused at the same rate as L-[^{35}S]methionine solutions. Blood samples were collected and prepared as described previously.

Plasma samples were deproteinized using the picric acid procedure of Stein & Moore (1954). The deproteinized samples were then adjusted to pH 7 with 1 M-lithium hydroxide, cysteine oxidized to cystine, adjusted to pH 7 with 1 M-hydrochloric acid and then stored at −40° (Stein & Moore, 1954).

Separate samples of whole blood (30 ml) were withdrawn during L-[^{35}S]methionine infusions at 6 and 8 h to isolate plasma albumin. The samples were immediately centrifuged as described previously and the plasma obtained stored in liquid N_2. On thawing, plasma albumin was isolated according to the procedure of Swick & Ip (1974). Following the final step of the procedure, albumin samples, dissolved in ethanol, were dried under vacuum for 24 h at 50°. The purity of albumin samples was confirmed using polyacrylamide gel electrophoresis (Laemmli, 1970). Bovine albumin (Sigma Chemical Co. Ltd) was used as a standard.

On the day preceding the L-[^{35}S]methionine infusion, two 250 × 250 mm mid-back patches of wool were clipped as closely as possible to the surface of the skin from each sheep. The clipped areas were kept covered until wool samples were collected 6 d later (the time taken for the radioactivity to appear above the skin surface (Downes, 1961)). The samples were washed with diethyl ether, ethanol and water and finally freeze-dried.

The samples of plasma albumin and wool were prepared using the performic acid procedure of Moore (1963) to oxidize sulphur amino acids to cysteic acid and methionine sulphone. Following oxidation the samples were hydrolysed with 6 M-HCl for 22 h at 110°. The hydrolysates were taken to dryness under reduced pressure, the residues redissolved in lithium citrate buffer (pH 2·2), filtered through a 0·22 μm filter (Millipore (UK) Ltd, Harrow) and stored at −40°.

Analytical techniques

Feed samples were analysed for total N using the Kjeldahl procedure (Association of Official Agricultural Chemists, 1965). The deproteinized plasma samples and the hydrolysates (see above) of plasma albumin and wool were analysed for cystine and methionine or cysteic acid and methionine sulphone respectively, on an LKB 4400 (LKB Biochrom Ltd) amino acid analyser using lithium buffers. Amino acid concentrations were calculated with reference to DL-norleucine (BDH Ltd) as an internal standard.

The deproteinized plasma samples and the hydrolysates of plasma albumin and wool were assayed for ^{35}S radioactivity in cystine and methionine or cysteic acid and methionine sulphone respectively. A preparative amino acid analyser operating on the lithium system of Atkin & Ferdinand (1970) and a fraction collector were used to separate S amino acids. The fractions of column effluent (1·2 ml) were mixed with 10 ml of a commercial scintillator solution (Fisofluor; Fisons Ltd, Loughborough) and counted on an Intertechnique SL 30

Table 2. *The effect of duodenal L-methionine infusion on plasma free methionine and cystine concentration in sheep*

(The values are means with their standard errors of the nine samples taken during L-[^{35}S]methionine infusion)

L-Methionine (g/d)	Infusion rate (μmol/h)	Plasma methionine (μmol/l)		Plasma cystine (μmol/l)	
		Mean	SE	Mean	SE
0	0	2·2	0·4	4·8	0·2
1	279	13·0	3·0	4·0	0·3
2	558	19·4	0·9	3·8	0·2
3	837	42·0	2·4	10·7	0·5
4	1117	33·8	5·3	4·7	0·2
5	1396	22·99	15·2	10·9	9·7

scintillation counter. The external standard channels' ratio method was used to correct for quenching.

Calculations

The constant intravenous infusions of L-[^{35}S]methionine and L-[^{35}S]cystine were used to determine methionine and cystine flux respectively. The mean plasma plateau specific activities of methionine and cystine were calculated from the values obtained for plasma samples collected at 6, 7 and 8 h after the beginning of the infusion. The flux was calculated by dividing the infusion rate of isotope by the plateau specific activity of methionine and cystine (Shipley & Clark, 1972).

The proportion of cystine derived from methionine was calculated as the plateau specific activity ratio, cystine-S : methionine-S during the L-[^{35}S]methionine infusion. This proportion and cystine flux during the L-[^{35}S]cystine infusion were used to calculate the amount of cystine synthesized from methionine. The amount of cystine synthesized from methionine was expressed on an equisulphur basis.

RESULTS

The plasma concentrations of methionine are presented in Table 2. A substantial increase in methionine concentration was noted when 5 g/d were infused, indicating that methionine was exceeding requirements. The plasma cystine concentrations were not markedly affected by L-methionine infusions (Table 2).

The specific activities of plasma methionine and those of plasma cystine during L-[^{35}S]methionine infusions apparently reached plateau well before 6 h. During L-[^{35}S]methionine infusions the maximum specific activity of plasma cystine recorded was $1{\cdot}5 \times 10^4$ disintegrations/min per μmol cystine-S and this fell to background levels before L-[^{35}S]cystine infusions, when the plateau specific activities (reached before 6 h) ranged from 5×10^4 to $1{\cdot}25 \times 10^5$ disintegrations/min per μmol cystine-S. Therefore it was assumed that carry-over effects on plasma cystine specific activity were minimal. Despite this it was considered unwise to attempt to use the same animal for several experimental periods (i.e. different duodenal methionine infusion rates), thus the replication of the experimental values was restricted.

The values of methionine and cystine flux through the plasma pool are presented in

Table 3. *The effect of* L-*methionine infusion on methionine and cystine flux, the percentage of cystine flux originating from methionine and the percentage of methionine flux converted to cystine in sheep, calculated from plasma free amino acid specific activities*

L-Methionine infusion level (g/d)	L-Methionine infusion level (μmol/h)	Methionine flux (μmol/h)	Cystine-sulphur flux† (μmol/h)	Percentage of cystine-S originating from methionine	Amount of cystine-S originating from methionine (μmol/h)	Percentage of methionine flux converted to cystine	Apparent whole-body protein synthesis* (g/kg body-weight$^{0.75}$ per d)	
							Methionine flux	Cystine flux
0	0	880	418	4·51	18·8	2·13	10·69	6·90
1	279	1198	1154	5·80	66·9	5·58	14·52	19·78
2	558	1070	930	8·24	76·6	7·15	9·87	12·09
3	837	1362	676	13·73	92·8	6·81	17·36	12·38
4	1117	1461	1306	13·04	170·3	11·65	19·58	24·82
5	1396	2166	992	18·53	183·8	8·48	27·61	18·20

* Calculated from the cysteine and methionine content of sheep muscle (Lawrie, 1974) but not corrected for total input of cystine or methionine from intestines or for oxidation of cystine or methionine as appropriate (see text).

† Calculated from plasma specific activity during L-[^{35}S]cystine infusion.

Table 3. The regression of methionine flux (Y, μmol/h) *v.* methionine infusion (X, μmol/h) was as follows:

$$Y = 0{\cdot}76\ (\text{SE}\ 0{\cdot}18)\,X + 819{\cdot}8 \quad (r\ 0{\cdot}89,\ P < 0{\cdot}05).$$

In contrast to the significant relation between methionine infusion and methionine flux, no apparent systematic changes in cystine flux could be detected ($P > 0{\cdot}30$). Surprisingly, in the studies of Williams & Leng (1972) the abomasal infusion of 2·5 g/d of DL-methionine increased the entry rate of cystine from 92 mg/h to 142 mg/h.

Increasing methionine infusion (Table 3) resulted in an increase in the percentage of plasma cystine originating from methionine. The values would indicate that between 4·59 (0 g infused) and 18·5% (5 g infused) of cystine originated from methionine. However, the regression of the percentage of cystine originating from methionine (Y, %) *v.* methionine infusion (X, μmol/h) was not significant:

$$Y = 0{\cdot}006\ (\text{SE}\ 0{\cdot}003)\,X + 7{\cdot}02 \quad (r\ 0{\cdot}70,\ P > 0{\cdot}05).$$

The regressions of both the amount of cystine synthesized from methionine (Y, μmol/h) and the conversion of methionine to cystine (Y, %) *v.* methionine infusion (X, μmol/h) were described by the following equations:

$$Y\ (\mu\text{mol/h}) = 0{\cdot}478\ (\text{SE}\ 0{\cdot}015)\,X + 19{\cdot}30 \quad (r\ 0{\cdot}96,\ P < 0{\cdot}01),$$
$$Y\ (\%) = 0{\cdot}005\ (\text{SE}\ 0{\cdot}001)\,X + 3{\cdot}42 \quad (r\ 0{\cdot}83,\ P < 0{\cdot}05).$$

It should be noted, however, that while increases in the amount of cyst(e)ine synthesized from methionine followed closely the increases in L-methionine infusion, the percentage of the methionine flux converted to cyst(e)ine did not show such a systematic change but was in the range of 2·1–11·6%. Caution should be taken in extrapolating the plasma results presented in Table 3 to the whole animal since it would have to be assumed that plasma free amino acid pools equilibrate with the intracellular pools of amino acids, and this is not always the case (see Reeds & Lobley, 1980).

Table 4. *The effect of* L-*methionine infusion on the percentage of cystine-sulphur originating from methionine in plasma albumin and wool*

(Mean values with their standard errors of four determinations (two samples prepared and assayed in duplicate

L-Methionine infusion level		Albumin (%)		Wool (%)	
(g/d)	(μmol/h)	Mean	SE	Mean	SE
0	0	14·81	0·94	67·23	1·67
1	279	16·52	3·87	—*	
2	558	42·66	2·82	65·99	1·66
3	837	50·97	2·05	54·94	2·29
4	1117	50·10	2·82	—*	
5	1396	44·56	1·44	81·59	3·28

* See below.

The percentage of cystine originating from methionine in the liver and the skin tissues, as indicated by plasma albumin and wool samples, was generally increased by L-methionine infusions (Table 4). However, the values for plasma albumin would indicate that, once this percentage reached the value of 40–50% (2–3 g L-methionine infused/d), transsulphuration capacity of the liver had been exceeded. The values obtained for wool samples suggested generally higher transsulphuration capacity of the skin compared with the liver. In addition, the skin appeared to have adequate capacity to convert methionine to cystine even at the highest L-methionine infusion rates (5 g/d). As only trace amounts of ^{35}S radioactivity were present in wool samples collected from sheep infused with 1 and 4 g L-methionine, we have no explanation for this.

DISCUSSION

In the present study, plasma methionine concentration responded to the post-ruminal L-methionine infusions as expected. At least as indicated by changes in plasma methionine concentration (Table 2), the methionine status of the experimental animals varied from being deficient to being in excess.

The elevation in plasma methionine concentration in sheep infused with the 5 g L-methionine/d suggests that the apparent supplemental methionine requirement in the present study was between 4 and 5 g/d. This value was slightly higher than 3·12 g (Chalupa & Chandler, 1972), 3·3 g (Reis *et al.* 1973) or the value of 2·62 g (Strath & Shelford, 1978) obtained in similar studies but with different diets. The changes in plasma methionine concentration may be compared with changes in methionine flux (Table 3). Methionine flux determined in the present study apparently responded to increasing L-methionine infusion rates, and the highest value of methionine flux was obtained at the highest rate of L-methionine infusion. The close resemblance between methionine duodenal supply and plasma methionine flux was previously reported in sheep (Fennessy *et al.* 1978; Gill & Ulyatt, 1979) and in cattle (Mathers & Miller, 1983).

In a simplified model of the whole-body amino acid metabolism (Garlick, 1980) the flux represents the rate at which an amino acid leaves the total free amino acid pool of the body by two ways: incorporation into protein and catabolism. This model has been used in studies on whole-body metabolism in sheep (Gill & Ulyatt, 1979) and cattle (Mathers & Miller, 1983). In the present studies, methionine flux was considered to represent the utilization

for protein synthesis and for conversion of methionine to cystine. Interpretations of these findings are complicated by the possible increase in total body protein synthesis as methionine uptake increases. Some values are available for the oxidation rates of the carbon component of methionine.

The studies of Gill & Ulyatt (1979) indicate that the percentage of methionine oxidized ranged from 13·3 to 19·9. It is also interesting to note that in calves infused with graded levels of methionine (Mathers & Miller, 1983), methionine oxidation was increased from 17 to 30% over the range of methionine supplied. An estimate of methionine to cystine conversion was made in the present study. First the percentage of cystine flux originating from methionine was estimated over the experimental range of L-methionine infusion rates (Table 3). The substantial increase in this value (4·51–18·53%) indicated increased catabolism of methionine via the transsulphuration pathway. In similar studies with steers (Buttery *et al.* 1984), the estimated percentage of cystine flux originating from methionine was 6·7. The percentage of total methionine flux that was converted to cystine was calculated on an equisulphur basis from the amount of cystine synthesized daily from methionine and methionine flux values. The values obtained were in the range of 2·13–11·65%.

It is generally accepted that the conversion of methionine to cystine is particularly important in sheep (Armstrong & Annison, 1973). As wool has high cystine and low methionine contents (Hogan, 1975), the absorbed methionine must be converted to cystine before incorporation into wool. The results obtained in the present study, both the proportion of cystine originating from methionine and methionine to cystine conversion, may suggest that the whole-body conversion of methionine to cystine is not efficient. As discussed by Reis *et al.* (1973) and also by Radcliffe & Egan (1978), this inefficiency is also consistent with the plasma free methionine and cystine concentrations. In both studies post-ruminal methionine infusion resulted in high plasma methionine concentration while only slight changes in cystine concentration were observed. Plasma free methionine and cystine profiles obtained in the present study (Table 2) closely resembled those previously reported. It seems probable that the limited capacity of the liver to convert methionine to cystine (as discussed later) may be an important factor.

It has been assumed that whole-body protein synthesis can be calculated from an essential amino acid flux, provided the amino acid composition of body protein is known and the flux is corrected for dietary input or amino acid catabolism (Reeds & Lobley, 1980). The apparent whole-body protein synthesis in the present study (Table 3) was calculated from methionine and cystine flux values assuming that the average methionine and cystine contents of sheep protein are 2·3 and 1·3% respectively (Lawrie, 1974). Whole-body protein synthesis calculated from methionine flux without any correction for methionine catabolism or methionine uptake from the gut determined at the 0, 1, 2, 3 and 4 g infusion levels was in the range of 10–20 g/kg body-weight$^{0.75}$ per d. The values obtained may be compared with the values of 15·5 and 15·7 g/kg body-weight$^{0.75}$ per d reported for adult sheep by Bryant & Smith (1982) and Reeds & Lobley (1980) using tyrosine and leucine flux values respectively. The values calculated during the present study do give some confidence in the techniques used in the present study to measure plasma flux but also illustrate the need to correct flux rates before using them to assess whole-body protein synthesis. The apparent whole-body protein synthesis calculated for the sheep infused with the 5 g L-methionine level was high (27·6 g/kg body-weight$^{0.75}$ per d), presumably because of the increase in methionine availability. Food intake and presumably methionine leaving the abomasum remained constant. This increased flux was also observed in calves infused with increasing amounts of methionine (Mathers & Miller, 1983).

We suggest that much of the variation in plasma cystine flux can largely be explained by technical difficulties in determining cystine specific activity.

The survey studies on the distribution of the enzymes of methionine metabolism in ruminant tissues (Radcliffe & Egan, 1974), clearly indicate that the methionine–cystine conversion pathway does not operate in skeletal muscle. This was confirmed using perfused ruminant diaphragm by Coward & Buttery (1982). Radcliffe & Egan (1978) studied hepatic enzymes of the transsulphuration pathway and implied that liver was the major site of methionine to cystine conversion. Downes *et al.* (1964), in studies on sheep, clearly demonstrated the activity of the transsulphuration pathway in the skin. As the liver and the skin are considered to be the main active sites of the transsulphuration pathway, the substantial proportion of cystine originating from methionine in plasma albumin and wool samples could be expected. The values obtained (Table 4) suggest that while the transsulphuration capacity of the liver was exceeded at the low duodenal L-methionine infusion rates (2–3 g) the transsulphuration capacity of the skin was not so easily exceeded. The limited transsulphuration capacity of the liver can be explained in terms of the metabolic regulation of methionine catabolism. It has been demonstrated (Radcliffe & Egan, 1978) that the inability of the sheep to convert excessive amounts of methionine to cystine was associated with decreased activity of methionine adenosyltransferase (*EC* 2.5.1.6) in the liver, resulting in reduced methionine entry into the transsulphuration pathway. The possibility of an alternative pathway of methionine catabolism also exists. As discussed by Steele & Benevenga (1978), the transamination pathway of methionine metabolism may contribute significantly to methionine catabolism, especially when the capability of transsulphuration has been exceeded or impaired

It was shown (Downes *et al.* 1964) that after an intradermal dose of L-[^{35}S]methionine had been given, about 90% of the ^{35}S incorporated into wool at the injection site was present as cystine. In addition, similar values of methionine and cystine as supplements for wool growth were demonstrated by Reis *et al.* (1973). The fact that the incorporation of ^{35}S radioactivity into wool samples (the 1 and 4 g L-methionine infusion levels) could not always be measured is difficult to explain. As reported by Downes (1961), the specific activity of cystine in wool samples reached a maximum on the 6th day after an intravenous injection of DL-[^{35}S]cystine.

In conclusion, these findings show that while the methionine to cysteine conversion is active in sheep, the pathway in the liver can be easily saturated. The results again illustrate the compartmentalization of free amino acids within individual tissues and also suggest that the capacity of the skin to synthesize cystine from methionine is high.

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