Utilisation of methionine by the mammary gland of the lactating goat

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ABSTRACT

The goat milk protein, α-lactalbumin, contains no methionine residues, hence if ^{35}S label is supplied to the gland in the form of labelled methionine only, the appearance of ^{35}S in α-lactalbumin gives a measure of transsulphuration of methionine to cysteine within the gland. Two lactating Saanen goats were surgically modified with catheters implanted in the mammary milk vein, femoral and external pudic mammary artery, and jugular vein to enable infusion of ^{35}S-methionine, into either the whole body (jugular) or directly into the gland, and repetitive blood sampling. Specific radioactivity of methionine in the blood and milk proteins was determined at hourly intervals for 9 hours. Radioactivity of ^{35}S was determined in α-lactalbumin when ^{35}S-methionine was supplied directly to the gland via the mammary artery, but could not be detected when supplied via the jugular vein. Analysis of mammary tissue from the latter experiment did however show the appearance of ^{35}S label in the intracellular cysteine pool which was derived from methionine. Both approaches suggested that transsulphuration within the gland, although small, may be important especially when supply of methionine to the gland is high.

The fractional extraction of plasma methionine by the gland for two goats was 46.4±2.7 % and 31.5±2.3 %, which is 11.5% and 13.5% of the whole body entry rate. The relative plateau methionine specific radioactivities (SRA; dpm/nmol methionine) at 9 hours in blood (612±32; 494±27), milk casein (449±2, 229±13) and individual whey proteins (eg ß-lactoglobulin; 830±50; 320±23), together with the rates of approach to plateau for the SRA of each pool, indicated that casein and whey proteins derive the methionine used in their synthesis from different precursor pools. The rise in casein - and ß-lactoglobulin - bound methionine specific radioactivity indicated half-lives' of 5.9±0.54 and 4.4 ±0.13 hours respectively.

Keywords: transsulphuration; milk protein; net uptake.

INTRODUCTION

The uptake of the sulphur amino acids (methionine and cysteine) by the lactating mammary gland is of significance as they appear to be taken up from the blood in insufficient quantities relative to their output in milk proteins (Clark *et al.* 1978; Davis and Collier 1985). Thus, they may be limiting for synthesis of milk proteins. However, there is increasing awareness that amino acid utilisation and channelling within the gland itself, including utilisation of amino acid from sources other than that supplied directly by the gland's blood supply, has important effects on milk protein output (Bequette *et al.* 1994, Metcalf *et al.* 1994). Recently we reported on the metabolism of the cysteine within the mammary gland and confirmed the low uptake of free cysteine from the blood pool (13% of whole body entry rate) and showed there was little indication of the gland utilising, directly, alternative sources of blood cysteine such as the peptide glutathione (Knutson et al 1994). An alternative supply of cysteine to the gland could be methionine which could be transsulphurated to cysteine either in the liver or within the gland itself. The goat milk protein α-lactalbumin contains no methionine residues, hence if the ^{35}S label is supplied directly to the gland in the form of labelled methionine only, the appearance of ^{35}S in α-lactalbumin indicates transsulphuration of methionine within the gland. The present study was designed to measure the uptake of methionine by the gland from the blood and the rate of its direct utilisation for protein synthesis relative to its use within the gland as cysteine via the transsulphuration pathway.

MATERIALS AND METHODS

Two Saanen goats in late lactation were surgically modified under general anaesthesia as described by Knutson *et al.* (1994) to place indwelling catheters in the jugular vein, caudal superficial epigastric vein (mammary vein), external pudic and saphenous arteries. Blood vessels between the two udder halves were ligated and a transit time ultrasonic flow probe fitted around the external pudic artery to determine blood flow. The goats were housed in metabolism crates , fed *ad libitum* hay and water and 1.5 kg/day of concentrate. The animals were milked twice daily. In experiment one (close infusion experiment), labelled ^{35}S-methionine (NEN research Products, Dupont, USA) and cold carrier (2 μM methionine) in 100 ml sterile saline was infused at 0.4 g/min (2.35μCi/min ^{35}S-methionine) for 3 hours directly into the right hand side mammary artery. In experiment two (whole body experiment) labelled methionine was infused into the jugular vein at 6.2 μCi/min ^{35}S-methionine for 9 hours. Experiment two took place approximately one month after the close infusion. In both experiments blood (approximately 10 ml) was sampled at hourly intervals from the femoral artery and the milk vein, simultaneously, by peristaltic pump over a

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10-20 min period. At the same time milk was collected by hand-milking, assisted using oxytocin administered intravenously (2 injections, 100 mIU at each milking), to ensure complete removal of milk. On completion of the 9-hour infusion mammary gland tissue, from the infused udder half was sampled from 5 sites after the goats were sacrificed by a lethal overdose of sodium pentabarbitone (TechVet, NZ).

All blood samples were collected into plastic tubes containing disodium-EDTA and immediately processed according to procedures described by Lee *et al.*(1995), except for samples of whole blood for subsequent methionine analysis. For the analysis of methionine concentration and specific radioactivity measurements in blood, 1 ml of deionised water was added to 1 ml of blood - to lyse the red cells - together with 0.1 ml of dithiothreitol and 0.05 ml of 2.987 mM nor-leucine internal standard. The complete sample was then centrifuged through a 10,000 MW membrane (Centrisart I, Sartorius, Gottingen, Germany) at 2,550 g for 90 min. Methionine was determined in the centrifuged filtrate after reverse phase separation of its phenylisothiocyanate derivative (Bidlingmeyer *et al.* 1984). Radioactivity of ^{35}S associated with methionine, cystathionine, cysteine and oxidation products were determined by ion-exchange HPLC and counting of ß-radioactivity using an on-line detector (Lee *et al.* 1995). Casein and whey protein concentrations in skim milk were determined by capillary zone electrophoresis (Paterson *et al.* 1995). Radioactivity of ^{35}S-methionine incorporated in individual whey proteins was determined by on-line liquid scintillation counting after separation by HPLC in conjunction with anion exchange chromatography (Mono-Q, Pharmacia, Sweden) (Andrews *et al.* 1985). Total casein was precipitated from skim milk as described by Andrews and Alichanidis (1983) and re-solubilised in 6M urea prior to HPLC and analysis for radioactivity in the casein peaks.

Concentration and radioactivity data were used to calculate specific radioactivity (SRA; DPM/nmole) of methionine in plasma, whole blood, mammary tissue and milk protein. The rise in SRA of methionine in plasma and that bound in casein and ß-lactoglobulin were described by a single exponential function: $y = A + B\,e^{-kt}$, where y is the SRA of methionine at time t (hours), A is the plateau SRA, B is the increase in SRA to plateau and k is the fractional rate constant determined for protein synthesis (per hour; $t_{1/2}$ (half-life) is equal to 0.693/k). Net uptake of methionine by the gland was calculated from arteriovenous concentration differences and blood flow, whilst total uptake by the gland (estimate of protein synthesis) was calculated from the difference in radioactivity of methionine in arterial and venous blood divided by the SRA of methionine in the arterial blood flow past the gland. Irreversible loss rate (ILR), to estimate whole body utilisation of methionine, was calculated as described by Lee *et al.* (1995).

RESULTS AND DISCUSSION

The HPLC distribution of ^{35}S radioactivity in the proteins, ß-lactoglobulin and α-lactalbumin in whey obtained from the milking 2 hours after the start of an infusion

FIGURE 1: Distribution of ^{35}S-radioactivity after separation by ion-exchange of α-lactalbumin, ß-lactoglobulin and various other components in goat whey collected from the right hand side mammary gland after two hours infusion of either ^{35}S-cysteine (————) and ^{35}S-methionine (----------) directly into the gland, or infusion of ^{35}S-methionine into the jugular vein (— — — —).

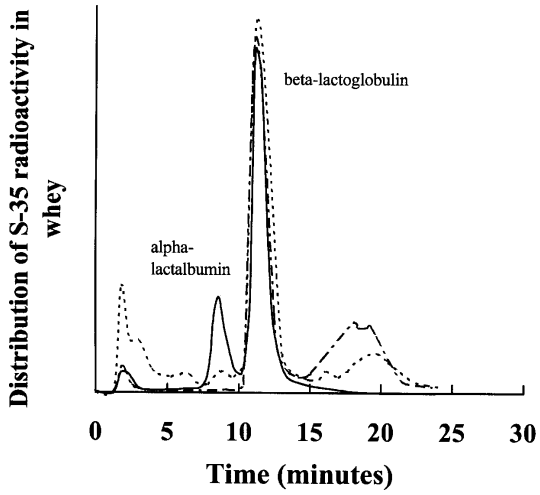
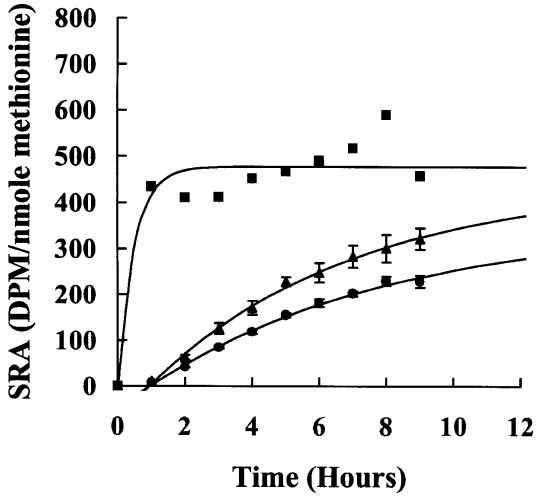


FIGURE 2: Specific radioactivity of methionine in plasma, casein and ß-lactoglobulin during a continuous infusion of ^{35}S-methionine into the jugular vein of goat 132. Values for the secreted milk proteins are the means for the right and left hand mammary gland. Plasma (■—■), casein (●—●), ß-lactoglobulin (▲—▲). Solid lines are for the function $y = A + B\,e^{-kt}$ (see Table 1 for predicted half-lives and plateau SRA's).



of ^{35}S - methionine directly into one half of the mammary gland (Expt. 1), is compared in Fig. 1 with that of whey collected at the same time, but after a whole body ^{35}S-methionine infusion (Expt.2). Also shown is the distribution of ^{35}S-cysteine in whey proteins after infusion of labelled cysteine (Knutson *et al.* 1994). The distribution of ^{35}S - cysteine is in proportion to the number of cysteine residues in the two whey proteins and their relative concentration in whey. Methionine residues are absent in goat α-lactalbumin, so the appearance of ^{35}S-label in the α-lactalbumin peak after infusion of labelled methionine directly into the gland is supportive of a small amount of transsulphuration (conversion of homocysteine in the transsulphuration pathway to cysteine via cystathionine

synthetase). This was estimated to be 10% of total label appearing in the whey proteins. There was no appearance of label in α-lactalbumin at the same time period when ^{35}S-methionine was infused into the jugular vein, even though the total radioactivity of methionine in the arterial supply to the gland was comparable in both experiments. The radioactivity in the casein region (15-25 min) was probably from residual casein in the whey preparation, whilst ^{35}S-label at the start of the chromatogram includes that in sulphate, methionine sulphoxide and minor milk proteins. Further indication of transsulphuration in the gland itself was obtained from measurements of ^{35}S-label in intracellular cysteine in mammary tissue extracts (data not shown): the SRA of which was estimated to be higher than that of cysteine in plasma. The latter includes methionine converted to cysteine in the liver together with possible export of cysteine from the gland. It was concluded that the free ^{35}S-cysteine in mammary tissue extracts was primarily derived from transsulphuration from within the gland, however further work will be required to quantify this.

Figure 2 shows the temporal changes in the SRA (DPM/nmol) of methionine in plasma, compared with that of methionine in total casein and ß-lactoglobulin, during a 9-hour jugular infusion of ^{35}S - methionine. Changes in the SRA of methionine with time in whole blood was similar to that in plasma. Mean SRA's of methionine between 7 and 9 hours of the infusion for the blood pool and individual milk proteins are given in Table 1, although the SRA's for the proteins had not reached plateau over this period. Table 1 also gives the predicted plateau SRA of bound methionine and estimated half-life for casein and ß-lactoglobulin calculated from fitting a single exponential to the rise in SRA of protein bound methionine (fig. 2). The predicted plateau SRA of methionine in the milk proteins from goat 123 are not in agreement with goat 132 and are also higher than the SRA for methionine in the plasma precursor pool, which may indicate underestimation of either the amount of protein or of bound methionine. However there is good agreement for the calculated half-lives of the respective proteins obtained from the two goats, with the half-life of ß-lactoglobulin about one hour shorter than casein in both goats. The half-life for casein synthesis calculated in the present study is in good agreement with the 1.5 -5.0 hours reported by Bequette *et al.*(1994) obtained from casein labelling with ^{13}C amino acids.

After only a short time (2-6 hours) the SRA of 'free' methionine in plasma had reached a 'pseudo' plateau, whereas the SRA of methionine in the milk proteins was still increasing. The SRA of methionine in ß-lactoglobulin increased at an appreciably faster rate than that of casein. As in the work of Bequette *et al.* (1994) with leucine, appearance of ^{35}S-label was noted after 1 hour, indicating a small pool size of newly synthesised casein. However the rise of labelled methionine in synthesised casein did not increase at the same rate as that of the blood precursor pool. This has been taken by others (Bequette *et al.* 1994) to infer, either, intracellular sources of precursor amino acid with slow turnover rates, or additions of amino acid other than that labelled. One of these source could be methionine containing peptides, derived either from plasma or from intermediate mixing pools within the gland as a consequence of general protein degradation. The latter is now thought to be as much half of synthesised protein in the gland, and has been discussed in more detail by Bequette *et al.* (1994). Further evidence for the contribution of non-labelled methionine channelled from degraded protein, or from other sources, is shown by the very low SRA of intracellular methionine extracted from gland tissue (Table 1). The presence of distinct precursor pools for charging tRNA and subsequent protein synthesis have been identified in a number of other cell systems and are reviewed by Baldwin *et al.* (1994).

Methionine concentrations in arterial and venous blood, together with net and total uptakes of methionine into the whole mammary gland, and the amount of methionine extracted by the gland as a proportion of whole body utilisation (ILR) are given in Table 2. Both the concentration and SRA of methionine in plasma and whole blood (Table 1) were similar, indicating fast exchange between red cells and the plasma pool. Net uptake of methionine by the gland was a high proportion of the arterial flux (46.4 and 31.5 % for the two goats respectively) but the net amount entering the gland was less than 20% of that utilised by the whole body as indicated by the ILR. The high net fractional extraction by the gland for methionine contrasts with that of cysteine reported by Knutson *et al.* (1994) which was less than 8% of the arterial flux, although absolute net uptake was similar (204µmol

TABLE 1: Specific radioactivity of methionine in plasma, whole blood, milk proteins and mammary tissue extracts after infusion of ^{35}S-methionine into the jugular vein for 9 hours. Results are the mean ± s.e.of the left and right hand sides of the gland for samples collected at 7-9 hours. Note that SRA's (DPM/nmole methionine) for the proteins are not at plateau at this time period (see Fig. 2). Predicted plateau SRA (A) and half lives of labelled protein were obtained from fitting the exponential function: $y = A + B\ e^{((-0.693/\text{half-life})*t)}$.

	SRA (7-9 hours)		Predicted plateau (SRA)		Half-life (hours)	
	132	123	132	123	132	123
Plasma	521±31	625±8	478	611	-	-
Whole blood	494±27	612±32	-[1]	-	-	-
ß- Lactoglobulin	320±20	830±50	442	1227	4.22	4.60
Casein	229±13	449±2	359	820	5.12	6.66
α-Lactalbumin	ND[2]	ND	-	-	-	-
Mammary tissue	49±22[3]	66±28[3]	-	-	-	-

[1] Not determined

[2] Not detected

[3] Possible oxidation of methionine to methionine sulphoxide resulting in underestimation of SRA

TABLE 2: Milk yield, blood flow and uptake of methionine by the mammary gland of lactating goats compared with whole body methionine utilisation. ILR calculated from the total activity of isotope infused divided by the specific radioactivity of methionine at steady state (7-9 hours of ^{35}S-methionine infusion). Uptake measurements are the means ± s.e. of 6 measurements over duration of the infusion.

	Goat 132	Goat 123
Milk yield (ml/hr)	66	45
Blood flow (ml/min)	550	374
Methionine in whole blood (μM)		
Femoral artery	17.7±2.9	26.9±0.84
Mammary vein	9.4±0.65	18.4±0.62
IRL (mmol/hr)	1.5±0.1	1.37±0.09
Net uptake methionine (μmol/hr)	274±34	191±17
Fractional extraction	46.4±2.7	31.5±2.3
% of ILR	18.1	13.8
Total uptake methionine (μmol/hr)	220±14	187±12
Fractional extraction	34.8±2.1	33.7±3.8

cysteine/hr compared with 274 and 191 μmol methionine/hr for the two goats used in this study). One particular source of error in quantifying fractional extraction rates from the blood supply to the gland is the estimation of blood flow. Problems associated with blood flow measurements are well recognised (Linzell 1974), however further discussion is beyond the scope of this paper.

Total uptake as measured by the fractional extraction of ^{35}S-methionine from arterial blood is dominated by protein synthesis, although it may also include contributions from oxidation, production of methionine peptides and transsulphuration. The difference between net uptake and total uptake is commonly taken to indicate the contribution of amino acid from other pathways, particularly degradation of both mammary structural and milk proteins and release of methionine from peptides. In this study there was little or no difference between the two (Table 2), which may indicate that release of methionine from protein degradation within the gland into the venous drainage is minimal. Furthermore, oxidation of methionine, measured as arteriovenous differences in labelled oxidation products (sulphate, methionine sulphoxide) was also shown to be minimal. However, the low SRA measurements (Table 1) for methionine in casein and in mammary tissue compared with those of the blood pool, indicated considerable mixing of non-labelled methionine. This may have arisen either directly from protein degradation or from unknown peptide sources. These sources may, therefore, be compartmentalised, and channelled directly into the immediate precursor pool for protein synthesis without mixing with labelled methionine from arterial uptake prior to release into venous blood.

Total methionine output in the gland required for milk protein synthesis from both the goats used in this study was approximately 4.7 μmol/min, which compares with 4.6 and 3.2 μmol/min for the net uptake of methionine for the two goats, respectively (Table 2). Assuming steady state conditions with respect to mammary tissue synthesis - this is reasonable as the goats were in late lactation - and negligible oxidation within the gland, it is evident that net uptake of methionine, as measured in this preliminary work, is only barely enough to match milk protein output.

CONCLUSIONS

The measurements given in this work suggest that the lactating mammary gland of the goat has the capacity for transsulphuration and, although shown to be low, may be of importance if cysteine supply is limiting. The cysteine requirement is higher than that of methionine for only few milk proteins, and, as shown previously (Knutson *et al.* 1994), the fractional extraction of cysteine from arterial blood is considerably lower than that shown here for methionine. Differential channelling of methionine (and other amino acids) from protein degradation or peptide release directly into the intermediate precursor pool for protein synthesis, together with compartmentalisation, as also inferred by Bequette *et al.* (1994), may partly explain why, in some studies, synthesis of particular milk proteins are affected more than others when dietary sources of amino acids are altered. Differential effects on protein synthesis of individual milk proteins when methionine extraction is limited by supply or other factors - or the converse of this when there is an over-supply of methionine - may impact on milk protein composition and subsequently, milk characteristics. We have shown that the net disappearance of methionine from blood supply to gland is barely enough to meet milk protein output, so that effects on milk protein synthesis will be exacerbated if demands of other tissues, either within the gland itself or from whole body tissues in general, are in conflict. This may be the case in early lactation.

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REFERENCES

Andrews, A. T.; Alichanidis, E. 1983. Proteolysis of caseins and the proteose-peptone fraction of bovine milk. *Journal of Dairy Research* **50:** 275-290.

Andrews, A. T.; Taylor, M. D.; Owen, A. J. 1985. Rapid analysis of bovine milk proteins by fast protein liquid chromatography. *Journal of Chromatography* **348:** 177-185.

Baldwin, R.L.; Calvert, C.C.; Hanigan, M.D.; Beckett, J. 1994: Modelling amino acid metabolism in ruminants. *In:* Amino acids in farm animal nutrition (ed J.P.F. D'Mello) CAB International, pp 281-306.

Bequette, B.J.; Backwell, F.R.; Dhaona, M.S.; Walker, A.; Calder, A.G.; Metcalf, J.A.; Wray-Cahen, D.; Sutton, J.D.; Beever, D.E.; Lobley, G.E.; MacRae, J.C. 1994: Kinetics of blood free and milk casein-amino acid labelling in the dairy goat at two stages of lactation. *British Journal of Nutrition* **72:** 211-220

Bidlingmeyer, B. A.; Cohen, S.A.; Tarvin, T.L. 1984. Rapid analysis of amino acids using pre-column derivatisation *Journal of Chromatography* **336:** 93-104.

Clark, J. H.; Speirs, H. R.; Davis, C. L. 1978. Uptake and metabolism of nitrogenous components by the lactating mammary gland. *Federation Proceedings* **37**: 1233-1238.

Davis, S. R.; Collier, R. J. 1985. Mammary flow and regulation of substrate supply for milk synthesis. *Journal of Dairy Science* **68:** 1041-58.

Knutson, R.J.; Lee, J.; Davis, S.R.; Harris, P.M.; Mackenzie, D.D.S.; McCutcheon, S.N. 1994: The utilisation of whole body cysteine by the mammary gland of the lactating goat. *Proceedings of the New Zealand Society of Animal Production*. **54:** 103-105.

Lee, J.; Harris, P.M.; Sinclair, B.R.; Treloar, B.P. 1995. Sulfur amino acid metabolism and protein synthesis in young sheep fed ryegrass pasture and two *Lotus* cultivars containing condensed tannin. *Australian Journal of Agricultural Science* **46**: 1587-1600.

Linzell, J.L. 1974: Mammary blood flow and methods of identifying and measuring precursors of milk. *In:* Lactation Vol I, (Eds. B. L. Larson and V. R. Smith) Academic press, New York. USA Pg 143.

Metcalf, J.A.; Beever, D.E.; Sutton, J.D.; Wray-Cahen, D.; Evans, R.T.; Humphries, D.J.; Backwell, F.R.C.; Bequette, B.J.; MacRae, J.C. 1994: The effect of supplementary protein on in vivo metabolism of the mammary gland in lactating dairy cows. *Journal of Dairy Science*. **77:** 1816-1827.

Paterson, G. R.; Hill, J. P.; Otter, D. E. 1995. Separation of ß-Lacto–globulin A, B and C variants of bovine whey using capillary zone electrophoresis, *Journal of Chromatography A*, **700:** 105-110.