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POST-RUMINAL AMINO ACID SUPPLEMENTATION TO SHEEP FED ROUGHAGE DIETS

Thesis submitted for the degree of

Doctor of Philosophy

by

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SUMMARY

The studies reported in this thesis investigated aspects of post-ruminal amino acid supplementation and amino acid utilisation in young sheep fed low to medium quality cereal straw and cereal hay roughage diets. All experiments utilised surgically prepared animals.

As a first step in defining the amino acid requirements of young sheep a simple supply-demand model of amino acid utilisation was set up. The model suggested that methionine (and cystine) were likely to be the "first-limiting" amino acids for sheep fed roughage diets. Subsequently positive responses in terms of intake or the efficiency of nitrogen utilisation to an abomasal supplement of methionine were recorded although all animals in the experiment did not exhibit positive responses. Although the changes in the plasma amino acid pattern to an altered supply of substrate (methionine or glucose) were considerable the changes did not give any clear indication as to which amino acids would be most likely to be limiting for protein synthesis.

A number of experiments were carried out using each animal as its own control in order to investigate between animal variability in the methionine response. Variability was particularly evident in terms of voluntary intake and in one experiment (two sheep) in which the repeatability of the responses was examined, the changes in intake, urine nitrogen excretion and plasma amino acid pattern were similar on two occasions. However there were no indications in the plasma or blood cell amino acid patterns as to possible

explanations for the different responses to the methionine supplement in different animals. Changes in plasma amino acid patterns and voluntary intake in sheep receiving threonine in addition to methionine pointed to interactions between these two amino acids at the metabolic level.

The variability in the response to methionine led to the hypothesis that no one amino acid would be limiting for all facets of metabolism and that consequently supplementation with groups of amino acids would give the greatest responses. Subsequently methionine was shown to be the most critical of a group of amino acids for improving the efficiency of nitrogen utilisation although in some sheep there was evidence that other amino acids (e.g. threonine, leucine) were also particularly important for efficient nitrogen utilisation. Consequently, the usefulness of the concept of a "first-limiting" amino acid must be questioned.

In experiments designed to examine aspects of the metabolism of methionine, some sheep received intravenous infusions of radioactively-labelled methionine and cystine after which they were slaughtered and tissue samples obtained. A simple compartmental model of hepatic methionine metabolism was developed. However, as a result of metabolic compartmentation of methionine and cystine within the liver, the estimates of protein synthesis and methionine catabolism were clearly erroneous in all sheep except those receiving very high rates of methionine per abomasum.

The experiments highlighted the complexity of amino acid utilisation in the sheep. This is particularly evident when the between animal variability in the response to a methionine supplement and the complexity of methionine metabolism are considered.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and that, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except when due reference is made in the text.

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AMINO ACID NOMENCLATURE

The abbreviations for the amino acids used throughout this thesis are:

TAU	taurine
THR	threonine
SER	serine
GLU	glutamic acid
GLY	glycine
ALA	alanine
ABU	∝-amino-n-butyric acid
VAL	valine
1/2 CYS	half-cystine
MET	methionine
CYSH	cystathionine
ILE	isoleucine
LEU	leucine
TYR	tyrosine
PHE	phenylalanine
ORN	ornithine
LYS	lysine
HIS	histidine
TRP	tryptophan
ARG	arginine
DOPA	dihydroxyphenylalanine

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INTRODUCTION

Despite the considerable economic importance of ruminants throughout the world some aspects of their nutrition are poorly understood. This is particularly so in relation to the nitrogen nutrition of the ruminant animal.

The amount of protein and the balance of amino acids available to the tissues of the animal are of considerable importance in determining the productive capacity of the animal. Although the supply of metabolisable energy to the animal is of prime importance in determining productive capacity, a relative inadequacy of the protein supply will greatly reduce the efficiency of utilisation of energy in terms of the animal production.

On two occasions in recent years specialist panels of the FAO/IAEA (1972; 1975) have drawn attention to the lack of knowledge concerning the protein nutrition of the ruminant, and recommendations for research have been published (1972; 1975). These fall within the two components of nitrogen nutrition, namely the provision of microbial protein as a product of rumen fermentation and the requirement for exogenous protein or amino acids which bypass the rumen to supplement the microbial protein in order to meet the animal's overall requirements (FAO/IAEA 1972).

The work to be reported in this thesis was carried out in an attempt to make some contribution to an improved knowledge of amino acid utilisation in the sheep.

REVIEW OF LITERATURE

Aspects of protein and amino acid nutrition and metabolism with reference to the ruminant

Introduction

1. Protein and amino acid supply in the ruminant

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INTRODUCTION

Many aspects of nitrogen metabolism in the ruminant have been discussed in recent reviews (Chalupa 1968; Hatfield 1970; Nelson 1970; Purser 1970a, b; Preston 1972; Armstrong & Annison 1973; Armstrong & Hutton 1975; Smith 1975). The aim of this review is to examine the patterns of nutritional and metabolic factors in ruminants as they contribute to gain, loss and change in the protein mass of the animal.

The review begins with a brief discussion of protein and amino acid supply in the ruminant, followed by a consideration of the adequacy of the protein and amino acid supply in relation to the requirements of the animal for growth. Aspects of the tissue metabolism of the amino acids as they affect the availability of amino acids for protein synthesis are also covered. The review concludes with a consideration of protein accretion itself, in terms of its components, namely protein synthesis and degradation.

The questions raised will often be discussed on the basis of physiological mechanisms for which evidence is provided from research on non-ruminant species. The intention is to indicate areas of importance in a consideration of amino acid metabolism in the ruminant. In reference to the ruminant species, the emphasis is principally on sheep, cattle being considered only to highlight particular aspects.

1. PROTEIN AND AMINO ACID SUPPLY IN THE RUMINANT

Harris & Mitchell (1941) first showed that non-protein nitrogen would support growth in sheep. This was in marked constrast to results obtained with rats (Kriss & Marcy 1940). Subsequently Loosli *et al.* (1949) provided conclusive evidence that amino acids were synthesised in the rumen of sheep fed a diet containing urea as the sole source of nitrogen. A few years later, Duncan *et al.* (1953) and Weller (1957) published data for the amino acid composition of ruminal digesta and rumen microbial preparations, while Black *et al.* (1957) and Downes (1961) suggested that, except for arginine, the same amino acids that were essential for the growing rat were essential for the lactating ruminant.

The rumen exerts a considerable modifying effect on the balance of nutrients available to the animal when compared with the composition of the feed consumed. Thus Egan (1974) showed that a twelve-fold range in dietary crude protein concentration was condensed to a four-fold range in the protein concentration of the organic matter digested by the animal. Dietary quality influences the protein/energy ratio of the nutrients available to the animal through both the efficiency of rumen microbial protein synthesis (Walker & Nader 1968; Egan *et al.* 1975) and the amount of dietary protein escaping degradation in the rumen (Egan 1974; Walker *et al.* 1975).

In terms of the amino acid composition of rumen microbial preparations there is little variability in the data reported from various laboratories (Purser 1970a). The composition of dietary grass and legume protein is also similar to that of rumen microbes. However, there is evidence that the true digestibility of bacterial

and protozoal proteins may differ (McNaught *et al.* 1954 ; Bergen *et al.* 1968) while Bergen *et al.* (1967) have suggested that the availability of amino acids from microbial protein may vary between different species of microbes. These factors may assume considerable importance in particular situations. The only protein available in animals fed diets with urea as the sole source of nitrogen is of microbial origin, while Schelling *et al.* (cited by Schelling & Hatfield 1968) have observed only a limited number of bacterial species and the virtual absence of protozoa in the rumen of animals fed purified urea-based diets. Thus the influence of the microbial population on the amino acid supply to the host animal may be an important variable in studies of the amino acid requirements of ruminants, with apparently small variations being of nutritional significance to the host animal.

2. THE ADEQUACY OF THE PROTEIN AND AMINO ACID SUPPLY

2.1 Theoretical estimates of amino acid adequacy

A number of workers have produced theoretical estimates of the relationships between the amino acid supply and the amino acid requirements of cattle or sheep in different physiological states (Hogan 1970; Purser 1970a; Hutton & Annison 1972; Armstrong & Annison 1973; Egan & Walker 1975).

The estimates of Hutton & Annison (1972) and Egan & Walker (1975) which were based on the factorial method used by Williams *et al.* (1954) for the estimation of the amino acid requirements of rats, pigs and chicks, are likely to be the best of the theoretical estimates since these workers allowed for differences in the

utilisation of the different essential amino acids. Both Hutton & Annison (1972) and Egan & Walker (1975) used the concept of a utilisation factor, this being the ratio of the calculated requirement of an amino acid for net protein gain to the dietary supply of an amino acid. The calculations included allowances for metabolic losses (metabolic faecal and urinary nitrogen).

Hutton & Annison (1972) calculated the amino acid requirements for a growing steer using the utilisation factors for the pig, and suggested that methionine synthesised in the rumen would be barely adequate for the needs of the animal, while for all of the other essential amino acids the supply would be expected to be adequate. They suggested that some dietary protein must escape ruminal fermentation for maximal growth of ruminants. However, experiments involving methionine supplementation in growing cattle have not given the clearcut results which could be expected from the estimates of Hutton & Annison (1972). Steinaker *et al.* (1970) obtained a small, though non-significant, response to post-ruminal methionine in cattle fed a urea-based diet, while Chalupa *et al.* (1972) detected an improvement in nitrogen utilisation in one experiment but were unable to confirm this subsequently. Sibbald *et al.* (1968) obtained a higher rate of liveweight gain in steers fed a supplement of encapsulated methionine (designed to protect the methionine from degradation in the rumen). Other workers have observed increases in nitrogen retention in steers given abomasal supplements of lysine (Devlin & Woods 1964; 1965) or the branched chain amino acids and tryptophan (Chalupa & Chandler 1972).

Egan & Walker (1975) compared their calculated utilisation factors (the ratio of the estimated utilisation in protein synthesis to the estimated supply) for the various amino acids with the ratios derived from published data for the rat, pig and chick. Where the calculated utilisation factor for the ruminant greatly exceeded that for the other species, the particular amino acid was considered likely to be limiting production. On this basis, these workers suggested that methionine/cystine, histidine, leucine and tryptophan were likely to be limiting for growth in the growing sheep, although it is possible that the ruminant may derive histidine from sources other than protein digested in the intestines. In a number of studies, methionine has been shown to be the first limiting essential amino acid for growth in young sheep (Nimrick *et al.* 1970a, b; Barry *et al.* 1973; Schelling *et al.* 1973).

A significant factor to be noted from the papers of both Hutton & Annison (1972) and Egan & Walker (1975) is the low utilisation factors for methionine and tryptophan in the data for the rat, pig and chick. This would suggest that these amino acids have roles in metabolism other than their requirement for incorporation into protein. This is the situation with methionine (Finklestein 1974). Aguilar *et al.* (1972; 1974) have shown that the apparent uptake of methionine in protein synthesis is considerably lower than for any of the other essential amino acids even when supplied at levels well below its requirement for maximum growth. However, the values for tryptophan were similar to those for the other essential amino acids, and the low utilisation factors

for tryptophan may indicate an overestimate of its requirement in the non-ruminant species.

Such theoretical estimates of the adequacy/inadequacy situation in amino acid supply for the ruminant provide a useful starting point in the study of amino acid requirements and metabolism in the ruminant.

2.2 Plasma amino acids as indices of protein and amino acid supply

Both total feed intake (Nimrick *et al.* 1971) and energy intake *per se* (Purser *et al.* 1966; Halfpenny *et al.* 1969) have a marked effect on plasma amino acid concentrations in the ruminant. The pattern of plasma amino acid changes (particularly the branched chain amino acids) observed by Nimrick *et al.* (1971) resembled that of changes associated with increases in dietary protein (Schelling *et al.* 1967), or increases in the supply of protein at the duodenum (Hogan *et al.* 1968). However, the disproportionate increases in the branched chain amino acid concentrations were probably a reflection of the inability of the liver to catabolise significant quantities of these amino acids (Sketcher *et al.* 1974).

Bergen *et al.* (1973) have proposed a unitary hypothesis concerning the effects of dietary level and source of nitrogen and other dietary factors on plasma amino acid levels in ruminants. These workers have used the reported effects of dietary amino acid insufficiency on plasma and tissue amino acid patterns from rats and pigs. They consider that changes in plasma amino acid patterns are primarily a consequence of the total amino acids reaching the

duodenum. In its simplest terms, the model predicts that during a period of protein deficiency (particularly in growing animals) the plasma total and branched chain amino acids and phenylalanine will decline, while the non-essential to essential amino acid (N/E) ratio will increase. In terms of protein supply, and perhaps the protein to energy ratio of the nutrients available to the animals, the model appears satisfactory.

Egan (1972) fed a wide range of diets to sheep and found that the logarithm of the ratio of glycine to all other plasma amino acids was highly correlated with the quanity of α-amino nitrogen at the duodenum.

2.3 Plasma amino acids in the estimation of amino acid requirements and as indices of limiting amino acids

Almquist (1954) suggested that the relationship between plasma amino acid (PAA) levels and dietary protein intake may provide a direct method for establishing protein requirements.

A dietary deficiency of a single essential amino acid may result in a severely depressed level of that amino acid in plasma (Almquist 1954; Clark *et al.* 1966). Consequently, numerous workers have used changes in the levels of plasma amino acids to predict the essential amino acid requirements for rats and chicks (Morrison *et al.* 1961; Zimmerman & Scott 1965; McLaughlin & Illman 1967; Mitchell *et al.* 1968; Bravo *et al.* 1970; Stockland *et al.* 1970; Ganguli *et al.* 1972). When increasing levels of a single amino acid are added to a basal diet, the plasma concentration of the added amino acid is increased in the fed animal, but exhibits a 'broken line' response which is interpreted as providing an estimate of the amino acid requirements. Such patterns of response have been described

in studies with rats by Stockland *et al.* (1970) and Young & Munro (1973). These workers suggest that the point of inflection in the plasma concentration occurs at a lower level of the amino acid in the diet, than the level at which maximal weight gain is achieved. The 'broken line' response technique has been used by several workers for the estimation of the methionine requirement in sheep (Wakeling *et al.* 1970; Nimrick *et al.* 1970b; Chalupa & Chandler 1972; Reis *et al.* 1973b). However, Chalupa & Chandler (1972) noted that the nitrogen balance technique gave much lower coefficients of variation than the plasma technique in their studies of methionine requirements.

Several other techniques and modifications of the above techniques have been developed in attempts to determine the amino acid requirements (McLaughlan 1964; McLaughlan & Illman 1967) and to predict the limiting amino acids in a dietary protein (Longenecker & Hause 1959; Smith 1966; McLaughlan & Morrison 1968).

Potter *et al.* (1972) infused different proteins into the duodenum of sheep and using different plasma amino acid ratio techniques attempted to identify the limiting amino acid in each of these proteins. Although one of the techniques apparently correctly predicted the limiting amino acid in each of the protein sources, the application of the technique to studies reported in the literature was less successful.

Munro (1956) concluded that the release of insulin must be considered an integral part of the mechanism of the plasma amino acid response in many situations, since dietary carbohydrate has been

shown to depress plasma amino acid concentrations whereas dietary fat does not (Munro & Thompson 1953; Munro *et al.* 1959). The reductions in the concentrations of plasma amino acids in response to the administration of glucose (Munro & Thompson 1953) or insulin (Lotspeich 1949) have been found to be in the same proportions as the amino acids in muscle protein. In addition glucose administration has been shown to enhance the incorporation of amino acids into muscle protein in rats (Munro *et al.* 1959; Knipfel *et al.* (1969), while insulin itself also stimulates muscle protein synthesis (Wool 1972).

Changes in plasma amino acids in response to a ruminal starch-glucose load (Purser *et al.* 1966) or an arterial infusion of glucose or acetic, propionic or butyric acids (Potter *et al.* 1968) have been used in experiments designed to define the limiting amino acids for ruminal diets. The energy loading experiments have not been supported by direct studies to determine the limiting amino acids in these situations. However, it is likely that protein synthesis is being stimulated by the additional energy, since Eskeland *et al.* (1974) found that depressions in plasma essential amino acids in response to an intravenous infusion of glucose or acetic, propionic or butyric acids were inversely related to the nitrogen balance responses.

In a similar manner to that proposed for an energy induced stimulation of protein synthesis, the changes in the plasma amino acid pattern in response to an infusion of the first limiting amino acid might also be expected to indicate the other amino acids likely to be limiting for protein synthesis. Using this reasoning,

Scott *et al.* (1972) suggested that threonine was the most limiting amino acid for sheep after methionine requirements had been met. In the studies of Nimrick *et al.* (1970b) plasma lysine, threonine and valine concentrations suffered the greatest depressions when methionine and glutamate were administered per abomasum compared with glutamate alone. Subsequently lysine and threonine were identified as the next limiting amino acids.

2.4 Experimental evidence as to the adequacy of protein and amino acid supply in ruminants

2.4.1 Effect of supplemental protein on nitrogen retention and intake

Cuthbertson & Chalmers (1950) showed that casein administered post-ruminally promoted significantly higher nitrogen retention than casein given into the rumen in sheep fed a roughage diet. Subsequently many workers have shown that post-ruminal supplementation with casein improves nitrogen retention (Chalmers *et al.* 1954; Egan 1965, b, c; Egan & Moir 1965; Little & Mitchell 1967). The quality of the supplementary protein is clearly important since Reis & Schinckel (1964) showed that casein was a more effective stimulant of wool growth than gelatin, while addition of sulphur amino acids to both infusates improved wool growth further. Egan (1965a, b, c) also showed that the voluntary intake of a low nitrogen roughage diet by sheep was considerably increased by post-ruminal protein supplementation. The increase in intake was mediated by a mechanism different to that which operated for urea supplementation, since the response was delayed when the latter supplement was provided (Egan & Moir 1965).

The simplest interpretation of these results is that circumstances exist in which growth and production of ruminants is limited by the supply of protein or amino acids. This may involve limitations in voluntary intake or the efficiency of nitrogen utilisation, which presumably involves amino acid metabolism at the tissue level.

2.4.2 Effect of supplemental amino acids on nitrogen retention and wool growth

In some experiments, dietary supplements of individual amino acids have improved the efficiency of nitrogen utilisation by the animal. However, if dietary amino acids are to have an effect they must either enhance the efficiency of microbial protein synthesis or provide a significant amount of amino acids which escapes ruminal fermentation to yield extra amino acids for absorption in the intestine. It is possible that in some experiments in which a dietary supplement of methionine has produced a positive response, the methionine may have been acting as a readily available source of sulphur (Starks *et al.* 1954; Albert *et al.* 1956) and improving the efficiency of microbial protein synthesis, although it is also possible that some of the methionine escaped ruminal degradation and became available to the tissues of the host animal. In this respect, dietary methionine has improved wool growth in some studies (Starks *et al.* 1954; Wright 1971; Doyle & Bird 1975).

Marston (1935) was the first to show that an increased supply of cysteine at the tissue level may result in an increase in the rate of wool growth in sheep. Subsequently Reis and Schinckel (1963; 1964) showed than an increased post-ruminal supply of methio-

nine or cyst(e)ine produced large responses in wool growth. This has been confirmed by many workers for sheep fed roughage diets (Reis 1967; Dryden *et al.* 1969; Langlands 1970; Downes *et al.* 1970; Barry & Andrews 1973; Barry *et al.* 1973; Dove & Robards 1974). High levels of methionine have been found to depress wool growth (Reis 1967; Reis *et al.* 1973), whereas high levels of cystine do not depress wool growth. At low levels of supplementation, methionine is a more effective stimulant of wool growth than an equivalent amount of cystine (on a sulphur basis) (Williams *et al.* 1972b). This may be related to metabolic functions of methionine apart from its roles as a precursor of cyst(e)ine and as a protein amino acid. Methionine is a methyl donor (Finklestein 1974) and is also required in the synthesis of polyamines (Pegg & Williams-Ashman 1969), which may have a role in the *de novo* synthesis of protein (Tanner 1967).

In several experiments with young sheep, abomasal supplementation with methionine has improved nitrogen retention (Nimrick *et al.* 1970a, b; Scott *et al.* 1972; Chalupa & Chandler 1972; Schelling *et al.* 1973). Intraperitoneal administration of methionine markedly increased the voluntary intake, liveweight gain and wool growth of young sheep fed silage (Barry *et al.* 1973). Evidence of a possible aminostatic control of feed intake in rats has been provided by Leung & Rogers (1969) and Peng & Harper (1970). It has also been shown that amino acid imbalance depresses intake in preruminant lambs (Rogers & Egan 1975).

The order of limiting essential amino acids for growth of young sheep fed urea-based purified diets has been defined as methionine, lysine, threonine (Nimrick *et al.* 1970a). Nimrick *et al.* (1970a Experiment 2) suggested that lysine rather than methionine may have been first limiting for growth of some lambs, indicating the possibility of between animal variability or animal x diet interactions affecting the requirement or relative supply of amino acids. However, in a subsequent experiment reported in the same paper, neither lysine nor lysine and methionine improved nitrogen retention whereas methionine alone produced a large response. The rate of growth of lambs in the latter experiment was slow; it is therefore possible that a lysine x rate of growth interaction was operative. It has been argued that the lysine requirement relative to that for methionine (+ cystine) should increase rapidly with an increasing rate of growth due to the relative decline in the sulphur amino acid demand for wool growth. Schelling & Hatfield (1968) reported a large nitrogen retention response to lysine in young lambs fed a purified diet, similar to that used by Nimrick *et al.*(1970a, b). In the work of Schelling & Hatfield (1968) it is possible that the amount of methionine administered (4.3g/day) may have been excessive, since Reis *et al.* (1973a) observed a depression in wool growth with an infusion of a similar quantity of methionine.

Zein, a lysine-deficient dietary protein for non-ruminants (Hill & Olsen 1963) is relatively insoluble and fairly resistant to microbial degradation in the rumen (McDonald 1954; Ely *et al.* 1967). Thus, not unexpectedly, abomasal supplementation with lysine increased nitrogen retention in sheep when zein was the source of dietary protein (Scott *et al.* 1969; Moore *et al.* 1970). In sheep fed whole wheat diets, low levels of abomasal methionine supplementation

adversely affect wool growth (Reis & Tunks 1974; Downes *et al.* 1976). This is in marked contrast to the situation with roughage diets and may be related to a difference in the pattern of amino acids at the duodenum (and consequently at the tissue level), possibly due to differences in the type of microflora, or to differences in the amino acid balance of the protein which escapes ruminal fermentation.

3. MODIFICATION OF THE AMINO ACID SUPPLY AT THE TISSUE LEVEL

3.1 Intestinal amino acid absorption

3.1.1 Apparent absorption of amino acids

The apparent uptake of amino acids from the small intestine of the sheep has been studied in several laboratories using measurements of amino acid flow at the duodenum and ileum obtained in sheep prepared with re-entrant canulae (Clarke *et al.* 1966; Coehlo da Silva *et al.* 1972; Harrison *et al.* 1973; MacRae & Ulyatt 1974). However, the net absorption in terms of the amino acid uptake in the blood draining the intestine has been investigated in only two studies (Hume *et al.* 1972; Wolff *et al.* 1974). This is perhaps not surprising in view of the problems of technique involved with the latter type of study. An accurate estimate of the blood flow is required and the animal must be in a steady state, while small errors in the analysis of the amino acid concentrations can lead to very large errors in the estimation of amino acid utilisation (Wolff *et al.* 1972).

In both of these studies with sheep, only the amino acid flow in the plasma compartment was considered. However, in particular circumstances the blood cells may play an important role in the

nitrogen transport of amino acids (Elwyn 1970; Felig *et al.* 1973). Despite this possible error, there was reasonable agreement between the net intestinal uptake of essential amino acids and the composition of essential amino acids and the composition of rumen microbial protein and dietary lucerne in the experiments of Wolff *et al.* (1972) and of Hume *et al.* (1972).

Hume *et al.* (1972) observed that in the sheep fed 12 times daily, the overall net absorption was positive, while in those fed once daily, the net absorption was negative. Since the same sheep and the same level of feeding was used in each case, some form of α-amino nitrogen other than plasma free amino acids must reach the peripheral tissues in order that the skeletal muscle is not depleted. Similar results have been reported by Elwyn *et al.* (1968) for dogs fed a single high-protein meal. Presumably the amino acids could also be transported free in the blood cells, as peptides (e.g. glutathione), as carrier-bound amino acids (e.g. cysteine, tryptophan) or as protein amino acids in the plasma proteins, while transport of the carbon skeleton of an amino acid as the keto acid could also be important.

3.1.2 Endogenous protein secretions and amino acid uptake

Dietary protein undergoing digestion in the intestine is diluted by inputs of endogenous proteins in amounts which Nassett *et al.* (1955) have suggested could have considerable physiological impact. Nassett has suggested that the hydrolysis of the endogenous protein may serve to protect the tissues of the organism from the immediate effects of the ingestion of an unbalanced protein since

amino acids derived from the endogenous protein dilute out those derived from the dietary source (Nassett & Ju 1961; Nassett 1965). Such a gut homeostasis constitutes an attractive hypothesis, particularly for diets of unbalanced proteins in the short-term, providing for a delay in the expression of an amino acid deficit at the tissue level.

The free amino acid pattern of the gut contents of rats fed a histidine-imbalanced diet was very similar to that of rats fed a histidinecorrected diet after three days (Nassett & Ju 1975). Nevertheless the histidine imbalance was reflected in the plasma concentration of histidine after three hours as has been shown by many other workers (see Harper *et al.* 1970). There thus seems little support for the suggestion that endogenous protein inputs into the intestine may protect the organism from the effects of an unbalanced dietary protein. This contentious issue has been discussed more fully by Elwyn (1970) and Nassett & Ju (1975).

Notwithstanding the previous discussion, there is a possibility that gut homeostasis may reduce aberrations in the actual amino acid absorption from the intestine which could conceivably result from an unbalanced dietary amino acid supply. Hume *et al.* (1972) administered 10g of leucine per day to sheep via an abomasal canula and obtained results which suggested that an excess of an amino acid could cause considerable shifts in the net absorption of other essential amino acids, particularly lysine. Johns & Bergen (1973) also reported that saturating concentrations of leucine markedly depressed lysine uptake in segments of sheep jejunum *in vitro*. Such an interaction between leucine and lysine in amino acid uptake from the small intestine could

be of considerable importance in ruminants fed maize diets containing zein. Considerable quantities of zein are likely to escape ruminal fermentation (Ely *et al.* 1967), resulting in a very high ratio of leucine to lysine in the small intestine.

Williams (1969) has concluded that the proportions of amino acids in the portal blood reaching the liver in different subjects need not be the same even though the amino acids in the intestine available for absorption are in similar proportions. It is possible that this could result in differences in the efficiency of utilisation of amino acids by different individuals.

3.2 Hepatic amino acid metabolism

Elwyn (1970) suggests that the liver may be the key organ in the regulation of amino acid supply to the tissues, since except for the intestine it has access to dietary amino acids ahead of the other tissues. In addition, catabolic homones which cause protein depletion in most tissues of the body may promote protein accretion in the liver.

The extent and direction of net protein synthesis or degradation in the liver is a factor in the regulation of blood amino acid concentration. As evidence of this, high concentrations of amino acids in arterial blood do not appear to stimulate net protein synthesis in the liver (Elwyn 1970). In both the perfused dog liver (McMenamy *et al.* 1962) and in the liver of the dog, *in vivo* (Elwyn *et al.* 1968), blood amino acid concentrations were lower in experiments in which either no net synthesis or protein depletion took place.

Lindsay *et al.* (1975) have interpreted the above statement of Elwyn (1970) as implying that amino acids supplied through the artery would be treated in a different manner from those arriving via the portal vein. Understandably, Lindsay and his coworkers could find no evidence for this in experiments in which sheep livers were perfused via the hepatic artery or portal vein.

Many factors other than the blood supply of amino acids are likely to be involved in the integration of hepatic amino acid metabolism. Glucocorticoids increase the levels of a number of catabolic enzymes, including tryptophan oxygenase (Greengard *et al.* 1963), serine dehydratase (Jost *et al.* 1968) and tyrosine aminotransferase (Hershko & Tomkins 1971). Hepatic serine dehydratase in rats is very sensitive to glucose, the administration of small amounts resulting in a much depressed rate of enzyme synthesis and an increased rate of degradation (Jost *et al.* 1968). Hormones need not be directly involved in increasing the enzyme levels, e.g. Peraino (1967) has concluded that cortisone is not involved in the primary induction of serine dehydratase but instead appears to alter the capacity of the regulatory systems for the enzyme to respond to amino acids.

The administration of unbalanced amino acid mixtures to rats and chicks frequently results in an increased level of many catabolic enzymes in the liver (Nakano *et al.* 1970; Pamart *et al.* 1974; Woodward *et al.* 1975). The induction of threonine dehydratase by high levels of methionine in the rat (Sanchez & Swendseid 1969; Daniel & Waisman 1969; Girand-Globa *et al.* 1972) and the resultant

increased threonine oxidation as has been reported in the chick (Katz & Baker 1975) could be of considerable importance were it to occur in the sheep. Scott *et al.* (1972) suggested that threonine may become the next limiting amino acid for protein synthesis in sheep after the requirement for methionine has been met. They suggested this on the basis of the considerable depressions in the level of plasma threonine with an increasing rate of methionine supplementation. This depression could also reflect an increased rate of threonine catabolism. Doonan *et al.* (1974) found that in sheep the intraperitoneal injection of only 7g of a protein hydrolysate per day increased the level of hepatic threonine dehydratase approximately 10-fold whereas 200g of dietary protein failed to have any effect. Since this quantity of dietary protein would be expected to increase considerably the amino acid yield at the duodenum (Egan 1974) and hence amino acid uptake, it seems likely that the response to the intraperitoneal protein was a stress response possibly mediated through an increased glucocorticoid secretion. Since enzymes which are induced by high levels of protein (Pitot & Peraino 1964; Bourdel *et al.* 1975) the apparent absence of a protein response in hepatic threonine dehydratase in the sheep would suggest that a methionine induction of this enzyme would also be unlikely, unless excess methionine in the sheep also resulted in indirect effects such as an increased glucocorticoid secretion.

The possibility that there are considerable differences in amino acid metabolism between the ruminant species and the rat and chick should also be considered, as both the patterns of metabolites absorbed and the patterns of metabolism developed to cope with these

could differ between species. For example, Carlson & Dyer (1970) found that tryptophan oxygenase failed to respond to large doses of glucocorticoids or tryptophan in sheep and cattle, in marked contrast to the response in the rat (Greengard *et al.* 1963). The detoxification of benzoic acid may account for a significant proportion of the liver glycine uptake in the sheep (Wolff & Bergman 1972b) a situation which would not arise in rats consuming a normal diet. Large quantities of ammonia may be absorbed from the rumen of sheep fed high protein diets. Lindsay *et al.* (1975) have suggested that the presence of ammonia may depress the hepatic catabolism of amino acids in the sheep. However, their experiments with perfused livers are confounded by the absence of a specific energy source in one of the perfusates, while that which contained ammonia also contained propionic acid.

Subcellular compartmentation of amino acids and metabolites may also be a particularly important factor in the channeling of an amino acid into a particular pathway. Such a functional heterogeneity of the tissue free amino acid pool has been proposed by Kipnis *et al.* (1961) who suggested that amino acids for protein synthesis are drawn from a pool which is in rapid equilibrium with the extracellular rather than the intracellular pool. The compartmentation of certain enzymes or metabolites within discrete cellular organelles, as is the situation with hepatic carbohydrate metabolism in the rat (Greenbaum *et al.* (1971), is a possibility. For example, the enzymes of the γ-glutamyl cycle are located in the cell membrane of several mammalian tissues (Meister 1974). Thus this pathway which could utilise glutathione taken up from the blood cells (Richman & Meister 1975) could enable glutathione to serve as a readily available source of cysteine as well as glycine and glutamic acid.

3.3 Amino acid catabolism

The amount of any amino acid which is catabolised is potentially a very important variable in determining the availability of an amino acid for protein synthesis.

For some amino acids, catabolism may be an important source of another compound, e.g. cystathionine and cysteine from methionine, tyrosine (and DOPA) from phenylalanine, nicotinic acid from tryptophan. The factors controlling the catabolism of any one amino acid, in particular the factors which determine the basal catabolism or irreducible wastage of a particular amino acid are clearly very complex. For example, Aguilar *et al.* (1972) have shown that methionine has a very high rate of catabolism in comparison with the other essential amino acids, even when supplied at a level well below its requirement. These workers suggested that the role of methionine as a methyl donor and as the sulphur source in cysteine and taurine may be responsible for the high oxidation rate of methionine, although cysteine supplementation of the diet appeared to have little effect on the catabolism of either the methyl or carboxyl carbon of methionine (Aguilar *et al.* 1974).

Gluconeogenesis is of very great importance in the roughage fed ruminant, and the provision of glucose could be expected to be a significant feature of the animal's amino acid utilisation. Reilly & Ford (1971) estimated that about 28% of the glucose turnover originated from the total amino acid pool in the fed sheep, while Wolff & Bergman (1972a) and Heitmann *et al.* (1973) could account for 11 and 8% of the plasma glucose as arising from the hepatic metabolism of the amino acids which they measured. These estimates of gluconeogenesis from amino acids are less than the earlier

estimates of 33-50% of the glucose carbon arising from amino acids in the lactating dairy cow (Black *et al.* 1968).

3.4 Interorgan amino acid transport

The supply of an amino acid to a tissue could also be controlled through independent transport capabilities of the plasma and the blood cells. Elwyn (1966) proposed differential roles for plasma and erythrocytes in interorgan transport in the dog. Elwyn *et al.* (1972) claimed that in the unanaesthetised dog, transport of free amino acids from the peripheral tissues to the liver occurred mainly via plasma, while transport of amino acids from the liver to peripheral tissues occurred mainly in the blood cells. However, there was considerable variability between animals, in that in the dog in which erythrocyte amino acid concentrations were high, amino acid transport in the blood cells was greater for some amino acids than transport in the plasma, while in the other dog with low erythrocyte amino acid concentrations, the blood cells made little net contribution to transport (Elwyn *et al.* 1972). Elwyn (1970) suggested that the erythrocyte transport probably represented a stage in recovery from the catheter implant surgery. However, it may well be a more general phenomenon evident during certain physiological states. As well as the erythrocytes leucocytes (Gupta & Agarwal 1973) and platelet cells could also be involved in blood amino acid transport.

Other possible mechanisms of interorgan amino acid transport also require attention. These include the possibility that tissues (e.g. muscle) may catabolise plasma proteins as a source of amino

acids, while blood cell glutathione and plasma protein bound cysteine could function as important sources of cysteine for the tissues.

4. PROTEIN TURNOVER

Net tissue protein accretion constitutes only a small proportion of total protein synthesis in the growing animal, since much of the protein synthesised is subsequently degraded. As there have been few studies concerned with protein turnover in the ruminent, this review draws upon studies in a range of species.

Of the classical nutritionists and physiologists of the 19th and early 20th centuries, only Magendie conceived of the body tissue existing in a dynamic state of turnover (Munro 1970). The principle was established when Schoenheimer's group used ^{15}N-labelled amino acids to show that the body proteins were in a continuous state of turnover (Schoenheimer 1942).

The continual synthesis and degradation of protein could appear to be a wasteful and energentically costly process. However, protein turnover permits redistribution of amino acids for the synthesis of functional proteins both within and between tissues. In various nutritional situations protein acts as a source of amino acid which may be sequestered into catabolic pathways to yield energy, into pathways of gluconeogenesis, into pathways of synthesis of specific compounds (e.g. DOPA from tyrosine, nicotinic acid from tryptophan) or into protein synthetic pathways to provide for new patterns of enzymes and other proteins associated with adaptation to nutritional and physiological conditions. Thus the price of

flexibility and adaptive capacity is the continuing degradation and resynthesis of proteins in the face of all patterns of nutrient provision.

4.1 Protein synthesis

The effect of amino acid supply on protein synthesis has been reviewed on several occasions (Munro 1969; 1970; Clemens 1972). Most work has involved studies of the control and regulation of protein synthesis in the liver. However, whole animal studies have shown that the effect of malnutrition (undernutrition or a protein free diet) is to increase the rate of liver protein synthesis (and degradation) while the rate of muscle protein synthesis is decreased (Millward *et al.* 1975a, b). Thus the interpretation of many of the studies in relation to the effects of amino acid supply on the rate of protein synthesis is complex.

The actual site of protein synthesis in the cell is the aggregated ribosome (i.e. polysome). Consequently many workers have examined the effects of various nutritional treatments on the hepatic polysome profile, and it has been claimed that an increase in the rate of protein synthesis is generally associated with an increase in the degree of ribosomal aggregation (Munro 1970). Much of the early work *in vivo* suggested that tryptophan may have a specific role in liver polysome formation (Fleck *et al.* 1965; Wunner *et al.* 1966; Sidransky *et al.* 1967) although Munro (1968) proposed that typtrophan normally regulates liver polysome formation only because it is usually the least abundant component of the free amino acid pool and is thus the limiting amino acid determining the

rate of protein synthesis. Subsequently it was shown that in rats fed imbalanced diets, the addition of the limiting amino acid promoted polysome aggregation (Pronczuk *et al.* 1970; Ip & Harper 1973; 1974).

Tryptophan may exert more specific effects in some particular circumstances. Administration of high doses of tryptophan increases the activity of several enzymes including tryptophan oxygenase (Schutz *et al.* 1975), tyrosine aminotransferase (Cihak *et al.* 1973) and serine dehydratase (Cihak *et al.* 1975). However, methionine may also increase the activity of tryptophan oxygenase, threonine dehydratase and arginase in similar situations (Nakano *et al.* 1970). Recently, it has also been shown that imbalanced amino acid mixtures lacking tryptophan actually induce a greater increase in the activity of tyrosine aminotransferase than either the complete amino acid mixture or tryptophan alone (Bourdel *et al.* 1975).

Methionine may also have a specific controlling role in protein synthesis since it is required for the formation of the protein synthetic initiation complex on the ribosome. Methionine is also required for the synthesis of polyamines (Pegg & Williams-Ashman 1969) which may have a specific role in *de novo* protein synthesis in the cell (Tanner 1967).

Some part of the effect of amino acid supply on the rate of protein synthesis is probably mediated through the influence of amino acids on the rate of synthesis and/or the degradation of RNA (Wannemacher 1972). The rate of RNA synthesis is reduced in

chicks fed amino acid mixtures devoid of tryptophan or methionine (Herbert *et al.* 1972). There is also evidence to suggest that the rate of RNA synthesis is depressed when the level of transfer RNA charging is depressed (Smulson & Thomas 1969; Vaughan & Hansen 1973).

It is likely that many of the ribosomes on the polysome are inactive (Munro *et al.* 1964; Scornik 1969) probably due to the presence of inhibitors of protein synthesis (Ragnotti & Aletti 1975). For example, von der Decken (1967) showed that if rats which had been fed a protein-free diet were allowed to consume ahigh-protein diet, the polysome profile was unchanged yet the ability of the system to incorporate amino acids was markedly enhanced. Similarly Henshaw *et al.* (1971) concluded that variations between individuals in the rate of protein synthesis were due entirely to differences in polyribosome activity. Murty *et al.* (1974) have suggested that translational factors may be involved in the regulation of protein synthesis in rats fed threoninedevoid diets, since the initiation factors in the livers of rats showed increased activity, associated with a higher rate of liver protein synthesis, while both initiation and elongation factors from skeletal muscle preparations exhibited decreased activity, associated with a reduced rate of protein synthesis in the muscle.

Although energy supply is likely to be an important factor in determining the rate of protein synthesis, any effects of energy *per se* are very difficult to isolate from other influences. Both insulin and glucose have been shown to be important in the aggregation of polysomes and amino acid incorporation into protein

(Wittman *et al.* 1969; 1971; Sidransky & Verney 1971; Wagle & Sampson 1975). Administration of the methionine analogue, ethionine, which depletes cellular ATP through formation of S-adenosyl ethionine (Farber 1973) is associated with a depressed level of protein synthesis in female rats (Baglio & Farber 1965) but not in male rats (Oler *et al.* 1969). These changes are paralleled by changes in polysome stability which appear to be hormone-dependent. Freudenberg & Mager (1971) have suggested that the inhibitory effects of ATP depletion on protein synthesis in cell culture *in vitro* are related to the inhibitory effects of an accumulation of ADP and AMP on peptide chain elongation, although in the longer term ATP depletion resulted in defects at the initiation step in protein synthesis.

The effects of the various hormones on the rate of protein synthesis in mammalian systems are complex (review by Manchester 1970). Some of the effects in relation to the glucocorticoid-induced elevation of certain hepatic enzymes have already been mentioned, although it must be remembered that an increase in the level of an enzyme may result from an increased rate of synthesis and/or a decreased rate of enzyme degradation. Several hormones are known to promote an increased rate of muscle protein synthesis (insulin, growth hormone, testosterone), although the mechanisms are obscure. Insulin promotes amino acid uptake by the muscle although this is probably not the cause of the increased rate of protein synthesis (Munro 1970; Wool 1972). Growth hormone has been shown to increase the rate of peptide chain elongation in a rat diaphragm system *in vitro* (Kostyo & Rillema 1971).

4.2 Protein degradation

The factors which control the rates of degradation of cellular proteins are poorly understood. Recent reviews on this subject are those by Schimke (1970), Recheigl (1971) and Goldberg & Dice (1974).

There is now general agreement that the kinetics of liver protein degradation are first order both for individual enzymes and other proteins and for intracellular organelles (see Schimke 1970). Dreyfus *et al.* (1960) suggested that muscle myosin may have a definite lifespan although this has been generally interpreted as an artifact resulting from reutilisation of the labelled amino acid. However, recently Millward and his coworkers (Millward *et al.* 1975a) have again raised the possibility that the kinetics of muscle protein degradation are non-random.

The simplest explanation for the vast differences in the rates of turnover of the various cellular proteins is that the degradation rates are determined by inherent differences in the susceptibility of the cell proteins to a general intracellular proteolytic system (Goldberg 1972). The lysosome system is a candidate for such a general proteolytic role, although its significance in normal protein turnover is still unclear (Lloyd & Beck 1974). Intracellular proteases, specific for the degradation of pyridoxal phosphate dependent enzymes, have recently been isolated (Katunuma 1973; Katunuma *et al.* 1975; Kominami *et al.* 1975; Banno *et al.* 1975). These proteases are not associated with the lysosome and are widely distributed in the tissues of the rat.

Cortisone administration causes a decline in total muscle mass, probably partly due to an increase in protein catabolism (Goldberg 1969). However, cortisone administration had no significant effect on any of the common lysosomal enzymes in the studies of Buchanan & Schwartz (1967), while the level of some intracellular free peptidases has been shown to increase in situations where protein degradation is increased (e.g. starvation, cortisone administration) suggesting that such intracellular peptidases may perform an important regulatory role in the control of protein metabolism (Schwartz *et al.* 1956; Rose *et al.* 1959). It is possible that the lysosomes may have an important role in the catabolism of plasma proteins, particularly in muscle. Such a mechanism could provide a valuable source of amino acids for the muscle.

Schinke (1970) has suggested that a given molecule exists in an equilibrium of various conformational states and that only in certain state(s) would it be subject to degradation by the relatively non-specific proteases and peptidases. Thus tryptophan stabilises tryptophan oxygenase by promoting the conversion of the apo-form of the enzyme to the more stable holo-form (Knox & Piras 1966). Litwack & Rosenfeld (1973) have proposed that the "tightness" of cofactor binding could be a major determinant of degrative rates *in vivo* since the relative rates of coenzyme dissociation correlated well with the half-life of several enzymes, e.g. serine dehydratase is stabilised by its cofactor, pyridoxal phosphate (Khairallah & Pitot 1968). Changes in physiological conditions could alter the availability of cofactors or the strength

of cofactor binding, inducing changes in the protein-ligand bond which would alter the equilibrium of the conformational states.

Several workers have shown that protein catabolism in liver slices is inhibited by conditions which inhibit energy metabolism (Simpson 1953; Steinberg & Vaughan 1956). Brostrom & Jeffay (1970) have shown that the ability of many inhibitors of energy metabolism to inhibit degradation is dependent on the level of tissue integrity, while Hayashi *et al.* (1973) have shown that ATP enhances the catheptic activity of intact lysosomes by promoting the uptake of proteins by the lysosomes. However, the role of the lysosome system in normal protein catabolism *in vivo* remains unclear.

4.3 Whole body protein turnover

The total turnover of protein in the animal body is the sum of the turnover of all of the individual tissue proteins. The various techniques used in the estimation of protein synthesis and degradation *in vivo* have been discussed by Schimke (1970) and Garlick & Millward (1972). An earlier review by Waterlow (1969) is concerned with the more general methodology of protein nutrition. Much of the experimental work in this field has been carried out by Waterlow's group, using rats as the experimental animal.

A number of factors may influence the extent of protein turnover in the various tissues, e.g. the distribution of protein synthesis between tissues may change with age (Millward & Garlick 1972). Thus in the young rat the synthetic rate of mixed muscle proteins is about 15% per day, whereas in the adult rat it falls to about 9%. In contrast the rate of liver protein synthesis changes

very little with age, but is greatly increased under poor nutritional conditions (Millward *et al.* 1975a, b).

In larger animals protein turnover in the skeletal muscle is probably a more important component than in the rat (Young 1970). The ratio of total muscle protein to liver protein in the rat is about 4:1 (Millward & Garlick 1972) whereas in the sheep the ratio is about 23:1 (Woodlock 1972) and in cattle about 30:1 (Tudor 1971). Buttery *et al.* (1975) have calculated the rates of protein synthesis in the sheep using a continuous infusion of ^{14}C-lysine. They have estimated that about 80g of muscle protein and 25g of liver protein are synthesised each day in a 50kg sheep. This is a ratio of liver to muscle protein synthesis of about 0.3 compared with a value of about 1.4 in the 300g adult rat (Millward & Garlick 1972) and 0.12 in the 75kg pig (Garlick & Swick 1974).

Drew & Reid (1975) have shown that there is a considerable loss of muscle protein in sheep fed at a low plane of nutrition in order to lose 12% of bodyweight over 5 weeks. In contrast, in the weanling rat, there is no detectable loss of muscle protein for up to 3 weeks even though bodyweight may fall by 30% (Millward 1972).

Such considerations highlight the problems in extrapolation of data derived from rats to larger animals, particularly in regard to protein turnover. However, Swick & Benevenga (1973) have shown that the turnover rates of some catabolic enzymes in the adult pig are very similar to those in the rat despite the fact that the fractional synthetic rates of liver protein are about 15% in the pig (Garlick & Swick 1974) and 50% in the rat (Millward & Garlick 1972).

Rats fed a protein-free diet exhibit a two-stage response in terms of protein turnover (D.J.Millward pers. comm). In the first stage there is a large loss of liver protein and an increase in the rate of degradation, while in the second stage albumin synthesis is greatly depressed and the turnover rate of liver proteins is increased to about 95% per day. Garlick *et al.* (1973) have studied protein turnover in the liver and muscle of meal-fed rats. There was no change in the rate of liver protein synthesis after eating nor after starvation for 48 hours yet liver protein mass was decreased indicating that the adjustment occurred in the rate of liver protein degradation. However, the rate of muscle protein synthesis increased after eating and declined during starvation over a 48 hour period. Millward (1970) had previously shown a reduced rate of synthesis of muscle proteins in starved rats or rats fed a protein-free diet. Although the myofibrillar proteins have a slower rate of turnover than the sarcoplasmic proteins, the rates of synthesis and degradation of the former are apparently more sensitive to protein and energy supply.

Goldberg (1969a, b) has studied protein turnover in work - and growth hormone-induced muscle hypertrophy and denervation - and cortisol-induced muscle atrophy. Both the technique used (pulse-chase) and the interpretation of the results, together with the fact that the animals were hypophysectomised, cast doubts on the interpretation of the data. However, Goldberg (loc cit) suggested that work-induced hypertrophy results from an increased rate of synthesis and decreased degradation whereas that induced by growth hormone results from an increased rate of synthesis only.

Both denervation and cortisol apparently caused an increased rate of degradation and reduced synthesis.

CONCLUSIONS

Of the areas discussed in this review, there are a number of aspects which deserve particular attention in ruminant studies.

The factors influencing the rate of increase of the muscle protein mass in relation to the rates of muscle protein synthesis and degradation in the ruminant are unknown. The studies of Eskeland and his coworkers (1973; 1974), in which sheep receiving intravenous infusions of glucose, acetic, propionic or butyric acids exhibited markedly different rates of nitrogen retention may be particularly relevant to these questions. Since the rate of protein turnover in the muscle is relatively high (Buttery *et al.* 1975), a slight reduction in the rate of protein degradation could result in a large increase in the rate of muscle protein accretion.

The effects of various hormones on the rates of muscle protein synthesis and degradation and on the partition of substrates between fat and protein synthesis in the animal is virtually an unknown area in studies of ruminant metabolism. The partition of nutrients into protein or fat synthesis (or heat production) is vital in terms of the production achieved from a ruminant animal, whether it be lactating or growing.

Contrary to a commonly held opinion that the composition of the body of the continuously growing ruminant is virtually unalterable by nutritional conditions, there is an increasing amount

of evidence that both the body protein content (Ørskov *et al.* 1971) and the body fat content (Tudor 1976) can be considerably affected by differences in the supply of nutrients reaching the duodenum, even in animals fed natural diets. The effects of providing protein and amino acid supplements which take the ruminant animal outside the nutritional constraints imposed by the rumen and the microbial population of the rumen and their bioenergetic limitations are still to be explored, although considerable effects of such supplementation on the voluntary intake of sheep have been reported (Egan 1965; Barry *et al.* 1973; see Section 2.4).

The timing of nutrient supply in relation to the efficiency of production is a further area in which very little is known. For example, decreasing the frequency of feeding of sheep fed wheat and/or roughage diets results in an increased rate of wool growth with little or no effect on the liveweight of sheep fed survival rations (Franklin 1952; Hill *et al.* 1968; Birrell & Bishop 1970; Langlands 1973). However it is quite possible that the body energy status of such animals is altered through changes in the body composition. Hill *et al.* (1968) suggested that the increased wool growth could be attributed to an increased flow of dietary protein to the intestines in the sheep fed once weekly as compared with those fed once daily. However, for a growing animal more frequent feeding may result in an improved efficiency of energy utilisation and an improved rate of growth (Reid *et al.* 1968).

The factors which determine the fate of an amino acid (e.g. gluconeogenesis, protein synthesis, catabolism, etc.), particularly in critical situations of amino acid supply, are poorly understood.

A knowledge of such factors is especially important in terms of an understanding of methionine metabolism in the sheep, since methionine has frequently been implicated as the "first-limiting" amino acid (see Section 2.4), both for wool growth and voluntary intake. Although the wool growth response to post-ruminal methionine supplementation is a well-established phenomenon, the studies have not generally been extended to investigations of the effects of methionine on voluntary intake, tissue protein accretion, methionine metabolism or the possible inter-relationships with other amino acids (in terms of metabolic interactions, as discussed for methionine and threonine in Section 3.2 and also in terms of possible effects when other essential amino acids, in addition to methionine, are also supplied). The studies to be reported in this thesis were initiated to investigate some of these aspects in sheep fed roughage diets and given post-ruminal supplements of methionine and other amino acids.

CHAPTER 1

The prediction of limiting amino acids for the sheep using a simple supply-demand model or the changes in plasma amino acids in response to a short-term intravenous glucose loading or a post-ruminal supplement of L-methionine.

INTRODUCTION

Apart from the many studies of wool growth responses to an increase in the tissue supply of the sulphur amino acids, methionine and cyst(e)ine (Marston 1935; Reis & Schinckel 1964; Barry 1973; Reis *et al.* 1973a) and the supplementation studies of Nimrick and his coworkers (Nimrick *et al.* 1970a, b), there has been very little work on the amino acid requirements of sheep.

As a first step in an investigation of responses to post-ruminal supplements of amino acids in sheep fed roughage diets, some predictions of the likely order of amino acid limitation were attempted. Three approaches were used. The first involved a simple model based on the supply of amino acids at the duodenum and a factorial estimate (Williams *et al.* 1954) of the amino acid requirement. A similar method has been used by Hutton & Annison (1972) and Egan & Walker (1975) to predict the amino acid requirements of ruminants in various physiological states. The other approaches utilised changes in the concentrations of plasma amino acids in response to a short-term intravenous glucose loading, or changes in response to an abomasal infusion of the probable "first-limiting" amino acid, methionine.

The experiments were designed to test the following hypothesis. The pattern of change in the plasma concentration of essential amino acids would reflect the pattern of induced amino acid deficiency when the animal was provided with an improved supply of substrate. The substrate supply was improved in two ways:

(i) increased supply of energy with an intravenous infusion of glucose;

(ii) an improved balance of amino acids by an abomasal infusion of the likely "first limiting" amino acid, methionine.

Glucose, rather than a volatile fatty acid, was chosen as the energy substrate for two reasons. In studies with sheep, an arterial infusion of glucose induced the greatest depressions in the plasma concentrations of essential amino acids when compared with infusions of acetic, propionic or butyric acids (Potter *et al.* 1968), while Eskeland *et al.* (1971) showed that an intravenous infusion of glucose promoted a higher nitrogen retention than an isocaloric infusion of any of the volatile fatty acids. It was recognised that glucose would be likely to induce an insulin response and that insulin promotes amino acid uptake by the muscle and also stimulates muscle protein synthesis (Manchester 1970). For this reason the glucose-induced removal of amino acids from the plasma may not provide a very accurate reflection of the effect of an improved energy supply *per se*, since muscle protein synthesis would be preferentially stimulated. Therefore a second plasma sample was taken some time after the completion of the glucose infusion. It was hypothesised that at this time, the pattern of change in plasma amino acids would provide a more accurate indication of the likely limiting amino acids,

since at this time any change would reflect an improved supply of energy rather than an insulin effect *per se*. The glucose infusion was calculated to provide a significant improvement in energy supply to the animal amounting to an increase of about 75% in the net energy supply to the tissues over the two hour period of the infusion.

Methionine was selected as the most likely amino acid to promote an improved nitrogen retention, since much published work has shown that an improved post-ruminal supply of methionine results in an improved animal performance in sheep fed roughage diets (Reis & Schinckel 1964; Barry *et al.* 1973).

EXPERIMENTAL

Animals

Eight Dorset x Merino wethers aged about 10 months, and of bodyweight 20-28 kg were used. Each had been surgically prepared with an abomasal canula and a ruminal fistula three months prior to the commencement of the experiment. The animals were housed indoors in individual pens.

Diets and feeding

The diets, each offered to four sheep, were a wheaten hay chaff (WHC) fed *ad libitum* and a chopped subterranean clover hay (CH) fed at a rate of 40g dry matter/$kg^{0.75}$/day. The feeds contained 1.3% and 3.7% nitrogen respectively, with apparent dry matter digestibilities of 55% and 78%, measured in a pretreatment period. The sheep received the experimental diets for at least one month prior to the commencement of the experiment.

The sheep were fed daily at 1030 hours (h) following the removal of residues. A mineral supplement (10g/day; Moir & Harris

1962) was sprinkled on top of the feed. Water was offered *ad libitum* to the sheep on the WHC diet and restricted to 1.2 litres/day for the sheep on the CH diet.

The feeding procedure was altered on days 8 and 11 of each period. At 1930h on days 7 and 10 residues (if any) were removed. On the following day all animals were fed hourly from 0730 to 1830h at a rate of 2g digestible dry matter/$kg^{0.75}$. Water (100 ml) was also offered hourly. On only two occasions during the experiment did any sheep fail to consume its complete hourly ration within the hour, and on virtually all occasions, it was consumed within 15 minutes. A quantity of feed and water to make up the feed offered to the normal daily amount was offered at 2000h.

Experimental design

The experiment consisted of three periods, each of 12 days. Period 1 was a control period in which each animal received an abomasal infusion of deionised water (140 ml daily). During periods 2 and 3, the sheep received an abomasal infusion of L-methionine at a rate of 0.08 and 0.16g L-methionine/$kg^{0.75}$/day respectively. The solutions were infused continuously using a peristaltic pump.

The glucose loading (I_0, no infusion; I_G, intravenous glucose) was carried out on days 8 and 11 of each period, two animals on each diet receiving the glucose infusion on day 8, with the treatments reversed on day 11. The glucose was infused at a rate of 0.5g D-glucose/$kg^{0.75}$ for two hours from 1215 to 1415h via a jugular catheter. The design and allocation of sheep to the treatments is given in Table 1.1. The catheters were inserted on the day preceeding the glucose loading, and kept patent with heparinised saline.

TABLE 1.1 Allocation of animals to the two glucose treatments (I_O no infusion, I_G intravenous glucose infusion for 2 hours at rate of 0.5g/kg$^{0.75}$/hour) in the three periods of the experiment. Samples were taken at three times (T_O, $T_{2.5}$, T_5) during days 8 and 11. The samples taken from the four animals on each diet and each methionine treatment were bulked by time of sampling and glucose treatment.

		Control		LMet		HMet	
Diet	Sheep	Day 8	11	8	11	8	11
WHC	(B	I_O	I_G	I_G	I_O	I_G	I_O
	(F	I_G	I_O	I_O	I_G	I_O	I_G
	(K	I_O	I_G	I_G	I_O	I_O	I_G
	(C	I_G	I_O	I_O	I_G	I_G	I_O
CH	(H	I_G	I_O	I_G	I_O	I_O	I_G
	(N	I_O	I_G	I_O	I_G	I_G	I_O
	(E	I_G	I_O	I_G	I_O	I_G	I_O
	(U	I_O	I_G	I_O	I_G	I_O	I_G

Blood samples were taken via the catheter at 1130 (T_0), 1400 ($T_{2.5}$), 1700 ($T_{5.5}$) and 1930h (T_8). The first 15 ml of blood taken from the catheter after the glucose infusion was completed were discarded. The blood was placed in heparinised tubes and centrifuged; the plasma was then drawn off and immediately frozen and stored at -15°C until required for analysis.

Infusion solutions

The L-methionine (Tanabe Seiyaku Co. Ltd., Tokyo) and D-glucose (BDH Chemicals) were made up to the appropriate concentrations with distilled water and then microfiltered using a Millipore filtration apparatus (0.22 μ filter). The sheep received 140 ml of the L-methionine solution daily and 60 ml of the D-glucose solution during the two hour glucose loading.

Measurements

The daily *ad libitum* dry matter intake was measured for the sheep fed the WHC diet.

The daily urine output of the sheep fed the CH diet was collected and measured and a subsample taken for nitrogen analysis on days 1 to 6 of each period. The urine was snap-frozen after flowing into containers packed in dry-ice. The urine was thawed and subsampled daily, and stored at -20°C until required for analysis for total nitrogen content.

Blood was sampled by catheter as previously described. Plasma glucose concentration was determined in individual samples, and plasma amino acids in samples bulked by times and treatments (i.e. 36 samples in all: 2 diets (WHC, CH) x 2 glucose infusions (I_0, I_G) x 3 sampling times (T_0, $T_{2.5}$, T_5) x 3 levels of methionine infusion

(control, 0.08g L-methionine/$kg^{0.75}$/day (LMet) and 0.16g L-methionine/$kg^{0.75}$/day (HMet)).

Analytical methods

The dry matter content of feed and faeces was determined by oven-drying at 85°C in a forced draft oven. Urine nitrogen was determined colourimetrically using a Technicon Auto-Analyser following digestion of the samples using a Kjeldahl technique (Munro & Fleck 1969).

Plasma glucose was measured in a Technicon Auto-Analyser using the standard ferricyanide technique. The plasma samples for amino acid analyses were treated with an equal quantity of 10% (w/w) trichloroacetic acid (TCA), centrifuged and the supernatant drawn off. The samples were then bulked on the basis of time and treatment, taking equal volumes of the sample from each animal. Plasma amino acid concentrations were determined by ion-exchange chromatography on a Technicon 'B' resin with a 20h separation using a sodium citrate buffer system. Norleucine (0.25 μ moles) was added as an internal standard.

Statistical analysis

No statistical analysis of the plasma amino acid data was possible since plasma samples had to be bulked for amino acid analysis due to limitations in the capacity of our analytical system.

CALCULATIONS AND RESULTS

Factorial Estimates of Amino Acid Requirements

Table 1.2 presents the basic intake and feed utilization data used in the calculations. The intake and digestibility data used

TABLE 1.2 Intake and utilisation data used in the calculations of amino acid supply and requirement

	Organic matter (g/day)			Nitrogen (g/day)			
	OMI	DOMI	OM apparently digested in stomachs[2]	NI	ADNI	Non-ammonia N[2] at duodenum	
A[1]	634	380	266	8.3	3.9	7.7	
B	732	439	306	9.6	4.5	8.8	(9.0)*
C	449	359	263	16.6	13.3	12.2	
D	449	359	263	16.6	13.3	12.2	(12.4)*

*includes 0.18g of infused methionine-N.

	Total apparently absorbed N (g/day)	Urine N (g/day)	N retention (g/day) Total	Wool	Tissue
A	3.9	3.3	0.6	0.8	-0.20
B	4.7	3.6	1.1	1.1	0
C	13.3	11.3	2.0	1.0	1.0
D	13.5	10.7	2.8	1.3	1.5

	Truly digestible amino acids at duodenum (g/day)[2]	Estimated total amino acid requirement to replace metabolic N losses and to support N gain. MFN[3]	EUN[4]	Tissue	Wool
A	30.6	15.0	7.5	-1.26	5.0
B	35.4 (37.4)*	17.5	7.5	0	6.9
C	48.8	7.5	7.5	6.25	6.25
D	48.8 (50.8)*	7.5	7.5	9.4	8.1

*includes 1.95g of infused methionine

OM - organic matter; DOMI - digestible OM intake;
N - nitrogen; ADNI - Apparently digestible N intake;
MFN - metabolic faecal N; EUN - endogenous urinary N.

1) The basal situations A and C are for a WHC diet and CH diet respectively. The situations B and D assume a response to an abomasal infusion of 0.16g L-methionine/kg$^{0.75}$/day in the sheep fed the diets WHC and CH respectively.
2) Values derived from the date of Egan (1974) for similar diets; values assumed to be proportional to intake.
3) MFN: calculated as 0.65g MF N/100g organic matter at the duodenum (Egan 1974); thus 2.4, 2.8, 1.2 and 1.2g N/day for situations A,B,C and D respectively.
4) EUN: assumed to be 100mg N/kg$^{0.75}$/day (ARC 1964) for a 28kg sheep.

in the calculations were those measured in an experiment in which two such diets were fed to young sheep. The situations A and C represented the basal diets, WHC and CH respectively. The situations B and D assumed a response to an abomasal infusion of 0.16g L-methionine/$kg^{0.75}$/day in sheep fed each of the diets. For the animals fed the WHC diet, the response was in terms of voluntary intake, overall nitrogen retention and wool growth, while for the sheep fed the CH diet, the response was in terms of nitrogen retention and wool growth.

The estimates of organic matter digestion in the stomachs and the non-ammonia nitrogen reaching the duodenum were calculated from the data of Egan (1974) for sheep for similar diets after correcting for differences in intake. The estimates of metabolic faecal nitrogen (MFN), and truly digestible amino acids at the duodenum were also derived from the data of Egan (1974). The basal nitrogen retention data were derived from values for similar sheep fed similar diets at the equivalent levels of intake. The estimates of the increse in nitrogen retention with methionine supplementation were based on data from a preliminary experiment. The values for wool growth were based on rates of wool growth published in the literature.

Table 1.3 gives the estimated values for amino acid supply and requirement for the four situations. The amino acid supply data were calculated from the data of Egan *et al.* (1975) for sheep fed similar diets. The values for tryptophan were from Fenderson & Bergen (1972). The amino acid composition data were from Block & Weiss (1956) and it was assumed that the tissue amino acid composition

TABLE 1.3 Calculated values for the supply of amino acids and the requirement and the ratio of the requirement to supply (R/S) for the various situations.

	Supply[2] (g/day)	Requirement (g/day) Tissue	Wool	Total[3]	R/S	
A[1]						
THR	1.76	1.02	0.33	1.35	0.77	
VAL	1.45	1.21	0.28	1.49	1.03	
CYS	0.17	0.30	0.50	0.80	4.7)	1.84[4]
MET	0.61	0.49	0.03	0.52	0.85)	
ILE	1.40	1.04	0.18	1.22	0.87	
LEU	2.38	1.81	0.40	2.21	0.93	
PHE	1.46	0.96	0.19	1.15	0.79	
TYR	1.42	0.74	0.25	0.99	0.70	
LYS	1.89	1.62	0.15	1.77	0.94	
HIS	0.60	0.57	0.05	0.62	1.03	
TRP	0.54	0.30	0.08	0.38	0.70	
ARG	1.52	1.36	0.47	1.83	1.20	
B[1]						
THR	2.03	1.20	0.45	1.65	0.81	
VAL	1.91	1.43	0.39[5]	1.82	0.95	
CYS	0.20	0.35	0.80[5]	1.15	5.8)	0.71[4]
MET	0.70 2.65[6]	0.58	0.04[5]	0.62	0.23)	
ILE	1.52	1.23	0.25	1.48	0.97	
LEU	3.12	2.13	0.54	2.67	0.86	
PHE	2.03	1.13	0.25	1.38	0.68	
TYR	1.66	0.88	0.34	1.22	0.73	
LYS	1.98	1.90	0.21	2.11	1.07	
HIS	0.80	0.68	0.07	0.75	0.94	
TRP	0.62	0.35	0.10	0.45	0.73	
ARG	1.61	1.60	0.64	2.24	1.39	

1) The basal situations A and C are for a WHC diet and CH diet respectively. The situations B and D assume a response to an abomasal infusion of 0.16g L-methionine/ $kg^{0.75}$/day in the sheep fed the diets WHC and CH respectively.

2) Supply of amino acids: calculated as truly digestible amino acids (see Table 1.2) using the amino acid pattern of duodenal digesta from Egan *et al.* (1975); diets A, B - mean of diets WS, WH; diets C,D - mean of diets LH, SC; (estimates of tryptophan - Fenderson & Bergen (1972).

TABLE 1.3 continued:

C[1]	Supply[2] (g/day)	Requirement (g/day) Tissue	Wool	Total[3]	R/S	
THR	2.82	1.02	0.41	1.43	0.51	
VAL	2.63	1.21	0.35	1.56	0.59	
CYS	0.27	0.30	0.63	0.93	3.44)	1.40[4]
MET	0.86	0.49	0.03	0.52	0.60)	
ILE	2.09	1.04	0.23	1.27	0.61	
LEU	4.30	1.81	0.49	2.30	0.53	
PHE	2.79	0.96	0.23	1.19	0.43	
TYR	2.29	0.74	0.31	1.05	0.46	
LYS	2.73	1.62	0.19	1.81	0.66	
HIS	1.10	0.57	0.06	0.63	0.57	
TRP	0.85	0.30	0.09	0.39	0.46	
ARG	2.22	1.36	0.58	1.94	0.87	
D[1]						
THR	2.82	1.17	0.53	1.70	0.60	
VAL	2.63	1.39	0.46[5]	1.85	0.70	
CYS	0.27	0.34	0.94[5]	1.28	4.74)	0.70[4]
MET	0.86	2.81[6] 0.56	0.05[5]	0.61	0.22)	
ILE	2.09	1.19	0.29	1.48	0.71	
LEU	4.30	2.07	0.64	2.71	0.63	
PHE	2.79	1.10	0.30	1.40	0.50	
TYR	2.29	0.85	0.40	1.25	0.55	
LYS	2.73	1.85	0.24	2.09	0.77	
HIS	1.10	0.66	0.08	0.74	0.67	
TRP	0.85	0.34	0.12	0.46	0.54	
ARG	2.22	1.56	0.76	2.32	1.05	

3) Amino acid composition of tissue proteins (mean of muscle and liver proteins) and wool: from Block & Weiss (1956). Metabolic nitrogen (MFN and EUN): assumed to derive from protein of the same composition as the tissue proteins.

4) Molar ratios on the basis of equivalent sulphur.

5) Methionine supplementation assumed to increase wool sulphur content from 2.76 to 3.20% based on the data of Barry & Andrews (1973).

6) Supplementation with 1.95g L-methionine/day.

was best represented as the mean of the values for liver and muscle. The metabolic faecal nitrogen and urine nitrogen losses were assumed to have arisen from protein of the same amino acid composition as the tissue proteins. Methionine supplementation was assumed to have increased the wool sulphur content from 2.76 to 3.20% (Barry & Andrews 1973) with methionine and cystine present in the same ratios.

The derived values for the ratios of requirement to supply (R/S) for the amino acids are given in Table 1.3. An R/S ratio of greater than 1 suggests that the amino acid requirement was greater than its supply and the highest ratio would be indicative of that amino acid which was relatively the most limiting in the particular situation.

In sheep fed either of the basal diets, the calculations suggested that the sulphur amino acids, methionine and cystine would be the amino acids most limiting for overall nitrogen retention (tissue and wool protein synthesis).

Experimental Results

Effects of methionine supplementation

The mean values for voluntary intake (WHC diet) or urine nitrogen excretion (CH diet) for the four sheep fed each diet and receiving the different levels of methionine infusion in three consecutive periods are given in Table 1.4. The individual animal values are given in Table 1.5.

If the changes in plasma amino acid patterns in response to methionine supplementation were to be interpreted in terms of the

TABLE 1.4 Mean values for the voluntary dry matter intake ($g/kg^{0.75}/day$) or urine nitrogen excretion (% of total N intake) for the four sheep fed each diet and receiving the different levels of methionine.

Infusion[1]	WHC Dry matter intake ($g/kg^{0.75}/day$)	CH Urine N excretion (% of feed+methionine N)
Control	62.4 ± 7.12[2]	0.705 ± 0.024
LMet	66.8 ± 10.86	0.698 ± 0.052
HMet	65.6 ± 11.28	0.687 ± 0.034

1) Control - water infusion; LMet - 0.08g L-methionine/$kg^{0.75}/day$; HMed - 0.16g L-methionine/$kg^{0.75}/day$.

2) Standard deviation

TABLE 1.5 Individual animal mean values for daily voluntary dry matter intake (g/kg$^{0.75}$/day) or urine nitrogen excretion (g/day) for the sheep fed each diet and receiving the different levels of methionine

Sheep	WHC diet: voluntary intake (g/kg$^{0.75}$/day) Control[1]	LMet	HMet
B	68.6 ± 2.3[2]	72.1 ± 2.7	66.7 ± 3.7
F	52.6 ± 2.4	56.1 ± 4.1	64.2 ± 2.9
K	66.6 ± 1.9	79.4 ± 1.6	79.4 ± 2.6
C	61.9 ± 7.0	59.6 ± 6.0	51.9 ± 3.3

Sheep	CH diet: urine N excretion (g/day) Control[1]	LMet	HMet
E	11.82 ± 0.18[2]	11.57 ± 0.05	11.59 ± 0.04
U	12.45 ± 0.19	11.52 ± 0.61	12.03 ± 0.08
H	11.35 ± 0.42	11.85 ± 0.11	11.69 ± 0.40
N	11.56 ± 0.19	11.91 ± 0.18	11.05 ± 0.41

1) Standard error of the mean (5 day values)

2) Control - water infusion; LMet - 0.08g L-methionine/kg$^{0.75}$/day; HMet - 0.16g L-methionine/kg$^{0.75}$/day.

original hypothesis (i.e. methionine stimulates protein synthesis), the animals receiving methionine would be required to have exhibited a positive response to the methionine supplement as compared with the control treatment in terms of voluntary intake and/or nitrogen retention, i.e. they would be required to have increased protein synthesis in response to a supplement of methionine.

The increase in the mean voluntary intake of the four sheep fed the WHC diet and the decline in the urine nitrogen excretion of the four sheep fed the CH diet when receiving the abomasal infusions of methionine may indicate a trend towards a positive response to the methionine. However on examination of the individual animal data (Table 1.5) it is apparent that two animals on each diet did exhibit marked positive responses to the additional methionine. This is taken as evidence that at least in some, if not in all, of the sheep, the abomasal supplement of methionine produced a positive response.

Plasma amino acids

Before considering the changes in plasma amino acid patterns brought about by the methionine and glucose treatments, the plasma amino acid levels in the control periods for animals fed the two diets may be examined (Table 1.6). The mean digestible energy intake for the animals fed the two diets was similar, although the protein yield at the duodenum in the animals fed the CH diet would have been about 70% higher than in those fed the WHC diet (Egan *et al.* 1975). This difference in the protein to energy ratio was associated with a higher level of both essential amino acids and total amino acids in the plasma of the sheep fed CH. Of the essential amino acids all but lysine and histidine were higher in animals fed the CH diet, while the plasma concentration of methionine was similar in both groups.

TABLE 1.6 Plasma concentrations of individual amino acids, the sum of essential (EAA), non-essential (NEAA), total and brached-chain (BrAA) amino acids and the glycine to other amino acid ratio (GLY/OAA) and the NEAA to EAA ratio (N/E) for the sheep fed the two diets. (Values are the means of three sampling times for bulked samples of plasma from four sheep on the control treatments on the days on which the sheep did not receive the glucose infusion).

	WHC	CH
	(Concentration μ moles/100ml plasma)	
THR	10.37	24.15
SER	12.24	12.40
GLU	22.46	21.94
GLY	56.37	62.58
ALA	16.01	21.78
VAL	18.65	31.29
MET	1.54	1.46
ILE	8.51	11.27
LEU	9.23	15.99
TYR	4.95	7.29
PHE	4.28	5.81
ORN	5.81	8.39
LYS	13.00	11.82
HIS	10.88	7.48
ARG	12.46	15.14
EAA	87.38	122.9
NEAA	117.8	127.1
Total AA	205.2	250.0
BrAA	36.39	58.55
GLY/OAA	0.379	0.334
N/E	1.35	1.03

EAA: THR, VAL, ILE, LEU, PHE, LYS, HIS, ARG.

NEAA: SER, GLU, GLY, ALA, TYR, ORN.

Total AA = EAA + NEAA, methionine omitted from all calculations.

TABLE 1.7 Ratios of concentrations of individual plasma amino acids in the methionine infusion periods to the concentrations in the control[1] period for the sheep fed the two diets. (Ratios are calculated from values which are the means of three sampling times for bulked samples of plasma from four sheep on the days in which the sheep did not receive the glucose infusion).

	WHC		CH	
	LMet	HMet	LMet	HMet
THR	73	83	77	53
VAL	80	77	83	64
ILE	83	66	81	70
LEU	71	54	82	65
TYR	96	82	96	76
PHE	99	100	90	84
LYS	75	59	80	62
HIS	77	61	95	87
ARG	95	92	84	82

[1] Actual concentrations of the amino acids in plasma during the control period are given in Table 1.6.
Control - water infusion; LMet - 0.08g L-methionine/$kg^{0.75}$/day; HMet - 0.16g L-methionine/$kg^{0.75}$/day.

Methionine infusion resulted in a depression in total plasma amino acids, the effect being more apparent for the essential amino acids (those classified as essential for the ruminant by Black *et al.* (1957) and Downes (1961). Figure 1.1 shows the mean effect of the abomasal methionine on the individual amino acids in plasma. Although the higher level of methionine usually resulted in a greater depression in the plasma amino acid concentrations, the mean values are shown in this graph. Table 1.7 gives the ratio of the concentrations of the essential amino acids in the plasma during the periods in which the sheep received the abomasal infusions of methionine, compared with the concentrations in the control period. The values are the means over the three sampling times on the days in which the sheep did not receive the glucose infusion.

Table 1.8 gives the values for the total plasma essential amino acids (EAA) for the sheep fed the two diets at the three sampling times and for the different methionine and glucose treatments.

The glucose infusions were administered over a 2 hour period and blood samples taken at the completion of the infusion must be compared with samples taken prior to the glucose loading. In this work, the effects of the glucose infusion were confounded with time since in some situations, there was a marked depression in plasma amino acid concentrations at $T_{2.5}$ in sheep not receiving the glucose infusion. This effect was particularly evident in the CH-fed animals and became more apparent with the two levels of methionine supplementation. By T_5 there was an apparent recovery in the plasma concentrations of these amino acids, although the level was still lower than at the T_0 sampling.

FIGURE 1.1 The effect of an abomasal supplement of L-methionine on the level of individual plasma amino acids (each value is the mean of three sampling times for plasma bulked from four sheep on the same treatments; data from the two methionine treatments have been included in the mean for the methionine treatment).

0----------0 control

0----------0 methionine infusion

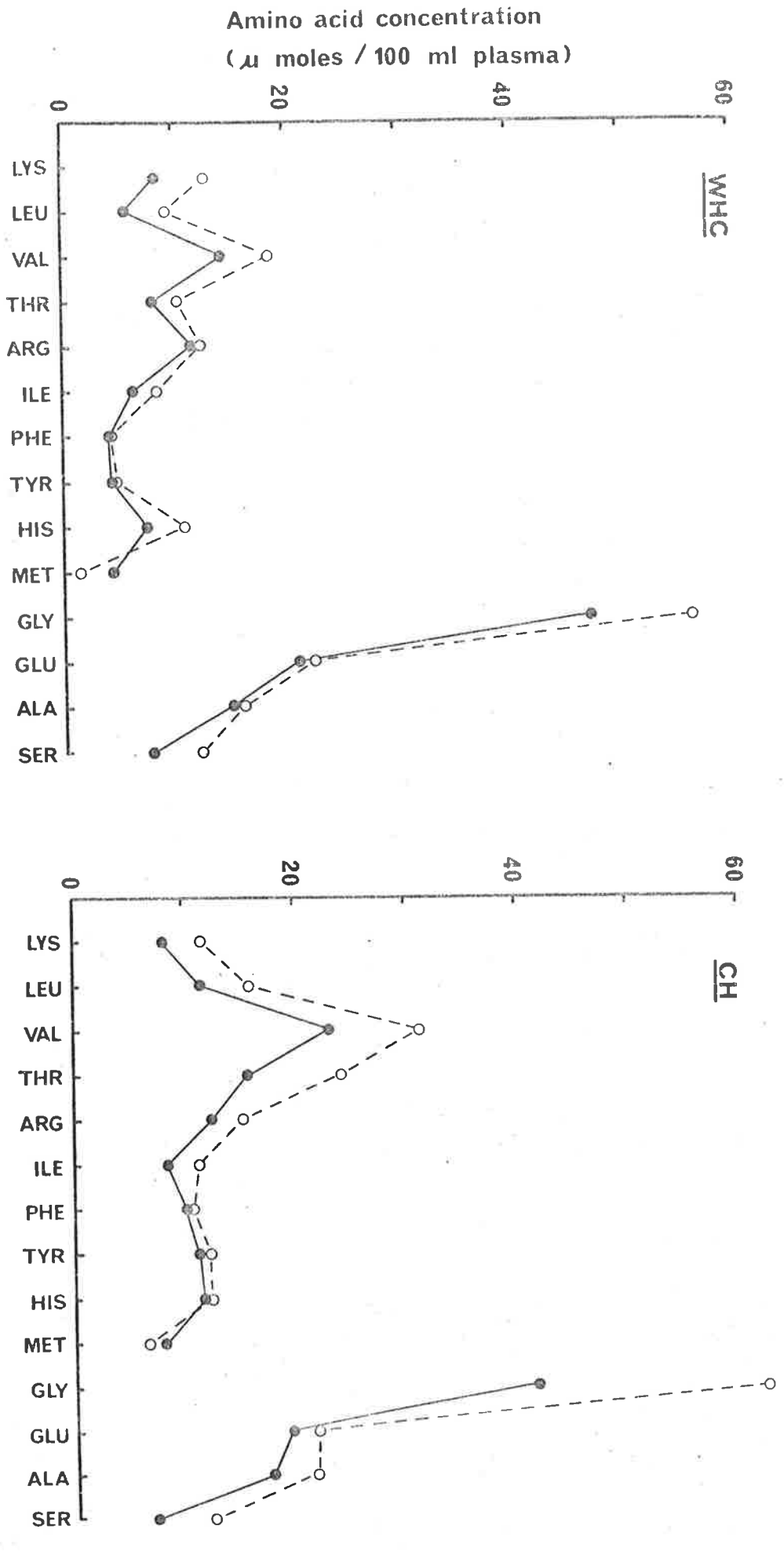

Amino acid concentration
(μ moles / 100 ml plasma)
0
20
40
60
LYS
LEU
VAL
THR
ARG
ILE
PHE
TYR
HIS
MET
GLY
GLU
ALA
SER
WHC
CH

TABLE 1.8 Total plasma essential amino acids (EAA[1]) for the sheep fed the two diets, at the three sampling times and for the different methionine and glucose treatments.

		WHC			CH		
		Sampling time					
		T_0	$T_{2.5}$	T_5	T_0	$T_{2.5}$	T_5
Control[2]	I_O[3]	85.1	90.2	86.9	124.7	115.0	129.2
	I_G	90.9	71.9	74.7	132.2	99.1	110.9
LMet	I_O	73.0	63.3	74.3	112.9	88.5	102.8
	I_G	78.4	68.9	58.8	94.8	64.8	72.6
HMet	I_O	62.8	61.3	66.8	106.1	67.0	74.3
	I_G	62.3	48.1	43.3	109.6	76.2	84.4

1) EAA: THR, VAL, ILE, LEU, PHE, LYS, HIS, ARG.

2) Control - water infusion; LMet - 0.08g L-methionine/$kg^{0.75}$/day;

3) I_O - no glucose infusion; I_G - intravenous glucose infusion.

The glucose loading during the period of water (i.e. no methionine) infusion resulted in a reduction in the concentration of most of the plasma amino acids, particularly the essentials. However, the glucose effect on total essential amino acids in the sheep receiving methionine was much less evident and in some cases (e.g. CH-high methionine) was apparently absent when compared with the no glucose infusion sample taken at the same time.

The relationship of the change in the plasma amino acid concentrations with glucose infusion to the amino acid composition of muscle was examined for each level of methionine infusion on each diet. The correlation coefficients for thse relationships for the sheep receiving and for those not receiving the glucose infusion are given in Table 1.9. The relationships for those animals given the glucose infusion but not receiving methionine are shown in Fig. 1.2.

The basic plasma amino data is given in Appendex Table 1.1.

DISCUSSION

Factorial Estimates of Amino Acid Requirements

For ruminants, factorial estimates of the amino acid requirement and the estimation of shortfalls in amino acid supply are arrived at only on the basis of many assumptions and are subject to many errors. However, the prediction of the likely order in which amino acids become limiting for productive functions in animals in a particular physiological state is a useful first step in the study of amino acid limitations to production. Certainly the attempt has highlighted a number of factors which point to deficiencies in the present knowledge.

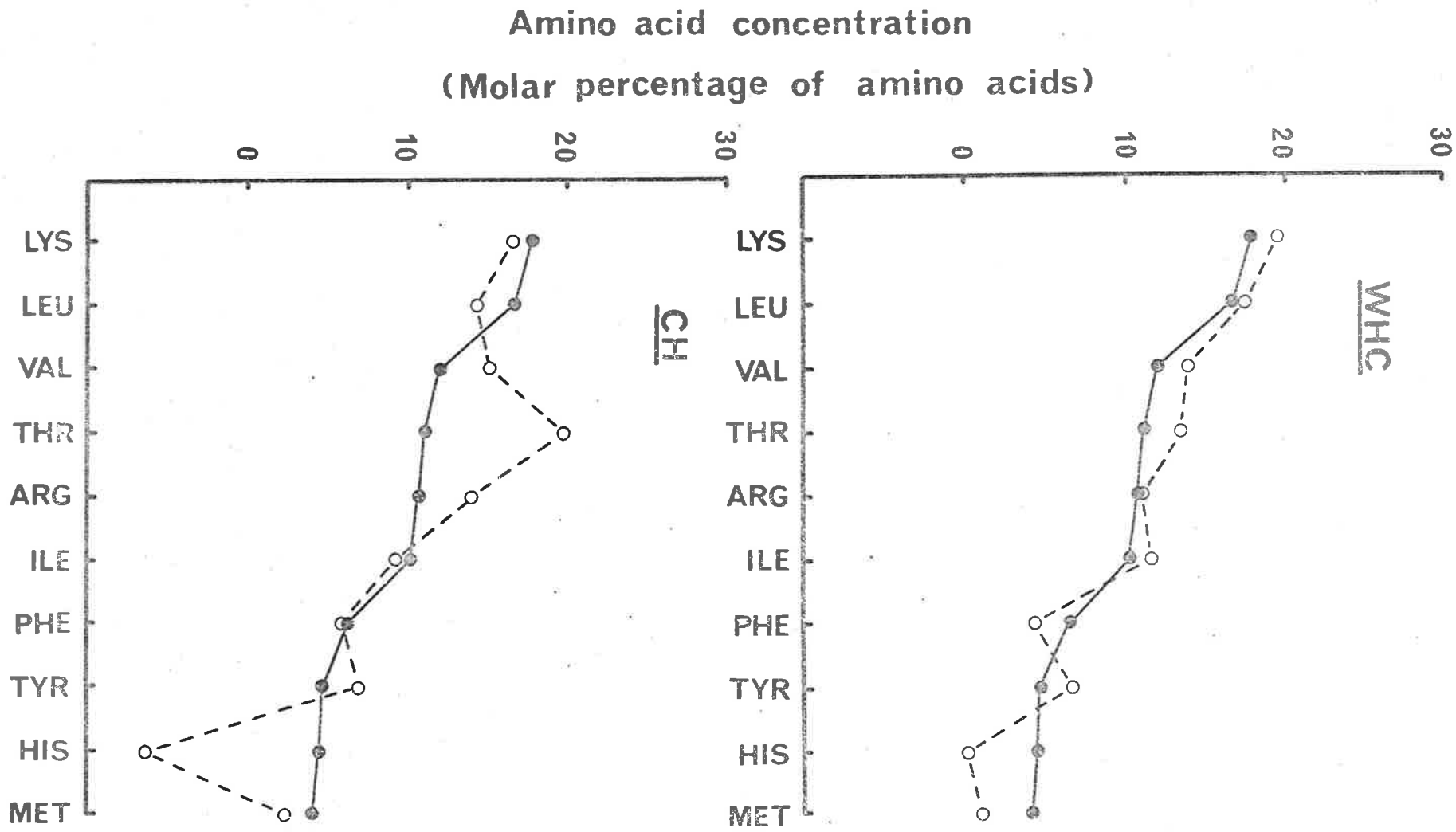
TABLE 1.9 Correlation coefficients for the relationships between the molar removal of amino acids from plasma and the molar amino acid composition of ovine muscle. (The molar removal of an amino acid is calculated as the difference between the concentration of the amino acid at T_0 and $T_{2.5}$. The amino acids included are lysine, leucine, valine, threonine, arginine, isoleucine, phenylalanine, tyrosine, histidine and methionine. All values are for bulked samples of plasma taken from the four sheep at any one time).

	WHC	CH
Sheep receiving glucose infusion (i.e. apparent glucose-induced removal of amino acids).		
Control[1]	0.955**	0.780**
LMet	0.452	0.646*
HMet	0.391	0.795**
Sheep not receiving glucose infusion (i.e. time-related apparent removal of amino acids).		
Control	-0.278	-0.167
LMet	0.079	0.499
HMet	-0.064	0.588

*P<0.05; **P<0.01

1) Control - water infusion; LMet - 0.08g L-methionine/$kg^{0.75}$/day; HMet - 0.16g L-methionine/$kg^{0.75}$/day.

FIGURE 1.2 Relationship between molar percentage composition of muscle (●——●) and the molar percentage removal of amino acids (0-----0) from plasma after the intravenous infusion of glucose in sheep fed the WHC and CH diets and not receiving methionine. (The molar removal of an amino acid is calculated as the difference between the concentration of an amino acid prior to the glucose infusion (T_0 sample) and the concentration in the sample taken at the end of the infusion ($T_{2.5}$ sample). The removal of an individual amino acid is then expressed as a percentage of the total removal of the amino acids, lysine, leucine, valine, threonine, arginine, isoleucine, phenylalanine, tyrosine, histidine and methionine. All values are from bulked samples of plasma taken from the four sheep at any one time).


Amino acid concentration
(Molar percentage of amino acids)
0
10
20
30
LYS
LEU
VAL
THR
ARG
ILE
PHE
TYR
HIS
MET
CH
WHC

In the present estimates there is no allowance for differences in the utilisation of amino acids. Such differences could arise from differential absorption of amino acids from the intestinal tract possibly due to interactions between amino acids at the site of absorption (Hume *et al.* 1972; Johns & Bergen 1973), or from differential catabolism of amino acids (Aguilar *et al.* 1972). The calculated requirement for individual amino acids could also be in error, due to differences in the amino acid pattern of endogenous losses (particularly MFN) as compared with the pattern of average tissue protein used in the calculations. Egan & Walker (1975) made allowance for some of these differences in the utilisation of the individual amino acids by comparing their calculated utilisation factor (requirement /supply) with those derived from published data for the rat, pig and chick when these species were fed a diet in which amino acids were provided in what was believed to be the optimal amounts and proportions.

The calculations suggested that the sulphur amino acids methionine and cyst(e)ine would be "first limiting" for production in the sheep fed the two diets. When additional methionine was provided (situations B and D), arginine was implicated as the next limiting amino acid. However, the animal probably has the capacity to synthesise some arginine in its own tissues (Black *et al.* 1957) in which case lysine would be implicated as the next limiting amino acid. For the sheep fed the CH diet, the low R/S ratios suggested that enery rather than any amino acid would be limiting production after the requirement for the sulphur amino acids has been met.

Experimental Data

General plasma amino acid patterns

Bergen *et al.* (1973) have proposed a unifying hypothesis to explain the relationship between plasma amino acids and diet in ruminants. Egan (1972) proposed that the glycine to other amino acid ratio (GLY/OAA) is a sensitive indicator of the yield of α-amino nitrogen at the duodenum. The differences between the CH- and WHC-fed animals in the present experiment lend general support to the observations of these workers, i.e. in the plasma of the animals fed the lower protein diet, there was a higher non-essential (NEAA) to essential (EAA) amino acid ratio (N/E), a higher GLY/OAA ratio, and a lower level of total amino acids, EAA, branched-chain amino acids (BrAA) and phenylalanine.

Methionine supplementation does appear to have modified some of the abovementioned relationships. Table 1.10 contains a summary of the relevant data for animals fed the two diets at the different levels of methionine supplementation (all values are the means of the three sampling times). The interpretation of the data for the WHC-fed animals may be complicated by a change in intake with methionine supplementation. In animals fed both diets, the plasma EAA, NEAA, total amino acids and BrAA were all depressed with the increasing level of methionine infusion. Phenylalanine concentrations were not altered in the sheep fed the WHC diet, but this was virtually the only plasma amino acid to be unaffected. In contrast the level of phenylalanine tended to decline in the CH-fed sheep, although this depression was entirely due to the marked depression in amino acid levels at $T_{2.5}$ and T_5 with methionine supplementation in the CH-fed sheep.

TABLE 1.10 Plasma amino acid parameters in the sheep fed the two diets at the three levels of methionine supplementation. (Each value is the mean of three sampling times (T_0, $T_{2.5}$, T_5) during the day on which the sheep did not receive a glucose infusion. Each sample is a bulked sample of plasma from the four sheep on each treatment at each time).

	Control[1]	LMet	HMet
Diet:WHC			
EAA[2]	87.4[8]	70.2	63.6
NEAA[3]	117.8	99.6	100.7
Total AA[4]	205.2	169.8	164.3
BrAA[5]	36.4	28.5	25.0
PHE	4.28	4.23	4.29
BrAA/OAA[6]	0.216	0.202	0.180
GLY/OAA[7]	0.379	0.387	0.405
N/E	1.35	1.42	1.58
Diet:CH			
EAA	122.9	101.4	82.5
NEAA	134.4	113.1	84.2
Total AA	257.3	214.5	166.6
BrAA	58.6	48.3	41.4
PHE	5.81	5.25	4.89
BrAA/OAA	0.295	0.291	0.330
GLY/OAA	0.321	0.307	0.252
N/E	1.09	1.12	1.02

1) Control - water infusion; LMet - 0.08g L-methionine/$kg^{0.75}$/day; HMet - 0.16g L-methionine/$kg^{0.75}$/day.
2) EAA: THR, VAL, ILE, LEU, PHE, LYS, HIS, ARG.
3) NEAA: SER, GLU, GLY, ALA, TYR, ORN.
4) Total AA: EAA + NEAA.
5) BrAA: VAL, ILE, LEU.
6) BrAA/OAA: BrAA/Total AA-BrAA.
7) GLY/OAA: GLY/Total AA-GLY.
8) AA concentrations in μ moles/100 ml plasma.

The effect of methionine on the plasma ratios tended to differ between diets. In the plasma of the sheep fed the WHC diet, the BrAA/OAA ratio tended to decline, while the GLY/OAA was unaffected and the N/E ratio tended to increase with the increasing level of methionine supplementation, due to the trend for the EAA to be depressed to a greater extent than the NEAA. However, apart from the GLY/OAA ratio (which declined with increasing methionine), neither of the other plasma relationships were consistently affected in the sheep fed the CH diet.

Although some of the plasma relationships were apparently altered by methionine supplementation, the unifying hypothesis of Bergen *et al.* (1973) has not been seriously challenged. The only point of dispute is the total AA levels since total AA levels were virtually identical in the sheep receiving the high rate of methionine infusion on both diets. Many of the plasma amino acid changes in the CH-fed sheep were due to the change in concentrations with time of sampling which were especially evident in the periods of methionine supplementation. The time of blood sampling may therefore be an important factor in interpreting plasma amino acid concentrations in relation to the hypothesis of Bergen and his coworkers (loc. cit.). However, the observation that the largest changes were at the highest level of methionine, while the changes with time in the control period were very small raises the possibility that the changes were a peculiar effect of methionine *per se*, rather than a general phenomenon associated with the diet. From a consideration of the general pattern of results, the N/E ratio would seem to offer the most potential as a reliable indicator of the protein to energy ratio

of the diet, although it would appear to bear little if any relationship to the amino acid balance of the diet.

Effects of methionine supplementation on plasma amino acids

In this section all of the data discussed refers to the changes in plasma amino acids in the periods in which methionine was infused compared with the control period. The data has been derived by taking means of the plasma concentrations for the three sampling times on the days in which glucose was not infused. Although no statistical analysis could be applied to the data, the changes in the concentrations of many of the amino acids in the plasma were considerable (Table 1.7).

Several mechanisms may have been responsible for the change in plasma amino acids with methionine supplementation. These include an increased withdrawal of amino acids from the plasma for protein synthesis, an increased catabolism of amino acids, hormonal effects or an effect on amino acid transport, possibly acting via hormonal influences. These possibilities will be briefly considered.

(i) Increased protein synthesis: this is a possibility since at least in some animals, there was an apparent positive response to the methionine supplement. A stimulation of tissue protein synthesis might be expected to result in the removal of essential amino acids from the plasma in approximately the same proportions as the amino acids are present in the tissue protein. These relationships were examined and the correlation coefficient for the relationship of the removal of essential amino acids with muscle composition was 0.61 ($P<0.05$) for the period of the low level of methionine infusion

and 0.59 ($P<0.06$) for the high methionine period in the sheep fed the WHC diet. For the animals fed CH, the correlations were lower and did not approach significance. The relationships were also examined using the amino acid composition of wool and the estimated overall amino acid composition of the whole body, but the correlation coefficients were low (<0.40). The results obtained for the animals fed the WHC diet in terms of the amino acid uptake/muscle composition relationship may provide some weak evidence that part of the change in plasma amino acids may have been due to a stimulation of protein synthesis through an increased supply of methionine.

(ii) Increased amino acid catabolism: methionine (as well as tryptophan or dietary protein) is known to induce a number of enzymes involved in amino acid catabolism in the rat and the chick (Harper 1968; Nakano *et al.* 1970). Tyrosine aminotransferase (Woodward *et al.* 1975; Bourdel *et al.* 1975) and threonine-serine dehydratase (Peraino *et al.* 1965; Cihak *et al.* 1975) have been well studied. However the amounts of methionine administered in these experiments were much higher (as a proportion of the diet or on a bodyweight basis when given intraperitoneally) than those administered to the sheep in the present experiment. An increased supply of protein *per se* probably does not induce threonine dehydratase in the sheep (see Review section 3.2), and therefore it is unlikely that methionine itself would have an effect on this enzyme. In conclusion, it is possible that an increase in amino acid catabolism may have been involved in some part of the depression observed in the plasma amino acid concentrations, although this awaits more detailed study.

(iii) Methionine administration results in an increased rate of corticosteroid secretion in rats (Girard-Globa *et al.* 1972) although the quantities administered were much higher than those used in the present work. Some part of the observed effect of methionine on the level of catabolic enzymes (Nakano *et al.* 1970) may be related to the increased level of corticosteroids (Girard-Globa *et al.*1972). A corticosteroid effect is most unlikely to have constituted an important component of the methionine response in the present experiment, since the corticosteroids increase muscle protein catabolism, which tends to result in an elevation in the level of most plasma amino acids (Lotspeich 1950; Kaplan & Shimizu 1963). Methionine does not appear to result in an increased level of plasma insulin in intact animals. Floyd *et al.*(1966) administered very high levels of methionine to adult humans and did not observe any change in plasma insulin in contrast to the effect of some of the other amino acids. Tao *et al.* (1974) fed sheep parenterally and found that with increasing levels of methionine, plasma insulin was initially reduced, but with further increases in methionine, insulin was unaffected. Growth hormone administration results in a depressed level of plasma amino acids and an increased uptake of amino acids by many tissues (Lotspeich 1950; Munro 1970), but any effects of methionine administration on growth hormone secretion have not been reported.

(iv) Amino acid transport: In addition to any possible involvement of hormones in the increased plasma uptake of amino acids in response to methionine, methionine may also influence the transport of other amino acids directly. For many years it has been known that methionine is involved in heteroexchange transport with other neutral amino acids (Christensen 1963; 1964), i.e. methionine has an affinity for two transport systems such that it is strongly concentrated by one mediator and then serves by exchange through the second mediator

(i.e. transport system) to drive the uphill transport of leucine and valine and similar amino acids. Such an effect of methionine could have been an important component of the plasma amino acid response in the present experiment, particularly in view of the marked effects of methionine on the level of the branched-chain amino acids in plasma.

Changes in the level of individual amino acids may have been more related to specific effects of methionine. The depressions in threonine and serine associated with methionine supplementation in animals fed both diets may have been due to the requirement for serine (which may be synthesised from threonine) in the synthesis of cystathionine from homocysteine in the catabolism of methionine via the transulphuration pathway. The greatly increased concentrations of taurine and cystathionine in the plasma of methionine - supplemented animals provide subjective evidence that the catabolism of methionine via the transulphuration pathway was increased in methionine-supplemented animals. The plasma concentration of methionine in supplemented animals was higher in those fed the WHC diet than in those fed CH diet. This may reflect a greater rate of methionine catabolism in animals fed the higher protein diet, particularly since Harper (1968) has shown that the level of enzymes which catabolise the essential amino acids are greatly increased at levels of dietary protein in excess of the requirement at least in the rat. However, Egan, Radcliffe and Fennessy (in preparation) were unable to detect any difference in the level of hepatic methionine adenosyl transferase between sheep given a poor quality low protein straw and those given a high protein lucerne chaff.

This is in marked contrast to the situation in rats where high protein diets do result in an increased activity of this enzyme (Finklestein 1967).

Effects of glucose infusion on plasma amino acids

Several workers have shown that the pattern of removal of essential amino acids from plasma following insulin (Lotspeich 1949) or glucose (Munro & Thomson 1953) administration is very similar to the amino acid composition of muscle. Glucose or propionic acid have similar effects in the sheep (Potter *et al.* 1968). These effects are not unexpected in view of the well-known effects of insulin on muscle protein synthesis although it is controversial as to whether the insulin induced amino acid uptake by muscle is independent of the effects of insulin on muscle protein synthesis (Munro 1970; Wool 1972).

The correlation between the glucose-induced removal of essential amino acids from plasma and the muscle composition was very high for the WHC-fed sheep not receiving methionine (r^2=0.91). The correlations for the CH-fed sheep were lower (r^2=0.42 to 0.63), although significant at all levels of methionine infusion. In contrast, in the WHC-fed animals receiving methionine the correlations were low and not significant. There are a number of possible reasons for the latter effect. The methionine-induced withdrawal of amino acids from plasma resulted in a greatly reduced concentration of several amino acids, perhaps to the extent that there was a much reduced capacity for the removal of amino acids from the plasma due to the involvement of the animal's own homeostatic mechanisms in maintaining plasma amino acid concentrations. A second possibility is that

methionine increased muscle protein synthesis to such an extent that there was little capacity for glucose to provide a further stimulation. Thus the factor limiting body protein synthesis for the sheep fed the WHC diet was probably protein *per se* rather than energy i.e. methionine had so improved the balance of amino acid supply to the tissues and hence the efficiency of amino acid utilisation, that for any further increase in protein synthesis to occur would have required more protein or more of one or more of the essential amino acids rather than more energy. The differences noted in the observations with the sheep fed CH as compared with those fed WHC may lend some support to these suggestions. In these sheep, the protein to energy ratio was such that energy rather than protein was probably limiting muscle protein synthesis in the methionine - supplemented animals. The high correlations between the glucose-induced removal of amino acids and muscle composition (Table 1.9) would tend to support this, although the marked depression at $T_{2.5}$ in the animals not receiving glucose must be borne in mind.

No explanation can be offered for the depression in the plasma amino acid concentrations at $T_{2.5}$ and to a lesser extent T_5 in the CH-fed animals receiving methionine ("time-related removal"). The sheep had received five feeds at hourly intervals prior to the T_0 blood sampling. On a highly fermentable diet such as the CH, the rate of fermentation and hence volatile fatty acid uptake from the rumen could be expected to have been relatively steady by this time. The methionine infusion was also provided continuously. The mechanism of the "time-related removal" was apparently the result of an interaction with the level of methionine infusion. The correlation coefficients for the "time-related removal" of the amino acids from plasma with the molar composition of muscle

(Table 1.9) were lower than those for the glucose-induced removal (r^2=0.25 and 0.35 compared with 0.42 to 0.63).

An indication as to whether the methionine- and glucose-induced changes in plasma amino concentrations were related to an increased rate of protein synthesis may be obtained from a comparison of the plasma amino acid changes brought about by these treatments.

The changes in the plasma concentrations of the essential amino acids evident in the first plasma sample, taken immediately following the glucose infusion in the control period, were strongly related to the muscle composition (Table 1.9 and Figure 1.2). Such a rapid response suggests a change in the uptake of amino acids and supports the suggestion that insulin was involved. However, any change at the later sampling when glucose levels in the plasma had returned to normal (Appendix Table 1.2) would be more likely to represent an effect of an improved energy supply *per se* rather than an insulin-mediated effect and therefore might be expected to reflect an uptake of amino acids into pathways of protein synthesis, rather than an effect on the transport of amino acids.

The correlation coefficients for the relationships between the delayed effect of glucose in the control period (i.e. T_5-T_0 expressed as the molar removal of each amino acid) and the effect of methionine (mean of LMet and HMet minus the control period values, each as a mean of three sampling times, and expressed as molar removal of each amino acid) are given in Table 1.11. The relationships plotted on the basis of the molar percentage removals of amino acids are shown in Figure 1.3.

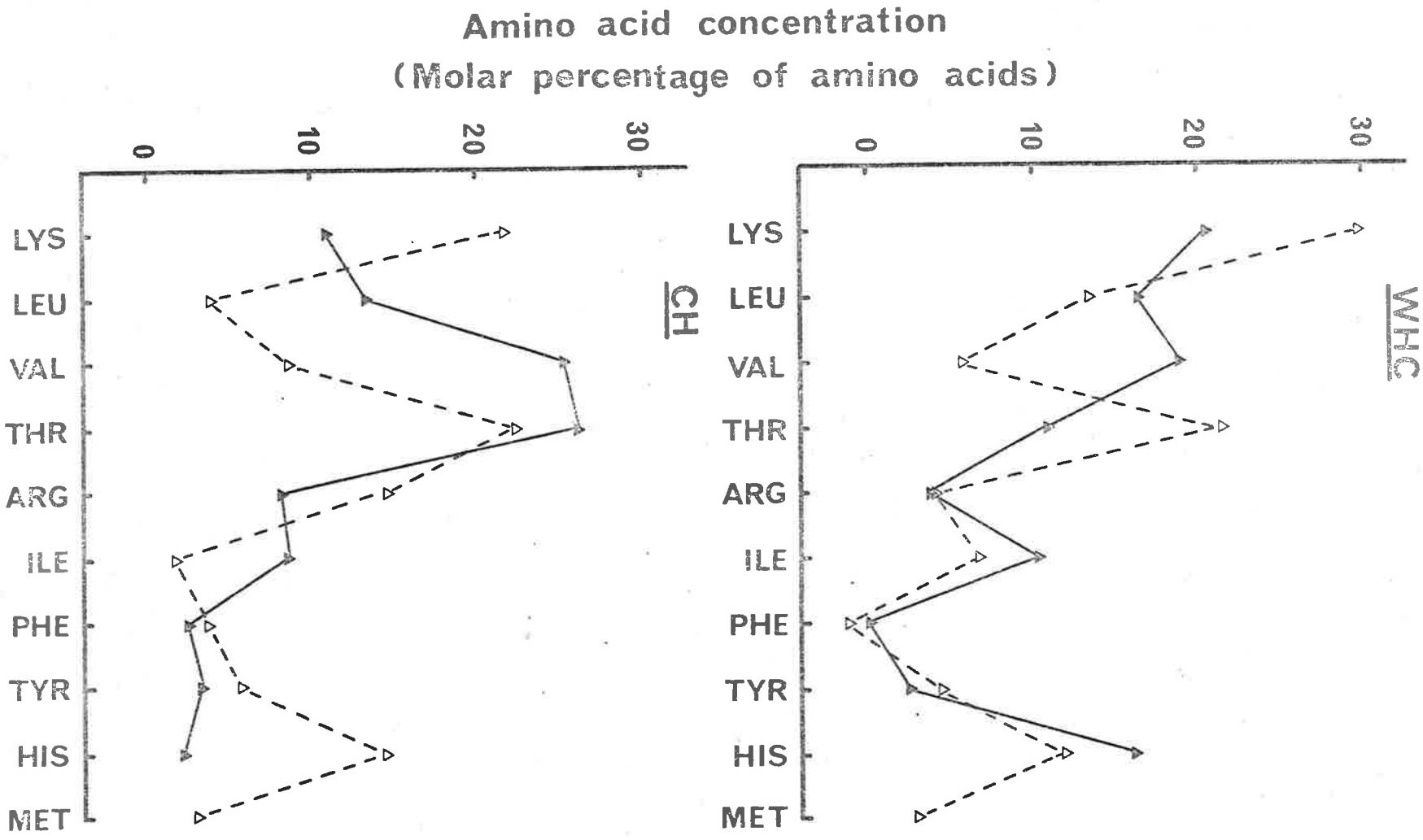
TABLE 1.11 Correlation coefficients for the relationship between the delayed effects of glucose on the molar removal of amino acids from plasma and the effect of abomasal methionine supplementation on the molar removal of amino acids from plasma. (The delayed effect of glucose on the molar removal of an amino acid is calculated as the difference between the concentration of the amino acid at T_0 and T_5 on the days in which the sheep not receiving the abomasal methionine infusion received the intravenous glucose infusion. The effect of methionine supplementation on the molar removal of an amino acid is calculated as the difference between the mean plasma concentration in the methionine infusion period and the concentration in the control period where the mean concentrations are the means of the three sampling times on the days on which the sheep did not receive the glucose infusion. The amino acids included are lysine, leucine, valine, threonine, arginine, isoleucine, phenylalanine, tyrosine and histidine. All values are for bulked samples of plasma taken from the four sheep at any one time).

Comparison of delayed glucose effect with	WHC	CH
LMet[1]	0.678*	0.376
HMet	0.637*	0.346
Mean Met	0.677*	0.553

1) LMet - 0.08g L-methionine/$kg^{0.75}$/day;
HMet - 0.16g L-methionine/$kg^{0.75}$/day;
Mean Met - mean of LMet and HMet.

* $P<0.05$

FIGURE 1.3 Relationship between the molar percentage removal of amino acids from plasma with methionine infusion (▲——▲) and the delayed effect of glucose on the molar percentage removal of amino acids from plasma (Δ-----Δ) in sheep fed the WHC and CH diets. (All calculations for methionine effects are performed on values, each of which is the mean of three sampling times, taken during the day in which the sheep did not receive the intravenous infusion of glucose. The methionine treatment is the mean of the two levels of methionine infusion. The molar removal of an amino acid is calculated as the difference between the methionine treatment and the control treatment. The delayed molar removal of an amino acid following glucose infusion is calculated as the difference between the concentration of an amino acid prior to the glucose infusion (T_0 sample) and the concentration in the sample taken 2.5 hours after the end of the infusion (T_5 sample). The molar percentage removal is calculated in the same manner as in Fig. 1.2 except that the effect on methionine is omitted from the methionine treatment. All samples are bulked samples taken from the four sheep in each treatment).


Amino acid concentration
(Molar percentage of amino acids)
0
10
20
30
LYS
LEU
VAL
THR
ARG
ILE
PHE
TYR
HIS
MET
CH
WHC

There was some suggestion of a relationship between the change in plasma essential amino acid patterns in these two situations for the animals fed the WHC diet (r^2=0.46) although the changes in lysine, valine and threonine, in particular, did differ quite considerably between the two treatments. However, the general correspondence between these changes does lend some support to the suggestion that the methionine-induced removal of amino acids from plasma in the sheep fed the WHC diet may have been partly associated with an increased rate of protein synthesis.

Plasma amino acids for the prediction of limiting amino acids

There are a number of possible methods of interpreting plasma amino acid changes for the prediction of limiting amino acids. In the interpretation of the present work two methods have been used after treatments in which the substrate supply to the tissues of the animal was altered.

The first method of interpretation is a simple plasma amino acid ratio (PAR) which is the ratio of the post-treatment concentration of an amino acid to the pre-treatment concentration, the amino acid with the lowest ratio being classified as "first limiting". The second method of interpretation involves calculation of the ratio of the molar removal of an amino acid (as a percentage of total removal of the selected essential amino acids) to the molar percent composition of muscle. The plasma amino acid depression method (PAD) thus defines the "first limiting" amino acid as that having the highest ratio. Ideally, the denominator used in the calculations for the PAD method should be the amino acid requirement, but this is clearly not possible for ruminant studies. The PAR and

PAD ratios for the essential amino acids in the sheep fed the WHC diet are given in Table 1.12. The values used in the calculations were those at T_5 following a glucose infusion (compared with T_0), and the mean plasma concentrations over the three sampling times and the two levels of methionine infusion (compared with the plasma concentrations for the sheep not receiving methionine, calculated as the mean of the three sampling times over the two levels of methionine supplementation on the days in which the animals did not receive the glucose infusion).

The two PAR predictions suggested that lysine and leucine would be the amino acids most likely to be limiting for protein synthesis in sheep fed the WHC diet whereas the PAD methods clearly implicated histidine as the likely "first limiting" amino acid. Even though it is probable that methionine supplementation did result in an improved nitrogen utilisation, the glucose loading did not reduce plasma methionine to such an extent that it was implicated as a likely limiting amino acid by the PAD method, although the PAR technique did suggest that methionine may have been "second limiting". However, for four of the amino acids the PAR ratios were all similar (0.66 to 0.75). The extent of the change in plasma histidine concentrations may have been an overestimate, since in the amino acid analytical system, the peak measured as histidine was not pure. The contaminant was not positively identified, but it was not tryptophan nor was it a methyl-histidine alone.

The accuracy of predictions of limiting amino acids for ruminants fed various diets and in different physiological states must be in question until such time as experiments designed to

TABLE 1.12 Plasma amino acid ratios (PAR[1]) and plasma amino acid - muscle composition ratios (PAD[2]) as indices of limiting amino acids for the sheep fed the WHC diet.

	PAR_G	PAR_M	PAD_G	PAD_M
THR	0.73	0.78	2.00	0.90
VAL	0.94	0.79	0.71	1.46
MET	0.71	-	0.69	-
ILE	0.85	0.75	0.18	0.95
LEU	0.75	0.63	0.23	0.90
PHE	1.05	1.00	0.57	0
LYS	0.66	0.67	1.22	1.08
HIS	0.81	0.69	3.08	3.19
ARG	0.94	0.94	1.36	0.33

1) PAR: The ratio of the post-treatment concentration of an amino acid to the pre-treatment concentration.

$$PAR_G = \frac{\text{(AA) Control } I_G\ T_5}{\text{(AA) Control } I_G\ T_O}$$

$$PAR_M = \frac{\text{(AA) Met mean}}{\text{(AA) Control}}$$

2) PAD: The ratio of the molar removal of an amino acid (as a percentage of the total removal of the selected essential amino acids) to the molar percentage composition of muscle.

$$PAD_G = \frac{[\text{(AA) Control } (T_5 - T_0)] \,/\, [\text{Total (AA) Control } (T_5 - T_0)]}{\text{(AA) Muscle / Total (AA) Muscle}}$$

$$PAD_M = \frac{[\text{(AA) Met mean - (AA) Control}] \,/\, [\text{Total (AA) (Met mean - control)}]}{\text{(AA) Muscle / Total (AA) Muscle}}$$

where (AA) is the concentration of the particular amino acid; total (AA) is the sum of the selected essential amino acids; Met mean is the mean concentration of the amino acid(s) for the two methionine treatments and the mean of the three sampling times in sheep not receiving the glucose infusion.

assess the accuracy of the predictions are carried out. Potter *et al.* (1972) have attempted to develop a technique for the assessment of limiting amino acids for sheep, based on duodenal infusion of different proteins. However when applied to the data of Nimrick *et al.* (1970), the technique failed to identify the limiting amino acids (as defined using nitrogen balance responses to abomasal infusions of amino acids).

Any prediction of the limiting amino acids for the sheep fed the CH diet in the present work is considered of little value since it is very likely that energy rather than any amino acid would be limiting protein synthesis following an increase in the supply of methionine.

CONCLUSIONS

The factorial estimates of amino acid requirements and the estimated deficiencies in the supply of amino acids, although subject to many errors, suggested that methionine (and cystine) were likely to be the "first-limiting" amino acids for the sheep fed the two diets in the present experiment. Subsequently, abomasal supplementation with methionine resulted in an apparent positive response in terms of an increased intake or a reduced urinary nitrogen excretion in some animals.

The changes in the plasma amino acid pattern in response to a change in the supply of substrate (i.e. glucose and/or methionine) were probably of metabolic significance. However the use of plasma amino acid changes to predict the likely limiting amino acids failed to provide any clear indication as to those amino acids most likely to be limiting for protein synthesis.

CHAPTER 2

Nitrogen retention and voluntary intake of young sheep fed roughage diets and given post-ruminal supplements of L-methionine and other amino acids.

INTRODUCTION

The experiments to be reported in this chapter arose out of the observation that there were apparent marked differences between animals in the response to low levels of methionine given per abomasum in the experiment reported in Chapter 1.

The possibility of between animal variability in responses to nutritional treatments is not usually considered. In traditional experimental designs (e.g. Latin square, factorials, etc.) any between animal variability in the measured parameter is usually allocated to the error term. This variability may be due to the effects of time, carryover effects of a previous treatment, or basic variability between animals. These experimental designs do not possess the capacity for testing whether the basic differences between animals in the parameters measured and in the responses of the animals to the treatment are indicative of real differences between the animals. The differences are regarded as indications of random variability and are allocated to the error term. In many cases, it would seem that the possibility that such variability is a real, rather than a random phenomenon merits special attention.

In the work to be reported here, each animal was used as its own control. However, this has the disadvantage that, due to the

likelihood of carryover effects between treatments, the control period must come first. Treatments must also be carried out such that carryover effects are minimised. For this reason, the amount of the methionine supplement was increased with time such that a period of high methionine supplementation followed a period in which methionine was given at a lower level. Similarly supplements of other amino acids were also added to the supplement given in the previous period. It must be noted that where animals act as their own control and receive stepwise increases in the treatment, time may be the factor producing the response. The more frequently repeated the response the more acceptable is the interpretation that the observed response is due to the treatment *per se*.

In order to evaluate the repeatability of the response to the treatment within any one animal, two sheep were given a sequence of increasing levels of methionine on two occasions. An attempt was also made to estimate the length of the time taken for the animals to re-establish at pre-infusion control levels following a sequence of methionine infusions. A dose response curve was obtained in this experiment and in a further experiment in which methionine was infused at levels from low to toxic amounts. In two experiments, the response of sheep to threonine or threonine and leucine in addition to a basal methionine infusion was examined.

Response criteria used in the various experiments included voluntary intake, nitrogen retention and urine nitrogen excretion.

EXPERIMENTAL

Animals, diets and feeding

Experiment 2/1: Two Dorset x Merino wethers were used. At the start of the experiment they were aged about 20 months and weighed 30 kg (Eli) and 31 kg (Eunuch). They were fed a diet of equal parts of wheaten straw and wheaten hay chaff *ad libitum* (0.75% nitrogen in mixture). These sheep had previously been used in the experiment reported in Chapter 1.

Experiment 2/2: Three Dorset x (Dorset x Merino) wethers, aged about 8 months and weighing 25.5 kg (761), 30.5 kg (663) and 29.5 kg (748) at the start of the experiment were used. They were fed the same diet *ad libitum* as the sheep in Experiment 2/1. They had been fitted with abomasal tubes (Appendix 2) at least one month prior to the start of the experiment.

Experiment 2/3: Three Dorset x (Dorset x Merino) wethers, aged about 10 months and weighing 34.0 kg (675), 34.5 kg (744) and 35.0 kg (769) at the start of the experiment were used. They were fed a diet of wheaten hay chaff *ad libitum* (1.05% nitrogen). They had been fitted with abomasal tubes at least one month prior to the start of the experiment.

Experiments 2/1, 2/2, 2/3: Water was freely available at all times. A mineral mix (8 g/day; Moir & Harris 1962) was sprinkled on top of the feed. Vitamins A and D_3 (APAC; Nicholas Pty. Ltd.) were given with the feed on day 1 of each period.

Feed was offered daily at 1000h following the removal of residues. Fresh allowances of c.20% in excess of the expected daily intake were offered. All sheep were housed indoors in metabolism pens and were fitted with faecel harnesses. All sheep were weighed at intervals during the experiments (prior to feeding in the morning).

Experimental design

Experiment 2/1: The experiment consisted of 13 periods which, except for period 7, were of 11 days duration. The two sheep received an abomasal infusion of 0, 0.7, 1.4 and 2.1g of L-methionine/day in periods 1, 2, 3 and 4 respectively. The sequence was repeated in periods 8 - 11. During the periods 5 - 7 the sheep did not receive any infusion. In period 12, the sheep received an infusion of 2.1g L-methionine plus 1.4g L-threonine/day while in period 13 an additional daily supplement of 2.1g L-leucine was administered with the methionine and threonine.

Experiment 2/2: The original design was of four periods, each of 28 days. Period 1 was a control period (water infusion) while in periods 2, 3 and 4 the animals received abomasal infusions of 1.4, 4.2 and 8.4g of L-methionine/day respectively. On day 17 of each period, the sheep were given an intravenous infusion of L-^{35}S-cystine and cystine entry rate measured, and on day 19, liver biopsy samples were obtained. The entry rate and biopsy data will be reported separately (Egan, Radcliffe and Fennessy, in preparation). Various modifications of the basic experimental design, involving threonine supplementation were made as the experiment progressed. Sheep 761 died during biopsy in period 3. A biopsy was not performed on sheep 748 in period 4.

Experiment 2/3: The experiment consisted of two periods, each of 16 days. Period 1 was a control period (water infusion) while in period 2, the sheep received an abomasal infusion of 1.6g of L-methionine/day.

Measurements

Experiment 2/1: The daily *ad libitum* dry matter intake was measured. Urine was collected daily into sulphuric acid (final urine pH<2), the urine weighed and a daily subsample taken and stored at -20°C. Faeces were collected and bulked over a 7 day period (days 6 - 12) and the apparent digestibilities of dry matter, organic matter and nitrogen determined. The nitrogen balances of the individual animals on the various treatments were calculated using the feed intake for days 3 to 9, the urine collected on days 5 to 11, and the faeces collected on days 6 to 12.

Experiment 2/2: Daily dry matter intake was measured, and urine and faeces collected as in Experiment 2/1, except that the nitrogen balances were calculated over a 10 day period before the biopsy and a 7 day period after the biopsy. The balance calculations used feed intake on days 5 to 14, the urine collected on days 7 to 16 and the faeces collected on days 8 to 17 for the pre-biopsy nitrogen balance, and the feed intake for days 20 to 26 and the collected urine and faeces on days 22 to 28, and 23 to 29 respectively for the post-biopsy balances.

Experiment 2/3: Daily *ad libitum* dry matter intake was measured and urine and faeces collected as in Experiment 2/2. Nitrogen

balance was calculated over the same period as the pre-biopsy balance in Experiment 2/2.

Composite samples of feeds and residues from each experiment were retained for analysis.

Analytical methods

The dry matter content of feed, faeces and residues was determined by oven drying at 85°C and organic matter by ashing at 550°C in a muffle furnace.

Nitrogen concentration in subsamples of dried feeds and residues and wet faeces and urine was determined colourimetrically using a Technicon Auto Analyser following digestion of the samples using a Kjeldahl technique (Munro & Fleck 1969).

Amino acid infusions

All amino acids used were obtained from Tanabe Seiyaku Co. Ltd., Tokyo. The amino acid solutions were replaced every 2 days, and adjusted to pH 3.5 with hydrochloric acid to reduce the risk of microbial contamination. The infusion lines were regularly disconnected and flushed with a sodium hypochlorite-sodium chloride solution after which they were washed with distilled water and the infusion recommenced.

RESULTS

Experiment 2/1

The voluntary intake and urine nitrogen excretion data for the two animals in each period of the methionine sequences are given

in Table 2.1. Figure 2.1 presents the mean voluntary intakes and the urine nitrogen excretion, the latter as a percentage of total nitrogen supply (intake plus infusate nitrogen).

The two sheep responded in different directions in terms of voluntary intake, Eunuch significantly increasing voluntary intake in response to methionine, while Eli's voluntary intake was depressed by the methionine infusions. The same general responses in intake and urine nitrogen excretion were apparent in each sequence, despite the differences in the voluntary intake between the two control periods.

Table 2.2 gives the mean daily nitrogen retention and the urine nitrogen excretion (ratio to apparently absorbed nitrogen) for the two animals in each period of the methionine sequences. The faeces nitrogen content was measured on bulked samples for each treatment and hence an estimate of error could not be derived. The ratio of urine nitrogen excretion to apparently absorbed nitrogen is an expression of the relative inefficiency of utilisation of absorbed nitrogen. However, it is also dependent on the faecal nitrogen output.

The values for the apparent digestibility of organic matter and nitrogen for the two animals in each period are given in Table 2.3. There was considerable variability in the estimates of nitrogen digestibility, this being reflected in the variability in the estimates of nitrogen balance and in the ratio of the urine nitrogen excretion to apparently absorbed nitrogen.

Table 2.4 presents the voluntary intake, urine nitrogen excretion and the apparent nitrogen retention data for the two sheep over the last three periods of the experiment (the infusions of

TABLE 2.1 (Experiment 2/1): Mean daily voluntary dry matter intake (DMI) and urine nitrogen (N) excretion for Eli and Eunuch during the two methionine infusion sequences.

Methionine infused (g/day)	Sequence A DMI (g/day)	Sequence A Urine N (g/day)	Sequence B DMI (g/day)	Sequence B Urine N (g/day)
Eli				
0	711 ± 4.3[1]	2.07 ± 0.081	603 ± 8.4	1.80 ± 0.113
0.7	654 ± 46.3	1.46 ± 0.056	578 ± 16.1	1.47 ± 0.143
1.4	643 ± 10.0	1.56 ± 0.051	596 ± 6.0	1.23 ± 0.032
2.1	593 ± 7.2	1.35 ± 0.028	551 ± 17.6	1.34 ± 0.019
Eunuch				
0	595 ± 9.9	2.30 ± 0.083	528 ± 11.1	2.22 ± 0.054
0.7	690 ± 8.4	1.41 ± 0.024	625 ± 14.9	1.63 ± 0.084
1.4	703 ± 13.5	1.48 ± 0.048	700 ± 5.0	1.42 ± 0.077
2.1	659 ± 15.3	1.32 ± 0.059	692 ± 19.4	1.32 ± 0.033

1) ± standard error of the mean (n = 7)

FIGURE 2.1 (Experiment 2/1): Effect of level of methionine infusion on (a) voluntary food intake (days 3 to 9 ± standard error of the mean) and (b) urine nitrogen excretion as a percentage of total nitrogen supply (supply is the sum of feed and infusate nitrogen; urine collected on days 5 to 11 ± standard error of the mean) in the two methionine infusion sequences (●——● sequence A; o----o sequence B).

FIGURE 2.1a

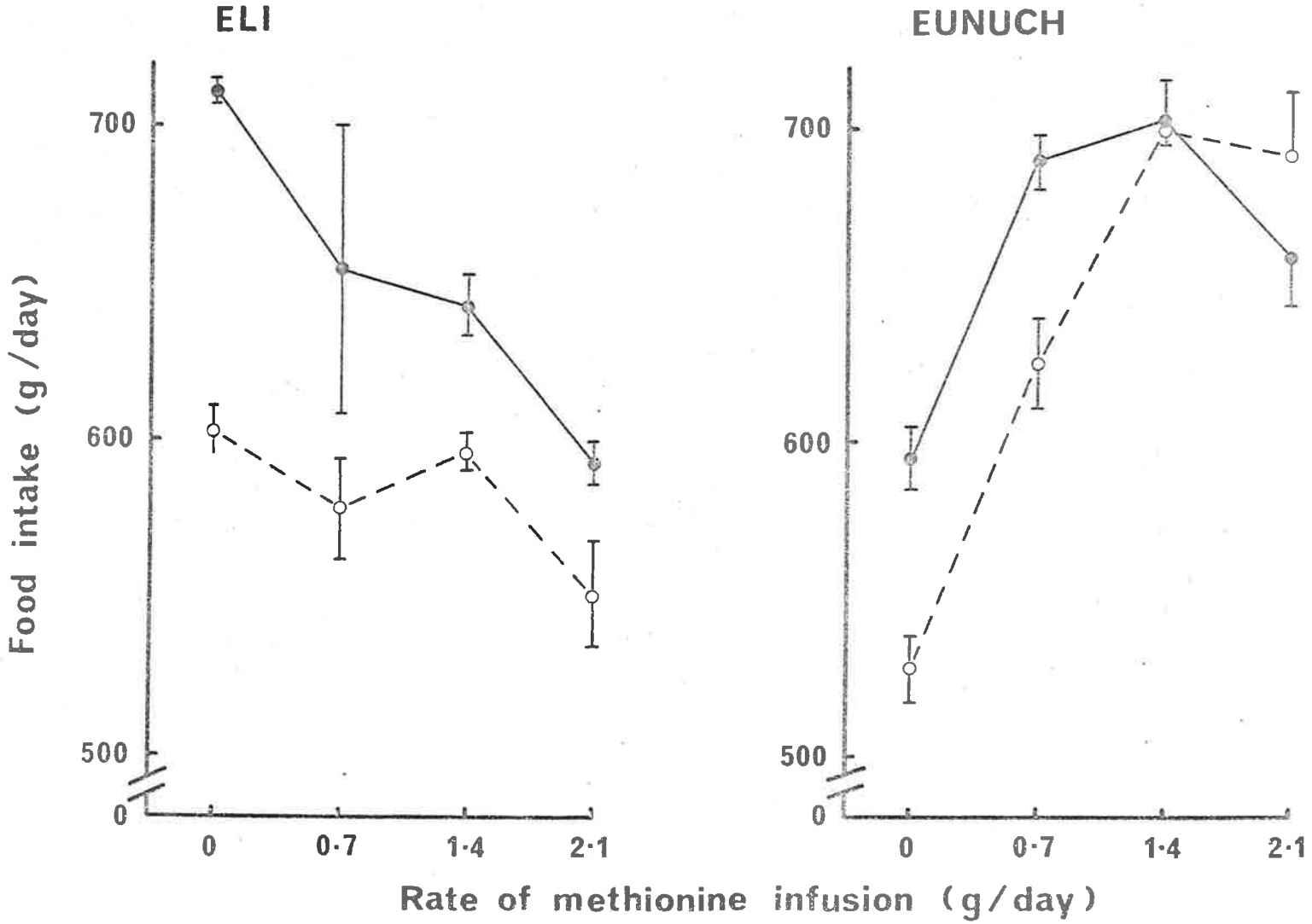

ELI
EUNUCH
700
600
500
0
Food intake (g/day)
0
0·7
1·4
2·1
Rate of methionine infusion (g/day)

FIGURE 2.1b

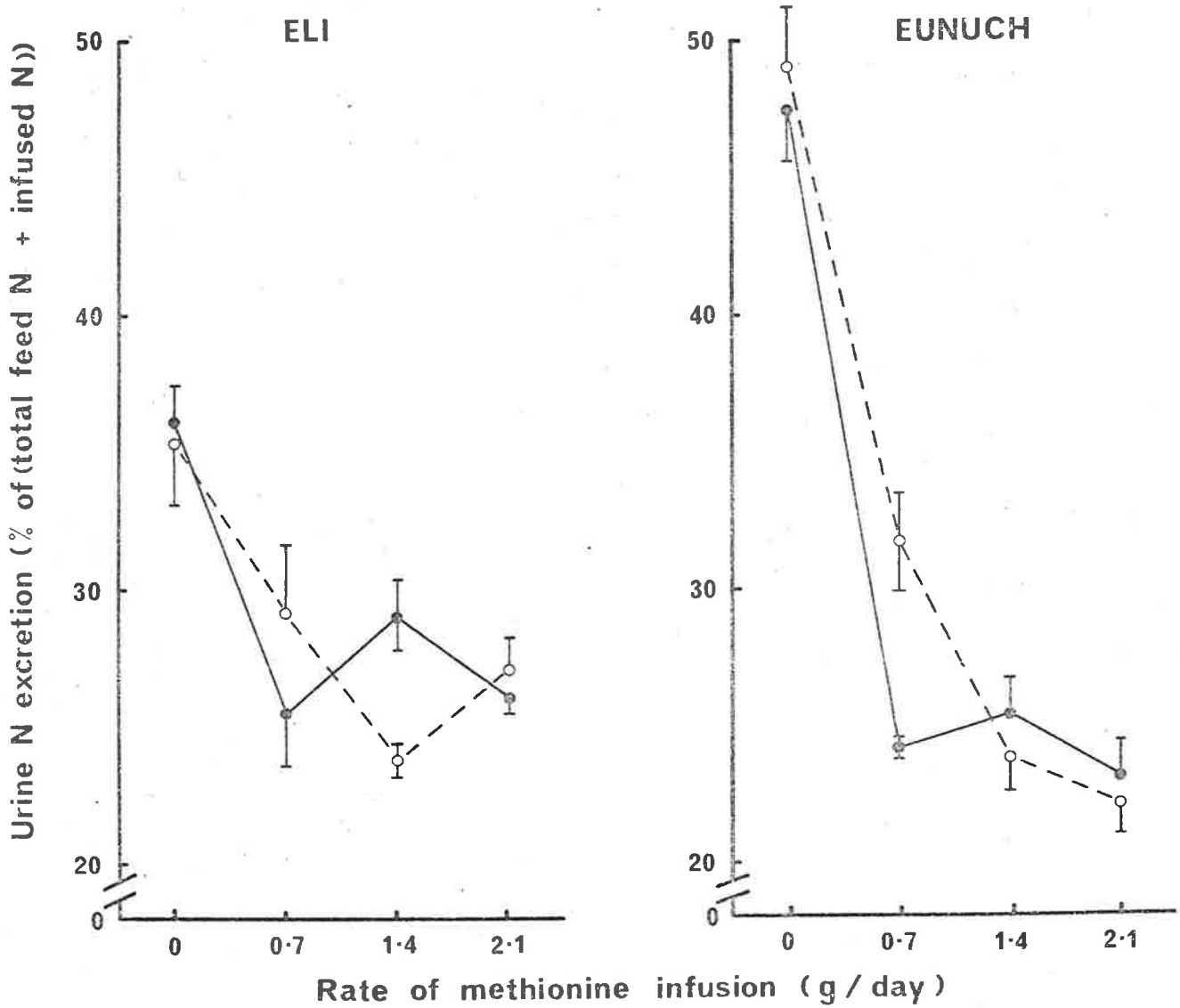


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TABLE 2.2. (Experiment 2/1): Nitrogen retention and urine nitrogen (N) excretion for Eli and Eunuch during the two methionine infusion sequences.

Methionine infused (g/day)	Sequence A N retention (g/day)	Sequence A Urine N[1] (ratio to abs. N)	Sequence B N retention (g/day)	Sequence B Urine N (ratio to abs. N)
	Eli			
0	-0.20	1.11	0.26	0.874
0.7	1.48	0.495	0.49	0.749
1.4	0.59	0.727	1.30	0.486
2.1	0.96	0.585	0.93	0.591
	Eunuch			
0	-0.26	1.13	-0.08	1.04
0.7	1.23	0.535	0.45	0.782
1.4	0.63	0.700	1.24	0.534
2.1	0.92	0.588	1.36	0.493

1) Urine N (ratio to absorbed N)

$$= \frac{\text{Urine N excretion}}{\text{(Feed N intake - faecal N) + methionine N}}$$

TABLE 2.3 (Experiment 2/1): Values for the apparent digestibility of feed organic matter (OM) and feed nitrogen (N) for Eli and Eunuch during the two methionine infusion sequences.

Methionine infused (g/day)	Sequence A Apparent digestibility of OM	Sequence A Apparent digestibility of N	Sequence B Apparent digestibility of OM	Sequence B Apparent digestibility of N
	Eli			
0	49.08	32.58	52.70	40.58
0.7	51.92	53.40	51.83	38.19
1.4	49.54	38.60	53.30	47.43
2.1	53.03	42.37	51.26	43.50
	Eunuch			
0	50.55	41.95	55.52	47.30
0.7	54.75	44.52	52.55	39.78
1.4	52.24	34.94	54.51	43.43
2.1	51.10	37.17	51.71	42.87

TABLE 2.4 (Experiment 2/1): Voluntary dry matter intake (DMI), urine nitrogen (N) excretion and nitrogen retention for Eli and Eunuch in periods 11 to 13.

Infusion[1]	DMI (g/day)	Urine N (g/day)	Urine N[2] (ratio to abs.N)	N retention (g/day)
	Eli			
M	551 ± 17.6[3]	1.34 ± 0.019	0.591	0.93
MT	569 ± 11.5	1.59 ± 0.081	0.547	1.31
MTL	623 ± 9.0	1.57 ± 0.065	0.492	1.66
	Eunuch			
M	692 ± 19.4	1.32 ± 0.033	0.493	1.36
MT	727 ± 6.3	1.50 ± 0.037	0.510	1.43
MTL	736 ± 8.6	1.64 ± 0.037	0.442	2.07

1) Infusion: M = 2.1g L-Met/day (0.20g N/day);
T = 1.4g L-Thr/day (0.16g N/day);
L = 2.1g L-Leu/day (0.22g N/day).

2) Urine N (ratio to abs. N) = $\frac{\text{Urine N excretion}}{\text{(Feed N intake - faecal N) + infusate N}}$

3) ± standard error of the mean (n = 7).

methionine, methionine + threonine and methionine + threonine + leucine). When compared with methionine + threonine the latter supplement resulted in a significantly increased voluntary intake by Eli, while the trend for an increased intake by Eunuch in response to threonine or threonine and leucine failed to reach significance. A higher intake could be expected to promote an increased nitrogen retention and this could partly explain the apparent increased retention by Eli in response to the leucine supplement. However an increased apparent nitrogen retention in the absence of any marked effect on urine nitrogen excretion must be interpreted with caution in view of the concern about the accuracy of the faecal nitrogen output data. Despite these reservations there was an apparent trend for threonine and leucine to increase nitrogen retention more so than threonine alone.

Residual effects of methionine administration

The effect of an earlier treatment on the subsequent animal performance is a potentially important source of error in nutritional studies. Figure 2.2 shows the mean values for nitrogen intake and urine nitrogen excretion in periods 1 through to 4, and then the three day mean values for nitrogen intake and urine nitrogen excretion for Eli and Eunuch through the periods in which either no infusion or a water infusion was given (periods 5 to 8).

Experiment 2/2

Table 2.5 gives the mean values for voluntary dry matter intake, urine nitrogen excretion and apparent nitrogen retention for sheep 761 in the control and 1.4g methionine infusion periods, and for sheep 663 and 748 for the control, 1.4g and 4.2g methionine

FIGURE 2.2 (Experiment 2/1): Nitrogen intake and urine nitrogen excretion for the sheep Eli and Eunuch during sequence A, and the 3 day mean values for nitrogen intake and urine nitrogen excretion through the periods in which either no infusion or a water infusion was given.

Feed nitrogen intake)
)
Methionine nitrogen infused) 7 day means
)
Urine nitrogen excretion)

□ Feed nitrogen intake)
) 3 day means
■ Urine nitrogen excretion)

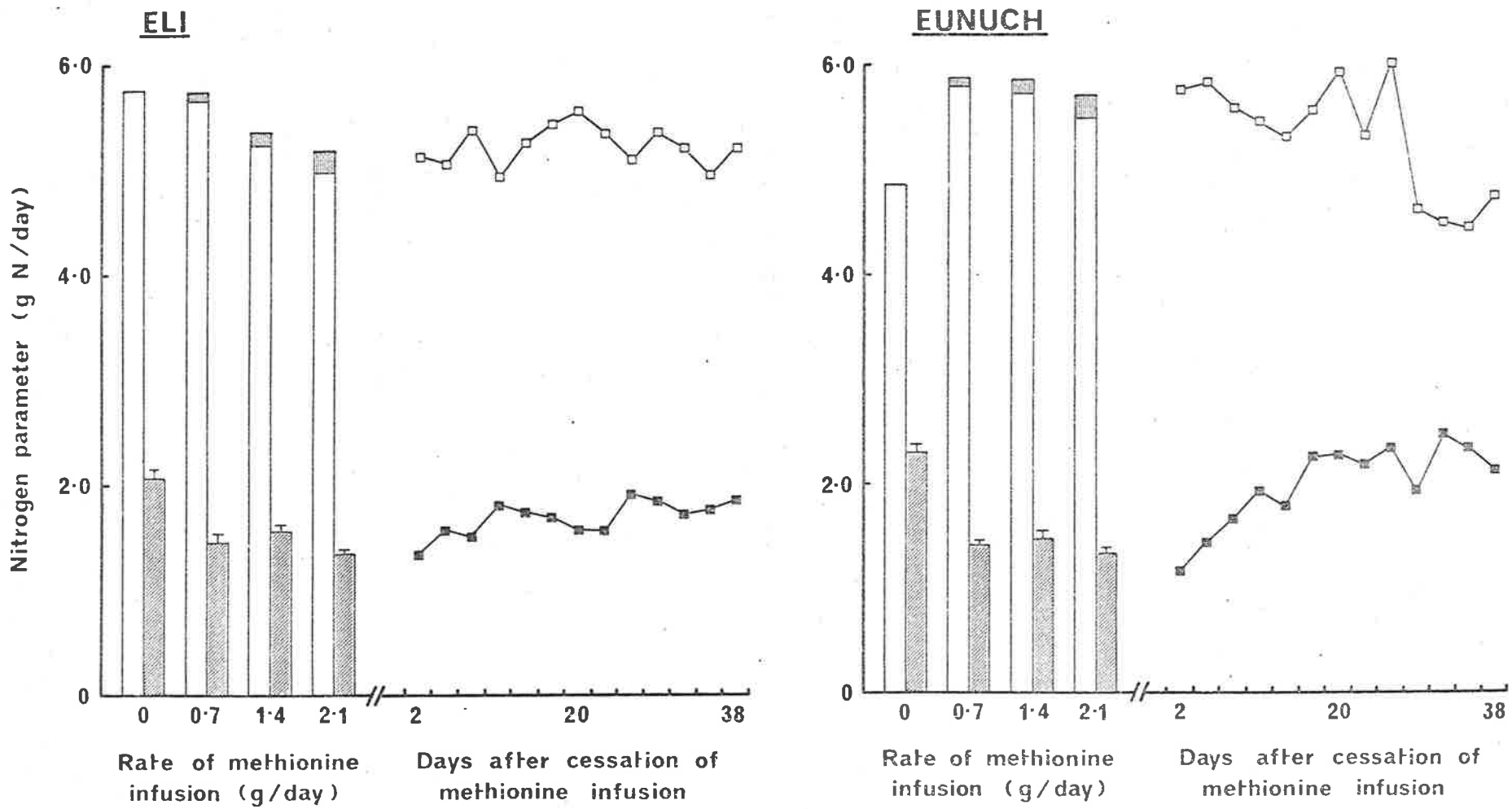

ELI
EUNUCH
Nitrogen parameter (g N/day)
6·0
4·0
2·0
0
0
0·7
1·4
2·1
2
20
38
Rate of methionine infusion (g/day)
Days after cessation of methionine infusion

TABLE 2.5 (Experiment 2/2): Voluntary dry matter intake (DMI), urine nitrogen (N) excretion and nitrogen retention for sheep 761, 663 and 748.

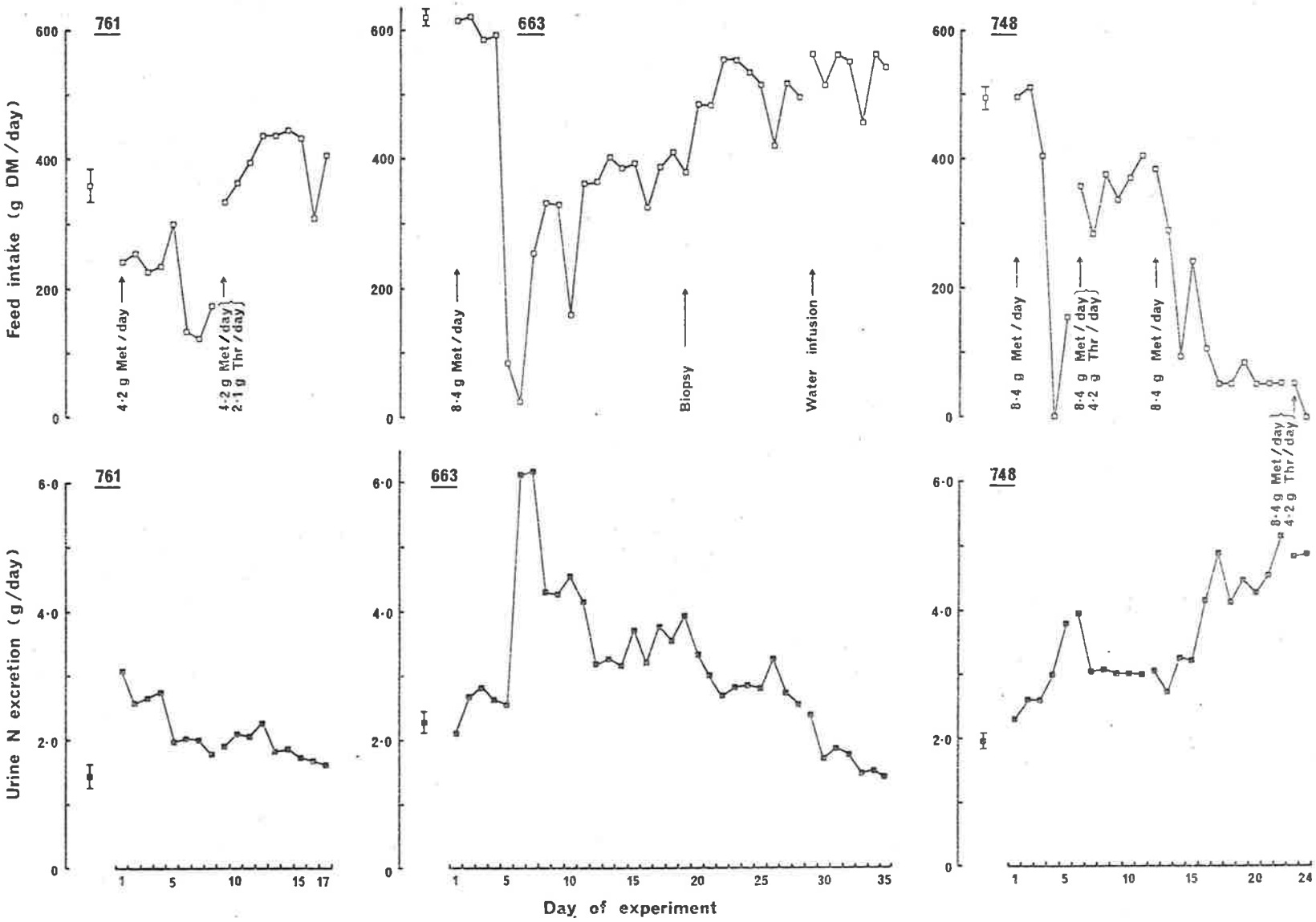
Sheep	Methionine infused (g/day)	DMI (g/day)	Urine N (g/day)	Urine N (ratio to abs. N)	N retention (g/day)
761	0	565 ± 12.0[2]	1.82 ± 0.089	0.956	0.08
	OB[1]	542 ± 13.2	1.55 ± 0.082	0.812	0.36
	1.4	454 ± 9.9	2.00 ± 0.084	1.10	-0.17
663	0	604 ± 24.6	2.36 ± 0.065	1.41	-0.68
	OB	563 ± 9.2	1.92 ± 0.045	1.26	-0.40
	1.4	644 ± 9.1	1.53 ± 0.045	0.780	0.43
	1.4B	604 ± 16.0	1.57 ± 0.029	0.792	0.41
	4.2	636 ± 19.9	2.12 ± 0.057	0.784	0.59
748	0	683 ± 12.6	2.49 ± 0.097	1.51	-0.84
	OB	646 ± 27.2	2.28 ± 0.143	1.13	-0.25
	1.4	717 ± 5.2	1.54 ± 0.048	0.538	1.33
	1.4B	661 ± 21.9	1.63 ± 0.049	0.640	0.92
	4.2	534 ± 5.9	1.91 ± 0.043	0.771	0.57

1) All B values are means for 7 day values post-biopsy; other values are means for 10 days.

2) ± standard error of the mean.

NB. Methionine N infused was approximately 0.13g and 0.39g/day for the 1.4 and 4.2g methionine infusions respectively.

FIGURE 2.3 (Experiment 2/2): Voluntary feed intake (o——o) and urine nitrogen excretion (●——●) for sheep 761 receiving a basal infusion of 4.2g L-methionine/day and for sheep 663 and 748 receiving basal infusions of 8.4g L-methionine/day. (The single value is the mean ± standard error of the mean for the voluntary feed intake and urine nitrogen excretion for the previous treatment post-biopsy subperiod for each animal; the arrows denote the addition to or removal from the infusion of L-threonine, the methionine to threonine ratio being maintained at 2:1).


761
663
748
Feed intake (g DM / day)
Urine N excretion (g/day)
4·2 g Met / day
4·2 g Met / day
2·1 g Thr / day
8·4 g Met / day
Biopsy
Water infusion
8·4 g Met / day
8·4 g Met / day
4·2 g Thr / day
8·4 g Met / day
8·4 g Met / day
4·2 g Thr / day
Day of experiment

infusion periods. Results are presented for both pre-biopsy (10 day) and post-biopsy (7 day) subperiods. Intake was consistently, though not always significantly, lower following the liver biopsy. As a result of the post-biopsy intake depressions the only valid comparisons would be for the treatment against the previous post-biopsy treatment (e.g. 1.4g methionine treatment compared with the post-biopsy control period).

Figure 2.3 presents the daily food intake and urine nitrogen excretion for each sheep during the infusions of 4.2g methionine (761) or 8.4g methionine (663, 748). The individual animal responses to each level of methionine infusion are given below.

Sheep 761: The voluntary intake of sheep 761 was depressed by about 16% ($P<0.01$) by the infusion of 1.4g methionine. On day 26 of this period the intake was further reduced, and remained at about this level until day 6 of the 4.2g methionine period when it was depressed by a further 50%, remaining low for 3 days. Threonine (2.1g/day) was infused with the methionine from the morning of day 9 onwards. This apparently stimulated intake, which rose steadily until about day 12 when it tended to plateau.

An elevation in urine nitrogen excretion was evident in the first collection taken after the start of the 4.2g methionine infusion. This high output of urinary nitrogen continued for a further 3 days after which it declined to a relatively stable level.

Sheep 663: A 14% increase ($P<0.01$) in voluntary intake was observed in response to the 1.4g methionine infusion; there was a further small non-significant increase in intake in response to the 4.2g methionine infusion. Intake was maintained at the post-biopsy

levels of the 4.2g methionine treatment for the first four days of the 8.4g methionine infusion. However, on day 5 and 6 intake fell to very low levels, but subsequently recovered slowly over the next 12 days. Following the biopsy on day 19, the voluntary intake increased, in contrast to the effects of biopsy during the previous treatments. Associated with the fall in intake, urine nitrogen increased more than two-fold and then subsequently declined. Urine nitrogen excretion was apparently inversely related to intake with a lag of about 24 hours. The withdrawal of methionine had no effect on the voluntary intake over the first 7 days, although the urine nitrogen excretion was considerably lower, probably associated in part with the cessation of the methionine infusion, since 8.4g methionine contributed about 0.8g of nitrogen per day.

Sheep 748: The voluntary intake of sheep 748 increased by 11% ($P<0.01$) in response to the infusion of 1.4g of methionine daily, but decreased by 19% ($P<0.01$) in response to the 4.2g methionine infusion. By day 3 of the 8.4g methionine infusion, intake was depressed and the animal consumed no feed at all on day 4, and only a small amount on day 5. Threonine (4.2g/day) was added to the infusate on day 6 prior to feeding. This was accompanied by an increased intake on day 6. Intake then rose slowly until the threonine infusion was stopped on the morning of day 12. The infusion of 8.4g methionine alone again resulted in a depressed intake. However, intake was not increased when the threonine infusion was resumed on day 23. On day 25, the animal was offered lucerne and immediately recommenced eating. Throughout the period of WHC feeding, there was an apparent inverse relationship between intake and urine nitrogen excretion, the latter lagging by about 24-48 hours.

Experiment 2/3

Mean values for voluntary dry matter intake, urine nitrogen excretion and apparent nitrogen retention for the control and methionine infusion periods are given in Table 2.6. Values for the apparent digestibility of organic matter and feed nitrogen are given in Table 2.7.

There was an apparent increase in voluntary intake in response to methionine in one animal (sheep 744) and when corrected for the change in liveweight over the experiment, the increase was about 8%.

DISCUSSION

Statistical analysis

There are several problems associated with the use of an animal as its own control as has been the case for the experiments reported in this chapter. Since it is a relatively insensitive form of analysis, a simple unpaired t-test (Snedecor & Cochran 1967) was selected as the most appropriate form of analysis for comparison between treatments.

In order to overcome the problem of carryover effects from one treatment to the next, a sequential approach was used in which the new treatment always included the previous treatment. Thus the two principal problems in the analysis of these experiments are (i) the confounding effect of treatments with time, and (ii) the non-independence of individual measurements within a treatment.

The confounding effect of time includes the effects of environment, feed quality, ag e, weight and physiological state of the animal.

TABLE 2.6 (Experiment 2/3): Voluntry dry matter intake (DMI), urine nitrogen excretion and nitrogen retention for sheep 675, 744 and 769 in the control period and in the period of methionine infusion (1.6g L-methionine/day).

	DMI (g/day)	DMI ($g/kg^{0.75}$/day)	Urine N (g/day)	Urine N (ratio to abs. N)[1]	N retention (g/day)
Control					
675	1011 ± 31.1[2]	70.7	3.35 ± 0.143	0.492	3.46
744	836 ± 13.5	57.6	2.76 ± 0.054	0.510	2.65
769	1041 ± 18.3	71.8	2.66 ± 0.081	0.416	3.72
Methionine					
675	961 ± 26.4	65.4	3.43 ± 0.221	0.631	2.01
744	941 ± 17.3	62.3	2.53 ± 0.059	0.465	2.91
769	1026 ± 17.4	70.3	2.82 ± 0.201	0.492	2.91

1) ± standard error of the mean (n = 10).

2) Urine N (ratio to abs. N) = $\frac{\text{Urine N excretion}}{\text{(Feed N - faecal N) + methionine N}}$

TABLE 2.7 (Experiment 2/3): Values for the apparent digestibility of feed organic matter and feed nitrogen for sheep 675, 744 and 769 in the control period and in the period of methionine infusion (1.6g L-methionine/day).

	Apparent digestibility of	
	Organic matter	Nitrogen
Control		
675	63.48	59.25
744	65.19	54.09
769	58.69	53.97
Methionine		
675	60.64	51.21
744	63.34	52.55
769	56.82	50.71

The environmental conditions, temperature, humidity and light were not controlled in the present experiment, but would be more likely to have affected results in the later periods of Experiments 2/1 and 2/2 when temperatures were higher. All feed for each experiment was well mixed prior to the start of the experiment in order to overcome any problems of feed variability. For Experiments 2/1 and 2/2 a maintenance diet was used in an attempt to eliminate any confounding effects of change in liveweight, and as a result the only apparent trend in liveweight was the considerable loss of weight (4-5kg) which accompanied the feed intake depressions in Experiment 2/2. However, in Experiment 2/3 the animals gained an average of about 80g/day in liveweight.

Two alternatives were considered in order to at least partly counteract the above problems. These were: (i) to use other animals as controls in time, and relate any treatment results to these time controls in relation to the experimental animal's own control parameters, or (ii) to repeat the same treatments on the same animals at a different time, i.e. to assess the repeatability of the response in an individual animal. The second alternative was chosen, since it required fewer animals, and it was expected that the results would be more clearcut. The repeatability experiment involved two animals whose intake responses to the methionine supplementation were in opposite directions, the responses being repeated in each of two experiments.

The non-independence of individual measurements within a treatment, although a theoretical problem, would be more likely to increase the variance, rather than decrease it. For example, a high

level of urine nitrogen excretion on one day would be likely to result in a reduced excretion on the following day providing that all fluctuations about the mean are relatively random.

Experiment 2/1

The responses of the two sheep, Eli and Eunuch to the methionine infusions were in marked contrast; the intake of the former was depressed, while the intake of the latter was increased. Although methionine infusion reduced urine nitrogen excretion in both animals the response was considerably greater for Eunuch, which, coupled with the increase in intake contributed to a considerable improvement in the efficiency of nitrogen utilisation (see Fig.2.1). That the marked variability between animals in the methionine response was a real effect, is strongly indicated from the results of the second part of the experiment, sequence B, in which the same trends in intake and urine nitrogen excretion as occurred in sequence A were apparent for each animal.

The improvements in nitrogen retention resulting from methionine supplementation were in the order of about 1.0g per day, of which about 0.3 - 0.4g could be due to an increase in wool growth (Robards 1971; Barry & Andrews 1973), the rest of the response being due to an increase in the rate of muscle or visceral protein accretion. The responses were preumsably the result of an increased supply of an amino acid which was limiting for some physiological function(s). Despite the marked contrast in the intake responses, the depressions in urinary nitrogen output and the resultant improvements in nitrogen retention were similar in both animals. These results do seem to raise some questions as to the concept of a first limiting amino acid.

It is apparent that an amino acid which is limiting for some physiological function (e.g. methionine for wool growth and tissue protein accretion), may not be limiting, and in fact, may produce an adverse effect on some other physiological function (e.g. intake). Methionine is involved in many aspects of metabolism in addition to its role as a protein amino acid. It has vital roles as a methyl donor and as a cystein precursor, and is involved in the initiation step in the mechanism of protein synthesis (Finklestein 1974; Smith & Henshaw 1975). For this reason, a response to methionine may reflect its influence on any number of metabolic processes, as well as possible interactions with other amino acids.

The responses to leucine (and threonine) may also be interpreted as the result of an increased supply of an amino acid limiting for some physiological function. However, as with methionine, these may not necessarily have occurred through an increased supply of an amino acid for protein synthesis. For example, leucine stimulates insulin release from the isolated pancreas *in vitro* (Milner 1969), and has also been shown to stimulate insulin secretion in man (Floyd *et al.* 1966) although the quantities used in this study were very high. Thus an effect of leucine could be mediated through an effect of insulin release, which may stimulate muscle protein synthesis (Manchester 1970).

Nimrick *et al.* (1970a,b) reported a nitrogen retention response to an abomasal infusion of methionine in young growing sheep fed a purified diet with urea as the sole nitrogen source, while Schelling *et al.* (1973) obtained similar results with sheep fed diets containing natural feedstuffs. In their studies Nimrick

et al. (1970a,b) found that "limiting order of essential amino acids was methionine, lysine, threonine".

The long term carryover effects of methionine administration on urine nitrogen excretion are apparent in Figure 2.2. This is in direct contrast to the results reported by Schelling & Hatfield (1968) following an abomasal infusion of casein to lambs. This is probably a result of the fact that the animals in the present experiment were fed *ad libitum*. while those of Schelling & Hatfield (1968) were fed at a constant level of intake. In early experiments these workers observed that feed intake and nitrogen retention did not drop completely back to control levels after a casein infusion in lambs fed *ad libitum*.

Experiment 2/2

In Experiment 2/2 major differences between animals in response to methionine were again evident. Sheep 761 suffered an intake depression on the 1.4g methionine infusion; the intake of sheep 748 was depressed at the 4.2g level while the intake of sheep 663 was not affected until the 8.4g methionine infusion.

As well as the intake depression of 16% associated with the 1.4g methionine infusion in sheep 761, the urine nitrogen excretion was increased and nitrogen retention decreased compared with the control treatment. This was in direct contrast to the improved nitrogen retention exhibited by Eli in response to the 2.1g methionine infusion, despite a similar depression in voluntary intake (sequence A). Methionine supplementation at 1.4 and 4.2g/day resulted in an increased nitrogen retention compared with the control treatment in both sheep 663 and 748, being particularly evident at the lower level.

These between animal differences in the response to the same treatment again raise questions as to the concept of a first-limiting amino acid.

Nimrick *et al.* (1970a) suggested that in some sheep fed purified diets in their experiments, lysine rather than methionine, may have been "first limiting". However, the limiting amino acid concept assumes that the animal's metabolic functions are in a simple sense, homogeneous, and fails to take into account that different amino acids may be limiting for different physiological functions. In strict terms an amino acid is only limiting for a metabolic function or functions, and to use the term "first limiting" in general terms implies some universal property of the amino acid. For this reason the concept of a "group of critical amino acids" rather than the concept of a "limiting amino acid" would appear to be more useful.

Experiment 2/3

The methionine supplement resulted in a small positive response in only one animal in this experiment, while in the other two sheep the apparent nitrogen retention was depressed mainly due to increases in the faecal nitrogen output. The ratio of the urine nitrogen excretion to the apparently absorbed nitrogen was low for all three animals in the control period, reflecting a very high efficiency of use of absorbed nitrogen. For this reason there was probably little capacity for an improved efficiency of nitrogen utilisation. However, there remains a possibility that sheep fed a higher quality roughage, as in this experiment, may be particularly sensitive to small quantities of additional methionine.

Methionine and voluntary intake

A stimulatory effect of methionine (given by intraperitoneal injection) on the voluntary intake of sheep fed a silage diet has been reported by Barry *et al.* (1973). However an intake response to supplementation with a specific amino acid in sheep fed a low quality roughage diet has not previously been reported although the response to postruminal protein *per se* is a well-known phenomenon (Egan 1965; Schelling & Hatfield 1968). Egan (1965) stressed the importance of a chemical regulatory effect on voluntary food intake, while Schelling & Hatfield (1968) suggested that such a chemical regulatory system "must key on specific nitrogen component(s) rather than on general nitrogen levels".

The correction of a dietary methionine deficiency in rats fed low protein diets results in an increased voluntary intake (Byington *et al.* 1972). Similarly, rats fed imbalanced diets (made deficient in one essential amino acid by the addition of a quantity of amino acids lacking this one essential amino acid) suffer an intake depression (Harper *et al.* 1970). Recently, this phenomenon has been reported in young pre-ruminant lambs (Rogers & Egan 1975). Leung & Rogers (1969) have shown that the intake depression is related to the change in the plasma concentration of the limiting amino acid and that the intake depression may be partly reversed by the infusion of a small amount of the limiting amino acid into the carot id artery but not into the jugular vein. These workers involed an aminostatic regulation of food intake centred on the brain rather than effects monitored elsewhere in the body to explain the intake depression resulting from the feeding of imbalanced diets. However,

there remains a considerable amount of controversy as to the factors controlling food intake in such situations (Harper *et al.* 1970; Noda 1975).

A mechanism for the control of food intake could be expected to be mediated through the brain, although the precise nature of such a mechanism is open to much debate. An increased supply of any amino acid could function through its effects on the transport of other amino acids into the brain, or possibly through effects on the hormone status of the animal, which could modify responses within the brain. The possible explanations for a stimulatory effect of an amino acid on voluntary intake are numerous, and any further speculation in the present context is not warranted.

The methionine-induced depression of voluntary intake is quite a different situation to that of the intake stimulation. In one animal (Eli) the depression was apparently corrected by a supplement of leucine administered with the abomasal supplement of methionine and threonine, although the time elapsed between the control period and this treatment period must be taken into account. In two other animals (sheep 761, 748) the administration of a threonine supplement resulted in a partial correction of the intake depression due to methionine. In this way, it is reminiscent of the response to the addition of the limiting amino acid in animals fed imbalanced diets (Harper *et al.* 1970). Sheep 663 adjusted to the high level of methionine and over a period of about 3 weeks its voluntary intake recovered to a level of about 85% of its intake on the previous treatment. It is possible that both sheep 761 and 748 would have adjusted given sufficient time, although there were

indications that both were considerably more sensitive to the high levels of methionine supplementation than was sheep 663.

An interaction of methionine and threonine metabolism within the brain is a possibility. Serine, which may be synthesised from threonine, is required for the synthesis of cystathionine from homocysteine via the transulphuration pathway (Finklestein 1974). It is possible that an accumulation of homocysteine within the brain could have altered the cellular milieu and consequently caused intake to be adversely affected. Both cystathionine and glycine (which may also be synthesised from serine) are neurotransmitter amino acids and intimately involved with brain and nervous system metabolism (Quastel 1974). It is thus not unlikely that excess methionine affected threonine-serine-glycine inter-relationships within the brain resulting in adverse effects on voluntary intake. Alternatively excess methionine may have induced a simple threonine deficiency, similar to the imbalance situation.

Methionine toxicity, particularly in rats and guinea pigs, has been studied in many laboratories in recent years. Although many aberrations of metabolism have been reported, there are no indications as to the actual cause of the adverse effects of methionine (see Benevenga 1974), although a toxic metabolite(s) has been suggested as a likely cause. However it seems that a uni-factorial approach may well be inadequate and that high levels of methionine may be exerting their effects at many levels. The possible effects include induced deficiencies of other amino acids, such as leucine and threonine (as discussed above); the toxic effects of possible metabolite(s) of methionine (e.g. methyl mercaptan,

Canellakis & Tarver 1953); effects of excess methionine on the intracellular ATP status (Farber 1973; see Review, Section 4.1); other possibilities include the effects of methionine accumulation in the blood and tissue pools which could have considerable effects, including adverse effects on the transport of amino acids.

CONCLUSIONS

The experiments reported in this chapter provided strong indications that between animal variability was a real phenomenon, and of sufficient magnitude (at least in terms of the response to methionine) that it could be an important variable in nutritional experiments. Therefore, it is a phenomenon that should be investigated although there are considerable problems in designing suitable experiments.

The changes in intake (both increases and depressions) which were recorded in response to the methionine supplement were probably mediated through the brain. However, the nature of the mechanisms and the reasons behind the variability between animals are open to considerable debate. Some of the possible effects of methionine, particularly at the metabolic level, have been considered in the discussion, but any interpretation awaits further study.

CHAPTER 3

Plasma and blood cell amino acid patterns in young sheep fed roughage diets and given post-ruminal supplements of L-methionine and other amino acids

INTRODUCTION

In several studies of amino acid nutrition and metabolism, particularly amino acid imbalance studies in rats and chicks, the plasma amino acid pattern has been shown to reflect the pattern of amino acids fed to the animals within a very short time after feeding (Leung *et al.* 1968; Harper *et al.* 1970). However, in all of this work groups of animals have been used. The presentation of mean responses and the general use of factorial designs for the experiments, preclude any close examination of the variability between animals in the response to the treatment.

The plasma amino acid patterns in the sheep in the present experiments were examined for a number of reasons. Do changes in the plasma concentrations of amino acids give any indication as to possible metabolic changes taking place in the animal? For example, what is the relationship of plasma methionine and threonine in the sheep given abomasal supplements of methionine, in view of the reported increases in threonine catabolism in rats and chicks given high levels of dietary methionine (Sanchez & Swendseid 1969; Katz & Baker 1975)? Do the plasma amino acid changes give any indication as to the possible reasons behind the differences between animals in the response to methionine?

Traditionally, the blood cells (erythrocytes, leucoytes, platelets) have been regarded as unimportant in amino acid metabolism in animals. However, in recent years Elwyn and his coworkers (Elwyn 1966; 1970; Elwyn *et al.* 1972) have shown that the erythrocytes are important while others (Felig *et al.* 1973; Aoki *et al.* 1974) have also shown that the blood cells are involved in amino acid transport in the intact animal. For this reason, the blood cell amino acid patterns were examined in one experiment likely to produce extreme results (i.e. Experiment 2/2 involving the infusion of high levels of methionine, ca.8g/day).

EXPERIMENTAL

The data from three experiments are reported.

Experimental details

Experiment 1/1: The details of this experiment are given in Chapter 1. Plasma amino acids were determined for each animal using a pooled sample from time T_0 on days 8 and 11 of each period.

Experiment 2/1: The details of this experiment are given in Chapter 2. Blood samples for plasma amino acid analyses were obtained by jugular puncture at 1430h on days 3 and 11 of periods 1 to 4 and 8 to 13.

Experiment 2/2: The details of this experiment are given in Chapter 2. Blood samples for plasma and blood cell amino acid analyses were taken at 1430h. Samples on day 17 were taken from a jugular catheter, while all other samples were obtained by jugular puncture.

Blood sampling and preparation of plasma and blood cells

The methods used in Experiment 1/1 are given in Chapter 1. In Experiments 2/1 and 2/2, blood samples were collected into heparinised tubes in ice, centrifuged at 4°C and the plasma drawn off. Plasma proteins were immediately precipitated using an equal volume of 10% W/V trichloroacetic acid (TCA). Following centrifugation, the protein free supernatant was drawn off and stored at -20°C until required for analysis. Immediately after removal of the plasma, the blood cells were washed with ice-cold isotonic saline, centrifuged and the supernatant drawn off. This procedure was carried out three times. The blood cells were stored at -20°C until required for preparation. The frozen blood cells (known amount) were thawed, haemolysed in an equal volume of distilled water, and then the same volume of 10% TCA was added to precipitate the proteins. The preparation was then shaken vigorously, centrifuged and the supernatant retained for analysis.

Plasma and blood cell amino acid concentrations were determined by ion-exchange chromatography on a Technicon 'B' resin using a sodium citrate buffer system with an 18 or 20h separation. Norleucine (0.25 μ mole) was added as an internal standard.

RESULTS AND DISCUSSION

Plasma and blood cell amino acid patterns which are not presented in the text are contained in Appendix Tables 3.1, 3.2 and 3.3. Throughout this chapter the sum of the essential amino acids (EAA) includes threonine, valine, isoleucine, leucine, phenylalanine, lysine, histidine and arginine while the non-essential amino acids (NEAA) are

serine, glutamic acid, glycine, alanine, tyrosine and ornithine. Methionine and its metabolites, taurine, α-amino-n-butyric acid, cystine and cystathionine, have been omitted from all calculations unless otherwise stated. The other amino acids (OAA) in the glycine to OAA ratio (GLY/OAA) is the sum of the EAA and NEAA minus glycine.

Effect of methionine infusion on plasma amino acids

The plasma amino acid data are summarised in Tables 3.1 (Experiment 1/1), Table 3.2 (Experiment 2/1) and Table 3.3 (Experiment 2/2). The data presented for Experiment 1/1 are for the three sheep fed the WHC diet for which the intake response could be defined.

The most obvious effect of methionine supplementation on the plasma amino acid pattern was the increase in the concentration of plasma methionine itself. The increases in plasma methionine (up to 150-200 fold at the 8.4g/day infusion) were similar to those reported for rats receiving high methionine diets (Sanchez & Swendseid 1969; Girard-Globa *et al.* 1972) and for sheep receiving high rates of abomasal methionine infusion (Reis & Tunks 1971; Reis *et al.* 1973b). Associated with the increase in plasma methionine were comparatively smaller, but consistent increases in the concentration of the methionine metabolites, taurine, cystine, and cystathionine, and to a lesser extent α-amino-n-butyric acid (ABU), all products of methionine metabolism via the transulphuration pathway (Finklestein 1974). In almost every case, any level of methionine supplementation depressed plasma total amino acids (excluding methionine) when compared with the "no infusion" control. The effect on the non-essential to essential amino acid ratio was variable. Some of the

TABLE 3.1 (Experiment 1/1): Plasma amino acid concentrations (μmoles/100 ml plasma) in three sheep (B,F,K) given abomasal infusions of water (OM), 0.08g L-methionine/$kg^{0.75}$/day (LM) and 0.16g L-methionine/$kg^{0.75}$/day (HM) in successive periods.

Sheep:	B			F			K		
	OM	LM	HM	OM	LM	HM	OM	LM	HM
TAU	0.66	1.58	15.33	1.72	3.18	19.86	2.05	2.42	3.64
THR	12.97	9.78	5.30	15.69	8.28	8.88	10.26	8.35	8.71
SER	13.03	10.21	6.53	14.53	6.17	5.25	13.41	8.87	8.93
GLU	19.58	17.80	19.44	26.15	18.67	19.33	22.30	13.59	19.09
GLY	66.43	58.00	38.75	63.59	40.94	37.25	60.36	42.50	42.53
ALA	16.28	13.99	11.67	17.74	14.85	15.83	15.07	11.21	12.04
ABU	tr.	1.13	0.83	0.57	0.83	0.83	0.89	0.83	0.69
VAL	22.18	16.70	13.51	20.28	13.97	13.10	18.23	14.01	12.82
MET	1.34	3.13	4.13	1.64	4.46	8.70	1.40	2.00	2.52
CYSH	ND	0.48	1.01	ND	0.87	1.46	ND	tr.	0.40
ILE	9.56	7.87	5.05	9.79	6.18	6.52	8.08	6.20	5.83
LEU	11.22	6.37	4.69	11.22	6.53	6.17	9.82	6.30	5.80
TYR	4.71	4.84	3.27	7.08	6.56	6.10	5.61	4.29	4.96
PHE	4.30	3.79	3.18	4.44	5.35	4.81	4.78	3.92	5.13
ORN	5.43	4.99	2.92	6.06	3.45	3.64	6.07	5.66	4.12
LYS	14.91	8.78	7.01	19.33	9.50	11.02	16.65	12.40	10.46
HIS	10.85	8.51	4.58	9.93	8.32	6.12	11.60	8.14	8.35
ARG	12.08	11.39	8.31	15.00	12.11	11.74	13.12	15.43	14.63
EAA[1]	98.07	73.19	51.63	105.7	70.24	68.36	92.54	74.75	71.73
NEAA[2]	125.5	109.8	82.58	135.2	90.64	87.40	122.8	86.12	91.67
GLY/OAA[3]	0.423	0.464	0.406	0.359	0.341	0.314	0.389	0.359	0.352
N/E[4]	1.28	1.50	1.60	1.28	1.29	1.28	1.33	1.15	1.28

1) EAA: THR, VAL, ILE, LEU, PHE, LYS, HIS, ARG.

2) NEAA: SER, GLU, GLY, ALA, TYR, ORN.

3) GLY/OAA: Ratio of GLY to (EAA + NEAA - GLY)

4) N/E: Ratio of NEAA to EAA.

TABLE 3.2 (Experiment 2/1): Mean values for the concentration of amino acids in the plasma of the sheep, Eli and Eunuch, during the two methionine infusion sequences.

Sheep: Eli

	Sequence A				Sequence B			
	Methionine infused (g/day)							
	0	0.7	1.4	2.1	0	0.7	1.4	2.1
TAU	4.00	4.10	8.29	23.45	2.47	2.19	12.95	19.52
THR	8.50	7.22	5.19	5.42	8.27	4.67	5.14	3.84
SER	18.22	16.68	9.73	10.03	16.46	14.59	10.43	9.12
GLU	40.47	39.56	32.44	29.60	36.84	30.74	27.06	23.15
GLY	109.0	93.46	59.46	52.36	88.14	89.04	54.97	45.44
ALA	19.74	20.87	19.48	19.35	20.20	24.90	21.67	22.06
ABU	1.27	2.51	2.21	2.63	1.37	1.77	1.65	1.64
VAL	14.36	12.67	10.02	9.69	13.59	11.73	10.62	9.71
½CYS	1.59	4.13	4.61	5.04	1.12	3.88	4.81	4.27
MET	1.70	3.68	5.91	12.75	1.66	3.95	8.66	25.32
CYSH	ND	0.35	1.13	1.84	ND	0.53	1.85	1.92
ILE	6.49	8.02	6.12	5.29	7.05	6.81	4.90	4.89
LEU	6.88	5.78	4.93	4.29	6.08	5.82	4.83	3.59
TYR	4.83	6.03	6.34	6.00	5.53	6.36	6.74	6.33
PHE	3.06	4.07	4.11	4.55	2.78	4.41	4.97	4.30
ORN	5.77	6.31	5.30	4.57	5.88	3.72	4.98	4.58
LYS	13.94	12.41	11.18	9.66	12.12	8.01	10.83	7.98
HIS	14.43	14.15	12.51	13.89	12.76	13.81	13.57	12.08
ARG	7.86	11.73	9.78	7.52	8.45	7.80	7.47	6.93
EAA[1]	75.52	76.05	63.84	60.31	71.10	63.06	62.33	52.32
NEAA[2]	198.0	182.9	132.8	121.9	173.1	169.4	125.9	110.7
GLY/OAA[3]	0.663	0.565	0.433	0.403	0.565	0.621	0.413	0.386
N/E[4]	2.62	2.41	2.08	2.02	2.43	2.69	2.02	2.08

1) EAA: THR, VAL, ILE, LEU, PHE, LYS, HIS, ARG.

2) NEAA: SER, GLU, GLY, ALA, TYR, ORN.

3) GLY/OAA: ratio of GLY to (EAA + NEAA - GLY)

4) N/E: ratio of NEAA to EAA.

TABLE 3.2 (Experiment 2/1) continued:

Sheep: Eunuch

	Sequence A				Sequence B			
	Methionine infused (g/day)							
	0	0.7	1.4	2.1	0	0.7	1.4	2.1
TAU	4.58	2.71	6.66	19.36	2.62	3.12	12.62	24.53
THR	14.76	10.67	10.19	8.39	13.51	11.08	9.88	7.21
SER	13.98	15.01	13.23	10.19	18.01	16.19	13.14	10.25
GLU	30.56	32.56	34.00	26.68	33.40	31.36	27.35	22.38
GLY	85.40	77.61	56.03	46.56	70.49	79.87	49.68	41.75
ALA	20.58	20.57	27.03	29.21	21.61	26.10	26.04	22.84
ABU	1.50	1.53	2.17	2.80	1.14	1.44	2.31	2.37
VAL	17.24	13.13	12.00	9.52	15.08	13.42	13.25	11.36
½CYS	1.94	3.21	3.49	4.41	1.37	2.81	3.81	3.64
MET	1.54	3.04	3.96	15.08	1.28	3.77	9.39	20.36
CYSH	ND	0.72	1.71	3.04	ND	0.21	1.93	2.65
ILE	7.34	6.81	6.26	4.81	7.41	6.86	7.14	6.13
LEU	7.21	5.45	6.72	4.95	7.19	5.23	5.29	4.14
TYR	5.51	5.87	7.07	7.24	5.89	6.59	7.49	7.04
PHE	4.08	4.15	4.33	4.99	3.59	4.58	4.74	5.38
ORN	7.20	6.82	4.45	4.20	6.82	5.35	5.47	5.10
LYS	14.75	11.09	9.25	8.91	12.58	12.08	11.64	9.65
HIS	14.10	16.77	16.13	18.14	13.37	17.28	11.14	15.06
ARG	9.07	7.45	11.59	7.52	7.22	10.11	6.58	4.10
EAA[1]	88.53	75.48	76.47	67.23	79.95	80.64	69.66	63.03
NEAA[2]	163.2	158.4	141.8	124.1	156.2	165.5	129.2	109.4
GLY/OAA[3]	0.513	0.497	0.345	0.322	0.426	0.480	0.333	0.319
N/E[4]	1.84	2.10	1.85	1.85	1.95	2.05	1.85	1.74

1) EAA: THR, VAL, ILE, LEU, PHE, LYS, HIS, ARG.

2) NEAA: SER, GLU, GLY, ALA, TYR, ORN.

3) GLY/OAA: ratio of GLY to (EAA + NEAA - GLY)

4) N/E: ratio of NEAA to EAA.

TABLE 3.3 (Experiment 2/2): Plasma amino acid concentrations (μmoles/100 ml plasma) during the period of the highest rate of methionine infusion in each sheep compared with the mean values for the concentrations in each preceeding period.

Sheep 761	MO[1] Mean[2]	M1.4 Mean	Day 3	M4.2 8	10[3]
TAU	0.89	1.99	9.77	18.56	9.45
THR	12.80	4.90	6.23	14.50	37.83
SER	17.44	7.92	7.32	9.16	8.27
GLU	21.59	14.20	13.52	6.03	9.10
GLY	83.66	49.50	74.06	68.59	44.96
ALA	20.58	10.12	7.41	9.68	12.31
ABU	0.75	0.25	3.28	5.11	1.69
VAL	15.11	5.90	7.67	10.92	8.67
½CYS	1.60	2.46	3.78	3.53	4.64
MET	1.61	1.59	4.39	172.4	159.1
CYSH	tr	0.46	2.65	3.25	2.03
ILE	7.27	3.28	3.17	2.94	3.27
LEU	6.84	2.67	4.24	4.61	4.38
TYR	4.63	2.40	4.49	3.22	3.84
PHE	3.58	2.29	4.49	3.08	2.86
ORN	6.53	2.55	4.61	3.87	4.28
LYS	15.28	6.46	8.43	25.74	13.13
HIS	16.82	11.36	13.54	13.52	8.81
ARG	11.30	6.46	7.76	11.02	9.59
EAA[4]	89.00	43.32	55.53	86.33	88.54
NEAA[5]	154.4	86.69	111.4	100.5	82.76
GLY/OAA[6]	0.524	0.615	0.797	0.580	0.356
N/E[7]	1.74	2.00	2.01	1.16	0.935

1) MO - control water infusion; M1.4 - 1.4g L-methionine/day; M4.2 - 4.2g L-methionine/day; M8.4 - 8.4g L-methionine/day.

2) All means are mean values for the samples taken on days 3 and 17.

3) Infusion of 4.2g L-methionine plus 2.1g L-threonine/day for approximately 30L prior to this sampling.

TABLE 3.3 (Experiment 2/2) continued:

Sheep 663	MO[1] Mean[2]	M1.4 Mean	M4.2 Mean	M8.4 Day 3	8	11	17
TAU	4.90	9.10	17.69	15.24	17.38	12.38	12.97
THR	13.19	5.89	8.40	12.78	10.44	6.44	7.04
SER	16.38	7.80	6.43	11.45	8.10	9.45	9.12
GLU	33.45	23.62	14.80	13.30	13.87	8.80	9.77
GLY	115.3	61.82	42.36	40.40	43.34	40.58	37.59
ALA	21.71	14.34	19.24	18.46	11.18	11.80	12.02
ABU	1.42	1.77	1.43	0.81	2.44	1.61	1.09
VAL	16.62	11.13	9.00	10.81	10.62	9.82	9.87
½CYS	2.48	4.08	3.98	7.22	3.94	5.34	5.44
MET	2.05	5.87	128.0	270.0	177.3	183.3	193.1
CYSH	ND	1.40	1.88	2.23	0.86	0.75	0.76
ILE	8.46	5.38	3.11	3.67	4.32	3.70	1.28
LEU	8.75	4.22	2.96	3.85	3.85	4.69	4.70
TYR	5.72	5.44	4.70	6.50	3.36	3.92	3.89
PHE	3.90	4.50	4.15	4.82	3.15	3.39	3.71
ORN	7.28	4.08	4.72	6.76	4.41	3.97	3.82
LYS	20.99	9.92	11.03	16.85	15.85	21.50	16.03
HIS	23.02	18.44	15.21	8.79	11.57	11.32	10.19
ARG	13.02	7.28	8.32	9.65	11.98	12.58	11.75
EAA[4]	107.9	66.75	62.16	71.22	71.78	73.44	64.57
NEAA[5]	199.8	117.1	92.25	96.87	84.26	78.52	76.21
GLY/OAA[6]	0.599	0.507	0.378	0.316	0.385	0.364	0.364
N/E[7]	1.85	1.75	1.48	1.36	1.17	1.07	1.18

4) EAA: THR, VAL, ILE, LEU, PHE, LYS, HIS, ARG.

5) NEAA: SER, GLU, GLY, ALA, TYR, ORN.

6) GLY/OAA: Ratio of GLY to (EAA + NEAA - GLY)

7) N/E: Ratio of NEAA to EAA.

TABLE 3.3 (Experiment 2/2) continued:

Sheep 748	MO[1] Mean[2]	M1.4 Mean	M4.2 Mean	Day 3	5	M8.4 8[8]	11[9]	17[10]
TAU	3.4	8.44	17.15	15.23	19.02	14.95	19.05	19.20
THR	17.31	7.48	7.46	10.14	8.88	105.1	84.77	6.96
SER	19.81	8.26	8.26	9.77	11.47	12.27	15.55	8.21
GLU	33.88	21.90	14.45	12.61	13.22	11.88	12.32	10.40
GLY	106.8	54.96	44.76	39.19	46.79	58.53	49.07	49.09
ALA	24.25	16.70	14.97	19.74	15.73	18.59	19.71	10.81
ABU	1.49	1.35	1.65	1.16	1.83	2.24	1.88	2.24
VAL	19.34	10.35	7.98	9.49	9.07	9.12	9.12	9.09
½CYS	2.28	4.25	4.71	9.23	5.78	6.56	5.69	2.66
MET	2.08	6.36	126.9	311.2	311.2	293.4	311.2	358.2
CYSH	ND	1.75	3.16	2.61	2.75	3.07	3.43	1.88
ILE	8.49	4.27	2.75	3.47	3.35	1.47	3.04	2.82
LEU	9.53	3.72	2.72	3.55	4.47	4.18	3.76	4.58
TYR	5.62	4.70	3.92	3.98	3.91	3.86	3.91	2.69
PHE	4.27	4.06	3.59	3.89	3.78	4.13	3.85	2.95
ORN	7.62	4.20	3.66	4.91	2.99	4.31	2.82	2.61
LYS	18.26	8.32	8.33	16.37	11.02	15.94	11.36	14.59
HIS	20.93	17.44	13.48	9.29	11.18	11.68	10.01	12.01
ARG	10.97	7.70	6.31	11.75	11.21	13.50	9.41	11.82
EAA[4]	109.1	63.34	52.62	67.95	62.96	165.1	135.3	64.82
NEAA[5]	198.0	110.7	90.02	90.20	94.11	109.4	103.4	83.81
GLY/OAA[6]	0.533	0.462	0.457	0.329	0.425	0.271	0.259	0.493
N/E[7]	1.81	1.75	1.71	1.33	1.49	0.663	0.764	1.29

8) Infusion of 8.4g L-methionine plus 4.2g L-threonine/day for approximately 54h prior to this sampling (i.e. from day 6).

9) See note 8.

10) Threonine infusion ceased on day 12, 5 days prior to this sampling.

possible factors involved in the methionine-induced depression of amino acids have been discussed in Chapter 1. At high levels of methionine, some of the essential amino acids were markedly elevated (see Table 3.3), possibly through effects of high levels of methionine on protein catabolism. This effect could possibly be mediated through the effects of methionine on corticosteroid secretion (Girard-Globa *et al.* 1972), resulting in an increased rate of protein catabolism. However, this seems unlikely in view of the elevation of the plasma threonine concentrations since if the response in sheep is similar to the response in the rat, corticosteroids could be expected to induce an increase in the level of the enzyme, threonine serine dehydratase (Peraino 1967), which could be expected to act against a rise in the plasma concentration of threonine. Such speculation does assume that changes in the plasma levels of amino acids do reflect changes in the tissue free amino acids. At low levels of methionine supplementation (e.g. 0.7g/day in Experiment 2/1), withdrawal of amino acids for protein synthesis may have been an important component of the change in the plasma amino acid pattern, but at higher levels of methionine, this is unlikely since there was little further increase in protein synthesis as estimated by nitrogen balance (Chapter 2), but a very marked shift in plasma amino acid levels. It is concluded that much of the change in plasma amino acids may not have been a result of an increased demand for protein synthesis, but rather a response to some metabolic effect of methionine, either on amino acid catabolism or transport. This is particularly so at the higher levels of methionine infusion. These changes have previously been discussed in Chapter 1 in relation to the methionine-induced removal of amino acids from plasma as a possible indicator of the likely limiting amino acids, and in

relation to the effects of a glucose-induced stimulation of protein synthesis. Generally the plasma results were similar to those reported by Reis *et al.* (1973b) for sheep receiving abomasal infusions of methionine, although the extent of the changes observed in the present work was usually greater.

There were very marked effects of methionine supplementation on the total essential and non-essential amino acid levels in plasma. However the effects on the GLY/OAA and N/E ratios differed between animals and were apparently unrelated to the animal's level of intake or to the type of intake response to methionine. The GLY/OAA ratio declined as a result of methionine supplementation in four of the five sheep in Experiments 2/2 and 2/3 while the effect on the N/E ratio was variable. This is in contrast to the situation in Experiment 2/1 where the GLY/OAA ratio was unaffected. Such alterations in the GLY/OAA ratio highlight the necessity for an understanding of glycine metabolism (perhaps with particular reference to methionine) if the ratio is to be useful as a metabolic index of amino acid absorption as originally proposed by Egan (1972).

The comparative effects of methionine infusion on plasma and blood cell amino acids (Experiment 2/2)

Table 3.4 gives the plasma and blood cell amino acid patterns in the blood samples taken on day 17 of each period in which methionine alone was infused.

Blood cell amino acid concentrations were much less affected by methionine supplementation than the plasma amino acids, although most changes were in the same direction for both plasma and blood cells.

TABLE 3.4 (Experiment 2/2): Amino acid concentrations in the plasma (µmoles/100 ml plasma) and in the blood cells (µmoles/100 ml blood cells) on day 17 of each period in which either water (MO) or methionine only (M1.4, 4.2, 8.4) was infused.

Sheep 761	Plasma		Blood cells	
	MO	M1.4	MO	M1.4
TAU	1.07	2.62	18.11	54.68
THR	9.68	4.09	17.26	8.80
SER	18.92	7.43	19.25	7.84
GLU	22.38	8.80	21.19	18.78
GLY	74.67	45.24	81.53	82.67
ALA	16.60	7.00	31.73	26.57
ABU	1.31	0.25	3.55	1.32
VAL	14.21	4.50	14.96	9.62
½CYS	1.47	2.25	ND[1]	2.33
MET	1.28	1.05	0.32	1.64
CYSH	tr.[1]	0.73	0.54	0.66
ILE	6.91	1.99	6.34	3.65
LEU	6.30	2.41	10.31	8.06
TYR	4.32	1.85	3.64	3.15
PHE	3.37	1.59	4.15	4.71
ORN	6.82	2.36	21.57	17.65
LYS	13.71	5.20	23.98	19.39
HIS	16.00	10.91	16.56	17.51
ARG	11.39	4.79	1.40	1.92

1) ND: not detectable; tr: trace.

TABLE 3.4 (Experiment 2/2) continued:

Sheep 663

	Plasma				Blood cells			
	MO	M1.4	M4.2	M8.4	MO	M1.4	M4.2	M8.4
TAU	3.40	15.52	19.58	12.97	24.68	54.62	34.47	65.25
THR	11.59	4.67	6.04	7.04	10.45	5.39	8.14	19.53
SER	16.33	6.48	6.03	9.12	12.10	6.50	8.62	119.0 [2]
GLU	30.77	22.28	10.59	9.77	28.05	18.12	14.82	26.80
GLY	104.3	41.66	33.77	37.59	88.91	51.09	33.09	63.08
ALA	19.14	13.30	13.47	12.02	28.59	22.91	18.15	28.82
ABU	1.32	1.23	1.04	1.09	3.01	3.49	0.86	1.21
VAL	12.32	9.71	8.02	9.87	14.30	11.45	8.93	15.03
½CYS	2.48	3.70	3.05	5.44	1.51	0.40	1.46	ND
MET	2.22	4.81	132.7	193.1	1.98	1.23	39.73	103.5
CYSH	ND	1.74	1.74	0.76	ND	0.20	0.39	1.04
ILE	8.38	4.51	2.45	1.28	6.38	5.39	2.10	5.17
LEU	8.09	4.02	2.70	4.70	15.49	12.90	10.27	27.51
TYR	4.79	4.25	4.19	3.89	4.82	4.73	5.06	9.95
PHE	3.31	4.63	3.74	3.71	4.42	5.38	4.37	7.96
ORN	7.30	3.52	3.24	3.82	4.88	3.33	2.72	3.19
LYS	22.22	8.50	7.48	16.03	28.44	19.57	15.34	22.65
HIS	22.96	15.99	7.93	10.19	19.04	17.87	11.81	27.42
ARG	13.21	5.50	6.19	11.75	24.52	19.13	17.97	46.41

2) Peak not pure; a major contaminant was present in the serine peak of this sample; see also Table 3.5 note 3.

TABLE 3.4 (Experiment 2/2) continued:

Sheep 748

	Plasma			Blood cells		
	MO	M1.4	M4.2	MO	M1.4	M4.2
TAU	2.89	15.72	18.05	39.33	51.86	55.16
THR	13.48	6.15	5.83	15.50	10.16	14.56
SER	19.80	7.75	7.27	15.75	15.50	15.90
GLU	29.11	17.76	11.69	22.60	21.98	19.75
GLY	96.2	40.95	37.16	73.04	59.47	48.10
ALA	19.36	17.45	14.19	29.67	32.55	32.98
ABU	1.23	1.25	1.52	2.13	1.62	0.78
VAL	17.37	10.69	7.14	16.73	15.36	14.29
½CYS	1.78	3.95	4.68	ND	3.93	2.07
MET	1.90	6.41	159.1	1.47	2.58	79.99
CYSH	tr.	2.46	2.99	ND	0.72	0.78
ILE	7.62	4.94	1.81	5.60	5.66	3.03
LEU	7.90	4.35	1.88	17.44	18.60	18.21
TYR	4.80	4.61	3.38	5.44	5.75	6.73
PHE	3.35	4.44	2.83	5.94	7.53	7.78
ORN	7.01	4.28	2.72	6.47	5.94	7.05
LYS	18.79	8.99	5.67	22.00	21.00	18.67
HIS	19.16	16.68	12.79	16.61	17.75	15.58
ARG	10.53	8.33	5.45	2.68	2.79	3.44

In particular the lysine and leucine levels in the blood cells were much less affected than the plasma levels in the periods in which methionine was infused. The effect on the other amino acids tended to differ between animals, e.g. the plasma serine concentration was markedly depressed by methionine supplementation in both sheep 663 and 748, but only in sheep 663 was the blood cell concentration of serine reduced. The blood cell methionine levels were always considerably less than the plasma concentrations, while for taurine the situation was reversed.

The effect of methionine infusion on blood cell amino acids

There were marked differences between animals in the effect of methionine infusion on blood cell total essential and non-essential amino acids (Table 3.5). In sheep 748, there was little or no effect of methionine on blood cell levels, while in sheep 761 and 663, methionine at a daily rate of 1.4g (and 4.2g) markedly depressed amino acid concentrations. However, at the high level of methionine (8.4g daily) in sheep 663, the blood cell amino acids were greatly increased.

Table 3.6 gives the mean values for the blood cell amino acid concentrations for the four or five blood samplings from each sheep when the lower rates of methionine (1.4 and 4.2g daily) were infused as compared with the periods in which the high rate of methionine (8.4g daily) was infused. The concentrations of most of the blood cell amino acids were significantly increased during the high methionine infusion in sheep 663, whereas only methionine, taurine and cystathionine were significantly higher in sheep 748. The only essential amino acid not increased by the high level of methionine in

TABLE 3.5 (Experiment 2/2): Blood cell total essential (EAA) and non-essential (NEAA) amino acid concentrations (μmoles/100 ml blood cells) in samples from sheep 761, 663 and 748 on each blood sampling day in each period.

	761		663		748	
	EAA[1]	NEAA[2]	EAA	NEAA	EAA	NEAA
MO day 17	95.0	178.9	123.0	167.4	102.5	153.0
N1.4 day 3	80.1	174.4	117.2	87.4	123.0	174.7
17	73.7	156.7	97.1	106.7	98.9	141.2
M4.2 day 3	64.6	135.0	80.5	97.6	86.2	135.7
8	98.7	143.6	NS[4]		NS	
10	104.8	129.9	NS		NS	
17	NS		78.9	82.5	95.6	130.5
M8.4 day 3	NS		152.7	178.9[3]	90.7	117.3
5	NS		NS		108.0	128.0
8	NS		174.1	224.5[3]	152.6	136.4
11	NS		159.7	203.1[3]	148.5	137.8
17	NS		171.7	250.8[3]	109.9	134.6

1) EAA - THR, VAL, ILE, LEU, PHE, LYS, HIS, ARG.

2) NEAA - SER, GLU, GLY, ALA, TYR, ORN.

3) A contaminant of serine or glutamic acid was present in these samples; assuming that serine concentration was 15 μmoles/100 ml and glutamic 28 μmoles/100 ml (calculated from the two samples with pure peaks), then the concentrations of the contaminant in samples from days 3, 8, 11 and 17 would be approximately 33, 81, 70 and 104 μmoles/100 ml respectively. If the total NEAA were corrected for the presence of the contaminant, the total NEAA would be 146, 144, 133 and 147 μmoles/100 ml in the four samples from days 3, 8, 11 and 17 respectively.

4) NS: no sample.

TABLE 3.6 (Experiment 2/2): Amino acid concentrations in the blood cells (µmoles/100 ml blood cells) of sheep 761, 663 and 748 on day 17 of the control period (MO) and the mean concentration in samples during the infusion of 1.4 and 4.2g L-methionine/day compared with the mean concentrations in samples during the infusion of 8.4g L-methionine/day.

Sheep 761	MO[1]	M1.4, 4.2[2]
TAU	18.11	39.46 ± 6.70
THR	17.26	13.13 ± 2.97
SER	19.25	10.36 ± 1.77
GLU	21.19	15.37 ± 2.09
GLY	81.53	75.78 ± 4.32
ALA	31.73	26.08 ± 4.20
ABU	3.55	2.00 ± 0.37
VAL	14.96	11.10 ± 0.99
½CYS	ND	ND[3]
MET	0.32	21.76 ± 20.18
CYSH	0.54	1.05 ± 0.17
ILE	6.34	4.02 ± 0.58
LEU	10.31	9.12 ± 0.75
TYR	3.64	3.55 ± 0.27
PHE	4.15	4.59 ± 0.16
ORN	21.57	21.25 ± 2.19
LYS	23.98	20.08 ± 2.58
HIS	16.56	16.50 ± 0.86
ARG	1.40	0.73 ± 0.40
EAA[4]	94.96	79.26 ± 7.22
NEAA[5]	178.9	152.4 ± 8.58

1) Values for day 17 of the control period; one sample only.

2) Mean of 4 values (days 3, 17 of M1.4; days 3, 8 of M4.2) ± standard error of the mean.

3) ND - not detectable; ½CYS detectable in only one sample.

4) EAA - THR, VAL, ILE, LEU, PHE, LYS, HIS, ARG.

5) NEAA - SER, GLU, GLY, ALA, TYR, ORN.

TABLE 3.6 (Experiment 2/2) continued:

Sheep 663	MO[1]	M1.4, 4.2[6]	M8.4[7]	t-test[8]
TAU	24.68	45.96 ± 4.36	55.92 ± 3.52	NS
THR	10.45	6.55 ± 0.68	15.92[9] ± 1.28	***
SER	12.10	7.02 ± 0.79	58.20[9]	-
GLU	28.05	13.69 ± 1.77	56.71[9]	-
GLY	88.91	44.50 ± 4.63	57.11 ± 3.25	NS
ALA	28.59	19.92 ± 1.11	29.84 ± 1.22	***
ABU	3.01	2.14 ± 0.69	1.15 ± 0.39	NS
VAL	14.30	10.69 ± 1.44	14.06 ± 0.45	NS
½CYS	1.51	1.64 ± 0.51	NM[10]	-
MET	1.98[10]	17.66 ± 9.67	107.1 ±13.18	**
CYSH	ND[10]	0.26 ± 0.06	0.90 ± 0.07	***
ILE	6.38	5.09 ± 1.05	4.70 ± 0.37	NS
LEU	15.49	11.69 ± 1.59	24.19 ± 1.18	***
TYR	4.82	5.26 ± 0.44	8.42 ± 0.78	*
PHE	4.42	4.92 ± 0.44	7.13 ± 0.41	*
ORN	4.88	3.16 ± 0.65	4.02 ± 0.46	NS
LYS	28.44	18.71 ± 1.55	30.07 ± 2.49	**
HIS	19.04	16.16 ± 2.52	25.56 ± 1.44	*
ARG	24.52	19.62 ± 1.88	42.92 ± 2.92	***
EAA[4]	123.0	93.43 ± 8.92	164.6 ± 5.04	***
NEAA[5]	167.4	93.55 ± 5.40	-	-

6) Mean of 4 values (days 3, 17 of M1.4 and M4.2) ± standard error of the mean.

7) Mean of 4 values (days 3, 8, 11, 17 of M8.4) ± standard error of the mean.

8) t-test for significance of difference between M1.4, 4.2 and M8.4, NS - not significant; *P<0.05; **P<0.01; ***P<0.001.

9) Peaks not pure; a major contaminant of the serine or glutamic acid peak was present in these samples.

10) NM - not measurable; ND - not detectable.

TABLE 3.6 (Experiment 2/2) continued:

Sheep 748	MO[1]	M1.4, 4.2[6]	M8.4[11]	t-test[8]
TAU	39.33	52.84 ± 0.83	63.76 ± 1.42	***
THR	15.50	13.31 ± 1.12	33.32 ±10.03	NS
SER	15.75	15.63 ± 0.51	16.22 ± 1.15	NS
GLU	22.60	19.43 ± 1.26	16.25 ± 1.08	NS
GLY	73.04	62.41 ± 7.76	53.22 ± 2.20	NS
ALA	29.67	34.05 ± 0.98	31.61 ± 1.08	NS
ABU	2.13	1.67 ± 0.54	1.80 ± 0.41	NS
VAL	16.73	15.60 ± 1.64	12.69 ± 0.55	NS
½CYS	ND[10]	3.40 ± 0.47	NM[10]	-
MET	1.47	34.58 ±19.07	156.3 ±10.57	**
CYSH	ND	0.51 ± 0.14	2.28 ± 0.53	*
ILE	5.60	4.56 ± 0.79	3.32 ± 0.28	NS
LEU	17.44	18.10 ± 1.36	19.28 ± 0.87	NS
TYR	5.44	6.85 ± 0.47	7.13 ± 0.32	NS
PHE	5.94	7.44 ± 0.48	7.20 ± 0.25	NS
ORN	6.47	7.06 ± 0.44	6.35 ± 0.42	NS
LYS	22.00	21.17 ± 2.11	23.88 ± 1.17	NS
HIS	16.61	17.83 ± 1.27	20.06 ± 1.30	NS
ARG	2.68	2.90 ± 0.37	2.18 ± 0.21	NS
EAA[4]	102.5	100.9 ± 7.85	121.9 ±12.17	NS
NEAA[5]	151.0	145.4 ± 9.97	130.8 ± 3.78	NS

11) Mean of 5 values (days 3, 5, 8, 11, 17 of M8.4) ± standard error of the mean. The samplings on days 8 and 11 cover the period in which threonine also was infused.

sheep 663 was isoleucine. This may be related to some particular facet of isoleucine metabolism in this animal, although this cannot be ascertained.

The high level of methionine administration was associated with the appearance of an additional major peak in the blood cell amino acid chromatogram of sheep 663. Unfortunately this peak (approximately 1-2 times the area of glycine) eluted with either serine or glutamic acid. It is possible that this unknown is glutamine or sarcosine (Hamilton 1963). Sarcosine (N-methyl glycine) is a possible product of methionine catabolism, particularly in animals receiving large amounts of methionine (Benevenga 1974).

The effect of the high methionine infusion in grossly elevating the blood cell amino acids in sheep 663 is a particularly interesting phenomenon, especially in view of the fact that there was no such effect apparent in sheep 748. Therefore, methionine brought about some alteration in amino acid transport, either through a stimulation of amino acid uptake (from plasma or tissues) or an inhibition of transport (i.e. increased retention) out of the blood cells. Unfortunately amino acid analyses were not performed on tissues from these animals. A methionine effect on amino acid transport could have operated through the heteroexchange phenomenon (Christensen 1963; 1964); i.e. methionine has an affinity for two transport systems, with the result that it is strongly concentrated by one mediator and then serves by exchange through the second mediator (i.e. transport system) to drive the uphill transport of leucine and valine and similar amino acids. However, there are many other possible mechanisms, perhaps involving changes in ion balance

(Zweig 1973; Christensen & Handlotgen 1975), which could conceivably have produced such an effect. Any further speculation is not warranted, although the results and the interpretation clearly indicate that the simplistic approach to blood cell amino acid concentrations and interpretations in terms of transport and availability of amino acids to cells must be backed up by much more information than exists at present.

Blood cell ornithine and arginine concentrations

The differences between animals in the blood cell concentrations of the urea cycle intermediates, arginine and ornithine were particularly striking (Table 3.7). Sheep 761 exhibited high levels of ornithine and low levels of arginine in the blood cells, while in sheep 663 the reverse situation was apparent. In sheep 748, both ornithine and arginine concentrations in the blood cells were comparatively low. Although citrulline concentrations are not presented in the present work (citrulline and proline were not consistently resolved in our amino acid separation), the total peak area of proline and citrulline was similar in all animals. These marked differences between animals in the ornithine and arginine concentrations may reflect differences in the activity of the urea cycle (including differences in the activities of the urea cycle enzymes) as well as differences in the transport of these amino acids.

Repeatability of plasma amino acid patterns (Experiment 2/1)

The apparent variability between animals in the response to an abomasal infusion of methionine has been discussed in Chapter 2. Further supportive evidence that this variability was both a real and a repeatable phenomenon may be obtained from an examination of plasma amino acid patterns and the changes in these patterns in

TABLE 3.7 (Experiment 2/2): Individual animal mean values for the blood cell concentrations (µmoles/100 ml blood cells) of ornithine and arginine.

Sheep	Concentration (µmoles/100 ml blood cells)[1] ORN	ARG	n
761	21.31 ± 1.69	0.87 ± 0.33	5
663	3.73 ± 0.38	30.52 ± 4.20	9
748	6.65 ± 0.28	2.52 ± 0.20	10

1) Mean value ± standard error of the mean; the mean over all samples in all periods.

response to methionine. It is this repeatability of the plasma amino acid patterns which should hold the most significance if the changes reflect physiological consequences of methionine administration which differ between animals.

The repeatability of the basal plasma amino acid pattern may be assessed from the correlation of the concentration of the individual amino acids between the two control periods. The correlation coefficients for these relationships are given in Table 3.8a. These within-animal between-period correlations ($t = 15.7$ and 18.3) were also higher than the between-animal correlation ($r = 0.895$; $t = 11.7$).

Table 3.8b presents the correlation coefficients for the relationships between the concentration of an amino acid in sequence A and sequence B. Except for histidine and arginine concentrations, the correlation coefficients were high. Also for most amino acids, the within-animal between-period correlation was higher than the between-animal correlation.

A further criterion of the repeatability of the response may be the relationship between the change in the concentrations of individual amino acids in the two sequences in response to each treatment. The correlation coefficients are summarised in Table 3.8c. However, in this case the between-animal correlations (except for the lowest rate of methionine supplementation) were at least as good as the within-animal between-period correlations.

The plasma methionine concentrations for the two sheep in the two dose response sequences are shown in Fig.3.1. Although the

TABLE 3.8 (Experiment 2/1): Correlation coefficients for the relationship between plasma amino acid patterns in the two methionine infusion sequences.

a) Relationship between the basal (control) values for the plasma essential amino acids (correlation between concentrations of the nine essential amino acids on two sampling days in period 1 and the concentrations in period 8).

	Within animals (n=18)	Between animals (n=36)
Eli	0.97^{**}, t=15.7	0.90^{**}, t=11.7
Eunuch	0.98^{**}, t=18.3	

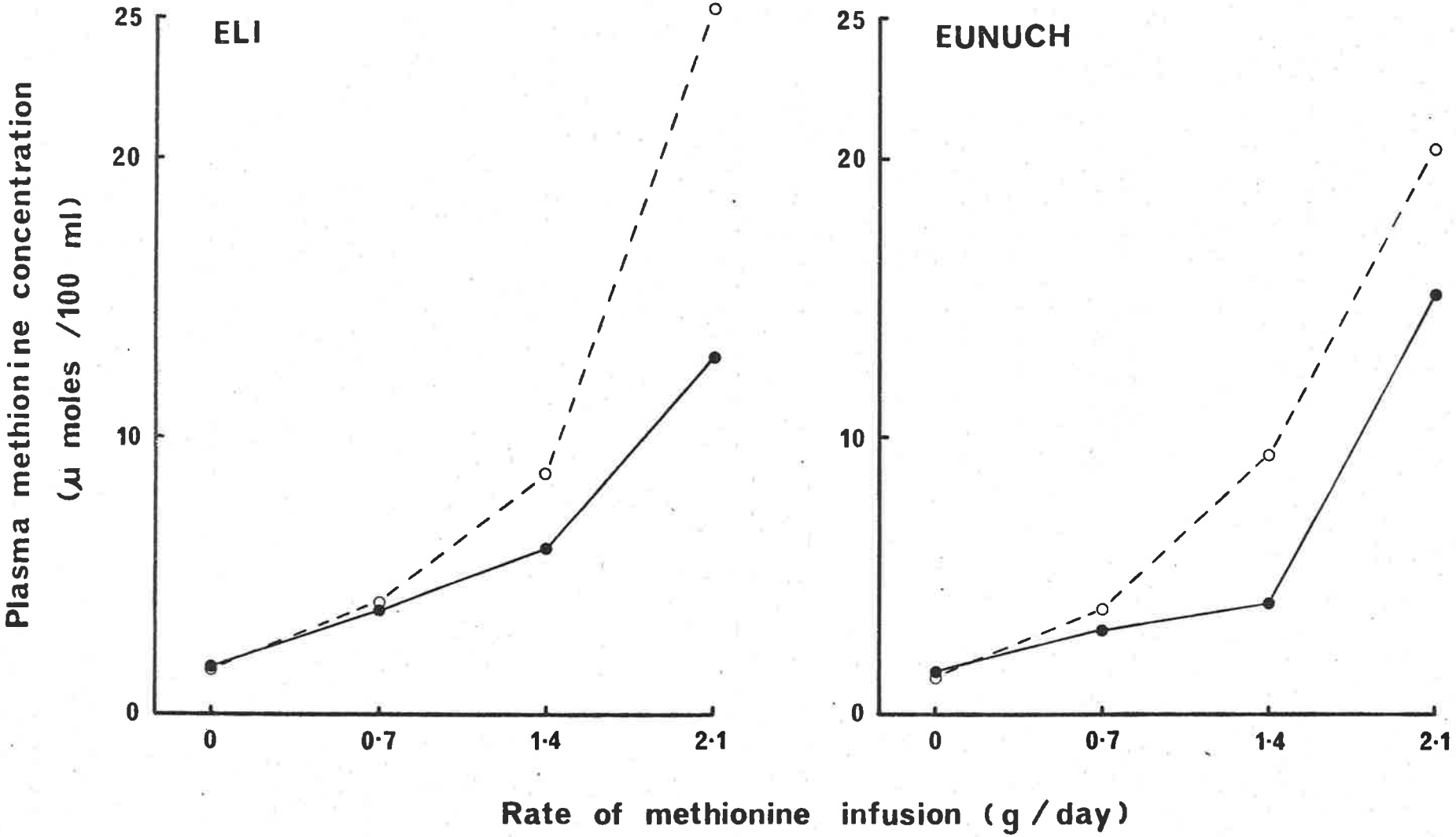
b) Relationship beteen all values for the concentration of each individual essential amino acid in sequence A and the values in sequence B (correlation between concentrations for two sampling days in each of four periods in sequence A and the concentrations in sequence B).

	Within animals (n= 8)		Between animals (n=16)
	Eli	Eunuch	
THR	0.72^{*}_{**}	0.79^{*}_{**}	0.74^{**}_{**}
VAL	0.81^{**}	0.92^{**}	0.62^{**}
MET	0.83^{NS}	0.87^{*}	0.93^{NS}
ILE	0.55^{*}	0.75^{*}	0.21^{NS}
LEU	0.76^{*}	0.79^{*}	0.43^{*}
PHE	0.80^{*}	0.79^{NS}	0.59^{NS}
LYS	0.77^{NS}	0.45^{NS}	0.25^{NS}
HIS	0.19^{NS}	0.19^{NS}	0.17^{NS}
ARG	0.38^{NS}	0.07^{NS}	0.21^{NS}

(c) Relationship between changes in the plasma concentration of essential amino acids (Thr, val, ile, leu, phe, lys) in sequence A and sequence B (change is expressed as the molar change in the mean concentration i.e. Control-Treatment).

	Within animals (n=6)		Between animals (n=12)
Methionine (g/day)	Eli	Eunuch	
0.7	0.73^{NS}_{*}	0.69^{NS}_{NS}	0.47^{NS}_{**}
1.4	0.81^{*}	0.58^{*}	0.81^{**}
2.1	0.94	0.80	0.87

FIGURE 3.1 (Experiment 2/1): Effect of level of methionine infusion on plasma methionine concentration (μmoles/100 ml) in the two sheep Eli and Eunuch in the two methionine infusion sequences (values are the means of days 3 and 11 in each period; ●——● sequence A; o----o sequence B).


ELI
EUNUCH
Plasma methionine concentration
(μ moles /100 ml)
25
20
10
0
0
0·7
1·4
2·1
Rate of methionine infusion (g / day)

trends with an increasing level of methionine supplementation were the same in each sequence, the actual plasma methionine concentrations were considerably higher during the infusion of 1.4 and 2.1g of methionine in sequence B than in sequence A. There are no obvious reasons for this variability between the two sequences.

The repeatability of the plasma amino acid patterns between sequences within each sheep is clearly evident, both for the plasma concentrations in the two control periods and for most of the essential amino acids over the different levels of methionine infusion. An indication as to whether the responses truly reflect variability between animals rather than a general response to methionine infusion may be obtained from a comparison of the correlation coefficients for the between-animal relationships as against the within-animal between-period correlation. The fact that the latter correlations were usually stronger is evidence that between-animal variability was greater than the within-animal variability.

Plasma amino acids and between-animal variability in the methionine response

Plasma amino acid patterns frequently reflect an imbalance or deficiency in the supply of amino acids (Leung & Rogers 1969; Harper *et al.* 1970). For this reason, plasma amino acid patterns were examined for differences between those sheep exhibiting a positive response (R+) in voluntary intake to a supplement of methionine, and those either not responding or exhibiting a negative response (R-) in terms of intake.

In Experiment 1/1 plasma leucine, lysine and threonine were depressed to the greatest extent in sheep B(R-) and F(R+), while in sheep K(R+), the plasma threonine concentration was much less affected than either leucine, lysine, valine or isoleucine. The results of Experiments 2/1 and 2/2 were similar. Taken over both methionine sequences in Experiment 2/1, the amino acid most depressed as a result of methionine treatment was threonine (62% of control for Eli (R-) and 68% for Eunuch (R+)). Plasma leucine, valine and lysine were all depressed to about 75% of the control values in each animal, although in sequence B, there was a tendency for plasma leucine to be more affected than either lysine or valine. In sheep 761 (R-) all plasma essential amino acids were greatly depressed by a daily infusion of 1.4g of methionine, although threonine, valine, leucine, isoleucine and lysine were particularly affected. These changes were very similar to those observed in the plasma pattern of sheep 663 (R+) and sheep 748 (R+) both of which exhibited positive responses to methionine supplementation in marked contrast to the adverse effects apparent in sheep 761. Overall there were no readily apparent differences in the plasma amino acid patterns of animals classified in the different response categories depending on their intake response to methionine.

The 2.1g methionine supplement given to Eli and Eunuch (Experiment 2/1) resulted in very marked depressions in plasma threonine and leucine (Table 3.9) and when these amino acids were administered abomasally, there was an apparent positive response in terms of voluntary intake and the efficiency of nitrogen utilisation (Chapter 2). As discussed in Chapter 2, it seems as though both animals responded to leucine plus threonine moreso than to threonine

TABLE 3.9 (Experiment 2/1): Plasma threonine, leucine and total essential (EAA) and non-essential amino acid (NEAA) concentrations (μmoles/100 ml) for the two sheep, Eli and Eunuch during periods 11 (2.1g L-methionine/day; M), period 12 (M + 1.4g L-threonine/day; MT) and period 13 (MT + 2.1g L-leucine/day; MTL).

	M		MT		MTL	
Day:	3	11	3	11	3	11
Eli						
THR	2.68	4.99	31.55	40.48	31.70	28.77
LEU	3.24	3.94	3.25	3.52	9.70	6.04
EAA[1]	50.92	55.67	77.62	89.97	85.11	63.12
NEAA[2]	112.9	108.4	106.7	115.3	108.2	72.76
Eunuch						
THR	6.03	8.39	47.60	46.54	55.42	54.09
LEU	3.41	4.86	4.92	4.94	12.27	11.52
EAA[1]	61.33	64.68	108.4	107.5	133.2	116.5
NEAA[2]	105.7	113.0	116.5	128.1	134.3	128.2

1) EAA: THR, VAL, ILE, LEU, PHE, LYS, HIS, ARG.

2) NEAA: SER, GLU, GLY, ALA, TYR, ORN.

alone. However, the usefulness of such changes in plasma amino acid patterns as predictive indices of the relative deficiencies of amino acids must await more definitive studies.

The only clearcut changes in the plasma amino acid pattern with threonine and leucine infusions were the increases in the concentrations of these amino acids themselves (except for the threonine effect on methionine concentrations to be discussed in the next section). The increase in threonine concentration in the plasma of Eli was much less than the increase apparent for Eunuch. Eunuch exhibited a higher level of plasma threonine than Eli throughout the experiment. Such a basic difference in threonine concentrations may be indicative of a difference in the activity of threonine-serine dehydratase.

The whole question of the interpretation of plasma amino acid patterns is in need of considerably more definitive study. In many cases, it seems that the pattern of changes reported in the literature are dependent on the imposition of quite extreme treatments (e.g. amino acid imbalance) or alternatively require large numbers of animals to show differences in treatments. Patterns of change in different individuals may reflect unique individual metabolic patterns, or perhaps the patterns may appear similar, and not reflect the real differences at the tissue level.

Effect of threonine on the plasma amino acid pattern

Table 3.10 gives the plasma concentrations of methionine and its metabolites in Eli and Eunuch during the methionine infusions of sequence B and during the infusion of threonine plus methionine. With

TABLE 3.10 (Experiment 2/1): The plasma concentrations of methionine and its metabolic products (μmoles/100 ml plasma) and the methionine to products ratio in the sheep Eli and Eunuch in periods 8 to 12.

	Methionine infused (g/day)								
	0	0.7		1.4		2.1		2.1 + Thr[2]	
Day	3,11	3	11	3	11	3	11	3	11
	Eli								
TAU	2.47	2.19	2.19	10.43	15.46	19.62	19.41	21.22	22.35
ABU	1.37	2.01	1.52	1.47	1.83	1.95	1.33	2.27	2.91
½CYS	1.12	3.40	4.36	4.82	4.79	4.04	4.49	4.29	5.71
MET	1.66	3.62	4.27	7.76	9.55	17.16	33.48	26.24	19.12
CYSH	ND	0.29	0.77	1.56	2.13	1.96	1.88	1.87	4.51
M/P[1]	0.666	0.635	0.642	0.989	1.09	2.16	4.35	3.11	1.46
	Eunuch								
TAU	2.62	3.68	2.56	3.71	21.52	23.53	25.53	22.54	26.81
ABU	1.14	1.49	1.38	2.63	1.98	2.68	2.06	2.62	2.46
½CYS	1.37	2.80	2.81	3.42	4.20	3.96	3.32	4.21	5.42
MET	1.28	3.77	3.77	8.27	10.51	18.22	22.50	16.57	12.89
CYSH	ND	ND	0.42	1.87	1.99	2.44	2.86	2.27	3.29
M/P[1]	0.510	0.879	0.818	1.20	1.29	2.01	2.73	1.82	1.15

1) M/P - methionine: products ratio, products being the sum of the concentration of ABU, ½CYS and CYSH.

2) L-threonine infused a rate of 1.4g/day.

an increasing level of methionine infusion, the plasma concentration of methionine and its metabolites tended to increase, although the increase in the methionine concentration was greater than that in the metabolites (excluding taurine). These changes are reflected in the M/P ratio (methionine to products ratio; the products being α-amino-n-butyric acid (ABU), cystine (expressed as half-cystine) and cystathionine).

Threonine supplementation was associated with a decrease in the concentration of plasma methionine and an increase in the level of ABU, half-cystine and cystathionine, all products of the transulphuration pathway of methionine metabolism (Finklestein 1974). This effect was more apparent on day 11 of the methionine and threonine infusion than on day 3. These results suggest that threonine may have increased the rate of methionine catabolism via the transulphuration pathway. Serine (a metabolite of threonine) is required for the formation of cystathionine, and it is possible that threonine simply increased the supply of serine and so increased the rate of formation of cystathionine from homocysteine.

In contrast to the effects observed in Experiment 2/1, the only readily apparent effect of threonine supplementation on the plasma amino acid pattern of sheep 761 and 748 was the elevation in plasma threonine levels. However, the samples analysed were all obtained within 5 days of the start of the threonine infusion.

CONCLUSIONS

The changes in plasma amino acid concentrations as a result of methionine supplementation were probably principally due to metabolic effects of methionine and did not reflect withdrawal of amino acids for protein synthesis.

Blood cell amino acid concentrations were much less affected by methionine supplementation than were plasma concentrations, although changes in both compartments were generally in the same direction. There was evidence of considerable variability between animals both in the blood cell concentrations of ornithine and arginine and in the effects of methionine on the concentrations of amino acids in the blood cells.

Further evidence that between animal variability was a real and repeatable phenomenon was apparent from the repeatability of the plasma amino acid patterns in the two methionine supplementation sequences. However there were no obvious differences between animals in the plasma amino acid patterns which could be related to differences in the type of response to the methionine supplement. There was evidence of an interaction of methionine and threonine metabolism in the changes in the plasma amino acid patterns. However the whole question of the interpretation of plasma (and blood cell) amino acid patterns is in need of considerably more definitive study.

CHAPTER 4

Post-ruminal supplementation with groups of essential amino acids in young growing sheep fed a roughage diet

INTRODUCTION

The apparent between animal variability in the response to an abomasal supplement of methionine in previous work (Experiment 1/1) led to the suggestion that no one amino acid would be likely to be limiting production for all of a group of sheep fed the same diet. It also seemed likely that even though an amino acid may be first limiting for one facet of production (e.g. cysteine for wool growth) it does not necessarily follow that the same amino acid would be limiting for other productive functions (e.g. muscle protein accretion).

These considerations led to the experiment to be reported in this paper. It is suggested that a group of amino acids when given as an abomasal supplement to a group of sheep would promote an increased protein synthesis (as determined by overall nitrogen retention) in all sheep. In contrast a supplement of a single amino acid would be likely to result in both positive and negative responses within a group of sheep. In practical terms this would mean that no one amino acid was "first-limiting" and that the concept of a "first-limiting" amino acid in terms of the whole animal is inadequate.

EXPERIMENTAL

Animals

Seven Dorset x (Dorset x Merino) wethers aged about 8 months and of bodyweight 23-29 kg at the start of the experiment were used.

Each had been surgically prepared with an abomasal tube (Appendix 2) at least three weeks prior to the commencement of the experiment. The animals were housed indoors in metabolism pens and fitted with faecal collection harnesses.

Diets and feeding

The sheep were offered a diet of wheaten hay chaff (1.05% nitrogen in the dry matter) *ad libitum*. They were fed daily at 1000h following the removal of feed residues; the amount of feed offered was c.15% in excess of the expected daily intake. A mineral supplement (8g/day; Moir & Harris 1962) was sprinkled on top of the feed. Vitamins A and D_3 (APAC: Nicholas Pty. Ltd.) were given with the feed on the first day of each period. Water was available at all times.

Experimental design

The experiment consisted of six periods, each of 16 days. The first and second periods (HC and NC) were control periods; a cyclic changeover design (Davis & Hall 1969) was used over the remaining four periods (A1 to A4) during which the amino acid mixtures were administered. The experimental design is given in Table 4.1.

In period HC, the sheep received an abomasal infusion of water, while in period NC they received an abomasal infusion of triammonium citrate which provided 2.6g of nitrogen/day (isonitrogenous with the infusions in periods A1 to A4). The cyclic changeover design (Davis & Hall 1969) permitted the use of seven amino acid treatments administered over only four experimental periods. However, the use of seven amino acids in the mixtures meant that three of ten amino

TABLE 4.1: The experimental design and the allocation of animals to the various treatments.

Sheep	648	768	655	669	664	759	760
Period							
HC	CONTROL - WATER INFUSION						
NC	CONTROL - TRIAMMONIUM CITRATE INFUSION						
A1	0	1	2	3	4	5	6
A2	3	4	5	6	0	1	2
A3	4	5	6	0	1	2	3
A4	1	2	3	4	5	6	0

1) Treatments: all solutions were administered at a rate of 220 ml/day into the abomasum.

HC - water infusion; NC - triammonium citrate infusion to provide 2.6g of nitrogen (N) daily.

The amino acid treatments are described by the amino acid which was omitted from the particular treatment (e.g. LEU - infusate contained threonine, valine, isoleucine, tryptophan, lysine, methionine and triammonium citrate):

0 - LEU; 1 - THR; 2 - VAL; 3 - ILE; 4 - TRP; 5 - LYS; 6 - MET.

2) Amino acids were supplied as the L-isomers at the following rates (g/day): leucine 2.3, threonine 1.6, valine 1.7, isoleucine 1.7, tryptophan 0.5, lysine hydroch loride 1.5 and methionine 1.6.

Triammonium citrate was added to make the nitrogen infusion up to 2.6g N/day.

acids usually considered as indispensable had to be omitted from the experimental treatments.

Histidine and arginine were omitted from the amino acid treatments since there is a considerable amount of work which suggests that these amino acids are synthesized in mammalian tissues (Krebs 1964). Phenylaline appeared to be the most suitable of the remaining eight indespensable amino acids for omission from the experimental treatments. As reported in Chapter 1 (Table 1.7), the methionine supplement did not affect plasma phenylalanine concentrations in the sheep fed the wheaten hay chaff diet. This was in marked contrast to the effects of methionine infusion on the other indispensable or essential amino acids. The effect of methionine on the plasma tyrosine concentrations was also very small. In addition the requirement to supply (R/S) ratios for phenylalanine and tyrosine (Table 4.2) were the lowest of all essential amino acids. Therefore histidine, arginine and phenylalanine were selected as the three amino acids most suitable for omission from the amino acid treatments. Thus the seven amino acids included were leucine, threonine, valine, isoleucine, tryptophan, lysine and methionine.

In an attempt to estimate the most appropriate amount of each amino acid to be included in the supplementary amino acid mixture, a simple supply-demand model, similar to that in Chapter 1, was set up. The calculations were similar to those for situation B in Chapter 1, except that the estimated tissue nitrogen retention was increased from 0 to 2g/day in anticipation of a greater nitrogen retention response to a group of amino acids than to methionine alone.

TABLE 4.2: Estimates of the tissue supply of amino acids, the requirement, the daily amino acid deficit and the ratio of the requirement to the supply for a 28kg sheep fed a wheatenhay chaff diet and receiving an abomasal infusion of 1.6g of methionine/day.

	Supply	Requirement			Deficit	R/S ratio
		Tissue	Wool	Total		
		(g amino acid/day)				
THR	2.03	1.80	0.45	2.25	0.22	1.11
VAL	1.91	2.14	0.39	2.53	0.62	1.32
CYS	0.20[2]	0.53	0.80	1.33	1.13	) 1.00[3]
MET	2.30[2]	0.88	0.04	0.92	-1.38	)
ILE	1.52	1.84	0.25	2.09	0.57	1.38
LEU	3.12	3.19	0.54	3.73	0.61	1.20
PHE	2.03	1.69	0.25	1.94	-0.09	0.96
TYR	1.66	1.31	0.34	1.65	-0.01	1.00
LYS	1.98	2.85	0.21	3.06	1.08	1.55
HIS	0.80	1.01	0.07	1.08	0.28	1.35
ARG	1.61	2.40	0.64	3.04	1.43	1.89
TRP	0.62	0.53	0.10	0.63	0.01	1.02

1) Details of methods of calculation are given in Tables 1.1 and 1.2. The only modifications are that the tissue protein accretion has been increased from 0 to 13.5g/day (i.e. 2g N/day) and the rate of methionine infusion reduced from 0.16 to 0.12g/kg $^{0.75}$/day.

2) Includes 1.60g methionine as abomasal supplement.

3) Molar ratios on the basis of equivalent sulphur.

The calculated data are presented in Table 4.2. On examination, it was considered that the calculated amino acid deficits would be too unreliable as values on which to base the quantities of supplementary amino acids to be supplied. It was therefore decided to supply the amino acids in the ratios of the requirement for the growing rat (Sowers *et al.* 1972), taking 1.6g of methionine as the base value. The only modification was that the quantity of lysine in the amino acid mixture for the sheep was reduced. Triammonium citrate was included as a source of non-specific nitrogen.

The amino acids were provided in groups of six (i.e. the complete mix minus one of the amino acids); i.e. the experiment was performed such that the effect of omitting one amino acid from the group of seven selected amino acids was determined. Thus four animals received each amino acid treatment over the periods A1 to A4. The daily supplement of six amino acids and triammonium citrate was equivalent to about 20g of amino acids (10g of essential amino acids and 10g of non-specific amino-nitrogen).

Measurements

The daily *ad libitum* dry matter was measured. Composite samples of feed and residues were retained for analysis.

Urine was collected into sulphuric acid (final urine pH<2.5), the amount of urine measured and a daily subsample taken and stored at -20°C. Faeces were collected and bulked over a ten day period (days 8-17) and stored at -20°C.

The ten day nitrogen balances for each sheep were calculated using the feed intake and infusion data for days 5 to 14, the urine

collected on days 7 to 16 and the faeces collected on days 8 to 17.

The apparent digestibilities of dry matter, organic matter and nitrogen were calculated using the data for feed and faeces as for the nitrogen balance estimates.

The sheep were weighed regularly prior to feeding in the morning and mean liveweights for each period calculated. The average growth rates were 60g/day during the control periods and 115g/day during the periods of amino acid infusion.

Analytical methods

The dry matter content of feeds, faeces and residues were determined by oven drying at 85°C and organic matter by ashing at 550°C in a muffle furnace.

The nitrogen content of dried subsamples of feeds and residues and wet subsamples of faeces and urine was determined colourimetrically using a Technicon Auto Analyser following manual digestion of the samples using a Kjeldahl technique (Munro and Fleck 1969).

Amino acid infusions

All amino acids used were the L-isomers (98% pure) obtained from Tanabe Seiyaku Co. Ltd., Tokyo. The triammonium citrate was the laboratory reagent grade (BDH Chemicals). The solutions were made up in distilled water and corrected to pH 4.5 with hydrochloric acid. The solutions were infused continuously using a peristaltic pump, and the solutions replaced every two days. The infusion lines were disconnected regularly and flushed with a sodium hypochlorite-sodium chloride solution after which they were washed with distilled water and the infusion recommenced.

RESULTS AND DISCUSSION

All intake and nitrogen retention data presented in this chapter have been adjusted to allow for changes in weight using metabolic bodyweight ($kg^{0.75}$).

Control periods

The two control periods (HC and NC) were included in the experiment in order to provide some basic data on voluntary intake and nitrogen retention in all seven sheep. The complete data for the individual animals are given in Appendix Table 4.1. The overall mean values for the parameters measured in the control periods are presented in Table 4.3. There were no significant differences between the two periods in any of the parameters although the difference in mean daily nitrogen retention was substantial. However, within each of the periods, nitrogen retention (NR, $g/kg^{0.75}$/day) was significantly ($P<0,01$) positively correlated with digestible organic matter intake (DOMI, $g/kg^{0.75}$/day). The regression equations ($y = bx + a$) were:

HC: NR = 0.0138DOMI - 0.457 (1)

Standard error (SE) of b = ± 0.0041; SE of a = ± o.146; r = 0.829; n = 7.

NC: NR = 0.0193DOMI - 0.586 (2)

SE of b = ± 0.0047; SE of a = ± 0.166; r = 0.877; n = 7.

Although the regression coefficients (b) of these equations were not significantly different ($F = 0.763$; df 1,10), the adjusted mean values were significantly diffent ($P<0.01$; $F = 12.2$; df 1,11).

The difference in the adjusted mean values for nitrogen retention indicates a nitrogen retention response to the triammonium citrate infusion. A small part of this response was probably due to the energy

TABLE 4.3: Mean values for organic matter intake (OMI), digestible organic matter intake (DOMI), apparent digestibility of OM and feed nitrogen, nitrogen retention and urine nitrogen excretion (as ratio to absorbed N) for the two control periods.

	HC[1]	NC
OMI ($g/kg^{0.75}$/day)	59.0 ± 1.9	57.7 ± 1.8
DOMI ($g/kg^{0.75}$/day)	35.0 ± 1.2	35.1 ± 1.1
App. dig. of OM (%)	59.28 ± 0.34	60.73 ± 0.49
App. dig. of feed N (%)	46.61 ± 1.43	47.39 ± 1.67
N retention ($g/kg^{0.75}$/day)	0.026 ± 0.020	0.089 ± 0.025
Urine N excretion (ratio to absorbed N)[2]	0.931 ± 0.067	0.830 ± 0.045

1) HC - water infusion; NC - triammonium citrate infusion.

2) Urine N (ratio to absorbed N) = $\frac{\text{Urine N excretion}}{\text{(Feed N intake - Faeces N) + Infusate N}}$

provided by the citrate; this would have been equivalent to an effective increase of about one gram of DOM/kg$^{0.75}$/day. The major part of the nitrogen retention response was probably due to an increased recycling of nitrogen to the rumen which would have stimulated microbial protein synthesis resulting in an increased flow of protein to the intestines and promoting an increased net protein synthesis in the tissues of the animal.

Analysis of amino acid infusion data

Throughout the following discussion the various amino acid treatments are described by the amino acid which was omitted from the infusate. The data for the periods of amino acid supplementation (periods A1 to A4) were analysed by the method of Davis and Hall (1969) which estimates the direct effect of treatments and any possible residual effects from the treatment in the previous period. The latter effects were small for all parameters analysed and in all cases were not significant. The error variance is therefore based on 12 degress of freedom after testing for residual effects and deciding to disregard them. The mean values for voluntary intake, the apparent digestibility of feed components and the nitrogen parameters are presented in Table 4.4. The values are presented as adjusted means having been adjusted for sheep differences. In Table 4.5 the comparative effects of the omission of the different amino acids on the various parameters are presented as the t-test values for the differences between adjusted means (e.g. the difference in intake between sheep receiving the LEU and LYS treatments was 8.7g OM/kg$^{0.75}$/ day, while the standard error of the difference between means was 3.22; the t-value was thus 2.70 indicating that the difference in intake was significant at the 5% level). Only t-values of 1.25 or greater are

TABLE 4.4: Adjusted treatment mean[1] values for organic matter intake (OMI), apparent digestibility of OM and nitrogen (N), N retention and urine N excretion (ratio to absorbed N) for the sheep on the different treatments. The treatments are described by the amino acid which was omitted from the infusion.

	LEU	THR	VAL	ILE	TRP	LYS	MET	S.E.D.[2]
OMI ($g/kg^{0.75}$/day)	54.5	56.4	59.1	58.8	56.3	63.2	52.1	3.2
App. dig. of OM (%)	61.14	60.65	61.43	59.52	59.85	60.89	59.53	1.02
App. dig. of feed N (%)	50.63	52.36	52.66	50.92	46.19	52.46	53.74	2.52
N retention ($g/kg^{0.75}$/day)	0.198	0.212	0.286	0.250	0.203	0.274	0.141	0.034
Urine N excretion (ratio to absorbed N)[3]	0.637	0.619	0.485	0.544	0.586	0.536	0.738	0.054

1) Adjusted for sheep differences over the periods A1 to A4 during which the sheep received the amino acid infusion treatments.

2) S.E.D. - standard error of the difference between means.

3) Urine N (ratio to absorbed N) = $\frac{\text{Urine N excretion}}{\text{(Feed N intake - Faeces N) + Infusate N}}$

TABLE 4.5: t-test values[1] for the difference between any two treatments in the adjusted mean values for organic matter intake, apparent digestibility of feed nitrogen (N), daily N retention and urine N excretion (ratio to absorbed N). The treatments are described by the amino acid which was omitted from the infusion mixture.

Organic matter intake

	LEU	THR	VAL	ILE	TRP	LYS
THR	-					
VAL	1.43	-				
ILE	1.35	-	-			
TRP	-	-	-	-		
LYS	2.70	2.12	1.27	1.34	2.12	
MET	-	1.32	2.17	2.10	1.32	3.44

Apparent digestibility of feed N

	LEU	THR	VAL	ILE	TRP	LYS
THR	-					
VAL	-	-				
ILE	-	-	-			
TRP	1.76	2.45	2.57	1.88		
LYS	-	-	-	-	2.49	
MET	-	-	-	-	3.00	-

Nitrogen retention

	LEU	THR	VAL	ILE	TRP	LYS
THR	-					
VAL	2.59	2.18				
ILE	1.53	-	-			
TRP	-	-	2.44	1.38		
LYS	2.24	1.82	-	-	2.09	
MET	1.68	2.09	4.26	3.21	1.82	3.91

Urine N (ratio to absorbed N)

	LEU	THR	VAL	ILE	TRP	LYS
THR	-					
VAL	2.81	2.48				
ILE	1.72	1.39				
TRP	-	-	1.87	-		
LYS	1.87	1.54	-	-	-	
MET	1.87	2.20	4.69	3.59	2.81	3.74

[1] t values $\geq$ 1.25 only are presented; t required for significance (12df) at level of 1% 3.06; 5% 2.18; 10% 1.78; 25% 1.25.

presented in the table. Although a probability value of 25% (i.e. t = 1.25) cannot be considered to approach significance, the inclusion of t-values which fell between the 5% and 25% levels provides an indication of possible trends in the effects of treatments when considered in the overall context of the results. The t-values for the differences in the digestibility of organic matter were all low and have been omitted from the table.

Amino acid supplementation and voluntary intake

The mean voluntary intake of the sheep given the MET treatment was lower than the mean intake of sheep given all other treatments although the difference was significant only when compared with the VAL ($P<0.05$) and LYS ($P<0.01$) treatments. Similarly the intake of the sheep on the LYS treatment was higher than the intake for all other treatments although the difference reached significance only in comparison with the LEU ($P<0.05$) and MET ($P<0.01$) treatments. The depressed intake which was particularly evident in the sheep given the MET treatment was reminiscent of the intake depressions brought about by a dietary amino acid imbalance in rats (Harper *et al.* 1970) or lambs (Rogers and Egan 1975). A mild imbalance response (i.e. a depression in intake followed by an intake recovery) was apparent in the intake patterns of at least two sheep when given particular amino acid treatments (sheep 768 when given the THR treatment and sheep 759 when given the MET treatment). These imbalance type responses reflect the importance of a balanced amino acid supply, while the very high intakes of the sheep given the LYS treatment may point to important interactions of lysine with other amino acids, perhaps one of the three "essential" amino acids omitted from the treatments (i.e. histidine, arginine, phenylalanine).

Effects of tryptophan on the apparent digestibility of feed nitrogen

The inclusion of tryptophan in the infusate resulted in a considerable increase in the apparent digestibility of feed nitrogen. The value for the TRP treatment was significantly ($P<0.05$) lower than the values for the MET, VAL, LYS and THR treatments while the differences between the TRP and the LEU and ILE treatments just failed to reach significance. The average response of nearly six percentage units in nitrogen digestibility represents a considerable reduction in faecal nitrogen loss; for a 30kg ($13kg^{0.75}$) sheep consuming 55g OM/$kg^{0.75}$/day (1.14% N in the OM) it would amount to a reduction of about 0.5g in daily faecal nitrogen output ($13 \times 55 \times 0.0114 \times 0.06 = 0.49g$). The tryptophan effect on nitrogen digestibility was not accompanied by any signficant effect on organic matter digestibility nor was there any indication in the mean values that the reduced faecal nitrogen loss contributed to an increase in nitrogen retention. However, on examination of the individual animal data there was evidence that for two sheep (648 and 664) the lower nitrogen digestibility recorded for the TRP treatment was associated with a reduced nitrogen retention. There is no obvious explanation for the effect of tryptophan on the apparent digestibility of feed nitrogen although it is possible that tryptophan increased the efficiency of amino acid absorption from the intestine or caused a reduction in pancreatic or intestinal protein secretion. An explanation for this effect of tryptophan must await further study.

Effect of amino acid infusion on nitrogen utilisation

The data for the nitrogen retention and urine nitrogen excretion fell into three broad groups (i) MET, (ii) LEU, THR, TRP and (iii) ILE, VAL, LYS. The group (iii) treatments were significantly

($P<0.01$) better than the MET treatment in terms of the efficiency of nitrogen utilisation. It is also apparent in Table 4.4 that the different amino acid treatments had marked effects on voluntary intake and that the lower nitrogen retention values were associated with the lower mean intakes. The regression equation for the relationship between nitrogen retention (NR, $g/kg^{0.75}/day$) and digestible organic matter intake (DOMI) using treatment mean values was:

$$NR = 0.0197\ DOMI - 0.457 \qquad (3)$$

SE of b = ± 0.0035; SE of a = ± 0.120; r = 0.931; n = 7.

Thus about 86% (r^2) of the variation in the mean values for nitrogen retention among the different amino acid treatments was accounted for by differences in digestible organic matter intake. This suggests that all amino acids were utilised for protein synthesis (as determined by nitrogen retention) with virtually the same efficiency and that the principle effect of the different treatments was on voluntary intake. This very strong relationship between nitrogen retention and digestible organic matter intake was partly due to the use of adjusted treatment mean values in the calculations and the relationship was somewhat weaker (r^2 = 50%, n = 28) when unadjusted values for all animals were used. The lower correlation was simply a consequence of between animal variability in the response to the different treatments although between period and residual effects would also have contributed.

The regression coefficients in equations (2) and (3) were almost identical while the intercepts differed by 0.129g nitrogen retained/$kg^{0.75}$/day. Thus at the same intake, the nitrogen retention of sheep receiving the amino acid infusions was $0.129g/kg^{0.75}$/day

greater than the sheep receiving an isonitrogenous infusion of triammonium citrate (disregarding any possible effects of time since the triammonium citrate and amino acid infusions were carried out in different periods). For all of the amino acid treatments the actual nitrogen retention was greater than expected nitrogen retention based on the isonitrogenous infusion calculated from equation (2). The actual and expected nitrogen retention values for each treatment are presented in Table 4.6. The differences between these two values were very similar for all treatments with a mean of 0.142 ± 0.007g/kg$^{0.75}$/day. Thus most of the difference in treatment mean values for nitrogen retention can be explained in terms of differences in intake rather than differences in the efficiency of nitrogen utilisation. Such a result would suggest that no one amino acid was "first limiting" in terms of the efficiency of nitrogen utilisation.

Implications for amino acid supplementation

As suggested previously in relation to the derivation of equation (3) the use of adjusted mean values may have contributed to the apparent lack of any difference between treatments in the efficiency of nitrogen utilisation after allowing for treatment - induced differences in intake. In other words the use of adjusted mean values may have hidden important differences between animals in the responses. The data presented in Table 4.7 indicate considerable between animal variability in the response to the amino acid treatments. For example: (i) two sheep exhibited markedly depressed intakes when given the MET (sheep 759) and THR (sheep 768) treatments yet the nitrogen retention values, although lower than for other treatments, represented a considerable net protein synthesis especially considering

TABLE 4.6: Mean values for digestible organic matter (DOMI) and nitrogen retention (g/kg$^{0.75}$/day) for the sheep given the different treatments, and the expected nitrogen retention calculated from equation (2) (which relates nitrogen retention to DOMI for the sheep during the period of triammonium citrate infusion). Treatments are described by the amino acid which was omitted from the infusion mixture.

	Control periods[1]		Amino acid treatments[2]						
	HC	NC	LYS	VAL	ILE	THR	TRP	LEU	MET
DOMI (g/kg$^{0.75}$/day)	35.0	35.1	38.5	36.3	35.0	34.2	33.7	33.3	31.0
N retention (g/kg$^{0.75}$/day)									
Actual	0.026	0.089	0.274	0.286	0.250	0.212	0.203	0.198	0.141
Expected[3]			0.156	0.114	0.090	0.074	0.065	0.057	0.012
Difference from expected[4]			0.118	0.172	0.160	0.138	0.138	0.141	0.129

1) Mean values for seven animals (Table 4.3).

2) Adjusted mean values (Table 4.4).

3) Expected nitrogen retention calculated from equation (2) which related nitrogen retention to DOMI during the infusion of triammonium citrate (isonitrogenous with amino acid infusion treatments).

4) Mean difference from expected = 0.142 ± 0.007 (standard error of mean).

TABLE 4.7: Individual animal values for digestible organic matter intake (DOMI) and nitrogen retention (NR, g/kg$^{0.75}$/day) during periods A1 to A4. Treatments are described by the amino acid which was omitted from the infusion mixture.

	648			768	
	DOMI	NR		DOMI	NR
LEU	36.8	0.339	THR	28.8	0.116
ILE	38.0	0.388	TRP	32.5	0.224
TRP	37.0	0.245	LYS	37.8	0.272
THR	36.6	0.301	VAL	38.1	0.266

	655			669	
	DOMI	NR		DOMI	NR
VAL	37.0	0.241	ILE	34.8	0.207
LYS	40.0	0.324	MET	31.3	0.203
MET	35.8	0.124	LEU	30.4	0.132
ILE	40.7	0.273	TRP	34.3	0.265

	664			759	
	DOMI	NR		DOMI	NR
TRP	29.8	0.146	LYS	39.4	0.235
LEU	30.6	0.238	THR	36.1	0.260
THR	33.8	0.226	VAL	35.5	0.253
LYS	35.5	0.223	MET	27.1	0.128

	760	
	DOMI	NR
MET	30.4	0.045
VAL	34.2	0.289
ILE	30.9	0.177
LEU	33.6	0.112

the reduced energy intake; (ii) sheep 655 and 760 suffered only slight depressions in intake (if depressions at all) when given the MET treatment yet their actual nitrogen retentions were much lower than those recorded when they consumed a similar quantity of feed on the VAL (655) and ILE (760) treatments; (iii) although the voluntary intake of sheep 669 and 760 was relatively unaffected by the LEU treatment the efficiency of nitrogen utilisation was lower on the LEU treatment than on comparable treatments in the same sheep.

In the context of preventing a depression in intake when providing supplemental mixtures of essential amino acids to sheep in the present experiment it may be argued that methionine should be considered as the "first-limiting" amino acid. However, at the individual animal level, the evidence for between-animal variability in the responses and the paradoxical effects of some of the treatments on intake and nitrogen utilisation would support the contention that no one amino acid can be considered "first-limiting" but rather that a group of amino acids should be considered as limiting for overall protein synthesis.

CONCLUSIONS

The results reported in this chapter provide support for the hypothesis proposed in the introduction, i.e. that no one amino acid was limiting production for all of a group of sheep fed the same diet and an amino acid which was limiting for intake was not necessarily limiting for efficient nitrogen utilisation. On the basis of the overall differences in intake between treatments and on a consideration of the individual animal responses it may be suggested that an appropriate amino acid supplement for sheep fed a diet such as that

in the present experiment should include methionine, threonine, leucine and tryptophan as well as a source of non-specific amino nitrogen. However, it is possible that an increased supply of microbial or plant protein *per se* may be the most satisfactory method of increasing net protein synthesis, since it is apparent that only slight changes in the balance of amino acids reaching the duodenum may have significant effects on the overall net protein synthesis in the animal, particularly through the effects of the amino acid balance on intake.

CHAPTER 5

Voluntary intake and nitrogen retention in young sheep fed low quality roughages and given post-ruminal supplements of L-methionine

INTRODUCTION

The results of the experiments reported in Chapter 2 of this thesis suggested that the response to methionine supplementation was quite variable among sheep fed the same diet, and that, for the two animals tested, the individual animal response to methionine in terms of voluntary intake, urine nitrogen excretion and plasma amino acid patterns (Chapter 3) was repeatable. There was also a suggestion that the adverse effects of methionine (when infused at approximately the same rate in Experiments 2/1, 2/2 and 2/3) were more apparent in the sheep receiving a higher quality roughage diet (Experiment 2/3 - *ad libitum* energy intake of about 1.6 times maintenance) than in the sheep fed the lower quality diet (Experiments 2/1, 2/2 - *ad libitum* energy intake of about 1.0 times maintenance).

In the experiments to be reported in this chapter, the responses to methionine supplementation in sheep fed very low quality roughages were examined (at *ad libitum* intakes, the energy intake provided about 40% and 70% of the maintenance requirement).

A number of animals were used in these experiments, each animal acting as its own control. The experiments were carried out in order to obtain further information on the variability between animals and on the range of the methionine response in a group of animals. The repeatability of the methionine response in sheep fed

two different diets was also examined. Very low quality roughages were selected as the experimental diets since it was believed likely that most sheep would exhibit positive responses to a methionine supplement.

EXPERIMENTAL

Animals

Twenty-one Dorset x (Dorset x Merino) wethers, aged about 7 months and of 21-34kg bodyweight were used. Each had been surgically prepared with an abomasal canula at least one month before the start of the experiments. The animals were housed indoors in metabolism pens and were fitted with faecal collection harnesses.

Diets and feeding

Two experiments were carried out. In Experiment 5/1, the sheep were offered a diet of hammermilled barley straw (0.43% nitrogen) *ad libitum* and in Experiment 5/2, a mixture of equal parts of the barley straw and wheaten hay chaff *ad libitum*, the nitrogen content of the mixed diet being 0.67% of the dry matter.

Feed residues were removed at 0930h. The fresh allowance of feed was offered at 1000h at 15-25% in excess of the estimated daily intake. The animals had been offered the diets *ad libitum* for 3 weeks prior to the start of the experiments.

Water was available at all times. A mineral supplement (8g per day; Moir & Harris 1962) was sprinkled on the feed daily. Vitamins A and D_3 (APAC, Nicholas Pty. Ltd.) were given with the feed on day 1 of each period and during the period between the experiments.

Experimental design

The experimental periods were all of 11 days duration. Experiment 5/1 was of two periods, and Experiment 5/2 of three periods. During the first period (control), the animals received 220 ml of distilled water via the abomasal canula; during the second period, they received an abomasal infusion of L-methionine (0.12g/kg$^{0.75}$/day) and in the third period (Experiment 5/2 only), they received an abomasal infusion of L-methionine (0.36g/kg$^{0.75}$/day). The infusions were administered continuously using a peristaltic pump.

Measurements

The sheep were weighed at the start of each experiment and at the end of each experimental period. The daily *ad libitum* dry matter intake was measured. Urine was collected into sulphuric acid (final urine pH $\leq$ 2.5), the amount of urine measured and a daily subsample taken and stored at -20^{o}C. Faeces were collected and bulked over a 7 day period (days 6-12) and the apparent digestibilities of dry matter, organic matter and nitrogen determined. The nitrogen balances for the treatments were calculated using the feed intake for days 3 to 9, the urine collected on days 5 to 11, and the faeces collected on days 6 to 12. Composite samples of feeds and residues were retained for analysis.

Analytical methods

The dry matter content of feed, faeces and residues was determined by oven drying at 85^{o}C and organic matter by ashing at 550^{o}C in a muffle furnace. Faeces samples were freeze-dried for nitrogen and organic matter determinations.

In order to reduce the number of analyses required the feed residues left by the animals were bulked on the basis of the proportion and amount of feed not consumed.

Nitrogen concentration in subsamples of urine, dried feeds, residues and freeze-dried faeces was determined colourimetrically using a Technicon Auto Analyser following digestion of the samples using a Kjeldahl technique (Munro & Fleck 1969).

L-Methionine infusions

The L-methionine (Tanabe Seiyaku Co. Ltd. Tokyo) solutions were replaced every 2 days, and were adjusted to pH 3.5 with hydrochloric acid to reduce the risk of microbial contamination. The infusion lines were regularly disconnected and flushed with a sodium hypochlorite-sodium chloride solution after which they were washed with distilled water and the methionine infusion recommenced.

RESULTS

The diets used in these experiments were of low quality and consequently most of the sheep lost weight during the experiments. These changes in liveweight introduce complications into the interpretation of individual animal responses.

For accurate between period comparisons of the voluntary intake of a particular animal within an experiment, the voluntary intake data should be corrected for the change in liveweight using an appropriate factor (e.g. $kg^{0.75}$). However in the present work, such an adjustment of the individual daily intakes was not considered justified since the liveweights of the sheep were measured only at

the beginning and end of each treatment period. For this reason, the treatment mean voluntary intakes for each animal (g dry matter/ $kg^{0.75}$/day) are presented without standard errors, while the actual mean intakes (g dry matter/day) are presented with standard errors. The voluntary intake data for the two experiments are presented in Tables 5.1 and 5.2.

In Experiment 5/1, when the voluntary intakes of the sheep in the methionine infusion period were compared with the intakes during the control period, eight sheep exhibited an increase in intake ($P<0.05$) while none exhibited a decrease ($P<0.05$), and the intakes of 13 sheep were not significantly affected. The possibility of an effect of time cannot be excluded, although despite a decline in liveweight, eight animals increased their intakes. However, in those animals in which intake was depressed, the effect cannot be separated from a decline in liveweight.

In Experiment 5/2, eight sheep had an increased intake in the period of the low rate of methionine infusion as compared with their intakes during the control period. Three animals exhibited a decrease in intake, and the intakes of the remainder were not significantly altered. In the period of the high methionine infusion, the intakes of three sheep were increased as compared with the control period, while the intakes of seven were depressed and the intakes of 11 were unchanged.

Tables 5.3 and 5.4 give the individual animal mean values for daily urine nitrogen excretion with their standard errors. The daily mean values for nitrogen supply (feed plus methionine nitrogen) and the

TABLE 5.1 (Experiment 5/1): Individual animal values for voluntary food intake for the sheep fed the barley straw diet in the control period (BC - water infusion) and in the period of methionine infusion (BM - 0.12g L-methionine/kg$^{0.75}$/day).

	Voluntary intake			
	BC (g DM/day)[1]	BM	BC (g DM/kg$^{0.75}$/day)	BM
574	209 ± 10.7	197 ± 9.8	19.2	19.3
582	328 ± 12.8	383 ± 8.4	27.1	32.2
378	342 ± 16.6	304 ± 11.9	28.7	26.4
408	438 ± 26.2	465 ± 11.0	36.8	39.7
581	277 ± 20.9	326 ± 17.5	20.6	24.5
365	321 ± 23.2	383 ± 11.9	25.9	30.9
393	414 ± 13.6	380 ± 14.0	33.2	30.9
402	369 ± 20.3	389 ± 22.2	30.0	32.1
567	383 ± 20.6	384 ± 12.0	27.2	28.5
389	418 ± 20.0	395 ± 9.0	29.9	28.6
583	288 ± 16.5	358 ± 10.9	22.1	28.2
577	248 ± 15.4	336 ± 23.7	20.5	28.7
580	199 ± 34.4	377 ± 26.8	17.0	32.8
558	277 ± 17.5	389 ± 8.5	26.1	37.0
462	202 ± 13.8	186 ± 10.8	17.7	16.9
612	219 ± 12.5	223 ± 8.6	21.0	22.3
568	255 ± 9.2	241 ± 5.2	19.5	19.3
508	353 ± 16.8	426 ± 9.4	32.7	39.4
445	296 ± 19.9	311 ± 8.3	25.5	28.0
585	295 ± 11.5	311 ± 15.6	25.0	27.0
564	140 ± 11.5	185 ± 9.0	12.8	17.8

1) Mean daily intake for days 3 to 9 (± standard error of the mean).
t-test for significance of difference in voluntary intake between the two periods:
increased intake ($P<0.05$) - 582, 365, 583, 577, 580, 558, 508, 564;
no change in intake ($P>0.05$) - 574, 378, 408, 581, 393, 402, 567, 389, 462, 612, 568, 445, 585.

TABLE 5.2 (Experiment 5/2): Individual animal values for voluntary food intake for the sheep fed the barley straw - wheaten hay chaff diet in the control period (WC) and in the periods of methionine infusion (WM - 0.12g L-methionine/$kg^{0.75}$/day; WH - 0.36g L-methionine/$kg^{0.75}$/day).

Sheep	Voluntary intake (g DM/day)[1]			(g DM/$kg^{0.75}$/day)		
	WC	WM	WH	WC	WM	WH
574	260 ± 5.6	285 ± 7.3	376 ± 9.5	28.0	30.6	39.6
582	428 ± 9.3	471 ± 7.7	453 ± 10.3	36.3	39.9	38.7
378	506 ± 6.9	479 ± 7.1	434 ± 17.8	44.0	41.7	38.1
408	603 ± 9.2	566 ± 12.8	601 ± 21.2	52.4	49.2	52.3
581	406 ± 10.4	435 ± 13.9	277 ± 30.6	32.0	34.5	23.1
365	561 ± 10.7	555 ± 15.8	557 ± 23.2	44.5	44.1	44.2
393	588 ± 13.8	667 ± 14.4	604 ± 14.3	47.8	53.4	48.7
402	528 ± 15.1	513 ± 7.8	432 ± 40.2	44.3	43.1	37.6
567	383 ± 24.8	399 ± 13.2	469 ± 21.2	29.5	31.7	39.4
389	538 ± 11.7	599 ± 13.3	416 ± 70.8	40.5	45.0	31.8
583	481 ± 5.8	516 ± 14.8	456 ± 21.2	38.8	41.3	36.8
577	305 ± 4.7	335 ± 6.9	274 ± 14.0	28.0	31.3	26.1
580	445 ± 12.8	515 ± 14.2	451 ± 18.5	37.7	45.2	40.3
558	598 ± 12.3	571 ± 17.3	498 ± 22.7	55.9	52.9	47.8
462	337 ± 10.3	334 ± 5.7	217 ± 42.3	32.1	31.8	22.0
612	302 ± 6.8	270 ± 4.2	284 ± 15.7	32.5	29.7	31.9
568	354 ± 14.7	326 ± 15.4	231 ± 27.2	30.2	28.1	20.8
508	646 ± 15.6	627 ± 23.9	593 ± 18.7	57.7	55.0	52.9
445	446 ± 15.2	461 ± 13.4	421 ± 19.2	41.0	41.9	39.0
585	387 ± 13.1	361 ± 15.1	345 ± 7.5	36.5	34.7	33.5
564	171 ± 9.7	217 ± 6.2	264 ± 6.3	18.0	23.3	28.7

1) Mean daily intake for days 3 to 9 (± standard error of the mean). t-test for significance of difference in voluntary intake between supplementation and control periods:

WM/WC increased intake ($P<0.05$) - 574, 582, 393, 389, 583, 577, 580, 564;
no change in intake ($P>0.05$) - 581, 365, 402, 567, 558, 462, 568, 508, 445, 585;
decreased intake ($P<0.05$) - 378, 408, 612.

WH/WC increased intake ($P<0.05$) - 574, (567), 564;
no change in intake ($P>0.05$) - 582, 408, 365, 393, (389), 583, 577, 580, (462), 612, 445;
decreased intake ($P<0.05$) - 378, (581), (402), 558, (568), 508, 585.

Note: Sheep numbers in parenthesis indicate those sheep which were apparently particularly sensitive to the high rate of methionine infusion, as evidenced by the effect of methionine supplementation on voluntary food intake and urine nitrogen excretion during this period.

TABLE 5.3 (Experiment 5/1): Individual animal values for urine nitrogen excretion for the sheep fed the barley straw diet in the control period (BC-water infusion) and in the period of methionine infusion (BM - 0.12g L-methionine/$kg^{0.75}$/day)

Sheep	Urine nitrogen excretion (g/day)[2] BC	BM	Significance of difference (t-test)[1]
574	2.84 ± 0.222	1.72 ± 0.194	**
582	2.01 ± 0.273	1.25 ± 0.114	*
378	1.74 ± 0.135	0.91 ± 0.053	**
408	1.52 ± 0.072	1.20 ± 0.029	**
581	2.23 ± 0.137	1.25 ± 0.042	**
365	2.30 ± 0.141	1.12 ± 0.035	**
393	1.58 ± 0.123	1.34 ± 0.073	NS
402	1.82 ± 0.118	1.13 ± 0.066	**
567	1.96 ± 0.112	1.56 ± 0.062	**
389	1.89 ± 0.089	1.31 ± 0.073	**
583	2.03 ± 0.082	1.15 ± 0.086	**
577	2.20 ± 0.157	1.19 ± 0.077	**
580	2.89 ± 0.152	1.53 ± 0.073	**
558	1.95 ± 0.179	1.42 ± 0.049	*
462	2.33 ± 0.257	1.76 ± 0.175	NS
612	2.04 ± 0.170	1.30 ± 0.094	**
568	2.20 ± 0.082	1.40 ± 0.067	**
508	1.54 ± 0.073	1.16 ± 0.073	**
445	1.91 ± 0.054	1.37 ± 0.056	**
585	1.59 ± 0.203	1.18 ± 0.083	NS
564	1.78 ± 0.095	1.10 ± 0.071	**

1) NS - not significant; * $P<0.05$; ** $P<0.01$.

2) Mean daily urine N excretion for urine collected on days 5 to 11 (± standard error of the mean).

TABLE 5.4 (Experiment 5/2): Individual animal values for urine nitrogen excretion for the sheep fed the barley straw-wheaten hay chaff diet in the control period (WC) and in the periods of methionine infusion (WM - 0.12g L-methionine/$kg^{0.75}$/day ; WH - 0.36g L-methionine/$kg^{0.75}$/day).

Sheep	Urine nitrogen excretion (g/day)[2]			Significance of difference (t-test)[1]		
	WC	WM	WH	WC/WM	WM/WH	WC/WH
574	1.64 ± 0.102	1.21 ± 0.123	1.28 ± 0.140	*	NS	NS
582	1.37 ± 0.153	0.84 ± 0.035	1.27 ± 0.057	**	**	NS
378	1.27 ± 0.032	0.77 ± 0.030	1.23 ± 0.071	**	**	NS
408	1.17 ± 0.106	1.05 ± 0.026	1.38 ± 0.045	NS	**	NS
581	1.40 ± 0.073	0.97 ± 0.056	2.10 ± 0.255	**	**	*
365	1.46 ± 0.153	0.95 ± 0.029	1.43 ± 0.078	**	**	NS
393	1.55 ± 0.071	1.01 ± 0.040	1.25 ± 0.105	**	*	**
402	1.37 ± 0.098	1.24 ± 0.050	2.66 ± 0.242	NS	**	**
567	1.57 ± 0.047	1.51 ± 0.076	2.08 ± 0.106	NS	**	**
389	1.49 ± 0.128	1.02 ± 0.043	2.26 ± 0.327	**	**	*
583	1.29 ± 0.060	0.94 ± 0.058	1.41 ± 0.076	**	**	NS
577	1.83 ± 0.059	1.15 ± 0.081	1.69 ± 0.165	**	*	NS
580	1.52 ± 0.059	1.00 ± 0.060	1.46 ± 0.101	**	**	NS
558	0.92 ± 0.048	0.86 ± 0.043	1.25 ± 0.100	NS	**	*
462	1.43 ± 0.063	1.15 ± 0.053	2.01 ± 0.240	**	**	*
612	1.53 ± 0.072	1.41 ± 0.062	1.54 ± 0.111	NS	NS	NS
568	1.30 ± 0.089	0.90 ± 0.029	2.53 ± 0.237	**	**	**
508	0.96 ± 0.022	0.88 ± 0.056	1.07 ± 0.058	NS	*	NS
445	1.29 ± 0.076	1.18 ± 0.124	1.59 ± 0.087	NS	*	*
585	1.41 ± 0.066	1.50 ± 0.089	1.64 ± 0.070	NS	NS	*
564	1.20 ± 0.028	1.01 ± 0.082	1.31 ± 0.062	*	*	NS

1) NS - not significant; * $P<0.05$; ** $P<0.01$.

2) Mean daily urine N excretion for urine collected on days 5 to 11 (± standard error of the mean).

urine and faeces nitrogen excretion are given in Appendix Tables 5.1 and 5.2. The values for nitrogen retention for each individual animal are given in Table 5.5. These are expressed as grams of nitrogen retained per kg liveweight to the power $^{0.75}$/day since this parameter represents the summation of changes in intake and the efficiency of nitrogen utilisation, while also allowing for changes in liveweight values adjusted on the basis of metabolic body size.

The mean values over all animals for voluntary intake and nitrogen retention (g/kg$^{0.75}$/day) and total nitrogen supply and urine and faecal nitrogen excretion (g/day) for all treatments are given in Table 5.6. The nitrogen loss was significantly reduced ($P<0.001$ and $P<0.025$) in the periods in which the animals received the low level of methionine supplementation as compared with the respective control periods. The reduction in nitrogen loss was the result of a reduced urine nitrogen excretion ($P<0.001$) even though nitrogen supply was increased, while the faecal nitrogen excretion was unchanged. At the high level of methionine supplementation (Experiment 5/2), the nitrogen loss was slightly though non-significantly elevated ($0.10>P>0.05$) compared with the lower level of methionine supplementation, although the loss was still less than that in the control period.

Figure 5.1 shows the distribution of the nitrogen retention data for each period of the two experiments. Figure 5.2 shows the distributions of the changes in nitrogen retention and the changes in voluntary intake in the methionine infusion periods as compared with the appropriate control periods.

TABLE 5.5 (Experiments 5/1 and 5/2): Individual animal values for nitrogen retention for the sheep in two experiments.

Sheep	Apparent nitrogen retention ($g\ N/kg^{0.75}/day$)				
	Experiment 5/1		Experiment 5/2		
	BC	BM	WC	WM	WH
574	-0.354	-0.267	-0.251	-0.132	-0.066
582	-0.219	-0.141	-0.097	-0.008	-0.032
378	-0.198	-0.126	-0.062	-0.003	-0.037
408	-0.193	-0.144	-0.031	-0.004	+0.018
581	-0.193	-0.114	-0.099	-0.021	-0.116
365	-0.240	-0.132	-0.054	+0.009	-0.004
393	-0.202	-0.168	-0.077	-0.006	-0.003
402	-0.216	-0.131	-0.067	-0.032	-0.135
567	-0.199	-0.156	-0.099	-0.091	-0.075
389	-0.192	-0.130	-0.076	-0.011	-0.077
583	-0.202	-0.121	-0.067	-0.006	-0.028
577	-0.231	-0.123	-0.142	-0.050	-0.118
580	-0.312	-0.169	-0.125	-0.017	-0.044
558	-0.238	-0.161	-0.006	+0.019	-0.036
462	-0.240	-0.192	-0.153	-0.066	-0.114
612	-0.255	-0.181	-0.156	-0.136	-0.118
568	-0.221	-0.161	-0.077	-0.022	-0.126
508	-0.232	-0.171	-0.037	+0.004	+0.010
445	-0.230	-0.165	-0.082	-0.043	-0.071
585	-0.193	-0.154	-0.127	-0.101	-0.091
564	-0.209	-0.137	-0.096	-0.083	-0.076

TABLE 5.6: Mean values over all animals for voluntary intake and nitrogen retention (g/kg$^{0.75}$/day), and total nitrogen supply, urine nitrogen excretion and faecal nitrogen excretion (g/day) for the various treatments (n = 21).

	Voluntary intake (DM) (g/kg$^{0.75}$/day)	Nitrogen retention	Total nitrogen supply	Nitrogen excretion urine (g/day)	faeces	total
BC	24.7 ± 1.32	-0.227 ± 0.0088	1.30	2.02 ± 0.081	1.99 ± 0.098	4.01 ± 0.092
BM	28.1 ± 1.42	-0.154 ± 0.0073	1.58	1.30 ± 0.046	2.05 ± 0.083	3.35 ± 0.087
Significance of differences[1] (t-test)	(*)	***		***	NS	***
WC	38.5 ± 2.17	-0.094 ± 0.0116	3.07	1.38 ± 0.046	2.74 ± 0.133	4.12 ± 0.127
WM	39.4 ± 1.97	-0.038 ± 0.0101	3.29	1.07 ± 0.046	2.62 ± 0.134	3.69 ± 0.125
WH	36.8 ± 2.05	-0.064 ± 0.0103	3.30	1.64 ± 0.099	2.36 ± 0.143	4.00 ± 0.119
Significance of differences (t-test) WC/WM	NS	***		***	NS	*
WM/WH	NS	(*)		***	NS	(*)
WC/WH	NS	(*)		*	(*)	NS

1) NS not significant; (*) P<0.10; *P<0.05; **P<0.01; ***P<0.001.

FIGURE 5.1 (Experiments 5/1 and 5/2): The pattern of distribution of values for nitrogen retention (mg N retained/$kg^{0.75}$/day at 30mg N intervals) for the sheep in the two experiments.
(Experiment 5/1 (barley straw diet): BC-water infusion; BM-0.12g L-methionine/$kg^{0.75}$/day.
Experiment 5/2 (barley straw/wheat hay chaff diet): WC-water infusion; WM-0.12g L-methionine/$kg^{0.75}$/day; WH-0.36g L-methionine/$kg^{0.75}$/day).

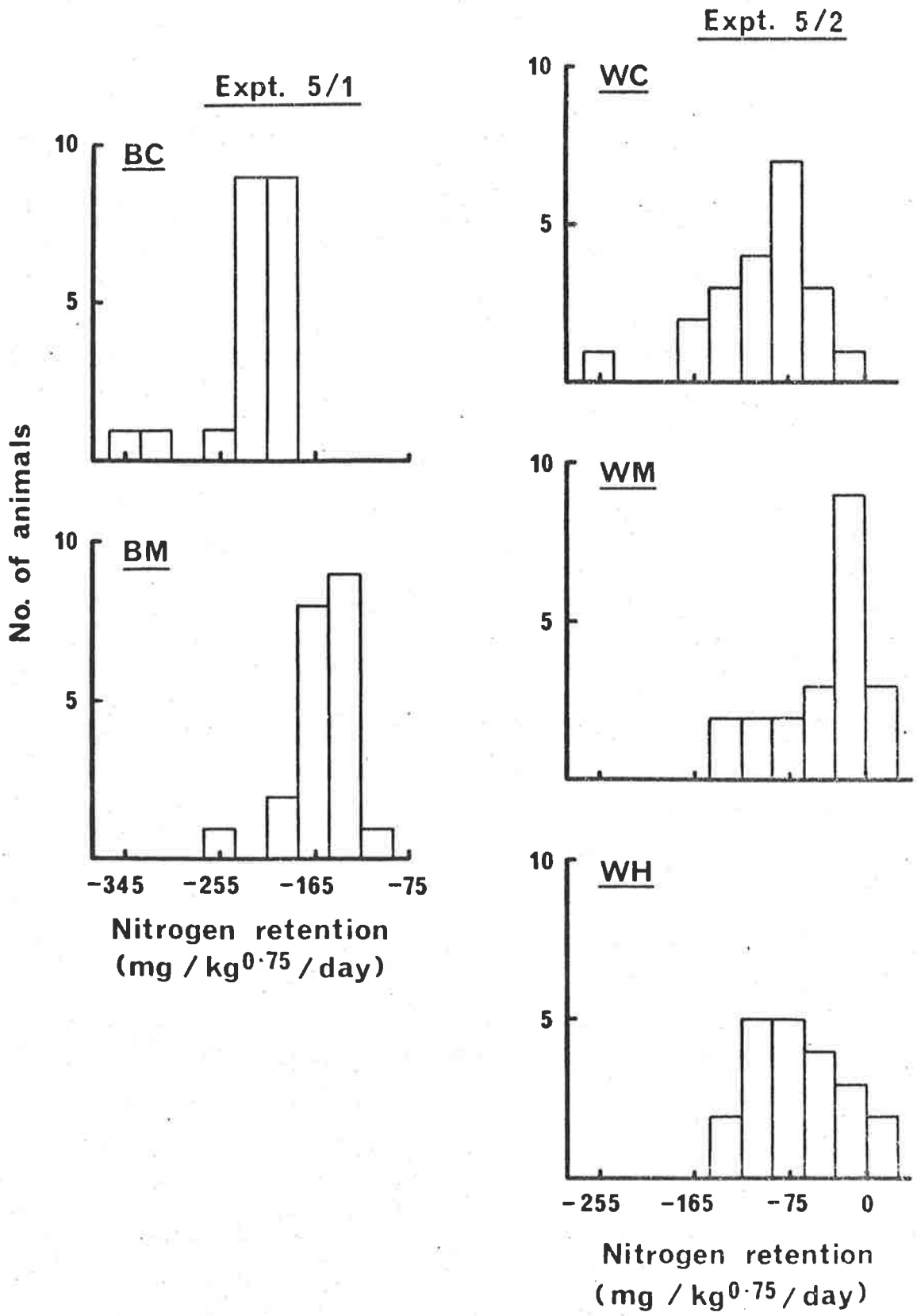
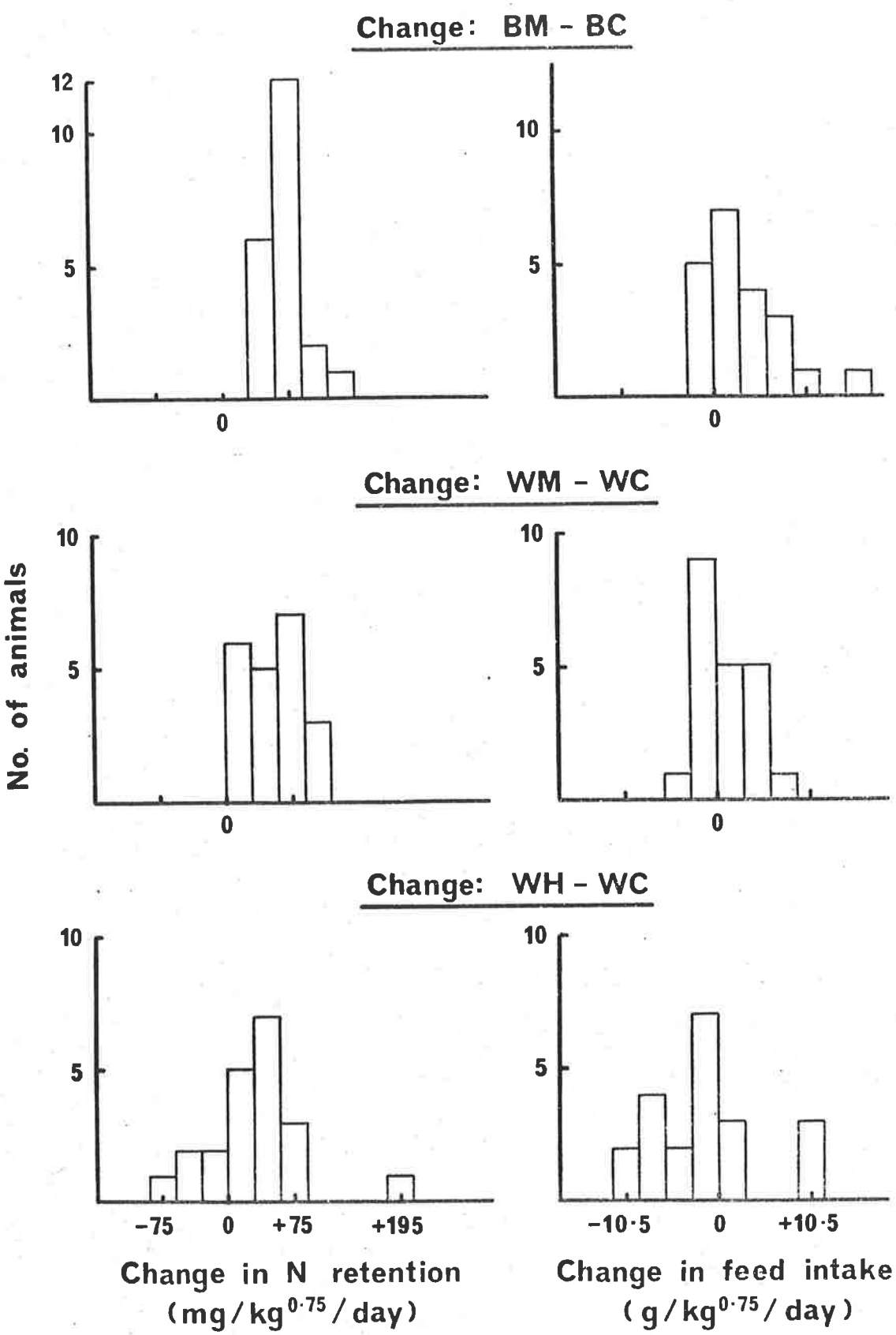

Expt. 5/1
Expt. 5/2
BC
BM
WC
WM
WH
10
5
No. of animals
-345
-255
-165
-75
0
Nitrogen retention
(mg / kg0·75 / day)

FIGURE 5.2 (Experiments 5/1 and 5/2): The pattern of distribution of changes in nitrogen retention (mg N retained/$kg^{0.75}$/day at 30mg N intervals) and changes in voluntary intake (gDM/$kg^{0.75}$/day at 3.0g intervals) with methionine supplementation for the sheep in the two experiments.

(Experiment 5/1 (barley straw diet): BC-water infusion; BM-0.12g L-methionine/$kg^{0.75}$/day.

Experiment 5/2 (barley straw/wheaten hay chaff diet): WC-water infusion; WM-0.12g L-methionine/$kg^{0.75}$/day; WH-0.36g L-methionine/$kg^{0.75}$/day).


Change: BM - BC
12
10
5
0
10
5
0
Change: WM - WC
No. of animals
10
5
0
10
5
0
Change: WH - WC
10
5
-75
0
+75
+195
Change in N retention
(mg/kg^0·75/day)
10
5
-10·5
0
+10·5
Change in feed intake
(g/kg^0·75/day)

The regression equations for the relationships between daily faecal nitrogen or faecal organic matter output and organic matter intake for each period for each diet are given in Table 5.7. All relationships were very highly significant ($P<0.001$). In Table 5.8 values derived from these equations for various levels of organic matter intake are presented.

Figure 5.3 shows the voluntary intake and the urine nitrogen excretion patterns for six of the sheep when given the high rate of methionine infusion in Experiment 5/2. The sheep were those which were particularly sensitive to the high rate of methionine infusion as judged by the effect on voluntary feed intake and urine nitrogen excretion. One of these sheep (567) initially increased intake in response to the high methionine infusion such that the mean intake over the period was significantly increased, but by day 6 intake had started to decline and urine nitrogen excretion was elevated.

DISCUSSION

Throughout this discussion differences in animal performance (i.e. voluntary intake, nitrogen retention, etc.) in the methionine infusion period(s) of each experiment as compared with the appropriate control period have been accepted as responses to an increased supply of methionine *per se*. The possibility that the differences were effects associated with time (i.e. environmental effects) has been discounted since, between animal variability in response notwithstanding, the responses observed were sufficiently large and occurred in a numberof sheep (as well as being observed on two diets) to suggest that the effects were real effects of the additional methionine.

TABLE 5.7: Regression equations for the relationships between daily faecal nitrogen output (FN) or faecal organic matter output (FOM) and daily organic matter intake (OMI) for the sheep fed the two diets with different levels of methionine supplementation (n = 21, all values in g/day).

		r	SE of b[1]
Diet:	Barley straw (B)		
	BC[2]: FN = 0.00563 OMI + 0.427	0.935	0.00049
	BM : FN = 0.00433 OMI + 0.713	0.882	0.00053
	BC : FOM = 0.549 OMI + 8.31	0.980	0.0256
	BM : FOM = 0.476 OMI + 22.8	0.940	0.0397
Diet:	Barley straw/wheat hay chaff (W)		
	WC : FN = 0.00482 OMI + 0.791	0.924	0.00046
	WM : FN = 0.00506 OMI + 0.516	0.942	0.00029
	WH : FN = 0.00563 OMI + 0.228	0.965	0.00035
	WC : FOM = 0.466 OMI + 20.1	0.984	0.0192
	WM : FOM = 0.444 OMI + 21.2	0.976	0.0229
	WH : FOM = 0.470 OMI + 1.00	0.977	0.0237

1) ± standard error of the slope.

2) C-control (water) infusion; M-low methionine, 0.12g L-Met/kg$^{0.75}$/day; H-high methionine, 0.36g L-met/kg$^{0.75}$/day.

3) Comparisons of slopes and adjusted means.

Treatment comparison	Relationship	F-test on Slopes (df1,38)	Adjusted Means (df1,39)
BC,BM	FN/OMI	NS	NS
	FOM/OMI	NS	NS
WC,WM	FN/OMI	NS	P<0.01
	FOM/OMI	NS	P<0.05
WC,WH	FN/OMI	NS	P<0.01
	FOM/OMI	NS	P<0.01
WM,WH	FN/OMI	NS	NS
	FOM/OMI	NS	P<0.05

TABLE 5.8: Predicted values for the faecal output of nitrogen (N) and organic matter (OM) for the sheep consuming the two basal diets and receiving the various methionine treatments calculated from the regression equations (Table 5.7) for different levels of organic matter intake.

Diet: Barley straw (B)

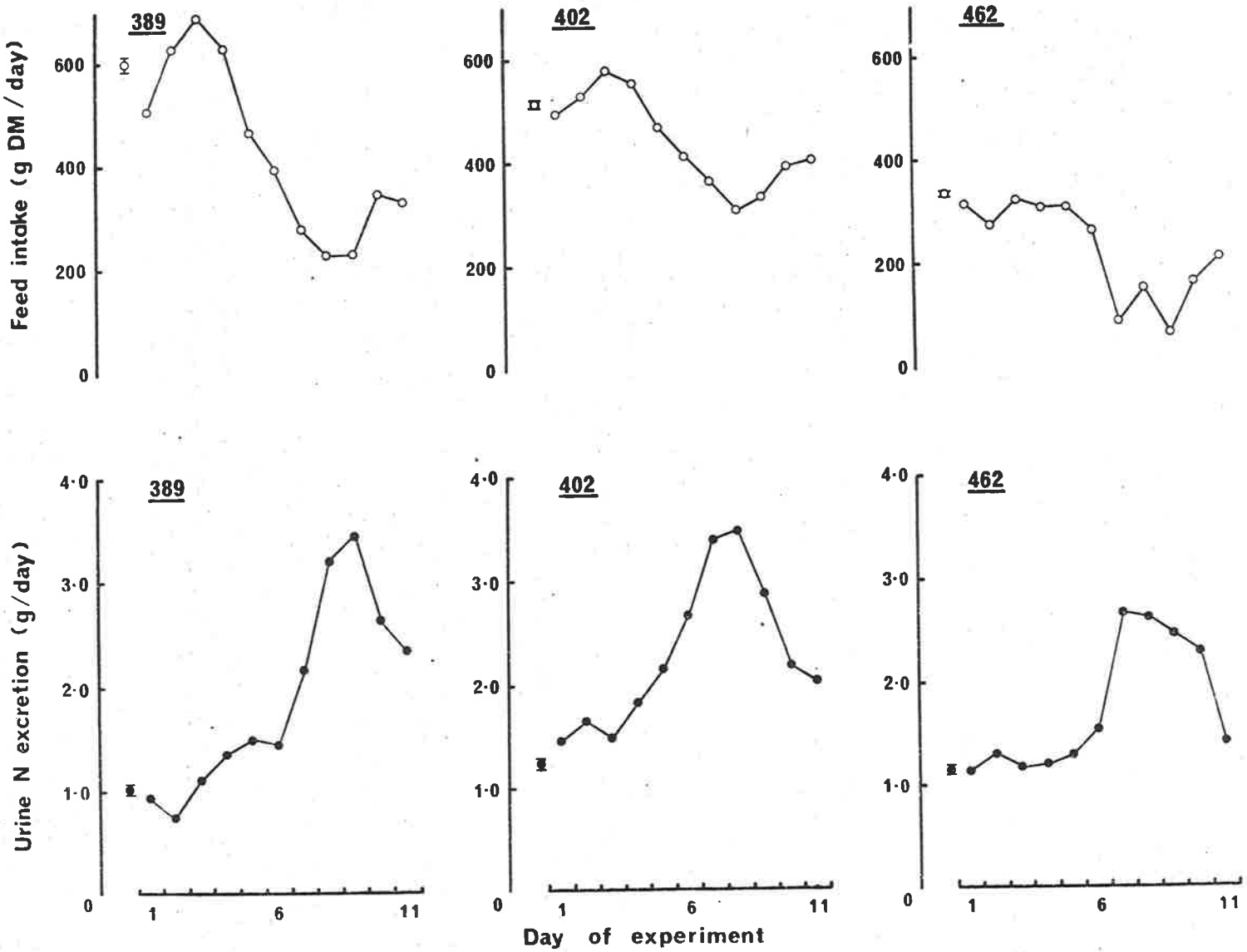
		Organic matter intake (g/day)		
		200	300	400
Faecal N: (g/day)	BC[1]	1.553	2.116	2.679
	BM	1.579	2.012	2.455
Faecal OM (g/day)	BC	118.1	173.0	227.9
	BM	118.0	165.6	213.2

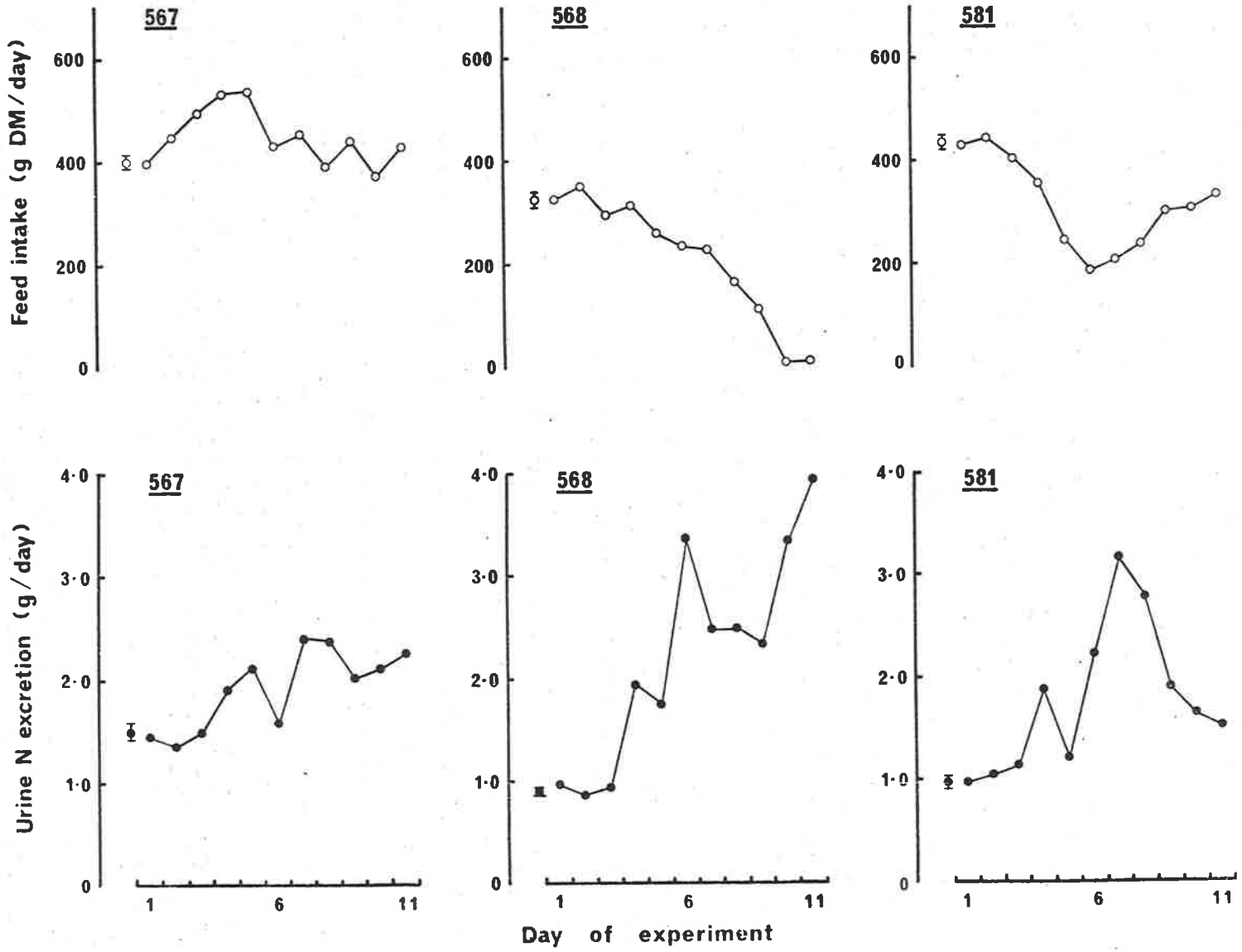
Diet: Barley straw/wheat hay chaff (W)

		Organic matter intake (g/day)			
		200	300	400	500
Faecal N: (g/day)	WC[1]	1.756	2.238	2.720	3.202
	WM	1.528	2.034	2.540	3.046
	WH	1.354	1.917	2.480	3.043
Faecal OM: (g/day)	WC	113.3	159.9	206.5	253.1
	WM	110.0	154.4	198.8	143.2
	WH	95.0	142.0	189.0	236.0

1) C-control (water infusion); M-low methionine, 0.12g L-met/kg$^{0.75}$/day; H-high methionine, 0.36g L-met/kg$^{0.75}$/day.

FIGURE 5.3 (Experiment 5/2): Voluntary feed intake (0——0) and urine nitrogen excretion (●——●) for six sheep fed the barley straw/wheaten hay chaff diet which were apparently particularly sensitive to the high rate of methionine infusion (0.36g L-methionine/$kg^{0.75}$/day) as evidenced by the effect of methionine supplementation on voluntary food intake and urine nitrogen excretion during this period. (The single values ⊥, ⊥ represent the mean ± standard error of the mean for the previous treatment period, i.e. methionine supplementation at a rate of 0.12g/$kg^{0.75}$/day).


389
402
462
Feed intake (g DM / day)
600
400
200
0
Urine N excretion (g/day)
4·0
3·0
2·0
1·0
0
1
6
11
Day of experiment


567
568
581
Feed intake (g DM/day)
600
400
200
0
♂
♀
Urine N excretion (g/day)
4·0
3·0
2·0
1·0
1
6
11
Day of experiment

The overall intake response to methionine infusion for the animals fed the lower quality diet (Experiment 5/1) was about 14% although this change failed to reach significance ($0.10>P>0.05$). However, some animals did exhibit a significant increase in intake ranging from 10 to 90%. There was no overall change in intake in response to the low level of methionine infusion in the animals fed the higher quality diet (Experiment 5/2), although some sheep did exhibit an increase in intake (8-30%), while in others intake was apparently depressed (part of the decrease in intake may have been associated with the reduced liveweight). In neither experiment was there any obvious relationship between the basal intake of an animal and the nature of its intake response to the methionine supplement.

The overall nitrogen retention responses for the sheep fed the two diets indicated that the low level of methionine supplementation resulted in a significantly reduced nitrogen loss. The response was greater (about 0.9g/day) when the sheep were fed the barley straw diet than when fed the mixed straw-wheaten chaff diet (about 0.65g N/day); the greater response was associated with the larger intake response to methionine. Since the animals fed both diets were in negative nitrogen balance, the increased supply of methionine had a protein-sparing effect, although the actual extent of this effect cannot be ascertained, since part of the nitrogen retention response would probably have been due to an increase in wool growth (Reis & Schinckel 1964; Dove & Robards 1974).

Excluding any effects of nitrogen supply (intake and infusate nitrogen), the nitrogen retention response had two components, a

reduction in urine nitrogen excretion and a reduction in faecal nitrogen excretion. The decrease in faecal nitrogen and organic matter excretion may be considered as a response through an improved efficiency of the digestive processes, while the reduced urine nitrogen excretion would reflect the protein-sparing and wool growth responses to methionine. This assumes that an increase in wool growth could not account for all of the observed improvement in nitrogen utilisation. A response in wool growth of this magnitude could be considered most unlikely in view of the reported responses in sheep fed low quality roughages (Dove & Robards 1974).

The consistent effect of methionine on faecal nitrogen and faecal organic matter outputs is difficult to explain. Such a response to postruminal or intraperitoneal administration of methionine or cyst(e)ine has been reported by other workers (Dryden *et al.* 1969; Barry 1971; Dove & Robards 1974). Methionine and methionine hydroxy analogue (MHA) have been found to increase cellulose and glucose fermentation and accelerate rumen bacterial growth *in vitro* (Salsbury *et al.* 1971; Gil & Shirley 1971; 1972). However even if the effect of methionine were to occur *in vivo* (as it does for MHA-Polan *et al.* 1970), this is unlikely to explain the present results, since it would require significant recycling or refluxing (by reverse digesta flow) of the abomasally infused methionine to the rumen. The response is thus probably due to an increased efficiency of digestion in and/or absorption from the small intestine, although a number of other possibilities cannot be excluded. These possibilities include a reduced input of pancreatic and other proteins into the intestine or an increased digestion in the hind-gut. However, both

of these possibilities could be expected to alter one of the faecal components more than the other; the first would markedly decrease faecal nitrogen and alter organ matter output by only a small amount while the second might be expected to increase nitrogen capture across the hindgut, while decreasing faecal organic matter output (i.e. increased organic matter digestion and volatile fatty acid production with an increased microbial protein synthesis). The effect of methionine on the apparent digestibility of the feed components is similar to the effects due to tryptophan in the experiment reported in Chapter 4. However, in the latter work the effect of tryptophan on nitrogen digestibility was considerable while any effects on organic matter digestibility were small and non-significant.

A significant protein-sparing effect of methionine occurred independent of any increase in intake and with only a slight change in faecal nitrogen output. Methionine (or a metabolite of methionine) must have altered the rate of protein catabolism and/or protein synthesis, the net result being a reduced net rate of body protein catabolism. However, the higher rate of methionine supplementation adversely affected nitrogen utilisation in some animals (sheep 389, 402, 462, 567, 568, 581) since the urine nitrogen excretion was increased by more than 0.4g/day compared with the same animals in the control period; in most of these animals intake was also depressed by the high level of methionine.

The overall responses to methionine in the sheep fed the two diets have been discussed. However, it is evident that there was considerable between animal variation in the response. In the

barley straw experiment, the methionine infusion apparently increased the efficiency of nitrogen utilisation in all animals (increased nitrogen retention), while no animals suffered significant depressions in voluntary intake. When these same animals were fed the mixed straw-wheaten chaff diet, the nitrogen retention respones to the low level of methionine were generally smaller and in some cases virtually non-existent, while voluntary intake was significantly depressed in three animals.

At the high level of methionine, the voluntary intake of a number of animals was depressed, although there was considerable variability between animals in their sensitivity to the methionine. Three animals (sheep 564, 567, 574) exhibited increased intakes of about 25% during the balance period in response to the high level of methionine, although in one (sheep 567) intake subsequently declined. In sheep 389 and 567, intake initially increased at the high level of methionine, but subsequently was markedly depressed (see Fig. 5.3).

The inverse relationship between voluntary feed intake and urine nitrogen excretion which was apparent in Experiment 2/2 was again evident in those animals adversely affected by the high level of methionine. Of the six animals which were clearly affected, four (sheep 389, 402, 462, 581) showed signs of adaptation, this adaptation being characterised by a subsequent increase in intake after a few days and reduced urinary nitrogen excretion. One animal (sheep 568) showed no sign of adaptation and its voluntary intake declined steadily until on days 10 and 11 of the infusion, intake was virtually zero.

There was no apparent relationship between the response of any one animal to the low level of methionine infusion on the two diets. This is in marked contrast to the repeatability of the methionine response in the two sheep fed a mixed chaff-straw diet in Experiment 2/1 (Chapter 2). However, the differences between the diets in the present experiment were very much greater than the differences between sequences in Experiment 2/1. The average amino acid yield at the duodenum in the sheep fed the barley straw diet would have been about 8g per day (calculated from Egan 1974), while the average yield in the animals fed the mixed straw-wheaten chaff diet in Experiment 5/2 would have been about 18g of amino acids per day. In contrast the differences in amino acid yield brought about by the difference in the basal intake in the two control periods in Experiment 2/1 would have been no more than about 15% while the protein to energy ratio would have been unchanged. The difference in digestible energy intake between the animals fed the two diets in the present experiment would have been about 75%, again in marked contrast to the situation in Experiment 2/1. Thus the basis of the between animal variability in the methionine response in these experiments may well have been an animal x diet interaction.

Despite the extent of between animal variability in the response to a post-ruminal supplement of methionine some general trends are apparent. Table 5.9 summarises the experiments in which sheep fed roughage diets received an abomasal infusion of methionine at a rate of 0.12g L-methionine/$kg^{0.75}$/day. These results suggest that the response to methionine was greatest with sheep fed the very low quality roughages, and that the extent of the response

TABLE 5.9: Values for intake and nitrogen utilisation parameters and the estimated amino acid yields at the duodenum for sheep fed roughage diets of varying quality and receiving an abomasal infusion of methionine (0.12g L-methionine/$kg^{0.75}$/day) in four experiments.

Diet	Expt.	Voluntary intake in control period ($gDM/kg^{0.75}$/day)	Apparent digestibility of organic matter (%)	N content of diet consumed (%)	[1]Estimated AA yield at duodenum (gAA/day)	[2]Ratio of *ad libitum* intake to maintenance	Nitrogen retention $g/kg^{0.75}$/day Control	Methionine	No.of observations
Barley straw	5/1	25	42	0.43	8	0.4	-0.227	-0.154	21
Barley straw/ wheaten hay chaff	5/2	39	48	0.70	18	0.7	-0.094	-0.038	21
Wheaten straw/ chaff	2/1, 2/2	47	52	0.83	26	1.0	-0.006	0.060	7
Wheaten hay chaff	2/3	67	62	1.10	50	1.6	0.227	0.176	3

1) Estimated amino acid (AA) yield at the duodenum calculated from the data of Egan (1974) for similar diets assuming that AA yield is directly proportional to intake.

2) Assuming that the digestible energy (DE) requirement for maintenance is 480 $kJDE/kg^{0.75}$/day.

decreased with increasing quality of diet until with sheep fed higher quality diets, nitrogen retention was adversely affected by the additional methionine. However, due to the small numbers of animals used in the experiments with the higher quality diets, these results must be treated with caution. When sheep were fed a similar diet to that used in Experiment 2/3 (Experiment 4, Chapter 4), methionine was shown to be an important component of the supplementary amino acid mixture which would support maximum nitrogen retention. It must also be borne in mind that the sheep in Experiment 2/3 were apparently more efficient in terms of nitrogen utilisation than those in Experiment 4 (0.227g N retained/$kg^{0.75}$/day compared with 0.089g N retained/$kg^{0.75}$/day in the control periods), and the differences in the apparent importance of methionine may be related to basic differences in the efficiency of nitrogen utilisation. However, the differences in the circumstances of methionine supplementation (i.e. as a single amino acid supplement as against part of a mixture) may also have been important.

On the basis of the above discussion of the results of the experiments reported in Chapters 2, 4 and 5, it may be suggested that the response to methionine in sheep fed roughage diets in these experiments was dependent on the interaction of the supplemental methionine or its metabolites with other amino acids. Any hypothesis concerning the responses to methionine must explain both between diet and within-diet between-animal variation in the response. This underlines the need to consider individual animal responses, and to look closely at results in which a high variability in the response outweighs differences between means. In addition to this, there is

the problem of carryover effects between periods when treatments are given in a factorial or Latin square sequence. It is therefore apparent that the experimental design for such experiments becomes the major problem.

CHAPTER 6

Methionine and Cystine metabolism in sheep given post-ruminal supplements of L-methionine

INTRODUCTION

In order to gain an understanding of methionine metabolism in sheep receiving abomasal supplements of methionine, and also to study aspects which might be related to the between animal variability in the response, some more detailed aspects of methionine metabolism were examined. The experiment to be reported in Chapters 6, 7 and 8 formed part of this study. In particular, the experiment was designed to provide quantitative data concerning the importance of the transulphuration pathway of methionine metabolism in liver and skeletal muscle, and also to provide information concerning the effects of methionine supplementation on the rate of protein synthesis in liver and muscle. For these purposes, an intravenous infusion of ^{35}S-labelled methionine and ^{3}H-labelled cystine was used.

The system involving the infusion of the two labelled amino acids was chosen for two principal reasons:

(i) the two cyst(e)ine labels could be expected to permit the derivation of quantitative data concerning methionine metabolism more effectively than the apparent proportional metabolism based on radioactivity ratios (transfer quotient approach - Klieber *et al.* 1962), in both the liver and muscle as well as in the whole animal;

(ii) it effectively provided three labelled amino acids (^{35}S-methionine, ^{35}S-cystine and ^{3}H-cystine) from which to derive estimates of the rate of protein synthesis.

Data relating to plasma, blood cell and tissue amino acid patterns, and activities of enzymes in the transulphuration pathway will be reported separately.

EXPERIMENTAL

Animals

Twelve Dorset x (Dorset x Merino) wethers, aged about 10 months were used in the experiments. The animals, each surgically prepared with an abomasal canula, had previously been used in the experiments reported in Chapter 5.

Diets and feeding

The sheep were fed a 2:1 mixture of wheaten hay chaff and barley straw in equal amounts at hourly intervals for 15 days. The quantity offered over 24 hours was 90% of the mean *ad libitum* intake for the 5 days prior to the start of hourly feeding. A mineral mix (10g/day; Moir & Harris 1962) and vitamins A and D_3 (APAC: Nicholas Pty. Ltd.) were given with the hourly feeds. Water was available at all times. Continuous lighting was maintained throughout.

Experimental design

Four animals were allocated to each level of abomasal methionine infusion (0, 0.12, 0.36g L-methionine/$kg^{0.75}$/day). The experiment was divided into 3 periods. The first 4 days of hourly feeding were used as an adjustment period. During the next 5 days

all sheep received an abomasal infusion of water (control period) while over the last 6 days, the water infusion continued or methionine was infused (treatment period). The animals were slaughtered on day 16. Six sheep (two from each treatment), were used in the radioisotope experiments. Each received an intravenous infusion of L-^{35}S-methionine (140-300 μCi) and L-3,3'-^{3}H-cystine (280-400 μCi; Radiochemical Centre, Amersham) for 16.5 hours prior to slaughter, which was at about 0930h on day 16 of the experiment. The infusates were given in sterile saline (without carrier) at a rate of 10 ml/hr via a jugula catheter. A second jugular catheter (in the alternate vein) was used for blood sampling.

Measurements

Urine was collected daily into sulphuric acid (final urine pH<2.5). The amount of urine was measured and a daily subsample taken and stored at -20°C. Faeces were collected and bulked by period. Nitrogen balances were calculated over 5 days for both the control and treatment periods.

Jugular whole blood and plasma samples for free amino acid analyses were obtained at the completion of both control and treatment periods. Liver and muscle samples were taken at slaughter for free amino acid analyses. Samples of liver, muscle and plasma proteins were prepared in order to derive an estimate of the fractional rate of protein synthesis in liver, muscle and plasma proteins in those animals receiving the isotope infusion (Chapter 7).

Blood sampling

Samples for whole blood and plasma amino acid analyses were taken from the jugular vein at 0800h on day 10 (water infusion) and on day 16. During the isotope infusion, blood samples (7 ml) were obtained at 8, 15, 30, 60 and 120 min. after the start of the infusion and thereafter at 2 hourly intervals until 14h. Further samples (12 ml) were taken at 15h, 15h10m and 15h20m. Samples (12 ml) were also taken from 3 sheep at 5h50m, 6h and 6h10m. A subsample for determination of packed cell volume was taken from all samples.

Whole blood and plasma samples for amino acid analyses were prepared with 10% (w/v) trichloroacetic acid (TCA) containing 2% (w/v) thiodiglycol; the latter was used in an attempt to reduce the extent of methionine oxidation during storage. The preparation was as follows: immediately after sampling, a subsample of blood was added to an equal volume of distilled water, and the cells lysed by vigorous shaking. A half-volume of TCA solution was then added to the lysed cells, the preparation shaken and centrifuged. The whole blood supernatant was drawn off, recentrifuged and then retained for analysis. A separate subsample of the whole blood was centrifuged and the plasma drawn off. The plasma proteins were then precipitated with an equal volume of TCA, the mixture shaken and centrifuged. The supernatant was drawn off and retained. The plasma protein pellets from the three samples at 15h, 15h10m and 15h20m were retained for three of the six sheep receiving the isotope infusion. In these cases, the pellet was washed twice with TCA, freeze-dried and retained for analysis. All sample solutions were stored at $-15^{\circ}C$.

Slaughter of animals and tissue sampling

About 16.5 hours after the start of the isotope infusion the animal was anaesthetised with sodium pentobarbitol (Nembutal). A lateral incision was then made immediately behind the last rib on the right side. Immediately the intestine was exposed it was clamped in several places to prevent digesta flow, so that samples could be obtained for another experiment. Blood samples were then obtained from the portal vein, hepatic vein and the aorta or renal artery. The samples of whole blood and plasma were prepared as described in the previous section.

The liver, kidney, spleen, diaphragm and heart were then removed and weighed. Samples were rapidly taken and immersed in liquid nitrogen. Samples of wool-free skin, intestine and muscle (*M.semitendenosus*) were also taken and treated in the same manner. Liver and muscle samples were obtained within 15 minutes of the start of the anaesthetic infusion. Respiration and cardiac function were maintained without assistance until the heart was excised. All samples were obtained prior to the removal of the heart. The animals which did not receive isotope were treated similarly except that only blood, liver and muscle samples were taken. However, the liver, kidney, spleen, diaphragm and heart were removed and weighed.

Sample preparation

The tissue samples in the liquid nitrogen were ground to a fine powder using a stainless steel pestle. The ground tissue was then retained as required, a portion of the powder being used for dry matter determination. Approximately 5g of liver and 5g of muscle

were added to tubes containing about 25 ml (weighed) of 2.5% TCA/2% thiodiglycol. The tubes were weighed and the amount of tissue calculated. The tissue was then homogenised using an Ultra-Turrax tissue homogeniser. The sides of the tube and the homogeniser were washed down with 0.5% TCA/0.4% thiodiglycol and the preparation weighed and centrifuged. The tissue supernatant was drawn off and stored at -15°C until prepared for analysis. The tissue pellet was washed twice with 0.5% TCA/0.4% thiodiglycol, freeze-dried, weighed and retained.

Analytical methods

Feed samples were oven dried at 85°C. Faeces and tissue samples were freeze-dried. Organic matter content of feeds and faeces was determined by ashing in a muffle furnace at 550°C. The nitrogen content of feed, faeces and urine was determined colourimetrically using a Technicon Auto Analyser following manual digestion according to a Kjeldahl technique (Munro & Fleck 1969).

Tissue preparation for amino acid analyses: Subsamples of the tissue homogenate and plasma protein pellets were prepared for amino acid analysis by hydrolysis for 16h in 6N hydrochloric acid under reflux in a flask purged and maintained with a nitrogen atmosphere (Mondino & Bongiovanni 1970). The tissue supernatants were concentrated by heating (36 hours at 55°C) followed by freeze-drying. The residue was then reconstituted in 0.1N hydrochloric acid.

Amino acid analyses: Amino acid analyses of liver and muscle tissue supernatants and protein hydrolysates were carried out by ion-exchange chromatography using an 18h separation and sodium citrate buffers on a

Technicon 'B' resin. The specific activity values for methionine and cystine in these samples were determined by counting the appropriate column effluent fractions and relating the counts to the concentration of the amino acid in the fraction. The methionine concentration in each individual fraction was determined by ninhydrin assay using 1% ninhydrin in acetate buffer, and hydrazine sulphate (0.025%). The cystine concentration in each individual fraction was determined using a slightly modified version of the technique of Gaitonde (1967) after reduction of the cystine to cysteine with dithiothreitol. For the cystine determination: 1 ml of dithiothreitol solution (0.308g dithiothreitol made up to 100 ml with 0.4M K_2HPO_4 - Williams *et al.* (1972a) was added to 1 ml of the column effluent and the mixture incubated for 60 minutes at 45°C to ensure complete reduction of cystine to cysteine; following incubation, 3 ml of the acid ninhydrin-acetic acid solution (Gaitonde 1967) was added and the preparations heated at 95°C in a water-bath for 15 minutes in covered tubes. The tubes were cooled and the colour measured at 560 nm. The colour was stable for at least 30 minutes. Ethanol was not added as recommended by Gaitonde (1967), as it caused colour fading. A range of standards was included in each run of both the methionine and cystine analyses.

The specific activities of methionine and cystine in whole blood and plasma were determined in the appropriate fractions after ion-exchange chromatography on a 35 x 0.5 cm column packed with Technicon 'A' resin, with a sodium citrate buffer (pH 3.5 containing 1% thiodiglycol) at a temperature of 60°C, and a flow rate of 25 ml/hour. In this system, cystine and methionine eluted after 82-95 and 126-140 minutes respectively. The methionine and cystine

concentrations were determined in the appropriate fractions as described above.

The specific activities of blood and plasma cystine and methionine measured after separation on the short column were within ±5% of the values determined using the 18h separation in the two samples analysed on both systems. However, the short column separation was not suitable for analysis of liver tissue supernatants due to contamination of the methionine peak with another ninhydrin-positive compound.

In all cases, methionine concentration has been expressed as the sum of methionine sulphoxide plus methionine. The methionine specific activity was calculated for the pure methionine peak only, and the concentration of methionine sulphoxide calculated from the ^{35}S activity associated with the methionine sulphoxide peak and the methionine specific activity assuming that the specific activities of methionine and methionine sulphoxide were identical. The concentration of methionine sulphoxide was derived in this manner since methionine sulphoxide did not elute as a pure peak in the short column system, being contaminated with another ninhydrin-positive compound. To maintain consistency in the expression of results the same method of calculation was used for samples separated on the Technicon 'B' system although the methionine sulphoxide did apparently elute as a pure peak in some of the samples analysed in this system.

Values for cystine concentration and for cystine specific activity have been expressed in relation to half-cystine.

In previous experiments reported in this thesis plasma and blood cell samples were prepared with TCA alone without the addition of thiodiglycol. In these samples the "aspartic acid" peak was considerably increased in size in samples from animals receiving a supplement of methionine. Co-chromatography of methionine sulphone and aspartic acid showed that under the conditions of our system these two amino acids eluted together. However, in plasma prepared with TCA and thiodiglycol there was no detectable methionine sulphone peak and no ^{35}S radioactivity eluted with the aspartic acid peak. In liver tissue supernatants, and to a lesser extent in muscle tissue supernatants, there were small amounts of both ^{35}S and ^{3}H activity eluting with a ninhydrin-positive peak which lay between aspartic acid and threonine (see Addendum to Chapter 6). It would thus appear that the thiodiglycol prevented the final oxidation step of methionine sulphoxide to methionine sulphone. However, Patureau-Mirand *et al.* (1973) found that 2% thiodiglycol in the TCA effectively prevented any oxidation of methionine since in such treated extracts they found only traces of methionine sulphoxide.

Radioactivity measurements

All isotope counting was carried out on a Packard Tri-Carb scintillation counter at 4°C. Aqueous solutions were prepared for counting using a toluenetriton scintillation mixture containing PPO and POPOP (Patterson & Green 1965). The window settings and gain controls were chosen so that no tritium registered in the second and third channels. The settings were:

Red (first) channel	20-500	100% gain
Green (second)	175-1000	15% gain
Blue (third)	300-1000	15% gain

The ratio of the counts registered in the blue and green channels indicated the degree of quenching, and correction factors for the efficiency of counting of each isotope were determined from standard curves. To derive the standard curves, standards containing known quantities of ^{3}H alone or ^{3}H plus ^{35}S, but with different amounts of carbon tetrachloride (0-100 µl per vial) as a quenching agent were prepared. The counting efficiencies were also checked on a number of samples by spiking pre-counted samples with known quantities of the isotopes. The formulae for the calculation of the amount of activity present in the samples are given below:

$$^{35}S \text{ dpm} = \frac{(\text{Green cpm} - \text{Background cpm})}{E\ ^{35}S \text{ green}}$$

$$^{35}S \text{ cpm in red channel} = {}^{35}S \text{ dpm} \times E\ ^{35}S \text{ red}$$

$$^{3}H \text{ dpm} = \frac{(\text{Red cpm} - \text{Background cpm}) - {}^{35}S \text{ cpm red}}{E\ ^{3}H \text{ red}}$$

where dpm = disintegrations per minute, cpm = counts per minute, green and red refer to the channels, background is the background counts in each channel, and E is the efficiency of counting of the ^{3}H or ^{35}S in the particular channel, the efficiency having been calculated from the blue:green ratio-efficiency relationships.

Calculations

For the estimation of entry rate of methionine and cystine in plasma, steady state conditions were assumed.

Entry rate (µ moles/min) is given by the formula

$$\frac{\text{rate of isotope infusion (dpm/min)}}{\text{plateau specific activity (dpm/µ mole)}}$$

where the rate of isotope infusion is the rate of infusion of the labelled amino acid in terms of the isotope itself, and where the

plateau specific activity is the specific activity of the infused amino acid in the plasma at plateau, i.e. after it has reached apparent equilibrium with the whole animal free pool of the amino acid. The entry rates of both methionine and cystine in terms of ^{35}S and ^{3}H respectively have been calculated using this formula (Waterlow 1969).

Since methionine is converted to cysteine (and hence cystine) in animal tissues, some of the ^{35}S-methionine will have been converted to ^{35}S-cystine. The transfer quotient (Kleiber *et al.* 1961) is the ratio of plasma specific activities of ^{35}S-cystine (^{35}S dpm/μ mole half-cystine) and ^{35}S-methionine, and indicates the proportion of plasma cystine which was apparently derived from plasma methionine. By applying this proportional factor to the total cystine entry rate (determined from the ^{3}H-cystine dilution) the actual quantity of cystine which was derived from methionine may be estimated. However, it is important to note that all of these parameters (entry rate, transfer quotient, etc.) provide estimates for only that part of the total body pool which was in equilibrium with the sampled pool, in this case the plasma pool.

The concentration and specific activity values for methionine and cystine in the blood cells were derived from the known concentration and specific activity values for these amino acids in the plasma and whole blood in the following manner (e.g. for methionine).

$$\text{Blood cell methionine (}\mu\text{ moles methionine/100 ml blood cells)} = \left\{ (\text{WBMet}) - \frac{(100 - \text{PCV\%})}{100} (\text{PMet}) \right\} \times \frac{100}{\text{PCV\%}}$$

where WBMet and PMet are the concentrations of methionine (μ moles/ 100 ml) in whole blood and plasma respectively, and where PCV% is the packed cell volume percentage.

$$\text{Blood cell methionine specific activity dpm/}\mu\text{ mole)} = \frac{\left\{ (\text{WBMet})(\text{WBMet SA}) - \frac{(100-\text{PCV\%})}{100} (\text{PMet})(\text{PMet SA}) \right\} \times \frac{100}{\text{PCV\%}}}{\text{Blood cell methionine concentration}}$$

where WBMet SA and PMet SA are the values for the specific activity (dpm/μ mole) of methionine in the whole blood and plasma respectively and the other expressions are as for the previous equation.

RESULTS AND DISCUSSION

Intake, digestion and nitrogen retention

Table 6.1 presents the mean values for the apparently digestible organic matter intake (g DOMI/$kg^{0.75}$/day) and apparent retention of nitrogen (g N/$kg^{0.75}$/day) for each animal in the control and treatment periods. Changes in the DOMI between the control and treatment periods reflect changes in the apparent digestibility of organic matter since the dry matter intake was the same in both periods. Between animal variability in the methionine response was clearly evident with one animal on each level of methionine exhibiting a reduced nitrogen retention when given the supplemental methionine, while the size of the response in the remaining six sheep was quite variable. The improvement in nitrogen retention ranged from 0.25g N/day (sheep 580) to 1.16g N/day (sheep 389). Two sheep which received the high level of methionine infusion (sheep 462, 378) had an increased apparent digestibility of organic matter in the methionine

TABLE 6.1: Apparently digestible organic matter intake (DOMI) and nitrogen retention for the twelve sheep in the control and treatment periods.

	DOMI ($g/kg^{0.75}/day$)		N retention ($g/kg^{0.75}/day$)	
	Control	Treatment	Control	Treatment
OMET[1]				
445	32.8	32.4	-0.020	0.004
582	26.0	24.2	-0.101	-0.106
365	30.6	30.9	0.099	0.129
564	--[2]	24.4	--[2]	-0.170
LMET				
580	30.1	28.9	-0.016	0.007
389	27.5	27.8	-0.067	0.018
558	28.3	26.0	-0.057	-0.064
393	25.7	26.5	-0.113	-0.064
HMET				
462	19.0	23.5	-0.129	-0.027
567	27.8	27.7	-0.076	-0.017
583	28.7	30.0	-0.021	-0.035
378	26.4	30.7	-0.082	0.014

1) Infusion during the treatment period; OMET-control, water infusion; LMET-low methionine, 0.12g L-met/$kg^{0.75}$/day; HMET-high methionine, 0.36g L-met/$kg^{0.75}$/day.

2) Urine and faeces discarded in error.

infusion period compared with the control period. Increases in the apparent digestibility of nitrogen were associated with increases in the apparent digestibility of organic matter (Appendix Table 6.1). The improved apparent digestibility in response to methionine has been discussed in Chapter 5, but remains unexplained.

The effect of methionine infusion on organ weights

The mean fresh weights of the liver, kidneys, spleen, diaphragm and heart taken at slaughter for the animals on each treatment are given in Table 6.2. The individual tissue weights and the liver dry weights are given in Appendix 6.2. The mean liver fresh weight of the sheep receiving the low level of methionine was significantly higher ($P<0.05$) than the mean weight of liver from the control animals. The same trend was also apparent in the animals receiving the high level of methionine, although the difference was not significant due to the low liver weight recorded for one animal (sheep 462). Changes in the weights of organs other than the liver exhibited no clear trends with methionine supplementation.

Excess methionine has been found to induce marked hypertrophy of the liver in rats (Sanchez & Swendseid 1969; Girard-Globa *et al.* 1972). In the latter work, the livers of rats fed diets devoid of the sulphur amino acids contained significantly less nitrogen than the livers of rats fed balanced diets. In the present work, if it is assumed that the nitrogen content of the dry liver was 12% (Fennessy unpublished), the daily liver nitrogen accretion accounted for about 0.13g, or about 20% of the mean increase in nitrogen retention due to methionine.

TABLE 6.2: Wet weights of tissues, expressed as percentage of the metabolic body size (liveweight $kg^{0.75}$) in sheep on the three levels of methionine.

	Liver	Kidneys	Spleen	Diaphragm	Heart
OMET	3.06^{1a2} ±0.055	0.797^{a} ±0.0397	0.445^{a} ±0.0417	1.07^{ab} ±0.051	1.34^{a} ±0.050
LMET	3.35^{b} ±0.072	0.700^{a} ±0.0354	0.619^{a} ±0.108	1.17^{a} ±0.048	1.18^{a} ±0.107
HMET	3.33^{ab} ±0.190	0.769^{a} ±0.0306	0.436^{a} ±0.0284	0.980^{b} ±0.0184	1.24^{a} ±0.082

1) mean value (n = 4) ± standard error of the mean

2) figures within columns with superscripts in common are not significantly different ($P<0.05$).

Plasma and blood cell methionine and cystine

The total ^{35}S and ^{3}H activity in the jugular plasma tended to increase with time during the period of the isotope infusion. After 12 hours there was an apparent tendency for the plasma activity to plateau (Appendix Table 6.3). Table 6.3 gives the correlation coefficients and the slopes of the regression equations for the relationships between ^{35}S activity and ^{3}H activity in plasma and the time of infusion over the period from 1 to 12 hours.

The values for the specific activity and concentration of methionine and cystine in the plasma and blood cells of samples taken at 6 hours (three sheep only) and 15 hours (six sheep) are given in Table 6.4.

Table 6.5 gives the values for the concentration and specific activity of plasma and blood cell methionine at five times during the isotope infusion in sheep 389. The cystine concentrations could not be determined accurately in the column effluent from the 4, 5 and 9 hour plasma samples due to the small amount of plasma available for chromatography. Figure 6.1 illustrates the contribution of methionine ^{35}S activity to total plasma ^{35}S activity during the period of the isotope infusion in sheep 389. This animal received an abomasal infusion of methionine at a rate of $0.12g/kg^{0.75}/day$.

On the basis of the results for sheep 582 and 389, it would appear that the specific activity of plasma methionine reached an apparent plateau at about 6 hours after the start of the infusion. However, the total ^{35}S activity in plasma continued to rise after this time (Appendix Table 6.3). This was due to an increase in the

TABLE 6.3: Values for the regression coefficients (slope of regression line) and correlation coefficients (r) for the relationship between plasma ^{35}S or ^{3}H activity (dpm x 10^3/ml plasma) and the time of infusion (hours) over the period of the infusion from 1 to 12 hours in the six sheep.

		^{35}S activity		^{3}H activity	
		Slope	r	Slope	r
OMET[1]					
	445	0.174	0.88	0.731	0.98
	582	0.219	0.99	0.529	0.94
LMET					
	580	0.190	0.94	0.762	0.98
	389	0.736	0.98	0.658	0.95
HMET					
	462	1.50	0.99	0.900	0.99
	567	1.29	0.99	0.746	0.99

1)
OMET - control, water infusion; LMET - low methionine, 0.12g L-methionine/$kg^{0.75}$/day; HMET - high methionine, 0.36g L-methionine/$kg^{0.75}$/day.
n = 7, samples at 1, 2, 4, 6, 8, 10 and 12 hours.

TABLE 6.4: Values for the concentration (u moles/100 ml) and specific activity (^{35}S or ^{3}H dpm x 10^3/u mole) of free methionine and cystine (expressed as half-cystine) in the plasma and blood cells in samples taken at 6 and 15L[1] after the start of the isotope infusion. (All specific activity values have been corrected to a standard total isotope infusion of 500 x 10^6 dpm of ^{35}S and 750 x 10^6 dpm of ^{3}H).

	Free methionine[3]		Free cystine (as ½ cys)		
	Concentration (u moles/100ml)	Sp. act. (^{35}S dpm x 10^3/u mole)	Concentration (u moles/100ml)	Sp.act(dpm x 10^3/u mole) ^{35}S	^{3}H
OMET[2]: Sheep 445					
Plasma 15h	4.39	52.5	1.74	10.1	293
Blood cells 15h	NM[4]	NM	ND[5]	-	-
Sheep 582					
Plasma 6h	2.90	51.5	1.89	2.37	184
15h	3.27	52.0	1.85	7.43	202
Blood cells 6h	NM	NM	ND	-	-
15h	NM	NM	ND	-	-
LMET: Sheep 580					
Plasma 15h	8.20	27.2	1.42	9.97	276
Blood cells 15h	10.20	13.9	ND	-	-
Sheep 389					
Plasma 6h	23.5	22.5	3.60	6.20	234
15h	24.6	23.3	2.99	5.23	133
Blood cells 6h	26.4	6.49	ND	-	-
15h	31.9	11.9	ND	-	-
HMET: Sheep 462					
Plasma 6h	106	9.48	2.28	5.17	248
15h	105	15.7	3.32	4.89	124
Blood cells 6h	88.4	2.65	ND	-	-
15h	67.1	9.02	ND	-	-

continued/..

TABLE 6.4: continued

	Free methionine[3]		Free cystine (as ½ cys)		
	Concentration (u moles/100ml)	Sp.act, (^{35}S dpm x 10^3/u mole)	Concentration (u moles/100ml)	Sp.act (dpm x 10^3/u mole) ^{35}S	^{3}H
HMET: Sheep 567					
Plasma 15h	188	8.46	3.12	3.01	128
Blood cells 15h	116	4.27	ND	-	-

1) Samples at 6 and 15h refer to bulked samples of TCA precipitated plasma and blood cell supernatants from samples of blood taken at the following times:
6h - 5h50m, 6h, 6h10m (3);
15h - 15h, 15h10m, 15h20m (3).

2) Infusion during the treatment period; OMET - control, water infusion; LMET - low methionine, 0.12g L-met/$kg^{0.75}$/day; HMET - high methionine, 0.36g L-met/$kg^{0.75}$/day.

3) Methionine as methionine plus methionine sulphoxide

4) NM - not measurable; although methionine was present in the blood cells, there were very large errors involved in the calculation of concentrations and specific activity values from the plasma and whole blood values.

5) ND - not detectable.

TABLE 6.5: Values for the concentration (μ moles/100ml) and specific activity (^{35}S dpm x 10^3/μ mole) of free methionine in the plasma and blood cells from samples of jugular blood from sheep 389 over the period of 4 to 15 hours after the start of the isotope infusion. (All specific activity values have been corrected to a standard total isotope infusion of $500x10^6$ dpm of ^{35}S and $750x10^6$ dpm of ^{3}H).

	Methionine concentration[1] (μ moles/100ml)		Methionine specific activity (^{35}S dpm x 10^3/μ mole)	
Time (h)[2]	Plasma	Blood cells	Plasma	Blood cells
4	23.8	67.0	19.3	1.92
6	23.5	26.4	22.5	6.49
9	28.2	51.9	20.8	5.99
13	31.9	45.2	24.6	10.7
15	24.6	31.9	23.3	11.9

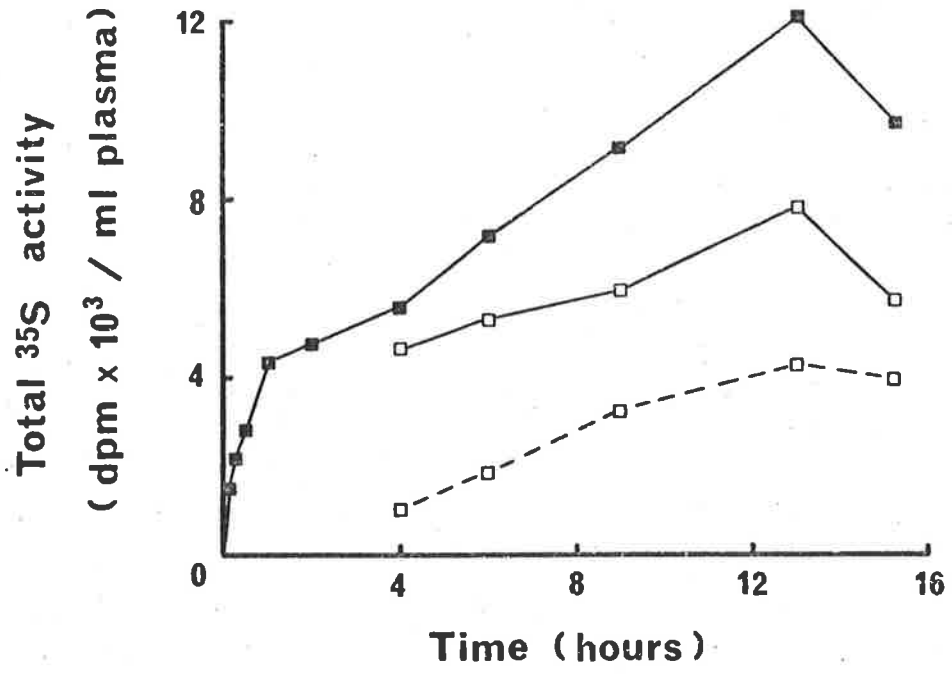
1) Methionine as methionine plus methionine sulphoxide.

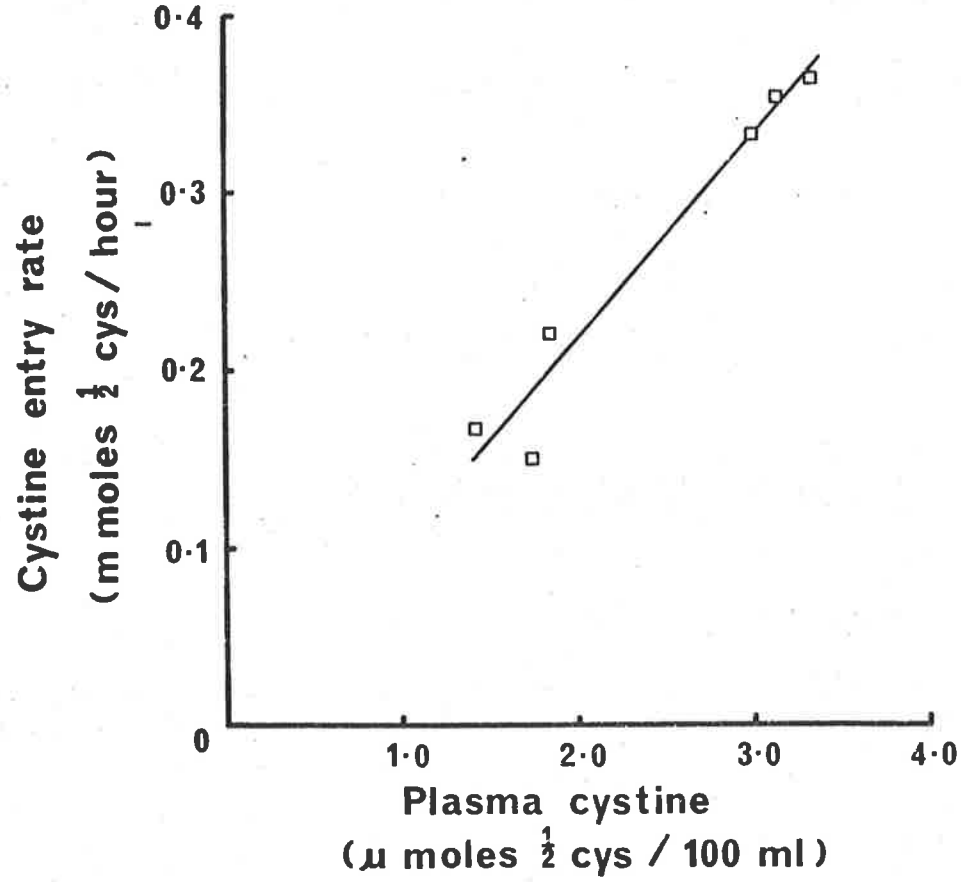
2) Samples at the various times refer to bulked samples of TCA precipitated plasma and blood cell supernatants from samples of blood taken at the following times:

4h - one sample at 4h;
6h - 5h 50m, 6h, 6h 10m, (3);
9h - 8h, 10h (2);
13h - 12h, 14h (2);
15h - 15h, 15h 10m, 15h 20m (3).

FIGURE 6.1: Total plasma ^{35}S activity (●———●), methionine ^{35}S activity (0———0) and the non-methionine ^{35}S activity (0-----0 calculated by difference) during the period of the isotope infusion in sheep 389, which received 0.12g L-methionine/kg$^{0.75}$/day. (All values have been corrected to a standard total ^{35}S infusion of 500 x 10^6dpm).

FIGURE 6.2: Relationship between the estimate cystine entry rate in plasma (m moles ½ cys/hour) and the concentration of cystine in plasma (u moles ½ cys / 100ml plasma). All values have been corrected to a standard total isotope infusion of 500 x 10^6 dpm of ^{35}S and 750 x 10^6 dpm of ^{3}H).


Total ^{35}S activity
(dpm x 10^3 / ml plasma)
12
8
4
0
4
8
12
16
Time (hours)


Cystine entry rate
(m moles ½ cys / hour)
0·4
0·3
0·2
0·1
0
1·0
2·0
3·0
4·0
Plasma cystine
(μ moles ½ cys / 100 ml)

concentration of plasma methionine and/or an increase in the proportion of non-methionine ^{35}S activity (i.e. products of methionine metabolism). It is apparent from Figure 6.1 that both of these factors were responsible for the continuing rise in plasma ^{35}S activity in sheep 389.

The specific activity of the plasma methionine in sheep 462 (which received the high level of methionine infusion) at 6h was much lower than the specific activity at 15h. The rate of increase in the total plasma ^{35}S activity in both sheep 462 and 567 was much greater than that in the plasma of the sheep receiving the infusions of water or the lower level of methionine (Table 6.3). These factors, together with the greatly increased plasma methionine concentrations and the lower values for the specific activity of plasma methionine in sheep 462 and 567, are indicative of a greatly increased free methionine pool in the sheep receiving the highest level of methionine infusion.

In all sheep, the specific activity of the blood cell methionine was considerably less than that of plasma, although the ratio of the specific activities tended to increase with time (e.g. for sheep 389 - 0.10 at 4h, 0.29 at 9h to 0.51 at 15h). It is apparent from the differences in the specific activity of methionine between plasma and blood cells and from the wide fluctuations in the blood cell methionine concentration with time (e.g. in sheep 389) that the blood cells could not have been drawing methionine from only the plasma pool. The pattern of these results tends to provide support for the suggestion of Elwyn (1970) (see Review Section 3.4) that the blood cells may have an important role in the interorgan transport of some

amino acids.

There was evidence of considerable variability in the specific activity of plasma cystine. In two sheep (389, 462) the ^{3}H-cystine specific activity in the 6h sample was about twice that of the 15h sample, while the ^{35}S-cystine specific activity was similar at both samplings. These data suggest marked fluctuations in the clearance of cystine from the plasma pool, although the time course of such fluctuations could be quite rapid, since the rate of ^{3}H-cystine infusion was sufficient to double the specific activity of plasma cystine in only five minutes assuming that there was no exchange of plasma cystine with a pool of lower ^{3}H-cystine specific activity. There was evidence of similar variability in the specific activity of plasma cystine in the sheep in the work of Williams & Leng (1972). These workers could observe no trends with time after about 4 hours of infusion (i.e. in the "plateau phase").

In the present work all of the free cystine present in the whole blood samples was accounted for as plasma cystine.

Entry rates of methionine and cystine in plasma

Table 6.6 gives the value for the entry rates of methionine and cystine in plasma calculated from the values for the specific activities measured in the 15h samples. The entry rate of methionine was markedly increased with methionine supplementation and the increase was apparently greater than the increase in methionine supply from the infusion. The very high entry rate of methionine calculated for sheep 567 may have been an overestimate since there is a possibility that the specific activity of plasma methionine had not reached a plateau by 15h.

TABLE 6.6: Entry rate of methionine and cystine in plasma and the estimated contribution of cystine derived from methionine to the entry rate of cystine in sheep given abomasal infusions of 0, 0.12 or 0.36g L-methionine/$kg^{0.75}$/day.

Sheep	Methionine infused ($g/kg^{0.75}$/day)	Methionine infused (m moles/h)	Entry rate (m moles/h) Methionine	Entry rate (m moles/h) Cystine (as ½ cys)	Contribution of cystine derived from methionine to cystine entry rate (m moles ½cys/h)
445	0	0	0.56	0.150	0.029
582	0	0	0.57	0.221	0.032
580	0.12	0.37	1.11	0.168	0.075
389	0.12	0.50	1.27	0.333	0.061
462	0.36	1.14	1.92	0.364	0.072
567	0.36	1.47	3.56	0.353	0.125

The quantity of digesta methionine (excluding the infused methionine) reaching the duodenum would be expected to have been about 0.14 to 0.20 μmoles/hour in the sheep in the present experiment (calculated from the data of Egan *et al.* 1975). Assuming that the net absorption of methionine from the intestine was similar to the estimated flow of methionine to the duodenum (excluding any contribution of endogenous protein to the net uptake of methionine from the intestine), the methionine entry rate was three to four times the expected absorption of the amino acid from the intestine in the sheep not receiving any supplement of methionine. This is in marked contrast to the situation with lysine in the human adult, where the lysine entry rate was approximately 50 times higher than the lysine intake (Waterlow 1967). However, the entry rate of lysine in the adult sheep in the studies of Buttery *et al.* (1975) was similar to the expected net absorption from the intestine.

The estimated cystine entry rates were very low (18 and 33mg/hour for sheep 445 and 582), although the higher value was similar to rates measured by Williams *et al.* (1972a) for sheep fed a similar quantity of lucerne. The entry rates were higher in three of the four sheep receiving the methionine supplement, while plasma cystine concentrations were also elevated. There was a highly significant correlation between the estimated entry rate of cystine and the plasma cystine concentration ($r = 0.98$; $df = 5$). This relationship is plotted in Figure 6.2. Williams & Leng (1972) reported a similar correlation, although the actual regression relationship was not given in their paper. The flow of cystine to the duodenum in the sheep in the present experiment would be expected to have been about 0.10 to 0.15 μmoles of half-cystine

/hour (calculated from the data of Egan *et al.* (1975). Thus the cystine entry rate was similar to or perhaps slightly higher than the estimated rate of cystine absorption. This is a similar situation to that reported by Williams *et al.* (1972a). These workers suggested that the small entry rate of cystine relative to the exogenous supply may indicate a substantial reutilisation of cystine within the cells. The liver to plasma ratios of ^{3}H-cystine (Table 6.9) lend support to the suggestion that this was the situation with liver cystine metabolism. This will be discussed in a later section.

The estimated contribution of cystine derived from methionine to the cystine entry rate in plasma is given in Table 6.6. More accurately, this is the apparent contribution to the plasma cystine entry rate of cystine derived from plasma methionine, and if cystine were derived from a methionine pool of lower specific activity than the plasma methionine, then this would be an underestimate. This was almost certainly the situation in the present work since (i) the liver methionine specific activity was lower than the plasma methionine specific activity (Table 6.9) and (ii) the difference in the cystine entry rates of sheep 389, 462 and 567 compared with the sheep not receiving methionine (sheep 445, 582) was greater than the estimated increase in cystine derived from methionine.

Plasma, blood cell and tissue free methionine and cystine

The values for the concentration and specific activity of free methionine in the plasma and blood cells (15h samples) and in the liver and muscle tissue free pools are presented in Table 6.7.

TABLE 6.7: Values for the concentration (u moles/100 ml) and specific activity (^{35}S or $^{3}H \times 10^{3}$ dpm/u mole) of free methionine and cystine (expressed as half-cystine) in plasma, blood cells (15h samples), and in liver and muscle tissue water. (All specific activity values have been corrected to a standard total isotope infusion of 500×10^{6} dpm of ^{35}S and 750×10^{6} dpm of ^{3}H).

	Free methionine[1]		Free cystine (as ½ cys)		
	Concentration (u moles/100ml)	Sp.act, (^{35}S dpm x 10^{3}/u mole)	Concentration (u moles/100ml)	Sp.act (dpm x 10^{3}/u mole) ^{35}S	^{3}H
OMET[2]: Sheep 445					
Plasma	4.39	52.5	1.74	10.1	293
Blood cells	NM[3]	NM	ND[4]	-	-
Liver	51.4	6.06	112	5.75	12.5
Muscle	14.2	17.6	ND	-	-
Sheep 582					
Plasma	3.27	52.0	1.85	7.43	202
Blood cells	NM	NM	ND	-	-
Liver	32.1	5.36	53.5	5.76	12.0
Muscle	10.4	14.6	ND	-	-
LMET: Sheep 580					
Plasma	8.20	27.2	1.42	9.97	276
Blood cells	10.2	13.9	ND	-	-
Liver	40.6	5.92	52.0	11.0	10.4
Muscle	12.2	18.6	ND	-	-
Sheep 389					
Plasma	24.6	23.3	2.99	5.23	133
Blood cells	31.9	11.9	ND	-	-
Liver	175	6.18	74.8	5.74	6.88
Muscle	50.8	13.0	ND	-	-

continued/..

TABLE 6.7: continued

	Free methionine[1] Concentration (u moles/100ml)	Free methionine[1] Sp.act. (^{35}S dpm x 10^3/u mole)	Free cystine (as ½ cys) Concentration (u moles/100ml)	Free cystine (as ½ cys) Sp.act. (^{35}S dpm x 10^3/u mole)	Free cystine (as ½ cys) Sp.act. (^{3}H dpm x 10^3/u mole)
HMET: Sheep 462					
Plasma	105	15.7	3.32	4.89	124
Blood cells	67.1	9.02	ND	-	-
Liver	711	10.7	105	3.48	4.73
Muscle	141	16.4	ND	-	-
Sheep 567					
Plasma	188	8.46	3.12	3.01	128
Blood cells	116	4.27	ND	-	-
Liver	1589	6.78	74.2	1.67	2.29
Muscle	199	8.67	ND	-	-

1) Methionine as methionine plus methionine sulphoxide.

2) Infusion during the treatment period; OMET - control, water infusion; LMET - low methionine, 0.12g L-met/kg$^{0.75}$/day; HMET - high methionine, 0.36g L-met/kg$^{0.75}$/day.

3) NM - not measurable; although methionine was present in the blood cells, there were very large errors involved in calculating the concentrations and specific activity values from the plasma and whole blood values.

4) ND - not detectable.

The liver and muscle free methionine concentration increased considerably in three of the four sheep receiving the supplemental methionine (sheep 389, 462, 567). Although the plasma methionine concentration in sheep 580 was increased as compared with those sheep not receiving the additional methionine (sheep 445, 582), the liver and muscle free methionine levels in all three sheep were similar. The increase in the free methionine levels was particularly evident in the two sheep receiving the highest level of methionine infusion (sheep 462, 567).

Table 6.8 gives the values for the proportion of "total methionine" actually present as true methionine in the analysed samples. The very high concentrations of sulphoxide in the liver of the two sheep which received the high level of methionine (sheep 462, 567) possibly indicate a metabolic pathway involving oxidation of the methionine, although it is not possible to determine whether this was the situation from a study of the data. It is probable that much of the methionine oxidation occurred in storage, although the thiodiglycol did apparently inhibit the degree of methionine oxidation compared with previous experiments (see Experimental). Girard-Globa *et al.* (1972) detected considerable increases in the levels of methionine sulphoxide in the plasma of rats fed high methionine diets, although the increase in the actual concentration of plasma methionine itself was much greater.

As was the case with the blood cells there was no detectable free cystine in the muscle tissue free pool. In contrast, the liver free cystine concentrations were 30 to 60 times higher than the plasma free cystine and the concentrations in the two pools were apparently

TABLE 6.8: Percentage values for the proportion of total methionine actually present as true methionine in the analysed samples.

Sheep	Plasma	Whole blood	Liver	Muscle
445	59	57	43	35
582	56	55	44	47
580	68	70	44	60
389	85	87	41	87
462	83	85	9	83
567	74	76	13	76

1) Total methionine as methionine plus methionine sulphoxide. The specific activity of methionine was calculated for the pure methionine peak only, and the concentration of methionine sulphoxide calculated from the ^{35}S activity associated with the methionine sulphoxide peak and the specific activity of the methionine peak assuming that the specific activities of methionine and methionine sulphoxide were identical.

unrelated. Although plasma cystine tended to increase with methionine supplementation, there was no such trend apparent for the liver free cystine. The highest concentration of liver free cystine was found in sheep 445, an animal which did not receive any supplemental methionine. The absence of any detectable free cystine in the muscle tissue supernatants is interesting in view of the fact that the enzyme, cystathionine-γ-lyase was not detectable in the skeletal muscle of sheep (Radcliffe & Egan 1974). However, small amounts of free cystathionine were found in muscle extracts from sheep receiving abomasal supplements of L-methionine (Fennessy unpublished).

Specific activity ratios between the free pools of methionine and cystine

Table 6.9 gives the ratios of the specific activities of liver and muscle free ^{35}S-methionine to plasma ^{35}S-methionine and the ratios of liver free ^{35}S-cystine and ^{3}H-cystine to plasma free ^{35}S-cystine and ^{3}H-cystine respectively. A ratio close to unity suggests a rapid equilibration of the amino acid between the two pools. A ratio far removed from unity suggests a poor equilibration, with dilution of the radioactivity by a source of cold amino acid, or perhaps simply poor mixing or poor communication between the two pools. These ratios are generally interpreted as indices of the degree of re-entry into the tissue free pool of amino acids derived from protein catabolism (Gan & Jeffay 1967). (Amino acid re-entry is defined as that proportion of the tissue free amino acid pool which is derived from protein catabolism, assuming that there is no re-entry of labelled amino acid from protein catabolism). With the hepatic free ^{35}S-cystine (which is synthesized from ^{35}S-methionine via the

TABLE 6.9: Values for the ratios of the specific activities of liver free methionine to muscle free methionine and liver free ^{35}S-cystine and ^{3}H-cystine to their respective plasma free cystine specific activities.

	Methionine specific activity ratios		Cystine specific activity ratios	
Sheep[1]	Liver / Plasma	Muscle / Plasma	Liver ^{35}S / Plasma ^{35}S	Liver ^{3}H / Plasma ^{3}H
445	0.115	0.335	0.567	0.043
582	0.103	0.281	0.775	0.059
580	0.218	0.684	1.10	0.038
389	0.266	0.558	1.10	0.052
462	0.682	1.04	0.712	0.038
567	0.801	1.02	0.555	0.018

1) Sheep 445, 582 - control (water) infusion; 580, 389 - methionine at a rate of 0.12g/$kg^{0.75}$/day; 462, 567 - methionine at a rate of 0.36g/$kg^{0.75}$/day.

transulphuration pathway), the ratio reflects not only re-entry from protein catabolism (as well as possible poor mixing of the liver and plasma free pools), but also differentials due to intracellular synthesis of cystine which are not reflected in the ratio through mixing with the plasma free cystine pool. The implicit assumption in these calculations is that the plasma pool is the circulating pool, i.e. the pool from which amino acids entering the cell are obtained.

To allow direct quantitative evaluation of re-entry, the ratios should have been calculated using the true intracellular specific activity rather than the overall tissue specific activity. However the extracellular spaces of the liver and muscle tissues were not measured in the present experiment, and corrections to allow the calculation of true intracellular specific activity have not been made. If it is assumed that the amino acids in the extracellular space had the same specific activity as the amino acids in the plasma, then the failure to make a correction for the extracellular space would have resulted in an overestimate of the intracellular specific activity.

The increase in the ratio of the specific activity of liver and muscle free methionine to plasma free methionine which occurred with the increase in the level of methionine supplementation was consistent with a reduced proportional re-entry of methionine into the tissue free pools as a result of the increased tissue concentrations of methionine.

In the muscle free pool of sheep 462 and 567 (which received the highest rate of methionine supplementation) the apparent re-entry of methionine was zero, i.e. in comparison with the turnover of

methionine in the muscle free pool and the rate of mixing with the plasma free methionine pool, the rate of re-entry of methionine from protein catabolism into the muscle free pool was negligible. In contrast, the ratio of the liver specific activity of methionine to that in the plasma did not reach unity even at the highest level of methionine supplementation (sheep 462, 567), despite the very large increases in the liver free methionine pool. The ratios calculated for these two sheep were consistent with a very high rate of liver protein degradation (such that protein degradation contributed 30% and 20% of the total liver free methionine pool in sheep 462 and 567 respectively) and/or a poor mixing of the intracellular free methionine pool and the plasma pool (assuming that this is the circulating pool). Since the first explanation would require either a very fast rate of liver protein turnover or alternatively a very slow rate of liver free methionine turnover, the second possibility (i.e. poor mixing of the two pools) would seem the more likely. However, a further alternative could be considered, namely that the plasma did not represent the circulating pool of free methionine, and that the blood cells were also involved, in which case the plasma to tissue specific activity ratios are inappropriate. Thus despite the very large increase in the pool size of liver free methionine, (as in sheep 462 and 567) there still appear to have been barriers to the complete mixing of the methionine within the cell (assuming that the jugular plasma methionine specific activity was virtually the same as that of the blood plasma flowing into the liver). This raises the question of compartmentation of methionine within the cell. Studies of such subcellular compartmentation in the case of the metabolites of carbohydrate metabolism in the liver (Greenbaum *et al.* 1971), and amino acid metabolism in the brain (Roach *et al.* 1974) have

been reported, although there is a possibility that compartmentation in the brain is associated with different cell types (Quastel 1974).

The very low values for the ratio of the specific activity of liver free ^{3}H-cystine to the plasma free ^{3}H-cystine indicated very little mixing of the intracellular cystine with the plasma cystine pool. The true ratios based on intracellular values would have been even lower due to the considerable concentration gradient between the liver and the plasma free cystine pools. However, the specific activities of ^{35}S-cystine in plasma were higher than those in the liver free pool in four out of the six sheep (sheep 445, 582, 462, 567). This is surprising in view of the likelihood that the liver does represent a major site of methionine catabolism (via the transulphuration pathway) in the sheep. The observations regarding the cystine specific activity ratios in the liver and plasma suggest that there was a considerable movement of cystine out of the cell (as represented by the ^{35}S-cystine specific activity in the plasma), while there was a much smaller movement in the reverse direction (i.e. from plasma into the cell, as represented by ^{3}H-cystine specific activity in very close communication with the plasma pool but in poor communication with the rest of the intracellular free cystine pool. However, this seems less likely if the studies on the location of the transulphuration pathway enzymes in the rat liver and human cell culture lines are applicable to the sheep. In studies with these tissues, Allsop & Watts (1975) have shown that methionine adenosyl transferase, cystationine synthase and cystathionine-γ-lyase are localised in the cytoplasm rather than in the membranes (which could be expected to provide an advantage in terms of access to the plasma pool).

The evidence for poor mixing of the free methionine and cystine pools in plasma with the tissue free pools and for metabolic compartmentation of the free amino acids raises questions as to the interpretation of amino acid entry rate data. In a comparative sense the rate of flux of an amino acid through the plasma pool (i.e. the entry rate) may provide some useful information although there are always problems in the interpretation of such data. The problems are not unlike those in the interpretation of plasma amino acid profiles, i.e. differences between individuals may reflect unique individual metabolic patterns, or perhaps the patterns may appear similar, and not reflect real differences at the tissue level. This is essentially the problem encountered by Williams *et al.* (1972a) in their investigation of cystine metabolism in sheep which were genetically different in wool production. Although there must have been considerable differences in the utilisation of the sulphur amino acids for wool growth, no differences in the metabolism of cystine were apparent from a study of the cystine entry rates.

CONCLUSIONS

Theoretically the methodology used in the experiment reported in this chapter should have provided useful information concerning the effects of methionine supplementation on the rate of methionine catabolism in the whole animal. However the results obtained were not readily interpretable. From a study of the specific activity ratios of methionine and cystine in the plasma and tissue pools, there was evidence of very poor mixing of the amino acids between these pools, except in those animals receiving the highest rate of abomasal methionine infusion in which the tissue free methionine pools were

grossly expanded. The metabolism of methionine and cystine is apparently very complex and as a result the interpretation of data is particularly difficult.

ADDENDUM

Several unidentified fractions collected during ion-exchange chromatography (18h) of liver homogenate TCA supernatants contained significant amounts of ^{35}S and/or ^{3}H. Some characteristics of these fractions are given below.

Fraction "A" - collected 35-55 minutes after the buffer start. These fractions included taurine and a compound not positive to ninhydrin eluting just after taurine or partly coincident with it. The pattern of elution suggests that taurine was not a major contributor to the total ^{35}S and ^{3}H in these fractions. In sheep 445 and 582 the ^{35}S component of these fractions represented about 9% and 3% of the total non-methionine free ^{35}S while in the four sheep given abomasal methionine, this fraction represented 40-50% of the non-methionine free ^{35}S. The comparable values for the ^{3}H component were 24% and 6% for the control sheep and 58-73% for the methionine-supplemented animals.

Fraction "B" - collected 75-85 minutes after the buffer start. This fraction was a significant component in only one animal, sheep 567, receiving the high rate of methionine supplementation. It was a compound not positive to ninhydrin and contained 37% of the non-methionine free ^{35}S and 6% of the free ^{3}H.

Fraction "C" - This fraction was coincident with a peak eluting between aspartic acid and threonine, usually not well separated from the latter. In control sheep, this fraction contained about 25% of the non-methionine free ^{35}S and 18% of the free ^{3}H, whereas in methionine-supplemented sheep, the corresponding values were 8% and 5% respectively.

Fraction "D" - This was a mixed fraction containing both ^{35}S and ^{3}H and eluting at positions coincident with two broad ninhydrin-positive peaks eluting between glutamic acid and glycine, and in some chromatograms running into the latter amino acid peak. It is likely that at least part of this fraction was oxidised glutathione (Hamilton 1963). The ^{35}S present in this fraction constituted about 35% of the total non-methionine-free ^{35}S, and the ^{3}H about 30% the total free ^{3}H, whereas in the methionine-supplemented animals, the corresponding values were 3-22% of the ^{35}S and 4-15% of the ^{3}H.

Fraction "E" - This fraction was coincident with a ninhydrin-positive peak eluting about 10-15 minutes before valine. It contained about 10% of the total non-methionine-free ^{35}S and 8% of the free ^{3}H. The proportion of counts tended to be higher in animals not receiving supplementary methionine.

Fraction "F" - This fraction was coincident with a ninhydrin-positive peak which eluted 10-15 minutes after valine. It contained about 9% of both the non-methionine-free ^{35}S and free ^{3}H in control animals and about 4% of each in the methionine-supplemented animals.

Fraction "G" - This fraction was coincident with a ninhydrin-positive peak which eluted between methionine and cystathionine. It contained both ^{35}S and ^{3}H in small amounts.

CHAPTER 7

Protein synthesis in the liver and muscle of sheep given post-ruminal supplements of L-methionine

INTRODUCTION

The rate of protein synthesis in a tissue may be estimated from the relative rate of incorporation of a radioactively labelled amino acid into the tissue proteins from a precursor amino acid pool of known specific activity (Waterlow & Stephen 1968; Gan & Jeffay 1971; Garlick & Millward 1972).

The use of this procedure involves many assumptions, and one major problem is associated with the measurement of the specific activity of the amino acid immediately prior to its incorporation into protein. In recent years there has been a great deal of controversy as to the identity of the free amino acid precursor pool for protein synthesis in the tissues, i.e. the pool from which amino acids are directly sequestered for protein synthesis. The various points of view have been summarised by Airhart *et al.* (1974) and van Venrooij *et al.* (1974). Kipnis *et al.* (1961) proposed that the amino acid pool involved in protein synthesis equilibrated very rapidly with the extracellular amino acid pool. Subsequently several workers have proposed that the extracellular amino acid pool best represents the precursor pool for protein synthesis (Hider *et al.* 1969; 1971; van Venrooij *et al.* 1972), while others (Morgan *et al.* 1971; Fern & Garlick 1973; Li *et al.* 1973) have favoured the concept of an intracellular precursor pool. Ideally, the best estimate of the specific activity of the precursor amino acid would be obtained from the measurement of the specific activity of the tRNA-bound amino acid or the specific activity

of the amino acid in the nascent peptide, i.e. during the synthesis of the polypeptide on the polysome. Recently Airhart *et al.* (1974) and van Venrooij *et al.* (1974) have isolated and measured the specific activity of the tRNA-bound amino acid and have proposed that a "membrane pool" (which is a mixed pool containing amino acids derived from both the extracellular and intracellular pools) best represents the precursor pool for amino acids entering protein.

For technical reasons it was not practicable to utilise the tRNA-bound amino acid approach in the present experiment and a less direct method was used, analogous to that of (Millward *et al.* 1975a). An infusion of the two labelled amino acids (^{35}S-methionine and ^{3}H-cystine) seemed to provide a very good alternative approach since it effectively provided three labelled amino acids (^{35}S-methionine, ^{35}S-cystine and ^{3}H-cystine) for incorporation into protein. In this approach it is assumed that the ratio of the specific activities of any two of the amino acids in the protein will reflect the ratio of the specific activities of the same two amino acids in the precursor pool, assuming that all amino acids for protein synthesis are drawn from the same pool. Even if this were not the case for methionine and cystine, it could be expected to be a valid assumption for the two cystine labels assuming that cystine synthesised from methionine behaves in the same manner as the cystine represented by the ^{3}H-labelled cystine. With a similar approach, Fern & Garlick (1974) have used two isotope labels and the amino acids, glycine and serine in an attempt to identify the precursor pool of amino acids for liver protein synthesis in rats.

It was hoped that the data from this experiment would provide information on the relationships (if any) between nitrogen retention,

the response to methionine and the rate of protein synthesis in the liver and muscle.

METHODOLOGY

Experimental

The measurement of amino acid concentrations and the specific activities of the amino acids in the free and protein amino acid pools of plasma has been described in Chapter 6.

The specific activity of plasma protein cystine was corrected for the presence of disulphide-bound cystine. The bound cystine was determined using the method of Downes (1961) for releasing the disulphide-bound cystine from the plasma proteins, and the measuring the cystine concentration and the specific activity of cystine as described in Chapter 6. The method of correction for the bound cystine was as follows:

$$\text{Protein cys} = \text{Total cys} - \text{Bound cys}$$

$$\text{Protein cys radioactivity} = (\text{Total cys} \times \text{sp. act.}) - (\text{Bound cys} \times \text{sp.act.})$$

$$\text{Protein cys sp. act.} = \frac{\text{Protein cys radioactivity}}{\text{Protein cys}}$$

where "protein cys" is the concentration of cystine in the plasma proteins, "total cys" is the total measured concentration of plasma protein cystine (includes both the protein and disulphide-bound cystine), "bound cys" is the disulphide-bound cystine, and "sp. act." within the brackets is the measured specific activity of the cystine in each of the respective compartments.

Specific activity of the precursor amino acids

Four possible precursor pools for protein synthesis have been considered.

Plasma and whole tissue free pools: These constituted two possible precursor pools of free amino acids and were the pools actually sampled in the experiment. The full details have been given in Chapter 6.

Intracellular free pool: The third pool was the intracellular free pool. The values for the amino acid concentrations and specific activities have been calculated assuming that 42% of the total liver tissue water and 23% of the total muscle tissue water was extracellular in origin. These values for extracellular space were derived from those used by Waterlow & Stephen (1968). It has been assumed that the extracellular pool was identical to that of the plasma in terms of the concentration and specific activities of the amino acids.

The concentration and specific activity of the amino acids in the intracellular pool have been calculated in the following manner (e.g. for liver methionine).

$$\text{Intracellular methionine concentration} = \frac{M_L - 0.42\ M_E}{(1.0-0.42)}$$

where M_L is total liver free methionine and M_E is plasma free methionine concentration, all values expressed in μ moles/100 ml tissue water or plasma.

$$\text{Specific activity of intracellular methionine} = \frac{M_L \rho M_L - 0.42\ M_E \rho M_E}{\rho M_L - 0.42 \rho M_E}$$

where M_{LI} and ρM_{LI} are the values for the specific activity of methionine in the liver free and plasma free pools respectively.

Membrane free pool: The fourth pool was an intermediate or "membrane" pool and is similar to that postulated by Airhart *et al.* (1974). This pool is considered to draw amino acids from both the extracellular and intracellular pools in proportion to the concentration of the

amino acid in each. Thus the specific activity has been calculated as follows (e.g. for liver methionine).

$$\text{Specific activity of membrane pool methionine} = \frac{M_E \rho M_E + M_{LI} \rho M_{LI}}{M_E + M_{LI}}$$

where M_{LI} and ρM_{LI} are the concentration and specific activity values for intracellular free methionine.

Calculations

With the use of certain assumptions, the fractional rate of protein synthesis in a tissue may be derived from the specific activity of the protein amino acid and the specific activity of the precursor amino acid determined on samples taken at the end of the isotope infusion. Thus an estimate of the daily fractional rate of protein synthesis of the tissue proteins is given by the following expression

$$\frac{\text{SA protein-AA}}{\text{SA precursor-AA}} \times \frac{24}{t}$$

where "SA protein-AA" is the specific activity of the amino acid in the protein at the end of the isotope infusion (i.e. time, t in hours), and "SA precursor-AA" is the mean specific activity of the amino acid at the site of protein synthesis over the period of the infusion.

The application of this method involves a number of assumptions:

(i) That the precursor pool of amino acids for protein synthesis can be correctly identified and characterised in terms of the specific activity of the amino acid. This assumption has been discussed in the introduction but is further considered in the discussion of the present work.

(ii) That the kinetics of the increase in the specific activity of the amino acid in the precursor pool can be defined in order than an estimate of the mean specific activity of the precursor amino acid over the period of the isotope infusion may be calculated.

(iii) That there is no loss of radioactive label from the protein over the period of the infusion. Therefore the specific activity of the protein amino acid at the time of sampling represents the total incorporation of the amino acid into the protein over the period of the infusion. This assumption is probably incorrect since the kinetics of protein degradation are apparently first order (i.e. random) (Schimke 1970 and Review Section 4.2). However, the possibility that this may not be the case for muscle proteins has again been raised by Millward *et al.* 1975a). Despite the question regarding this assumption any error is likely to be very small, although the greatest error would occur if protein degradation were non-random and the most newly synthesised proteins were degraded preferentially.

(iv) That the muscle protein as sampled be free of any contamination by the more highly radioactively labelled plasma proteins. Since muscle samples were not washed free of blood in the present experiment, such contamination of the muscle protein would have resulted in an overestimate of the specific activity of the protein amino acids and hence to an overestimate of the rate of protein synthesis. The likely extent of this overestimate is considered in the discussion. Plasma protein contamination of the liver proteins is not considered as constituting a significant problem since the specific activity of the protein amino acids in the plasma proteins and liver proteins were similar.

With slight modifications the assumptions involved in the measurement of protein synthesis in the present work are analogous to those used by Garlick and Millward and coworkers (Garlick & Millward 1972; Garlick *et al.* 1973). These workers have produced mathematical expressions to define the rate of change of the specific activity of the precursor amino acid. For the calculation of the synthesis rate, measured values for the specific activity of free and protein-bound amino acids (samples obtained at slaughter) are inserted in the appropriate equations and the fractional synthesis rate derived (Garlick *et al.* 1973).

The method of calculation of the mean precursor pool specific activity will now be considered. For four of the sheep (sheep 445, 582, 580 and 389), it was assumed that the specific activities of the amino acids in all pools reached a plateau specific activity instantaneously (i.e. at zero time). This assumption would have resulted in an overestimate of the precursor amino acid and hence an underestimate of the rate of protein synthesis. For sheep 462 and 567 (which received the highest rate of methionine infusion), the estimate of the precursor specific activities of the ^{35}S labelled amino acids has been adjusted in an attempt to allow for a delay in the attainment of a ^{35}S-methionine specific activity plateau which was apparent for sheep 462 (see Chapter 6 - Table 6.4; 6h compared with 15h samples). Although the only measurement of methionine specific activity in the plasma of sheep 567 was obtained from the samples taken at 15h, it is also very probable that there was a delay in reaching a ^{35}S-methionine plateau specific activity in this animal. This suggestion receives support from a comparison of the rates of increase of total plasma ^{35}S

activity in sheep 567 and 462 as compared with those for sheep 445, 582, 580 and 389 (see Chapter 6 - Table 6.3). The correction factor for the precursor specific activity in sheep 462 and 567 was calculated (using the data for sheep 462) as follows: the mean specific activity was calculated from the area under the line drawn through the 6h and 15h value for the methionine specific activity in plasma over the period 0 to 16.5h and the area (dpm - h/μmole) divided by the length of period of the infusion (16.5h) and the mean specific activity calculated. The value so calculated was 70% of the plasma methionine specific activity at 15h. For this reason a correction factor of 0.7 was used to adjust the specific activities of the precursor ^{35}S-methionine and ^{35}S-cystine. The use of this estimate will also result in an overestimate of the precursor amino acid specific activity since it is assumed that the specific activity of the precursor amino acid lies on the extrapolated 6 to 15h specific activity line from time zero.

The extent of the bias in the calculation of the fractional synthetic rate of protein, having assumed that the plateau specific activity was attained instantaneously, may be calculated for sheep 389, using the data presented in Chapter 6 (Table 6.5). In this case the area under the methionine specific activity curve was also overestimated by about 13% and hence the rate of protein synthesis would have been underestimated by about 11%.

RESULTS AND DISCUSSION

Specific activities

The values for the specific activities of methionine and cystine (expressed as half-cystine) in the four alternative precursor pools are given in Table 7.1. The specific activities of methionine and cystine

TABLE 7.1: Values for the specific activity of methionine and cystine in the various precursor pools as used in the calculations of the rate of protein synthesis.

	[1]Specific activity (dpm x 10^3/μ mole)			
	LIVER			MUSCLE
	Methionine ^{35}S	Cystine (as ½ cys) ^{35}S	^{3}H	Methionine (^{35}S)[1]
OMET[2]: Sheep 445				
Plasma[3]	52.5	10.1	293	52.5
Whole tissue	6.06	5.75	12.5	17.6
Intracellular	4.34	5.72	10.7	14.9
"Membrane"	6.69	5.76	13.2	22.1
Sheep 582				
Plasma	52.0	7.43	202	52.0
Whole tissue	5.36	5.76	12.0	11.6
Intracellular	3.27	5.74	9.20	14.6
"Membrane"	6.11	5.77	13.0	20.0
LMET: Sheep 580				
Plasma	27.2	9.97	276	27.2
Whole tissue	5.92	11.0	10.4	18.6
Intracellular	3.95	11.0	7.37	17.0
"Membrane"	6.59	11.0	11.6	20.9
Sheep 389				
Plasma	23.3	5.23	133	23.3
Whole tissue	6.18	5.74	6.88	13.0
Intracellular	5.11	5.75	4.73	11.7
"Membrane"	6.55	5.74	7.68	15.1
HMET: Sheep 462				
Plasma	11.0	3.42	124	11.0
Whole tissue	7.48	2.44	4.73	11.5
Intracellular	7.25	2.42	3.11	11.6
"Membrane"	7.57	2.47	5.34	11.3
Sheep 567				
Plasma	5.92	2.11	128	5.92
Whole tissue	4.75	1.17	2.29	6.07
Intracellular	4.68	1.08	0.051	6.11
"Membrane"	4.76	1.10	3.15	6.02

See next page for footnotes 1,2 and 3.

TABLE 7.1: Footnotes continued

1) All free amino acid ^{35}S specific activity values for sheep 462 and 567 have been adjusted for the slow isotope equilibration (of ^{35}S) using a factor of 0.70 (i.e. actual value x 0.70; see "Calculations" section for details).

2) Infusion during the treatment period; OMET - control, water infusion; LMET - low methionine, 0.12g L-met/$kg^{0.75}$/day; HMET - high methionine, 0.36g L-met/$kg^{0.75}$/day.

3) Precursor amino acid pools; see "Methodology" section for details. Plasma - plasma amino acid pool. Whole tissue - whole tissue free amino acid pool. Intracellular - tissue free amino acid pool corrected for the presence of amino acids in the extracellular pool. "Membrane" - the pool assumed to be intermediate between the plasma and intracellular free amino acid pools.

in the proteins of liver and muscle are given in Table 7.2. The specific activities of both methionine and cystine in the liver proteins exhibited a marked decline with the increasing level of methionine while there was no apparent trend in the specific activity values in muscle proteins.

Specific activity ratios in the free and protein pools

The specific activity ratios for cystine ^{35}S: cystine ^{3}H, methionine ^{35}S: cystine ^{35}S and methionine ^{35}S: cystine ^{3}H in the various precursor pools and in the mixed liver and muscle proteins are given in Table 7.3. Since there was no detectable free cystine present in the muscle tissue extracts, the appropriate ratios for the muscle free pool could not be derived.

The ratios of the specific activities of the three labelled amino acids to one another did not give any clear indication as to which, if any, of the proposed precursor pools best represented the true pool from which methionine and cystine were drawn for incorporation into the liver proteins. However, some general observations are relevant. In general, the ratios in the liver protein were more closely related to ratios for the liver pools than to the ratios for the plasma pool. This was particularly evident for the cystine ^{35}S: cystine ^{3}H ratios. In this case, it may be that the whole liver or liver membrane free pools represented the true precursor pool reasonably well, since any differences between the ratios in either of these free pools and the liver protein were apparently within the order of the accuracy of the estimates and do not indicate a consistent bias towards either higher or lower ratios.

TABLE 7.2: Values for the specific activity of methionine (^{35}S dpm x 10^3/μ mole) and cystine (^{35}S or ^{3}H dpm x 10^3/μ mole of half-cystine) in the proteins of liver and muscle.

	Specific activity (dpm x 10^3/μ mole)					
	LIVER			MUSCLE		
Sheep[1]	Methionine ^{35}S	Cystine (as ½ cys) ^{35}S	Cystine (as ½ cys) ^{3}H	Methionine ^{35}S	Cystine (as ½ cys) ^{35}S	Cystine (as ½ cys) ^{3}H
445	3.53	2.24	2.85	0.237	0.148	0.609
582	3.53	1.05	1.67	0.141	0.049	0.405
580	2.84	0.964	1.58	0.359	0.371	0.549
389	1.09	0.649	1.06	0.233	0.173	0.417
462	0.844	0.679	1.18	0.228	0.092	0.525
567	0.560	0.402	0.984	0.112	0.055	0.606

1) Sheep 445, 582 - control (water) infusion; 580, 389 methionine at rate of 0.12g/kg$^{0.75}$/day; 462, 567 - L-methionine at rate of 0.36g/kg$^{0.75}$/day.

TABLE 7.3: Values for the ratios of the specific activity of cystine ^{35}S to cystine ^{3}H, methionine ^{35}S to cystine ^{35}S and methionine ^{35}S to cystine ^{3}H in the alternative precursor pools and in the liver and muscle proteins.

	Precursor pools				Proteins	
Sheep[1]	Plasma	Liver whole	Liver intracellular	Liver "membrane"	Liver	Muscle
	Ratio of Cystine ^{35}S to Cystine ^{3}H					
445	0.034	0.459	0.535	0.435	0.786	0.243
582	0.037	0.480	0.624	0.443	0.629	0.121
580	0.036	1.05	1.50	0.948	0.610	0.676
389[2]	0.039	0.834	1.21	0.747	0.612	0.415
462[2]	0.028	0.515	0.778	0.462	0.575	0.175
567[2]	0.016	0.510	21.1	0.349	0.409	0.091
	Ratio of methionine ^{35}S to cystine ^{35}S					
445	5.20	1.06	0.758	1.16	1.58	1.60
582	7.00	0.930	0.570	1.06	3.36	2.88
580	2.73	0.538	0.358	0.599	2.95	0.968
389[2]	4.49	1.08	0.888	1.14	1.68	1.35
462[2]	3.21	3.07	2.99	3.07	1.24	2.48
567[2]	2.81	4.06	4.36	4.33	1.39	2.04
	Ratio of methionine ^{35}S to cystine ^{3}H					
445	0.179	0.484	0.405	0.506	1.24	0.389
582	0.257	0.447	0.356	0.468	2.11	0.348
580	0.099	0.567	0.536	0.568	1.80	0.654
389[2]	0.175	0.897	1.08	0.853	1.03	0.559
462[2]	0.088	1.58	2.33	1.42	0.715	0.434
567[2]	0.046	2.07	91.8	1.51	0.569	0.185

1) Sheep 445, 582 - control (water) infusion; 580, 389 - L-methionine infusion at rate of $0.12g/kg^{0.75}$/day; 462, 567 - L-methionine infusion at rate of $0.36g/kg^{0.75}$/day.

2) All free amino acid ^{35}S specific activity values for sheep 462 and 567 have been adjusted for the slow isotope equilibration (of ^{35}S) using a factor of 0.70 (i.e. actual value x 0.70; see "calculations" section for details).

The specific activity ratios of methionine ^{35}S to cystine ^{35}S in the liver free pools were less than those in the liver proteins in four of the six animals, while in the other two (sheep 462 and 567 which received the highest rate of methionine supplementation), the ratios in the free pools were considerably higher than those in the protein. Thus in four of the animals, it seems likely that the methionine utilised in protein synthesis was derived from a pool having a higher specific activity than that of the whole liver methionine, or that the cystine was derived from a pool of lower specific activity than the whole liver cystine. Presumably as a result of the greatly increased liver free methionine concentrations and methionine specific activities (compared with plasma), the situation was reversed for sheep 462 and 567. This is further supportive evidence for the thesis that there is a considerable degree of metabolic compartmentation of methionine and cystine in the normal liver, and that the greatly increased methionine concentrations resulted in a breakdown of some of these barriers (whether chemical or physical). This change in the ratio relationship in the two sheep receiving the highest level of methionine compared with the other four sheep would also suggest that none of the four proposed precursor pools adequately represents the true precursor pool for methionine and cystine entering protein synthesis.

Although the methionine ^{35}S to cystine ^{3}H ratios in the liver proteins were more closely related to the liver free pools than to the plasma pool, no clear relationship to any of the three alternative pools is obvious.

The absence of a detectable free cystine pool in muscle made any comparisons involving a cellular precursor pool impossible. Thus the only possible comparison was that of the muscle protein with the plasma free pool. No obvious relationships for any of the ratios were apparent. A comparison of the ratio of the specific activity of muscle free methionine to plasma cystine with this ratio in muscle protein also failed to reveal any relationship. The origin of the cystine which was utilised in muscle protein synthesis is obscure. It is possible that there did exist a very small free cystine pool possibly localised at the site of transfer RNA charging which was simply too small to be detected with the techniques used in the present experiment. Although no cystathionine-γ-lyase was detected in sheep muscle in the studies of Radcliffe & Egan (1974), it is also possible that the enzyme is present at a very low activity and that an active transulphuration pathway is present in the skeletal muscle. However, there are other possibilities to be considered. Glutathionine (γ- glutamylcysteinylglycine) could serve as a ready source of cystine, particularly if the γ-glutamyl cycle enzymes were localised in the muscle membrane as in many other mammalian tissues (Meister 1974). Cysteine may also be transported bound to the plasma proteins. The plasma proteins themselves may also function as a source of amino acids for the muscle in that it is possible that some of the plasma proteins could be degraded within the skeletal muscle yielding amino acids for metabolism by the muscle cell (see Review Section 3.4). However the cystine ^{35}S: cystine ^{3}H ratios in the plasma proteins of the three sheep for which data are available (total plasma protein cystine including disulphide-bound cysteine) bore no resemblance to the ratios in muscle (0.34, 0.38, 0.34 in the plasma proteins compared with 0.24, 0.68 and 0.09 in the muscle proteins).

Estimated rates of tissue protein synthesis

Table 7.4 gives the calculated daily fractional synthetic rates of the liver and muscle proteins for the six sheep. The apparent rates of liver protein synthesis calculated from the specific activities of ^{35}S-methionine and ^{35}S-cystine and ^{3}H-cystine in the four alternative precursor pools are given. For muscle proteins, only the rates calculated from the methionine specific activity values are given.

Liver proteins: There was no apparent relationship between the estimates of liver protein fractional synthetic rates using the different radioactively labelled amino acids. Considering the methionine precursor alone, there was a decline in the apparent rate of protein synthesis (calculated on the basis of the liver free pools) with the increase in the level of methionine supplementation. This is reflected in the highly significant negative correlation ($r = -0.97$) between the logarithm of the liver methionine concentration and the logarithm of the fractional synthetic rate of liver protein.

The apparent rates of protein synthesis calculated from the specific activity of the liver cystine showed a significant positive correlation with the tissue cystine concentration, the correlation coefficients being 0.84 for the ^{35}S-cystine and 0.36 for the ^{3}H-cystine. The latter correlation improved considerably ($r = 0.94$) if the data for sheep 567 were omitted. It is important to note that there was no correlation between the liver cystine concentration and the rate of methionine supplementation or tissue methionine concentration (see Chapter 6).

TABLE 7.4: Estimated values for the fractional synthesis rate (FSR, $days^{-1}$) of mixed liver "domestic" proteins and mixed muscle protein. (The rates have been calculated from the ratio of the specific activity of the protein amino acid to the specific activity of the precursor pool amino acid[3]).

	Fractional synthesis rate ($days^{-1}$)[3]			
	Liver "domestic" protein Precursor amino acid			Muscle protein Precursor amino acid
Precursor pool	^{35}S-Met	^{35}S-Cys	^{3}H-Cys	^{35}S-Met
OMET[1]: Sheep 445				
Plasma[2]	0.097	0.322	0.014	0.0065
Whole tissue	0.843	0.565	0.330	0.0196
Intracellular	1.18	0.568	0.386	0.0231
"Membrane"	0.764	0.564	0.312	0.0155
Sheep 582				
Plasma	0.098	0.205	0.023	0.0039
Whole tissue	0.954	0.264	0.202	0.0141
Intracellular	1.56	0.265	0.263	0.0176
"Membrane"	0.837	0.263	0.186	0.0102
LMET: Sheep 580				
Plasma	0.151	0.140	0.008	0.0191
Whole tissue	0.695	0.127	0.219	0.0280
Intracellular	1.04	0.127	0.311	0.0306
"Membrane"	0.603	0.127	0.198	0.0249
Sheep 389				
Plasma	0.068	0.180	0.012	0.0145
Whole tissue	0.310	0.164	0.222	0.0260
Intracellular	0.256	0.164	0.323	0.0289
"Membrane"	0.242	0.164	0.199	0.0223
HMET: Sheep 462				
Plasma	0.111	0.288	0.014	0.0301
Whole tissue	0.164	0.404	0.361	0.0288
Intracellular	0.169	0.407	0.549	0.0285
"Membrane"	0.162	0.399	0.320	0.0292
Sheep 567				
Plasma	0.137	0.277	0.008	0.0274
Whole tissue	0.171	0.499	0.623	0.0268
Intracellular	0.173	0.542	28.0	0.0266
"Membrane"	0.170	0.530	0.453	0.0270

1) Infusion during the treatment period; OMET - control, water infusion; LMET - low methionine, 0.12g L-met/$kg^{0.75}$/day; HMET - high methionine, 0.36g L-met/$kg^{0.75}$/day.

2) Precursor amino acid pools; see "Methodology" section for details. Plasma - plasma amino acid pool. Whole tissue - whole tissue free amino acid pool. Intracellular - tissue free amino acid pool corrected for the presence of amino acids inthe extracellular pool. "Membrane" - the pool assumed to be intermediate between the plasma and intracellular free amino acid pools.

See next page for footnote 3.

TABLE 7.4: Footnote 3 continued

3)

$$FSR = \frac{\text{SA protein - AA}}{\text{SA precursor - AA}} \times {}^{24}/_{t} \quad (\text{days}^{-1})$$

where "SA protein - AA" is the specific activity of the amino acid in the protein at the end of the isotope infusion (i.e. time, t in hours), and "SA precursor - AA" is the mean specific activity of the amino acid at the site of protein synthesis over the period of the infusion.

These relationships between the apparent fractional synthetic rates of the liver proteins and the concentrations of the precursor amino acids raise some questions as to the interpretation of the fractional synthetic rate data. It may be postulated that the calculated fractional synthetic rates were either the true rates of liver protein synthesis or were artifacts related in some way to the concentration of the precursor amino acid. These possibilities will be considered, firstly with respect to methionine. The calculated fractional synthetic rates may have been accurate estimates in which case the increased tissue methionine concentration depressed the rate of protein synthesis (and presumably degradation). The estimated fractional synthetic rates of protein synthesis in sheep 445, 582 and 580 were very high, ranging from 70-100% of liver protein being synthesised daily. These rates were similar to the actual fractional synthetic rates for liver proteins measured in malnourished rats (Millward *et al.* 1975b), which were twice as high as those measured in well-fed rats. If it is accepted that the true fractional synthetic rates of the mixed liver proteins in the sheep were close to the 16% calculated from sheep 462 and 567, then by comparison with the rat data, the very high estimates of fractional synthetic rate for sheep 445, 582 and 580 were gross overestimates. For this reason, it is likely that the apparent fractional synthetic rates for liver proteins were not the true rates, particularly for sheep 445, 582 and 580 and possibly for sheep 389, but were rather artifacts related to the tissue concentration of the precursor amino acids. The situation is similar for the estimates of fractional synthetic rates derived from the cystine precursors, although the range in the estimated rates was smaller, and the relationship was in the

opposite direction to that of methionine (i.e. the fractional synthetic rate was positively correlated with the cystine concentration).

The greatly expanded liver free methionine pools in sheep 462 and 567, and the associated increase in the specific activity of the liver free methionine in relation to the plasma methionine reflected the improved mixing of the extracellular and intracellular methionine pools in those animals receiving the highest rate of methionine supplementation. For this reason it is likely that the fractional synthetic rates of liver proteins in these two sheep calculated on the basis of the liver methionine free pools were relatively accurate. Assuming steady state conditions, these rates indicate that 16-17% of the liver protein was turning over each day. The small increase in liver size (see Chapter 6) would require that the rate of synthesis was about one percentage unit higher than the rate of degradation. These values are similar to those reported by Buttery *et al.* (1975) for liver protein synthesis (10%/day) in mature wethers, measured using a continuous infusion of lysine.

In the absence of any compelling evidence that the plasma pool did best represent the true precursor pool supplying amino acids for protein synthesis in the liver, and on the basis of the work reported by Airhart *et al.* (1974) and van Venrooij *et al.* (1974), it appears that an intermediate pool (i.e. drawing amino acids from both the extracellular and intracellular free pools) would be likely to best represent the true precursor pool for protein synthesis in the present work. However, the evidence for compartmentation of methionine and cystine within the liver free pool, and the strong correlations between liver amino acid concentrations and the apparent rates of protein synthesis suggest that even an intermediate

precursor pool (i.e. the whole liver free pool or the "membrane" pool) did not adequately represent the true precursor pool.

Thus it must be concluded that there is a functional heterogeneity of the liver methionine and cystine pools, such that amino acids which are utilised for protein synthesis, are drawn from only a small part of the tissue pool (perhaps similar to that originally suggested by Kipnis *et al.* (1961)). However, it does seem likely that amino acids are drawn from both the extracellular and intracellular free pools, and that neither pool represents the sole source of amino acids for protein synthesis.

Differences in the types of experiment which have been conducted in an attempt to define the precursor pool for protein synthesis in a tissue have probably contributed to much of the confusion which surrounds the topic. Some of the problems in interpretation as they relate to differences in the amino acids used in different studies, and in particular to *in vitro* versus *in vivo* situations, have been discussed by van Venrooij *et al.* (1974).

The technique involving the continuous infusion of a radioactively labelled amino acid has been used by many workers for the measurement of tissue protein synthesis *in vivo*. Lysine (Waterlow & Stephen 1968; Gan & Jeffay 1971), tyrosine (Garlick *et al.* 1973; Millward *et al.* 1975a, b) glycine and serine (Fern & Garlick 1974) and leucine (Fern *et al.* 1971) have been used as the labelled precursor amino acid. In all of these reports, the largest contribution of an amino acid from intracellular protein catabolism was the 60% value reported by Gan & Jeffay (1971) for lysine in the liver of rat s (i.e. the specific activity of the liver free lysine

pool was 40% of that of the plasma free lysine pool, the difference reflecting that proportion derived from intracellular sources). In contrast the specific activity ratios of liver intracellular ^{35}S-methionine and ^{3}H-cystine to their respective plasma specific activities were 0.08 and 0.06 for methionine and 0.04 and 0.05 for cystine in the two sheep not receiving the methionine supplement in the present experiment (sheep 445 and 582). This is supportive evidence for the suggestion that there are likely to be major differences in the metabolism of different amino acids and perhaps for any particular amino acid in different species.

Plasma protein synthesis: The liver export proteins (i.e. plasma proteins) are synthesised on the membrane-bound ribosomes of the liver (Redman 1968; Williams & Ganoza 1970) and are secreted into the plasma.

The fractional rate of liver protein synthesis estimated from the specific activity of the liver proteins represented the synthesis of the liver "domestic" proteins. Samples of plasma proteins were obtained from three of the six sheep in the present experiment. The estimated fractional synthetic rates for the plasma proteins are summarised in Table 7.5.

Except for sheep 567, (which received the highest level of methionine infusion) the rates of protein synthesis estimated from the three labelled amino acids bore little resemblance to each other. However in the animal with the greatly expanded liver methionine pool (sheep 567), the rates of protein synthesis estimated from all three amino acids were very similar. The similarity between the fractional rates of synthesis calculated from the methionine "membrane" pool in the three animals (0.25 to 0.30) may lend some

TABLE 7.5: Estimated values for the fractional synthesis rate (FSR, $days^{-1}$) of plasma proteins (i.e. liver "export" proteins). (The rates have been calculated from the ratio of the specific activity of the protein amino acid to the specific activity of the precursor pool amino acid[2,3,4]).

Precursor Pool	Fractional synthesis rate ($days^{-1}$) Precursor amino acid		
	^{35}S-Met	^{35}S-Cys	^{3}H-Cys
Sheep 445			
Plasma[1]	0.038	0.047	0.005
Whole tissue	0.329	0.082	0.112
Intracellular	0.459	0.082	0.131
"Membrane"	0.298	0.082	0.106
Sheep 580			
Plasma	0.061	0.045	0.004
Whole tissue	0.279	0.041	0.112
Intracellular	0.419	0.041	0.158
"Membrane"	0.251	0.041	0.100
Sheep 567			
Plasma	0.221	0.140	0.007
Whole tissue	0.276	0.252	0.365
Intracellular	0.280	0.273	16.4
"Membrane"	0.275	0.268	0.266

1) Precursor amino acid pools; see "Methodology" section for details. Plasma - plasma amino acid pool. Whole tissue - whole tissue free amino acid pool. Intracellular - tissue free amino acid pool corrected for the presence of amino acids in the extracellular pool. "Membrane" - the pool assumed to be intermediate between the plasma and intracellular free amino acid pools.

2) $$FSR = \frac{\text{SA protein - AA}}{\text{SA precursor - AA}} \times {}^{24}/_{t} \quad (days^{-1})$$
where "SA protein - AA" is the specific activity of the amino acid in the protein at the end of the isotope infusion (i.e. time, t in hours), and "SA precursor - AA" is the mean specific activity of the amino acid at the site of protein synthesis over the period of the infusion.

3) The spe cific activity of plasma protein cystine was corrected for the presence of disulphide-bound cystine; see "Methodology" section for details.

4) Specific activity (dpm x $10^3/\mu$ mole) of the plasma protein amino acids for sheep 445, 580 and 567 respectively were:

^{35}S-Met	1.37, 1.14, 0.903;
^{35}S-Cys (as ½ cys)	0.325, 0.310, 0.203;
^{3}H-Cys (as ½ cys)	0.967, 0.801, 0.576.

support to the suggestion that, at least for methionine incorporated into plasma proteins, the "membrane" pool may best represent the true precursor pool. Also in terms of the functional arrangement of the liver cell, with the plasma proteins being synthesised on the membrane-bound ribosomes, this would seem a reasonable suggestion.

Muscle proteins: The rates of muscle protein synthesis were calculated assuming that an intermediate pool of methionine (i.e. the whole tissue free pool or the "membrane" pool) best represented the true precursor pool. The fractional synthetic rates ranged from about 1.4 to 2.9% per day. These values, for the *M.semitendinosus* muscle, were similar to those reported by Buttery *et al.* (1975) for the *Longissimus dorsi* and *gastrocnemius* muscles in mature sheep. The higher rates of synthesis measured in the present work occurred in animals receiving the abomasal supplement of methionine. However, there was no overall correlation between the rate of muscle protein synthesis and the nitrogen balance. Millward *et al.* (1975a) have shown that higher rates of both muscle protein synthesis and degradation are associated with higher rates of net muscle growth, and it may have been that in methionine supplemented animals there was a higher rate of muscle growth, despite the fact that this was not reflected in the nitrogen balance data.

The error in the estimate of the rate of muscle protein synthesis due to plasma protein contamination may be estimated. If it is assumed that 5% of the wet weight of muscle is blood, plasma proteins then represent about 0.35% of the wet weight or about 1.5% of the muscle dry weight or about 2% of the muscle protein*. If the

*5% of wet weight is blood = 3.5% of wet weight is plasma (30% blood cells) = 0.35% of wet weight as plasma proteins (i.e. 10% of plasma) = 1.5% of muscle dry weight (23% dry matter in muscle) = 2.0% of muscle protein (75% muscle dry weight is protein).

specific activity of plasma protein methionine is six times that of the total muscle proteins (mean value for sheep 445, 580 and 567), then the true specific activity of the muscle proteins would be about 90% of the measured value ((0.02 x 6) + 0.98y = 1, where y is the true specific activity of muscle proteins).

The specific activity ratio of muscle intracellular methionine to plasma methionine was higher than the ratio of specific activity of liver intracellular methionine to plasma methionine in each of the six sheep. This fact reflects either a lower proportional contribution of unlabelled amino acids derived from protein catabolism to the muscle free pool as compared with liver, or simply a better mixing between the circulating pool and muscle free pool than between the circulating pool and the liver free pool (assuming that plasma is the circulating pool). Both of these factors were probably involved.

Whole body protein degradation

The entry rate or flux of an amino acid through the plasma pool represents the turnover of that portion of the amino acid which is in equilibrium with the plasma pool. It thus represents a fraction of the total turnover of that amino acid in the whole body.

An estimate of the proportion of the total amino acid turnover represented by the plasma entry rate for any one tissue is given by the ratio of the specific activity of the amino acid in the intracellular pool to that in the plasma pool. Thus a ratio of 0.20 suggests that 20% of the intracellular pool is in equilibrium with the plasma pool or that the entry rate of the amino acid in plasma represents 20% of the total flux of the amino acid through the tissue free pool.

An estimate of the equilibrium of an amino acid in the plasma with the intracellular (IC) free pool of the amino acid is given by the expression (IC-AA/Plasma AA).

$$\frac{\sum\left(\frac{\text{IC-AA sp.act.}}{\text{Plasma-AA sp.act.}} \times \text{IC-AA pool size}\right) \text{ of all tissues}}{\sum \text{(IC-AA pool size) of all tissues}}$$

The value so derived gives an estimate of the degree of communication of the plasma pool with the whole body intracellular tissue free pool of the particular amino acid. A small error in the estimate could arise from the omission of the relative rates of turnover of the amino acid pools in the different tissues. However, the allowance for the turnover rate would have to be included in both the numerator and denominator, and hence any error would be small.

The derivation of the above value permits the calculation of the whole body turnover of a free amino acid, since the value obtained represents that part of the whole body free pool which is in equilibrium with the plasma, and hence is represented in the entry rate of the amino acid in plasma. Therefore the whole body turnover of an amino acid is given by the expression:

$$\frac{\text{Entry rate of the amino acid in plasma}}{\text{IC-AA/Plasma AA}}$$

However, in the present work, data was obtained for only two tissues, the liver and the skeletal muscle, and the full calculations could not be carried out. For this reason it has been assumed, for the purposes of calculation of the whole body turnover of methionine, that one-half of the body tissue free methionine pool behaved in a manner similar to the muscle free methionine pool and that one-half behaved in a manner similar to the liver free methionine pool.

The whole body turnover of an amino acid which is not synthesised in the animals own tissues represents amino acid absorption from the digestive tract and amino acid release in protein degradation. Thus the amino acid released in protein degradation is equal to the whole body turnover of the amino acid less the net absorption of the amino acid from the digestive tract. If the size of the whole body protein amino acid pool is also known then the proportion of the whole body protein which is degraded each day is given by the expression (days^{-1}, fractional degradation rate):

$$\frac{\text{AA released in protein degradation (in moles/day)}}{\text{Whole body protein AA pool (in moles)}}$$

These calculations have been carried out in order to estimate the fractional degradation rate (FDR) of the whole body protein pool using the data obtained for methionine. The details of the calculations for the six sheep, and the estimated values for the whole body FDR are given in Table 7.6. The mean FDR was 0.098 ± 0.015 days^{-1}, which suggests that approximately 10% of the whole body protein was degraded each day. However, the estimate was very crude having been based on values for only two tissues, although theoretically the basic method is sound.

CONCLUSIONS

The identification of the precursor pool of amino acids for protein synthesis is the major problem in deriving estimates of the rate of protein synthesis in a tissue from the ratio of the specific activities of the precursor and protein amino acid. Theoretically the use of three isotopically labelled amino acids

TABLE 7.6: Data for the calculation of the whole body protein fractional degradation rate (FDR, days^{-1}) and the calculated FDR for the six sheep.

Sheep	Live-weight (kg)	[1]Total body protein (kg)	[2]Total body protein MET (m moles)	[3]Entry rate of MET (m moles/day)	[4]Proportional MET re-entry into plasma pool	[5]Whole body MET turnover (m moles/day)	[6]Net intestinal MET absorption (m moles/day)	[7]MET released in protein degradation (m moles/day)	[8]Whole body FDR (days^{-1})
445	21.5	3.31	510	13.5	0.183	73.5	3.6	69.9	0.137
582	29.0	4.41	679	13.7	0.143	96.0	3.4	92.6	0.136
580	24.3	3.74	576	26.7	0.384	69.5	12.3	57.2	0.099
389	32.5	4.95	762	30.4	0.362	83.9	16.1	67.8	0.089
462	24.6	3.77	581	46.1	0.830	55.6	30.2	25.4	0.044
567	29.9	4.56	702	85.4	0.895	95.4	39.1	56.3	0.080

1) Total body protein calculated from the regression relationship of protein on empty bodyweight from the data of Fennessy (1971)
2) Assuming 2.3g methionine (MET) 100g protein (Fennessy unpublished).
3) Entry rate of methionine in plasma/(m moles/day) $= \frac{\text{rate of } ^{35}S \text{ isotope infusion (dpm/h) x 24}}{\text{plasma MET specific activity at plateau (dmp/m mole)}}$
(See Chapter 6, Table 6.6).
4) Proportional re-entry of methionine into the plasma pool is that part of the whole body tissue free methionine pool which is in communication with the plasma pool and is thus represented in the calculated entry rate of methionine in plasma. It has been assumed that in half of the body tissues, methionine re-entry into the plasma pool was in the same proportion as in liver, and in the open half of the body tissues, re-entry was in the same proportion as in muscle; Thus proportional re-entry $= 0.5 \left(\frac{\text{liver intracellular MET sp.act.}}{\text{plasma MET sp.act.}} + \frac{\text{muscle intracellular MET sp.act.}}{\text{plasma MET sp.act.}} \right)$
5) Whole body methionine turnover $= \frac{\text{Entry rate of methionine (m moles/day)}}{\text{Proportional MET re-entry into plasma}}$
6) Net intestinal methionine absorption = digesta methionine + infused methionine; digesta methionine calculated from the data of Egan *et al.* (1975) for sheep fed similar diets (i.e. assuming 0.19g methionine/100g digestible organic matter intake and 85% of this was absorbed; assuming that the abomasally infused methionine was 100% absorbed).

(see next page for footnotes 7 and 8).

TABLE 7.6: Footnotes continued

7) Methionine released in protein degradation (m moles/day) =

(Whole body MET turnover - Net intestinal MET absorption).

8) Fractional degradation rate ($days^{-1}$) of whole body protein

$$= \frac{\text{Methionine released in protein degradation (m moles/day)}}{\text{Total body protein methionine (m moles)}}$$

should have been an excellent method for the identification of the precursor pool. Consequently an accurate estimate of the fractional rate of protein synthesis in a tissue should have been obtainable. However this was not the case and the accuracy of the estimates of the fractional synthetic rates is open to question. It is likely that metabolic compartmentation of methionine and cystine is of considerable importance in sulphur amino acid metabolism in the liver of the sheep (and probably also in other tissues including the skin) and that this compartmentation was the reason for the problems in the estimation of the fractional rates of protein synthesis.

CHAPTER 8

A compartmental model for methionine and cystine metabolism in the sheep

INTRODUCTION

The estimates of methionine and cystine entry rate, discussed in Chapter 6, provided limited information concerning the metabolism of these amino acids. In an attempt to obtain more detailed information about the rate of methionine and cystine turnover within a particular tissue and the rate of methionine catabolism via the transulphuration pathway, some simple compartmental models were devised.

METHODOLOGY

Experimental

The details of the experiment and the preparation of samples and calculation of the cellular concentration and specific activity values for methionine and cystine are given in Chapter 6.

Models

Three compartmental models of ascending order of complexity were set up. They are shown diagramatically in Figure 8.1. Each model contains three basic pools.

(i) Pool A is the circulating pool which is assumed to be the plasma free pool, which both supplies the amino acids to the cell and draws amino acids from the cell; for the purposes of the model the extracellular tissue space is assumed to be part of this pool. However, the validity of these assumptions is open to question since it is possible that the red blood cells may play a sginificant role in the

FIGURE 8.1: Diagrammatic representation of the compartmental Models I, II and III.

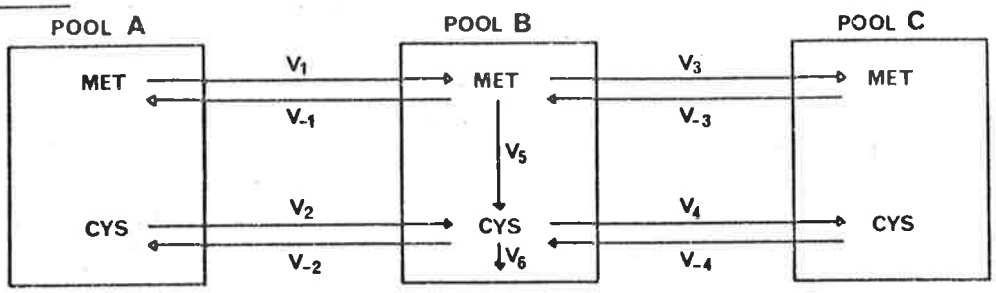
Pool A is the circulating pool, which is assumed to be the plasma free pool, which both supplies amino acids to the cell and draws amino acids from the cell.

Pool B is the intracellular free amino acid pool.

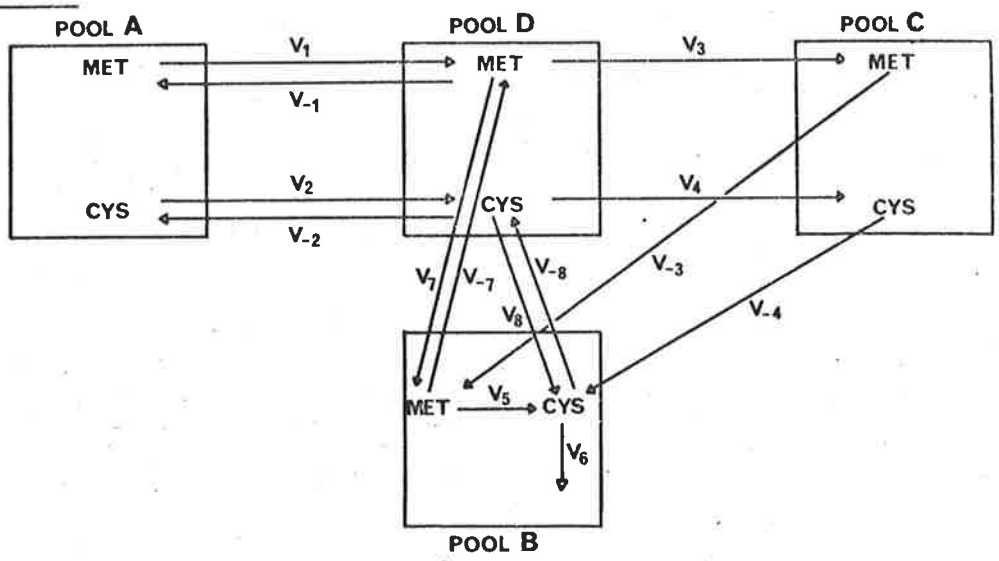
Pool C is the intracellular protein pool.

Pool D is the "membrane" pool through which all amino acids must pass on their passage into or out of the intracellular free pool and from which amino acids are taken for protein synthesis.

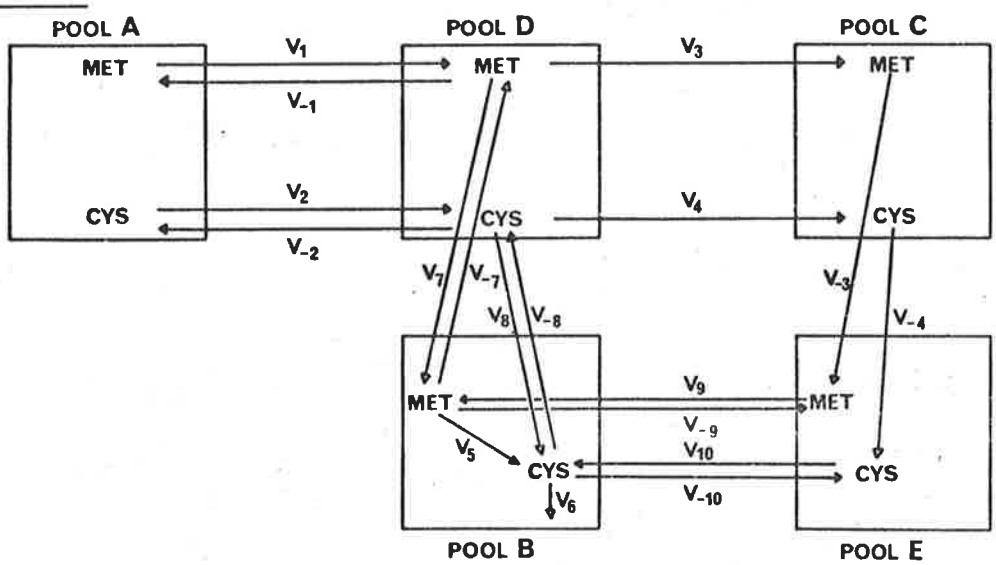
MODEL I



MODEL II



MODEL III



interorgan transport of amino acids (Elwyn 1970 and see Review, Section 3.4).

(ii) Pool B is the intracellular free amino acid pool.

(iii) Pool C is the intracellular protein pool.

In addition, Model II has a further pool, pool D, a "membrane" pool through which all amino acids must pass on their passage into or out of the intracellular free pool, and from which free amino acids are taken for protein synthesis. In Models I and II, the intracellular free pool is assumed to be a homogeneous pool to which amino acids released in the catabolism of the intracellular proteins are added. However, in Model III, a further pool, pool E, is considered as the pool to which amino acids derived from protein catabolism are added.

Several assumptions are involved in the application of these models in addition to those mentioned with regard to the particular models.

(i) That there is no compartmentation of the amino acids within the cell (except as specified for Models II and III above).

(ii) That the animal is in a steady state, and that all tissues are in a steady state, and that no pool (either protein-bound or free) is expanding or contracting in size, and that the specific activity of all components is at a plateau.

(iii) That no isotopically-labelled amino acid is released in protein catabolism. This is assumed in the calculation of the rate of protein synthesis (see Chapter 7), and in the mathematical development of the models.

(iv) That all of the proteins synthesised remain within the cell, and that the rate of amino acid relese from catabolised protein is equal to the rate of amino acid uptake in the

synthesis of protein.

The previous assumption (iv) is not required for the algebraic development of the models, but is only required for the mathematical solution of the models and the calculation of the flux rates. In fact assumption (iv) is not valid for the synthesis and catabolism of liver proteins. As discussed in Chapter 7, there are two categories of liver protein synthesis, the synthesis of the "domestic" or endogenous tissue proteins, and the synthesis of the "export" or plasma proteins. The rates of liver protein synthesis as calculated in the previous chapter represent the rate of synthesis of the "domestic" proteins only.

Calculations

The symbols used in the mathematical treatment of the models are: V = the flux of amino acid defined as the rate of entry into, or the rate of exit from, the tissue pools, expressed as μ moles of methionine or half-cystine per gram of tissue protein per hour;

ρ = the specific activity of the amino acid in the particular pool; the first subscript refers to the amino acid, i.e. M for methionine and C for half-cystine; the second subscript denotes the amino acid pool; the third subscript (for half-cystine only) refers to the isotopic label, i.e. H for tritium-labelled and S for the ^{35}S-labelled amino acid; e.g. ρ_{MA} = the specific activity of methionine in pool A; ρ_{CAH} = the specific activity of half-cystine in pool A in relation to the tritium label.

The rate of amino acid (AA) incorporation into protein (V_3,V_4) is calculated from the fractional synthetic rate (FSR hours^{-1}) of

tissue protein, and the concentration of the amino acid in the protein*

Rate of amino acid incorporation = FSR (h^{-1}) x AA (g/g protein).

It is assumed that $V_3 = V_{-3}$ and $V_4 = V_{-4}$ (i.e. assumption (iv)).

The mathematical solution of each model to enable the calculation of the rate of amino acid entry into the tissue and the rate of methionine catabolism via the transulphuration pathway may now be attempted. The development of the models involves the algebraic derivation of relationships between flux rates and specific activities.

Model I

A relationship which will permit the calculation of the methionine flux (V_1) from the plasma free pool, pool A, to the intracellular free pool, pool B, is first derived.

The first steps (equations (1) and (2)) in the development of this relationship require that the amount of methionine in pool B is constant, and the specific activity of methionine in pool B is constant, so that the amount of methionine activity in pool B is constant, such that the activity entering = the activity leaving the pool (i.e. the steady-state assumption, assumption (ii)).

The methionine flux about pool B may therefore be expressed as:

$$V_1 + V_{-3} = V_{-1} + V_3 + V_5 \qquad (1)$$

and $V_1\rho_{MA} + V_{-3}\rho_{MC} = V_{-1}\rho_{MB} + V_3\rho_{MB} + V_5\rho_{MB}$ (2)

But assumption (iii) is that $V_{-3}\rho_{MC}$ is zero.

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*Amino acid concentrations (in μ moles/100g protein) in proteins used in the calculations were: liver proteins - methionine 14.1, cystine 11.0,; plasma proteins - methionine 2.68, cystine 14.2 (Fennessy, unpublished).

Therefore

$$V_1\rho_{MA} = \rho_{MB}\ (V_{-1} + V_3 + V_5) \tag{3}$$

By substituting the right hand side (RHS) of equation (1) into equation (3), the latter now becomes:

$$V_1\rho_{MA} = \rho_{MB}\ (V_1 + V_{-3}) \tag{4}$$

Therefore

$$\frac{V_1}{V_{-3}} = \frac{\rho_{MB}}{\rho_{MA} - \rho_{MB}} \quad \text{or} \quad V_1 = \frac{V_{-3}\ \rho_{MB}}{\rho_{MA} - \rho_{MB}} \tag{5}$$

But V_{-3} is known since V_3 is known and $V_{-3} = V_3$ (assumption (iv)). Therefore the value for V_1 may be calculated.

Using the same assumptions as in the derivation of equations (1) and (2), the cystine flux about pool B may be expressed as:

$$V_2 + V_{-4} + V_5 = V_{-2} + V_4 + V_6 \tag{6}$$

In terms of ^{35}S flux, the cystine flux about pool B is:

$$V_2\rho_{CAS} + V_{-4}\rho_{CCS} + V_5\rho_{MB} = V_{-2}\rho_{CBS} + V_4\rho_{CBS} + V_6\rho_{CBS} \tag{7}$$

But assumption (iii) is that $V_{-4}\rho_{CCS}$ is zero.

Therefore

$$V_2\rho_{CAS} + V_5\rho_{MB} = \rho_{CBS}(V_{-2} + V_4 + V_6) \tag{8}$$

By substituting the RHS of equation (6) into equation (8), the latter now becomes:

$$V_2\rho_{CAS} + V_5\rho_{MB} = \rho_{CBS}\ (V_{-2} + V_4 + V_5) \tag{9}$$

Therefore

$$V_2 = \frac{V_{-4}\rho_{CBS} + V_5(\rho_{CBS} - \rho_{MB})}{(\rho_{CAS} - \rho_{CBS})} \tag{10}$$

Similarly in terms of ^{3}H flux, the cystine flux about pool B is (note: there is no synthesis of methionine from cystine, and therefore there is zero flux of ^{3}H from methionine):

$$V_2\rho_{CAH} + V_{-4}\rho_{CCH} = V_{-2}\rho_{CBH} + V_4\rho_{CBH} + V_6\rho_{CBH} \quad (11)$$

But assumption (iii) is that $V_{-4}\rho_{CCH}$ is zero. Therefore by substituting the RHS of equation (6) into equation (11), the latter now becomes:

$$V_2\rho_{CAH} = \rho_{CBH}(V_2 + V_{-4} + V_5) \quad (12)$$

Therefore

$$V_2 = \frac{\rho_{CBH}(V_{-4} + V_5)}{(\rho_{CAH} - \rho_{CBH})} \quad (13)$$

Both equations (10) and (13) provide expressions for V_2, so equating these expressions:

$$\frac{V_{-4}\rho_{CBS} + V_5(\rho_{CBS} - \rho_{MB})}{(\rho_{CAS} - \rho_{CBS})} = \frac{\rho_{CBH}(V_{-4} + V_5)}{(\rho_{CAH} - \rho_{CBH})} \quad (14)$$

Therefore

$$\frac{V_5(\rho_{CBS} - \rho_{MB})}{(\rho_{CAS} - \rho_{CBS})} - \frac{V_5\rho_{CBH}}{(\rho_{CAH} - \rho_{CBH})} = \frac{V_{-4}\rho_{CBH}}{(\rho_{CAH} - \rho_{CBH})} - \frac{V_{-4}\rho_{CBS}}{(\rho_{CAS} - \rho_{CBS})} \quad (15)$$

In adjusting equation (15) the LHS becomes:

$$\frac{V_5\left[(\rho_{CBS} - \rho_{MB})(\rho_{CAH} - \rho_{CBH}) - \rho_{CBH}(\rho_{CAS} - \rho_{CBS})\right]}{(\rho_{CAS} - \rho_{CBS})(\rho_{CAH} - \rho_{CBH})}$$

and the RHS becomes:

$$\frac{V_{-4}\left[\rho_{CBH}(\rho_{CAS} - \rho_{CBS}) - \rho_{CBS}(\rho_{CAH} - \rho_{CBH})\right]}{(\rho_{CAH} - \rho_{CBH})(\rho_{CAS} - \rho_{CBS})}$$

Therefore transposing

$$\frac{V_5}{V_{-4}} = \frac{\rho_{CBH}(\rho_{CAS} - \rho_{CBS}) - \rho_{CBS}(\rho_{CAH} - \rho_{CBH})}{(\rho_{CBS} - \rho_{MB})(\rho_{CAH} - \rho_{CBH}) - \rho_{CBH}(\rho_{CAS} - \rho_{CBS})} \quad (16)$$

But V_{-4} is known since V_4 is known and $V_{-4} = V_4$ (i.e. assumption (iv)). Therefore the value of V_5, the rate of catabolism of methionine to cysteine, may be calculated. Having calculated V_5, and V_{-4} being known the value of V_2, the flux of cystine from pool A to pool B, may

be calculated. Substituting the values of V_1, V_{-3}, V_3 and V_5 into equation (1), and solving the equation gives the value of V_{-1}, the rate of flux of methionine from pool B to pool A.

Model II

A relationship which will allow the calculation of the methionine flux (V_7) from the membrane free pool, pool D, to the intracellular free pool, pool B, is first derived. The derivation is analogous to that for deriving V_1 in Model I.

The methionine flux about pool B may be expressed as:

$$V_{-3} + V_7 = V_5 + V_{-7} \quad (17)$$

or

$$V_{-3}\rho_{MC} + V_7\rho_{MD} = V_5\rho_{MB} + V_{-7}\rho_{MB} \quad (18)$$

But assumption (iii) is that $V_{-3}\rho_{MC}$ is zero.

Therefore

$$V_7\rho_{MD} = V_5\rho_{MB} + V_{-7}\rho_{MB} \quad (19)$$

By multiplying equation (17) through by ρ_{MB}, the following expression is obtained:

$$V_{-3}\rho_{MB} + V_7\rho_{MB} = V_5\rho_{MB} + V_{-7}\rho_{MB} \quad (20)$$

Both equations (19) and (20) provide expressions for ρ_{MB} $(V_5 + V_{-7})$, so equating these expressions:

$$V_7\rho_{MD} = V_{-3}\rho_{MB} + V_7\rho_{MB} \quad (21)$$

Therefore

$$\frac{V_7}{V_{-3}} = \frac{\rho_{MB}}{(\rho_{MD} - \rho_{MB})} \quad \text{or} \quad V_7 = \frac{V_{-3}\rho_{MB}}{(\rho_{MD} - \rho_{MB})} \quad (22)$$

But V_{-3} is known since V_3 is known and $V_{-3} = V_3$ (i.e. assumption (iv)). Therefore the value of V_7 may be calculated.

A relationship which will allow the calculation of cystine flux (V_8) from the membrane free pool, pool D, to the intracellular free pool, pool B, is derived in the following manner.

The cystine flux about pool B may be expressed as:

$$V_{-8} + V_6 = V_5 + V_{-4} + V_8 \quad (23)$$

or in terms of the ^{35}S flux as:

$$V_{-8}\rho_{CBS} + V_6\rho_{CBS} = V_5\rho_{MB} + V_{-4}\rho_{CCS} + V_8\rho_{CDS} \quad (24)$$

But assumption (iii) is that $V_{-4}\rho_{CCS}$ is zero.

Therefore

$$\rho_{CBS}(V_{-8} + V_6) = V_5\rho_{MB} + V_8\rho_{CDS} \quad (25)$$

By substituting the RHS of equation (23) into equation (25), the latter now becomes

$$\rho_{CBS}(V_5 + V_{-4} + V_8) = V_5\rho_{MB} + V_8\rho_{CDS} \quad (26)$$

Therefore

$$V_5 = \frac{V_8(\rho_{CDS} - \rho_{CBS}) - V_{-4}\rho_{CBS}}{(\rho_{CBS} - \rho_{MB})} \quad (27)$$

Similarly the cystine flux in terms of ^{3}H about pool B may be expressed as (note: there is no synthesis of methionine from cystine, and therefore there is zero flux of ^{3}H from methionine):

$$V_{-8}\rho_{CBH} + V_6\rho_{CBH} = V_{-4}\rho_{CCH} + V_8\rho_{CDH} \quad (28)$$

But assumption (iii) is that $V_{-4}\rho_{CCH}$ is zero.

Therefore

$$\rho_{CBH}(V_{-8} + V_6) = V_8\rho_{CDH} \quad (29)$$

By substituting the RHS of equation (23) into equation (29), the latter now becomes:

$$\rho_{CBH}(V_5 + V_{-4} + V_8) = V_8\rho_{CDH} \quad (30)$$

Therefore

$$V_5 = \frac{V_8(\rho_{CDH} - \rho_{CBH})}{\rho_{CBH}} - V_{-4} \qquad (31)$$

Both equations (27) and (31) provide expressions for V_5, so equating these expressions:

$$\frac{V_8(\rho_{CDS} - \rho_{CBS}) - V_{-4}\rho_{CBS}}{(\rho_{CBS} - \rho_{MB})} = \frac{V_8(\rho_{CDH} - \rho_{CBH})}{\rho_{CBH}} - V_{-4} \qquad (32)$$

Therefore

$$\frac{V_8(\rho_{CDS} - \rho_{CBS})}{(\rho_{CBS} - \rho_{MSB})} - \frac{V_8(\rho_{CDH} - \rho_{CBH})}{\rho_{CBH}\ (\rho_{CBS} - \rho_{MB})} = \frac{V_{-4}\rho_{CBS}}{(\rho_{CBS} - \rho_{MB})} - V_{-4} \qquad (33)$$

In adjusting equation (33), the LHS becomes:

$$\frac{V_8\left[\rho_{CBH}(\rho_{CDS} - \rho_{CBS}) - (\rho_{CBS} - \rho_{MB})(\rho_{CDH} - \rho_{CBH})\right]}{CBH\ (\rho_{CBS} - \rho_{MB})}$$

and the RHS becomes:

$$\frac{V_{-4}(\rho_{CBS} - \rho_{CBS} + \rho_{MB})}{(\rho_{CBS} - \rho_{MB})}$$

Therefore

$$\frac{V_8}{V_{-4}} = \frac{\rho_{MB} \cdot \rho_{CBH}}{\rho_{CBH}(\rho_{CDS} - \rho_{CBS}) - (\rho_{CBS} - \rho_{MB})(\rho_{CDH} - \rho_{CBH})} \qquad (34)$$

But V_{-4} is known since V_4 is known and $V_{-4} = V_4$ (i.e. assumption (iv). Therefore the value of V_8 may be calculated.

An estimate of V_5 (the rate of methionine catabolism to cysteine) may be obtained by substituting the value of V_8 obtained from equation (34), into equation (27) or equation (31). Having calculated the value of V_5, the rate of movement of methionine (V_{-7}) from the intracellular pool, pool B, to the membrane pool, pool D may be calculated using equation (17) or equation (19), since now V_7, V_{-3} and V_5 are all known.

The rate of movement of methionine (V_{-1}) from the membrane pool, pool D, into the plasma pool, pool A, may now be calculated. The methionine flux about pool D may be expressed as:

$$V_7 + V_3 + V_{-1} = V_1 + V_{-7} \quad (35)$$

or $$V_7 \rho_{MD} + V_3 \rho_{MB} + V_{-1}\rho_{MD} = V_1\rho_{MA} + V_{-7}\rho_{MB} \quad (36)$$

Therefore

$$V_{-1}\rho_{MD} - V_1\rho_{MA} = V_{-7}\rho_{MB} - V_7 \rho_{MD} - V_3 \rho_{MD} \quad (37)$$

Now multiplying equation (35) through by ρ_{MA} gives

$$V_{-1}\rho_{MA} - V_1\rho_{MA} = V_{-7}\rho_{MA} - V_7\rho_{MA} - V_3\rho_{MA} \quad (38)$$

Both equations (37) and (38) provide expressions for $V_1\rho_{MA}$, so equating these expressions:

$$V_{-1}(\rho_{MD} - \rho_{MA}) = V_{-7}(\rho_{MB} - \rho_{MA}) - V_7(\rho_{MD} - \rho_{MA}) - V_3(\rho_{MD} - \rho_{MA}) \quad (39)$$

Therefore

$$V_{-1} = \frac{V_{-7}(\rho_{MB} - \rho_{MA})}{(\rho_{MD} - \rho_{MA})} - V_7 - V_3 \quad (40)$$

Therefore the value of V_{-1} may be calculated, and by substituting known values for V_3, V_7, V_{-7} and V_{-1} into equation (35), the value of V_1, the rate of movement of methionine from the plasma pool, pool A, to the membrane pool, pool D, may be calculated.

Model III

Although this five-compartment model represents the next step in an attempt to obtain meaningful information about the intracellular metabolism of methionine and cystine, insufficient equations could be generated to enable any estimate of the rate of methionine catabolism using the data obtained in the present experiment.

Intracellular recycling

The proportion of the intracellular free pool of amino acid which is derived from the circulating pool (i.e. plasma) may be estimated from the ratio of the specific activities of the intracellular and plasma free amino acid at plateau (Gan & Jeffay 1967). For example, the proportion of intracellular methionine derived from plasma is given by ρ_{MB}/ρ_{MA}. This assumes that plasma is the circulating free pool from which the tissue draws the amino acid. However, the possibility that the blood cells may be a source of amino acid of different specific activity cannot be excluded. It is generally considered that that proportion of the intracellular free pool which is unlabelled is derived from the catabolism of unlabelled intracellular proteins (Gan & Jeffay 1967; Aub & Waterlow 1970), assuming that there is no re-entry of labelled amino acid from protein catabolism. In the case of amino acids which may be synthesized by the animal (e.g. cysteine from methionine) there is clearly another source of unlabelled amino acid. The use of two labelled amino acids as in the present experiment permits an estimate of the proportion of the intracellular amino acid which is derived from catabolism of unlabelled intracellular protein assuming that this and methionine catabolism are the only two sources of cystine other than plasma. Thus tru re-entry from protein catabolism will be the mean of the two estimates (i.e. ^{3}H - and ^{35}S - cystine) since both labelled (i.e. ^{35}S - cystine) and unlabelled (i.e. in terms of ^{3}H - cystine) is being added from the same source (i.e. a ^{35}S - methionine pool). Therefore the proportional re-entry of cystine from protein catabolism is given by the expression:

$$0.5\left[\left(1 - \frac{\rho_{CBH}}{\rho_{CAH}}\right) + \left(1 - \frac{\rho_{CBS}}{\rho_{CAS}}\right)\right] \qquad (41)$$

In the foregoing discussion it is assumed that there is a single homogeneous intracellular free amino acid pool (i.e. the Model I situation).

The proportion of the total flux of an amino acid through the intracellular free pool which is derived from catabolism of intracellular protein may be determined from the calculated flux rates. Thus the proportional re-entry of methionine from protein catabolism (R_M) is given by the following equations:

$$R_M \text{ (Model I)} = \frac{V_{-3}}{V_{-3} + V_1} \text{ or } \frac{V_{-3}}{V_3 + V_{-1} + V_5} \quad (42)$$

and $$R_M \text{ (Model II)} = \frac{V_{-3}}{V_{-3} + V_7} \text{ or } \frac{V_{-3}}{V_5 + V_{-7}}. \quad (43)$$

Similarly the expressions for cystine re-entry from protein catabolism (R_C) are:

$$R_C \text{ (Model I)} = \frac{V_{-4}}{V_{-4} + V_2 + V_5} \quad (44)$$

and $$R_C \text{ (Model II)} = \frac{V_{-4}}{V_{-4} + V_8 + V_5} \quad (45)$$

An estimate of the internal intracellular reutilisation of an amino acid may also be derived from the calculated flux rates. Reutilisation may be defined as that proportion of the amino acid flux through the tissue which is reutilised (either in protein synthesis or amino acid catabolism) without first equilibrating with the circulating pool. Thus the reutilisation of methionine (R_{u_M}) and cystine (R_{u_C}) for the Model I situation is given by the following expressions:

$$R_{u_M} = \frac{V_3 + V_5}{V_{-3} + V_1} \text{ or } \frac{V_3 + V_5}{V_3 + V_5 + V_{-1}} \quad (46)$$

and $$R_{u_C} = \frac{V_4 + V_6}{V_4 + V_6 + V_{-2}} \text{ or } \frac{V_2 + V_{-4} + V_5 - V_{-2}}{V_{-4} + V_5 + V_2} \quad (47)$$

Reutilisation of methionine and cystine in this context has limited meaning in terms of Model II, since the amino acids for protein synthesis are assumed to be obtained from a mixed pool.

Intracellular free amino acid pool turnover

An estimate of the turnover time (hours) of the intracellular free pool of an amino acid is obtained by dividing the pool size (i.e. concentration of free amino acid/g tissue protein) by the rate of flux (μ moles/g tissue protein/hour) through the pool. Thus the turnover times for methionine and cystine in Models I and II may be calculated as follows (where the concentration of the free amino acid is expressed in terms of μ moles/g tissue protein/hour, this being calculated using the ratio of intracellular space to tissue protein and the intracellular concentration of the amino acid).

Model I:

The turnover time of methionine is equal to:

$$\frac{\text{Methionine concentration}}{V_3 + V_5 + V_{-1}} \qquad (48)$$

Similarly the turnover time of cystine is equal to:

$$\frac{\text{Cystine concentration}}{V_{-2} + V_4 + V_6} \qquad (49)$$

Model II:

The turnover time of methionine is equal to:

$$\frac{\text{Methionine concentration}}{V_{-7} + V_5} \qquad (50)$$

Similarly the turnover time of cystine is equal to:

$$\frac{\text{Cystine concentration}}{V_{-8} + V_6} \qquad (51)$$

But

$$V_{-8} + V_6 = V_8 + V_{-4} + V_5 \text{ (equation 23).}$$

Therefore the turnover time of cystine may be derived using the following expression:

$$\frac{\text{Cystine concentration}}{V_8 + V_{-4} + V_5} \qquad (52)$$

RESULTS

The plasma and whole tissue free pools were the amino acid pools sampled in the present experiment and hence all other values for concentrations and specific activities have been derived on the basis of certain assumptions. The method of derivation of the values is given in Chapter 7. The concentration and specific activity values for methionine and cystine used in the calculations are given in Table 8.1. These values have previously been presented in full.

The calculations were based on the rate of amino acid release in protein catabolism and the specific activities of the amino acids in the various pools. The rate of amino acid release in protein catabolism was assumed to be equal to the rate of amino acid uptake in protein synthesis (assumption (iv)).

The values of V_3 and V_4 have been derived using the fractional synthetic rates of the liver "domestic" proteins, assuming that an intermediate ("membrane") pool best represents the true precursor pool of amino acids for protein synthesis. However, the fractional synthetic rate has been estimated using the data for methionine only, since in the circumstances this was probably the best estimate of the rate of protein synthesis available. As discussed in Chapter 7 the estimated rate of liver protein synthesis for sheep 462 and 567, (the sheep receiving the highest rate of methionine infusion) may have

TABLE 8.1: Values for the specific activity (dpm x 10^3/μ mole) of methionine (ρ_M) and cystine (ρ_C, expressed as half-cystine) in the three free amino acid pools, pool A (plasma pool), pool B (intracellular pool) and poo D ("membrane" pool), and the values for the intracellular concentrations of methionine and cystine (μ moles/g liver protein) used in the calculations.

Sheep[2]	ρ_{MA}[1]	ρ_{MB}	ρ_{MD}	Methionine (μ moles/g liver protein)
445	52.5	4.34	6.69	1.63
582	52.0	3.27	6.11	1.28
580	27.2	3.95	6.59	1.37
389	23.3	5.11	6.55	6.09
462	15.7	10.4	10.8	23.0
567	8.46	6.69	6.81	55.2
	ρ_{CAS}	ρ_{CBS}	ρ_{CDS}	Cystine (μ moles ½cys/g liver protein)
445	10.1	5.72	5.76	3.68
582	7.43	5.74	5.77	2.20
580	9.97	11.0	11.0	1.89
389	5.23	5.75	5.74	2.72
462	4.89	3.46	3.49	3.56
567	3.01	1.54	1.58	2.53
	ρ_{CAH}	ρ_{CBH}	ρ_{CDH}	
445	293	10.7	13.2	
582	202	9.20	13.0	
580	276	7.37	11.6	
389	133	4.73	7.68	
462	124	3.11	5.34	
567	128	0.051	3.15	

1) The second subscript denotes the free pool (e.g. ρ_{-A}, pool A) and the third subscript denotes the position of the radioactive label on the cystine (e.g. ρ_{--S} for the ^{35}S label and ρ_{--H} for ^{3}H label).

2) Sheep 445, 582 - control (water) infusion; 580, 389 - L-methionine infusion at rate of 0.12g/$kg^{0.75}$/day; 462, 567 - L-methionine infusion at rate of 0.36g/$kg^{0.75}$/day.

been reasonably accurate.

The estimated values for methionine and cystine flux, turnover times of the intracellular amino acid pools and the estimated proportional re-entry of methionine and cystine from protein catabolism are given in Tables 8.2, 8.3 and 8.4. Figure 8.2 gives a diagramatic representation of the methionine and cystine flux through the amino acid pools of the liver in sheep 462, given the high (0.36g L-methionine/$kg^{0.75}$/day) level of methionine.

DISCUSSION

The negative values for the estimates of methionine catabolism (V_5) in the liver in sheep 445, 582, 580 and 389, are clearly in error. The source of this error was an incorrect identification of the methionine pool which served as the cystine precursor and/or an incorrect identification of the product (i.e. cystine) pool. The most probable cause of this error was the subcellular compartmentation of methionine and/or cystine such that the general intracelullar pools of these two amino acids, as defined in the models did not adequately represent the true situation.

Evidence that the mixed intracellular pool of free cystine did not represent the cystine derived from methionine comes from the observation that in four of the six sheep, the ^{35}S specific activity of plasma cystine was actually higher than that of the mixed intracellular cystine, when the only possible source of plasma ^{35}S-cystine was an intracellular ^{35}S-methionine pool. However, this pool need not necessarily have been a liver pool, since other tissues (e.g. skin and spleen) are known to possess the rate-limiting enzymes for

TABLE 8.2: Values for the flux rates (μmoles amino acid/g tissue protein/hour) and the turnover times (hours) of methionine and cystine (expressed as half-cystine) in the liver as derived using Model I.

Sheep[1]	Flux rate (μ moles amino acid/g liver protein/hour)						Turnover time (hours)	
	Methionine			Cystine (as ½cys)				
	V_1	V_3[2]	V_5	V_2	V_4	V_5	Met	Cys
445	0.404	4.49	-16.0	-0.472	3.50	-16.0	0.333	-0.284
582	0.330	4.92	-9.09	-0.250	3.84	-9.09	0.244	-0.400
580	0.623	3.67	-4.46	-0.044	2.87	-4.46	0.319	-1.16
389	0.400	1.42	-9.64	-0.316	1.11	-9.64	3.35	-0.308
462	1.85	0.95	0.36	0.028	0.74	0.36	8.23	3.14
567	3.80	1.01	0.23	0.0004	0.78	0.23	11.5	2.50

1) Infusion: sheep 445, 582 - control (water) infusion; 580, 389 - low methionine, 0.12g L-met/$kg^{0.75}$/day; 462, 567 - high methionine - 0.36g L-met/$kg^{0.75}$/day.

2) Assumption (iv): $V_3 = V_{-3}$ and $V_4 = V_{-4}$.

TABLE 8.3: Values for the flux rates (μ moles amino acid/g tissue protein/hour) and the turnover times (hours) of methionine and cystine (expressed as half-cystine) in the liver as derived using Model II .

Sheep[1]	Methionine Flux rate (μ moles/g liver protein/hour)						Methionine Turnover time (hours)
	V_1	V_{-1}	V_3	V_5	V_7	V_{-7}	
445	1.47	17.4	4.49	-16.0	8.27	28.7	0.128
582	1.22	10.3	4.92	-9.09	5.67	19.7	0.121
580	1.74	6.21	3.67	-4.47	5.49	13.6	0.150
389	1.39	11.0	1.42	-9.65	5.02	16.1	0.946
462	2.07	1.71	0.95	0.36	22.0	22.6	1.00
567	3.34	3.11	1.01	0.23	59.0	59.8	0.920

	Cystine Flux rate (μ moles ½cys/g liver protein/hour)			Cystine Turnover time (hours)
	V_4	V_5	V_8	
445	3.50	-16.0	-52.6	-0.056
582	3.84	-9.09	-12.6	-0.124
580	2.87	-4.47	-2.80	-0.430
389	1.11	-9.65	-13.7	-0.122
462	0.74	0.36	1.55	1.34
567	0.78	0.23	0.017	2.46

1) Infusion: sheep 445, 582 - control, water infusion;
580, 389 - low methionine, 0.12g L-met/$kg^{0.75}$/day;
462, 567 - high methionine, 0.36g L-met/$kg^{0.75}$/day.

2) Assumption (iv): $V_3 = V_{-3}$ and $V_4 = V_{-4}$.

TABLE 8.4: Estimated values for the proportion of the total flux of methionine or cystine through the intracellular pool which is derived from the catabolism of intracellular protein (i.e. proportional re-entry).

	Estimated proportional re-entry					
	Methionine			Cystine		
Sheep[1]	From plateau sp.act.[2]	Model I eq.(42)	Model II eq.(43)	From plateau sp. act.	Model I eq.(44)	Model II eq. (45)
445	0.917	0.917	0.352	0.699	-0.270	-0.054
582	0.937	0.937	0.465	0.591	-0.698	-0.215
580	0.855	0.854	0.401	0.434	-1.76	-0.652
389	0.780	0.780	0.220	0.432	-0.125	-0.050
462	0.340	0.339	0.041	0.634	0.652	0.279
567	0.209	0.210	0.017	0.745	0.770	0.757

1) Infusion: sheep 445, 582 - control, water infusion;
580, 389 - low methionine, 0.12g L-met/kg$^{0.75}$/day;
462, 567 - high methionine, 0.36g L-met/kg$^{0.75}$/day.

2) Proportional re-entry of amino acid from protein catabolism calculated from the specific activity of the amino acid at plateau (Gan & Jeffay 1967) $= \left(1 - \frac{\text{intracellular specific activity}}{\text{plasma specific activity}}\right)$.

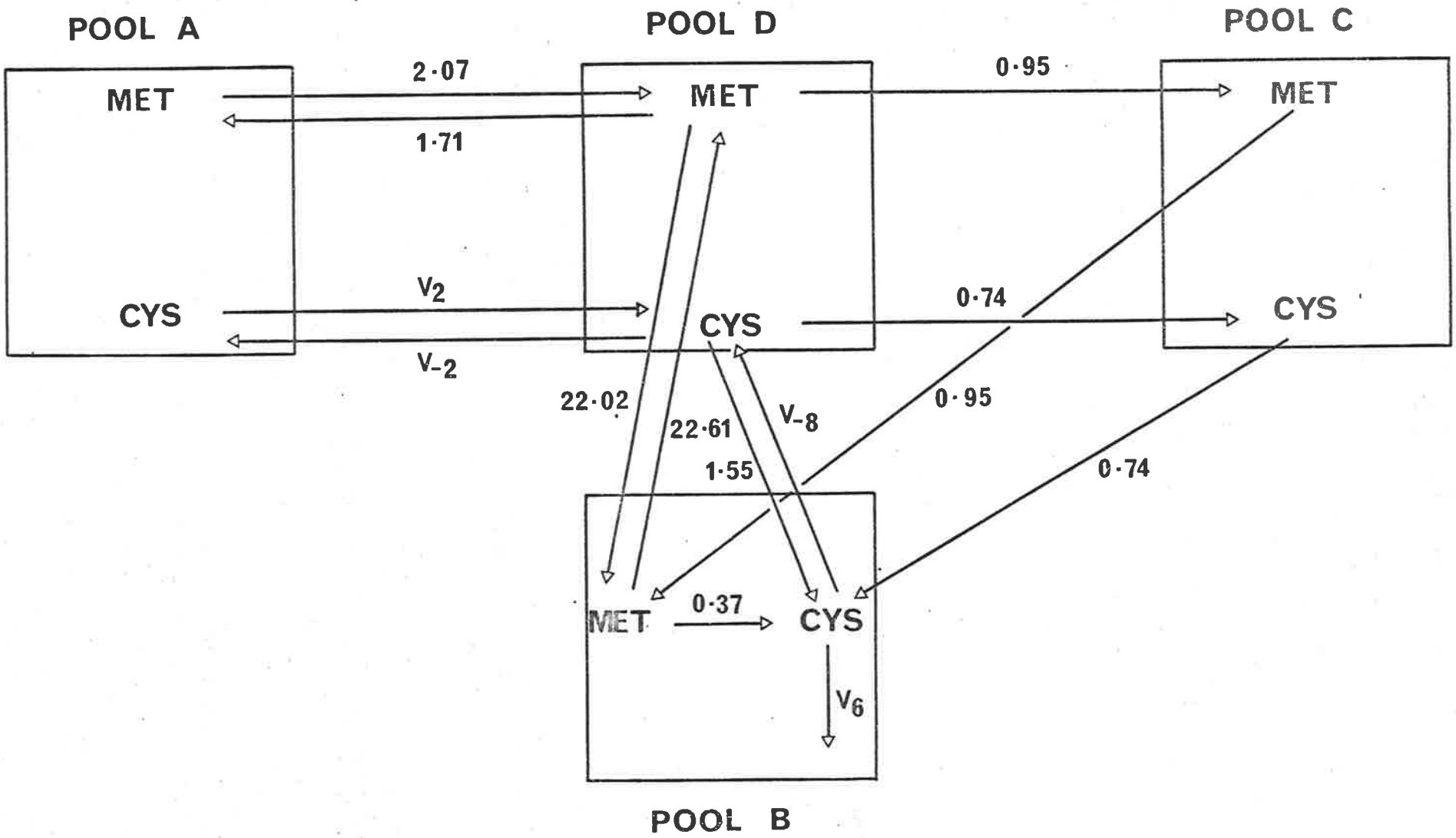
FIGURE 8.2: Diagrammatic representation of the flux of methionine (μ moles/g liver protein/hour) and cystine (μ moles ½ cys/g liver protein/hour) through the amino acid pools of the liver in sheep 462 as derived using Model II.

Pool A - the plasma free amino acid pool.

Pool B - the intracellular free amino acid pool.

Pool C - the intracellular protein amino acid pool.

Pool D - the "membrane" free amino acid pool.


POOL A
POOL D
POOL C
MET
CYS
MET
CYS
MET
CYS
2·07
1·71
0·95
V_2
V_{-2}
0·74
22·02
22·61
V_{-8}
1·55
0·95
0·74
MET
0·37
CYS
V_6
POOL B

the transulphuration pathway (methionine adenosyl transferase and cystathionine synthase (Radcliffe & Egan (1974) and unpublished). However, this seems unlikely in view of the general importance of the liver in amino acid metabolism. Further supportive evidence that compartmentation of the sulphur amino acid pools within the liver was the cause of the abberant calculated flux parameters of the models comes from the fact that in the sheep given the high rate of methionine supplementation, the liver free methionine pools were grossly increased in size compared with the control animals and those receiving the low rate of methionine supplementation. In the two sheep with the very large pools (462, 567), the ^{35}S specific activity of the intracellular cystine was lower than that of the intracellular methionine, whereas in the sheep with the small liver free methionine pools, the situation was reversed. It is likely that the high methionine loading resulted in a much greater intracellular methionine flux resulting in greatly reduced differences between the subcompartments.

Compartmentation of metabolites and different parts of metabolic pathways within a tissue has been shown for hepatic carbohydrate metabolism (Greenbaum *et al.* 1971), although it is a poorly studied phenomenon in terms of amino acid metabolism, with the exception, perhaps, of some aspects of amino acid metabolism in the brain (Roach *et al.*1974) and the compartmentation of amino acids for protein synthesis in the cell (Kipnis *et al.* 1961; Airhart *et al.* 1974; van Venrooij *et al.* 1974). However the extent of the error generated in the data derived in the present work is alarming in terms of an understanding of methionine (and cystine) metabolism in the sheep.

Little can be said about the rest of the predicted values, the estimates of proportional re-entry and turnover times being so dependent on the estimate of methionine catabolism (V_5). However for two sheep (those receiving the high level of methionine) the general intracellular pool may have represented a reasonable approximation of the methionine precursor pool, although the problems in identifying the product pool were still very important. In these sheep, the estimates of hepatic methionine catabolism were equivalent to about 0.10g of methionine per day, representing about 30% of the estimated whole body cystine production from methionine (see Chapter 6, Table 6.6).

The estimates of methionine re-entry (i.e. the proportion of the total methionine flux through the intracellular pool which is derived from protein catabolism) derived from the Model II data in the sheep 462 and 567 were markedly different from those calculated from the Model I data, and from the plasma specific activity, both of which assumed a single homogeneous intracellular free amino acid pool. The very low values of 4.1 and 1.7% derived using Model II suggest that only a very small part of the intracellular pool was derived from re-entry of methionine from protein catabolism. This suggests that much of the high apparent re-entry (calculated from the plasma specific activity) from protein catabolism simply reflected poor mixing of the intracellular pool with the circulating pool. This is further evidence for the existence of a considerable degree of metabolic compartmentation, and shows that despite the greatly expanded pool size of methionine, there was still a considerable proportion of the intracellular pool which was in poor communication with the plasma.

As discussed previously, the fractional synthetic rate of liver proteins represented only the synthesis of domestic proteins. The effects of including allowances for plasma protein synthesis and degradation on the calculated rates of methionine and cystine flux will now be considered for two of the sheep (sheep 462 and 567 which received the high rate of methionine infusion).

The rate of plasma protein synthesis was measured in only three animals, the mean fractional synthetic rate being 0.275 $days^{-1}$. Applying this rate to the estimated total body plasma protein pool in sheep 462 and 567 gave values for synthesis of 23.8 and 28.9g of plasma proteins/day respectively. The rates were converted to the uptake of methionine and cystine (μ moles/g liver protein/hour) using the appropriate factors and liver weights (see footnotes to Table 8.5 for full calculations). There is considerable doubt regarding the site of plasma protein catabolism (Rothschild *et al.* 1970), although it is usually stated that the liver is not an important site for the catabolism of these proteins. For this reason, the data were derived using two alternative estimates of plasma protein degradation in the liver, namely that 20% of that synthesised was ultimately degraded in the liver (situation B), or that 50% was degraded in the liver (situation C). The estimated values for methionine and cystine flux and the turnover times of the intracellular free amino acid pools for sheep 462 and 567, together with the data previously derived (situation A) are given in Table 8.5.

The most important result of including the plasma proteins in the calculations was the increase in the calculated rate of methionine catabolism to cystine, this being very dependent on the estimate of cystine re-entry into the intracellular pool from protein catabolism

TABLE 8.5: Values for the flux rates (μ moles amino acid/g tissue protein/hour) and the turnover times (hours) of methionine and cystine (expressed as half-cystine) in the liver of sheep 462 and 567 as derived using Model II but including or excluding allowances for the synthesis and degradation of plasma proteins (i.e. liver "export" proteins).

Sheep[1]	Methionine Flux rate (μ moles/g liver protein/hour) V_1	V_{-1}	V_3	V_{-3}	V_5	V_7	V_{-7}	Methionine Turnover time (hours)
462A[2]	2.07	1.71	0.95	0.95	0.36	22.0	22.6	1.00
B	2.26	1.31	1.40	1.04	0.60	24.1	24.5	0.918
C	2.54	1.37	1.40	1.18	0.95	27.3	27.5	0.809
567A	3.34	3.11	1.01	1.01	0.23	59.0	59.8	0.920
B	4.42	3.78	1.37	1.08	0.35	63.4	64.1	0.857
C	4.86	4.16	1.37	1.19	0.52	69.9	70.5	0.777

	Cystine Flux rate (μ moles ½cys/g liver protein/hour) V_4	V_{-4}	V_5	V_8	Cystine Turnover time (hours)
462A	0.74	0.74	0.36	1.55	1.34
B	3.10	1.21	0.60	2.53	0.820
C	3.10	1.92	0.95	4.02	0.517
567A	0.78	0.78	0.23	0.017	2.46
B	2.67	1.16	0.35	0.025	1.65
C	2.67	1.72	0.52	0.037	1.11

1) Sheep 462 and 567 received infusions of 0.36g L-methionine/$kg^{0.75}$/day.
2) A - plasma protein synthesis and degradation not included.
B - plasma protein synthesis and degradation were derived in the following manner: 50% of plasma proteins synthesised in the liver were assumed to have been ultimately degraded in the liver. The rate of plasma protein synthesis (g/g liver protein/hour) were calculated as follows (assuming a mean fractional synthesis rate for plasma proteins of 0.275 $days^{-1}$ (see Table 7.5), blood volume as 6% of liveweight (Fennessy unpublished) and plasma proteins as 8.8g/100 ml plasma)); sheep 462 - 23.8g plasma proteins synthesised/day which for a liver weight of 82.6g (72% protein) is 0.0167g plasma proteins/g liver protein/hour; sheep 567 - 28.9g plasma proteins synthesised/day which for a liver weight of 125.2g (72% protein) is 0.0134g plasma proteins/g liver protein/hour; and assuming concentrations of 0.4g methionine/100g protein (26.8 μ moles/g) and 1.7g cystine/100g protein (142 μ moles half-cystine/g).
C - plasma protein synthesis calculated as for B; 20% of plasma proteins synthesised in the liver were assumed to have been ultimately degraded in the liver.

(V_{-4}) and the rate of flux of cystine from the membrane pool to the intracellular pool (v_8). This is apparent from an examination of equations (31) and (34). At the highest rate of methionine catabolism (situation C), an estimated 0.3g of methionine was converted to cystine in the liver. This value is a little less than the estimated whole body synthesis of cystine from methionine in these two sheep (see Chapter 6, Table 6.6). However, as discussed in Chapter 6, the estimated entry rate of cystine was low, and the intracellular ^{3}H - cystine specific activity (in the liver) was very low compared with the plasma free pool, both factors which suggest that the infused labelled cystine was not mixing well with the intracellular cystine pool, which would result in an underestimate of the cystine entry rate in plasma. In contrast, there was evidence of considerable mixing of the intracellular cystine pool with the plasma pool, this being apparent from the high ratios of intracellular ^{35}S-cystine specific activity to the specific activity in the plasma pool.

The complexity of methionine and cystine metabolism in the sheep is clearly evident from the foregoing discussion. In order to gain a greater understanding of methionine and cystine metabolism, and in particular to obtain information concerning the metabolic compartmentation of these amino acids much more data than obtained in the present experiment would be required. If adequate data could be obtained a dynamic model of methionine metabolism within a tissue could be developed. An approach which could yield useful data would utilise the various steps in the catabolism of methionine and other steps in the utilisation of methionine within the tissue. Such an approach would involve sequential tissue and blood sampling

from animals receiving infusions of radioactively-labelled methionine and cystine (e.g. methyl-^{14}C-methionine, ^{35}S-methionine, ^{35}S-cystine) in order to define the shape of the specific activity curves for the various compounds. Where possible the concentration and specific activity of methionine, S-adenosylmethionine, tRNA-bound methionine, cystathionine, cysteine and tRNA-bound cysteine in the tissue and circulating free pools would be determined. The specific activity of the amino acids in the plasma and tissue proteins would also be determined. Having obtained such data, relevant information concerning the various methionine sub-pools would be obtained from the concentration and specific activity values for extracellular and intracellular methionine, protein-precursor methionine (i.e. tRNA-bound methionine), methionine for catabolism (S-adenosylmethionine), and the extent of mixing of cysteine derived from methionine with the intracellular free cysteine pool. The rate of tissue protein synthesis could also be derived using the specific activity values of the tRNA-bound amino acids and the protein amino acids.

The information so obtained would be used in the development of a dynamic model of methionine metabolism using similar concepts (but including additional intracellular pools) to those used in the models developed in the present chapter.

CONCLUSIONS

The compartmental models were set up in an attempt to obtain more detailed information about methionine and cystine metabolism in the tissues of the sheep. When applied to the data obtained in the present experiment, the calculated values for hepatic methionine catabolism were obviously incorrect in four of the six sheep. The

errors were apprently due to metabolic compartmentation of the amino acids and their metabolites within the tissue; i.e. the models set up were too simple to adequately define the situation *in vivo*. However, for two sheep (those receiving the highest rate of methionine infusion) the estimated rates of methionine catabolism via the transulphuration pathway may have been reasonably accurate. In these sheep, which had greatly expanded methionine pools, there was evidence of a much greater degree of mixing of the circulating (plasma) pool with the intracellular free methionine pool. As a result of the increased pool size the degree of metabolic compartmentation within the liver was greatly reduced. The extent of the error in the estimates of methionine metabolism is alarming in view of the attempts to gain an understanding of sulphur amino acid metabolism in the sheep.

GENERAL DISCUSSION

The experiments reported in this thesis have highlighted the complexity of amino acid utilisation and metabolism in the sheep. There are several aspects of methionine utilisation which illustrate this complexity. These aspects include the very marked between animal variability in the response to a post-ruminal supplement of methionine and the peculiarities in the metabolism of methionine and cystine as evidenced by the studies using the radioactively-labelled amino acids.

The variability between animals in the methionine response is an especially interesting phenomenon. In the two animals in which the dose response sequence was repeated, the trends in the responses were similar on both occasions. This repeatability lends support to the suggestion that the differences in the responses reflected real differences in the utilisation of the additional methionine at the tissue level. However the fact that the individual responses were often quite different on the two diets examined in Experiment 5 points to a possible interaction of methionine or its metabolites with other amino acids. The possibility that animals fed higher quality diets may be more susceptible to the adverse effects of methionine would, if confirmed, lend support to the theory that it is the interaction of methionine with other amino acids which is the important factor in determining the methionine response, rather than an effect of excess methionine *per se*. In this respect, the partial alleviation of the methionine-induced intake depressions by threonine (Chapter 2) and the effect of threonine supplementation on the concentration of methionine and its metabolites in plasma (Chapter 3) suggest an interaction of methionine and threonine at the tissue level.

Any hypothesis concerning the responses to methionine must explain both between-diet and within-diet between-animal variation. This underlines the need to consider individual animal responses and to look closely at results in which a high variability in the response outweighs differences between means. In addition there is the problem of carryover effects between periods when treatments are given in a factorial or Latin square sequence. It is therefore apparent that the experimental design and the statistical analysis of such experiments becomes a major problem.

The variability between animals in the methionine response and the contrasting effects of methionine on the different parameters raise questions as to the usefulness of the concept of a "first-limiting" amino acid. In the strict sense an amino acid may be "first-limiting" for the synthesis of a particular protein (e.g. threonine for the synthesis of the γ chain of haemoglobin - Hunt *et al.* 1969) or for a particular metabolic function (e.g. tyrosine as a precursor of DOPA). However when all such limitations are integrated at the whole animal level problems arise in the applicability of the concept. For example in some animals in the present experiments, methionine was apparently limiting for protein synthesis in the overall sense such that methionine increased nitrogen retention yet at the same time voluntary intake was depressed. This suggests that methionine altered some aspect of metabolism related to the mechanisms of intake regulation such that intake was adversely affected. It is also likely that in some situations methionine promoted an increase in wool growth (although this was not measured in the present work) while overall nitrogen retention was adversely affected. A similar situation may have occurred with respect to the liver hypertrophy reported in the

present work (Chapter 6). In respect of between animal comparisons the multiple supplementation experiment (Chapter 4) is also relevant. In this work there was some evidence that amino acids other than methionine were particularly important for efficient nitrogen utilisation in some animals.

Although the changes in the plasma amino acid pattern in response to methionine were repeatable in the two animals examined (Chapter 3) there were no obvious differences between animals in the plasma or blood cell amino acid patterns which could point to the reasons for differences in the response to methionine. The relevance and interpretation of plasma and blood cell amino acid patterns is in need of considerably more definitive study. The patterns of change in different individuals may reflect unique individual metabolic patterns or perhaps the patterns may appear similar and not reflect real differences in amino acid utilisation at the tissue level.

The complexity of methionine metabolism was also evident in the experiment in which six sheep received intravenous infusions of radioactively-labelled methionine and cystine. Theoretically the methodology involving infusion of ^{35}S-methionine and ^{3}H-cystine should have provided accurate data on the rate of methionine catabolism and the rate of protein synthesis both in individual tissues and in the whole animal. However there was evidence of very poor mixing between the circulating free pools of methionine and cystine and the tissue free pools of these amino acids except in the two sheep which received the high rate of abomasal methionine infusion. The very poor mixing apparently reflects metabolic compartmentation of methionine and cystine within the liver. Consequently it was only in those two

sheep receiving the high rate of methionine infusion (and as a result having greatly expanded tissue methionine pools) that the calculated rates of liver protein synthesis and the hepatic catabolism of methionine are likely to be accurate.

A P P E N D I C E S

APPENDIX 1

APPENDIX TABLE 1.1: Amino acid concentrations (μ moles/100ml) in bulked samples of plasma.

	Diet: WHC			Methionine: OM (Control)		
	I_O			I_G		
	T_0	$T_{2.5}$	T_5	T_0	$T_{2.5}$	T_5
TAU	2.40	2.32	2.30	2.69	2.11	2.20
THR	10.03	10.46	10.63	13.86	11.07	10.08
SER	14.02	11.95	10.75	12.65	9.97	9.75
GLU	17.42	24.43	25.53	15.16	15.99	25.72
GLY	61.72	54.48	52.92	58.66	53.66	54.88
ALA	15.15	15.14	17.75	13.71	13.19	17.17
ABU	0.75	0.30	1.17	0.66	0.65	1.34
VAL	15.55	19.00	21.39	17.17	14.29	16.16
MET	1.87	1.44	1.31	1.82	1.59	1.29
CYSH	tr.	ND	tr.	tr.	tr.	ND
ILE	8.16	8.43	8.94	8.21	5.78	7.01
LEU	9.11	9.35	9.23	9.41	5.79	7.05
TYR	5.29	4.81	4.76	5.00	3.61	4.22
PHE	4.39	4.20	4.26	4.05	3.15	4.26
ORN	6.47	5.59	5.36	5.42	4.88	5.85
LYS	14.54	13.87	10.60	15.23	11.21	10.01
HIS	11.44	10.49	10.72	11.09	11.11	8.99
ARG	11.85	14.40	11.14	11.83	9.54	11.12

I_O - no glucose infusion; I_G - intravenous glucose infusion at a rate of 0.5g D-glucose/$kg^{0.75}$/hour for 2 hours.

Methionine treatments: OM - control water infusion;
LMet - 0.08g L-methionine/$kg^{0.75}$/day;
HMet - 0.16g L-methionine/$kg^{0.75}$/day.
tr - trace; ND - not detectable; NM - not measurable.

APPENDIX TABLE 1.1 continued:

	Diet: WHC			Methionine: LMet.		
	I_O			I_G		
	T_o	$T_{2.5}$	T_5	T_0	$T_{2.5}$	T_5
TAU	4.68	2.02	6.52	7.08	5.73	5.80
THR	7.91	6.44	8.36	9.77	10.39	7.73
SER	9.53	7.09	8.73	6.96	7.82	6.30
GLU	15.88	20.15	24.10	14.85	12.30	23.03
GLY	49.76	45.79	46.69	46.16	41.16	35.89
ALA	13.92	14.52	15.26	13.34	11.11	14.16
ABU	0.93	0.59	1.06	0.81	0.86	0.81
VAL	14.60	14.28	15.81	13.69	11.40	12.77
MET	3.66	3.14	3.70	3.76	3.32	2.57
CYSH	0.65	0.53	0.71	0.48	0.52	0.46
ILE	6.56	7.15	7.48	7.69	5.93	5.85
LEU	6.50	6.20	7.00	6.82	4.73	4.92
TYR	5.15	4.54	4.61	4.88	4.18	3.79
PHE	4.72	3.87	4.10	4.58	4.05	4.00
ORN	4.94	3.48	4.58	4.36	4.41	4.22
LYS	10.59	7.63	10.92	12.63	11.71	7.26
HIS	10.18	7.14	7.78	9.93	10.39	7.25
ARG	11.97	10.62	12.87	13.30	10.28	8.79

APPENDIX TABLE 1.1 continued:

	Diet: WHC			Methionine: HMet		
	I_O			I_G		
	T_0	$T_{2.5}$	T_5	T_0	$T_{2.5}$	T_5
TAU	16.72	16.02	17.12	17.71	13.85	17.17
THR	9.26	6.90	9.54	9.81	6.19	4.88
SER	5.95	6.40	8.42	7.94	6.55	4.56
GLU	14.47	21.00	32.01	13.55	12.79	20.27
GLY	42.35	39.06	60.61	37.44	31.29	31.53
ALA	12.28	13.86	20.98	11.31	7.80	12.28
ABU	1.06	1.20	1.51	0.68	0.88	0.90
VAL	11.99	13.01	18.24	11.04	8.01	8.47
MET	6.20	4.82	6.27	5.92	4.10	4.26
CYSH	1.15	1.15	1.13	1,12	0.97	1.06
ILE	5.46	5.85	5.44	4.82	3.41	3.67
LEU	4.93	5.45	4.71	4.73	3.35	3.74
TYR	4.47	3.97	3.67	3.91	2.75	2.92
PHE	4.21	4.39	4.26	4.28	3.55	3.77
ORN	3.91	4.09	4.54	2.91	3.31	2.91
LYS	8.55	7.17	7.17	8.32	6.68	5.21
HIS	6.64	6.48	6.74	8.39	8.81	5.85
ARG	11.72	12.06	10.67	10.88	8.12	7.78

APPENDIX TABLE 1.1 continued:

	Diet: CH			Methionine: OM (Control)		
	I_O			I_G		
	T_0	$T_{2.5}$	T_5	T_0	$T_{2.5}$	T_5
TAU	2.87	2.44	2.40	3.04	2.80	2.90
THR	24.56	22.85	25.04	27.98	20.69	22.74
SER	12.33	12.26	12.60	12.84	9.63	10.73
GLU	18.40	21.20	26.23	15.07	12.72	19.77
GLY	62.89	57.53	67.31	59.78	52.06	55.94
ALA	21.06	20.84	23.43	21.94	15.55	18.99
ABU	0.99	0.94	1.42	0,45	0.81	0.84
VAL	30.42	29.04	34.42	31.11	25.49	29.10
MET	1.52	1.37	1.50	2.21	1.24	1.53
CYSH	0.22	tr.	tr.	tr.	ND	tr.
ILE	11.06	10.93	11.81	11.35	7.85	10.93
LEU	15.75	15.19	17.02	16.25	10.94	15.35
TYR	8.17	6.62	7.07	8.08	5.49	6.75
PHE	6.00	5.47	5.96	6.44	4.19	5.57
ORN	9.74	7.57	7.87	9.42	7.02	5.43
LYS	11.65	10.33	13.48	14.06	7.91	8.99
HIS	9.25	6.15	7.04	9.32	11.56	5.92
ARG	15.99	15.02	14.40	15.68	10.43	12.25

APPENDIX TABLE 1.1 continued:

	Diet: CH			Methionine: LMet		
	I_O			I_C		
	T_0	$T_{2.5}$	T_5	T_0	$T_{2.5}$	T_5
TAU	3.40	2.57	3.33	3.72	3.21	2.82
THR	21.04	16.41	18.08	14.81	11.09	10.11
SER	11.88	8.45	8.09	7.03	5.01	4.68
GLU	18.85	19.77	22.81	15.41	11.36	21.20
GLY	55.24	46.03	49.95	43.69	32.49	30.66
ALA	19.81	17.66	21.00	15.52	12.29	14.35
ABU	1.45	0.98	1.09	1.27	0.72	0.81
VAL	28.41	22.63	27.28	24.38	15.58	18.55
MET	3.11	2.30	2.28	2.05	1.56	2.17
CYSH	0.50	0.28	0.32	0.40	tr.	tr.
ILE	9.50	8.11	9.88	7.18	4.42	7.09
LEU	14.02	11.86	13.33	11.50	6.41	9.87
TYR	8.02	6.15	6.80	5.90	3.65	5.18
PHE	5.91	4.59	5.26	5.63	3.68	4.85
ORN	7.97	5.41	5.39	7.24	4.03	3.89
LYS	11.19	7.85	9.38	9.48	5.95	5.77
HIS	8.09	6.34	6.97	8.62	9.04	6.15
ARG	14.75	10.69	12.65	13.19	8.61	10.20

APPENDIX TABLE 1.1 continued:

	Diet: CH			Methionine: HMet		
		I_O			I_G	
	T_0	$T_{2.5}$	T_5	T_0	$T_{2.5}$	T_5
TAU	10.60	6.38	8.88	9.34	8.12	8.64
THR	17.01	10.49	11.23	22.30	15.69	14.52
SER	6.73	4.33	4.56	6.12	5.63	6.17
GLU	16.84	17.40	21.60	14.85	14.38	24.88
GLY	39.59	30.29	30.02	40.19	34.00	35.35
ALA	18.84	14.02	15.01	18.56	14.86	17.77
ABU	1.45	0.95	1.26	1.11	1.02	1.23
VAL	24.85	16.79	18.87	23.16	17.27	20.06
MET	4.98	2.76	3.30	4.82	3.43	4.42
CYSH	0.82	0.76	0.62	0.63	0.65	0.74
ILE	10.12	6.15	7.45	9.10	6.27	8.22
LEU	13.59	8.41	9.26	11.90	7.56	9.68
TYR	7.40	4.61	4.69	6.40	4.59	5.60
PHE	6.62	3.72	4.33	6.28	4.44	5.21
ORN	7.31	4.13	5.10	6.39	5.04	4.18
LYS	9.78	5.52	6.51	12.73	6.81	6.72
HIS	8.62	5.31	5.55	8.62	7.90	7.48
ARG	15.48	10.60	11.14	15.55	10.21	12.47

APPENDIX TABLE 1.2: Plasma concentrations of glucose (mg glucose/100ml plasma) for each individual animal at each sampling during the experiment.

		T_0		$T_{2.5}$		T_5		T_8	
		I_0	I_G	I_0	I_G	I_0	I_G	I_0	I_G
Diet:	WHC								
Control	B	51.0	57.0	61.0	148	57.5	49.0	58.0	54.0
	F	43.0	57.0	58.0	159	56.5	87.0	75.0	69.5
	K	61.0	55.0	60.5	156	61.5	60.0	71.0	72.5
	C	55.0	65.0	63.5	156	60.0	86.5	69.0	48.0
LMet	B	59.0	63.5	65.0	157	55.5	66.0	63.5	59.5
	F	62.0	48.0	65.0	157	69.5	57.0	65.5	62.5
	K	63.0	55.0	54.5	156	55.0	60.0	67.0	55.5
	C	65.0	51.0	69.0	153	62.5	52.0	65.5	53.0
HMet	B	63.5	57.5	58.0	147	62.0	42.0	64.0	55.0
	F	46.0	52.0	62.0	155	58.0	62.0	60.0	65.0
	K	56.5	61.5	59.0	156	57.5	55.5	59.0	57.0
	C	54.5	54.0	57.0	156	59.0	59.5	58.5	48.0
Diet:	CH								
Control	E	55.0	60.0	65.5	157	66.5	60.5	65.0	61.0
	U	61.5	66.0	71.5	150	64.5	56.5	73.5	65.5
	H	54.0	71.0	63.5	157	62.0	55.5	65.0	56.5
	N	43.0	67.0	51.0	139	52.0	56.5	63.0	52.5
LMet	E	58.0	73.0	77.5	159	63.0	56.5	69.0	63.0
	U	58.0	68.0	70.5	123	64.0	55.0	54.0	58.0
	H	51.5	57.5	51.5	156	46.0	48.0	62.0	53.0
	N	59.0	50.0	68.5	155	60.0	49.0	63.5	61.0
HMet	E	64.0	74.5	69.5	156	69.5	54.0	69.5	56.5
	U	56.5	55.5	65.0	157	67.5	62.0	65.0	62.0
	H	48.0	59.0	66.0	157	70.5	64.0	61.5	56.0
	N	61.0	59.5	59.5	157	57.5	51.0	60.5	49.5

Control - water infusion; LMet - 0.08g L-methionine/$kg^{0.75}$/day; HMet - 0.16g L-methionine/$kg^{0.75}$/day. I_0 - no glucose infusion; I_G - intravenous glucose at rate of 0.5g D-glucose/$kg^{0.75}$/hour for 2 hours between $T_{0.5}$ and $T_{2.5}$. WHC - wheat hay chaff; CH - clover hay.

APPENDIX 2

Surgical preparation of sheep with intra-abomasal tubes

The traditional method of abomasal canulation severely inhibits the mobility of the abomasum within the gut cavity, and the abomasal tube preparation was intended to provide a less severe surgical preparation. Although a numberof animals were maintained with abomasal tubes for periods of six months or longer without any apparent trouble, three animals rejected the preparation following a period of infection which did not respond to antibiotic treatment. Consequently the intra-abomasal tube was much less satisfactory than the traditional method of abomasal canulation.

The details of the surgical preparation follow.

Tube: The tube used was a heavy-walled PVC (external diameter 5.5mm and internal diameter 3.0mm) of about 40cm in length. The tube was secured to a piece of Dacron felt (Spec. No. 54, Permeability 7l/cm; Troy Melb. New Hampshire) using a Silastic* adhesive (Silastic Adhesive B). An oval portion of Dacron felt (6cm in length and 2cm in width) with a small opening (through which to pass the tube) about 2cm from one end was used. The tube was passed through the opening such that about 5cm would be left free in the abomasum. The tube was then secured to the Dacron felt. The abomasal end of the tube was slit in a number of places to permit flow of infusate should the end of the tube become blocked with digesta.

Surgery: Anaesthesia was induced and maintained with sodium pentobarbitone (Nembutal) administered via the jugular vein.

A lateral incision was made on the right side of the sheep about 4cm behind the last rib. The muscle layers were parted by blunt disection and the peritoneum exposed. The peritoneum was clamped with Mosquito forceps at each end of the incision and then cut along the line between the forceps. The abomasum was then exteriorised. A suitable position for the implantation of the tube was selected and a ring of Murphy sutures about 1cm in diameter placed in the serosal layer of the abomasal wall using Chromic 4/0 gut. A small slit was then cut within the suture ring, the tube inserted into the abomasum and the ends of the suture drawn up firmly and tied. The Dacron felt was then sutured into the serosal layer of the abomasal wall. The free end of the tube was then passed out through a stab wound in the side of the animal and the peritoneum, muscle layers and skin sutured.

The sheep were returned to their pens and resumed eating within 1-3 hours. By the second or third day following surgery, intakes had usually returned to normal levels. Routine post-operative care was carried out and all sheep received a course of antibiotics over the three days after surgery.

APPENDIX 3

APPENDIX TABLE 3.1 (Experiment 2/2): Plasma amino acid concentrations in samples taken on day 3 of the control (MO) and methionine infusion M1.4, M4.2) periods.

	Sheep 761		Sheep 663			Sheep 748		
	MO	M1.4	MO	M1.4	M4.2	MO	M1.4	M4.2
TAU	0.71	1.35	6.39	2.67	15.79	3.94	1.11	16.25
THR	15.91	5.71	14.78	7.11	10.76	21.13	8.81	9.08
SER	15.95	8.41	16.42	9.12	6.82	19.82	8.76	9.24
GLU	20.80	19.60	36.13	24.96	19.01	38.64	26.03	17.20
GLY	92.64	56.45	126.2	81.97	50.94	117.4	68.97	52.39
ALA	24.55	13.23	24.28	15.38	25.01	29.13	15.94	15.75
ABU	tr.	0.25	1.52	2.31	1.82	1.74	1.45	1.78
VAL	16.00	7.29	20.92	12.55	9.98	21.31	10.01	8.81
½CYS	1.72	2.66	2.48	4.46	4.91	2.77	4.54	4.73
MET	1.94	2.13	1.88	6.92	123.3	2.25	6.31	94.74
CYSH	tr.	tr.	tr.	1.05	2.02	ND	1.03	3.33
ILE	7.63	4.56	8.53	6.24	3.76	9.35	3.59	3.69
LEU	7.38	2.93	9.40	4.42	3.21	11.16	3.08	3.55
TYR	4.94	2.95	6.65	6.62	5.20	6.43	4.80	4.46
PHE	3.78	2.99	4.48	4.37	4.56	5.19	3.68	4.34
ORN	6.24	2.74	7.26	4.63	6.20	8.22	4.11	4.59
LYS	16.85	7.72	19.75	11.34	14.57	17.71	7.64	10.98
HIS	17.64	11.81	23.07	20.88	22.48	22.70	18.19	14.17
ARG	11.20	8.13	12.82	9.06	10.44	11.41	7.07	7.16

ND - not detectable; tr. - trace.

APPENDIX TABLE 3.2 (Experiment 2/2): Blood cell amino acid concentrations (μ moles/100ml blood cells).

	Sheep 761				Sheep 663				
	M1.4 3	M4.2 3	M4.2 8	M4.2 10	M1.4 3	M4.2 3	M8.4 3	M8.4 8	M8.4 11
TAU	22.51	43.37	37.28	38.10	44.47	50.28	57.41	51.22	49.78
THR	11.46	10.35	21.89	33.43	5.44	7.21	15.76	13.67	14.72
SER	7.18	11.63	14.78	13.36	5.04	7.92	17.50	11.81	84.49*
GLU	19.18	11.55	11.95	12.90	11.74	10.08	61.33*	109.4*	29.32
GLY	83.20	71.76	65.50	48.42	40.93	52.89	52.07	62.36	50.95
ALA	37.76	19.30	20.70	23.85	18.32	20.30	33.47	28.67	28.38
ABU	2.64	2.64	1.40	0.91	3.18	1.03	0.65	0.49	2.23
VAL	11.69	9.44	13.63	13.23	14.40	7.96	13.36	14.60	13.25
½CYS	ND	ND	ND	0.89	2.85	1.85	NM	NM	NM
MET	1.19	1.91	82.31	53.67	1.64	28.04	133.4	72.04	119.4
CYSH	1.38	0.89	1.26	1.60	0.20	0.35	0.99	0.84	0.72
ILE	5.71	3.65	3.05	2.70	5.87	7.01	5.07	4.95	3.59
LEU	9.89	7.69	10.84	9.30	15.47	8.12	22.05	24.09	23.10
TYR	3.64	3.13	4.29	4.79	6.54	4.70	9.45	7.64	6.63
PHE	4.82	4.12	4.71	4.12	5.92	4.00	7.55	6.91	6.10
ORN	23.39	17.58	26.38	26.53	4.83	1.74	5.10	4.46	3.32
LYS	18.54	15.09	27.29	23.29	22.56	17.38	31.85	33.25	32.52
HIS	17.57	13.94	16.98	18.54	22.52	12.43	21.96	28.31	24.54
ARG	0.37	0.32	0.32	0.20	25.01	16.37	35.14	48.27	41.86

* Peaks not pure; a major contaminant of the serine or glutamic acid peak was present in these samples.

APPENDIX TABLE 3.2 (Experiment 2/2) continued:

	Sheep 748						
	M1.4 3	M4.2 3	M8.4 3	M8.4 5	M8.4 8	M8.4 11	M8.4 17
TAU	52.89	51.46	59.43	66.35	67.25	63.64	62.11
THR	15.18	13.32	17.13	16.72	59.28	56.47	17.00
SER	16.78	14.34	13.25	15.30	16.08	16.18	20.31
GLU	20.00	15.97	15.59	16.25	13.81	20.25	15.33
GLY	84.87	57.60	45.75	51.16	57.62	54.27	57.29
ALA	36.90	33.76	29.67	32.30	33.57	33.98	28.53
ABU	3.20	1.07	0.99	0.90	1.77	3.14	2.22
VAL	20.21	12.54	10.51	13.05	13.35	13.47	13.05
½CYS	4.19	3.40	NM	NM	NM	NM	NM
MET	3.50	52.26	129.3	139.8	168.7	154.5	189.1
CYSH	0.27	0.25	3.22	2.17	1.19	1.07	3.74
ILE	6.15	3.41	3.09	3.71	3.44	4.00	2.35
LEU	21.09	14.51	16.04	20.23	18.89	20.60	20.63
TYR	8.06	6.86	6.25	6.95	7.63	8.06	6.78
PHE	8.35	6.10	6.94	7.51	7.04	8.00	6.52
ORN	8.11	7.14	6.56	6.08	7.70	5.08	6.33
LYS	27.18	17.82	20.07	23.08	26.16	23.57	26.53
HIS	21.40	16.57	15.16	21.33	21.90	19.75	22.17
ARG	3.47	1.89	1.72	2.38	2.55	2.60	1.65

NM - not measurable.

APPENDIX TABLE 4.1: Individual values for organic matter intake (OMI), apparent digestibility of organic matter and feed nitrogen, nitrogen retention and urine N excretion (ratio to absorbed N) for all periods of the experiment. Amino acid treatments are described by the amino acid which was omitted from the infusion.

	OMI ($g/kg^{0.75}$/day)	App. digestibility of OM(%)	Feed N(%)	N retention ($g/kg^{0.75}$/day)	Urine N (ratio to absorbed N)
HC					
648	55.8	60.22	52.16	0.068	0.790
768	60.3	59.87	40.48	0.035	0.867
655	64.6	58.96	49.43	0.063	0.821
669	65.6	60.37	47.03	0.091	0.729
664	54.6	58.60	46.33	-0.049	1.190
759	59.2	59.08	47.14	0.006	0.981
760	52.9	57.88	43.67	-0.035	1.140
NC					
648	58.5	60.52	46.89	0.139	0.723
768	55.3	61.36	40.80	0.063	0.868
655	62.3	58.68	45.85	0.066	0.872
669	64.3	62.83	53.83	0.205	0.640
664	54.2	61.42	48.89	0.026	0.949
759	58.6	60.15	51.43	0.109	0.792
760	50.9	60.15	44.02	0.017	0.966
A1					
648 (LEU)	60.4	60.90	58.59	0.339	0.459
768 (THR)	47.3	60.79	47.60	0.116	0.771
655 (VAL)	64.1	57.77	47.61	0.241	0.560
669 (ILE)	57.0	61.06	47.23	0.207	0.552
664 (TRP)	49.6	60.04	41.98	0.146	0.681
759 (LYS)	63.8	61.79	52.58	0.235	0.590
760 (MET)	52.6	57.82	43.21	0.045	0.908
A2					
648 (ILE)	64.2	59.24	60.39	0.388	0.408
768 (TRP)	54.0	60.18	41.84	0.224	0.515
655 (LYS)	68.3	58.60	54.90	0.324	0.498
669 (MET)	50.6	61.83	56.18	0.203	0.623
664 (LEU)	49.3	61.99	56.34	0.238	0.579
759 (THR)	60.7	59.42	54.44	0.260	0.560
760 (VAL)	56.6	60.42	48.30	0.289	0.488

APPENDIX TABLE 4.1 continued:

	OMI ($g/kg^{0.75}$/day	App. digestibility of OM(%)	Feed N(%)	N retention ($g/kg^{0.75}$/day)	Urine N (ratio to absorbed N)
A3					
648 (TRP)	61.9	59.71	54.27	0.245	0.558
768 (LYS)	60.1	62.91	49.22	0.272	0.513
655 (MET)	61.9	57.86	50.84	0.124	0.771
669 (LEU)	47.3	64.33	46.34	0.132	0.701
664 (THR)	53.4	63.32	56.72	0.226	0.588
759 (VAL)	56.8	62.43	54.54	0.253	0.524
760 (ILE)	54.4	56.71	43.00	0.177	0.645
A4					
648 (THR)	60.6	60.44	58.79	0.301	0.484
768 (VAL)	61.4	61.99	50.49	0.266	0.510
655 (ILE)	68.4	59.49	52.90	0.273	0.525
669 (TRP)	54.2	63.34	52.70	0.265	0.470
664 (LYS)	59.2	59.93	52.37	0.223	0.601
759 (MET)	45.7	59.37	58.91	0.128	0.733
760 (LEU)	57.5	58.35	43.42	0.112	0.770

APPENDIX 5

APPENDIX TABLE 5.1 (Experiment 5/1): Individual animal values for nitrogen supply (feed N intake + methionine N infused) and urine and faecal nitrogen excretion for the sheep fed the barley straw diet in the control period (BC) and in the period of methionine infusion (0.12g L-methionine/$kg^{0.75}$/day).

	Nitrogen parameter (g/day)					
	BC			BM		
	Supply	Urine	Faeces	Supply	Urine	Faeces
574	0.93	2.84	1.95	1.00	1.72	2.00
582	1.42	2.01	2.06	1.78	1.25	2.21
378	1.48	1.74	2.10	1.44	0.91	1.98
408	1.90	1.52	2.68	2.14	1.20	2.62
581	1.20	2.23	1.55	1.55	1.25	1.82
365	1.40	2.30	2.07	1.79	1.12	2.30
393	1.79	1.58	2.73	1.79	1.34	2.51
402	1.59	1.82	2.43	1.81	1.13	2.27
567	1.66	1.96	2.50	1.82	1.56	2.37
389	1.80	1.89	2.60	1.85	1.31	2.34
583	1.24	2.03	1.84	1.69	1.15	2.08
577	1.08	2.20	1.68	1.57	1.19	1.82
580	0.87	2.89	1.62	1.75	1.53	2.16
558	1.19	1.95	1.76	1.79	1.42	2.06
462	0.89	2.33	1.28	0.94	1.76	1.29
612	0.95	2.04	1.56	1.03	1.30	1.54
568	1.13	2.20	1.83	1.19	1.40	1.80
508	1.53	1.54	2.49	1.96	1.16	2.65
445	1.29	1.91	2.05	1.48	1.37	1.94
585	1.28	1.59	1.97	1.48	1.18	2.07
564	0.64	1.78	1.14	0.94	1.10	1.26

N supply for days 3 to 9; urine N for urine collected on days 5 to 11; faeces N for faeces collected on days 6 to 12.

APPENDIX TABLE 5.2 (Experiment 5/2): Individual animal values for nitrogen supply (feed N intake + methionine N) and urine and faecal nitrogen excretion for the sheep fed the barley straw/wheaten hay chaff diet in the control period and in the periods of methionine infusion (WM - 0.12g L-methionine/$kg^{0.75}$/day; WH - 0.36g L-methionine/$kg^{0.75}$/day).

	Nitrogen parameter (g/day)								
	WC			WM			WH		
	Supply	Urine	Faeces	Supply	Urine	Faeces	Supply	Urine	Faeces
574	1.91	1.64	2.60	2.14	1.21	2.16	2.92	1.28	2.27
582	2.97	1.37	2.74	3.40	0.84	2.66	3.56	1.27	2.66
378	3.48	1.27	2.92	3.44	0.77	2.70	3.31	1.23	2.50
408	4.13	1.17	3.32	4.05	1.05	3.05	4.57	1.38	2.98
581	2.81	1.40	2.67	3.21	0.97	2.51	2.48	2.10	1.77
365	3.88	1.46	3.10	4.01	0.95	2.95	4.31	1.43	2.93
393	4.03	1.55	3.43	4.79	1.01	3.86	4.65	1.25	3.44
402	3.62	1.37	3.05	3.71	1.24	2.85	3.49	2.66	2.38
567	2.65	1.57	2.37	2.96	1.51	2.60	3.68	2.08	2.49
389	3.69	1.49	3.21	4.29	1.02	3.41	3.53	2.26	2.28
583	3.33	1.29	2.87	3.70	0.94	2.84	3.60	1.41	2.54
577	2.16	1.83	1.88	2.43	1.15	1.82	2.34	1.69	1.89
580	3.10	1.52	3.05	3.69	1.00	2.88	3.58	1.46	2.61
558	4.13	0.92	3.27	4.12	0.86	3.06	3.86	1.25	2.98
462	2.35	1.43	2.53	2.43	1.15	1.97	2.00	2.01	1.12
612	2.14	1.53	2.06	2.06	1.41	1.89	2.31	1.54	1.82
568	2.49	1.30	2.09	2.46	0.90	1.81	2.22	2.53	1.09
508	4.45	0.96	3.90	4.50	0.88	3.58	4.49	1.07	3.53
445	3.12	1.29	2.72	3.34	1.18	2.63	3.37	1.59	2.55
585	2.73	1.41	2.67	2.68	1.50	2.23	2.80	1.64	2.10
564	1.42	1.20	1.13	1.77	1.01	1.53	2.22	1.31	1.61

N supply for days 3 to 9; urine N for urine collected on days 5 to 11; faeces N for faeces collected on days 6 to 12.

APPENDIX 6

APPENDIX TABLE 6.1: Values for the apparent digestibility of organic matter (dig. OM%) and the apparent digestibility of nitrogen (dig. N%) for the twelve sheep in the control and treatment periods.

	Dig. OM%		Dig. N%	
	Control	Treatment	Control	Treatment
[1] OMET				
445	54.45	53.82	36.10	37.36
582	53.95	50.30	36.82	26.53
365	56.83	57.36	39.08	39.08
564	- [2]	60.12	-	29.16
LMET				
580	54.53	52.26	31.58	31.23
389	56.54	57.10	35.63	39.57
558	59.22	54.30	40.66	33.59
393	54.30	55.95	29.78	35.38
HMET				
462	49.08	60.73	26.60	46.29
567	59.19	58.75	38.08	38.81
583	56.25	58.82	36.46	40.43
378	54.80	63.80	32.67	45.23

1) Infusion during the treatment period:
OMET - control water infusion;
LMET - low methionine 0.12g L-met/$kg^{0.75}$/day;
HMET - high methionine 0.36g L-met/$kg^{0.75}$/day.

2) Urine and faeces samples discarded in error.

APPENDIX TABLE 6.2: Weights of organs and tissues taken from the twelve sheep at slaughter (weights expressed as a percentage of the metabolic bodysize ($kg^{0.75}$)).

	Liveweight $kg^{0.75}$	Weight (percentage of liveweight $kg^{0.75}$) Dry weight Liver	Organ Wet Weights Liver	Kidneys	Spleen	Diaphragm	Heart
[1]OMET							
445	10.0	0.851	2.99	0.740	0.520	1.05	1.33
582	12.5	0.827	3.00	0.768	0.432	1.20	1.31
365	12.3	0.811	3.02	0.764	0.333	1.07	1.24
564	10.5	0.880	3.22	0.914	0.495	0.952	1.48
LMET							
580	10.9	0.861	3.37	0.734	0.560	1.07	0.927
389	13.6	0.943	3.51	0.596	0.926	1.18	1.15
393	12.7	0.927	3.35	0.717	0.417	1.14	1.45
558	8.9	0.834	3.16	0.753	0.573	1.30	1.19
HMET							
462	11.0	0.751	2.79	0.818	0.382	0.964	1.07
567	12.8	0.978	3.62	0.781	0.453	1.03	1.30
378	12.5	0.971	3.57	0.680	0.400	0.944	1.44
583	11.8	0.918	3.34	0.797	0.508	0.983	1.15

1)
Infusion during the treatment period;
OMET - control water infusion;
LMET - low methionine, 0.12g L-met/$kg^{0.75}$/day;
HMET - high methionine - 0.36g L-met/$kg^{0.75}$/day.

APPENDIX TABLE 6.3: Total plasma ^{35}S and ^{3}H activity at various times during the isotope infusion (all values corrected to standard total isotope infusions of 500 x 10^6 dpm ^{35}S and 750 x 10^6 dpm ^{3}H).

	OMET[1]		LMET		HMET	
	445	582	580	389	462	567
Time	Total plasma ^{35}S activity (dpm x 10^3/ml)					
8m	1.29	1.29	0.58	1.55	1.81	0.53
16	1.91	1.53	1.27	2.23	2.62	0.98
30	1.27	1.81	1.53	2.79	3.01	2.01
60	1.57	2.01	2.20	4.36	4.18	2.25
2h	2.55	2.14	2.52	4.78	6.15	5.18
4	2.29	2.29	3.44	5.58	10.25	7.21
6	2.68	2.82	3.80	7.15	12.36	9.12
8	2.71	3.48	3.52	7.75	15.23	12.68
10	3.02	3.80	3.97	10.48	18.90	14.87
12	4.23	4.35	4.60	12.73	20.55	16.84
14	5.46	3.98	5.28	11.44	21.65	19.04
15	4.53	4.28	5.37	9.62	21.33	19.81
	Total plasma ^{3}H activity (dpm x 10^3/ml)					
8m	2.04	2.63	1.09	2.10	2.02	0.70
16	2.72	2.87	2.04	3.37	2.73	1.24
30	2.75	3.70	1.91	4.45	3.77	2.41
60	4.30	3.99	4.15	6.55	4.51	2.76
2h	6.90	4.35	5.52	6.17	6.18	4.42
4	6.75	5.32	7.80	6.85	8.45	5.87
6	8.29	8.47	8.96	8.78	10.66	6.80
8	9.82	7.47	9.62	8.60	11.72	9.00
10	11.21	9.07	10.86	11.78	13.36	9.51
12	13.43	9.51	13.54	13.67	14.68	11.60
14	17.33	10.13	14.90	15.35	17.76	12.41
15	15.27	11.77	14.64	13.10	17.03	13.41

1) Infusion during the treatment period; OMET - control, water infusion; LMET - low methionine, 0.12g L-met/$kg^{0.75}$/day; HMET - high methionine, 0.36g L-met/$kg^{0.75}$/day.

APPENDIX TABLE 6.4: Urine outputs of ^{35}S activity and ^{3}H activity as a percentage of the total infusion of each isotope over the period of the isotope infusion.

	OMET		LMET		HMET	
	445	582	580	389	462	567
^{35}S	2.99	8.29	4.66	6.32	6.12	9.73
^{3}H	1.68	5.75	1.93	2.20	1.99	2.67

APPENDIX TABLE 6.5: Total radioactivity counts (dpm x 10^3/g dry tissue) in samples of tissue taken at slaughter.

	OMET		LMET		HMET	
	445	582	580	389	462	567
	Tissue radioactivity ^{35}S x 10^3/g dry tissue					
Pancreas	1132	821	1296	730	530	358
Intestine	903	686	592	413	368	240
Liver	679	465	611	449	651	484
Kidneys	586	463	477	271	295	213
Skin	98.3	133	264	209	191	121
Heart	154	139	122	107	171	120
Diaphragm	74.2	57.1	78.8	55.3	144	126
Muscle	35.0	32.4	66.2	46.4	109	89.2
	Tissue radioactivity ^{3}H x 10^3/g dry tissue					
Pancreas	1744	1248	1655	1463	1261	1192
Intestine	772	661	782	431	536	496
Liver	463	303	476	312	398	357
Kidneys	914	643	713	450	560	515
Skin	187	273	386	337	321	185
	Ratio of radioactivity ^{35}S: ^{3}H					
Infusate	0.67	0.67	0.67	0.67	0.67	0.67
Pancreas	0.65	0.66	0.78	0.50	0.42	0.30
Intestine	1.17	1.04	0.76	0.96	0.69	0.48
Liver	1.47	1.53	1.28	1.44	1.64	1.36
Kidneys	0.64	0.72	0.67	0.60	0.53	0.41
Skin	0.53	0.49	0.68	0.62	0.60	0.65

Note: Samples were freeze-dried and prepared for counting by the method of Mahin & Lofberg (1966). The values for the ^{3}H activity in the samples of skeletal muscle, heart and diaphragm were highly variable and for this reason are not presented.
All values have been corrected to a standard total infusion of 500 x 10^6 dpm of ^{35}S and 750 x 10^6 dpm of ^{3}H.

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