SULPHUR METABOLISM AND EXCRETION STUDIES IN RUMINANTS

V.* RUMINAL DESULPHURATION OF METHIONINE AND CYST(E)INE

By P. R. BIRD†

[Manuscript received 26 May 1971]

Abstract

The concentrations of volatile sulphides in the rumen fluid of sheep were determined at intervals after giving single intraruminal infusions of DL-methionine, L-cystine, or L-cysteine. The basal ration fed contained 0·1% sulphur.

In the first experiment one sheep was given 3·0 g sulphur as methionine, cystine, or cysteine immediately after feeding (200 g dry matter) on days 1, 3, and 5, respectively. Peak concentrations of total sulphides, at 2 hr after infusion, were 1, 47, and 43 μg S/ml, respectively.

In the second experiment four sheep were dosed with methionine (≡ 1 g sulphur) for 20 days then given methionine (≡ 3·0 g sulphur) on the 21st day. There was no adaptation response to methionine.

In the third experiment, on the first day two sheep were given methionine (≡ 2·0 g sulphur) and two sheep a control water infusion. On the third day the treatments were reversed. The infusions were made 2 hr after feeding (800 g dry matter). There was a small increase in the concentration of CH_3SH ($P < 0·01$) and of H_2S ($P < 0·05$) due to methionine. The mean increment in CH_3SH and H_2S concentrations above basal for the period 0·5–12 hr post-infusion was 1·60 and 0·66 μg S/ml. The rate of decline of CH_3SH concentration in the rumen was proportional to the rate of dilution of a soluble marker, further indicating that a portion of the infused methionine flowed from the rumen undegraded.

I. INTRODUCTION

The amino acids ingested by ruminants are, in general, extensively degraded by the ruminal microflora (e.g. see Chalmers and Synge 1954). The nutritional cost incurred in the catabolism and subsequent resynthesis of amino nitrogen by the bacteria may be considerable (Clarke, Ellinger, and Phillipson 1966). A notable limitation imposed by this process is on the effectiveness of dietary sulphur-containing amino acid supplements to stimulate wool growth. The concentrations of sulphur in wool is about 3–4% (e.g. see Reis *et al.* 1967) compared with about 1% in bacteria (Roberts *et al.* 1955), hence the wastage of other absorbed amino acids is minimized and wool growth maximized if supplemental methionine or cystine is absorbed unaltered. Subcutaneous injections of cysteine were shown by Marston (1935) to increase wool growth, and subsequently methionine infused into the abomasum has been found to be equally effective (e.g. Reis and Schinckel 1963).

* Part IV, *Aust. J. agric. Res.*, 1971, **22**, 443–52.

† Department of Animal Science and Production, Institute of Agriculture, University of Western Australia, Nedlands, W.A. 6009.

Although dietary methionine (Colebrook *et al.* 1968) and cystine (e.g. Marston 1932) may both fail to stimulate wool growth, the rate of degradation of methionine in the rumen is apparently considerably slower than that of cysteine or cystine, judged by the rate of ammonia production during *in vitro* incubation (Lewis 1955; Lewis and Emery 1962). If this situation prevails *in vivo* then significant amounts of dietary methionine might pass intact to the intestines. Supplements of methionine given in the diet have in some instances given wool growth responses (e.g. Starks *et al.* 1954; Graceva 1969), although Downes *et al.* (1970) have shown that dietary methionine was extensively degraded compared with abomasal infusions of methionine.

The generalized reaction described for the decomposition of cysteine by aerobic bacteria (e.g. Desnuelle, Wookey, and Fromageot 1940; Fromageot and Kiun 1941), and by an anaerobic rumen coccus (Barker 1961) was

$$HS\text{–}CH_2\text{–}CH(NH_2)\text{–}COOH \xrightarrow{\text{sulphydrase}} NH_3 + H_2S + \text{pyruvate}.$$

Soil bacteria have been shown to degrade methionine to thiomethane (CH_3SH), ammonia, and α-aminobutyrate (e.g. Mitsuhashi 1949), while Zikakis and Salsbury (1969) and Salsbury *et al.* (1971) found that CH_3SH, and often traces of dimethyl sulphide, were produced from *in vitro* incubation of L-methionine by ruminal microorganisms. According to Segal and Starkey (1969) and Ruiz-Herrera and Starkey (1970), methionine was degraded by some aerobic bacteria by the following reactions:

$$H_3CS\text{–}(CH_2)_2\text{–}CH(NH_2)\text{–}COOH \xrightarrow{+O} H_3CS\text{–}(CH_2)_2\text{–}CO\text{–}COOH + NH_3 \qquad (1)$$

$$H_3CS\text{–}(CH_2)_2\text{–}CO\text{–}COOH \xrightarrow{+2H} H_3C\text{–}CH_2\text{–}CO\text{–}COOH + CH_3SH \qquad (2)$$

$$H_3C\text{–}CH_2\text{–}CO\text{–}COOH \xrightarrow{+O} CO_2 + H_2O$$

$$CH_3SH \xrightarrow{-2H} \tfrac{1}{2}(CH_3)_2S_2$$

Similar reactions may occur in the rumen, despite the anaerobic environment. The proposed reaction scheme shows oxidative deamination (1) preceding demethiolation (2), a conclusion suggested by the fact that some of the bacterial cultures partially decomposed methionine without demethiolation occurring. Oxidation of methyl mercaptan (thiomethane) to dimethyl disulphide was accomplished by a few of the cultures. Other volatile sulphur-containing products did not occur. According to Ruiz-Herrera and Starkey, in some bacteria the enzyme system responsible for demethiolation (2) is inducible.

In view of differences that exist between the sulphur-containing amino acids in rates of deamination *in vitro*, the present experiments were performed to compare the effects of intraruminal infusions of equivalent amounts of sulphur as cysteine, cystine, or methionine on the concentration of sulphides in the ruminal liquor; the possibility of adaption to methionine by the ruminal microorganisms was also examined. An experiment was also performed to determine whether both CH_3SH and H_2S concentrations in rumen liquor were elevated by intraruminal infusions of methionine.

A method for the sampling of rumen fluid and for the collection and differentiation of the volatile sulphides is presented.

II. Materials and Methods

(a) *Diets*

The basal ration used for all experiments was a mixture of 60% oaten hulls, 36% lucerne chaff, and 4% minerals. It contained 0·1% sulphur.

(b) *Sheep*

Rumen-fistulated Merino wethers weighing between 30 and 45 kg were used. The animals were confined to metabolism cages during each experiment.

(c) *Experimental Plans*

(i) *First Experiment.*—On days 1, 3, and 5, 3·0 g sulphur either as L-cysteine or L-cystine or DL-methionine was infused intraruminally as a single dose to sheep 186 immediately after presentation of the ration. In each instance feed intake was restricted to 200 g daily and rumen fluid samples were taken at intervals over the 24 hr following the infusion, in order to follow the changes in concentration of sulphides produced from the infused amino acid. No prior infusions of methionine or cyst(e)ine were given. The ration was eaten within 2 hr of presentation on each occasion.

(ii) *Second Experiment.*—For 20 successive days four sheep (186, 41, 62, and 67) were each given DL-methionine (≡ 1·0 g sulphur) per rumen immediately after feeding. On the 21st day, DL-methionine (≡ 3·0 g sulphur) was infused as a single dose after feeding and samples of rumen fluid were taken at intervals over the following 24 hr to examine the effect of possible adaptation to methionine by the ruminal microflora. The sheep ate the daily ration (800 g) within 12 hr of presentation.

(iii) *Third Experiment.*—Four sheep (18, 22, 20, and 32) were used in a crossover design experiment. No prior adaptation to methionine infusion was given. Sheep 18 and 22 received DL-methionine (≡ 2·0 g sulphur) as a single dose and sheep 20 and 32 received a control infusion of water on the first day. On the third day the treatments were reversed. The infusions were made 2 hr after giving the daily ration (800 g). The rations were consumed within 12 hr of presentation. The concentrations of chromium, CH_3SH, and H_2S were determined in samples of rumen fluid obtained before and up to 12 hr after the infusions were made.

(d) *Infusion of Solutions*

The sulphur-containing amino acids were dissolved, or suspended in water (400 ml) and infused as a single dose into the rumen via the cannula. In the third experiment the infusion solution also contained a soluble marker.

(e) *Determination of Rumen Volume and Dilution Rate*

The chromium–ethylenediaminetetraacetic acid (Cr–EDTA) complex was prepared basically as described by Binnerts, Vant Klooster, and Frens (1968). The chromium concentration in strained rumen fluid was determined by atomic absorption spectroscopy. The procedures described by Warner and Stacey (1968) were used to estimate the rumen volume and half-life of marker in the rumen.

(f) *Sampling Procedure*

Samples of rumen liquor were obtained using a permanent tube extending through the cannula bung into the rumen contents. A tared 10-ml glass syringe, fitted with a three-way tap, was used to withdraw the fluid. The first two 10-ml samples were discarded and the next sealed within the syringe preparatory to weighing and analysis of sulphides. At normal ruminal pH sulphide is present in the gas space, hence this method of sampling minimized losses of sulphide from the rumen by diffusion, as well as minimizing oxidation of sulphides in the samples.

(g) *Apparatus for Collection of Volatile Sulphides*

Each distillation unit consisted of a sample tube, gas-washing tube, and collecting tube in series. The sample tube had a stopper through which passed nitrogen gas inlet and outlet lines and a sample-inlet needle. The carrier gas inlet extended to the bottom of the tube. The outlet

gas line passed into the sealed washing tube and then into the open collecting tube which contained the absorbing solution. Prior to use, 10 ml of 5N HCl was put in the sample tube and oxygen flushed from the system with nitrogen gas.

The sample was injected into the sample tube and the tap on the inlet needle closed. A slow stream of nitrogen was bubbled through the tubes for 20–25 min and the sulphides evolved passed through the contents of the washing tube and into the absorbing solution. The emptied syringe was reweighed to determine the weight of samples delivered.

(*h*) *Separation and Analysis of Sulphides*

H_2S and CH_3SH can be separated by their differential solubility in acidic $CdCl_2$ solution (Shaw 1940). In the present experiment H_2S, but not CH_3SH, was precipitated when passed through 25 ml of an aqueous solution (pH 3·2) containing 20 g $CdCl_2$ and 0·4 ml 5N HCl per litre.

CH_3SH can be collected in alkali, as the mercaptide $NaCH_3S$, therefore the possibility of employing the rapid titrimetric method used for H_2S analysis (Bird and Fountain 1970) for CH_3SH analysis was examined. Duplicate 1-ml aliquots of a solution of CH_3SH in NaOH were added either directly to 10 ml of 1N NaOH and titrated with mercuric acetate (using dithizone indicator), or treated with 5N HCl prior to collection and titration, as follows:

Treatment	Gas washing agent	Sulphur content (μg/ml)
Direct titration	—	65
5N HCl, distillation	—	61
5N HCl, distillation	acid, 2% $CdCl_2$	63

The acid released CH_3SH and any H_2S present in the solution. The direct titration includes both H_2S and CH_3SH sulphur. Most of the CH_3SH in the sample was recovered after passage through the washing agent. It was assumed that the titration reactions were

$$2\ CH_3SH + Hg^{2+} \rightarrow (CH_3S)_2Hg + 2H^+$$

$$H_2S + Hg^{2+} \rightarrow HgS + 2H^+$$

This assumption was tested by determination of the total sulphur and thiomethane sulphur content of $Hg(CH_3S)_2$ produced by trapping CH_3SH in Hg^{2+} solution. Total sulphur determination, following oxidation of the samples and subsequently reducing the sulphate so formed to H_2S (see Bird and Fountain 1970), and thiomethane sulphur determination (after treating with 5N HCl as previously described) differed by 3%, thus validating the method. The thiomethane titration endpoint is satisfactory only in the range 1–50 μg sulphur, giving a range of 0·1–5 μg sulphur per millilitre of rumen fluid.

The first step in determining the concentration of H_2S and CH_3SH in samples of rumen liquor is to collect CH_3SH and H_2S together in NaOH solution and the second is to collect only CH_3SH in NaOH solution. The H_2S concentration is obtained after regarding the CH_3SH titration volume as the blank. The CH_3SH concentration is obtained after subtraction of the usual titration blank, and consideration that Hg^{2+} precipitates twice as much sulphur from CH_3SH than from H_2S. The volatile $(CH_3)_2S$ and $(CH_3)_2S_2$ are insoluble in NaOH and are not precipitated by Hg^{2+} ions. Consequently these compounds are not included with the sulphides determined in these experiments.

III. Results

The results from the first experiment are shown in Figure 1. The very low concentrations of sulphides resulting from the infusion of methionine contrast with those from cyst(e)ine.

The results of the second experiment are shown in Figure 2. Comparing the concentrations of sulphide found in the rumen fluid here with those in experiment 1

(Fig. 1) it is evident that the adaptation period has not substantially altered the response to methionine infusions. The ruminal concentration of sulphides observed in this experiment could, in fact, arise largely from ingestion of the rations alone (see Fig. 4, expt. 3).

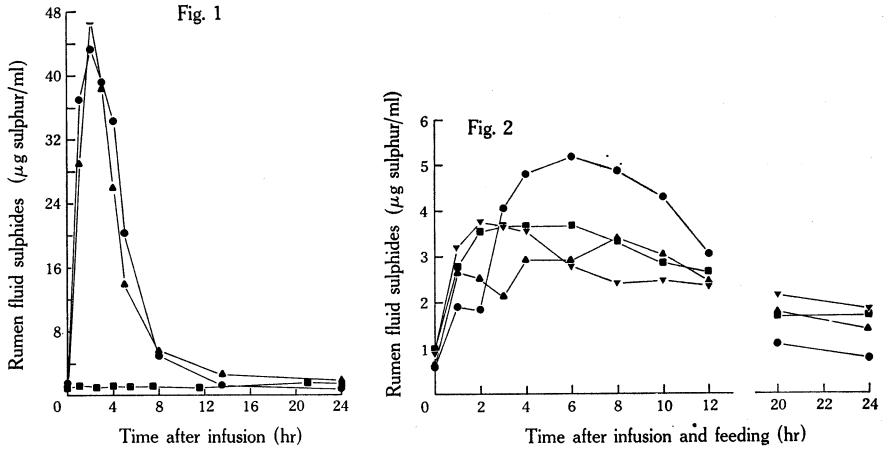
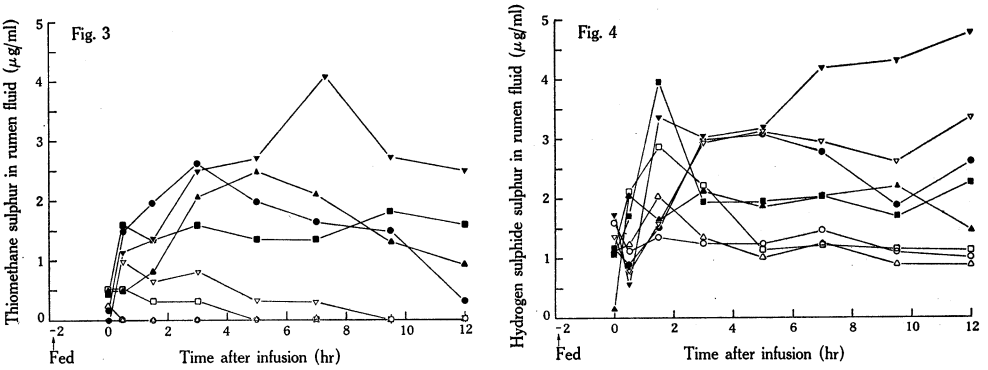


Fig. 1.—Effect of a single intraruminal infusion of 3·0 g L-cysteine sulphur (●), 3·0 g L-cystine sulphur (▲), or 3·0 g DL-methionine sulphur (■) on the concentration of sulphides in the rumen liquor of sheep No. 186.

Fig. 2.—Effect of a single intraruminal infusion of 3·0 g DL-methionine sulphur on the concentration of sulphides in the rumen after 20 days adaptation to methionine infusions. ● Sheep No. 186. ▲ Sheep No. 41. ■ Sheep No. 62. ▲ Sheep No. 67.

The relationship between time of sampling and the concentration of CH_3SH and of H_2S in the rumen liquor of the various sheep (expt. 3) are shown in Figures 3



Figs. 3 and 4.—Effect of single intraruminal infusions of 2·0 g DL-methionine sulphur on the concentration of thiomethane (Fig. 3) and hydrogen sulphide (Fig. 4) in rumen liquor (solid symbols) compared with basal concentrations derived from the ration (open symbols). ●, ○ Sheep 1. ■, □ Sheep 2. ▲, △ Sheep 3. ▼, ▽ Sheep 4.

and 4, respectively. In both cases the sulphide levels due to the basal diet are shown. There were no significant differences in the concentration of either CH_3SH or H_2S

found in rumen fluid between sampling times 0·5–12 hr post-infusion. The individual observation for CH_3SH and for H_2S, respectively, were therefore pooled for statistical purposes (see Table 1). The concentrations of CH_3SH following methionine infusions were significantly elevated above those for the basal diet ($P<0·01$), as also were the concentrations of H_2S ($P<0·05$). Between-sheep differences occurred only in the concentration of H_2S in rumen fluid ($P<0·05$).

TABLE 1

EFFECT OF INTRARUMINAL INFUSION OF METHIONINE ON THE MEAN CONCENTRATIONS OF HYDROGEN SULPHIDE AND THIOMETHANE IN THE RUMEN LIQUOR COMPARED WITH BASAL VALUES

DL-Methionine equivalent to 2·0 g sulphur infused. Values given are means of seven observations taken from 0·5 to 12 hr after infusion, and are expressed as μg sulphur per millilitre of rumen liquor. Within each column significant statistical differences due to treatment are indicated as follows: * $P<0·05$, ** $P<0·01$, *** $P<0·001$

	Sheep 18		Sheep 22		Sheep 20		Sheep 32		Mean	
	H_2S	CH_3SH	H_2S	CH_3SH	H_2S	CH_3SH	H_2S	CH_3SH	H_2S	CH_3SH
Water infusion	1·23*	0·00***	1·70	0·16***	1·25*	0·03**	2·72	0·45**	1·72*	0·16**
Methionine infusion	2·26*	1·64***	2·22	1·52***	1·92*	1·45**	3·12	2·44**	2·38*	1·76**

The measured rumen volumes and half-life of the marker in the rumen in the third experiment were 1283, 3810, 2470, and 2210 ml and 7·0, 10·9, 12·1, and 19·0 hr, respectively, for sheep 18, 22, 20, and 32. It may be estimated, therefore, that if none of the infused methionine was absorbed from or degraded in the rumen, and that it flowed from the rumen at the same rate as water, then at 12 hr post-infusion the percentage methionine remaining in the rumen would be 14·0, 45·0, 50·5, and 68·5 for sheep 18, 22, 20, and 32, respectively.

IV. DISCUSSION

The data show that intraruminal infusions of DL-methionine do not result in high concentrations of sulphides in the rumen, even after a period of adaptation (see Figs. 1 and 2). Bosman (1965) showed that more sulphide was formed *in vitro* from cyst(e)ine or glutathione than from either sulphate or methionine. Halverson, Williams, and Paulson (1968) provided evidence that the rate of sulphide production from methionine was about one-third of that from sulphate, which was in turn about one-half of that from an equivalent amount of cystine sulphur. Further *in vitro* work of Nader and Walker (1970) broadly supports those results for cysteine and methionine. In addition, cystine fed to sheep has been shown to produce higher concentrations of sulphide in the rumen than sulphate (Bird and Hume 1971).

Cystine, despite its insolubility, was not degraded at a rate slower than was cysteine, although Lewis and Emery (1962) showed that cystine was deaminated more slowly *in vitro* than was cysteine.

The increased production of H_2S resulting from methionine infusions is surprising, since H_2S has not been reported as a product of methionine degradation

by microorganisms. Nader and Walker (1970) assumed that the volatile sulphide arising from the anaerobic decomposition of methionine was all H_2S. The sulphides evolved in that experiment were not differentiated but collected in a 10% H_2O_2–1·5% NH_3 solution and estimated as sulphate. In the present experiment there was a parallelism between CH_3SH and H_2S concentrations (Figs. 3 and 4). Thus, the regression H_2S concentration (y, μg S/ml) on CH_3SH concentration (x, μg S/ml) for data taken from the methionine treatments 0·5–12 hr post-infusion was $y = 1·08 + 0·738x$ ($r = 0·58$, $P < 0·01$) suggesting that CH_3SH may be demethylated to form H_2S.

Although the increases in ruminal concentrations of CH_3SH due to methionine infusions were highly significant (expt. 3), the magnitude of the mean increment above those of the basal diet for the period 0·5–12 hr after infusion was only 1·60 μg S/ml. The mean increment in H_2S concentration was 0·66 μg S/ml. The decline in CH_3SH concentrations with time (Fig. 3) may be due, in part, to a loss of substrate methionine in the flow of fluid from the rumen. The sheep (No. 32) having the slowest dilution rate had maintained the highest concentration of CH_3SH, and conversely, the sheep (No. 18) with the greatest dilution rate had the lowest concentration of CH_3SH. In a system where the rate of substrate decomposition is slow (compare methionine with cyst(e)ine in Fig. 1), the rate of flow of substrate from the rumen will primarily determine the extent of degradation. The present data suggest that significant amounts of methionine leave the rumen undegraded. There are, however, several additional and largely unknown factors which require consideration before such a conclusion is justified:

(1) The absorption of methionine from the rumen.

(2) The differential rates of CH_3SH and H_2S absorption from the rumen.

(3) The rate of CH_3SH utilization by ruminal microorganisms.

(4) The production of more oxidized forms of sulphide in the rumen.

(5) The occurrence of deamination without demethiolation.

(6) The rate of methionine uptake by the ruminal microorganisms.

Kurilov *et al.* (1969) claimed that [^{35}S]methionine was absorbed and retained in the rumen wall and, depending upon relative concentration gradients, was subsequently either absorbed into the blood or returned to the rumen. If such a mechanism operates the animal could derive portion of its tissue methionine requirements by absorption from the rumen. Conversely, this gain could be offset by a reduction in the proportion of ingested methionine that passes undegraded to the abomasum.

Clearly, if the rates of absorption or utilization or both of CH_3SH exceed that of H_2S then a comparison of ruminal sulphide concentrations derived from cyst(e)ine and methionine (Fig. 1) will not truly indicate relative rates of substrate degradation. There is no *in vivo* evidence concerning CH_3SH absorption but the *in vitro* work of Halverson, Williams, and Paulson (1968) indicates that the rate of production of sulphide from methionine is considerably slower than from cystine. Either *O*-succinylhomoserine or *O*-acetylhomoserine may be conjugated with CH_3SH to form methionine in bacteria (Flavin and Slaughter 1967) or *Neurospora* (Moore, Thompson, and Smith

1969). Zikakis and Salsbury (1969) claimed, on the basis that in their *in vitro* system CH_3SH concentrations declined after reaching a peak, that CH_3SH was utilized by the ruminal bacteria. Since CH_3SH may be oxidized to $(CH_3)_2S_2$, and this product was not accounted for, their conclusion is equivocal. However, if some species preferentially incorporate CH_3SH, then the accuracy of Walker and Nader's (1968) method for estimating microbial synthesis from H_2S incorporation could be adversely affected.

If the CH_3SH derived from methionine is oxidized to $(CH_3)_2S_2$ or to $(CH_3)_2S$, these products would not have been recorded in the present experiment, nor in that of Halverson, Williams, and Paulson (1968), then the estimate of methionine decomposition would be too low. It does not seem likely that these oxidation reactions would occur rapidly in the reduced atmosphere of the rumen, although dimethyl sulphide has been found in the rumen fluid and milk of cows ingesting alfalfa (Dunham *et al.* 1968*a*). Dimethyl sulphide may also be found in grass and corn silage (Morgan and Pereira 1962). A possible precursor is dimethylthetin, or related thetin compounds, which occur in plants (Zikakis and Salsbury 1969). Dunham *et al.* (1968*b*) have, however, also shown that oral doses of methionine produce small but significant increases in the concentration of dimethyl sulphide in milk.

Possibly the most serious disadvantage of using sulphide production as an index of methionine decomposition is the possibility that deamination may occur but not demethiolation. According to the reaction scheme proposed by Segal and Starkey (1969), α-keto-γ-methylmercaptobutyrate would accumulate under these circumstances. On the other hand, H_2S production in the rumen should be a sensitive index of cyst(e)ine decomposition.

An explanation for the comparatively slow rate of methionine deamination, compared with that of cystine (Lewis and Emery 1962), may be that ruminal bacteria, like *Escherichia coli* (Roberts *et al.* 1955), might absorb methionine passively, but cystine actively. Bacterial deaminases being intracellular enzymes would require absorption to occur prior to ammonia release.

Finally, it must be recognized that methionine *per se* may be incorporated by the bacteria, or more probably by the protozoa which ingest preformed amino acids (Coleman 1967). Landis (1963) estimated that from 75–83% of dietary methionine could be so incorporated; the more recent estimate of Nader and Walker (1970) is a maximum of 11% direct incorporation by mixed ruminal microorganisms. It is possible that continued feeding of methionine may lead to an increased number of protozoa in the rumen and thereby increase the proportion of methionine that is directly utilized, but the data from Williams and Moir (1951) tend to negate this conjecture.

Either, or all, of the abovementioned factors might account for the apparent failure, in terms of wool growth stimulus, of dietary methionine supplements to reach the intestine unmodified. Conversely, a response to dietary methionine might occur under circumstances when the outflow of fluid from the rumen is rapid.

V. Acknowledgments

This work was supported by the Australian Meat Research Committee and Sulphur Institute funds. Criticism of the work by Professor R. J. Moir is acknowledged.

VI. References

Barker, H. A. (1961).—In "The Bacteria". (Eds. I. C. Gunsalus and R. Y. Stanier.) Vol. 2. (Academic Press: New York.)

Binnerts, W. J., Vant Klooster, A. Th., and Frens, A. M. (1968).—*Vet. Rec.* **82**, 470.

Bird, P. R., and Fountain, R. D. (1970).—*Analyst, Lond.* **95**, 98.

Bird, P. R., and Hume, I. D. (1971).—*Aust. J. agric. Res.* **22**, 443.

Bosman, M. S. (1965).—*Jaarb. Inst. Biol. scheik. Onderz. LandbGewars.* 97.

Chalmers, M. E., and Synge, R. L. M. (1954).—*Adv. Prot. Chem.* **9**, 93.

Clarke, E. M., Ellinger, G. M., and Phillipson, A. T. (1966).—*Proc. R. Soc.* B **166**, 63.

Colebrook, W. F., Ferguson, K. A., Hemsley, J. A., Hogan, J. P., Reis, P. J., and Weston, R. H. (1968).—*Proc. Aust. Soc. Anim. Prod.* **7**, 397.

Coleman, G. S. (1967).—*J. gen. Microbiol.* **47**, 433.

Desnuelle, P., Wookey, E., and Fromageot, C. (1940).—*Enzymologia* **8**, 225.

Downes, A. M., Reis, P. J., Sharry, L. F., and Tunks, D. A. (1970).—*Br. J. Nutr.* **24**, 1083.

Dunham, J. R., Ward, G., Bassette, R., and Reddy, M. C. (1968*a*).—*J. Dairy Sci.* **51**, 44.

Dunham, J. R., Ward, G., Bassette, R., and Reddy, M. C. (1968*b*).—*J. Dairy Sci.* **51**, 199.

Flavin, M., and Slaughter, C. (1967).—*Biochim. Biophys. Acta* **132**, 400.

Fromageot, C., and Kiun, T. P. (1941).—*Bull. Soc. Chim. Biol.* **23**, 1471.

Graceva, L. V. (1969).—*Ovcevodstvo* No. 2. p. 34. [cf. *Nutr. Abstr.* **39**, No. 8185.]

Halverson, A. W., Williams, G. D., and Paulson, G. D. (1968).—*J. Nutr.* **95**, 363.

Kurilov, N. V., Ptashkin, A. A., Potashevskü, N. D., and Solovev, A. M. (1969).—*Selskokhoz. Biol.* **4**, 77. [*Biol. Abstr.* 108287 (1970).]

Landis, J. (1963).—*Z. Tierphysiol. Tierernähr. Futtermittelk* **18**, 357.

Lewis, D. (1955).—*Br. J. Nutr.* **9**, 215.

Lewis, T. R., and Emery, R. S. (1962).—*J. Dairy Sci.* **45**, 765.

Marston, H. R. (1932).—*Aust. J. exp. Biol. med. Sci.* **9**, 235.

Marston, H. R. (1935).—*J. agric. Sci., Camb.* **25**, 113.

Mitsuhashi, S. (1949).—*Jap. J. exp. Med.* **20**, 211.

Moore, D. P., Thompson, J. F., and Smith, I. K. (1969).—*Biochim. Biophys. Acta* **184**, 124.

Morgan, M. E., and Pereira, R. L. (1962).—*J. Dairy Sci.* **45**, 467.

Nader, C. J., and Walker, D. J. (1970).—*Appl. Microbiol.* **20**, 677.

Reis, P. J., and Schinckel, P. G. (1963).—*Aust. J. biol. Sci.* **16**, 218.

Reis, P. J., Tunks, D. A., Williams, O. B., and Williams, A. J. (1967).—*Aust. J. biol. Sci.* **20**, 153.

Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. J., and Britten, R. J. (1955).—In "Studies of Biosynthesis in *Escherichia coli*". [Publ. Carnegie Inst. Wash. No. 607.]

Ruiz-Herrera, J., and Starkey, R. L. (1970).—*J. Bact.* **104**, 1286.

Salsbury, R. L., Marvil, D. K., Woodmansee, C. W., and Haenlein, G. F. W. (1971).—*J. Dairy Sci.* **54**, 390.

Segal, W., and Starkey, R. L. (1969).—*J. Bact.* **98**, 908.

Shaw, J. A. (1940).—*Analyt. Chem.* **12**, 668.

Starks, P. B., Hale, W. H., Garrigus, U. S., Forbes, R. M., and James, M. F. (1954).—*J. Anim. Sci.* **13**, 249.

Walker, D. J., and Nader, C. J. (1968).—*Appl. Microbiol.* **16**, 1124.

Warner, A. C., and Stacey, B. D. (1968).—*Br. J. Nutr.* **22**, 369.

Williams, V. J., and Moir, R. J. (1951).—*Aust. J. scient. Res.* B **4**, 377.

Zikakis, J. P., and Salsbury, R. L. (1969).—*J. Dairy Sci.* **52**, 2014.