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The Effect of Diet and of Methionine Loading on Activity of Enzymes in the Transulfuration Pathway in Sheep

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Abstract

In three experiments, activity of hepatic enzymes associated with metabolism of methionine through the transulfuration pathway were studied with respect to possible effects of diet and methionine infusion per abomasum.

In experiment 1 no differences in methionine adenosyltransferase (MAT) or cystathionine γ-lyase (CGL) were detected between lucerne and wheaten straw diets, or between effects of fasting for 48 and 96 h after feeding lucerne chaff as opposed to fasting after feeding wheaten straw. Fasting for 96 h resulted in a trend toward increasing CGL and MAT specific activities on both diets.

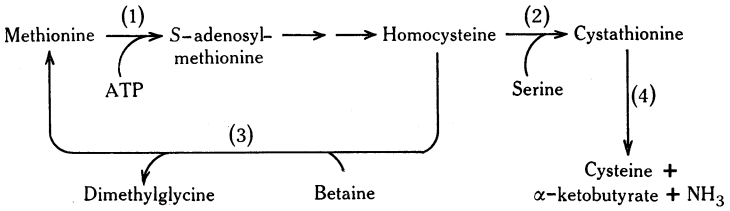
In experiment 2 MAT was depressed significantly by infusion of methionine at 1·4 g/day and to a greater extent by infusion at 4·2 g/day, whilst CGL was not significantly affected.

In experiment 3 MAT specific activity decreased significantly in response to both levels of methionine supplementation.

Betaine–homocysteine methyltransferase activity was increased by methionine infusion. CGL decreased in all treatments but there was a larger decrease in those animals receiving methionine infusion. No significant changes were observed in relation to other enzymes examined which included cystathionine β-synthase and threonine dehydratase. These observations are consistent with the hypothesis that in sheep the increase in methionine in blood plasma which occurs when methionine is absorbed in increased amounts may be due to reduced entry into the transulfuration pathway because of a repression of MAT activity.

Introduction

Methionine is an essential amino acid in the diet of mammals. Cysteine is generally considered to be dispensable, insofar as it can be synthesized from methionine via the transulfuration pathway which may be summarized thus:



Cysteine cannot completely replace methionine, but the amount of methionine required in the diet can be substantially reduced if cysteine is also supplied (Womack *et al.* 1937; Womack and Rose 1941; Rose *et al.* 1955). Calculations (Egan and Walker 1975) and supplementation studies (Schelling *et al.* 1973; Fennessy 1976) indicate that methionine is frequently an amino acid limiting for efficient *N*-retention

in sheep. Because of this, and because of the high cystine content of wool, the metabolism of the sulfur-containing amino acids is of special importance in the sheep. Although transulfuration occurs in the skin (Downes *et al.* 1964) and enzymes of the transulfuration pathway are present in muscle and most other tissues examined in the sheep (Radcliffe and Egan 1974), the present study has been concentrated on hepatic enzymes of the transulfuration pathway since methionine infusion per abomasum results in a greater elevation of methionine concentration in the portal blood than in the peripheral circulation (W. A. Hoey, personal communication), thus exposing the liver to a greater methionine load.

In the sheep when the normal amount of methionine supplied by the digesta is supplemented by abomasal or duodenal infusion, there appears to be a limit to the rate of its conversion to cysteine, as indicated by greatly increased methionine concentration in the plasma (Reis *et al.* 1973; Egan, Rogers and Fennessy, unpublished data). In the rat the activities of some enzymes in the transulfuration pathway are affected by a number of factors, including protein and methionine content of the diet, hormones and age (Finkelstein 1970). We have investigated the activities of the hepatic enzymes involved in methionine transulfuration and the changes which occur in response to dietary changes and to additional methionine provided by abomasal infusion. The enzymes examined were: (1) methionine adenosyltransferase (EC 2.5.1.6), (2) cystathionine β-synthase (EC 4.2.1.22), (3) betaine–homocysteine methyltransferase (EC 2.1.1.5), (4) cystathionine γ-lyase (EC 4.4.1.1) and threonine dehydratase (EC 4.2.1.16), an enzyme associated with the catabolism of serine (an amino acid which may influence methionine–cysteine conversion) and threonine.

Materials and Methods

Animals, Diets and Experimental Procedures

Experiment 1

Four mature Suffolk–Merino crossbred sheep (one ewe and three wethers) were pen-fed *ad libitum* once daily at 0930 h. Diets of lucerne chaff or wheaten straw were chosen from materials examined by Egan (1974) and Egan *et al.* (1975) and shown to yield an estimated 8·0% (wheaten straw) and 23·4% (lucerne chaff) of the total digestible energy as crude protein digested in the intestines.

Diets were supplemented with a complete mineral mix (12 g/day; Moir and Harris 1962) and vitamin A and D mix (0·5 g/day; Apac, Nicholas Products). Water was freely available at all times.

The experiment was carried out on a cross-over design. Two animals were fed on each diet for 21 days; intakes were measured on the last 7 days. The initial liver biopsy sample was taken on day 22. On the day of the biopsy the animals were not fed until after the operation at about 1000 h. After sampling, the animals remained on the same diet for a further 5 days, and then were fasted for 96 h. Liver biopsy samples were taken at 48 and 96 h after commencement of the fast. Diets for the two groups of sheep were then reversed and offered for 21 days before liver samples were taken. In this period the sheep remained on the same diets after biopsy for a further 8 days before being fasted for 96 h with liver biopsies performed at 48 and 96 h.

Experiment 2

Three 8-month-old Dorset Horn × Merino wethers (Nos 663, 748 and 761) weighing 25–30 kg were prepared with abomasal cannulae. Each was fed a mixed wheaten hay diet *ad libitum*, supplemented with mineral mix and vitamin A and D mix as in experiment 1. Residues from the preceding day were removed at 0900 h and a fresh allowance of feed presented. Water was freely available at all times.

Each sheep received, in successive periods, abomasal infusions of 250 ml/day containing 0, 1·4 or 4·2 g L-methionine (Tanabe Seiyaku Co., Osaka, Japan) in distilled water with pH adjusted to 3·5 with HCl. One sheep (663) also received 8·4 g L-methionine/day in an additional period. Infusates were pumped at a steady rate over 24 h/day. Experimental periods were of 28 days. Daily voluntary intake of feed was measured throughout, and a liver sample was taken by biopsy for enzyme assays on day 19. Jugular blood samples were taken on days 3 and 17 of each period at 1430 h and the plasma deproteinized with an equal volume of 10% TCA. Methionine and cysteine concentrations were determined using a Technicon amino acid analyser involving a 21-h separation.

Experiment 3

Twelve 8-month-old Dorset Horn × Merino wethers weighing 20–26 kg were prepared with abomasal cannulae. A large (approximately 2 g) 'pinch' biopsy sample of liver was taken during the surgical implantation of abomasal cannulae to establish pretreatment enzyme levels. Animals were fed a mixed wheaten hay diet (1·35% N) with supplements as in experiment 2.

The sheep were divided randomly into three groups of four animals. Each group received abomasal infusions of 280 ml distilled water/day containing 0, 0·12 or 0·36 g methionine per kilogram$^{0.75}$ live weight per day (equivalent to 0, 1·4 or 4·2 g/day respectively in a 26-kg sheep). After a prefeeding period of 14 days, methionine was infused for 32 days. The animals were then slaughtered and tissue samples collected for assay of enzymic activity and tissue free-amino acid levels. Jugular blood samples were taken at weekly intervals as in experiment 2.

Liver Biopsies

Liver samples in experiments 1 and 2 were taken by biopsy procedure using a modification of the technique described by Phillipo (1973) under local anaesthesia (paravertebral block using 2% Lignocaine with adrenaline). The animal was placed on its left side and a 1-cm incision made through the skin to permit insertion of the endoscope (Phillips, Aust.) through the abdominal wall, at a site 3 cm behind the last rib in a mid-flank position. The abdomen was then inflated with CO_2 through the outer jacket of the endoscope, and the endoscope was manipulated to bring the liver into view. A second incision was then made in the 10th intercostal space and a trochar and cannula (Dick 1952) inserted vertically into the abdominal cavity and manipulated until the cannula was in contact with the liver surface. A 20-ml syringe was attached to the cannula, suction supplied, and the cannula pushed through the lobe of the liver. The liver sample was withdrawn and placed in ice-cold 0·9% KCl containing 0·1 mM Na_2EDTA. The wet weight of the liver sample obtained was usually in the range 200–500 mg.

The endoscope permitted effective placement in the peripheral areas of the lobe and allowed the operator to avoid previously sampled regions. In all cases little bleeding occurred during observation for some 60 s after withdrawal of the cannula.

Sample Preparation and Enzymic Assays

Tissue samples were homogenized in 3 volumes ice-cold 0·9% KCl (containing 0·1 mM Na_2EDTA) as described previously (Radcliffe and Egan 1974). Cystathionine γ-lyase (CGL) and, in experiments 1 and 2, methionine adenosyltransferase (MAT) were assayed as previously described (Radcliffe and Egan 1974). In experiment 3, MAT was assayed by measurement of the *S*-adenosyl methionine formed using a Beckman 119 amino acid analyser with a short column of PA-35 resin (12·5 by 0·9 cm) according to the method of Gaull *et al.* (1969). Cystathionine β-synthase was assayed by the method of Gaull *et al.* (1969) with the exception that the preliminary chromatography on Dowex 50 was deleted since it was found that cystathionine and homocysteine could be sufficiently resolved by using a column of PA-28 resin (8 by 0·9 cm) and 0·2 M sodium citrate buffer at pH 3·08.

Betaine–homocysteine methyltransferase was assayed by the method of Finkelstein and Mudd (1967). This method depends on the formation of [*methyl*-^{14}C]methionine from [*methyl*-^{14}C]betaine and the separation of substrate and product by short-column ion-exchange chromatography. The incubation was carried out in 0·05 M tricine buffer (pH 8) containing 1 mM dithiothreitol.

Threonine dehydratase was activated and assayed according to the method of Doonan *et al.* (1974).

Nitroprusside–cyanide reagent was used as a qualitative test for the presence of disulphide compounds in urine (Dawson *et al.* 1969). Protein was determined by the Biuret method (Dawson *et al.* 1969) with bovine serum albumin as standard. Data were analysed by analysis of variance.

Results

Experiment 1

The specific activities of hepatic MAT and CGL in the sheep in experiment 1 are shown in Table 1. MAT and CGL activities were not significantly different for the two diets. Both showed a trend to be reduced after 48 h fast and to be greater at 96 h of fasting than at 48 h of fasting.

Table 1. Hepatic methionine adenosyltransferase and cystathionine γ-lyase activities from sheep fed wheaten straw or lucerne chaff

Values given are means of four sheep

Feeding regime	MAT (units/mg protein ± s.e.m.)[A]		CGL (units/mg protein ± s.e.m.)[B]	
	Lucerne	Wheaten straw	Lucerne	Wheaten straw
Fed	67·0 ± 13·8	50·7 ± 21·7	4·2 ± 1·1	4·3 ± 1·2
Fasted 48 h	26·3 ± 6·8	38·3 ± 5·2	4·0 ± 2·0	4·0 ± 1·0
Fasted 96 h	88·0 ± 31·4	162 ± 88	6·3 ± 2·1	6·3 ± 1·9

[A] Nanomoles of *S*-adenosyl methionine formed per milligram of protein per 30 min.
[B] Nanomoles of cysteine formed per milligram of protein per 30 min.

Experiment 2

Plasma methionine and cysteine concentrations in sheep receiving abomasal supplements of L-methionine are given in Table 2. Plasma methionine levels had increased markedly and highly significantly ($P < 0·001$) by day 17 of the infusion period when the animals received 4·2 g methionine/day. Cysteine concentration was also slightly and significantly higher ($P < 0·01$).

Table 2. Experiment 2: concentrations of cysteine and methionine in the plasma of sheep fed wheaten hay and receiving abomasal supplements of L-methionine

Infusion level (g/day) (mmol/day)		Plasma concentration (μmol/100 ml) in: Sheep 663		Sheep 748		Sheep 761	
		Day 3	Day 17	Day 3	Day 17	Day 3	Day 17
0	½Cys	2·4	2·5	2·8	1·8	1·5	1·7
	Met	1·8	2·2	2·2	1·9	1·3	1·9
1·4 (9·4)	½Cys	4·5	3·7	4·4	3·8	2·7	2·2
	Met	6·9	4·8	6·2	6·3	2·1	1·0
4·2 (28)	½Cys	4·9	3·0	4·6	4·6	3·8	4·6[A]
	Met	12·3	132·7	93·2	141·3	4·4	159·1[A]
8·4 (56)	½Cys	7·2	5·4	—	—	—	—
	Met	270·0	192·1	—	—	—	—

[A] For sheep 761 these are day-10 values: threonine was included in the infusate from day 8.

The specific activities of MAT and CGL in liver biopsy samples from the sheep in this experiment are given in Table 3. CGL activity was little changed under any of the treatments; MAT activity, however, decreased progressively with increasing

amounts of methionine to one-fourth the control values at 4·2 g methionine/day ($P < 0·01$). At 8·4 g/day, MAT activity was scarcely detectable in the one sheep (No. 663) that was sampled. With sheep Nos 748 and 761 a decline in food intake and other signs of metabolic disturbance occurred at 4·2 g methionine/day, which appeared to indicate methionine toxicity, and no higher dose rate was given.

Table 3. Experiment 2: mean dry matter intake for days 13–18 and hepatic methionine adenosyltransferase and cystathionine γ-lyase activities from sheep fed wheaten hay and receiving abomasal infusions of L-methionine

Infusion level (g/day) (mmol/day)	Sheep No.	Dry matter intake (g/day)	MAT (nmol per mg protein per 30 min)	CGL (nmol per mg protein per 30 min)
0	663	613	142	13·3
	748	672	141 } 191·0±49·8	10·0 } 9·2±2·6
	761	570	291	4·4
1·4 (9·4)	663	642	68	6·6
	748	682	79 } 79·0±6·4	5·7 } 7·7±1·5
	761	383	90	10·7
4·2 (28)	663	632	30	7·9
	748	544	50 } 45·0±7·6	7·3 } 7·8±0·3
	761[A]	399	55	8·2
8·4 (56)	663	385	2·5[B]	8·0

[A] Sheep 761 received 2·1 g threonine in addition to methionine.
[B] MAT activity was at the lowest level discernable by this technique.

Experiment 3

Table 4 shows the specific activities of some hepatic enzymes before and after infusion of methionine for 32 days.

MAT specific activity declined significantly after infusion of methionine at both levels ($P < 0·01$), while those animals receiving the water infusion showed little change. The mean change for animals on the water infusion was $+11·0 \pm 12·6\%$, while that for animals on 0·12 and 0·36 g per $kg^{0·75}$ per day was $-34·5 \pm 18·6$ and $-41·0 \pm 12·5\%$ respectively.

Cystathionine β-synthase specific activity showed no significant changes in response to the methionine supplement. Betaine–homocysteine methyltransferase activity was assayed in post-infusion samples only and was higher in the methionine-infused animals than in the controls ($P < 0·01$). CGL activity decreased in all three treatments, but with a larger mean decrease (70%) in the two methionine treatments ($P < 0·05$) than in the control (32%). Threonine dehydratase activity was low and showed no difference between treatments.

Plasma methionine concentrations are shown in Table 4 for each sheep prior to and after 32 days of receiving the infusions. For those sheep receiving methionine at 0·12 g per $kg^{0·75}$ per day plasma methionine concentrations showed a significant ($P < 0·05$) increase with the infusion and were significantly higher ($P < 0·05$) than for those sheep receiving water infusions. With infusion of 0·36 g per $kg^{0·75}$ per day, the plasma methionine level was further increased, being significantly higher than both the water ($P < 0·001$) infusion and the lower level of methionine infusion ($P < 0·01$). Nitroprusside–cyanide tests for disulphide compounds in urine were negative for all treatments.

Discussion

In sheep MAT and CGL activities in liver are normally of a similar order to those in other tissues. Absorbed methionine passes to the liver in portal blood at concentrations greater than in the peripheral circulation under normal dietary conditions (Wolff *et al.* 1972), and also during abomasal methionine infusion (W. A. Hoey, personal communication). Because of this the assay of the hepatic transulfuration pathway enzymes was considered an important first step in defining the effects of dietary differences on *S*-amino acid metabolism, and in studying the apparent limitation to methionine metabolism as manifested by greatly elevated plasma methionine levels which occurred when methionine was infused at 2–3 times the animal's estimated level of requirements (Reis *et al.* 1973*b*; Egan, Rogers and Fennessy, unpublished data).

Table 4. Experiment 3: specific activities of some hepatic enzymes in sheep fed wheaten hay, before and after abomasal infusion of L-methionine at two levels

Each value is the mean for four sheep ± s.e.m.

	Methionine infusion rate (g per $kg^{0.75}$ per day)		
	0	0·12	0·36
Plasma methionine (μmol/100 ml)			
Pre-infusion	0·5±0·3	0·6±0·1	0·8±0·3
Day 32	0·8±0·27	15·3±4·9	88·3±13·4
Methionine adenosyltransferase (nmol per mg protein per h)			
Pre-infusion	97±20	127±39	176±23
Day 32	100±10	68±9	104±13
% Change	+11±13	−35±19	−41±13
Cystathionine β-synthase (nmol per mg protein per 2 h)			
Pre-infusion	214±49	214±63	206±15
Day 32	268±56	182±18	194±11
Betaine–homocysteine methyltransferase (nmol per mg protein per 20 min)			
Day 32	1·6±0·3	2·5±0·4	4·3±0·7
Cystathionine γ-lyase (nmol per mg protein per 30 min)			
Pre-infusion	16·6±6·1	20·4±8·1	25·4±4·4
Day 32	10·5±3·6	5·5±1·6	6·7±1·6
Threonine dehydratase (μmol per g fresh tissue per 10 min)			
Pre-infusion	1·0±0·5	1·0±0·1	0·8±0·1
Day 32	0·2±0·1	0·4±0·1	0·6±0·1

For experiment 1, at the levels of intake achieved on each diet, 20–40 g of crude protein would reach the duodenum each day on the wheaten straw diet, compared with 180–200 g/day on the lucerne chaff diet [calculated from data of Egan (1974) and Egan *et al.* (1975)]. This would contain about 0·3–0·6 g/day of methionine and 0·1–0·3 g/day of cyst(e)ine for the wheaten straw diet, and 2·5–2·8 g/day of methionine and 0·8–0·9 g/day of cyst(e)ine for the lucerne chaff diet. The wheaten straw diet would be methionine-deficient, but the lucerne chaff diet would adequately meet methionine requirements according to the calculations of Egan and Walker

(1975). Despite these estimated differences in supply of methionine, and in contrast to Finkelstein's (1967) observation in rats, no effects of diet on activities of MAT and CGL were observed in the sheep. Finkelstein (1967) also observed that the effect of fasting on enzyme levels in the rat was dependent upon the protein content of the previous diet (or the protein status of the animal). No such interaction between effects of fasting and effects of diet were found in sheep.

When large amounts of methionine are infused, plasma methionine concentration builds up (e.g. experiments 2 and 3) indicating an incapacity of the animal to accommodate the methionine load in anabolic or catabolic pathways. Reis *et al.* (1973*b*) attributed the inability of the sheep to convert a large influx of methionine to cysteine to an observed reduction in plasma serine concentration, presumably related to reduced intracellular serine availability, and to a postulated decrease in cystathionine β-synthase activity. The observed decrease in plasma serine was not, however, a specific effect of methionine loading since a similar decrease occurred when cystine was infused.

Observations presented herein show that hepatic cystathionine β-synthase activity remained constant during the infusion of 1·4 or 4·2 g methionine/day. An infusion level of about 4·2 g methionine did, however, result in a large increase in plasma free methionine concentration (Table 4). Consequently this increase in plasma free methionine cannot be attributed simply to a metabolic block at the cystathionine β-synthase step in the transulfuration pathway in the liver. It remains possible that cystathionine β-synthase activity may be reduced in other tissues which are significantly more important in transulfuration and that this is the site where restriction of transulfuration takes place. However, if catabolism of methionine were blocked at the cystathionine β-synthase step, it would be reasonable to expect that a build-up of homocystine and homocysteine–cysteine mixed disulfide would occur in plasma and urine in analogy to human homocystinuria [cystathionine β-synthase deficiency (Gerritsen and Waisman 1972)]. In these and other studies on methionine metabolism involving states of methionine toxicity in sheep in this laboratory, the presence of these compounds has never been detected in either plasma amino acid analyses or in nitroprusside–cyanide tests of urine (unpublished observations). Further, Finkelstein *et al.* (1975) reported that in rats *S*-adenosyl methionine acts as an activator of cystathionine β-synthase. On the assumption that intracellular *S*-adenosyl methionine concentration increases in response to methionine administration (Lombardini and Talalay 1971), it appears unlikely that methionine loading would result in a decrease in CTS activity.

Interest in threonine dehydratase was related to its activity in the catabolism of both serine and threonine (Nishimura and Greenberg 1961). If threonine dehydratase affects serine availability at the intracellular level, cystathionine synthesis could be affected even where cystathionine β-synthase is not reduced in activity. Girard-Globa *et al.* (1972) found that methionine increased the activity of hepatic threonine dehydratase in the rat, with the effect that a secondary deficiency of threonine and serine occurred; this was followed by a subsequent decrease in threonine dehydratase to normal levels. Doonan *et al.* (1974) demonstrated in sheep that intraperitoneal injections of protein hydrolysate resulted in an increase in hepatic threonine dehydratase. Activities of this enzyme in experiment 3 reported herein were somewhat lower and showed less variability than those reported by Doonan *et al.* (1974). No increase in threonine dehydratase levels was observed as a result of

methionine infusion in experiment 3. Thus, these results do not support the idea that decreased availability of serine and threonine may occur because of a methionine-induced increase in threonine dehydratase activity.

In experiment 2 reduced hepatic MAT activity was associated with plasma methionine build-up in methionine-supplemented animals. MAT specific activity declined with successive increasing supplements of methionine, an effect suggesting repression of MAT by increasing availability of methionine at the cellular level, though cellular methionine concentrations were not measured. Caboche (1976) has reported that MAT specific activity decreases dramatically when baby hamster kidney cells are grown in media containing methionine at concentrations greater than 1 μM.

For experiment 3, the method of assay for MAT described by Gaull *et al.* (1969) was applied using short-column amino acid analysis to quantitate *S*-adenosyl methionine. Use of this technique avoided the difficulty caused by the high background counts that sometimes occurred using the [^{14}C]ATP assay in experiments 1 and 2. Animals showed a wide range of MAT activities on the basal diet. When allocated to the infusion treatments, each treatment group included animals with relatively low and relatively high MAT activities. For those animals receiving water infusion only, small increases or decreases in MAT activity were observed and were regarded as indications of the order of random variability with time or with such physiological changes which occur with abomasal water infusion. The infusion of methionine resulted in a significant decrease in MAT activity at both treatment levels. On an individual animal basis, those animals with an initial low specific activity remained low in response to methionine infusion, while those that were initially high decreased dramatically.

There was also a small but consistent decrease in CGL activity. Betaine–homocysteine methyltransferase was the only hepatic enzyme assayed that showed increased activity in response to methionine supplementation. Since this enzyme regenerates methionine from homocysteine, both the observed changes are in a direction to contribute to the hypermethioninaemia that occurs in response to methionine loading. Together, the observed decrease in MAT and increase in betaine–homocysteine methyltransferase would also be effective in preventing the accumulation of homocysteine, which, as stated earlier, has not been detected in plasma or urine in this study. Protection against homocysteine accumulation may be of physiological significance since several studies have suggested that homocysteine is a potentially toxic metabolite of methionine, and has been implicated in such diverse conditions as abnormal acceleration of skeletal growth, myointimal hyperplasia (Gerritsen and Waisman 1972; Clopath *et al.* 1975), schizophrenia (Beaton *et al.* 1975), and coronary artery disease (Wilcken and Wilcken 1976).

Decreases in MAT activity with increasing supply of methionine also could, in another sense, have a protective effect, since Farber (1973) has suggested that, at least in the guinea pig, depletion of ATP by excessive *S*-adenosyl methionine synthesis can lead to a breakdown in cellular integrity.

The observations reported herein demonstrate that reduced entry into the transulfuration pathway due to a decrease in hepatic MAT activity is likely to be a causative factor in the increase in plasma methionine levels that occurs when extra methionine is supplied to the sheep. It remains to be seen what, if any, effect increased

methionine levels have on transulfuration pathway enzymes in other tissues, especially skin and wool follicles.

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