A STUDY OF THE AMINO ACID STATUS OF SHEEP FED SILAGE, WITH PARTICULAR REFERENCE TO METHIONINE

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by

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ABSTRACT

The amino acid status of sheep fed silage appears to be an important factor in controlling voluntary intake. The basic aim of the experiments described in this thesis, was to study the digestion and utilisation of silage, in response to increased availability of methionine and protein. The treatments chosen to increase protein availability, were formalin-treatment of silage (Expt. 1) and supplements of formaldehyde-treated casein and a readily available energy source (Expt. 2). Methionine availability on untreated silage was increased by I/P infusion in Expt. 1, and methionine availability on all three treatments was increased by duodenal infusion in Expt. 2. These treatments provided from 0.8 to 6.0g/day of methionine available for absorption, and from 33 to 167g/day of total amino acids entering the duodenum. The response to increased methionine availability was measured in terms of N balance, plasma amino acid concentration and the percentage of L-methionine-C14 (U) oxidised to carbon dioxide.

Intake was restricted to near maintenance, but the amount offered was seldom completely eaten. There were no treatment differences in intake in Expt. 1, but intake of the basal silage diet decreased in Expt. 2. This decrease was partly prevented by the two supplements, and intake was increased by duodenal infusion of L-methionine on all treatments. The differences in amino acid intake accounted for 67% of the difference in flow of amino acids into the duodenum in the data from both experiments. In addition, the energy supplement increased the flow of amino acids into the duodenum, apparently by increasing the conversion of non-protein-nitrogen to protein-nitrogen in the rumen. An adequate supply of amino acids to the tissues is required for efficient utilisation of a feed. Thus, high amino acid and water soluble sugar

contents will improve the nutritive value of silage, by increasing the flow of amino acids into the duodenum.

N balance increased, and total plasma amino acid concentration decreased, in response to methionine infusion on all three treatments in Expt. 2. These results suggest that methionine was limiting tissue protein synthesis. Intake, was also decreased, but to a greater extent at lower levels of duodenal amino acid flow in Expt. 2. However, there was no depression of intake in Expt. 1, despite low flow rates of amino acids into the duodenum. This appeared to be due to a lower requirement for methionine in the sheep in Expt. 1, compared to Expt. 2.

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ABBREVIATIONS

AA	amino acid
C	carbon
DAP	2:6 diaminopimelic acid
DM	dry matter
DNA	deoxyribonucleic acid
I/P	intraperitoneal
ME	metabolisable energy
MHA	methionine - hydroxy - analogue
N	nitrogen
NA	nucleic acid
NAAN	non-amino acid-N
NPN	non-protein-N
OM	organic matter
RNA	ribonucleic acid
S	sulphur
SA	specific activity
WSC	water soluble carbohydrates
w/v	weight per volume
w/w	weight per weight

The use of domestic animals to provide resources for the human population has been a part of agriculture for some 10,000 years. However, the increase in the world's population and hence the relative decrease in land and food resources emphasise the need for increasing the efficiency of food production.

Ruminants make an important contribution to the efficient utilisation of resources, by the digestion of plant cell wall carbohydrates and by the conversion of non-protein-nitrogen (NPN) compounds to meat, wool and milk. The initial conversion of NPN to protein is achieved by microbes in the rumen. However, the efficiency of microbial protein synthesis in satisfying the amino acid requirements of ruminant tissues has only recently become the subject of detailed study. The main reason for the delay in studying ruminant amino acid requirements was this ability of the microbes to modify dietary protein. Hence, amino acid requirements must be specified at a duodenal rather than a dietary, level for ruminants. This has only recently become possible, through the use of sheep prepared with re-entrant cannulae in the proximal duodenum (Ash, 1962).

Numerous workers have suggested that methionine is the first limiting amino acid for live-weight gain and wool growth on a variety of ruminant diets (Nimrick et al., 1970; Hutton and Annison, 1972; Armstrong and Annison, 1973; Harrison et al., 1973). In addition, Barry et al. (1973) reported that the intake of some silages was increased in response to intraperitoneal injections of methionine. Hence, the availability of methionine on such feeds appears to limit the efficiency of their utilisation.

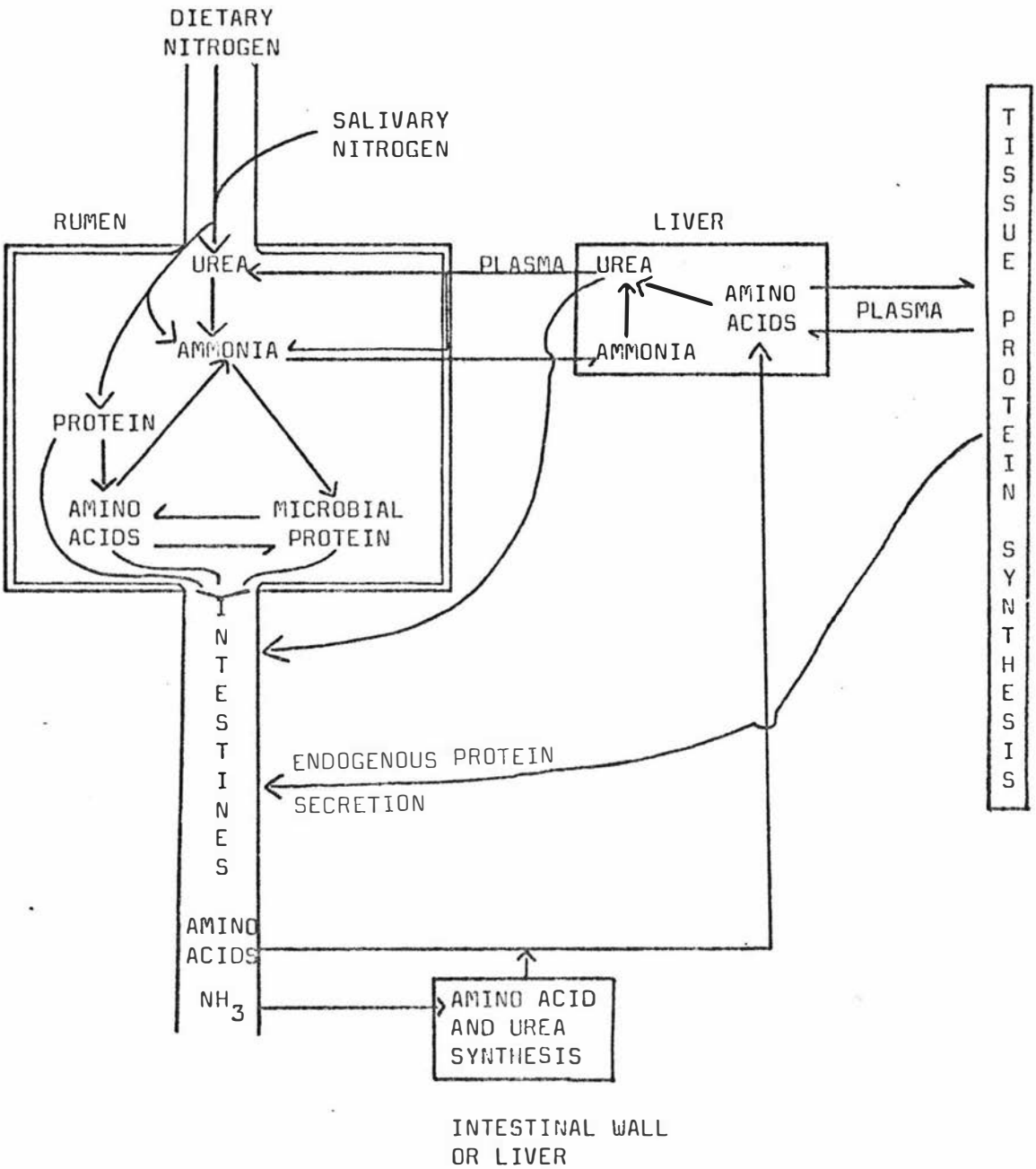
This thesis will examine the utilisation of nitrogen and amino acids, with particular emphasis on methionine, by sheep fed silage,

alone, and with supplements to increase the availability of methionine and total amino acids to the tissues.

CHAPTER 1

LITERATURE REVIEW

Fig. 1.1 N recycling in ruminants



1.1 Nitrogen metabolism in the rumen

The metabolism of nitrogen (N) in ruminants has received considerable attention from biochemists, microbiologists and nutritionists, and numerous reviews on various aspects of the subject have been written (e.g. Waldo, 1968; Smith, 1969; Allison, 1970; Helmer and Bartley, 1971). In addition, quantitative models of N metabolism in sheep have been proposed (Nolan, 1975; Nolan et al., 1976; Mazanov and Nolan, 1976). Thus, only a brief outline of the aspects of N metabolism which are pertinent to the present experiments will be given here.

A simplified diagram of the main N transactions in sheep is given in Fig. 1.1. The main reactions occurring in the rumen can be summarised as follows.

A. N entering the rumen

N may enter the rumen in the diet, in saliva or by transfer across the rumen wall. The major components of the N fractions of herbage are amino acids, present either as free amino acids or as part of protein molecules. However, silage differs from fresh feeds in having relatively high ammonia, amine and amide contents (Hughes, 1970). The N entering the rumen in saliva is also mainly in the form of NPN, with urea accounting for 60-70% of the total N secreted (Somers, 1961a). However, the proportion of urea in total saliva-N is affected by N intake (Somers, 1961c). Somers (1961b) had earlier shown that while daily secretion of urea-N was linearly related to N intake, daily secretion of total saliva-N did not follow a simple linear relationship. Saliva urea-N concentration appeared to follow blood urea-N concentration, but no simple relationship between these two factors was evident

(Somers, 1961b). Nolan et al. (1973) and Thornton (1970) stressed the relative importance of saliva-N compared to transfer of urea across the rumen wall. Houpt and Houpt (1968) found a positive linear relationship between net transfer of urea across the rumen wall and the concentration gradient between plasma and rumen, but other workers have reported limits to this transfer (Weston and Hogan, 1967; Vercoe, 1969).

B. Protein and amino acid degradation

The dietary protein may either be degraded to amino acids, fatty acids and ammonia, or it may leave the rumen undegraded. Differences in the rate of ruminal fermentation of proteins have been recorded by McDonald (1952) and Henderickx and Martin (1963), who showed that high solubility was correlated with a rapid rate of fermentation. The solubility of dietary protein, and hence the rate of ruminal degradation has been decreased by heat treatment of the protein (Tagari et al., 1962) and by treatment with tannins or formaldehyde (Tagari et al., 1965; Ferguson et al., 1967; Offer et al., 1971). Degradation of dietary protein releases amino acids, but only very low levels of free amino acids have been found in the rumen (Lewis, 1955; Wright and Hungate, 1967). Amino acids may be deaminated with release of ammonia (Lewis and Emery, 1962) or assimilated directly into bacterial protein (Nolan et al., 1976). Some amino acids are more rapidly deaminated than others (Lewis and Emery, 1962; Chalupa, 1974) and deamination rates also differ with the strain of bacteria involved (Scheifinger et al., 1975). Scheifinger et al. (1975) also reported that while individual strains of bacteria do not deaminate all the amino acids, methionine was unique in being metabolised by all strains. It was degraded by 25% of the strains tested, and synthesised by the other 75%. In addition, Salisbury

et al. (1970) suggested that methionine could be stored by bacteria through conversion to the sulphoxide when methionine concentrations were relatively high, with subsequent reconversion to methionine when the methionine concentration decreased.

C. Microbial protein synthesis

Microbial protein may be synthesised directly from amino acids or from ammonia. There is still considerable dispute over the relative importance of these precursors, with some workers favouring synthesis from ammonia (Bryant and Robinson, 1963; Portugal and Sutherland, 1966; Walker and Nader, 1968) while others support the importance of assimilation of amino acids (Prescott, 1961; Landis, 1963; Nolan *et al.*, 1976). Various methods for measuring the rate of protein synthesis have been proposed, and these will be discussed in Chapter 2.

An adequate supply of energy is also required for efficient protein synthesis, and to prevent breakdown of protein which has already been synthesised. Henderickx (1961) suggested that breakdown of microbial protein within the rumen might provide energy for protein synthesis during periods of low energy availability. The inefficiency of this process of recycling of N within the rumen was pointed out by Nolan and Leng (1972) and Nolan *et al.* (1976).

The efficiency of an energy source in increasing microbial protein synthesis will depend on its rate of utilisation by the microbes, relative to the rate of ammonia release from the N source (Helmer and Bartley, 1971). Thus, starch has been found to be more effective than xylan, pectin or the simple sugars, which in turn are more effective than cellulose or hemicellulose in promoting urea utilisation *in vitro* (Belasco, 1956; Henderickx and Martin, 1963). However, differences

exist between starch from different sources. Bloomfield et al. (1958) reported a 98% increase in urea utilisation with the addition of corn starch, while wheat starch only produced an 81% increase, and soluble starch a 55% increase. Arias et al. (1951) suggested that the rumen microbial population utilises urea more efficiently if small amounts of readily available energy such as sucrose or starch are provided, when cellulose is the main energy source. Lewis and McDonald (1958) found starch or grass levan to provide for efficient utilisation of ammonia released from casein. They stressed the importance of a steady release of energy at a rate similar to the rate of ammonia production. Djordevic et al. (1974) commented on the high degree of plant protein degradation at a low level of ~~dietary~~ digestible energy (2300kcal/kg diet). They found maximum protein synthesis at ~~an~~ a digestible energy level of 2900kcal/kg diet. At higher energy levels protein synthesis decreased again, but no adequate explanation for this was given.

D. N leaving the rumen

N may leave the rumen in the form of microbial protein, dietary protein, amino acids and other NPN compounds. The modification of dietary protein by the microbes in the rumen decreases the importance of dietary amino acid composition in the nutrition of the ruminant, and places more emphasis on the composition of the amino acids leaving the rumen. After leaving the rumen, nutrients flow into the omasum and abomasum prior to entering the duodenum. While some changes in the nitrogenous components of the digesta will take place in the abomasum, due to lysis of protozoa (Beever et al., 1973) and the addition of endogenous secretions (Harrop, 1974), most of the work on intestinal digestion of amino acids has studied the flow of amino acids into the

duodenum. This is due to the introduction of re-entrant cannulae which can be inserted in the proximal duodenum (Ash, 1962).

1.2 N entering the duodenum

Since the introduction of duodenal re-entrant cannulae, attention has been given to the proportion of dietary-N in the total-N entering the duodenum on natural feeds such as pasture and hay (Hutton et al., 1971; Coelho da Silva et al., 1973; Sutton et al., 1975; Walker et al., 1975). Harrison et al. (1973) reported a range of 37 - 85% of bacterial-N in the total-N at the duodenum on fresh, dried and frozen diets. Walker et al. (1975) reported a range of 42 - 74% microbial non-ammonia-N (as a percentage of total N entering the duodenum) in a comparison of seven pasture and hay diets. Proud (1973) compared total amino acid-N entering the duodenum on grass fed fresh, as silage or after drying. He recorded 18.8g amino acid-N/day entering the small intestine of sheep fed dried grass, and only 12.3 g/day for sheep fed wilted silage. Armstrong (1973) suggested that the low value for silage was due to inefficient use of silage-N within the rumen.

Differences in the N and energy composition of herbage, due to variety and stage of maturity have been found to have a marked effect on the digestion of N by ruminants (Egan, 1974; Ulyatt and MacRae, 1974; MacRae and Ulyatt, 1974; Hume and Purser, 1974). Increased dietary-N content, and a high concentration of water soluble carbohydrates generally increased the yield of microbial protein/100g OM apparently digested in the stomachs. However, an increased rate of microbial protein synthesis is only beneficial if the total protein entering the duodenum is increased. Thus, on diets with a high ratio of protein : NPN, increased digestion of dietary protein in the intestines may be

more beneficial than increased ruminal digestion.

Chalmers and Synge (1954) studied the effect of duodenal supplementation of protein on the efficiency of N utilisation. They found differences between different protein sources, suggesting that the quality of protein entering the duodenum had an important effect on N metabolism in the animal. Thus, changes in the balance of dietary and microbial protein entering the duodenum might be expected to alter the quality of protein available to the animal. However, the essential amino acid compositions of herbage and microbial protein are similar, and thus the amino acid composition of duodenal digesta from sheep fed different herbages is also similar (Clarke et al., 1966; Hogan and Weston, 1970; MacRae et al., 1972; Harrison et al., 1973; Ulyatt et al., 1975). Microbial protein does have a higher content of methionine and lysine than herbage, and the concentrations of these amino acids have been shown to increase on diets supporting a high rate of microbial protein synthesis (Coelho da Silva et al., 1972; Harrison et al., 1973; Ulyatt et al., 1975). However, Poley and Trenkle (1963) and Schelling (1968) did report differences in duodenal amino acid composition where the feed protein was very different in composition to microbial protein.

The quality of protein at the duodenum will depend on its capacity to fulfill the metabolic requirements of the animal, but since few individual amino acid requirements for ruminants have so far been determined, any definition of a high quality protein can only relate to monogastric animals. However, pork and mutton amino acid compositions are similar (USDA, 1968) which suggests that the proportions of amino acids required for tissue protein synthesis by pigs and sheep are also similar. Hence, a protein which is of a high quality for pigs, would

presumably also be of a high quality for ruminant tissue protein synthesis, when supplied at a duodenal level.

1.3 Amino acid metabolism within the animal

A. Introduction

The metabolism of individual amino acids has been studied in considerable detail since the work of Rose (1938) showed that a dietary supply of leucine, isoleucine, valine, lysine, methionine, phenylalanine, tryptophan, threonine, histidine and arginine was required for optimal growth, or the maintenance of ^{N} balance in rats. These amino acids have generally been referred to as the "essential amino acids", since their requirement for protein synthesis cannot be met by mammalian tissue synthesis. This classification of essential amino acids led to the adoption of the term "non-essential" to refer to amino acids which could be synthesised at a rate sufficient to meet the requirement. However, Christensen (1964) pointed out that "essential" amino acids were only of prime importance when in short supply, and some of the "non-essential" amino acids play more dominant roles in metabolism. Harper (1974) suggested that "dispensable" amino acids was a more appropriate term than "non-essential", and he referred to the N of these amino acids as "non-specific"-N.

It has been known for more than twenty years that ruminal micro-organisms could synthesise the ten essential amino acids (Loosli et al., 1949). Tissue amino acid metabolism in the ruminant was assumed to be similar to that of monogastrics, until Downes (1961) verified that nine of the ten amino acids essential for rat growth could not be synthesised by ruminant tissues. However, some synthesis of arginine did take place. Since then, postruminal administration of amino acids has confirmed

some of the assumed similarities between ruminants and monogastrics. However, species differences do exist. Results from monogastric species other than the rat, indicate that dietary glycine may be required by the chick (McDonald et al., 1969), while dietary arginine may not be necessary for humans (Irwin and Hegsted, 1971).

Thus it is apparent that false assumptions may be made by extrapolating results from one species to another. However, owing to the lack of data on ruminant amino acid metabolism, the following discussion will include references to monogastric metabolism.

B. Factors affecting concentrations of amino acids in plasma

The concentrations of individual amino acids in plasma are the net result of inputs such as intestinal absorption and tissue protein breakdown, and utilisation for tissue protein synthesis and metabolic reactions. The relative rates of these reactions are affected by many factors, which can be broadly classified into:

(1) physiological,

(2) nutritional.

(1) Physiological factors: Metabolism of amino acids by the liver and uptake of amino acids by the individual tissues for protein synthesis, results in different concentrations of free amino acids in blood samples taken from various parts of the body (Reilly and Ford, 1971; Wolff et al., 1973). Thus concentrations in portal plasma are high, while those of jugular or carotid plasma are lower. Differences within blood, between plasma-free and cell amino acid concentrations have also been found (Buraczewska et al., 1972) indicating either different rates of metabolism between the two compartments, or selective uptake by the cells. A distinction should also be made between "free" and protein-bound amino acids in the plasma. The following discussions will refer

to free plasma amino acids unless otherwise specified.

The utilisation of amino acids by the tissues is markedly affected by the action of hormones (Harper, 1969; Munro and Portugal, 1972) since certain hormones can influence:

(a) the rate of protein synthesis and metabolic interactions within the tissue,

(b) the rate of uptake of amino acids by the tissues,

(c) the rate of protein degradation,

(d) the rate of synthesis of non-essential amino acids within the tissue (Munro and Portugal, 1972).

Most attention has been given to the effects of growth hormone, thyroxine and insulin (Munro, 1964; Harper, 1969; Cahill et al., 1972). Growth hormone and insulin increased protein synthesis, thus increasing the utilisation of amino acids from the plasma, and hence decreasing their concentration. Thyroxine caused loss of body protein, with release of amino acids into the plasma, increasing plasma concentrations.

Differences in the rate of uptake of individual amino acids by the tissues as a result of age of an animal might also be expected to affect plasma amino acid concentrations. Oltjen et al. (1969) found marked sex and age differences in the plasma amino acid concentrations of calves, while Davey et al. (1973) found significant age effects only in leucine and lysine concentrations. However, differing rates of growth between animals of similar age have been shown to affect plasma amino acid concentrations (Windels et al., 1971; Boling et al., 1972). Total plasma amino acids were negatively correlated with live-weight gain.

(2) Nutritional factors: The absorption of amino acids into the blood-stream would be expected to affect plasma concentrations, but some changes occur due to utilisation of amino acids by the gut tissue (Hume et al., 1972; Wolff et al., 1973). Adaptation to high intakes of individual amino acids has also been reported, since initial responses disappeared with continuous infusion of the amino acid. However, repeated administration of methionine led to persistent changes in the plasma aminogram (Synderman et al., 1968). The effect of changes in total protein intake would also be expected to alter plasma amino acid concentrations, and numerous workers have reported increased plasma amino acid concentrations in response to increased intake in monogastrics (Windels et al., 1971; Davey et al., 1973; Itoh et al., 1974).

Increased plasma amino acid concentrations in response to increased absorption of protein in ruminants has also been recorded, with the change in essential amino acid concentrations being greater than that of dispensable amino acids (Amos et al., 1972; Potter and Bergen, 1974).

Evidence for protein synthetic mechanisms adapting to the supply of amino acid substrates has been reported for monogastric animals (Munro, 1969; Clemens, 1972; Wannemacher, 1972) but this response may be mediated by a hormonal response to diet. A decrease in catabolism of liver proteins appears to occur in conditions of protein deficiency (Stephen and Waterlow, 1966), and Waterlow (1969) proposed that protein homeostasis is the result of the balance between rates of catabolism and synthesis of body protein.

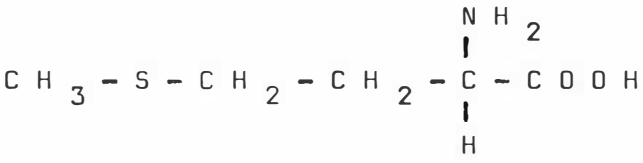
The rate of protein synthesis in monogastrics is stimulated by an increase in carbohydrate intake, with a consequent decrease in plasma essential amino acid concentrations (Munro and Thomson, 1953; Swendseid et al., 1967). A similar response to intravenous and intra-arterial

energy infusion has also been reported for sheep (Potter et al., 1968; Eskeland et al., 1974) with differing responses to different energy sources. Glucose resulted in the greatest decrease in plasma essential amino acid concentration, followed by propionate, acetate and butyrate respectively. Eskeland et al. (1974) reported a close correlation between decreased essential amino acid concentration and increased N balance values, suggesting that the decrease in plasma amino acids resulted from increased protein synthesis. They suggested that, since different tissues metabolise each energy source at a different rate (Mayfield et al., 1968), glucose and the volatile fatty acids would stimulate protein synthesis to a different degree.

The effect of energy in stimulating protein synthesis may occur through a reduced requirement for gluconeogenesis, and hence the release of amino acids for protein synthesis. Since very little glucose, per se, is absorbed from the ruminant digestive tract (Lindsay, 1970), production of glucose from propionate and amino acids is essential. Bergman et al. (1970) have shown that only 20 to 40% of the daily glucose production was obtained from absorbed propionate, although both Bergman et al. (1970) and Leng et al. (1967) showed that 50% of glucose carbon could come from propionate during periods of peak rumen fermentation. Wolff and Bergman (1973) reported between 11 and 30% of glucose being derived from plasma amino acids in fed sheep. Reilly and Ford (1971) reported an approximate value of 28% of glucose derived from amino acids. They also found an increase in total glucose production rate, and in glucose production rate from amino acids, in response to an increased rate of amino acid absorption. Thus, amino or organic acids could be expected to make an important contribution to gluconeogenesis, particularly during fasting, or on near-maintenance intakes.

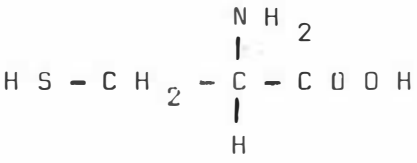
Fig. 1.2 structure of sulphur amino acids and related compounds

(a)



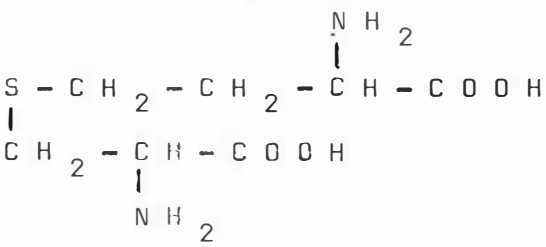
METHIONINE

(b)



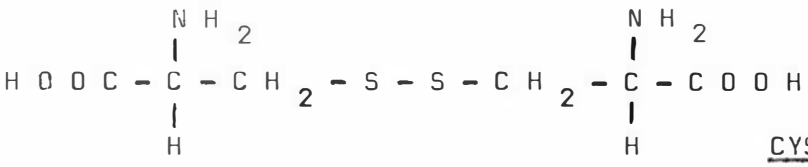
CYSTEINE

(c)



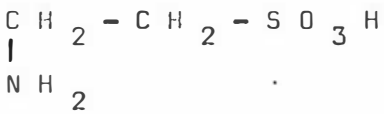
CYSTATHIONINE

(d)



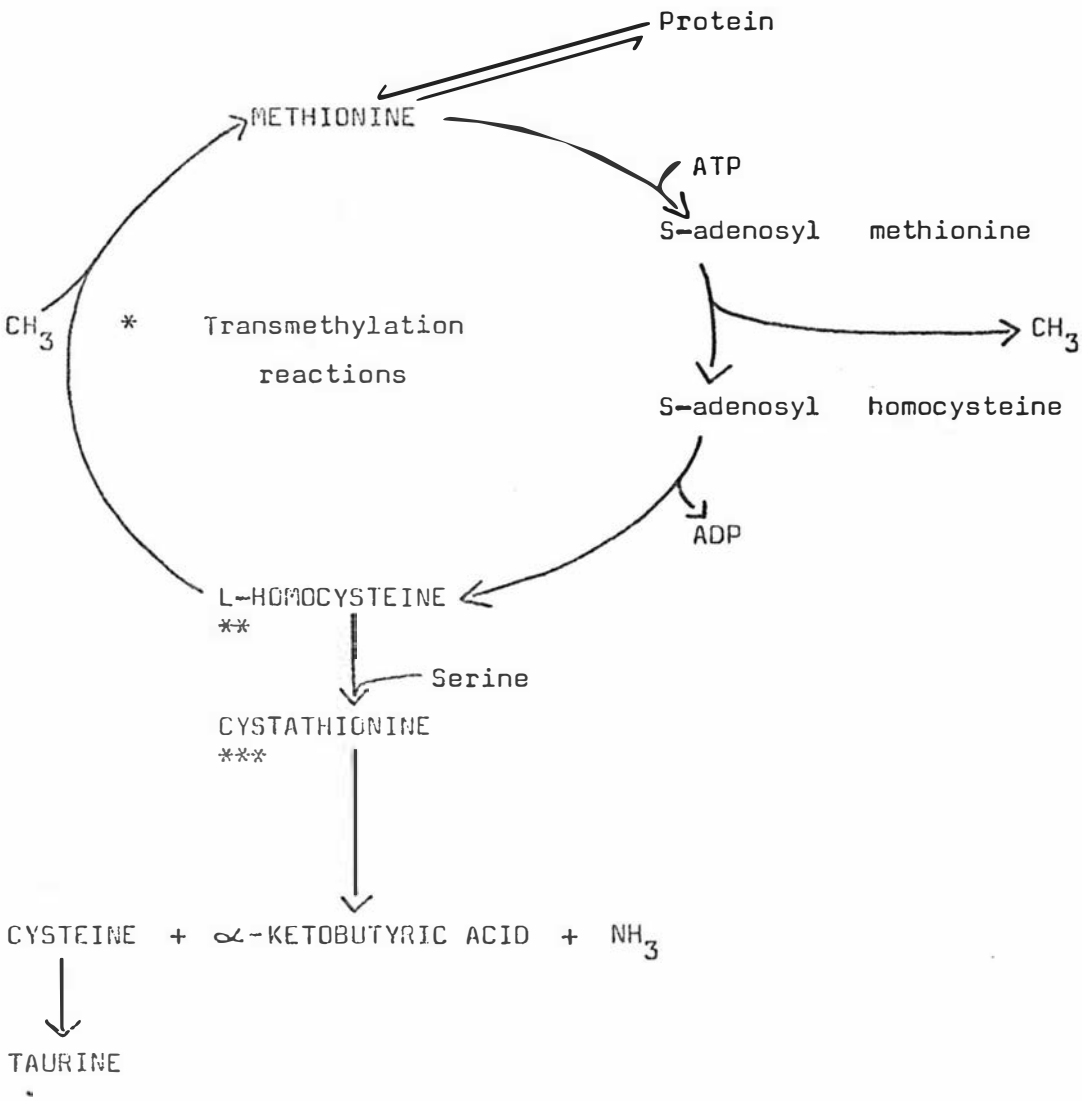
CYSTINE

(e)



TAURINE

Fig. 1.3 conversion of methionine to cysteine



* enzymes are N5-methyltetrahydrofolate homocysteine methyltransferase and betaine-homocysteine methyltransferase

** enzyme is cystathionine synthase

*** enzyme is cystathionine γ-lyase

1.4 Metabolism of S-containing amino acids

Methionine and cyst(e)ine are the main S-amino acids which occur naturally in proteins. Cystine is ~~incorporated~~ formed into protein by oxidation of the thiol group of cysteine. This leads to the formation of di-sulphide bridges between protein chains (Mahler and Cordes, 1971). Methionine has a five carbon (C) structure (Fig. 1.2a) which cannot be synthesised by mammalian tissue. Cysteine has only three C atoms (Fig. 1.2b) and can be synthesised from methionine. The pathway for cysteine synthesis from methionine involves the following steps (Fig. 1.3):

(1) an initial transmethylation reaction, with activation of methionine to S-adenosyl methionine (catalysed by methionine adenosyltransferase), followed by the formation of L-homocysteine,

(2) homocysteine then undergoes an irreversible reaction with serine, to form cystathionine, which contains two amino groups (Fig. 1.2c) (catalysed by cystathionine synthase),

(3) cystathionine is then converted to cysteine, with loss of one molecule of ammonia, and formation of α-ketobutyric acid (catalysed by cystathionine -γ- lyase),

(4) oxidation of cysteine to cystine (Fig. 1.2d) may take place,

(5) conversion of cysteine to taurine (Fig. 1.2e) is an alternative reaction for cysteine.

While detailed studies on the enzymes regulating these pathways relate mainly to rat liver preparations, these enzymes have also been found in the tissues of ruminants (Mudd et al., 1965; Radcliffe and Egan, 1974).

A. Metabolism of methionine

Methionine is required for three main functions:

(1) protein synthesis,

(2) conversion to S-adenosyl methionine, which is the dominant methyl group donor in biological systems,

(3) conversion to cysteine via cystathionine.

The distribution of methionine between these functions will depend on the availability of other amino acids. Thus, as protein intake increases, the absolute requirement for methionine for protein synthesis also increases. However, the percentage requirement for methionine decreases (Grau and Kamei, 1950). This decrease is presumably due to the requirement for protein synthesis increasing at a greater rate in response to increased protein intake, than the extra-protein functions of methionine (i.e. 2 and 3 above). The level of cysteine in the diet has also been shown to affect methionine requirement. This was first demonstrated by Womack and Rose (1941) who showed a "methionine sparing" effect, whereby cysteine could partially replace the dietary methionine in maintaining the growth rate of rats. Womack et al. (1953) found a similar "methionine sparing" effect of cysteine when measuring requirements in terms of N balance, but no effect on methionine requirement by any other dispensable amino acid was apparent.

B. Regulation of methionine metabolism

The conversion of methionine to cysteine involves the formation of homocysteine, prior to its conversion to cystathionine (Fig. 1.3). Supplementation of low methionine diets with cysteine has been shown to decrease the concentration of cystathionine synthase in the liver, while the concentration of betaine-homocysteine methyltransferase was

unaffected (Finkelstein and Mudd, 1967; Shannon et al., 1972). betaine-homocysteine methyltransferase is one of two enzymes which catalyse re-methylation of methionine from homocysteine (Fig. 1.3) and the above result would suggest an increased recycling of homocysteine to methionine (1b, Fig. 1.3). The relative decrease in cystathionine synthase would decrease the proportion of methionine converted to cysteine, and thus explain the "methionine sparing" effect of cysteine. Finkelstein and Mudd (1967) had suggested that methylation of homocysteine by N^5-methyltetrahydrofolate (N-mTHF) was not very important, and therefore did not assay this enzyme. However, in a later paper (Finkelstein et al., 1971) they concluded that N-mTHF activity was significantly affected by dietary metabolites and could contribute to regulation of methionine metabolism. Finkelstein et al. (1971) found that high protein diets caused decreased synthesis of this enzyme, and Cello and Finkelstein (1975) reported an increased synthesis of N-mTHF enzyme in response to low protein diets. This suggests an effect of protein in decreasing methionine regeneration during high availability of amino acids.

The response of liver enzymes to amino acid supply has mainly been studied using in vitro preparations, however, isotopes have been used for in vivo studies on the regulation of methionine metabolism. Edwards et al. (1963) found that considerable recycling of methionine can occur in the liver, since very little label from methionine-CH_3-^{14}C was incorporated into tissue protein. They suggested that approximately half of the methionine entering tissue protein first underwent trans-methylation reactions, regaining a methyl group from endogenous methionine. A high percentage of the methyl label was recovered in the liver, because some of the methyl groups donated by methionine are likely to be used for the synthesis of choline and creatinine (Stekol

et al., 1958). Finkelstein and Mudd (1967) also found a decrease in the concentration of methionine activating enzymes in the liver of rats fed diets low in methionine. These enzymes are involved in the initial formation of S-adenosyl methionine, which acts as a methyl donor. Finkelstein and Mudd (1967) suggested that these changes in enzyme concentrations regulated the distribution of methionine between protein synthesis and transmethylation.

C. Methionine as a donor of methyl groups and sulphur

The relative importance of the extra-protein functions of methionine, compared to those of other amino acids, was shown by Aguilar et al. (1972) who studied the efficiency of utilisation of the essential amino acids in rats. They found that labelled methionine gave the lowest values for incorporation into tissue protein, and the highest percentage oxidation, when fed at a given proportion of its requirement. A comparison of the methyl-and carboxyl-labelled isotopes of methionine showed a higher percentage oxidation of the carboxyl- than the methyl-C. In addition, heavy labelling of serine from methyl-labelled methionine indicated the importance of methionine as a methyl group donor (Aguilar et al., 1974).

Methionine also acts as a source of S for the synthesis of other S-containing organic compounds (Fig. 1.3). Reis et al. (1973) reported increased plasma concentrations of methionine, cystine, taurine and cystathionine in response to abomasal infusion of methionine in sheep. However, Tao et al. (1974) did not observe any significant increase in plasma cystine after methionine infusion into the jugular vein of sheep. Sheep plasma-free cystine concentrations have generally been found to be low: 0.14 - 0.48 mg/100ml plasma (Williams et al., 1972; Tao et al., 1974), and were not increased by infusions of large amounts of cystine (Reis et al., 1973). This suggests that the sheep is able to remove

large amounts of cystine from the plasma quite rapidly. This may be partly due to rapid binding of cystine to plasma proteins which can later be released as required (Downes,1961). Part of the increased cystine load appears to be converted to taurine, since taurine concentration increased markedly in response to cystine infusion. However, the increase in taurine in response to methionine infusion was less marked, indicating that the sheep was unable to rapidly convert excess methionine to cystine (Reis et al., 1973). Reis et al. (1973) suggested that the conversion of methionine to cysteine was limited by decreased availability of serine (reaction 2, Fig. 1.3) since plasma concentration of serine decreased with methionine infusion.

While the conversion of methionine to cysteine is important for the synthesis of wool protein, only one specific physiological function of taurine has been recorded. Taurine is known to conjugate with bile acids, which are important in the intestinal absorption of fat (Jacobson and Smith, 1968). Taurine was shown to be present in most organs of the body (Sturman, 1973), but supplemental taurine given to humans, was largely excreted in the urine (Sturman et al., 1975). Whittle and Smith (1974) showed that taurine synthesis was increased by S restriction in rats, but the additional taurine was also largely excreted in the urine.

D. Influence of methionine on the plasma concentrations of other amino acids

The effect of plasma infusions of methionine on the concentrations of other amino acids in the plasma has been studied by a number of workers. While the response measured, largely depends on the quantity and quality of dietary protein available to the animal, some specific

actions of methionine are apparent. Thus, an increased concentration of α-aminobutyric acid, in response to methionine infusion, is thought to indicate synthesis of this amino acid from methionine, and some evidence of the reverse reaction occurring in rats has also been presented (Synderman et al., 1968; Edwards et al., 1972). Synderman et al. (1968) also reported decreased plasma concentrations of the branched chain amino acids (valine, isoleucine and leucine), tyrosine and phenylalanine in response to methionine in rats. However, Canolty and Nasset (1975) showed increased plasma leucine and isoleucine and decreased histidine and threonine. Work with sheep and lambs has shown decreases in plasma concentrations of threonine, glycine, lysine, serine, glutamic acid, valine, leucine and isoleucine (Schelling, 1968; Reis and Tunks, 1971; Reis et al., 1973; Tao et al., 1974). However, the quantity and quality of protein available to the animal will also affect plasma amino acid concentrations, and expressing individual amino acids as ratios to, or percentages of other amino acids may be more meaningful. Thus, Papas et al. (1973) reported the effect of oral and abomasal supplements of methionine in terms of plasma methionine:valine ratios. These were unaffected by oral methionine, or MHA, while abomasal infusion significantly increased the methionine:valine ratio.

1.5 Amino acid requirements of ruminants

Knowledge of the nutrient requirements of an animal enables the provision of nutrients to be optimised, between maximum production and economic input. Qualitative assessment of the amino acid(s) most likely to limit production may lead to uncontrolled supplementation of a diet and this may lead to problems of toxicity. Thus, quantitative assessment of requirements is necessary for efficient animal production.

While the amino acid requirements of non-ruminants have received

considerable attention (e.g. A.R.C., 1967; Irwin and Hegsted, 1971) the capacity of the ruminal micro-organisms to utilise NPN in the synthesis of amino acids (Loosli et al., 1949) negates the relevance of dietary amino acid requirements for ruminants. Hence, some knowledge of the amino acids available for absorption, is a pre-requisite for the assessment of requirements. This has only been achieved recently, through the use of sheep prepared with re-entrant cannulae in the proximal duodenum and terminal ileum (Brown et al., 1968). Interest in the assessment of amino acid requirements for ruminants has also been stimulated by recent work on the manipulation of rumen fermentation. Three main methods have been used for controlling the quantity and quality of protein entering the duodenum:

(1) nutrient by-pass of the rumen, using the oesophageal-groove reflex (Ørskov and Benzie, 1969),

(2) protection of proteins from microbial attack, e.g. by reaction with formaldehyde or tannins (Tagari et al., 1965; Ferguson et al., 1967; Offer et al., 1971),

(3) promoting natural factors which decrease microbial attack, e.g. increased flow out of the rumen.

Thus, a quantitative assessment of the amino acids required for metabolism by ruminant tissues, is essential to maximise post-ruminal protein utilisation.

Definition and classification of the amino acids into essential and dispensable, has been discussed above, and between species differences in amino acid metabolism were also considered. While the requirements for essential amino acids must be individually specified, the non-specificity of dispensable amino acids allows for their requirement to be stated solely in terms of N and C. Hence only the requirements for essential amino acids will be discussed below.

A. Response to amino acid imbalanced diets

Harper (1964) defined amino acid imbalance as "a change in the proportions of amino acids in a diet, which results in a depression in food intake or growth rate, that can be completely prevented by a supplement of the indispensable amino acid present in least amount in the diet in relation to the amount required for optimum growth". This definition ignores any change in the proportional requirements for amino acids, in response to differing levels of protein intake. The metabolism of methionine has been shown to change with increasing availability (Finkelstein et al., 1971; Aguilar et al., 1974). In addition, Peng et al. (1975) reported that intake depression in rats fed amino acid imbalanced diets, increased with increased overall protein synthesis. Hence, discussion of amino acid imbalance in the present work, will assume the definition of Harper (1964), with an additional proviso that amino acid imbalance may arise in response to a change in the rate of tissue protein synthesis and/or the level of protein availability.

Harper et al. (1970) showed that decreased voluntary intake and growth rate in rats resulted from the addition of some amino acids to a low protein diet. The addition of these amino acids decreased the percentage of the first limiting amino acid in the diet. Addition of this amino acid restored the balance, and thus increased intake. While various theories on the mechanism of the intake response to amino acid imbalanced diets have been proposed, no one theory has yet been accepted. Simson and Booth (1974) suggested that the deleterious effects of a diet low in histidine, could initially be buffered by the utilisation of histidine from endogenous protein, but this would later accentuate the deficiency response to a histidine-free diet. Simson and Booth (1974) also suggested that biochemical changes in response to

an imbalanced diet occur within 2h of its ingestion, and these changes were involved in later conditioned rejection of the diet. Peng and Harper (1969) showed that changes in liver concentrations of amino acids were not responsible for decreased intake of imbalanced diets, since, while liver tryptophan concentration decreased on a tryptophan-deficient diet, liver threonine concentration increased on a threonine-deficient diet. Intake decreased in both cases.

Noda (1975) proposed that blood ammonia levels regulated the intake of amino acid imbalanced diets, but further proof is still required. Noda (1975) had suggested that detoxification of ammonia prior to its entry into the urea cycle could be a rate-limiting step, and thus increase the concentration of ammonia in the blood. Prior et al. (1975) did find a three to four fold increase in blood urea during the feeding of an amino acid imbalanced diet.

Rogers and Leung (1973) implicated the prepyriform cortex and medial amygdala of the brain in monitoring the factors responsible for the depression of food intake in response to diets deficient in an amino acid, but not to diets with excess amino acids. While most of this work on amino acid imbalanced diets has been recorded using non-ruminant animals, Rogers and Egan (1975) showed that pre-ruminant lambs responded in a similar manner to amino acid imbalanced diets. However, with ruminants the imbalance must occur at a duodenal, rather than a dietary level.

Barry et al. (1973) reported that low intake of silage by sheep could be increased by intraperitoneal supplementation with methionine. This suggests that fermentation of silage in the rumen produced an amino acid imbalance in the sheep, but that balance could be restored by

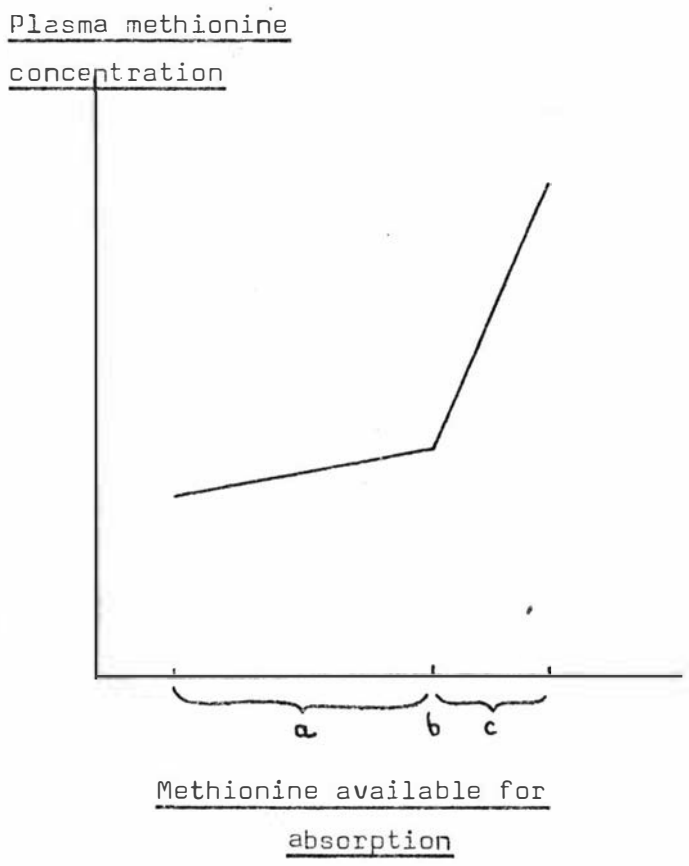


Fig. 1.4 Plasma response curve

addition of methionine. Thus, the quantity of methionine entering the duodenum appeared to be limiting the utilisation of other amino acids with these sheep. Other workers have also reported methionine as limiting protein synthesis in sheep and lambs (Reis, 1967; Graceva, 1969; Wakeling and Lewis, 1970; Dove and Robards, 1974). These results are further supported by comparisons of tissue protein analysis with duodenal amino acid flow, which showed that the duodenal flow of S-amino acids was most likely to limit tissue protein synthesis (Hogan, 1970; Hutton and Annison, 1972; Armstrong and Annison, 1973).

B. Methods for quantitative assessment of amino acid requirements

The ARC (1965) manual on nutrient requirements for ruminants does not give individual amino acid requirements, since these cannot be expressed in dietary terms. However, the ARC (1967) manual on pig requirements does give amino acid requirements, expressing individual amino acid concentrations as a percentage of the DM in the diet. These values are defined as giving maximum response, but the writers concluded the section by suggesting the use of response curves to define amino acid requirements over a range of responses. The response curves suggested, are plots of the response, expressed in terms of one chosen criterion, versus increasing absorbable levels of the amino acid in question (Fig. 1.4). While the level of absorbable amino acid is usually taken to mean dietary intake in monogastrics, it relates to amino acids entering the duodenum in ruminants. In most instances, such curves are composed of two lines, having different slopes, and the "break-point" where these two lines intersect, is defined as the requirement. The criterion used to measure the response will depend on the sensitivity required, and the degree of complexity of the experiment to be undertaken.

The criteria usually considered are as follows:

(1) production responses such as wool growth, live-weight gain and milk yield,

(2) factorial assessment by carcass analysis,

(3) N balance,

(4) plasma amino acid concentration,

(5) the use of isotopically labelled amino acids.

(1) Production responses: While some attention has been given to assessing individual amino acid requirements by measurement of production responses (Chung et al., 1973) these criteria are not very sensitive, and a considerable time delay is involved. These methods are therefore of more use for the detection of limiting amino acids, and for assessing the overall quality of a protein (Jacquot and Peret, 1972).

(2) Factorial assessment: A more rapid method for assessing individual amino acid requirements for production, is the calculation of the amount of each amino acid deposited in the meat, wool or milk protein synthesised. Williams et al. (1954) compared estimates of amino acid requirements of monogastrics, calculated either by analysis of the whole carcass, or by nutrition experiments. They found good agreement between the two sets of values for rats, but fewer data were available for pigs. The amino acids which showed closest agreement were leucine, isoleucine, threonine and valine, which have no function other than synthesis of tissue protein, while results for cysteine and methionine were consistently lower when estimated from carcass analysis. This might be expected because of the high requirements for methionine for extra-protein functions e.g. methylation reactions. Hutton and Annison (1972) calculated factors for the utilisation of amino acids by pigs,

from the carcass concentration expressed as a percentage of food requirement for individual amino acids. They then used these factors to calculate duodenal amino acid requirements for the young steer, from the concentrations of amino acids in the carcass. However, the use of conversion factors from pigs, ignores any differences in the metabolism of amino acids by ruminant and non-ruminant tissues. Armstrong and Annison (1973) also used a factorial approach to calculate requirements for methionine, cysteine and threonine for 45kg sheep, taking maintenance, wool growth and tissue protein deposition into account. Their approach used the apparent digestion of energy to estimate tissue protein synthesis, and required numerous assumptions regarding the utilisation of energy. In addition there is probably not sufficient evidence on the metabolism of these amino acids in ruminants, to allow estimation of their requirement for maintenance by this method.

(3) N balance: A deficiency of an amino acid which limits tissue protein synthesis will lead to an excess of the remaining essential amino acids, which will be converted to urea in the liver, and subsequently excreted in the urine (Prior et al., 1975). Thus, measurement of plasma urea concentration, or the daily amount of N retained by the animal, can give some indication of the efficiency of utilisation of the amino acids. Walker and Kirk (1975) used N balance measurements to assess methionine requirements of lambs fed cow's milk, and found that methionine supplementation was necessary to obtain maximum N balance on low or medium protein intakes.

(4) Plasma amino acid concentration: Schelling (1968) proposed a working model for plasma essential amino acid concentrations incorporating three increasing levels of response:

(a) a basal, or minimal, concentration (which is different for each amino acid) indicating a deficiency of that amino acid, which decreases the efficiency of protein synthesis,

(b) the optimal concentration, which supports the most efficient utilisation,

(c) excess concentration, which again decreases efficiency of protein synthesis.

Fitting these concentration levels to a response curve as described in the introduction to this section (Fig. 1.4), places (a) on the first intersecting line, below the requirement, (b) at the point of intersection, i.e. meeting the requirement and (c) on the second intersecting line, above the requirement. Several workers have used plasma amino acids for predicting requirement, by measuring the response to gradually increasing levels of: intake for rats (Zimmerman and Scott, 1965), and postruminal amino acids for calves (Williams and Smith, 1974) and sheep (Chalupa and Chandler, 1972; Armstrong and Annison, 1973; Tao et al., 1974). However, Wakeling and Lewis (1970) criticised the lack of sensitivity of this method, since numerous other factors will also affect plasma amino acid concentrations.

(5) The use of isotopically labelled amino acids: ^{14}C-labelled amino acids have been used to measure the degree of oxidation of an amino acid to carbon dioxide, since excess concentrations of an amino acid in the plasma, will result in an increase in oxidation. Chalupa and Chandler (1972) stated the advantages of this technique over other methods of assessing amino acid requirements: it takes less time than the measurement of N balance or production responses, and it is less affected by transient changes in plasma amino acids than direct measurement of plasma amino acid concentrations.

Brookes et al. (1973) estimated the lysine requirement of sheep by plasma amino acid concentrations, and ^{14}C oxidation. They found similar values for each method. Brookes et al. (1973) used a single injection of lysine-^{14}C and measured oxidation over the following six hours. Mitchell et al. (unpublished observations cited Armstrong and Annison, 1973) used the same principle to measure methionine requirement, but used a continuous infusion of ^{14}C-labelled amino acids for 5-6 hours, and plotted specific radioactivity of $^{14}CO_2$ against time, to find the asymptotic value (Annison et al., 1967) and the percentage of infused radioactivity expired during the infusion period. Plotting these values against absorbable methionine, gave estimates of requirement which were in good agreement with those from plasma amino acid measurements (Armstrong and Annison, 1973). Neale and Waterlow (1974) further refined the use of ^{14}C-labelled amino acids for estimating leucine and lysine requirements in rats, by determining total loss of label from the body, under conditions approaching maintenance. However, their estimates were higher than those obtained by growth or N balance methods. Annison (1975), while discussing the use of ^{14}C-labelled amino acids, pointed out that the sensitivity and accuracy of this technique would depend on the pathways of oxidation of each amino acid, and large differences in the rates of oxidation of individual amino acids ~~by sheep~~ have been shown (Black et al., 1968).

^{35}S-labelled methionine has also been used to assess methionine requirement, since surplus methionine may be converted to taurine and excreted in the urine. Mercer and Miller (1973) used this method to estimate methionine requirement of growing lambs, and found similar results to those determined by plasma urea measurements.

Table 1.1 Estimates of methionine requirements by sheep

Method of estimation	Requirement ($mg/kg^{.75}/day$)	Reference
Plasma amino acid concentration	105–125	Wakeling et al. (1970)
	207[1]	Reis et al. (1973)
Oxidation to $^{14}CO_2$	120–130	Armstrong & Annison (1973)
	130–160	Annison (1975)
^{35}S excretion	170	Mercer and Miller (1973)

[1] refers to infused methionine only.

C. Estimates of methionine requirements of sheep

Various estimates of the duodenal methionine requirements of sheep have been made, and these are compared in Table 1.1. While the effect of metabolic body weight is taken into consideration in stating these requirements, considerable variability might be expected to arise from different environmental conditions and rate of wool growth between the experiments. With regard to this latter aspect, it is interesting to note that the Australian workers (Reis et al., 1973) recorded a higher requirement than the British workers. The importance of wool to the Australian sheep farmer has probably led to selection of Australian sheep for a high rate of wool production.

Methionine can be converted to cyst(e)ine (Fig. 1.3) and thus the requirement for methionine will also depend on the availability of cyst(e)ine relative to its requirement. Thus the requirements for methionine and cyst(e)ine are frequently expressed as total S-amino acid requirements. Armstrong and Annison (1973) used the known values for apparently digested energy to calculate tissue protein deposition and hence a factorial estimate of S-amino acid requirements, on a variety of diets. These calculations gave values of 82 - 127 $mg/kg^{.75}/day$, and from these values, Armstrong and Annison (1973) suggested that while S-amino acids should not limit wool growth on forage diets, this may not be true for silage diets. The accuracy of this technique has already been criticised, but the relative differences between diets do support other studies in suggesting that silage may be a feed in which the S-amino acids are particularly limiting.

1.6 Formaldehyde - treatment of proteins

A. The theory for the reaction of proteins with formaldehyde

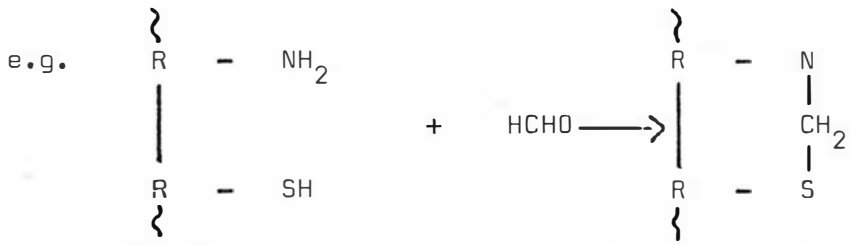
Treatment of proteins with aldehydes is a common practice in the Leather Industry, where the reaction of aldehydes with the surface of the fibrous proteins results in a tougher material, with decreased water sensitivity and increased resistance to the action of enzymes and chemical reagents (Walker, 1964). French and Edsall (1945) stressed the variety and complexity of possible reactions between formaldehyde and proteins, with the optimum conditions for formation and breakdown of the bonds, being dependent on pH and temperature. They quoted evidence for three types of bonding between formaldehyde and protein:

(1) a loose reversible combination, where formaldehyde can be removed by washing,

(2) bonds which can be broken by continuous washing over a period of days,

(3) a stable chemical complex which does not readily dissociate.

French and Edsall (1945) could not specify the various chemical groups involved in each type of bonding, but they did review evidence for the existence of methylene bridges between reactive groups such as the free amino group of lysine, and sulfhydryl groups.



Walker (1964) also suggested the formation of methylene bridges, and he pointed out that such formaldehyde-protein complexes would be less stable under acid than alkaline conditions. Thus, liberation of formaldehyde by distillation with dilute acid is often used to determine the degree of combination with protein. However, acid distillation is

not always reliable, since some irreversible binding may take place. Bowes et al. (1965) estimated bound formaldehyde by isotopic methods, and reported that a small proportion of the formaldehyde became more firmly bound during acid treatment. This fraction of the formaldehyde appeared to be bound to only one group, since the degree of crosslinking was markedly decreased. Bowes et al. (1965) found that, in contrast to earlier reports, reversal of the bonds with the amino groups did occur, and Neissner (1968) reported that after reversal of formaldehyde-binding, by boiling in water, there appeared to be no change in the casein molecule. Theis and Lams (1943) showed a pH reversibility with 45% release of binding when the pH was decreased from eleven to eight and 66% when the pH was decreased from eleven to two. However, Mellon (1958) reported that binding of amino groups to formaldehyde could also take place at an acid pH under some conditions. It would thus appear, that both the reactivity of the protein, and the conditions for its reaction with formaldehyde, can affect the type of bond formed and thus the ease of reversibility.

The reversal of formaldehyde-protein bonds with decreasing pH was the basis for formaldehyde-treatment of proteins for feeding to ruminants. Formaldehyde-treatment would decrease the solubility of the protein in the rumen, but the bonds would be reversed in the low pH of the abomasum and thus permit enzymic digestion in the intestines (Ferguson et al., 1967). However, the effect of differing reaction conditions on the type of bond formed, has led to detailed studies on the reversibility of formaldehyde-protein bonds. Hemsley et al. (1973) studied the effects of varying the reaction conditions, and found that the optimum degree of binding was with 1% formaldehyde. Below this level, there was increasing microbial attack in the rumen, while a higher degree of binding led to decreased N digestibility in the

intestines. The rate and extent of the protection reaction were found to increase with concentration of formaldehyde solution, with decreasing volume of formaldehyde used, and with increasing reaction time.

Hemsley et al. (1973) also noted unpublished observations by D.M. Walker where formaldehyde-treated casein was fed to rats. No difference in growth rate was recorded, compared to untreated casein, when the level of bound formaldehyde was less than 1%. These results are in contrast with those of Wachira et al. (1974) who fed casein which had been treated with 0.8g formaldehyde/100g crude protein, to rats, and found a decrease in growth rate with the treated versus untreated casein. However, these workers applied the formaldehyde in very low volumes, and did not measure the amount of bound formaldehyde. Hove and Lohrey (1976) also reported decreased digestibility of formaldehyde-treated casein in their rat growth trials. These workers suggested that more stable secondary complexes had been formed during the drying process included in the formaldehyde-treatment of the protein. However, MacRae et al. (1972) fed this same formaldehyde-treated protein to sheep, and observed a significant increase in apparent absorption of amino acids from the small intestine with formaldehyde-treatment, resulting in a higher overall retention of N. With reference to the earlier evidence of French and Edsall (1945), who suggested three gradations in the stability of bonding between formaldehyde and protein, this secondary complexing may be reversible in the sheep, but not in the rat, due to physiological differences between the two digestive systems.

Thus, while the chemistry of the reactions involved in formaldehyde-treatment of proteins is not yet clear, the following summary can be made.

(1) There appears to be an optimum level of formaldehyde-treatment to ensure adequate protection from microbial attack in the rumen, without affecting the digestibility in the intestines. Hemsley et al. (1973) proposed an optimum level of 1% bound formaldehyde.

(2) The concentration of formaldehyde solution, volume of application and degree of drying have also been shown to affect binding and reversibility (Hemsley et al., 1973; Hove and Lohrey, 1976).

(3) The reversal of formaldehyde-binding will be affected by the degree of binding, and is probably also dependent on pH and time spent in the digestive tract (French and Edsall, 1945).

B. Animal production responses to formaldehyde-treated proteins

The work by Ferguson et al. (1967) which restimulated interest in the use of treated proteins, reported a response in wool growth to formaldehyde-treatment of casein. Since then, various workers have recorded similar increases in wool growth (Hughes and Williams, 1970; Langlands, 1971; Barry, 1972). Barry et al. (1973) reported increased wool growth with formaldehyde-treatment of silage. However, reports of no response to formaldehyde-treatment also appear in the literature (e.g. Clark et al., 1971; Schmidt et al., 1972; Wachira et al., 1974). Positive responses appear to be less predictable at higher levels of energy intake, and when the untreated diet has a high crude protein content (Sharma et al., 1974; Wachira et al., 1974). In addition, Pisulewski and Rys (1975) pointed out the importance of considering the quality of a protein in relation to the animal's amino acid requirements. These workers found no beneficial effect on N retention in sheep, from formaldehyde-treatment of Horse Beans.

MacRae et al. (1972) found that the daily flow of amino acids into the duodenum was increased with formaldehyde-treated over untreated

casein. Apparent amino acid absorption from the intestines was also increased. Sharma et al. (1974) found similar results with formaldehyde-treated casein, but very little effect on flow from formaldehyde-treated rapeseed meal. They suggested that this could be due to the high flow rate of amino acids into the duodenum on untreated rapeseed meal. A depression in overall digestion in the rumen when feeding formaldehyde-treated supplements has been reported (Faichney and Weston, 1971; Hemsley et al., 1970). Faichney (1975) found a decrease in the mean retention time in the rumen, with formaldehyde-treatment of a concentrate diet, but both the solid and particulate phases were retained longer in the abomasum and small intestines. The importance of abomasal pH was suggested by Faichney (1974), who found a higher percentage of amino- and peptide-N in the abomasal-N of a sheep with a lower abomasal pH, whereas a higher degree of formaldehyde-protein complexing was found in a sheep with a higher abomasal pH. This data might suggest that pH is a major factor in the reversal of formaldehyde-binding, but the data recorded were only obtained from two sheep.

The overall effect of formaldehyde-treatment appears to be a decreased solubility in the rumen, resulting in increased flow of protein into the duodenum. The advantages of this increase will obviously depend on: the availability of the amino acids for absorption, i.e. reversal of binding; the flow of amino acids on the untreated diet; and the proportions of individual amino acids presented for absorption, in relation to the protein requirements of the animal.

1.7 The fermentation of silage and its effect on intake

The efficient use of herbage for ruminant production requires its conservation during periods of rapid growth, to enable supplementary feeding during periods of restricted growth. The main aims are to start

with a crop of high nutritive value, and to maintain this quality during the conservation process. The two main conservation processes in current practice are:

(1) field drying of a crop for storage as hay,

(2) anaerobic fermentation to produce silage.

Since one of the key factors in the efficient conservation of grass is the weather, silage has the advantage over hay, in that the grass can be cut and removed from the field in one operation. However, a high quality feed is of little value unless it is acceptable to the animal, and the voluntary intake of silage by ruminants has frequently been found to be lower than that of hay made from the same crop (e.g. Murdoch, 1964; Thomson, 1966; Wellman, 1966; Bishop and Kentish, 1970; Demarquilly and Jarrige, 1970). While considerable research on the ensilage process has led to an appreciation of the optimum conditions for fermentation, the factors involved in limiting intake have not been clearly established.

A. The fermentation of silage

Barnett (1954) outlined four main phases in ensiling:

(1) the respiration of plant cells, leading to the formation of carbon dioxide and water from water soluble carbohydrates,

(2) the production of acetic acid by bacterial action,

(3) the production of lactic acid by bacteria utilising soluble carbohydrates,

(4) a quiescent phase.

These four may be followed by a fifth phase of clostridial fermentation if the crop is poorly fermented. *Clostridium spp.* convert amino acids to ammonia and amines, and lactic acid to butyric acid, with a resultant increase in pH (Whittenbury *et al.*, 1967). Thus, a high

quality silage has been classified as having:

(1) a low butyric acid concentration (less than 0.3%),

(2) a low ammonia concentration (less than 8% of total N),

(3) a pH of approximately 4.2 or less (McDonald and Whittenbury, 1973).

While a low ammonia concentration represents little breakdown of amino acids, 40% of the original protein may be broken down, even in a well preserved silage (McDonald et al., 1969). The initial proteolysis appears to be due to autolysis by plant enzymes (Kemble, 1956) and this can be limited by rapid exclusion of oxygen from the silo. An anaerobic environment also favours a lactic acid fermentation, and this will rapidly decrease pH, thereby preventing clostridial growth (Macpherson and Violante, 1966; Ruxton et al., 1975). Degradation of the free amino acids is brought about mainly by clostridia with L-serine and L-arginine being the only amino acids to be significantly degraded by lactic acid bacteria (McDonald and Whittenbury, 1973). Decarboxylation of amino acids by clostridia leads to the formation of amines, and Hughes (1970) found the amines, histamine, tryptamine, tyramine, ethanolamine, ornithine, putrescine and cadaverine present in silage.

The effectiveness of a decrease in pH in inhibiting clostridia will be increased by a low moisture content of the herbage (Watson and Nash, 1960) and a high concentration of water soluble carbohydrate (Jones, 1970). The ratio of water soluble carbohydrate (WSC):protein in the herbage is also important, since a low WSC:protein ratio will provide substrates which favour the growth of clostridia rather than lactobacilli. This would lead to the undesirable production of ammonia and butyric acid. Kemble (1956) successfully ensiled grass of a high protein content by addition of glucose in combination with an innoculum of lactobacilli.

The main considerations for successful ensiling of herbage are thus to ensure an anaerobic environment and to prevent clostridial proliferation. The latter can best be achieved by ensiling herbage of a high DM percentage, and a high WSC:protein ratio. However, numerous chemical additives have been suggested as a means to facilitate successful fermentation.

B. Chemical aids to ensiling

McDonald and Whittenbury (1973) divided silage additives into two main groups:

(1) stimulants,

(2) inhibitors.

(1) Stimulants: Stimulants are compounds added prior to ensiling with the aim of promoting a lactic acid type of fermentation. Examples of these are compounds high in carbohydrate (e.g. molasses), or innocula of lactobacilli. The advantages of these additives have already been discussed in relation to their effect on the WSC:protein ratio of the herbage.

(2) Inhibitors: Inhibitors on the other hand, may either completely inhibit bacterial activity, or selectively prevent deleterious fermentation. The main inhibitors in common use are sodium metabisulphite, formic acid and the more recently introduced formaldehyde.

(a) Solium metabisulphite. This is used at concentrations which do not completely inhibit fermentation. Zelter (1960) found that while metabisulphite considerably reduced butyric acid production, it did not markedly prevent proteolysis. Murdoch et al. (1956) reported that metabisulphite decreased the formation of volatile acids and bases, but had little effect on the production of lactic acid.

(b) Formic acid. The use of mineral acids to produce a rapid initial

drop in pH, and thus inhibit clostridial fermentation, has now largely been replaced by application of formic acid to the herbage. In the UK, 85% formic acid is normally used, at 0.27% of the herbage DM. This decreases pH to 4.6 - 4.8 (McDonald and Whittenbury, 1973). Henderson and McDonald (1971) compared three levels of formic acid addition to grass: 0.22%, 0.34% and 0.51%. At higher levels of application, the formic acid appeared to affect the structure of the grass. No intake response to formic acid-treatment was observed, but the control silages were themselves readily consumed. Waldo et al., (1970); and Castle and Watson, (1973) found the following advantages of formic acid added prior to ensiling: decreased pH, increased digestibility, decreased levels of butyric acid, acetic acid and ammonia, and increased voluntary intake. However, formic acid is expensive, and under some conditions, losses of up to 50% of the acid have been reported (Henderson and McDonald, 1971).

(c) Formaldehyde. Formaldehyde is a substance in common use in Medicine and in the Leather Industry, because of its bacteriostatic properties, and its ability to react with proteins and thus protect them from microbial fermentation. Formaldehyde has been used to protect dietary proteins from degradation in the rumen, since the bonds are subsequently reversed at the low pH in the abomasum. Thus, the protein is available for digestion and absorption in the intestines (Ferguson et al., 1967). Hence formaldehyde has been applied to herbage in an effort to prevent protein degradation during ensiling. However, response to formaldehyde treatment of silage has been varied. Increased intake in response to formaldehyde-treatment of silage was reported by Barry et al. (1973) and Valentine and Radcliffe (1975), while Brown and Valentine (1972) reported decreased intake at high levels of application. Brown and Valentine (1972) suggested that the lack of response in their experiment could have been due to free formaldehyde present in the silage, since

Neumark et al. (1964) noted that free formaldehyde present in or added to silage decreased intake. Some free formaldehyde might be expected to occur in all formaldehyde-treated silages, since Theis and Lams (1943) reported that reversal of formaldehyde-binding with protein occurred with a gradual decrease in pH. Hence the decrease in pH in the silage stack might be expected to release free formaldehyde and protein.

Barry and Fennessy (1973) reported that secondary fermentation on opening of the stack was greater in formaldehyde-treated than untreated silages. Combinations of additives have been suggested to overcome this problem of secondary fermentation, and Barry (1975) compared the effects of formaldehyde, and formaldehyde/acid (formic or sulphuric) mixtures. Intake was highest on formaldehyde/formic acid-treated silages, and while intake of untreated and formaldehyde-treated silages decreased with time after opening the stack, addition of formic acid prevented this decrease. However, secondary fermentation was still in evidence. Valentine and Brown (1973) also found a significant increase in intake of formaldehyde/formic acid silage over untreated silage, while neither formaldehyde nor formic acid alone significantly increased intake over untreated silage.

C. Factors affecting intake of silage

(1) General: Much of the work on silage quality has been confined to biochemical and microbiological studies of fermentation within the silage stack, and has not considered voluntary intake by the animal. Thus, while a high quality silage has been defined as having a pH of 4.2 or less (McDonald and Whittenbury, 1973) various workers have reported that silages with a low pH adversely affect intake (Hutton et al., 1970; McLeod et al., 1970; Hutchinson and Wilkins, 1971; Wilkins et al., 1971). This appears to be partly due to a direct effect

of high acid concentration (McLeod et al., 1970), but a low pH also indicates considerable fermentation of water soluble carbohydrates (Brown and Radcliffe, 1972), and this may adversely affect the balance of N and energy metabolism in the rumen.

(2) The effect of form and concentration of N and energy on silage intake: The composition of the N fraction of silage differs from that of pasture and hay, in having relatively high ammonia, amine and amide contents (Hughes, 1970). High ammonia concentrations in silage have been correlated with low intakes (Harris and Raymond, 1963; Wilkins et al., 1971). Castle and Watson (1969) found that total intake of a mixed ration was also affected by silage-N content. They compared voluntary intake of silages of low (8%) and high (16%) digestible crude protein content, and found that total DM intake was lower on the silage with the higher N content. The effect of the N content of the concentrate part of mixed rations was studied by Murdoch (1964). He found that intake of silage decreased to a lesser extent with a high protein than a low protein concentrate. Since the N content of silage is usually of a low true protein percentage, these results suggest that the form, rather than the quantity of N intake, appears to exert a major effect on silage intake.

Efficient utilisation of NPN in the rumen is dependent on the availability of energy (Henderickx and Martin, 1963). Since the concentration of water soluble carbohydrates in silage is generally low (McDonald et al., 1969), increased utilisation of N may be achieved by increased energy availability. Thus, increased N retention in response to supplementation of silage with energy has been recorded (Durand et al., 1968; Thomson, 1968; Griffiths et al., 1973). The effect of silage-N and energy contents on silage intake, thus appears to be due

to the promotion of microbial protein synthesis and hence the total amount of protein entering the duodenum.

(3) The effect of duodenal protein availability on silage intake: Egan (1965) suggested that voluntary intake of low protein roughages may primarily be limited by a deficiency of protein absorbed by the animal. Increased growth rates in response to protein supplementation of animals fed silage have been reported (Forbes and Irwin, 1970; Drennan, 1973). However, Hutchinson et al. (1971) found no intake response to duodenally infused casein on a 1.86%N silage, although N retention was increased.

Increased intake in response to formalin-treatment of herbage prior to ensiling has been recorded (Barry et al., 1973). Since the main effect of formalin-treatment is to prevent protein degradation during ensiling, this result suggests a positive intake response to increased protein availability. Increased intakes of silage in response to intraperitoneal methionine have also been reported (Barry et al., 1973; Kelly and Thomas, 1975; Barry, 1976). This result suggests that feeding the untreated silages to sheep induced a state of methionine imbalance. Methionine has frequently been suggested as the first limiting amino acid for ruminants (Nimrick et al., 1970; Hutton and Annison, 1972; Armstrong and Annison, 1973; Harrison et al., 1973). Hence the availability of amino acids, and particularly methionine, appears to limit intake in some silages.

1.8 Conclusion

Methionine appears to be the first limiting amino acid for live-weight gain and wool growth in ruminants on a variety of feeds. In particular, some silages produce a very low level of S-amino acids entering the duodenum. Since low intake of silage has in some cases

been increased by supplemental methionine, it is suggested that feeding silage may produce a methionine imbalance. An imbalance state indicates that the complement of amino acids absorbed from the duodenum does not correspond to the complement required by the tissues. Thus, silage appears to have a marked effect on either the composition of duodenal amino acids, or the requirement for methionine relative to total amino acid requirements. The amino acid composition of duodenal digesta remains relatively constant in sheep fed natural diets, since microbial protein is of relatively constant amino acid composition and is similar to that of herbage protein. Feeding silage may change the composition of duodenal amino acids, but a more likely explanation of the imbalance state, is a change in the relative requirements for methionine and other amino acids, due to a decreased total availability in the small intestine. The percentage of the available methionine which is used for protein synthesis, compared to the percentage used for transmethylation reactions and conversion to cysteine, changes with the availability of methionine and other amino acids. Hence, a decrease in the availability of total amino acids may increase the percentage of methionine required.

Thus, the experiments to be reported were undertaken to study the effects of increasing the availability of total protein, and methionine alone, to sheep fed silage. The effects were studied in terms of the utilisation of N and amino acids by the sheep. The availability of total protein was increased in three ways: by formalin-treatment of the silage; supplementation with formaldehyde-treated casein; and supplementation with a readily available energy source, to stimulate microbial protein synthesis and thus increase the total flow of amino acids into the duodenum. Methionine availability was increased by infusion into the peritoneal cavity or duodenum.

CHAPTER 2

TECHNIQUES FOR STUDYING AMIND ACID METABOLISM IN RUMINANTS

2.1 Introduction

The preceding chapter has shown that a study of amino acid metabolism in ruminants requires measurement of the daily flow of amino acids into the duodenum. The accuracy of measuring the flow of digesta into the duodenum has been greatly facilitated by the use of sheep prepared with re-entrant cannulae in the proximal duodenum (Ash, 1962). These cannulae enable total collection of digesta and thus a representative sample can be obtained. Calculation of flow rate also requires reference to an indigestible marker since collection procedures frequently depress flow. The reasons for the choice of specific sampling system used in the experiments to be reported will be discussed below.

Analysis of the amino acid composition of the samples obtained, was carried out on a standard amino acid analyser employing the ion-exchange principle of amino acid analysis, first introduced by Moore and Stein (1951). Since the first chromatographic conditions were reported, numerous modifications have been made, and specific procedures for the measurement of groups of amino acids have been proposed (e.g. Jeppsson and Karlsson, 1972). In the experiments to be described, measurement of methionine specific radioactivity was required. These analyses were also conducted on an amino acid analyser and the reasons for this choice will be discussed below.

The amino acids entering the duodenum will be supplied either by dietary protein which has escaped rumen fermentation, or by microbial protein synthesised in the rumen. The effect of energy and N metabolism in the rumen on microbial protein synthesis has already been discussed, and thus the advantages of being able to measure its rate of synthesis are apparent. Numerous methods for quantifying microbial

protein synthesis have been proposed, but there are still considerable drawbacks to their routine use, and these will also be discussed.

2.2 Measurement of duodenal flow

The measurement of flow of nutrients into the duodenum has two main requirements. The first is obtaining an accurate measurement of digesta flow, and the second is the collection of a representative sample of digesta. Several reviews of the techniques involved in measuring flow into the duodenum have been published recently (Kotb, 1972; Engelhardt, 1974; MacRae, 1974; MacRae, 1975; Faichney, 1975). Thus, only the main considerations affecting the choice of method for the experiments to be reported, will be discussed.

A. General considerations

The collection of samples for measuring the composition of digesta entering the duodenum must be made prior to any absorption or secretion within the small intestine. However, since the abomasum is a sacular organ, with numerous folds dividing the contents, collection of a representative sample of abomasal digesta is difficult. Therefore, the most suitable sampling site appears to be immediately after the entry of digesta into the duodenum, prior to entry of secretions of bile and pancreatic juice. The ease of collection of digesta and measurement of flow has been greatly increased by the preparation of sheep with re-entrant cannulae in the proximal duodenum (Ash, 1962). These cannulae enable the collection of the total digesta entering the duodenum, the removal of a subsample and the return of digesta via the distal cannula. Failure to return digesta to the animal has been shown to increase flow (Hogan and Phillipson, 1962). Harris and Phillipson (1962) therefore suggested that an indigestible marker be used, to correct total flow to

100% recovery of the marker. They also suggested that the volume of subsample taken for analysis be replaced with donor digesta prior to return of the collected digesta to the animal. However, a number of workers have reported that this method of total collection tends to depress digesta flow (Topps et al., 1968; MacRae and Armstrong, 1969; Nicholson and Sutton, 1969; MacRae et al., 1972). Klooster et al. (1972) found that this depression in flow was only temporary, and continuation of the collection for 72h showed a recovery in flow rate. Since the collection and return of digesta is extremely tedious, several attempts to collect and sample digesta automatically have been made. Very little data from the use of automatic samplers has as yet been published (Tas et al., 1974; MacRae, 1975) and these samplers are not generally available.

An alternative procedure is to take spot samples. These have the advantage over continuous sampling techniques in that they are less traumatic to the animal and less laborious for the experimenter. MacRae and Ulyatt (1972) compared the use of spot and continuous sampling techniques and found close agreement between the two methods, although more variability was apparent when estimating flows from spot sampling. In general, spot sampling techniques are used for sheep prepared with simple T-shaped cannulae. These cannulae are easier to implant and maintain than re-entrant cannulae. However, sheep with re-entrant cannulae were used for the present experiments (Chapters 3 and 4), and a variation of the spot sampling technique was employed (Walker et al., 1975). Twelve spot samples were collected over a period of three days. There were four sampling times per day, with a 6h interval between each sampling time. Daily groups of sampling times were separated by an 8h interval. Hence, if samples on the first day were collected at 10 am,

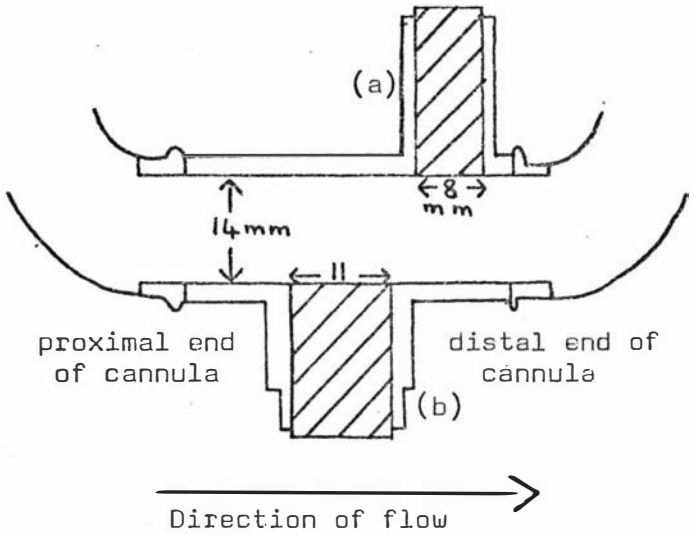


Fig. 2.1 Duodenal cannula between sampling

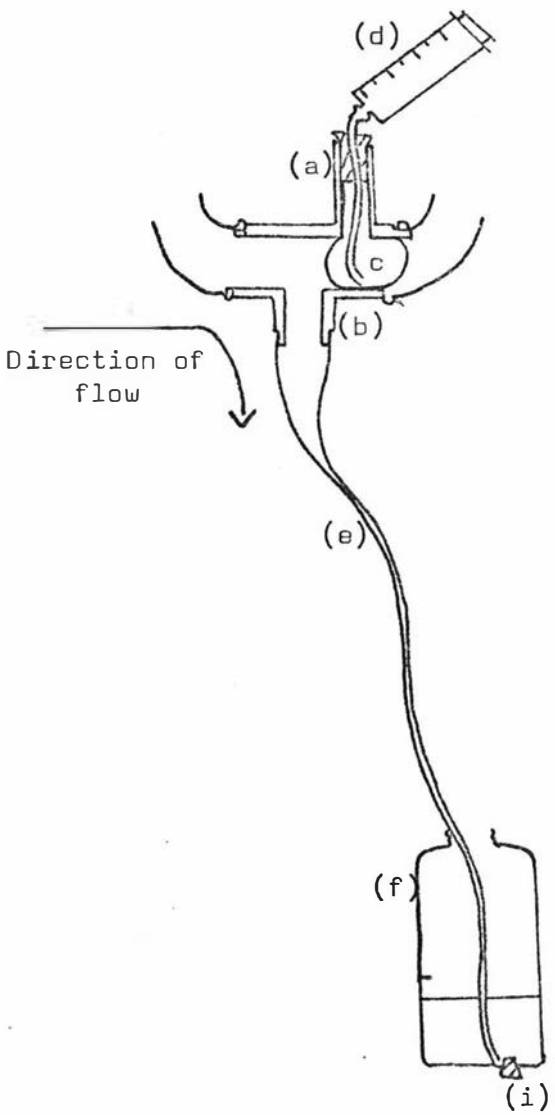


Fig. 2.2 Collection of digesta sample

4 pm, 10 pm and 4 am, the next sample would be collected at 12 noon.

The use of re-entrant cannulae for spot sampling enables the collection of the total digesta flowing during the sampling period. As the duodenum is a tubular organ, it was considered that there would not be a marked difference in flow rate between the solid and liquid phases of digesta. Hence it was considered that only one marker that associated with either the liquid or solid phase, was required to determine both DM and total flow rates. Since some difficulty had earlier been experienced in finding suitable solid phase markers, (MacRae and Ulyatt, 1972), a liquid phase marker (Cr EDTA) was chosen. (Weller and Pilgrim, 1974; Walker et al., 1975). Cr was analysed by atomic absorption spectrophotometry.

B. Accuracy of method and modifications introduced

In Expt. 1, spot samples of 40g were taken at each sampling time and then bulked. This technique was modified prior to Expt. 2, to obtain a more representative sample. This modification involved replacement of the tubing connecting the two ends of the re-entrant cannula, with a Perspex connecting piece. This had two side-arms, one for collection and one for return of digesta (Fig. 2.1). Samples could thus be collected without disconnection of the cannula at each sampling time. To ensure collection of total contents, a balloon (Fig. 2.2 c) was inserted through (a) and inflated with air from a syringe (d). This syringe was attached to a short length of tubing which was enclosed within the balloon. Inflating the balloon blocked off the distal end of the cannula forcing digesta to flow through (b). A length of Latex colostomy tubing (e) was attached to (b) and carried the duodenal digesta into a plastic collection bottle (f). The tubing collapsed when empty,

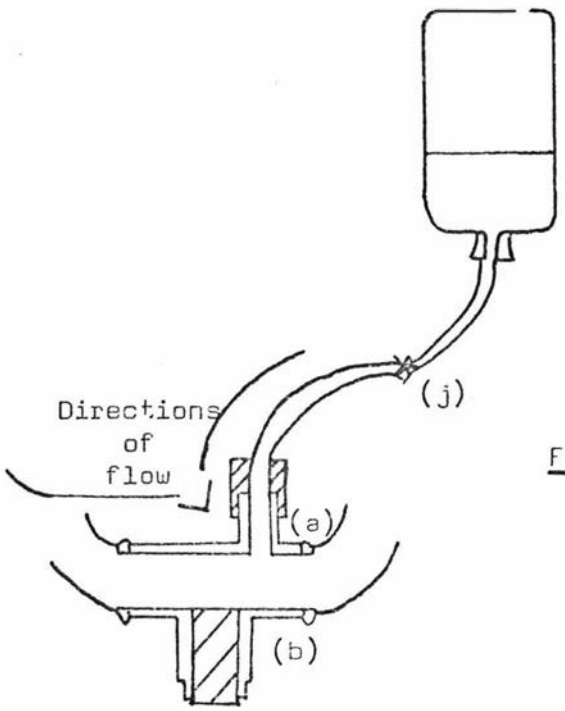


Fig. 2.3 Return of contents after subsampling

and thus simulated the conditions of flow through the duodenum. After 250ml of digesta had collected in (f), the balloon was removed and the rubber plugs replaced in (a) and (b). The digesta was mixed and a 25ml subsample taken. The collection bottle was then stoppered and placed in a basin of warm water, to reheat the digesta to 39°C, prior to return to the animal. The collection bottle (f) was then attached to a flexible plastic tube (Fig. 2.3) which in turn was connected to the re-entry arm of the connecting piece (a) by a rubber sleeve. Return of the digesta was thus achieved by inverting the plastic bottle and removing the small rubber stopper (i). The rate of return of digesta was controlled by a clamp (j). This technique enabled the collection of a larger volume of sample, which could then be subsampled to reduce the error associated with each sample. By this procedure only 25g digesta were removed at each sampling time, and in total this comprised less than 1.5% of the daily flow. It was considered that replacement of the volume sampled with donor digesta was not necessary, since 6h elapsed between ~~each~~ successive sampling times. In addition, the return of donor digesta which has been frozen and thawed, thus denaturing the protein, might have a marked effect on amino acid digestion and absorption.

The samples collected in Expt. 1, were bulked overall for each sheep on each treatment, giving only three estimates of flow per treatment. Considerable variation between replicates was observed in Expt. 1, and samples in Expt. 2 were bulked on a daily basis. This should have enabled estimation of the repeatability of measurements. However, daily intakes during flow measurements varied considerably and this produced large differences in the flow estimates recorded. On the control diet, intake varied by as much as 200g between consecutive days.

Thus, estimation of the accuracy of flow measurement was only calculated for sheep 1157 on replicates 1 and 2, and for sheep 1303 on replicates 3 and 4, when the intake of these sheep remained approximately constant. Since these two sheep were both being supplemented with formaldehyde-treated casein during the specified periods, a combined standard error (SE) was calculated. The SE obtained with a mean of 429gDM/day was ± 9.2 g/day which indicates a high degree of repeatability.

C. Analysis of CrEDTA

Prior to the start of the first experiment, two methods of preparing digesta samples for atomic absorption analysis of Cr were tested. In the first method, the samples were ashed in a furnace, prior to oxidation with potassium bromate, and they were subsequently dissolved in phosphoric acid-manganese sulphate solution (Williams et al., 1962). In the second method, the supernatant was prepared by centrifugation of total digesta at 38,000g, followed by filtration through Whatman No. 1 filter paper. Preparation of the sample by the ashing method of Williams et al. (1962) only gave a 71.3% (± 4.0) recovery of added Cr. Analysis of Cr in the supernatant, prepared by centrifugation gave a recovery of 96.6% (± 4.1), and after washing the pellet three times in water, and ashing, no Cr was detected. Hence further tests were conducted on supernatant material.

The routine standards used a potassium dichromate solution, where Cr is present in a different oxidation state (Cr^{6+}) from Cr complexed to EDTA (Cr^{3+}). To test the validity of the dichromate standards, alternative standards were made up from chromium potassium sulphate (Cr^{3+}) but no difference was detected. Measurement of Cr with and

without EDTA also had no effect. Williams et al. (1962) suggested the addition of 1000ppm calcium to standards and samples, to override any interference from calcium present in the digesta. The effect of calcium during these tests was found to be variable, but the presence of calcium appeared to maintain a constant recovery.

The following guidelines for the measurement of Cr by atomic absorption spectrophotometry were adopted:

(1) preparation of samples by centrifugation (38,000g) followed by filtration,

(2) dilution of samples and standards with calcium, to a final solution of 1000ppm calcium,

(3) measurement of the corresponding infusates with each batch of samples.

2.3 Amino acid analysis and measurement of specific activities

A. General amino acid analysis

Routine measurement of amino acid composition is now mainly conducted on automated amino acid analysers. A wide range of instruments are available, with adaptations for special analytical requirements (Porter et al., 1968). The basic principle of these analysers is the differential separation of individual amino acids on a column of ion-exchange resin, by elution with buffers of differing pH and ionic concentration. The chromatographic conditions are changed to achieve the particular analysis required (e.g. protein hydrolysate or physiological fluid analysis). However, analysis of specific amino acids by such automated procedures requires considerable manipulation of the chromatographic conditions to achieve separation in a minimum time. In addition the volume of sample which can be analysed, and the

range of concentration which can be accurately detected, are limited by the specifications of the instrument. Thus, for analysis of methionine specific activities in the experiments to be described, consideration was first given to other methods of measuring methionine concentration.

B. Measurement of methionine specific activity

The concentration of methionine in sheep plasma is very low, and since the isotope used in the present experiments (L-methionine-C14 (U)) was very expensive, the specific activity (SA) of methionine in the plasma samples was also very low. Thus a technique capable of analysing a relatively large volume of sample was required.

Specific methods for the determination of S-amino acids have been proposed (LaRue, 1965; Kyriacou, 1966; Rublev, 1973). However, since there are numerous transformations between individual amino acids, the measurement of methionine SA requires prior separation from the other amino acids. Thus, the choice of method for determining SA is limited to the various chromatographic methods. With paper and thin layer chromatography and electrophoresis, amino acid concentration is measured by a colour reaction on the stationary phase. The most commonly used reagent for colour development is ninhydrin. However, this reaction releases CO_2, the loss of which would affect the SA of ^{14}C labelled methionine. Thus, identification of the labelled amino acid either involves the running of two identical chromatograms, of which one is developed and so enables location of the amino acid position on the second, which can then be used for scintillation counting, or alternatively, autoradiography can be used to detect radioactivity. After detection of the position required, that amino acid is eluted from the support medium and the activity measured in a scintillation counter (Meister, 1965). The problem of running two chromatograms can be

avoided by thin layer chromatography of the dansyl derivatives of amino acids (Airhart et al., 1973), since their concentration can be measured by fluorescence under UV light (Weber, 1952). However, only small volumes of sample can be applied to paper or thin layer plates and thus these methods are not suitable for analysis of methionine SA in the present samples.

The use of ion-exchange or gas chromatography to measure SAs has the advantage that the amino acids are eluted from the stationary phase prior to colour development. The eluent can therefore be split into two streams, one for colour development, and one for radioactive counting. Thus, since standard amino acid analysers were available, it was decided to measure methionine SA using the specific S-amino acid procedure of Jeppsson and Karlsson (1972). The Beckman analyser at Applied Biochemistry Division, D.S.I.R., which was used for routine amino acid analysis cannot be readily modified to collect part of the column effluent for counting of radioactivity. Thus, a Jeol (JLC 6AH) amino acid analyser at Ruakura Animal Research Centre, Hamilton was used for the measurement of methionine SAs.

C. Discrepancy between amino acid analysers

The chromatographic procedure of Jeppsson and Karlsson (1972), which was used for measuring methionine SA on the Jeol, only separated the following amino acids: valine, cysteine, methionine, isoleucine, leucine, tyrosine and phenylalanine. Therefore a complete physiological fluid analysis for duplicate plasma samples for each sheep from each replicate was also run. The details of Expt. 1 are given in Chapter 3 (Expt. 1), but the basic design involved three treatments (untreated silage, formaldehyde-treated silage, and untreated

Table 2.1 A comparison of the observed plasma methionine concentrations as measured on a Jeol or a Beckman (in parentheses) amino acid analyser. (μmole/l)

	Rep. 1	Rep. 2	Rep. 3
Untreated[1] silage	9.6±1.7[2] (7.5)	6.7±0.7 (6.0)	11.2±1.6 (9.6)
Treated silage	9.6±1.9 (7.2)	12.4±2.0 (10.3)	13.7±2.5 (9.4)
Untreated silage + met	11.8±1.2 (19.0)	11.7±2.1 (16.4)	20.0±1.2 (25.5)

[1] for experimental design, see Chapter 3, Expt. 1.

[2] ± the SE of the mean.

Table 2.2 A comparison of the corrected plasma methionine concentration for the Jeol amino acid analyser, with the observed value from the Beckman amino acid analyser (in parentheses).

	Rep. 1	Rep. 2	Rep. 3
Untreated silage	9.2[1] (7.5)	5.5 (6.0)	11.3 (9.6)
Treated silage	9.2 (7.2)	12.8 (10.3)	14.5 (9.4)
Untreated silage + met	12.1 (19.0)	11.9 (16.4)	22.6 (25.5)

[1] values from Jeol amino acid analyser, corrected with the regression equation:

$Y = 1.281\ X - 3.062$ where Y is the concentration measured on the Beckman

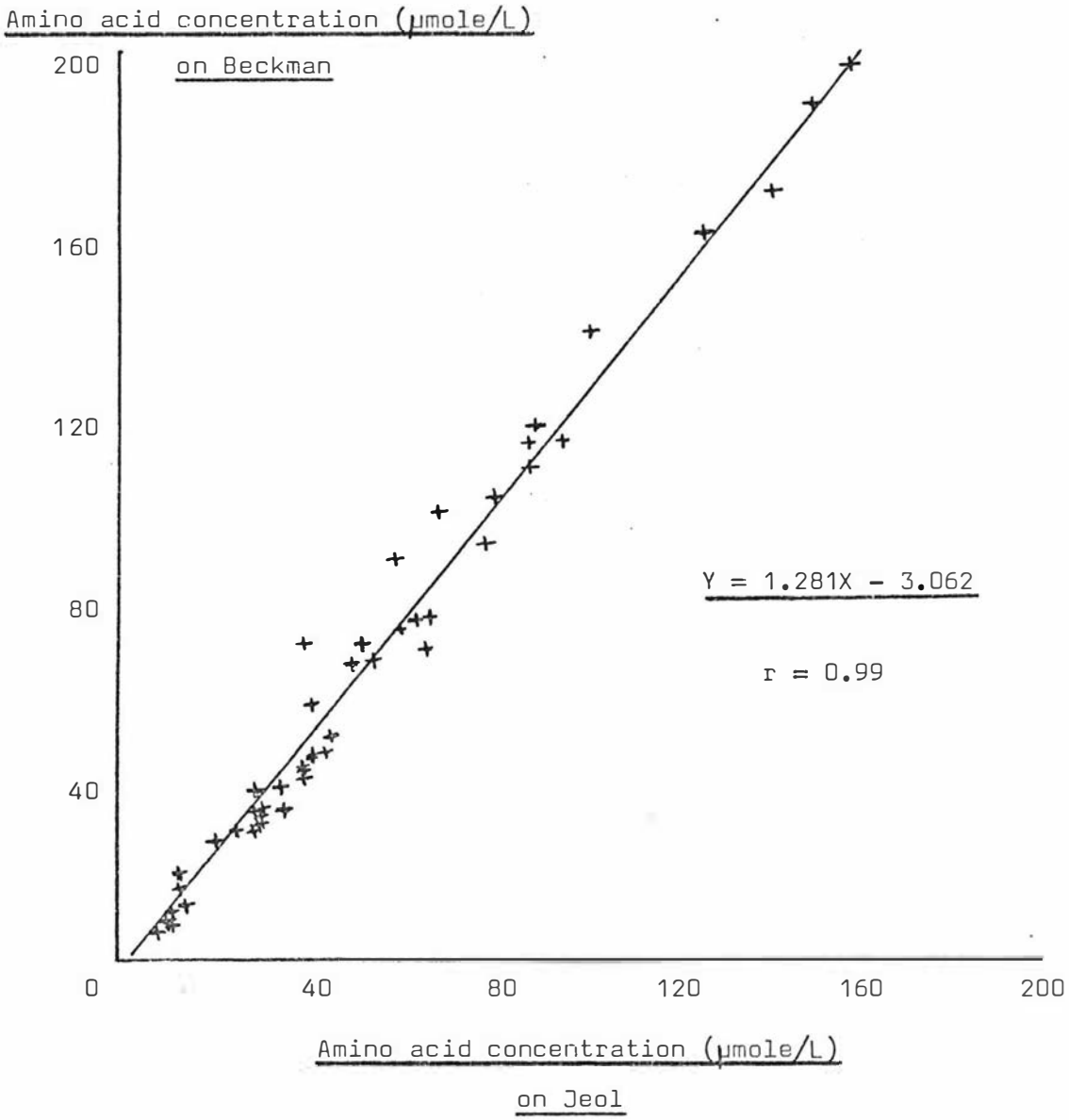


Fig. 2.4 Regression relationship between plasma amino acid concentrations measured on a Jeol or Beckman amino acid analyser.

silage + intraperitoneal (I/P) methionine infusion), with three sheep per treatment in a Latin Square design, giving nine separate samples. The physiological fluid analysis was carried out on a Beckman 120C analyser using a two column procedure. However, the concentrations of the six amino acids (cysteine concentrations were too low to measure) measured on both analysers differed markedly with the instrument used. The values obtained on the Beckman were higher than those obtained on the Jeol, for all amino acids except methionine in the plasma of sheep on two of the treatments. A regression relationship was fitted between the Beckman values (Y) and the Jeol values (X), using the concentrations of each of the six amino acids, from each of the nine samples analysed. This regression relationship is given in Fig. 2.4. The correlation coefficient of 0.99 indicates a constant relationship between the two analysers, independent of the amino acid compared. However, the concentration of methionine in the plasma of sheep receiving I/P methionine infusion did not follow the general trend (Table 2.1). This is not apparent from Fig. 2.4, owing to the low concentration of methionine in sheep plasma. Correction of plasma methionine concentrations using the regression relationship ($Y = 1.281X - 3.062$) (Table 2.2) produced little change in the actual value. Thus, the methionine concentration in the plasma of sheep fed treated and untreated silage remained higher from the Jeol than from the Beckman, this difference being only slightly greater than the SE. By contrast, the plasma methionine concentration in sheep receiving I/P infusion of methionine, remained higher on the Beckman than the Jeol. Thus, there appear to be two separate aspects of the differences between analysers. The first is a difference in all amino acids which can be described by the regression relationship in Fig. 2.4. The second is a

Table 2.3 The effect of freeze-drying on plasma amino acid concentration (μmole/1)

	Untreated silage + met (Rep. 1)		Untreated silage (Rep. 2)	
	Before	After	Before	After
Valine	85.4	84.0	111.6	104.4
Methionine	18.3	18.4	4.9	5.6
Isoleucine	40.8	45.7	42.4	42.8
Leucine	69.0	75.2	65.1	60.0
Tyrosine	27.4	30.6	30.4	32.4
Phenylalanine	34.9	41.0	21.9	24.3

Table 2.4 Comparison of colour factors, denoting the efficiency of ninhydrin reaction from Jeol and Beckman amino acid analysers

	Beckman	Jeol 1974	Jeol 1976
Valine	1.05 ± .028	0.92 ± .048	1.44 ± .118
Methionine	1.02 ± .039	0.87 ± .046	0.84 ± .071
Isoleucine	0.84 ± .108	0.82 ± .057	1.16 ± .107
Leucine	1.01 ± .046	0.75 ± .056	1.02 ± .069
Tyrosine	1.01 ± .040	0.79 ± .050	1.01 ± .089
Phenylalanine	1.01 ± .051	0.82 ± .031	1.01 ± .076

treatment effect on the difference in methionine concentration between analysers.

To try and find the reasons for these differences, the following points of procedure will be discussed relative to each amino acid analyser:

(1) method of sample preparation,

(2) sample storage time,

(3) method of calculating results,

(4) different chromatographic conditions.

(1) Method of sample preparation: The volume of sample applied to the resin on the Jeol amino acid analyser is a constant 0.8ml. The samples for analysis of methionine SAs were therefore first freeze-dried, and then re-constituted in 1ml of 0.01N HCl to enable the analysis of a larger volume of plasma. The samples applied to the Beckman columns were not freeze-dried. However, analysis of duplicate samples before and after freeze-drying indicated no marked effect of this treatment (Table 2.3).

(2) Time of storage: All plasma samples were stored at -20^{o}C for at least six months prior to analysis. The complete physiological fluid analysis (Beckman) was conducted some months after the measurement of methionine SAs (Jeol). However, the concentrations measured on the Jeol were lower than those measured on the Beckman, and thus loss of amino acids during storage cannot explain the overall concentration difference between analysers. Storage loss could explain the slight difference between analysers in the methionine concentrations in the plasma of sheep fed treated and untreated silages, since methionine has been shown to be particularly susceptible to degradation on storage.

However, the methionine concentration in the plasma of sheep receiving I/P methionine was higher on the Beckman than the Jeol, and cannot therefore be attributed to loss with storage.

(3) Calculation of results: Reaction of amino acids with ninhydrin is recorded as a series of peaks, the areas of which represent the concentration of the corresponding amino acids. Calculation of the areas under the peaks was carried out by a computer for the methionine SA analysis (Jeol) and by hand for the physiological fluid analysis (Beckman). Recalculation by hand of randomly chosen computed analyses from the Jeol, indicated no consistent differences between computer and hand calculation. Calculation of the amino acid concentrations from the peak area, involved the use of a conversion factor, related to the area of an internal standard (norleucine). Norleucine (NL) (0.100μmole) was applied to the column with each sample, and also with a standard mix of amino acids. This standard mix contained 0.100μmole of each amino acid required. The relationship between the peak area for a specific amino acid in the standard and the area under the peak for NL, gave the efficiency of ninhydrin reaction for each amino acid, relative to NL. The ratio of amino acid area to NL area in the sample could then be divided by the ratio in the standard, and thus corrected for the efficiency of ninhydrin reaction for each amino acid. The ratios obtained from chromatographing a standard mix of the six amino acids relevant to this discussion, should be close to 1.00. The actual values obtained for each analyser are presented in Table 2.4. The values obtained at the time of analysis on the Jeol (1974) were generally lower than those obtained on the Beckman, and later values obtained on the Jeol. The difference in colour factors for the Jeol as measured in 1974

and 1976, together with the lower concentrations determined on the Jeol than the Beckman suggests that the colour factors used in 1974 may not have been valid. The differences between the two Jeol standards are not consistent for all the amino acids, and thus cannot explain the regression relationship found for all amino acids between the two analysers. However, the colour factor for methionine was not significantly different as measured in 1974 or 1976, and thus the methionine SA measurements appear to be valid, at least for the sheep on treated and untreated silage.

(4) Chromatographic procedure: The Jeol analyses used two buffers of pH 2.82 and 3.66, with no temperature change. The Beckman analyses for the acidic and neutral amino acids used buffers of pH 3.25 and 4.30, with a temperature change from 30 - 62.5^{o}C. Thus the elution times for the individual amino acids differed between the two instruments. Hence, if unidentified ninhydrin-positive compounds were also present in the plasma samples, they might have been eluted from the two instruments in different regions of the chromatograph. The elution of such a compound with methionine, in plasma from sheep receiving I/P methionine, on the Beckman, might explain the higher concentration measured for sheep receiving this treatment, on the Beckman compared to the Jeol. Oxidation of the S-amino acids prior to analysis did not reveal an additional peak in place of methionine. In addition, attempts to separate and re-chromatograph the methionine peak from the Beckman analysis were unsuccessful, partly as a result of the very low concentration of methionine in the plasma. Thus, the presence of an impurity contributing to the methionine peak in the physiological fluid analysis from sheep receiving I/P methionine infusion, remains a possible explanation.

No definite conclusions on the discrepancy in amino acid concentrations as determined on the Beckman and Jeol amino acid analysers can be given. However, the most likely explanation for the difference in methionine concentrations appears to be partly due to a storage effect, and partly to an unidentified compound contributing to the methionine concentration in sheep receiving I/P infusion as determined on the Beckman. Hence the concentrations determined for methionine SA appear to be valid. The concentrations of the other amino acids were determined to study the effect of the various treatments, and thus comparisons between samples determined on the same analyser are also valid, with the qualification that the methionine peak may not be pure.

2.4 Measurement of microbial protein synthesis

Several methods for measuring the proportion of microbial-N in the total protein available to the animal at the duodenum have been proposed. Direct estimation may be achieved by measuring the degree of incorporation of a specific component into microbial protein. This estimation requires accurate measurement of the concentration of the component chosen, in a pure sample of rumen micro-organisms. This sample must not be contaminated by plant material and must be representative of the micro-organisms present in the rumen. Separation is achieved by differential centrifugation of a large volume of digesta. Resuspension and recentrifugation of the bacterial layer is necessary to ensure purity, and examination with a microscope to check for contamination with plant material should also be made. Impure samples must be discarded. The separation of a pure sample is a difficult, but essential part of any method for measuring microbial protein synthesis.

The microbes are usually separated from rumen digesta, since it is more difficult to obtain an acceptable preparation of duodenal digesta, due to the small size of plant particles (Ulyatt et al., 1975) and to lysis of micro-organisms in the acid conditions of the abomasum (Beever et al., 1974). The sample should also be prepared from fresh digesta rather than digesta which has been frozen, with consequent lysis of microbial cells.

The concentration of the marker in total digesta is also measured, and thus the proportion of microbial-N in total digesta-N is given by:

$$\frac{\text{concentration of marker/ g digesta-N}}{\text{concentration of marker/ g microbial-N}}$$

The main markers which have been used are:

(1) 2:6 diaminopimelic acid (DAP),

(2) nucleic acids (NA),

(3) labelled S groups (^{35}S).

(1) Diaminopimelic acid: The presence of the amino acid DAP in bacterial cell walls was first recorded by Work (1951). Synge (1953) then reported that DAP was not present in plant material and this led to the use of DAP as a marker for rumen microbial protein synthesis. However, DAP is not present in protozoa (Rose, 1968) and thus only bacterial protein synthesis is measured. In addition, there is considerable variation in the concentration of DAP between bacterial species (Weller et al., 1958). Further, DAP is absent from some bacteria (Rose, 1968), thus, a high proliferation of these bacteria could lead to an underestimation of synthesis.

(2) Nucleic acids: Smith and McAllan (1970) showed that the amounts of NAs entering the small intestine of ruminants bore little relation to dietary intakes of NAs. They observed almost complete breakdown of

dietary NAs in the rumen, and concluded that duodenal NAs were almost entirely microbial in origin. Smith (1975) pointed out that the variation in RNA concentration of bacteria was much lower than the variation in DAP concentration. The concentration of RNA also appeared to be less variable than the concentration of DNA (McAllan and Smith, 1972) and thus the use of RNA as a marker was preferred to DNA or total NAs. However, this method assumes complete degradation of dietary NA in the rumen. This hypothesis was tested by addition of pure NAs to the rumen (Smith and McAllan, 1970) but the fate of dietary NAs in insoluble plant material has not been clearly established.

(3) Labelled S: Nader and Walker (1970) showed that bacterial synthesis of S-amino acids was more important than direct incorporation of the preformed S-amino acids into bacterial protein. This finding was the basis of methods using infusion of $Na_2^{35}SO_4$ to measure rumen microbial protein synthesis (Beever et al., 1974; Walker and Nader, 1975). However, other workers have suggested that amino acids per se, including the S-amino acids, are an important precursor of microbial protein (Landis, 1963; Nolan et al., 1976). Thus, further work appears to be necessary before an evaluation of the validity of techniques using ^{35}S label can be made.

Beever et al. (1974) used a continuous infusion of $Na_2^{35}SO_4$ to label the methionine synthesised in microbial protein. They sampled duodenal digesta and measured SA of methionine in total digesta and in a sample of microbes, prepared from duodenal digesta. Knowledge of the percentage of methionine in the microbes, and in total duodenal digesta allowed calculation of the proportion of microbial protein as described above. However, separation of a pure and representative sample of microbes from duodenal digesta is a very difficult procedure, as

Table 2.5 Calculation of microbial protein synthesis in sheep fed silage, using ^{35}S marker (for Expt. 1).

Treatment	^{35}S SA in Digesta (dpm/g wet wt.)	^{35}S SA in Microbial S (dpm/µg S)	Rumen Volume (L)	Turnover Constant (d-1)	Microbial Protein Flow (g/day)	Total Flow of Amino Acids (g/day)
Untreated Silage						
Rep. 1	382	11.2	5.4	1.95	29.2	47.8
Rep. 2	378	4.5	4.7	1.94	62.2	46.2
Rep. 3	926	8.1	1.6	2.18	32.4	32.8
Treated Silage						
Rep. 1	777	12.3	4.2	0.58	12.5	57.6
Rep. 2	399	5.7	4.0	2.42	55.1	67.8
Rep. 3	565	4.6	5.6	1.93	107.8	72.0
Untreated Silage + Methionine						
Rep. 1	562	7.3	5.3	1.92	39.6	65.2
Rep. 2	743	6.4	2.4	1.35	30.6	52.9
Rep. 3	428	3.2	2.7	2.18	64.0	33.0

[1] For experimental design, see Chapter 3, Expt. 1

discussed earlier, and the determination of methionine SAs is a complex and tedious process.

Walker and Nader (1975) also measured microbial protein synthesis by continuous infusion of $Na_2^{35}SO_4$. They measured ^{35}S SA in total rumen contents and in a pure microbial preparation. The turnover constant for ^{35}S in rumen digesta was calculated from the rate of decrease in SA after termination of the labelled infusion. Rumen volume was also determined and hence total microbial-S flow/day was calculated as:

$$\frac{^{35}S \text{ SA /g wet wt. rumen contents} \times \text{rumen volume (l)} \times \text{turnover constant}}{^{35}S \text{ SA / µg microbial-S}} \quad (d^{-1})$$

Walker and Nader (1975) then assumed a constant N:S ratio in microbial protein of 13:1, and thus calculated microbial protein flow per day. However, there is dispute over the constancy of this ratio, and values ranging from 10.7:1 to 21.1:1 have been reported (Henderickx et al., 1972; Bird, 1973). Another source of error in this method is the contribution of recycling of labelled S to and within the rumen during the decay period. This would give a low turnover constant and hence underestimate protein synthesis.

Dr D.J. Walker had been working at Applied Biochemistry Division, D.S.I.R. just prior to the start of the present work, and hence the method of Walker and Nader (1975) was used to estimate microbial protein flow during Expt. 1 (Chapter 3). The results obtained are reported in Table 2.5 to highlight some of the possible sources of error in this method. Four separate figures are required for calculation of microbial protein flow by this method. An error in any one of these measurements will affect the final result. The results showed considerable variation with some values being higher than the total flow of protein per day,

and hence being impossible. Overestimation of microbial protein synthesis may be due to impure microbial preparations which would give a low value for the SA of the microbes and hence a high value for total rumen-S, and thus microbial protein synthesis. The value for microbial synthesis on treated silage Rep. 1 is considerably lower than any of the others, reflecting a low turnover constant for this animal. This may indicate a high degree of S recycling in this animal.

Various comparisons of microbial protein synthesis as estimated by some of these methods within the same experiments have been made (Hogan and Weston, 1972; Harrison et al., 1973; Beever et al., 1974; McAllan and Smith, 1974; Walker and Nader, 1975). The results showed considerable variation. Hence, since these methods also involve complex and time-consuming procedures, and use assumptions which may not be valid, no attempt to estimate microbial protein synthesis was made in Expt. 2.

CHAPTER 3

THE EFFECT OF FORMALIN-TREATMENT AND SUPPLEMENTATION OF SILAGE DIETS ON THE DIGESTION OF SILAGE BY SHEEP

3.1 Introduction

Numerous workers have reported low intakes of silage compared to hay made from the same pasture (Murdoch, 1964; Campling, 1966; Thomson, 1966; Wellman, 1966; Bishop and Kentish, 1970). However, while acetate, butyrate, ammonia and amines have all been suggested as possible intake depressants (Harris and Raymond, 1963; Neumark et al., 1964; McLeod et al., 1970; Wilkins et al., 1971), no single component of silage has been found to be a common factor in limiting intake.

Barry et al. (1973) studied the intake of pasture silage by sheep, and reported an increased intake in response to both intraperitoneal (I/P) methionine, and formalin-treatment of silage. Formalin (formaldehyde + formic acid)-treatment of silage was introduced as a means of protecting the herbage protein from degradation during ensiling, and thus of increasing the protein intake of the animal. Since formaldehyde has also been applied to proteins to protect them from degradation in the rumen (Ferguson et al., 1967), formalin-treatment of silage might also be expected to increase the flow of protein into the duodenum. Hence the flow of methionine into the duodenum would also be increased by formalin-treatment of silage. The results of Barry et al. (1973) suggest therefore, that feeding untreated silage to sheep may adversely affect the relationship between the percentage of methionine in total amino acids entering the duodenum, relative to the percentage of methionine required by the tissues.

The first experiment to be reported was therefore undertaken to study the effects of formalin-treatment and I/P methionine infusion, on N digestion in sheep fed a basal silage diet. The main effect of formalin-treatment was to increase the flow of amino acids into the duodenum. In Expt. 2, other methods of increasing the flow of amino acids into the duodenum were studied in sheep fed silage. The two

treatments chosen were: (a) supplementation with formaldehyde-treated casein and (b) infusion into the rumen, of a readily available energy source. The formaldehyde-treated casein was chosen to ensure that a high level of amino acids entered the duodenum. The energy source was chosen as a possible means of increasing microbial protein synthesis, since the water soluble carbohydrate fraction of silage is low, compared to that of herbage (McDonald et al., 1969). The three treatments (silage alone, silage + protein and silage + energy) were aimed at producing three different flow rates of amino acids into the duodenum. This would enable N digestion and utilisation to be compared at different levels of duodenal flow rate, and thus give some indication of optimum levels of supplementation.

The effect of these three treatments (Expt. 2) in increasing the flow of methionine into the duodenum was also studied (see following Chapter), and thus each of the treatments was also given in combination with duodenal infusion of L-methionine.

3.2 Methods

Sheep: Mature New Zealand Romney wethers were used in both experiments. The weight range was 28-44kg in Expt. 1 and 31-41kg in Expt. 2. The sheep were kept in metabolism crates under continuous artificial light, and at a constant temperature of 18 $\pm$ 2^{o}C, with free access to water. Each sheep was prepared with a rumen cannula and a re-entrant cannula in the proximal duodenum (Brown et al., 1968).

Feed: The two silages for Expt. 1, untreated and formalin-treated, were prepared at Invermay Research Centre, Mosgiel. The formalin-treated silage was prepared by application of 40% (w/v) formaldehyde (at a rate of 4.76ℓ/ton of fresh grass) and 85% (w/w) formic acid (at a rate of 1.00l/ton of fresh grass), to grass during harvesting. The

Table 3.1 Design of Expt. 1.

	Sheep numbers		
Silage	Rep. 1	Rep. 2	Rep. 3
Untreated	786	647	1157
Treated	731	786	441
Untreated + methionine	647	731	786

Fig. 3.1 Sequence of sampling, Expt. 1.

14 days	Preliminary period for adjustment to feed.
7 days	Balance collection.
4 days	Sampling of plasma and expired air.
9 days	Infusion of CrEDTA and collection of duodenal digesta samples.

silage used in this experiment was sent to Palmerston North in bulk, and weighed into daily rations of 3.2kg on arrival. These rations were stored at -20°C until one day prior to use. Refusals were weighed daily and stored at -20°C prior to determination of DM. The sheep had free access to salt licks during the experiment.

The silage used in Expt. 2 was cut and ensiled at Massey No. 3 Dairy Unit. The silage to be used was cut from the stack, packed into daily rations of 3.2kg, and stored at -20°C all in the same day. The daily rations were removed from the freezer one day prior to feeding. 20g of a mineral mix (Summit Products) was applied to the feed each day. Daily refusals were dried at 110°C for 48h prior to weighing. These weights were corrected for the loss of 18g volatile acids per 100g oven-DM (volatile acids measured by toluene distillation), to calculate true DM intake.

The daily ration of silage for each experiment was spread on a continuous belt feeder between 8 and 9 a.m. each day. This delivered approximately equal amounts of feed each hour.

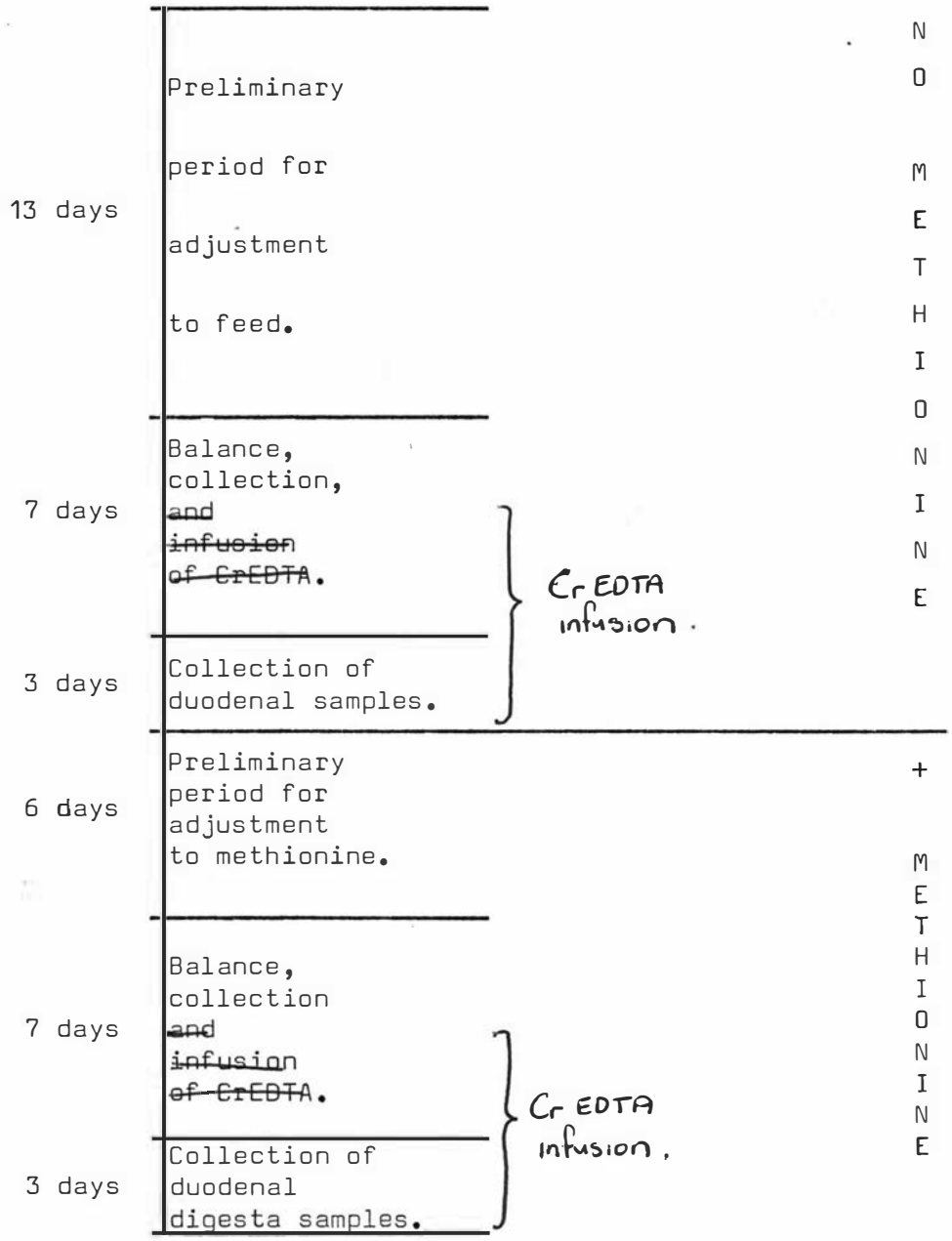
Design: In the first experiment, a 3X3 Latin Square was chosen, with three treatments (untreated silage, formalin-treated silage and untreated silage plus I/P methionine), and three sheep per treatment. However, after the second replicate had been completed, sheep 647 and 731 were losing considerable quantities of duodenal fluid from around their re-entrant cannulae. Sheep 647 died under anaesthesia during an attempt to rectify this. Sheep 731 was also replaced for the third replicate, since the loss of fluid would have introduced error in flow measurement. Therefore a balanced Latin Square was not possible. Table 3.1 gives the layout of sheep numbers in each replicate.

Table 3.2 Design of Expt. 2.

The treatments were: Control (C)
Control + formaldehyde-treated casein (C + P)
Control + starch/sucrose infusion (C + E)
Methionine infusion (+ M)

Sheep Nos.	821	1157	1303
Rep. 1	C	C + P	C + E
Rep. 2	C + M	C + P + M	C + E + M
Rep. 3	C + E	C	C + P
Rep. 4	C + E + M	C + M	C + P + M
Rep. 5	C + P	C + E	C
Rep. 6	C + P + M	C + E + M	C + M

Fig. 3.2 Sequence of sampling, Expt. 2.



Each replicate lasted for 34 days, with a 14 day "recovery" period of ad lib. feeding between each replicate when each sheep was fed either fresh pasture or dried grass. The sequence of sampling within each replicate is given in Fig. 3.1.

The second experiment consisted of two superimposed 3X3 Latin Squares. The first Latin Square involved feeding the control silage plus supplements. The second Latin Square was a repeat of the same treatments, but with an additional duodenal supplement of L-methionine to each sheep. The experimental design is given in Table 3.2.

The first replicate of one Latin Square was immediately followed by the first replicate of the second Latin Square. This replicate was followed by a "recovery" period of ad lib. feeding for 14 days (lucerne chaff), and the sequence then repeated for the next two replicates. The first Latin Square (no methionine) consisted of replicates 1, 3 and 5, each of which lasted 23 days. The second Latin Square (+ methionine) consisted of replicates 2, 4 and 6, each of which lasted 16 days. The sequence of sampling within replicates is given in Fig. 3.2.

In Expt. 1, the balance collections were made after an adaptation period of fourteen days. A preliminary period of this length was not possible in Expt. 2, owing to the shortage of feed. Hence, the adaptation period to feed in replicates 1, 3 and 5 was limited to thirteen days, and the adaptation period to methionine in replicates 2, 4 and 6 was limited to six days. The validity of this adaptation period was assessed by monitoring the daily changes in plasma amino acid concentrations and these results are presented and discussed in the next chapter.

Supplements: The two supplements given in Expt. 2 were formaldehyde-treated casein and an infusion of readily available energy. The formaldehyde-treated casein was from the same batch as that used by MacRae et al. (1972). The daily ration of formaldehyde-treated casein (45gDM)

was spread evenly over the silage on the belt feeder. However, an estimated 5g/day were not eaten by the sheep, and this amount increased with increasing silage refusals. The daily supplement of energy was a 1:1 mix of starch and sucrose. Starch has been shown to be more effective than simple sugars, xylan or pectin in promoting urea utilisation in the rumen (Henedrickx and Martin, 1963). The supplement was infused continuously into the rumen, both to ensure an accurate supply of energy and to prevent possible microbial degradation on contact with the silage. The soluble starch used (TB 870, NZ Starch Co.) formed a gel at a concentration greater than 5% (w/v water). Thus, to avoid infusing large volumes of water, sucrose (BDH, Lab. Grade) was substituted for 50% of the total energy infused per day. The infusion was prepared every second day, by adding 100g starch and 100g sucrose to 2l of boiling water. This solution was infused into the rumen at approximately 750ml/day with a peristaltic pump. The sheep on the other two treatments received a similar volume of water per day, to equalise any effects of this volume on the animal.

The two ~~levels~~ forms of supplement given, were chosen to provide two different flow rates of amino acids into the duodenum, relative to the control. MacRae et al. (1972) reported that the complete 60g of formaldehyde-treated casein supplement reached the duodenum on a dried grass diet. The 45g given in this experiment, was approximately equal to the basal flow of amino acids into the duodenum on untreated silage in Expt. 1. ~~There is little quantitative information on the utilisation of energy supplements to silage, and thus the infusion rate on this treatment was chosen as being isocaloric with the formaldehyde casein.~~

Methionine infusion: The L-methionine in Expt. 1 was infused intraperitoneally. An I/P catheter (1.5mm internal diameter, silastic) was

inserted as follows. The sheep was anaesthetised, and a small incision made through the skin, in the region of the lower abdomen. One end of the catheter was introduced into the peritoneal cavity through a 12 gauge needle and the free end was then drawn subcutaneously, by means of a 250cm long stainless steel needle, to emerge through the skin level with the backbone. The incision was then sutured. A continuous infusion was maintained with a peristaltic pump, using tubing calibrated to deliver 150ml/day. The infusate was made up in 4 l batches, each sterilised by millipore filtering. The L-methionine was dissolved in 0.74% saline, to give a solution isotonic with blood. The concentration of methionine (6.7g/l) was calculated to provide 1g L-methionine/day. In practice, the following amounts were infused:

Sheep 647 in replicate 1 received 0.97g/day
" 731 " " 2 " 0.94g/day
" 786 " " 3 " 1.07g/day

The L-methionine in Expt. 2 was infused into the distal end of the duodenal re-entrant cannulae. The methionine was infused as a 2% solution which was made up in 6l batches. Each batch was sterilised by millipore filtering, and divided into separate flasks for each sheep. These were kept in a refrigerator at -4^{o}C during infusion. The infusion was maintained with a peristaltic pump and tubing calibrated to deliver 85ml/day (i.e. 1.7g methionine /day). However, the pump did not maintain a constant rate in replicates 2 and 4 and there were fluctuations in the daily methionine infusion. The average values for each sheep during the collection of samples were:

1.96g/day for sheep 821)
1.77g/day for sheep 1157) Rep. 1
1.97g/day for sheep 1303)

1.64g/day for sheep 821)
1.47g/day for sheep 1157) Rep. 2
1.64g/day for sheep 1303)

1.88g/day for sheep 821)
1.85g/day for sheep 1157) Rep. 3
1.84g/day for sheep 1303)

Balance collections: Faeces and urine were collected for a period of seven days. Faeces were collected using a harness and collection bag, while urine was allowed to run freely into a collection vessel containing 25ml of concentrated hydrochloric acid, to prevent loss of ammonia. A 10% subsample of faeces was taken daily and bulked for analysis. The remainder was dried in an oven at 110^{o}C to determine DM. Daily subsamples (1%) of urine were also taken. These subsamples were bulked over the seven day period, and stored at -20^{o}C prior to analysis.

Expired air was collected in Expt. 1 using the respiration hood of Ulyatt (unpublished) and methane determined on an aliquot using an Aerograph 660 gas chromatograph. This allowed calculation of metabolisable energy (ME) intake. Methane production in Expt. 2 was calculated from the regression equation of Blaxter and Clapperton (1965) i.e. Methane production (kcal/100kcal intake) = 3.67 + 0.062O where O is the apparent digestibility coefficient for energy.

Marker and digesta sample collection: The techniques used are given in detail in Chapter 2, but a brief summary will be given here. Chromium ethylene diamine tetra acetic acid (CrEDTA) (Binnerts et al., 1968) was continuously infused into the rumen over a period of six days. Twelve steady state duodenal samples were collected as described in Chapter 2. All twelve samples were bulked in Expt. 1, but the samples in Expt. 2 were bulked on a daily basis. DM percentage was determined

on 10g aliquots by drying for 24h at 110°C. Further aliquots were centrifuged twice at 38,000g for 15 min and the supernatant stored for chromium analysis. The remaining sample was freeze-dried and stored at -20°C. Rumen samples were collected on the last day of each replicate in Expt. 1, and on the last day of replicates 2, 4 and 6 in Expt. 2. The samples were strained through cheese-cloth, and preserved by addition of 3ml 10N H_2SO_4 to 15ml rumen liquor, prior to storage at -20°C.

Chemical analysis: Analysis of OM, N, energy, lactic and acetic acid contents for Expt. 1 were conducted at Invermay (see Barry, 1975). The remainder of the analyses for Expt. 1 silages, and the analysis of the control silage for Expt. 2, were carried out at Palmerston North. DM percentages of the silages were measured by distillation with toluene (Barry, 1974). A sample of fresh silage was squeezed through cheesecloth and the juice used for the determination of pH. Subsamples of the feeds were freeze-dried and ground, prior to further chemical analysis. Water soluble sugars were analysed by the anthrone method of Bailey (1964). Analysis for OM, N and energy was conducted in a similar manner for the feeds, duodenal contents and faeces. All analyses were conducted on ground, freeze-dried material. The DM percentages of this material were determined by drying in an oven at 110°C for 24h. OM was determined by ashing for 5h in a furnace at 550°C. Total N was determined on dried samples (0.14g) and on 0.5ml urine samples with a Technicon autoanalyser, after prior digestion by the Kjeldahl method. Gross energy was determined on an adiabatic bomb calorimeter (Gallenkamp). Urine samples (10ml) were freeze-dried prior to determination of energy. The amino acid content of feed and duodenal digesta was measured on hydrolysates of the freeze-dried

material, with a Beckman 120C amino acid analyser. Hydrolysis was carried out with excess 6N HCl at 110°C for 22h, in sealed evacuated tubes, and after rotary evaporation, the hydrolysates were taken up in 5ml pH 2.2 buffer. Chromium determinations were made on the supernatants of duodenal digesta, with an atomic absorption spectrophotometer (Techtron). The samples were diluted 1 in 3 with 1500ppm calcium. Rumen ammonia was measured by the microdiffusion method of Conway (1957) on strained samples of rumen contents, preserved by the addition of 10N H_2SO_4 saturated with $MgSO_4$. Total volatile fatty acid concentration was determined by titration with N/25 NaOH after distillation in a Markham still. The molar proportions of volatile fatty acids were determined on an Aerograph 660 gas chromatograph.

Calculation of duodenal flow: The daily flow of DM into the duodenum was calculated from the known rate of infusion of chromium (Xppm/day) and the measured concentration of chromium at the duodenum (Yppm/ml supernatant).

$$\text{i.e. DM flow (g/day)} = \frac{X \times DM\%}{Y(100-DM\%)}$$

Statistical analysis: The replacement of two of the sheep in the third replicate of Expt. 1 gave a total of five sheep used in this experiment. Thus, four degrees of freedom must be allocated to the sums of squares for animal variation. The total degrees of freedom in a 3 X 3 Latin Square are eight, and with two degrees of freedom for the effect of replicates, and two degrees of freedom for treatment effects, no degrees of freedom are left for the error term. Hence comparisons between treatments were confounded by ~~would require~~ the use of animal effects as the error term. This assumes that the effect of animal variation is not significant. This assumption is invalidated by the results of Expt. 2, where significant differences between animals were recorded. Thus, no valid

Table 3.3 Analysis of variance for Expt. 2.

Effect	Degrees of freedom
Replicates	4
Treatments (T)	2
Methionine (M)	2
Interaction (TXM)	1
Animals	2
Error	6
TOTAL	17

Table 3.4 The composition of the silages fed in Expts 1 and 2.

	Expt. 1		Expt. 2
	Untreated Silage	Treated Silage	Control Silage
Toluene DM%	20.0	20.3	21.9
Organic matter (% DM)	87.9	86.9	87.7
Nitrogen (% DM)	2.42	2.24	2.51
Amino acid N (% total N)	50.9	90.7	74.6
Energy (kcal/g)	4.48	4.34	4.27
Water soluble sugar (% DM)	1.33	1.94	2.68
Acetic acid (% DM)	3.6	2.4	ND
Lactic acid (% DM)	3.1	2.5	ND
pH	4.6	4.5	4.1

Table 3.5 The amino acid composition of the silages fed in Expts 1 and 2, compared with the amino acid composition of the formaldehyde-treated casein and an average herbage (expressed as g individual amino acids/100g total amino acids).

	Expt. 1		Expt. 2		
	Untreated Silage	Treated Silage	Control Silage	Treated Casein	Herbage[1]
arg	3.4	5.6	2.8	3.5	4.8
his	4.2	2.0	3.2	2.6	2.1
ile	6.9	5.5	5.4	4.9	5.6
leu	11.7	9.9	9.1	8.9	8.8
lys	3.5	3.6	4.6	6.6	7.2
met	1.7	1.8	1.8	2.6	2.4
phe	7.0	6.4	7.2	5.0	5.7
thr	3.6	6.3	5.8	4.0	5.9
val	8.8	6.5	7.1	5.7	6.4
ala	11.7	8.0	10.8	2.7	7.1
asp	10.2	11.3	11.4	7.1	11.1
glu	9.7	11.2	10.1	23.2	12.8
gly	7.2	6.9	7.4	1.8	6.1
pro	5.7	6.7	5.6	10.0	4.2
ser	4.6	5.4	5.0	5.9	5.5
tyr	TR.	3.1	2.8	5.4[2]	4.2

[1] Data from MacRae and Ulyatt (1974) for average of three herbages.

[2] Value for tyrosine taken for untreated casein, since the presence of formaldehyde decreases the recovery of tyrosine after hydrolysis. (MacRae et al., 1972).

statistical analysis could be carried out for Expt. 1.

Expt. 2 consisted of two, 3 X 3 Latin Squares, one with methionine supplementation and the other with no methionine supplementation. Significant differences were detected by Analysis of Variance as detailed in Table 3.3.

Individual differences between protein and energy supplements and the control, were assessed by comparing the "least significant difference" (LSD) with the treatment means. The LSD (at $P < 0.05$) was calculated as: $\sqrt{\frac{2\ SE}{r}} \times t_{0.05}$

where SE is the standard error of the treatment means,

r is the number of means used to compute the SE,

$t_{0.05}$ is the "t" value at the 5% level of significance.

3.3 Results

The chemical compositions of the silages fed in Expts. 1 and 2 are given in Table 3.4. The water soluble sugar content of the herbage ensiled in Expt. 1 was 16.2% (Barry, 1975). A comparison of this concentration compared to those of the treated and untreated silages (Table 3.4), indicates that considerable fermentation of the carbohydrate fraction took place in both silage stacks. The amino acid-N in the total N fraction was markedly higher on the treated than untreated silage. The amino acid-N concentration of the control silage in Expt. 2 was between those of the treated and untreated silages (Expt. 1).

The amino acid compositions of the silages from both experiments and for the formaldehyde-treated casein supplement (Expt. 2) are given in Table 3.5. Since the amino acid compositions of the original herbages were not available, the silage compositions are compared with the average amino acid composition of three herbages, taken from

Table 3.6 The DM, ~~energy~~, nitrogen, ~~non-amino acid N~~, and amino acid intakes of sheep fed silage (g/day).

Expt. No.	Treatment	Methionine	Silage DM intake	Mean	Total DM intake	Nitrogen intake	Mean	Amino acid intake	Mean
1	Untreated	-							
	Rep. 1		574			13.9		45	
	Rep. 2		631	599	599	15.3	14.5	49	47
	Rep. 3		593			14.4		46	
	Treated	-							
	Rep. 1		480			10.8		61	
	Rep. 2		561	564	564	12.6	12.7	72	72
	Rep. 3		650			14.6		83	
	Untreated	+							
	Rep. 1		640			15.5		49	
	Rep. 2		633	620	620	15.3	15.0	49	48
	Rep. 3		587			14.2		45	
2	Control	-	413		413	10.4		48	
	+ protein	-	651		688	22.2		114	
	+ energy	-	576		669	14.5		65	
	Control	+	587		587	14.7		68	
	+ protein	+	672		710	23.0		118	
	+ energy	+	647		734	16.2		74	
	SE		17.4			0.71		2.5	
	Treatment significance		**			**		**	
	Methionine significance		**			**		**	
	Animal significance		**			*		**	

* $p < 0.05$

** $p < 0.01$

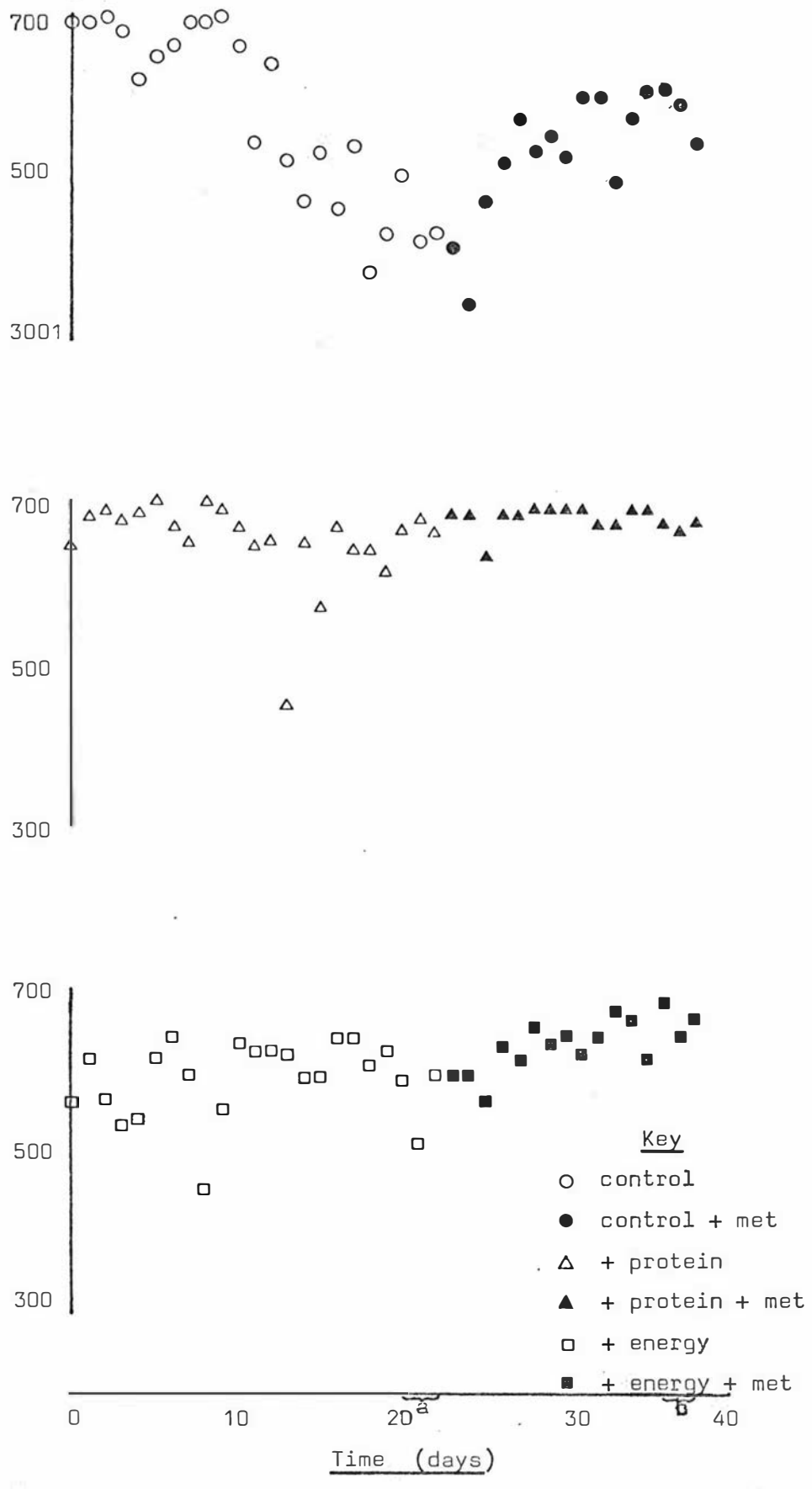


Fig. 3.3 Change in voluntary DM intake with time - Expt. 2

MacRae and Ulyatt (1974). The composition of the treated silage (Expt. 1) was closer to that of herbage than to the untreated silage. Considerable differences between the composition of the control silage (Expt. 2) and formaldehyde-treated casein, were recorded for alanine, aspartate, glutamate, glycine and proline.

The DM, N and amino acid (AA) intakes for both experiments are given in Table 3.6. The main difference in Expt. 1 was an increased AA intake on treated silage, due to the higher concentration of amino acids in the silage.

The intakes in Table 3.6 represent the mean daily intakes calculated from the total intake over the last three days of each replicate. This comparison was chosen, because the intakes in Expt. 2 changed with time after the start of each replicate. Separate plots for the mean daily DM intake on each treatment, are given in Fig. 3.3. The intakes (Table 3.6) for replicates 1, 3 and 5 were calculated over time (a) and for replicates 2, 4 and 6 over time (b). It can be seen from Fig. 3.3 that there was a gradual decrease in intake of the control silage after the first ten days of feeding. This decline continued until the start of the L-methionine infusion, when intake began to increase. However, during the time of infusion measured, intake did not return to the level at the start of the experiment. Supplementation with protein and energy maintained intake at a higher overall level, but some decrease in intake still occurred prior to methionine infusion. DM intakes in Table 3.6 showed a significant increase in response to supplementation ($P < 0.01$) and to methionine ($P < 0.01$). There was also a significant effect of animal variation on DM intake ($P < 0.01$). The individual data for each sheep are given in Appendix 1. The day to day variation in intake resulted in differences in intake between periods of balance collection and duodenal flow measurement (see

Table 3.7 The metabolisable energy intake of sheep fed formalin-treated silage, untreated silage, and untreated silage diets supplemented with formaldehyde-treated casein, energy and postruminal (I/P and duodenal) methionine compared to the estimated maintenance requirement.

Expt. No.	Treatment	Methionine	ME intake (kcal/day)		CH_4 excretion[1] (kcal/100kcal GE intake)	CH_4 excretion[2] (kcal/100kcal GE intake)		M/D (Mcal/ kgDM)	Maintenance requirement (kcalME)
				Mean	Mean		Mean	Mean	Mean
1	Untreated	-							
	Rep. 1		1644			5.3			
	Rep. 2		1614	1586	7.9	9.2	6.0	2.6	1350
	Rep. 3		1501			3.5			
	Treated	-							
	Rep. 1		1627			8.1			
	Rep. 2		1435	1577	7.8	6.2	5.7	2.8	1320
	Rep. 3		1670			2.8			
	Untreated	+							
	Rep. 1		1562			7.9			
	Rep. 2		1573	1567	8.0	5.4	5.7	2.5	1350
	Rep. 3		1565			3.7			
2	Control	-		1235	8.2		ND	3.0	1295
	+ protein	-		1829	8.4		ND	2.8	1320
	+ energy	-		1889	8.4		ND	3.3	1295
	control	+		1458	8.3		ND	2.5	1350
	+ protein	+		2000	8.5		ND	3.0	1295
	+ energy	+		1983	8.4		ND	3.1	1295

[1] Calculated from the regression equation of Blaxter and Clapperton (1965):

CH_4 excretion (kcal/100kcal GE intake) = 3.67 + 0.0620 where O is the apparent digestibility coefficient for energy.

[2] measured in respiration apparatus for Expt. 1

[3] calculated from ARC (1965) Nutrient requirements for ruminants using the M/D values for the feeds.

ND not determined

Table 3.8 The effect of formalin-treatment of silage and supplementation of untreated silage diets with formaldehyde-treated casein, energy, and postruminal (I/P or duodenal) methionine on rumen ammonia concentration and volatile fatty acid concentration and molar proportions.

Expt. No.	Treatment	Methionine	Total VFA concn. (meq/l)		Molar proportions (%)				NH_3-N concn. (mg/100ml)
					Acetate		Propionate	Butyrate	
				Mean		Mean	Mean	Mean	
1	Untreated	-							
	Rep. 1		59.5		79.2		11.1	1.8	
	Rep. 2		75.8	66.0	74.3	77.8	16.2 13.3	9.5 8.9	ND
	Rep. 3		62.6		80.0		12.5	7.5	
	Treated	-							
	Rep. 1		69.0		83.5		11.0	5.5	
	Rep. 2		74.2	68.5	86.2	85.6	7.5 8.8	6.4 5.7	ND
	Rep. 3		62.3		87.1		7.8	5.2	
	Untreated	+							
	Rep. 1		72.6		86.9		6.7	6.4	
	Rep. 2		82.9	80.7	76.6	81.4	15.1 10.8	8.7 7.9	ND
	Rep. 3		86.7		80.7		10.7	8.7	
2	Control	+	69.0		66.6		21.5	9.3	14.7
	+ protein	+	77.2		60.8		22.0	12.8	14.6
	+ energy	+	85.5		61.8		22.0	11.9	13.4
	SE		1.1		1.8		1.1	0.8	0.87
	Treatment significance		**		NS		NS	NS	NS
	Animal significance		**		NS		NS	NS	**

ND not determined

NS not significant

Table 3.9 The effect of formalin-treatment of silage, and supplementation of untreated silage diets with formaldehyde-treated casein, energy and postruminal (I/P and duodenal) methionine on the percentage of OM, N and energy intakes apparently digested in the stomach of sheep.

Expt. No.	Treatment	Methionine	Organic matter	Mean	Nitrogen	Mean	Energy	Mean
1	Untreated	-						
	Rep. 1		52.7)		15.8)		51.7)	
	Rep. 2		59.2)	60.9	28.1)	29.9	55.9)	57.7
	Rep. 3		70.7)		45.8)		65.6)	
	Treated	-						
	Rep. 1		35.9)		-37.0)		30.2)	
	Rep. 2		45.7)	44.7	-21.4)	-22.7	39.7)	39.9
	Rep. 3		52.4)		- 9.6)		49.8)	
	Untreated	+						
	Rep. 1		46.5)		3.9)		45.4)	
	Rep. 2		59.5)	57.9	17.0)	21.8	56.5)	56.9
	Rep. 3		67.6)		44.4)		68.8)	
2	Control	-	50.3		-10.3		44.7	
	+ protein	-	54.7		15.0		51.4	
	+ energy	-	57.7		- 8.7		53.1	
	Control	+	54.7		4.8		49.9	
	+ protein	+	50.7		3.3		43.8	
	+ energy	+	48.7		-22.4		40.9	
	SE		3.4		1.7		2.8	
	Treatment significance		NS		*		NS	
	Methionine significance		NS		NS		NS	

Appendix 1). Hence direct comparisons between balance and flow data cannot be made.

The adequacy of the various diets in meeting the energy requirements of the sheep for maintenance are given in Table 3.7. The maintenance requirements for metabolisable energy (ME) were calculated from tables in the ARC (1965) manual on nutrient requirements of ruminants. These depend on the M/D value (McalME/kg DM) of the feed. It would appear that the only treatment which did not provide the sheep with a greater than maintenance intake of energy, was the control silage in Expt. 2 and this was very close to maintenance.

ME intakes in Expt. 2 were calculated using a value for methane excretion calculated from the regression equation of Blaxter and Clapperton (1965). This is compared with the measured value for methane excretion in Expt. 1. The difference recorded contributes only a 3% error to the ME intake estimated.

Table 3.8 presents the effect of formalin-treatment of silage and supplementation with formaldehyde-treated casein, energy and postruminal methionine infusion on rumen ammonia concentrations and volatile fatty acid (VFA) concentrations and molar proportions. Total VFA concentration was significantly increased by supplementation ($P < 0.01$) in Expt. 2, and the effect of animal variation was also significant ($P < 0.05$). There was a marked difference in the molar proportions of VFAs between the two experiments. The proportion of acetate was higher and the proportion of propionate lower in Expt. 1 than Expt. 2. There were no significant differences in rumen ammonia concentration in Expt. 2.

The effect of the treatments on the percentage of nutrients digested in the stomach region is given in Table 3.9. The main difference in both experiments was in the digestion of N in the stomach.

Table 3.10 The effect of formalin-treatment of silage and the supplementation of untreated silage diets with formaldehyde-treated casein, energy and postruminal (I/P and duodenal) methionine on the intake and duodenal flow of non-amino acid -N (NAAN).

Expt. No.	Treatment	Methionine	NAAN intake	Duodenal NAAN Mean	Duodenal NAAN: NAAN intake Mean
1	Untreated	-			
	Rep. 1		6.7)	4.1)	0.61)
	Rep. 2		7.5) 7.1	3.6) 3.4	0.48) 0.49
	Rep. 3		7.0)	2.6)	0.37)
	Treated	-			
	Rep. 1		1.0)	5.6)	5.6)
	Rep. 2		1.1) 1.2	4.1) 4.9	4.1) 4.2
	Rep. 3		1.5)	3.0)	3.0)
	Untreated	+			
	Rep. 1		8.1)	4.5)	0.56)
	Rep. 2		8.1) 7.9	4.2) 3.8	0.52) 0.48
	Rep. 3		7.5)	2.6)	0.35)
2	Control	-	2.7	3.1	1.16
	+ protein	-	3.6	1.3	0.35
	+ energy	-	3.7	3.9	1.01
	Control	+	3.8	4.4	1.14
	+ protein	+	3.7	2.1	0.56
	+ energy	+	4.1	5.1	1.23
	SE		0.15	0.35	0.11
	Treatment significance		*	**	**
	Methionine significance		**	**	NS
	Interaction significance		NS	NS	NS
	Animal significance		**	NS	NS

Table 3.11 The effect of formalin-treatment of silage and the supplementation of untreated silage diets with formaldehyde-treated casein, energy and postruminal (I/P and duodenal) methionine on the flow of N and amino acids (AA) into the duodenum.

Expt. No.	Treatment	Methionine	AA intake		Duodenal AA		Duodenal AA: AA intake		Duodenal AA: N intake		Duodenal N		Duodenal AA as % total N	
				Mean		Mean		Mean		Mean		Mean		Mean
1	Untreated	-												
	Rep. 1		45		47.8		1.06		3.4		11.7		65.4	
	Rep. 2		49	47	46.2	42.2	0.94	0.90	3.0	2.8	11.0	10.2	67.2	66.6
	Rep. 3		46		32.8		0.71		2.1		7.8		67.3	
	Treated	-												
	Rep. 1		61		57.6		0.94		5.3		14.8		62.3	
	Rep. 2		72	72	67.8	65.8	0.94	0.92	5.4	5.2	15.3	15.4	70.9	68.4
	Rep. 3		82		72.0		0.88		4.9		16.0		72.0	
	Untreated	+												
	Rep. 1		46		65.2		1.42		4.2		14.9		70.0	
	Rep. 2		45	42	52.9	50.4	1.18	1.13	3.5	3.3	12.7	11.8	66.6	67.8
	Rep. 3		42		33.0		0.79		2.3		7.9		66.8	
2	Control	-	48		49.6		1.07		4.9		11.0		72.1	
	+ protein	-	114		110.5		0.97		5.1		18.8		94.0	
	+ energy	-	67		76.6		1.14		5.4		15.1		81.2	
	Control	+	65		59.5		0.88		4.1		14.0		68.9	
	+ protein	+	118		125.7		1.07		5.6		21.8		92.3	
	+ energy	+	74		98.6		1.36		6.3		21.0		75.1	
	SE		2.5		6.0		0.05		0.33		0.92		1.3	
	Treatment significance		**		**		**		NS		**		**	
	Methionine significance		**		NS		NS		NS		**		*	
	Interaction significance		NS		NS		*		NS		NS		NS	
	Animal significance		**		NS		**		*		NS		**	

A STUDY OF THE AMINO ACID STATUS OF SHEEP FED SILAGE, WITH PARTICULAR REFERENCE TO METHIONINE

E. MARGARET GILL

The units omitted from Tables 3, 10 and 3.11 should be g/day

However, the digestion of OM and energy in the stomachs in Expt. 1 were also decreased with treated compared to untreated silage. There was a net addition of N to the rumen on treated silage and a net loss on untreated silage in Expt. 1. In Expt. 2 there was a significant difference between the effects of protein and energy supplementation on N digestion in the rumen ($P < 0.05$). Energy supplementation resulted in net addition of N to the rumen, while the protein supplement resulted in a net loss.

Comparisons of the intakes of amino acids and non-amino acid-N (NAAN), with their flow into the duodenum are given in Tables 3.10 and 3.11. The main treatment difference in Expt. 1 was the decrease in NAAN between the mouth and duodenum on untreated silage, compared to the increase in NAAN between mouth and duodenum on treated silage. Thus, the ratio of NAAN at the duodenum to the NAAN intake was considerably higher on treated than untreated silage. Formalin-treatment of silage also increased the total flow of amino acids into the duodenum over untreated silage, despite lower intakes of total N on treated silage. However, there was no treatment effect on the percentage of amino acids in the total N entering the duodenum. The ratio of duodenal amino acids to amino acid intake also showed no difference between treatments.

The regression relationship between amino acid intake and the flow of amino acids into the duodenum was plotted for the data from both experiments (Fig. 3.4). The correlation coefficient (r) was 0.82 indicating that 67% (r^2) of the variability in duodenal amino acid flow was due to variation in amino acid intake.

In Expt. 2, the formaldehyde-treated casein significantly decreased the ratio of the NAAN entering the duodenum to NAAN intake ($P < 0.01$) while energy supplementation had no effect (Table 3.10). ~~Methionine~~

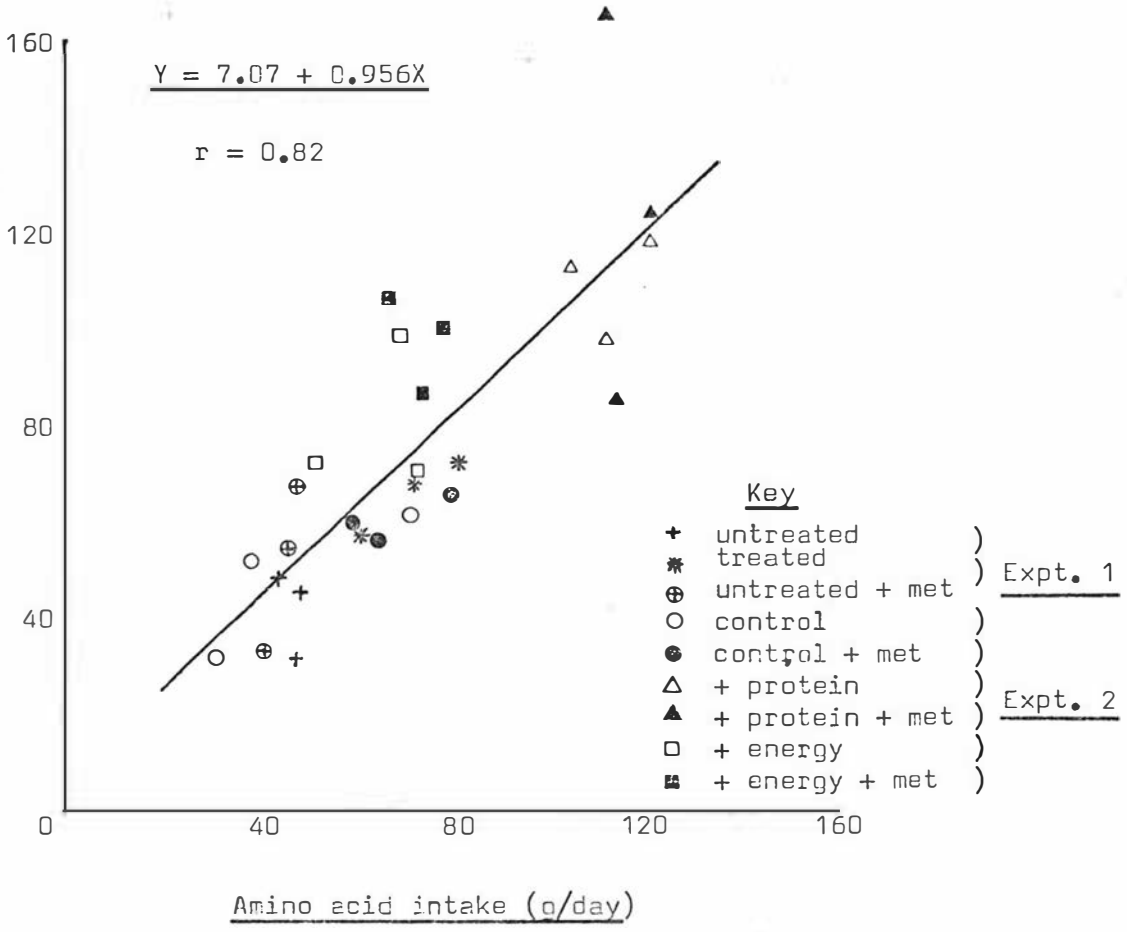


Fig. 3.4 The relationship between amino acid intake and duodenal amino acid flow for all treatments in Expts. 1 and 2

Table 3.12 The amino acid composition of microbial protein, and the effect of formalin-treatment of silage, and supplementation of untreated silage diets with formaldehyde-treated casein, energy and postruminal (I/P and duodenal) methionine on duodenal amino acid composition (Individual amino acids as a % of the total amino acids).

	Expt. 1			Expt. 2										
	Untreated	Treated	Untreated + met	Control	+ Protein	+ Energy	Control + met	+ Protein + met	+ Energy + met	SE	Treatment Significance	Methionine Significance	Bacteria[1]	Protozoa[1]
arg	4.3	4.9	4.5	4.7	4.4	4.6	4.8	4.3	4.8	0.06	**	NS	5.0- 5.3	3.7- 4.8
his	1.9	1.8	2.0	2.2	2.4	2.9	2.3	2.4	2.6	0.14	NS	NS	1.8- 2.0	1.6- 2.2
ile	5.9	5.8	5.7	5.7	5.2	5.4	5.2	5.1	5.2	0.09	NS	NS	5.2- 6.3	5.8- 6.3
leu	8.9	9.2	8.9	8.5	8.7	8.1	8.8	8.4	8.2	0.21	NS	NS	7.7- 8.7	7.4- 8.2
lys	6.8	5.9	6.9	6.5	6.7	6.7	6.3	6.6	6.6	0.09	NS	NS	7.6- 9.0	9.4-13.0
met	2.1	2.2	2.1	2.3	2.4	2.4	2.4	2.5	2.4	0.04	NS	NS	2.0- 2.2	1.3- 1.9
phe	6.1	6.2	6.0	6.2	5.6	5.8	6.2	5.4	5.8	0.09	**	NS	4.4- 5.1	5.0- 6.0
thr	5.6	5.6	5.8	6.5	5.4	6.3	6.2	5.2	6.0	0.16	**	NS	6.0- 7.8	4.8- 6.5
val	6.5	6.5	6.4	6.2	6.4	6.2	6.3	6.3	6.3	0.09	NS	NS	4.7- 5.2	4.0- 4.2
ala	7.3	7.1	7.1	6.2	5.3	7.3	6.7	4.9	7.2	0.13	**	NS	7.2- 7.6	4.4- 6.1
asp	11.2	11.2	11.2	11.7	9.7	11.8	11.3	9.5	11.7	0.21	**	NS	11.1-12.1	11.9-13.5
glu	12.5	12.6	12.3	12.8	17.8	13.1	12.5	18.0	13.0	0.20	**	NS	13.5-14.4	15.0-16.3
gly	6.3	6.3	6.1	6.6	4.7	6.8	6.5	4.6	6.6	0.18	**	NS	6.0- 7.6	4.1- 5.5
pro	5.4	5.6	5.5	4.7	6.8	4.1	5.2	7.4	4.5	0.17	**	*	2.4- 2.9	2.9- 6.0
ser	5.3	5.4	5.7	4.8	5.0	4.8	4.4	4.7	4.8	0.04	**	**	4.2- 5.1	4.4- 5.1
tyr	3.6	3.6	4.0	5.3	4.6	4.7	4.6	4.6	4.9	0.15	NS	NS	4.1- 4.5	4.2- 5.2

[1] Taken from Bergen et al. (1968)

The ratio of duodenal AA to AA intake was significantly decreased by ~~infusion significantly decreased the ratio of duodenal AA to AA intake~~. supplementation ($P < 0.01$) also ~~($P < 0.05$)~~ and there was a significant treatment/methionine interaction ($P < 0.01$), i.e. while duodenal AA to AA intake decreased in response to methionine on the control silage alone, it increased in response to methionine when the silage was supplemented with either energy or protein. The percentage of amino acids in the total N entering the duodenum was significantly increased by protein and energy supplementation ($P < 0.01$) and significantly decreased by methionine infusion ($P < 0.05$).

The amino acid composition of duodenal digesta in Expts. 1 and 2 is compared with the amino acid composition of microbial protein (Bergen et al.,1968) in Table 3.12. There were no marked treatment differences in duodenal amino acid composition in Expt. 1. In Expt. 2, protein supplementation of the control silage resulted in significant decreases in the percentages of arginine, phenylalanine, threonine, alanine, aspartate and glycine ($P < 0.01$) and significant increases in glutamate, proline and serine ($P < 0.01$). Energy supplementation resulted in a significant decrease in the percentage of phenylalanine ($P < 0.05$) and a significant increase in the percentages of alanine and serine ($P < 0.01$). Methionine infusion gave a significant increase in the percentage of proline ($P < 0.05$) and a significant decrease in the percentage of serine ($P < 0.01$). There was also a significant interaction between methionine and the supplements in the percentage of serine ($P < 0.05$). However, absolute changes were small and were probably of little biological significance. The composition of the microbial protein was included to give an indication of the relative contribution of microbial and feed protein at the duodenum.

Table 3.13 The effect of formalin-treatment of silage and supplementation of untreated silage diets with formaldehyde-treated casein, energy and postruminal (I/P and duodenal) methionine on the percentage of DM, OM, N and energy, and the percentage of duodenal N, digested in the intestines.

Expt. No.	Treatment	Methionine	DM (% intake)	Mean	OM (% intake)	Mean	Energy (% intake)	Mean	N as % intake	Mean	N as % duodenal N	Mean
1	Untreated	-										
	Rep. 1		28.6		18.7		17.1		67.1		69.8	
	Rep. 2		20.0	20.4	14.1	10.9	14.2	10.7	52.3	47.5	63.0	58.2
	Rep. 3		12.5		0		0.8		23.2		41.8	
	Treated	-										
	Rep. 1		50.8		37.6		39.4		100.0		73.0	
	Rep. 2		38.7	41.8	20.3	25.7	22.6	27.6	72.2	81.4	59.5	65.6
	Rep. 3		35.8		19.1		20.7		71.9		64.4	
	Untreated	+										
	Rep. 1		35.8		27.6		25.6		51.8		61.5	
	Rep. 2		25.3	26.9	13.8	15.8	11.2	13.1	42.5	37.9	59.1	52.2
	Rep. 3		19.6		6.0		2.4		19.4		35.9	
2	Control	-	40.3		28.4		27.9		76.3		68.6	
	+ protein	-	29.1		22.8		23.7		62.2		73.1	
	+ energy	-	29.3		25.7		22.6		77.5		70.0	
	Control	+	32.4		22.1		25.8		65.0		68.1	
	+ protein	+	36.5		28.9		31.1		72.5		73.9	
	+ energy	+	42.9		32.3		28.0		90.4		80.6	
	SE		3.1		1.8		3.2		7.5		2.2	
	Treatment significance		NS		NS		NS		NS		NS	
	Methionine significance		NS		NS		NS		NS		NS	
	Animal significance		*		**		NS		NS		NS	

Table 3.14 The effect of formalin-treatment of silage and supplementation of untreated silage diets with formaldehyde-treated casein, energy and postruminal (I/D and duodenal) methionine on the apparent digestibility of DM, OM, energy and N, and on N balance.

Expt. No.	Treatment	Methionine	DM%		OM%		Energy%		N%		N balance (g/day)	
				Mean		Mean		Mean		Mean		Mean
1	Untreated	-										
	Rep. 1		68.8		71.3		68.8		67.8		3.1	
	Rep. 2		70.6	68.5	73.3	71.2	70.1	68.4	70.8	67.6	-1.4	1.1
	Rep. 3		66.0		69.1		66.4		64.3		1.6	
	Treated	-										
	Rep. 1		70.6		73.7		69.6		63.0		3.3	
	Rep. 2		63.6	67.4	66.2	70.5	62.3	66.4	51.0	58.1	1.6	2.5
	Rep. 3		68.1		71.5		67.3		60.4		2.6	
	Untreated											
	Rep. 1	+	70.9		74.0		71.0		70.9		3.2	
	Rep. 2		70.5	70.5	73.4	73.7	67.8	70.0	69.6	69.3	1.9	2.8
	Rep. 3		70.0		73.6		71.1		67.5		3.2	
2	Control	-	67.8		75.5		72.6		66.0		-0.6	
	+ protein	-	71.7		79.3		76.3		74.8		3.5	
	+ energy	-	72.9		79.61		75.7		66.4		0.1	
	Control	+	70.8		76.7		75.1		69.7		2,3	
	+ protein	+	72.3		79.8		77.1		75.8		5.3	
	+ energy	+	73.7		80.1		76.9		67.5		4.0	
	SE		0.97		0.58		0.81		1.25		0.67	
	Treatment significance		NS		**		NS		**		*	
	Methionine significance		NS		NS		NS		NS		**	

Table 3.13 gives the effect of formalin-treatment of silage and supplementation of untreated silage with protein, energy and methionine on the percentage of nutrient intake and duodenal N which was digested in the intestines. The percentage of N entering the duodenum, which was digested in the intestines in Expt. 1, was similar on all treatments in replicates 1 and 2, but there was a marked decrease on untreated silage alone and with methionine infusion in replicate 3. This difference in replicate 3 was also apparent in the percentage of intake which was digested in the intestines, for all nutrients. The values for digestion of OM and energy were particularly low and suggest that measurement of duodenal flow in these two sheep was inaccurate. However, formalin-treatment of silage still appeared to increase the percentage of DM, OM, energy and N intake which was digested in the intestines.

In Expt. 2 there was no effect of supplementation or methionine infusion on the percentage of intake digested in the intestines. However, there was a significant difference between animals in the percentage of DM ($P < 0.05$) and OM intake ($P < 0.01$) digested in the intestines.

The apparent digestibilities and N balance of the silage diets and supplements are given in Table 3.14. In Expt. 1, N digestibility was lower on treated silage than untreated silage. The average N balance appeared to increase in response to both treatment of silage and methionine, but there was considerable variation within treatments.

In Expt. 2, there was no significant effect of methionine infusion on N digestibility. Energy and protein supplements both increased OM digestibility ($P < 0.01$) and protein increased N digestibility. N balance was significantly increased by supplements ($P < 0.05$) and by

methionine ($P < 0.01$).

3.4 Discussion

A. Introduction

The report by Barry et al. (1973) that both formalin-treatment of silage and I/P methionine supplementation of untreated silage increased intake in sheep, suggests that the amino acid status of sheep fed silage may be an important factor in controlling intake. Thus, the main aim of Expt. 1 was to compare the effects of formalin-treatment of silage and I/P methionine supplementation on the digestion and utilisation of N by sheep. The flow rates of amino acids into the duodenum in Expt. 1 only ranged from 33-72g/day and thus two other methods of further increasing total amino acid availability were studied in Expt. 2. These were: supplements of formaldehyde-treated casein, to ensure a high flow of amino acids into the duodenum, and ruminal infusion of readily available energy, to stimulate microbial protein synthesis. An increased flow of total amino acids into the duodenum is usually associated with an increased flow of methionine. Thus, the effect of increasing methionine through increasing the total flow of amino acids, was also compared with direct infusion of methionine into the peritoneal cavity (Expt. 1) or duodenum (Expt. 2).

B. Feed composition

Formalin-treatment of silage in Expt. 1 did not appear to decrease the fermentation of soluble carbohydrates, since the water soluble sugar content of both silages was low compared to that of herbage (Table 3.4). However, the higher amino acid content of treated silage indicated inhibition of protein and/or amino acid degradation during ensiling. The amino acid composition of the original herbages ensiled was not available, but since the amino acid composition of herbages is fairly constant (MacRae and Ulyatt, 1974), the composition of the treated and

untreated silages was compared with the composition of protein from similar herbage (Table 3.5, Ulyatt et al., 1975). This comparison indicated that the amino acid complement of treated silage was probably closer to that of herbage than to untreated silage. Hence formalin-treatment did appear to be effective in protecting herbage protein from degradation during ensiling. The amino acid composition of the control silage (Expt. 2) was closer to that of untreated silage (Expt. 1) than to herbage (Table 3.5).

The main difference between the control silage (Expt. 2) and the untreated and treated silages (Expt. 1), was in the concentration of amino acids (Table 3.4). Thus, while only 50.9% of the total N in untreated silage was in the form of amino acids, 74.6% of the N was in the form of amino acids in the control silage (Expt. 2). The main effect of formalin-treatment on silage composition was to increase the amino acid content. Such differences in amino acid content between the two different untreated silages, may partially explain some of the reported variability in responses to formalin-treatment of silage (Valentine and Brown, 1973; Barry and Fennessy, 1973). However, the silages used in Expts. 1 and 2 were made from different herbages, and the amino acid content of the original herbages was unknown.

The relative proportions of protein and free amino acids in the silage are not reported, since contradictory results were obtained. Extraction of the silage "protein" with sodium dodecylbenzenesulphonate (SDS) was attempted, but no protein was detected. On the other hand, extraction of the "NPN" in alcohol indicated that the total amino acid content was derived from protein.

C. Intake

In both experiments, intakes were restricted, so that comparisons between treatments could be made at the same level of intake. The

limit on silage intake was chosen to provide nutrients at a level slightly above maintenance. However, the daily ration was seldom completely eaten, refusals being particularly high on the control silage in Expt. 2. Metabolisable energy (ME) intakes were compared with maintenance requirements for ME, as proposed by the ARC (1965) manual on the nutrient requirements for ruminants (Table 3.7). This indicated that intakes in Expt. 1 were still slightly above maintenance, while the intake of the control silage in Expt. 2 was very near to maintenance. The supplements in Expt. 2 increased intake to greater than maintenance.

The daily output of methane was measured in Expt. 1 but not Expt. 2. Methane output was calculated for both experiments and compared with the observed values from Expt. 1. While there was some difference between the calculated and observed values, the error which this would introduce into the calculation of ME intake, would be small (approx. 3%) due to the low level of methane output compared to total ME intake.

Refusals in Expt. 1 were low and no treatment differences in DM intake were apparent (Table 3.6). However, Barry (1975, 1976) did report increased intake of these same silages in response to formalin-treatment and I/P methionine injection. The hypothesis that the amino acid status of individual sheep will have a marked effect on the intake of silage, is discussed in Chapter 4. The increased concentration of amino acids in treated silage did result in a higher intake of amino acids relative to untreated silage.

In Expt. 2, there was a significant effect of both supplementation and methionine infusion on silage DM intake ($P < 0.01$). The intake on the control silage was maintained near to the maximum for the first ten days (Fig. 3.3) and then showed a marked decline until the start of methionine infusion. During infusion, intake increased rapidly, but

under the limits imposed by the experiment, intake did not return to the same level as at the start of silage feeding. The decline in intake was significantly reduced, but not eliminated by energy and protein supplementation (Fig. 3.3) and increased intake in response to methionine was still present. A similar decline in intake with time on untreated silage diets was recorded by Barry et al. (1973) and Barry (1976). This decline was at least partially prevented by I/P methionine and formalin-treatment of silage. These results suggest that the percentage of methionine in the total amino acids absorbed by the animal on the control silage, did not correspond to the percentage of methionine in the total amino acids required by the animal. Thus, an amino acid imbalance was produced. Balance appeared to be partially restored at higher levels of amino acid availability, i.e. with protein and energy supplementation.

There was a significant difference between sheep in voluntary DM intake in Expt. 2 ($P < 0.01$, Appendix 1). This difference may be due to differences in amino acid status between sheep and this point is discussed in the following chapter, in relation to the absence of a treatment effect on intake in Expt. 1, compared to the treatment effect on intake in Expt. 2.

The intake differences in Expt. 2 might be expected to have an important effect on other parameters measured, through a direct influence on the rate of flow of nutrients through the digestive tract (Grovum and Hecker, 1973; Ulyatt and MacRae, 1974). Hence, the effect of the three supplements in increasing intake should be remembered during the following discussion on the digestion of silage.

D. Digestion of silage

(1) Rumen volatile fatty acid and ammonia concentrations

There were no marked treatment differences in rumen volatile fatty acid (VFA) concentration in Expt. 1 (Table 3.8). In Expt. 2, total VFA concentration in the rumen was significantly increased by both energy and protein supplementation ($P < 0.01$). The higher intakes on these supplements would have contributed to this increase in VFA concentration and in addition, the energy supplement would have been rapidly degraded to VFAs. There was no significant treatment effect on rumen ammonia concentration, but there was a significant difference between animals in both ammonia and VFA concentration ($P < 0.01$). The total VFA and ammonia concentrations were lower than those recorded by Bath and Rook (1965) and Chalmers (1963) also for silage diets. This may be due to the relatively low intakes, and to the continuous feeding system used in this experiment.

The high ruminal proportion of acetate on silage compared to other diets (e.g. acetate 60% on pasture, Ulyatt and Henderson, 1968) may affect the amino acid nutrition of ruminants, since the rate of tissue protein synthesis has been found to vary in response to different energy sources (Potter et al., 1968; Eskeland et al., 1974). Intra-arterial infusion of propionate led to a greater decrease in plasma essential amino acid concentration than a similar infusion of acetate or butyrate. However, amino acids rather than energy appear to limit the rate of live-weight gain of animals fed silage (McCarrick, 1966). Hence the high proportion of acetate may only be ~~important~~ relevant on silages which promote a high flow of amino acids into the duodenum.

There were marked differences between the molar proportions of acetate and propionate between Expts. 1 and 2 with acetate being higher and propionate lower in Expt. 1 compared to Expt. 2. These may reflect differences in the VFA composition of the two silages. However, complete analysis of the volatile acids was not conducted on the second silage, since the method was not available at Palmerston North. The change in the proportion of propionate in the total VFAs might also have had an effect on the amino acid nutrition of the animals, since gluconeogenesis plays a vital role in energy metabolism in ruminants. Bergman et al. (1970) have shown that 20 - 40% of daily glucose production was obtained from absorbed propionate, and a decrease in propionate availability might be expected to increase the contribution of amino acids to glucose production. Glucose production from amino acids has been reported to range from 11 - 30% (Wolff and Bergman, 1973; Reilly and Ford, 1971). However, data on gluconeogenesis in ruminants fed silage is not yet available.

(2) Apparent digestion of nutrients in the stomachs

The percentage of N intake which was digested in the stomach region in Expt. 1, appeared to differ between the treated and untreated silages (Table 3.9). On the treated silage there was a net addition of N to the rumen, while on the untreated silage, there was a net loss. A similar effect of formalin-treatment of silage was reported by Thomson et al. (1973). Despite the differences in the amino acid-N proportion in total N in the two silages, the percentages of amino acid-N in total N entering the duodenum were not appreciably different between treatments. A comparison of the amino acid-N (AAN) and non-amino acid-N (NAAN) consumed, and entering the duodenum, showed that the main effect of formalin-treatment on N digestion in the stomachs, occurred in the NAAN

fraction. The ratio of NAAN at the duodenum to NAAN in the feed, was higher on the treated silage than untreated silage. This may be due to the low NAAN intake on the treated silage (1.2g/day, Table 3.10) compared to the untreated silage (av. 7.2g/day). A possible explanation might be that ammonia production in the rumen from treated silage was low, and might therefore favour the transfer of urea across the rumen wall from the plasma to the rumen (Houpt and Houpt, 1968). The higher intake of NAAN on the untreated silage, on the other hand, would lead to a higher ammonia concentration within the rumen, and this might result in absorption of ammonia from the rumen.

The apparent digestion of OM and energy in the stomach region, also appeared to be lower on formalin-treated than on untreated silage. This might suggest that some free formaldehyde was present in the silage, thus depressing microbial activity within the rumen, or, alternatively, the rate of passage through the rumen might have been increased. However, there was considerable variability within treatments.

In Expt. 2, there was a significant effect of supplements on the percentage of N intake digested in the stomachs ($P < 0.05$, Table 3.9). This was due to a significant difference between the net addition of N to the rumen with the energy supplement, and a net loss of N from the rumen with the protein supplement. Neither supplement was significantly different from the control silage alone. Comparing the ratios of AAN and NAAN in feed and at the duodenum (Tables 3.10 and 3.11) showed that the main effect of protein supplementation was on digestion of NAAN. The formaldehyde casein supplement significantly decreased the flow of NAAN relative to control silage alone ($P < 0.01$ Table 3.10). The flow of NAAN into the duodenum on the control plus protein diet was also less

than the corresponding NAAN intake, suggesting that formaldehyde-casein might have increased the absorption of ammonia from the rumen. However, no evidence for this is available. The increased net loss of NAAN in the stomach region on casein supplemented silage, contributed to the significant increase in percentage of amino acids in the total N entering the duodenum ($P < 0.01$) relative to both the control and energy supplemented animals. The percentage obtained (mean 93%) was considerably higher than the 79.5% obtained by MacRae et al. (1972) who supplemented a dried grass diet with 60g/day of the same formaldehyde-treated casein.

Energy supplementation in Expt. 2 appeared to increase the ratio of duodenal AA to AA intake, but the difference was not significant. There was a significant difference between animals ($P < 0.01$, Table 3.11). This might suggest that the energy supplement was stimulating microbial protein synthesis and evidence for this comes from the increased proportion of amino acids in the total N entering the duodenum (Table 3.11). This ratio was significantly increased by energy supplementation relative to the control, indicating that the starch/sucrose infusion was effective in increasing the microbial utilisation of NAAN within the rumen.

There was also a significant interaction between the effect of supplementation with energy and protein, and the effect of methionine infusion on the ratio of AA entering the duodenum to AA intake ($P < 0.01$). This ratio decreased in response to methionine infusion in sheep fed control silage alone, and increased in response to methionine infusion in sheep fed supplemented silage diets. This result suggests different effects of methionine on N recycling to the rumen, on supplemented and unsupplemented diets. This in turn might

reflect changes in the distribution of methionine between its requirement for protein synthesis, and for extra-protein functions (Aguilar et al., 1972), dependent on the level of amino acids available to the tissues. However, no definite explanation can be given.

(3) Flow of nitrogenous compounds into the duodenum

Formalin-treatment of silage (Expt. 1) increased the flow of total N and amino acids into the duodenum compared to untreated silage. The only appreciable difference in intake between the treated and untreated silages was in amino acid intake, with the energy intake of treated silage being slightly lower (Table 3.6). Thus, the increased flow of amino acids into the duodenum on treated silage appears to result from both the increased amino acid intake, due to protection of the herbage protein and/or amino acids, from degradation during ensiling, and to the decreased percentage of N intake apparently digested in the stomach region.

The results from Expt. 1 do not provide any evidence as to whether the formalin-treatment continued to protect the herbage protein from microbial degradation in the rumen. Protection of protein by formaldehyde is generally taken to mean reversible binding of the formaldehyde to reactive groups in the protein. Three types of bond are formed between formaldehyde and protein, and at least one of these can be reversed by a decrease in pH (French and Edsall, 1945). While protection of dietary proteins by reaction with formaldehyde has been found to protect the protein from rumen microbial degradation (Ferguson et al., 1967; MacRae et al., 1972), the decrease in pH during ensiling might be expected to reverse some of the formaldehyde bonds. However, acid treatment of formaldehyde-bound proteins has also been found to bind part of the protein more firmly (Bowes et al., 1965). The effect of the decrease in pH during ensiling, on formalin-treatment of silage may thus

release some free formaldehyde and protein, while another part of the protein may become irreversibly bound. Hence, Thomson et al. (1973) found that formaldehyde-treatment of silage did increase the dietary N reaching the intestines, but at the expense of decreased microbial protein synthesis and decreased availability of this N for digestion in the intestines. Attempts to measure protein synthesis in Expt. 1 were reported in Chapter 2, but the results were inconclusive. However, decreased digestibility in the rumen was recorded (Table 3.9). The digestibility of N in the intestines (Table 3.13) will be discussed in detail below but no deleterious effect of formalin-treatment was apparent. The level of formalin-treatment used by Thomson et al. (1973) was not reported. However, Brown and Valentine (1972) reported that formalin application rates of 3.2% of the DM decreased ad lib. intake relative to untreated silage, and these workers suggested that free formaldehyde present in the silage could have had an adverse effect on the rumen microbial population. Reversal of binding in the silage stack would also have increased the content of free formaldehyde in the silage and could have adversely affected digestibility in the intestines as discussed earlier. The fate of free formaldehyde within the digestive tract does not appear to have been studied. Lower levels of formalin application to silage have been reported to increase liveweight gain and wool growth (Barry et al., 1973; Barry, 1975). Thus deleterious effects of the reversal of formaldehyde-binding in the silage stack may only be apparent at high rates of application. The level of application used in Expt. 1 (40% at 4.76l/ton fresh grass) did increase the flow of amino acids into the duodenum (Tables 3.11) and did not affect the digestion of N in the intestines (Table 3.13). This suggests that the main effect of the formalin-treatment of silage for this experiment was in increasing amino acid intake through protection

of the herbage protein from degradation during ensiling.

In Expt. 2, the flow of total N into the duodenum was significantly increased by both supplements and methionine infusion ($P < 0.01$, Table 3.11). The flow of amino acids into the duodenum was also significantly increased by both supplements relative to the control, and by the protein supplement relative to the energy supplement ($P < 0.01$). Methionine infusion had no significant effect on the flow of amino acids into the duodenum. Hence the proportion of amino acids in total N at the duodenum decreased in response to methionine infusion ($P < 0.05$). The reasons for this decrease are unknown. It might be suggested that increased intake could have influenced this ratio by affecting retention time in the rumen (Grovum and Hecker, 1973), but the within treatment differences in the ratio were low, despite a high variation in intake (Appendix 1).

The effect of intake on the flow of amino acids into the duodenum was also studied through the ratios of duodenal AA to AA intake, and duodenal AA to N intake (Table 3.11). The latter ratio showed more variability than the former, suggesting that the intake of amino acids had a greater effect on the flow of amino acids into the duodenum, than did the total N intake. The low range of the ratio duodenal AA to AA intake over the two experiments, led to the plotting of the regression between amino acid intake and duodenal amino acid flow (Fig. 3.4). The positive relationship indicated that increased amino acid intake increased the flow of amino acids into the duodenum. However, this relationship applied only to between treatment differences, and not to within treatment differences. The correlation coefficient for the ~~regression~~ relationship, $r = 0.82$, indicated that ~~differences~~ variation in amino acid intake ~~accounted for~~ was associated with 67% of the ~~differences~~ variation in amino acid flow into the

Table 3.15 The concentration of amino acids in feeds, the ratio of duodenal amino acids: amino acid intake, and the DM intake required to produce 100g amino acids entering the duodenum per day.

	Silages (Expts. 1 and 2)									
	Untreated	Treated	Control	Low N Hay[1]	Dried Lucerne[1]	Dried Grass[2]	Dried Grass[3]	Manawa Ryegrass[4]	White Clover[1]	Red Clover[1]
Amino acid concentration in feed (% of DM)	7.8	12.7	11.7	6.0	15.0	13.0	18.7	14.5	22.1	14.4
Ratio of $\frac{AAD}{AAI}$	0.70	0.92	1.03	2.7	0.88	0.93	1.02	0.95	0.63	1.05
DM intake required to produce 100g amino acids at duodenum (gDM/d)	1425	856	830	667	758	827	525	742	718	661

1 Data from Harrison et al. (1973)

2 " " MacRae et al. (1972)

3 " " Coelho da Silva et al. (1974)

4 " " MacRae and Ulyatt (1974)

duodenum. The regression was calculated for all treatments in Expts. 1 and 2, since no treatment effects were significantly different from the overall regression. However, energy supplementation in Expt. 2 did appear to increase the conversion of NAAN to microbial protein, as discussed earlier. Thus, variation in energy intake would also have contributed to the differences in flow of amino acids into the duodenum.

The ratios of duodenal amino acids to amino acid intake on fresh and dried pasture and hay diets (MacRae et al., 1972; Harrison et al., 1973; MacRae and Ulyatt, 1974; Coelho da Silva et al., 1973) are compared with the data from Expts. 1 and 2, in Table 3.15. The ratio varied from 0.6 on a diet of white clover, to 2.7 on a diet of low N hay supplemented with urea. The values obtained for silage in these experiments were similar to those obtained on Manawa ryegrass (MacRae and Ulyatt, 1974); dried grass (MacRae et al., 1972 ; Coelho da Silva et al., 1973) and on fresh red clover (Harrison et al., 1973). Calculation of the DM intake required to produce 100g amino acids entering the duodenum was made using these ratios and the concentration of amino acids in the individual feeds (Table 3.15). This calculation was limited to the feeds having similar ratios to silage, and to the two feeds with extreme ratios of duodenal amino acids to amino acid intake. The calculation showed that the DM intake required to produce 100g amino acids entering the duodenum per day, was considerably less on the fresh feeds and hay than on silage, especially untreated silage. Hence, the total intake of DM required to provide the animals with the same amount of protein, would have to be higher with silage, than the other feeds compared. Silages with a high amino acid content, or a ratio of duodenal amino acids to amino acid intake, which is greater than one (e.g. due to a high WSC content), would appear to maintain the amino acid status of the animal at a balanced level. However, silages

of a low amino acid content, or a low ratio of duodenal AA to AA intake, would limit the availability of amino acids to the animal. If these silages also contain components which are likely to depress intake, e.g. high acetate, butyrate or ammonia levels (McDonald and Whittenbury, 1973), the amino acid status of the animals would be further decreased, and an amino acid imbalance might occur (see Chapter 4).

(4) Composition of duodenal amino acids

The composition of duodenal amino acids was not markedly affected by formalin-treatment of silage in Expt. 1 (Table 3.12). The proportions of amino acids in duodenal digesta from Expt. 1, were similar to those of microbial protein and/or the feed amino acid content. The proportion of methionine in the total duodenal amino acids of sheep fed untreated silage (2.1%) was within the range, of 1.1 - 2.9%, reported by Harrison et al. (1973) for four fresh and dried forage diets and hay. This suggests that the production of a methionine imbalance in sheep fed silage, is due to an increase in the proportion of methionine in the total amino acids required by the animal, rather than to a decrease in the proportion of methionine available to the animal.

A method for estimating the relative contributions of microbial and dietary protein to the amino acids entering the duodenum, based on the known amino acid profiles of feed, microbial and endogenous protein was recently proposed by Evans et al. (1975). The relevant amino acid profiles were fed into a computer, and used to find the relative proportions of dietary and microbial protein which would generate the known amino acid composition of duodenal digesta. The possibility of individual dietary amino acids reaching the duodenum would introduce error into this computation. This programme was not available. However, a comparison of duodenal digesta amino acid composition with the

composition of microbial (Bergen et al., 1968) and feed protein (Table 3.5) can give some qualitative indication of the balance between microbial and dietary protein at the duodenum. A comparison of the duodenal amino acid composition on the control silage (Expt. 2) with that of the untreated silage (Expt. 1, Table 3.12), showed the main differences to be increases in the concentration of threonine and tyrosine, and decreases in the concentration of proline and alanine. Alanine and proline concentrations were higher in the feeds than in microbial protein, while threonine and tyrosine concentrations were lower in untreated silage than in microbial protein or the control silage. This might suggest a higher percentage contribution of microbial protein to the duodenal amino acid content of the control silage. However, the rate of microbial protein synthesis was not measured in Expt. 2, and the data for microbial protein synthesis in Expt. 1 were inconclusive (see Chapter 2).

Protein supplementation in Expt. 2 had a significant effect on the concentrations of arginine, phenylalanine, threonine, alanine, aspartate, glycine, glutamate, proline and serine in duodenal digesta ($P < 0.01$, Table 3.12). Most of these changes can be traced to the composition of the casein, compared to the amino acid composition of the silage (Table 3.5). The changes in arginine and serine concentration correspond to differences between casein and microbial protein composition (Bergen et al., 1968). Thus, there is further evidence to indicate that a high proportion of the formaldehyde-treated casein was reaching the duodenum. The magnitude of these differences was greater than those recorded by MacRae et al. (1972) who used the same formaldehyde-treated casein to supplement a dried grass diet.

These differences in amino acid composition due to the formaldehyde-treated casein might suggest that supplementation of silage diets with

formaldehyde-treated proteins of composition chosen to complement microbial protein in meeting the amino acid requirements of the animal, would increase the efficiency of utilisation of the amino acids absorbed.

(5) Intestinal digestion

In Expt. 1, the percentage of nutrient intake digested in the intestines was increased by formalin-treatment of silage (Table 3.13). This would be expected from the decreased digestion within the stomachs (Table 3.9). The apparent digestibility of the N entering the duodenum was high, and not adversely affected by formalin-treatment of the silage, suggesting that irreversible binding of formaldehyde to the herbage protein was not an important factor.

In Expt. 2, there were no significant effects of either supplementation or methionine on the percentage of DM, OM, energy or N intake digested in the intestines. However, there was a significant effect of animal variation on the percentage of DM ($P < 0.05$) and OM ($P < 0.01$) intake digested in the intestines.

The apparent digestibility of the N entering the duodenum was not significantly different between treatments (Table 3.13). While this might suggest that the formaldehyde-binding to casein was completely reversible, the large difference in the percentage of amino acids in the total N entering the duodenum, between the control and protein supplemented diets, might have masked any difference between treatments, since amino acids are more digestible than non-amino acid-N in the intestines (MacRae et al., 1972).

(6) Apparent digestibility in the whole digestive tract

In Expt. 1, there was a decrease in apparent digestibility of N in response to formalin-treatment of silage (Table 3.14). A similar decrease was reported by Barry and Fennessy (1973). This decrease in apparent digestibility of N, appeared to be due to a decrease in the N digested in the stomachs. There was a net addition of N to the rumen on treated silage compared to untreated silage (Table 3.9). Digestion of N in the intestines was not adversely affected by formalin-treatment (Table 3.13). Thus, since formalin-treatment increased the flow of amino acids into the duodenum (Table 3.11), treatment of silage would appear to increase the absorption of amino acids, relative to untreated silage. Amino acid-N will be utilised more efficiently than NPN absorbed from the rumen, and hence treatment of silage might be expected to increase N balance. However, the difference between treatments was very slight. This parameter will be discussed in more detail in the following chapter. No treatment effect on the apparent digestibility of DM, OM or energy was recorded in Expt. 1.

In Expt. 2, the overall digestibility of OM was significantly ($P < 0.01$) increased by both energy and protein supplementation (Table 3.14). This increase is probably due to the digestible nature of the supplements. However, no significant difference in energy digestibility was recorded in response to either energy or protein supplementation. This may be due to the higher SE for energy than OM digestibility.

Protein supplementation significantly increased N digestibility ($P < 0.01$). Since intestinal digestibility of N showed no significant treatment difference, increased N digestibility is presumably due to the increased loss of non-amino acid-N from the rumen when silage was supplemented with formaldehyde-treated casein (Table 3.10). N balance

was also significantly increased by protein supplementation ($P < 0.05$) but N intake was considerably greater with the protein supplemented diet. Thus, the increased N intake contributed by the formaldehyde-treated casein was not utilised as efficiently as the basal silage-N. This point and the effect of methionine infusion in increasing N balance will be discussed in detail in the next chapter.

CHAPTER 4

THE EFFECT OF FORMALIN-TREATMENT AND SUPPLEMENTATION OF SILAGE DIETS ON THE UTILISATION OF N BY SHEEP.

4.1 Introduction

The hypothesis that methionine is the first limiting amino acid on a variety of ruminant diets, has been proposed by a number of workers (e.g. Nimrick *et al.*, 1970; Hutton and Annison, 1972; Armstrong and Annison, 1973; Harrison *et al.*, 1973). Evidence for increases in live-weight gain and wool growth in response to supplemental methionine administered postruminally, has been reported (Reis, 1967; Wright, 1971; Dove and Robards, 1974). Production responses such as these, indicate that methionine may limit efficient utilisation of amino acids. In addition, Barry *et al.* (1973) have reported increased intakes of silage in response to intraperitoneal injection of methionine. Such a response suggests that feeding silage to sheep may place the animal in a state of amino acid imbalance. Since microbial protein is of relatively constant composition and is similar to herbage protein, the amino acid composition of duodenal digesta remains relatively constant in sheep fed different natural diets (Clarke *et al.*, 1966; Hogan and Weston, 1970; Harrison *et al.*, 1973; Ulyatt *et al.*, 1975). Hence, the amino acid status of ruminants appears to depend more on the quantity, than the quality of amino acids available to the animal. The flow of amino acids into the duodenum of sheep fed silage, is generally lower than pasture-fed animals (Armstrong, 1973; Thomson *et al.*, 1973). Silage, therefore, appears to be a suitable basal diet for a study of the metabolism of methionine, relative to the availability of other amino acids in sheep. Thus, the experiments to be reported compared the effect of changing the absolute and percentage (of total amino acids) availability of methionine to sheep. The utilisation of methionine was measured by various physiological parameters which have been used to study amino acid metabolism in non-ruminants.

In Expt. 1, the methionine available to the sheep was increased either by I/P infusion of methionine, or by feeding formalin-treated silage. Formalin-treated silage has been shown to increase intake and N retention over untreated silage (Barry et al., 1973) suggesting that it increased the flow of amino acids into the duodenum (see Chapter 3). The response to these treatments was measured in terms of N balance, free plasma amino acid concentrations and the oxidation of methionine to carbon dioxide. In addition, L-methionine-C14 U, was used to obtain information on methionine metabolism in ruminants. The second experiment was undertaken to provide additional data over a wider range of duodenal amino acid flow rates. The three treatments chosen to provide the varying levels of methionine were: a basal untreated silage diet, and supplementation of this diet with either formaldehyde-treated casein or a readily available energy source. In addition, each of these treatments was supplemented with a duodenal infusion of L-methionine. Only N balance and free plasma amino acid concentrations were used to study response to these treatments, due to the cost of L-methionine-C14 (U).

4.2 Methods

Details of the sheep, treatments, collection and analysis of digesta samples have been given in Chapter 4. The only additional methods used were related to the measurement of methionine metabolism.

Collection of plasma and expired air samples: Collection of plasma and expired air samples (Expt. 1) followed immediately after the period of collection of faeces and urine samples for balance measurement. L-methionine-C14(U) (New England Nuclear) was infused continuously into the right jugular vein. A sampling catheter was inserted in the left jugular vein. The catheters were 45cm long (1mm internal diameter, Portex) and 10cm were inserted into the vein on the day prior to

infusion. The catheters were filled with heparanised saline (250IU/ml) to prevent the formation of blood clots within the catheter. The free end of the catheter was attached to wool on the back of the sheep. Samples could thus be taken without disturbing the animal. The infusion solutions contained approximately 80 μC_i L-methionine-C14 (U) in 200ml sterile saline and were infused over 12h (see section on methionine metabolism). All tubing used in the infusion was flushed with alcohol, followed by sterile saline prior to use. Peristaltic pumps with tubing calibrated to deliver 150ml/day, were used to administer the infusion. The infusion solution was placed on a balance to record the infusion rate ~~between~~ within each sampling period. During an infusion, the sheep was placed in a metabolism crate with its head enclosed in a Perspex respiration hood, sealed by a rubber collar (Ulyatt, unpublished). The sheep had free access to water, and was able to stand or sit. Hourly rations were given to the animal through the top of the hood. The total flow rate of expired air was measured with a gas flowmeter (Parkinson Cowan). Temperature, vapour pressure and atmospheric pressure were measured continuously and used to correct gas volumes to standard temperature and pressure. Carbon dioxide content was determined on an aliquot, with an Infrared gas analyser (Grubb Parsons). A second aliquot was bubbled through 1N NaOH in a Dreschel wash bottle, to absorb carbon dioxide for the measurement of specific activity (SA). The wash bottles were changed at time of withdrawal of blood samples, i.e., at 5, 15, 30, 60, 90, 120, 150, 180, 210, 240 min after the start of the infusion, and thereafter at hourly intervals for a further 8h. Blood samples (10ml) were withdrawn into heparinised tubes and centrifuged at 6,000g for 20 min to separate plasma. The plasma samples from Expt. 1 were stored at $-20^{o}C$ prior to protein precipitation. However, in the first replicate of Expt. 2, this method

Table 4.1 The effect of blood sampling on the packed cell volumes of the sheep (Expt. 1)

Sheep No.	Rep. 1		Rep. 2		Rep. 3	
	0h	12h	0h	12h	0h	12h
647 (441, Rep. 3)	29.5	28.5	17.5	23.0	24.0	21.5
731 (1157, Rep. 3)	29.5	29.5	32.5	32.0	28.0	26.0
786	25.0	28.5	25.0	25.0	25.0	22.5

of storage was found to result in the disappearance of free cysteine from the plasma. Hence, later samples were separated into protein-free plasma, and protein precipitate prior to storage. In all cases, protein was precipitated by addition of an equal volume of 10% sulphosalicylic acid to the plasma. The protein precipitate was then removed by centrifugation at 6,000g for 15 min, and the supernatant stored at -20^{o}C.

The total volume of blood sampled from each sheep during a replicate in Expt. 1 was approximately 200ml. In order to check whether removal of this volume affected the animal, packed cell volumes were determined at 0 and 12h after the start of infusion for each sheep. These are given in Table 4.1. Sampling had little effect on packed cell volume.

In Expt. 2, catheters were inserted into a jugular vein of each sheep, in a similar manner to those in Expt. 1, three days prior to the start of methionine infusion. Samples (10ml) were taken on each of the following two days, and daily after the start of methionine infusion.

Plasma amino acid concentration: The total free plasma amino acids in samples from Expt. 1 were determined on a Beckman 120C amino acid analyser using a two column procedure. Prior to Expt. 2, this instrument was modified by the addition of a Locarte automatic loader. The loader necessitated the use of one column procedures, and while this modification had been completed for protein hydrolysate analysis, the resin for physiological fluid analysis was not available at the start of Expt. 2. Thus, plasma amino acid analyses in Expt. 2 were carried out on the physiological acidic and neutral resin from the original Beckman amino acid analyser. Separate determination of the basic amino acids was not possible, due to incompatability between the Locarte system and the Beckman basic amino acid column.

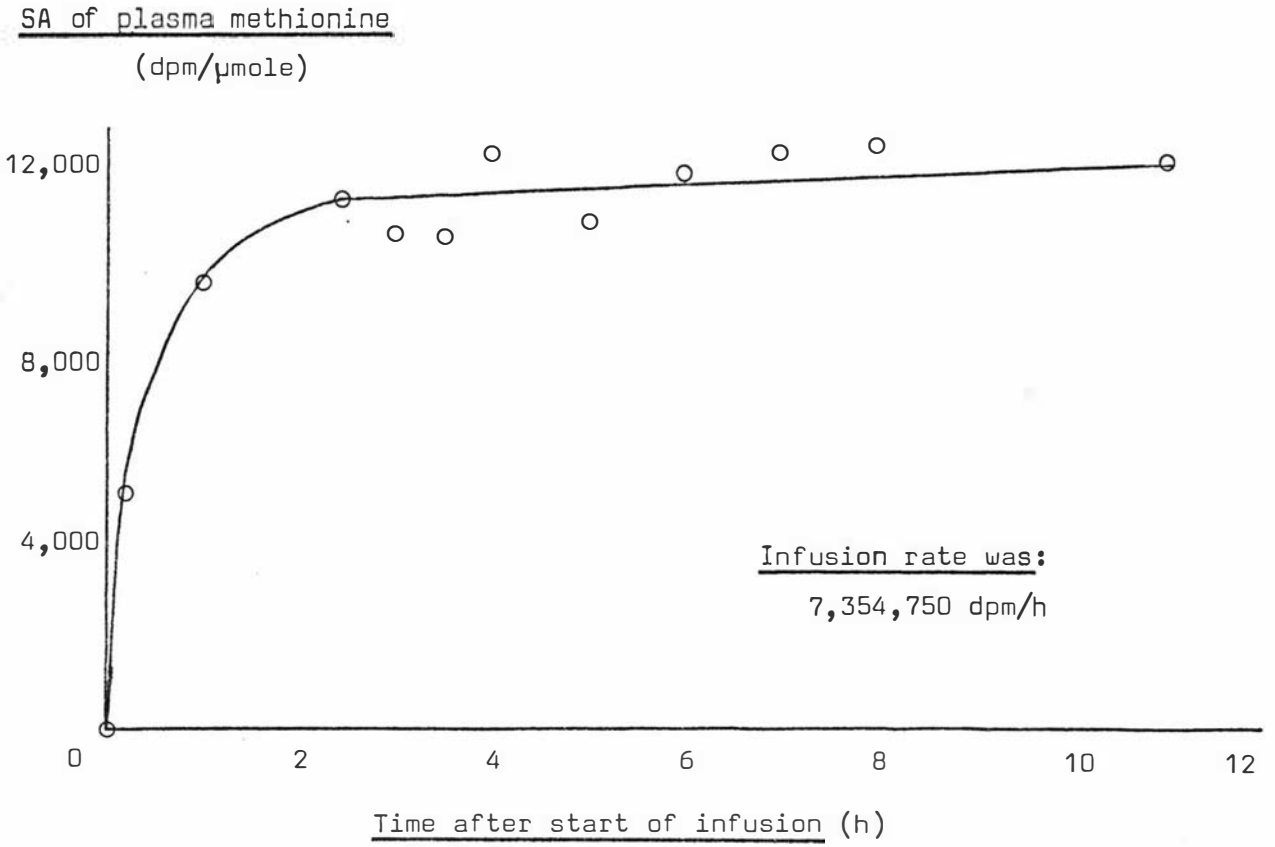


Fig. 4.1 The change in SA of plasma methionine with time after start of infusion of L-methionine-C14 (U)

The methionine SAs in Expt. 1 were measured on a Jeol-JLC 6AH amino acid analyser, using the specific S-amino acid procedure of Jeppson and Karlsson (1972). This analysis was discussed in Chapter 2.

Plasma urea concentration: Plasma urea concentration was measured by reaction with diacetyl monoxime on a Technicon autoanalyser.

Scintillation counting: CO_2 trapped in 1N NaOH from the gas wash bottles was precipitated as $BaCO_3$, by addition of excess $BaCl_2$. The precipitate was collected on Whatman No. 541 paper. After washing with hot water and drying with acetone, a weighed sample of the $BaCO_3$ (approximately 150mg) was suspended in the gel scintillation mix of Cluley (1962) (0.3% (w/v) diphenyloxazole (PPO) in toluene, +0.4% Aerosil), and ^{14}C activity counted in a Packard liquid scintillation counter. The efficiency of counting for the set weight of $BaCO_3$ was calculated as detailed by Cluley (1962).

The radioactivity in plasma methionine was determined on fractions of the column effluent from the Jeol amino acid analyser. The fractions were added to one of the following scintillation mixes, and counted on a Beckman or Packard liquid scintillation counter.

Scintillation mix 1: 0.5% PPO + 0.02% POPOP in Triton/Toluene (1:2)

Scintillation mix 2: 0.4% Omnifluor in Triton/Toluene (1:2)

An external standard of Hexadecane-C14 was used to plot the degree of quenching for each counter, and thus the efficiencies of counting were calculated.

Calculations

Methionine metabolism: SAs of plasma methionine were plotted against time for each sheep. The plot for sheep 647, replicate 1 is given in Fig. 4.1 (the complete data for all sheep is given in Appendix 3).

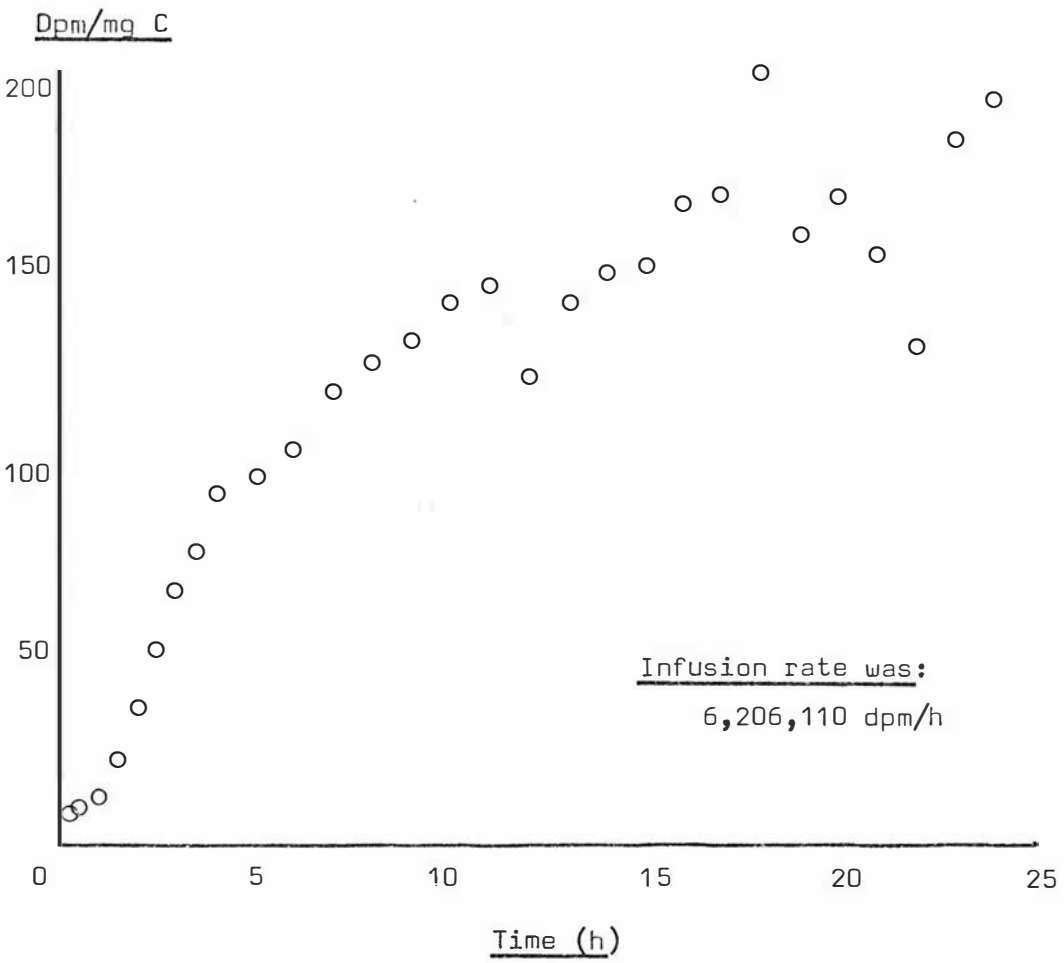


Fig. 4.2 The change in SA of CO_2 with time after the start of infusion of L-methionine-C14 (U)

While it can be seen that some of the data in the early part of this particular curve are missing (due to loss of samples on the amino acid analyser), it would appear that SA increased exponentially with time, to a plateau at approximately 3h. The small increase above this plateau value can be attributed to the recycling of labelled methionine within the animal. The shape of this curve can then be defined by two parameters which describe methionine metabolism. The first is the plateau SA (SA_∞, dpm/μmole), and from this the methionine turnover rate can be calculated.

i.e. Turnover rate = $\frac{\text{Infusion rate of L-methionine- C14 (U) (dpm/h)}}{\text{SA (dpm/μmole)}}$

The second is the rate constant (k). This was calculated as the slope of the curve represented by $\ln(SA_\infty - SA_t)$ versus time (t) (Riggs, 1963). Where SA_t is the SA at time t. The size of the methionine pool was also calculated from the relationship: Pool size = Turnover rate / k.

Oxidation of methionine: The first sheep was infused continuously for 24h to try and determine the time taken to reach a plateau value of $^{14}CO_2$ SA (Fig. 4.2). A plateau was not reached within 24h indicating recycling of ^{14}C within the animal. However, the rate of increase appeared to change at 11 - 12h. Thus, to obtain a standard comparison, subsequent infusions were limited to 12h duration, and SA_∞ calculated as the average of the values for the last three hours of infusion. The SA_∞ of CO_2 and the SA_∞ of methionine were then used to calculate the percentage of expired CO_2 which was derived from methionine.

i.e. % of CO_2 derived from methionine = $\frac{SA_\infty \text{ of expired } CO_2 \text{ (dpm/mgC)}}{SA_\infty \text{ of plasma met (dpm/mgC)}}$

and % methionine oxidised to CO_2 = $\frac{\text{dpm } ^{14}C \text{ expired/min}}{\text{dpm } ^{14}C \text{ infused/min}} \times 100$

Table 4.2 The flow of amino acids into the duodenum in sheep fed formalin-treated silage, untreated silage, and untreated silage diets supplemented with formaldehyde-treated casein, energy and postruminal (I/P and duodenal) methionine.

Expt. No.	Treatment	Methionine	Flow of amino acids into the duodenum (g/day)		Methionine available for absorption (g/day)		% methionine in total amino acids	
				Mean		Mean		Mean
1	Untreated	-						
	Rep. 1		47.6)		1.2)		2.4)	
	Rep. 2		46.2)	42.2	0.8)	0.9	1.7)	2.1
	Rep. 3		32.8)		0.8)		2.4)	
	Treated	-						
	Rep. 1		57.6)		1.4)		2.4)	
	Rep. 2		67.8)	65.8	1.5)	1.5	2.2)	2.3
	Rep. 3		72.0)		1.7)		2.4)	
	Untreated	+						
	Rep. 1		65.2)		2.5)		3.8)	
	Rep. 2		52.9)	50.4	1.9)	2.1	3.6)	4.4
	Rep. 3		33.0)		1.9)		5.8)	
2	Control	-	49.6		1.1		2.2	
	+ protein	-	110.5		2.7		2.4	
	+ energy	-	74.1		1.7		2.3	
	Control	+	60.3		3.2		5.3	
	+ protein	+	125.7		5.0		4.0	
	+ energy	+	98.6		4.2		4.3	
	SE		6.0		0.18		0.06	
	Treatment significance		**		**		NS	
	Methionine significance		NS		**		**	

* $p < 0.05$
** $p < 0.01$
NS not significant

Availability of methionine for absorption: The methionine available for absorption from the intestines was calculated by adding the daily amount of methionine infused, to the daily flow of methionine into the duodenum. The I/P methionine was assumed to be absorbed to the same extent as the methionine entering the duodenum, since no data on this were available.

Statistical analysis: This was identical to the analysis described in Chapter 3.

4.3 Results

The main aim of Expt. 1 was to compare the effects of formalin-treatment of silage, and intraperitoneal infusion of L-methionine on the utilisation of N by sheep. In Expt. 2 the effects of supplementing an untreated silage diet with formaldehyde-treated casein and energy were compared with the effect of duodenal infusion of L-methionine on the utilisation of N.

The effect of the treatments on the flow of amino acids into the duodenum, and on the total methionine available for absorption, are given in Table 4.2. In Expt. 1, the flow of total amino acids into the duodenum was higher on formalin-treated than untreated silage, while methionine infusion appeared to have no effect. The percentage of methionine in the total absorbable amino acids remained the same on treated and untreated silages, but was increased by I/P infusion. In Expt. 2, the flow of total amino acids into the duodenum was significantly increased by both protein and energy supplementation relative to unsupplemented silage ($P < 0.01$), while protein also significantly increased the flow of amino acids into the duodenum relative to energy supplementation ($P < 0.01$). Methionine infusion directly increased

Table 4.3 The effect of formalin-treatment of silage, and supplementation of untreated silage diets with formaldehyde-treated casein, energy and postruminal (I/P and duodenal) methionine on the concentration of methionine and total amino acids in sheep plasma

Expt. No.	Treatment	Methionine	Plasma methionine concentration (μmole/l)	Mean	Total[1] plasma amino acid concentration (μmole/l)	Mean	% met in total plasma amino acids	Mean
1	Untreated	-						
	Rep. 1		7.5)		3084)		0.2)	
	Rep. 2		6.7)	7.9	2240)	2684	0.3)	0.3
	Rep. 3		9.6)		2728)		0.4)	
	Treated	-						
	Rep. 1		7.2)		3626)		0.2)	
	Rep. 2		10.3)	9.0	2761)	3151	0.4)	0.3
	Rep. 3		9.4)		3066)		0.4)	
	Untreated	+						
	Rep. 1		19.0)		1868)		1.1)	
	Rep. 2		16.4)	20.3	2075)	2000	0.8)	1.0
	Rep. 3		25.5)		2056)		1.3)	
2	Control	-	14.2		2036[1]		0.7	
	+ protein	-	16.9		1987		0.9	
	+ energy	-	14.3		2046		0.7	
	Control	+	31.4		1673		2.0	
	+ protein	+	33.4		1366		2.5	
	+ energy	+	35.7		1617		2.3	
	SE		1.8		66		0.13	
	Treatment significance		NS		NS		NS	
	Methionine significance		**		**		**	
	Replicate significance		*		NS		*	

[1] "Total" plasma amino acids in Expt. 2 only represents free acidic and neutral amino acids.

Table 4.4 The effect of formalin-treatment of silage and the supplementation of untreated silage diets with formaldehyde-treated casein, energy and postruminal (I/D and duodenal) methionine on the percentage concentrations of individual amino acids in the total plasma amino acids.

	Expt. 1								
	Untreated			Treated			Untreated + met		
	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
ornithine	8.2	4.6	5.0	9.9	8.5	6.8	4.3	4.8	6.3
lysine	6.7	5.8	7.6	11.7	6.9	7.6	5.0	6.5	8.2
histidine	2.7	5.5	3.3	4.4	2.4	2.6	3.7	5.0	4.5
arginine	5.8	6.1	6.3	13.1	6.1	7.1	9.7	6.8	9.9
taurine	0.3	0.7	0.8	0.2	0.2	0.1	3.0	2.4	2.1
threonine	3.8	2.5	2.8	2.2	4.7	4.1	2.2	1.4	1.9
serine	10.1	4.9	5.1	4.6	5.8	6.8	3.5	4.3	4.0
proline	2.1	3.1	5.1	2.8	4.0	4.1	6.0	2.6	4.2
glutamic acid	9.6	16.0	11.7	7.0	6.9	7.9	16.1	9.6	13.1
citrulline	5.3	4.8	3.9	3.6	5.9	4.2	6.5	5.9	6.0
glycine	23.6	28.6	28.7	24.8	28.2	27.2	20.5	32.7	16.8
alanine	7.6	7.4	7.5	6.1	6.3	7.6	9.0	6.5	8.7
valine	6.5	5.0	6.5	4.8	7.4	6.5	5.0	4.9	5.7
cysteine	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.
cystathionine	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	0.4	0.2	0.6
methionine	0.2	0.3	0.4	0.2	0.4	0.4	1.1	0.8	1.3
isoleucine	3.1	2.1	3.0	2.0	3.0	2.6	2.4	2.3	3.5
leucine	3.9	3.0	4.4	3.4	4.3	4.6	3.9	2.7	3.6
tyrosine	1.6	1.5	1.6	1.2	1.8	2.2	1.6	1.4	2.0
phenylalanine	1.0	1.2	1.4	0.9	1.3	1.6	2.1	1.8	2.0

Table 4.4 continued

	Expt. 2										
	Control		+ protein		+ energy			Significance			
	- met	+ met	- met	+ met	- met	+ met	SE	Treatment (T)	Methionine (M)	Replicate	Interaction (T×M)
taurine	0.6	5.0	0.6	3.7	0.6	4.9	0.49	NS	**	NS	NS
threonine	3.0	1.9	5.6	3.1	4.3	2.2	0.21	**	**	NS	NS
serine	8.1	5.0	6.4	5.7	7.1	4.2	0.35	NS	**	**	*
proline	4.8	3.7	7.9	8.7	4.1	4.2	0.35	**	NS	NS	NS
glutamic acid	4.5	8.3	4.3	5.2	4.8	6.1	1.26	NS	NS	NS	NS
citrulline	5.0	7.8	5.6	7.4	5.1	7.0	0.39	NS	**	NS	NS
glycine	45.8	37.9	35.8	25.6	41.8	36.4	1.51	**	**	NS	NS
alanine	11.0	11.3	8.8	12.1	11.4	13.9	0.51	NS	*	*	NS
valine	6.2	5.5	10.9	9.6	8.7	6.5	0.29	**	**	NS	NS
cysteine	1.3	1.6	1.2	2.0	1.0	1.8	0.10	NS	**	**	*
cystathionine	Tr.	0.8	Tr.	0.6	Tr.	0.6	0.04	NS	**	*	NS
methionine	0.7	2.0	0.9	2.9	0.7	2.3	0.13	NS	**	*	NS
isoleucine	2.3	2.6	3.1	3.0	3.0	3.0	0.06	**	NS	**	NS
leucine	3.9	2.9	5.2	4.6	4.0	3.1	0.08	**	**	**	NS
tyrosine	2.2	3.3	3.6	4.9	2.8	3.6	0.22	**	**	NS	NS
phenylalanine	1.8	2.6	2.1	3.4	1.7	2.6	0.17	NS	**	NS	NS

available methionine, but did not significantly increase the total flow of amino acids. The percentage of methionine in total absorbable amino acids was therefore increased by methionine infusion, but it was unaffected by supplementation with protein or energy.

The data on free plasma methionine and total amino acid concentrations are presented in Table 4.3. Plasma methionine concentration was increased by I/P infusion, but unaffected by formalin-treatment of silage in Expt. 1. The concentration of total amino acids in the plasma was lower in response to methionine infusion compared to the concentration in sheep fed treated silage, but the concentration on untreated silage was between the values for the other two treatments.

The free plasma amino acid concentrations from Expt. 2, in Table 4.3, are the average of samples taken two days prior to, and the 15th and 16th days after, the start of methionine infusion. Plasma methionine concentration was significantly increased by duodenal methionine infusion ($P < 0.01$), but unaffected by protein or energy supplementation. "Total" plasma amino acid concentration in Expt. 2 refers only to acidic and neutral amino acids. This was significantly decreased by methionine infusion ($P < 0.01$), and unaffected by either of the supplements. The percentage of methionine in the "total" plasma amino acids was significantly increased by duodenal methionine infusion ($P < 0.01$).

Table 4.4 gives the effect of formalin-treatment and supplementation of silage on the percentage contributions of individual amino acids to the total amino acids in the plasma. The only marked consistent differences in response to intraperitoneal methionine in Expt. 1 were increases in the percentages of methionine, taurine, cystathionine, citrulline, and phenylalanine and a decrease in the percentage of

Table 4.5 The effect of duration of methionine infusion on the percentage of individual amino acids in the total plasma amino acids - Expt. 2 (Rep. 1).

Day	0	1	2	3	4	5	6
Control							
met	0.8	2.2	2.5	3.3	3.2	4.6	4.8
ser	7.4	6.1	5.8	5.4	4.0	3.7	5.5
ile + leu	7.0	5.8	5.5	7.6	7.7	7.4	6.4
val	7.6	5.5	5.2	6.3	7.1	7.1	6.8
tyr + phe	4.0	3.3	4.2	5.8	5.6	6.9	5.1
Control + protein							
met	0.8	1.9	2.3	2.8	3.0	3.4	3.3
ser	6.4	4.9	5.8	5.8	5.3	5.4	5.1
ile + leu	9.0	8.4	9.4	10.8	9.6	10.0	10.2
val	11.6	10.6	10.4	10.6	11.6	10.5	11.3
tyr + phe	6.0	6.9	7.2	8.2	8.8	8.9	9.2
Control + energy							
met	0.8	2.1	2.9	3.7	3.5	ND	5.3
ser	7.6	6.4	5.7	5.1	5.0	ND	5.6
ile + leu	7.0	5.1	3.6	5.0	5.1	ND	6.1
val	9.2	6.1	6.3	5.9	8.5	ND	7.1
tyr + phe	4.4	4.5	3.6	4.8	5.9	ND	7.6

Table 4.6 The effect of formalin-treatment of silage and I/P methionine infusion to sheep fed untreated silage, on metabolism of methionine (Expt. 1).

Treatment	Expt. 1								Calculated	
	Methionine Turnover Rate (mmole/h)	Mean	% Methionine Oxidised to CO_2	Mean	% CO_2 Derived From Methionine	Mean	Methionine Pool Size (μmole)	Mean	Total Plasma Methionine (μmole) Mean	Total Muscle Methionine (μmole) Mean
Untreated										
Rep. 1	0.43		16.0		0.070					
Rep. 2	0.54	0.51	14.7	15.3	0.050	0.066	477	562	19.9	576
Rep. 3	0.55		15.3		0.078		646			
Treated										
Rep. 1	0.53		13.3		0.073		631			
Rep. 2	0.67	0.64	17.5	15.6	0.108	0.100	245	605	18.7	558
Rep. 3	0.72		16.0		0.119		939			
Untreated + met										
Rep. 1	0.68		18.3		0.101					
Rep. 2	0.86	0.78	18.8	19.0	0.132	0.130	1089	1026	23.5	599
Rep. 3	0.79		19.9		0.156		963			

serine. In Expt. 2, 15 days of duodenal methionine infusion significantly increased the percentages of taurine, citrulline, cystathionine, cysteine, methionine, tyrosine and phenylalanine ($P < 0.01$) and significantly decreased the percentages of threonine, serine, glycine, valine and leucine ($P < 0.01$). The percentage of alanine was significantly increased ($P < 0.05$). There was a significant effect of replicates on the percentages of serine, cysteine, leucine and iso-leucine ($P < 0.01$) and alanine, and methionine ($P < 0.05$). There was also a significant interaction between the effects of supplementation and methionine on the percentage of serine and cysteine ($P < 0.05$).

The concentration of individual amino acids in the plasma after one day of methionine infusion was also measured, and is given in Appendix 4. One interesting feature of these concentrations is the disappearance of taurine from the plasma after 24h of methionine infusion.

The effects of duration of infusion of methionine on the percentages of individual amino acids in the plasma was measured during the first replicate of Expt. 2 (Table 4.5) to assess the validity of only six days preliminary period for adjustment to methionine infusion. Only the percentages of methionine, serine and the branched chain and aromatic amino acids are given, as they were considered to be representative of all the amino acids.

An infusion of L-methionine-C14 (U) was used to estimate parameters of methionine metabolism in Expt. 1. The results are given in Table 4.6. The rate of turnover of methionine, the percentage oxidised to CO_2, the percentage of CO_2 derived from methionine and the methionine pool size were all increased by I/P infusion of methionine relative to

Table 4.7 The effect of formalin-treatment of silage, and supplementation of untreated silage diets with formaldehyde-treated casein, energy and postruminal (I/P and ducdenal) methionine on urinary N, daily N retention and plasma urea concentration.

Expt. No.	Treatment	Methionine	N intake (g/day)		Urinary N (g/day)		N retention (g/day)		Plasma urea concn. (mgN/100ml)	
				Mean		Mean		Mean		Mean
1	Untreated	-								
	Rep. 1		13.9		7.2		3.1		15	
	Rep. 2		15.3	14.5	12.3	9.3	-1.4	1.1	12	14
	Rep. 3		14.4		8.4		1.6		14	
	Treated	-								
	Rep. 1		10.8		5.9		3.3		12	
	Rep. 2		12.6	12.7	5.7	5.9	1.6	2.5	12	13
	Rep. 3		14.6		6.1		2.6		14	
	Untreated	+								
	Rep. 1		14.8		7.3		3.2		9	
	Rep. 2		14.8	14.0	8.4	6.8	1.9	2.7	12	10
	Rep. 3		13.2		4.8		3.2		10	
2	Control	-	10.4		8.7		-0.6		13	
	+ protein	-	21.8		12.3		3.5		16	
	+ energy	-	14.1		10.2		1.5		12	
	Control	+	14.7		7.5		2.3		14	
	+ protein	+	22.6		11.8		5.3		17	
	+ energy	+	15.9		7.1		4.0		13	
	SE		0.64		0.36		0.67		0.94	
	Treatment significance		**		**		*		NS	
	Methionine significance		NS		**		**		NS	

untreated silage alone. The values obtained on formalin-treated silage were intermediate between untreated silage alone, and intraperitoneal methionine infusion. Individual sheep SA data and the values for the turnover constants are given in Appendix 3. Calculation of the size of the methionine pool from these data, could only be made for seven of the sheep. This was due to loss of some plasma samples collected during the early part of the infusion. The pool sizes obtained, were compared with the calculated values for total plasma and muscle methionine. These were calculated by assuming blood volume to be 5% of body weight and total muscle weight to be 45% of empty body weight (Harper, 1969). Plasma methionine concentrations were measured, and the methionine content of muscle was taken as 0.58% (USDA, 1968). Total plasma methionine was considerably lower than the values obtained from the data, while total muscle methionine was one thousand times higher. The approximate pool sizes for plasma and muscle methionine were calculated assuming blood volume to be 5% of body weight, and total muscle weight to be 45% of empty body weight (Harper, 1969). ~~Plasma methionine concentrations were measured, and the methionine content of muscle was taken as 0.58% (USDA, 1968).~~

Urinary-N, daily N retention and plasma urea concentration on formalin-treated silage and untreated silage supplemented with protein, energy and methionine are given in Table 4.7. Urinary-N appeared to be decreased by formalin-treatment of silage in Expt. 1, but N retention and plasma urea did not show any consistent difference between treatments. In Expt. 2, supplementation of untreated silage with protein significantly increased both urinary-N ($P < 0.01$) and N retention ($P < 0.05$) but had no significant effect on plasma urea concentration.

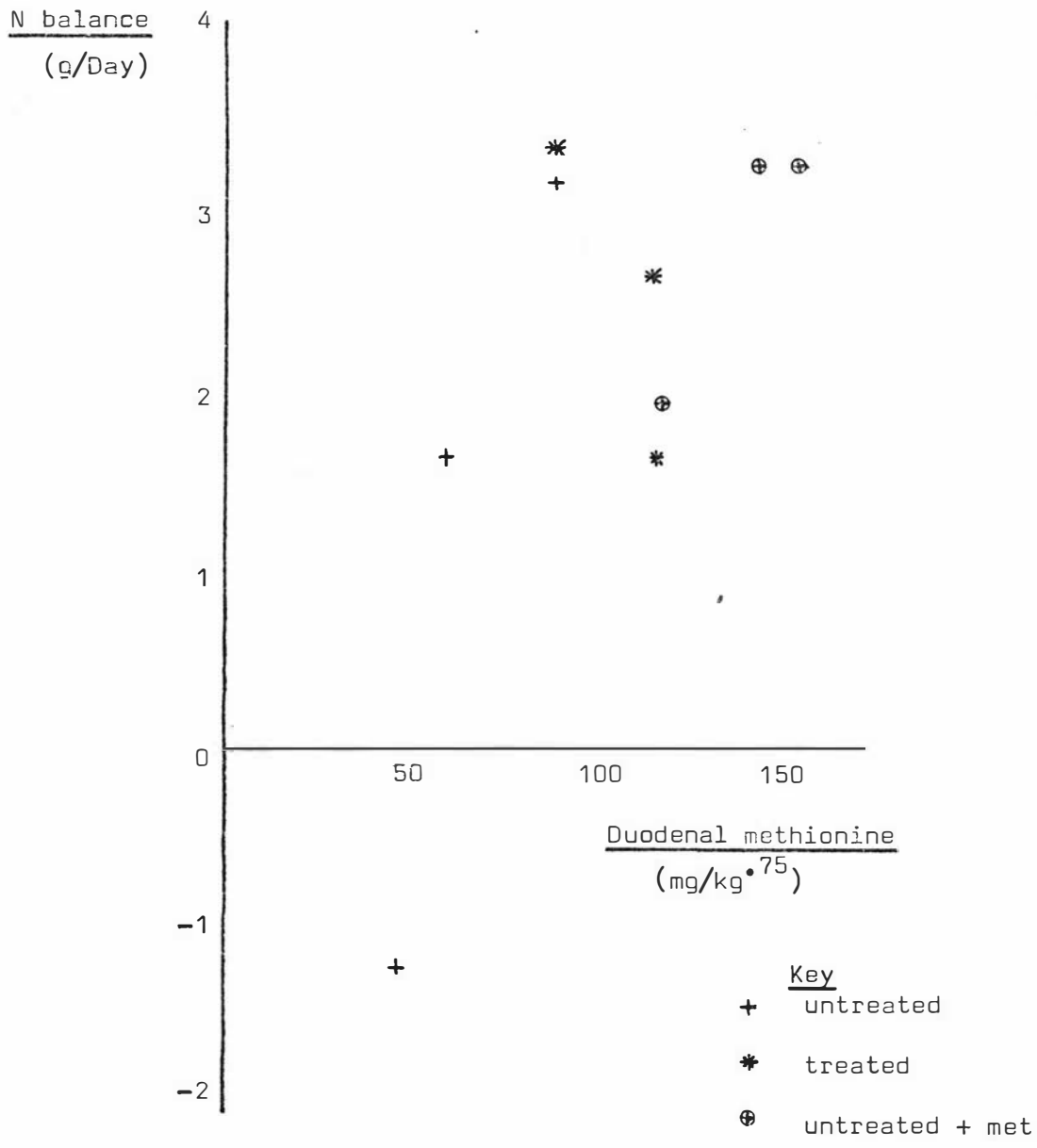


Fig. 4.3 N balance response to increasing methionine flow into the duodenum (Expt. 1)

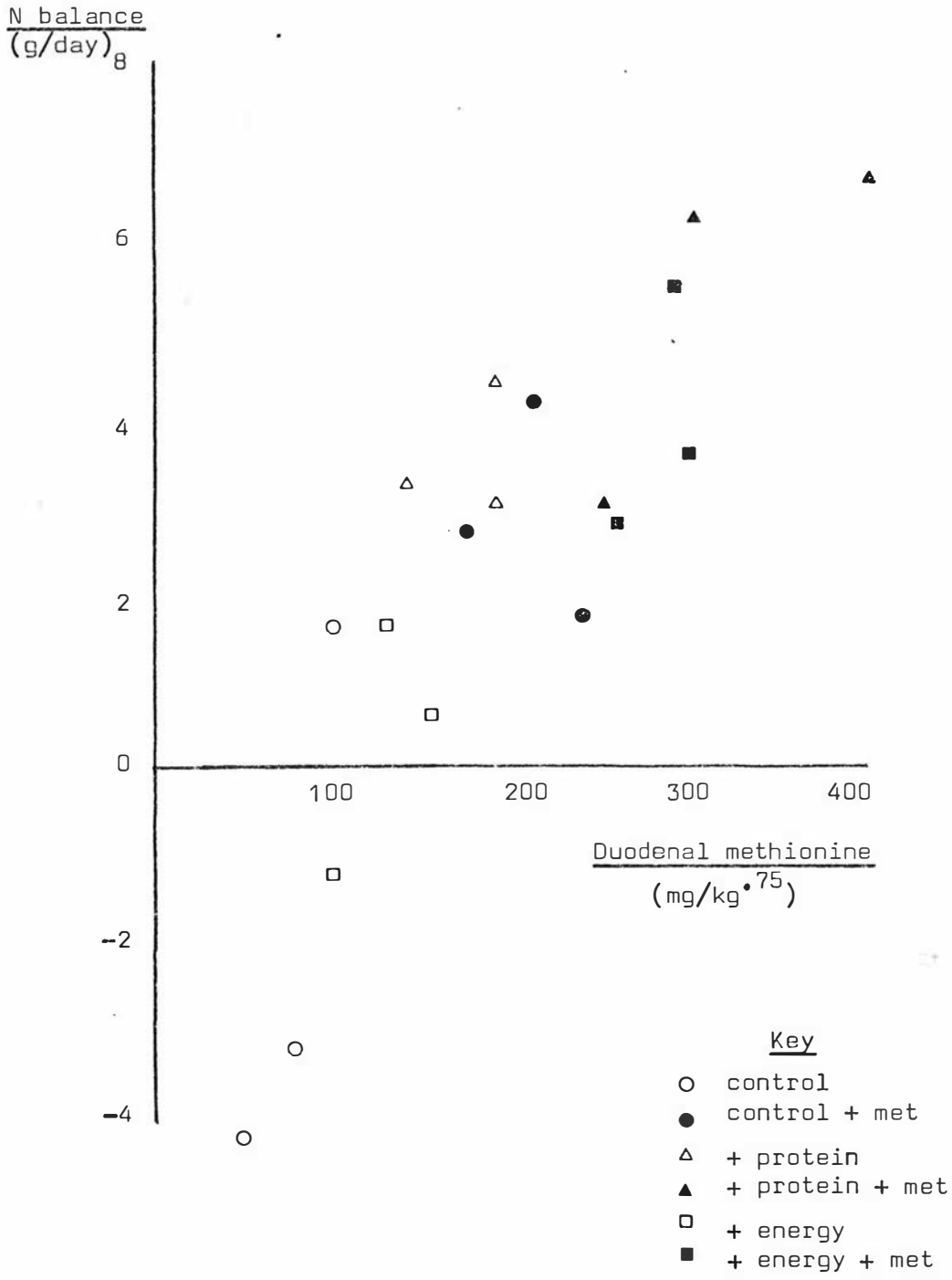


Fig. 4.4 N balance response to increasing methionine flow into the duodenum (Expt. 2)

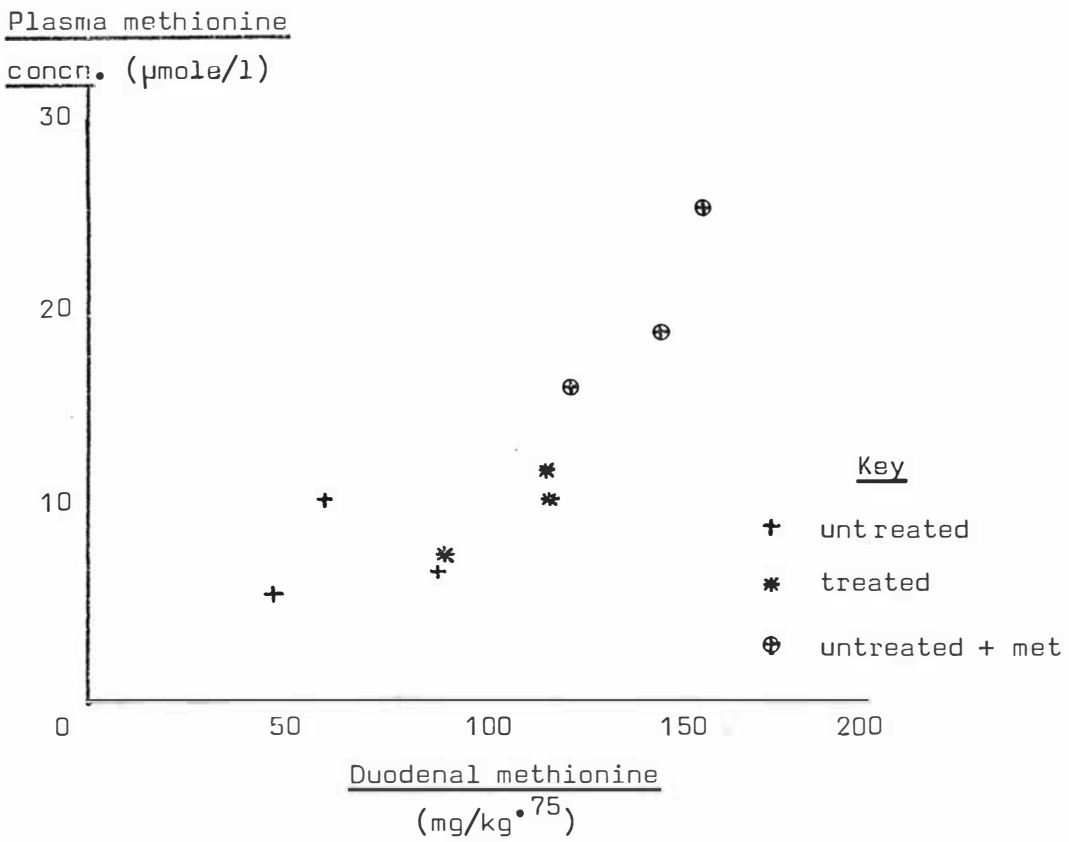


Fig. 4.5 Plasma methionine concentration response to increasing duodenal methionine (Expt. 1)

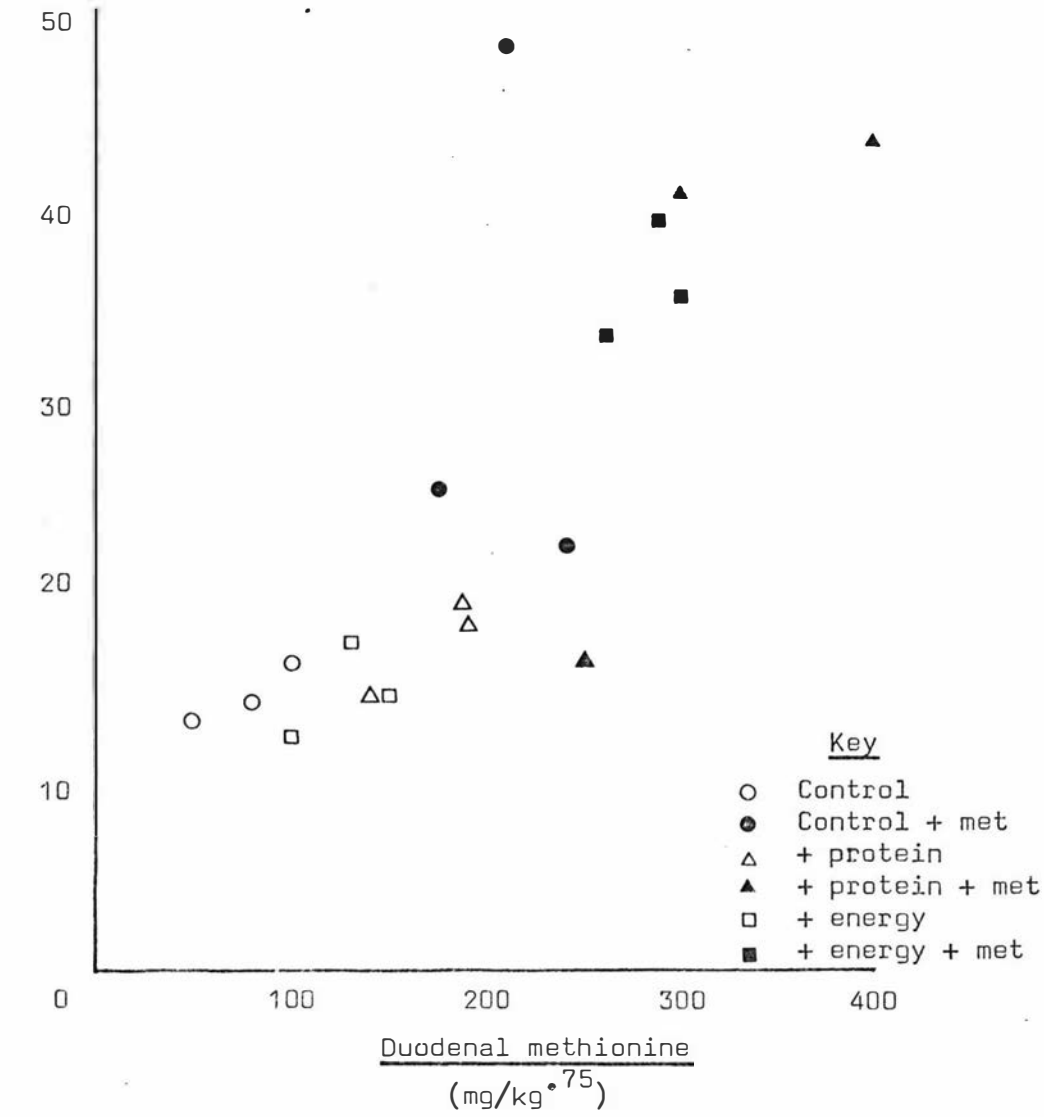


Fig. 4.6 Plasma methionine concentration response to increasing duodenal methionine (Expt. 2)

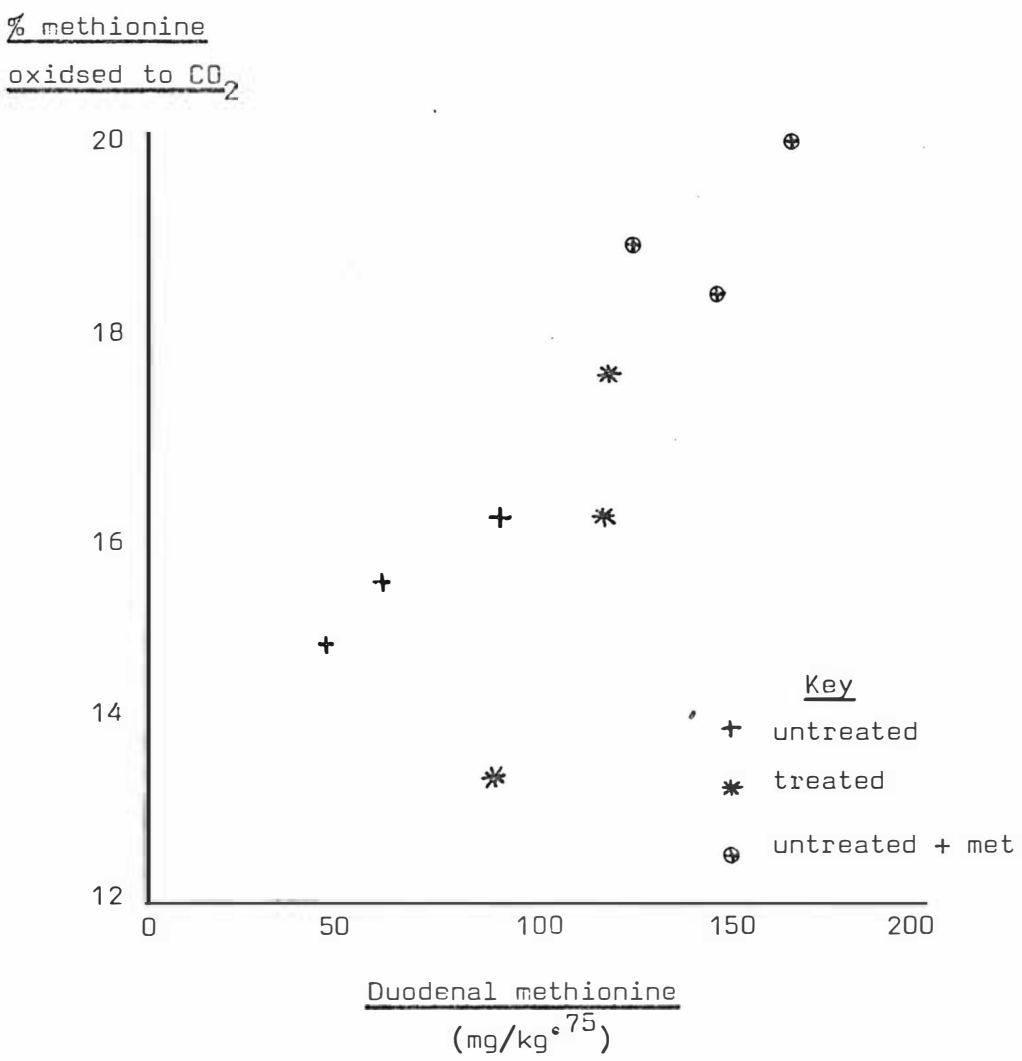


Fig. 4.7 Percentage of methionine oxidised to CO_2 in response to increasing methionine flow into the duodenum (Expt. 1)

Energy supplementation had no significant effect on urinary-N, N retention or plasma urea concentration. Duodenal infusion of methionine significantly decreased urinary N ($P < 0.01$) and significantly increased N retention ($P < 0.01$), but had no significant effect on plasma urea concentration.

Figs. 4.3 - 4.7 give the response in N balance, plasma methionine and percentage of methionine oxidised to CO_2, to increasing levels of methionine/$kg^{.75}$ entering the duodenum. All the parameters measured increased in response to increasing methionine availability. However, mathematical analysis of the curves did not detect any points of inflection. N balance (Figs. 4.3 and 4.4) increased from negative to positive values in response to increasing duodenal methionine in both experiments. The data were considered too variable for accurate prediction of the methionine required to produce zero N balance, but this appeared to fall within a range of 50 - 100mg duodenal methionine/$kg^{.75}$, in Expt. 1, and 100 - 200mg duodenal methionine/$kg^{.75}$ in Expt. 2. The declining intakes during Reps. 1 and 3 in Expt. 2 resulted in markedly different intakes during periods of balance and flow measurements. Thus, N balance values for Fig. 4.4 were recalculated on the basis of actual N intake during flow measurements.

4.4 Discussion

A. Introduction

Expt. 1 was undertaken to compare the effects of formalin-treatment of silage, and I/P supplementation of untreated silage, on the digestion and utilisation of N in sheep. These treatments had been shown to increase the voluntary intake of a basal silage diet, and the main aim of the experiment was to investigate possible factors controlling this increase. Since the main effects of formalin-treatment and methionine

infusion were to increase the methionine and total amino acids available to the sheep, alternative methods of further increasing amino acid availability were studied in Expt. 2. Thus, formaldehyde-treated casein and an intra-ruminal infusion of readily available energy, were used to supplement a basal silage diet. The effects of the various treatments on the digestion of silage have already been discussed (Chapter 3), and thus the following discussion will centre on the utilisation of methionine, relative to the presence of other amino acids.

B. Methionine and total amino acids before and after absorption

In Expt. 1, the total flow of amino acids into the duodenum was increased by formalin-treatment of silage and the absorption of methionine alone, was increased by I/P infusion of methionine to sheep fed untreated silage (Table 4.2). However, the total flow of amino acids only ranged from 32.8 - 72.0 g/day, and the methionine available for absorption ranged from 0.8 - 2.5 g/day. In Expt. 2, the daily flow of amino acids into the duodenum on untreated silage was increased by both formaldehyde-treated casein and intraruminal infusion of energy (Table 4.2). Each treatment was also supplemented with a duodenal infusion of methionine. The range of flow of total amino acids and of the methionine available for absorption were both greater than for Expt. 1. i.e. 33.1 - 167.0g amino acids/day, and 0.8 - 6.0g methionine/day.

The quantity of amino acids absorbed from the intestines was not measured, but it was assumed that the amount absorbed would increase with increasing flow rates of amino acids into the duodenum. Armstrong (1973) did report a significant decrease in the apparent digestibility of methionine in the small intestine when intake was increased from

900 - 1400 g/day. However, the decrease in digestibility on ground and pelleted grass was only 7.5%, and thus the absolute amount absorbed would still have increased. Formaldehyde-binding to silage (Expt. 1) and casein (Expt. 2), appeared to be largely reversed prior to digestion in the intestines, since total intestinal N digestion was not adversely affected by the formaldehyde-treatment (Chapter 3, Table 3.13). During absorption of amino acids from the duodenum, some changes in concentration occur, due to the utilisation of amino acids by the gut tissue (Hume et al., 1972; Wolff et al., 1973). In addition, metabolism of amino acids by the liver, and uptake of amino acids by the individual tissues for protein synthesis, result in different amino acid concentrations in plasma taken from portal, jugular or carotid blood vessels (Reilly and Ford, 1971; Wolff et al., 1973). Jugular blood samples were taken in the present work, and so metabolism of amino acids in the tissues prior to measurement in jugular plasma will affect the concentrations relative to the amount absorbed.

Utilisation of amino acids by the tissues can be broadly divided into two categories.

(1) The first is protein synthesis, which requires a particular complement of amino acids, corresponding to the composition of the protein formed. The rate of protein synthesis will be limited by the availability of the amino acid which is present in the lowest concentration relative to the concentration required. Thus, an inadequate supply of one amino acid will limit the utilisation of others. Rate of tissue protein synthesis has also been found to vary in response to total amino acid availability (Munro, 1969; Clemens, 1972), and the actions of growth hormone, insulin and thyroxine (Munro, 1964; Cahill et al., 1964).

(2) The second category covers the requirements for individual amino acids for specific functions. Methionine is required as a donor of methyl groups and also for conversion to cysteine (Finkelstein and Mudd, 1967).

The factors controlling the distribution of specific amino acids between these functions have not yet been clearly defined. However, some of the dietary factors involved in the regulation of methionine metabolism have been studied. Increased availability of cyst(e)ine has been found to decrease conversion of methionine to cysteine (Shannon et al., 1972); low amino acid intake increased regeneration of methionine from homocysteine (Finkelstein and Mudd, 1967); and low methionine intake decreased the use of methionine as a methyl group donor (Finkelstein and Mudd, 1967). These studies were conducted on in vitro liver preparations from monogastric animals. The importance of cyst(e)ine in wool growth (Ross, 1961) would be expected to produce marked differences in S-amino acid metabolism in sheep, compared to monogastrics. However, the results quoted do emphasise that a number of factors influence methionine metabolism, and that our understanding of these factors is still incomplete.

In Expt. 1, plasma methionine concentration appeared to be increased by I/P methionine infusion, but not by formalin-treatment of silage (Table 4.3). Total plasma amino acids were lower on untreated silage plus methionine, than treated silage, while there was considerable variability in the values for untreated silage alone. These results suggest that the quantity of methionine available to the tissues on untreated silage was limiting tissue protein synthesis. Methionine infusion possibly stimulated protein synthesis by removing amino acids from the plasma.

In Expt. 2, neither the concentration of methionine, nor "total" amino acids in the plasma was affected by supplementation with energy or protein. However, plasma methionine was increased ($P < 0.01$) and "total" plasma amino acid concentration was decreased ($P < 0.01$) by duodenal methionine infusion, on all treatments. The decrease in "total" plasma amino acid concentrations on all treatments suggests that maximum tissue protein synthesis was still limited by methionine availability at 2.7g methione/day (+ protein, Table 4.2). The concentration of "total" amino acids in the plasma was not significantly affected by treatment despite a two-fold increase in the flow of amino acids into the duodenum. This suggests that the additional amino acids were either being efficiently utilised or rapidly deaminated.

The above results suggest that the availability of methionine was lower than that required for maximum tissue protein synthesis, even at the higher flow rates of amino acids into the duodenum. Barry et al. (1973) found no beneficial effect of I/P methionine supplementation of formalin-treated silage on intake or live-weight gain, but wool growth was increased. The high methionine requirement for wool growth by sheep, may increase their susceptibility to methionine imbalance, at low levels of amino acid availability. I/P injection of methionine to sheep fed untreated silage produced a greater increase in wool growth than formalin-treatment of silage, but formalin-treatment produced a greater increase in intake than methionine infusion (Barry et al., 1973). This suggests that feeding untreated silage to sheep may produce an imbalance situation which can be partially alleviated by methionine infusion. Amino acid balance can also be improved by increased availability of total amino acids. Thus, an imbalance situation is more likely to occur at low flow rates of total amino acids into the duodenum. This might indicate that the extra-protein require-

ment for methionine is proportionally higher, than at higher flow rates of total amino acids into the duodenum (Grau and Kamei, 1950), or alternatively that higher availability of total amino acids reduces the effect of a low methionine proportion. However, in Expt. 2, intake still decreased slightly, at higher flow rates of duodenal amino acids. This may suggest that the extra-protein requirements for methionine were higher for the sheep in Expt. 2 than in Expt. 1, where no intake response was recorded. This will be discussed later, in relation to N balance.

The plasma methionine concentrations on the basal silage diet were higher in Expt. 2 than Expt. 1. Since the decreased intake in Expt. 2 (Chapter 3) suggests that methionine was more limiting in Expt. 2 than Expt. 1, this difference may reflect either a greater adaptation to low methionine in Expt. 1, or a decrease in methionine loss with storage of plasma samples. This latter possibility arises from the time delay between collection and analysis of samples in Expt. 1 (1 year) compared to Expt. 2 (1 month). The "total" amino acid concentration in plasma also differed between the two experiments, but this was due to the omission of the basic amino acids in Expt. 2.

C. The effect of treatments on individual amino acid proportions in the plasma

The effect of treatments on the concentration of individual amino acids in the plasma was also measured (Table 4.4). Since plasma amino acid concentrations are readily affected by numerous nutritional and physiological factors (Swendseid et al., 1967; Synderman et al., 1968; Oltjen et al., 1969; Amos et al., 1972), few conclusions can be drawn from changes in individual plasma amino acid concentrations. However,

observations on the effect of increased methionine availability on the concentration of other plasma amino acids may give some indication of specific actions of methionine.

The differences in percentage concentrations of serine, alanine, cystathionine, methionine, leucine and isoleucine between replicates in Expt. 2 were significant. Taurine concentration was too low to be measured in the plasma samples from replicate 1, Expt. 2, but its concentration could be measured after four to seven days of methionine infusion. Taurine was present in the plasma of the sheep prior to methionine infusion in replicates 2 and 4. These results suggest that there was a carryover effect from the preceding methionine infusion periods. This effect appeared to be similar on all treatments, and thus it would have had no effect on the treatment comparison as analysed by the Analysis of Variance.

The percentages of cysteine, cystathionine and taurine were significantly increased by methionine infusion ($P < 0.01$). This suggests that considerable conversion of methionine to cysteine did take place. Cystathionine is an intermediary in the conversion of methionine to cysteine, and taurine is a product of the metabolism of cysteine (Chapter 1, Fig. 1.2). The plasma amino acid concentrations after one day of methionine infusion were also measured in Expt. 2 (Appendix 5). These were interesting in respect of the change in taurine concentration. In replicates 2 and 4, taurine was present prior to methionine infusion, but had disappeared from the sample taken 24h after the start of infusion. It reappeared after four days of infusion.

There was also a significant interaction between supplements and methionine infusion on the percentage of cysteine and serine in the plasma

($P < 0.05$). The decrease in serine percentage in response to methionine infusion, was less on the protein supplemented diet, than on the other two treatments. Since serine is required for the conversion of methionine to cysteine, this might suggest a decreased percentage conversion of methionine to cysteine at higher flows of duodenal amino acids. However, the plasma cysteine percentage gave a greater response to methionine infusion on the protein supplemented than the control diet. Reis et al. (1973) also reported decreased plasma serine concentrations, and increased taurine and cystathionine concentrations, in response to postruminal methionine infusion. Similar changes to those recorded for the other amino acids have also been reported (Synderman et al.,1968; Tao et al., 1974).

Supplementation with protein and energy (Expt. 2) had a significant effect on the percentage of threonine, glycine, valine, isoleucine, leucine and tyrosine ($P < 0.01$). These changes generally reflect the differences in duodenal amino acid proportions between treatments. However, the increases in valine, isoleucine and leucine are not related to duodenal amino acid flow. Since the main requirement for these amino acids is for the synthesis of tissue protein, these increases may indicate a change in the rate of tissue protein synthesis relative to amino acid availability, as duodenal amino acid flow rates increase.

The changes in the proportions of plasma amino acids with time after the start of methionine infusion were also measured (Table 4.5) in Expt. 2, Rep. 1. The greatest changes appeared to occur during the first two days of infusion, and the percentages were relatively constant after four days of infusion. This suggests therefore, that the period of six days adjustment to methionine infusion was of sufficient length.

D. Metabolism of methionine

Methionine metabolism was studied through the use of labelled methionine. Continuous infusion of L-methionine-C14 (U) over a period of 12h, enabled estimation of methionine turnover rate, the percentage of methionine oxidised to CO_2, the percentage of CO_2 derived from methionine and the size of the pool with which infused methionine was in equilibrium (Table 4.6). The I/P methionine infusion supplied an average of 1.2g methionine/day (i.e. 0.33mmole/h) more than the untreated silage alone. This infusion increased the methionine turnover rate by an average of 0.27 mmole/h. The treated silage provided an additional 0.6g/day (i.e. 0.17mmole/h) which increased methionine turnover by an average of 0.13mmole/h. These figures support the hypothesis that methionine absorption increased with both increased flow of amino acids into the duodenum, and infusion of methionine into the peritoneal cavity.

Estimation of the size of the methionine pool, from plasma methionine SAs, could only be made for seven of the sheep. However, the size of pool appeared to increase from 562 - 1026μmole, in response to I/P methionine infusion. In order to gain some understanding of the source of methionine contributing to this pool, estimates of the total plasma and muscle methionine were made, using data on body weight and plasma methionine concentration. The average value for total plasma methionine was 21μmoles. This suggests that the turnover rates measured (Table 4.6) did not refer solely to turnover of methionine in the plasma, but that an additional pool of methionine was also involved. The methionine pool size obtained from the data was only 0.1% of total muscle methionine (av. 578mmole). However, muscle appears to consist of both metabolically active and inactive amino acid pools (Munro,

1964). Hence, the methionine pool to which the turnover rate applies, may be composed of plasma methionine, liver methionine and/or free muscle methionine.

The percentage of methionine which was oxidised to CO_2 was also measured, and was increased by I/P infusion of methionine. This may indicate either that methionine was being supplied in excess of requirement, or that utilisation of methionine for non-protein functions was increased with increased methionine availability. During the conversion of methionine to cysteine, the carboxyl moiety is converted into α-ketobutyric acid, which may then be oxidised and $^{14}CO_2$ expired. Aguilar et al. (1974) reported changes in the metabolism of methionine by rats with increasing percentage of methionine in the diet. They observed that incorporation of the methyl group of methionine into proteins increased as methionine increased, while incorporation of the carboxyl group remained unchanged. Thus, oxidation of the carboxyl group increased more rapidly than oxidation of the methyl group which was incorporated into other amino acids and hence into proteins.

The percentage of CO_2 derived from methionine also increased with I/P infusion. The average for all treatments was 0.1% at a turnover rate of 0.01g/h. Corresponding values for glucose (Bergman et al., 1963) were a turnover rate of 4g/h giving a percentage of CO_2 derived from glucose of 10%. Thus, oxidation of methionine makes a comparatively minor contribution to total oxidation in the sheep.

E. Overall N metabolism

The changes in the rate of tissue protein synthesis which have already been discussed, will also affect daily N retention by the sheep. In Expt. 1, formalin-treatment of silage resulted in net addition of N

to the rumen, compared to net absorption on untreated silage (Chapter 3, Table 3.9). Thus, the balance of amino acids absorbed, to ammonia absorbed would have been higher on the treated than untreated silage. Hence, urinary-N appeared to decrease in sheep fed untreated compared to treated silage. However, N retention and plasma urea concentration did not show any consistent treatment difference (Table 4.7).

In Expt. 2, urinary-N was significantly increased by supplementation with formaldehyde-treated casein over untreated silage alone. This was possibly due to increased digestion of N in the stomach region in response to the protein supplement (Chapter 3, Table 3.9). In addition, the utilisation of absorbed amino acid-N is limited by its balance of amino acids, relative to the proportions required for tissue protein synthesis. The effect of methionine infusion in increasing tissue protein synthesis has already been discussed. Thus the decrease in urinary-N in response to methionine infusion, can be attributed to increased efficiency of utilisation of amino acids for tissue protein synthesis. Urinary-N on the protein supplemented diet was only slightly decreased by duodenal methionine infusion, but N intake had increased on all treatments. N balance was significantly increased by both protein supplementation ($P < 0.05$) and by methionine infusion ($P < 0.01$). This result gives further evidence that tissue protein synthesis was increased by both increased total amino acid, and methionine, availability.

Energy supplementation had no significant effect on N balance or urinary-N, despite an increased flow of amino acids into the duodenum. This may reflect decreased absorption of ammonia from the rumen, due to more efficient utilisation of NPN in the rumen (Chapter 3).

There were no significant differences in plasma urea concentration. This result suggests that plasma urea concentration is not a sufficiently sensitive parameter for assessing the N status of ruminants. This was reinforced by daily measurement of plasma urea concentration after the start of methionine infusion. The effect of duration of methionine infusion on the percentage concentration of amino acids in the plasma has already been discussed (Table 4.5) and these showed a gradual change with time after the start of infusion. However, plasma urea concentrations did not show any consistent trends.

F. Requirements for methionine

One of the aims of these experiments was to estimate methionine requirement for the sheep. The parameters to be used for this estimation were:

(a) N balance,

(b) plasma methionine,

(c) oxidation of methionine to CO_2.

These parameters have been used to estimate amino acid requirements in non-ruminants (Zimmerman and Scott, 1965; Neale and Waterlow, 1974) by plotting increasing amino acid intake versus response. These "response curves" generally take the form of two intersecting lines, with the point of intersection being designated as the optimum requirement. Above this point, an increased response in terms of N balance requires a greater increment in methionine intake, than the same size of increase below the point of intersection. However, the results reported here for ruminants, emphasise the limits of such methods for estimating methionine requirement by highlighting two factors in particular, which should be defined for any estimation of requirement.

(1) Physiological state: A comparison of the daily N retention between Expt. 1 and 2, showed a marked difference in the quantity of N retained at low levels of methionine availability. This may be seen from Figs. 4.3 and 4.4. In Expt. 1, duodenal flow rates of 47 and 60 mg methionine /$kg^{.75}$/day, gave N balances of −1.2 and +1.6g/day respectively. In Expt. 2, the declining intake of the control silage in reps. 1 and 3, resulted in markedly different intakes during collection of faeces and urine samples for balance measurements, compared to intakes during collection of duodenal digesta samples for flow measurements. However, intakes in rep. 5 were similar during both periods, and a flow rate of 97mg/$kg^{.75}$/day gave a value of −1.2g N retained/day. Such differences between experiments may indicate that the sheep in Expt. 2 required more methionine to reach a positive N balance, than the sheep in Expt. 1. This possibility could explain the observed intake response in Expt. 2, while no such response was apparent in Expt. 1. A higher requirement for methionine would have been more likely to result in an imbalance situation.

No definite evidence for a higher methionine requirement in Expt. 2 is available, but two factors which could have contributed, will be considered. The first is the season of the year during which the experiments were conducted, in relation to wool growth. Expt. 1 was conducted from May to September, a period of relatively ~~high~~ low wool growth, and Expt. 2 from September to January, a period of relatively ~~low~~ high wool growth (Ryder and Stephenson, 1968). Since wool growth has a high requirement for cyst(e)ine (Ross, 1961), the requirement for methionine for conversion to cysteine (at a constant cysteine availability) would be expected to increase.

A second factor which could have influenced requirement between experiments, was a difference in the previous nutritional history of the

animals. The sheep in Expt. 2 were subjected to considerable nutritional deprivation six weeks prior to the start of the experiment. This was caused by the sheep refusing to eat an alternative batch of silage. Six sheep were offered this particular silage, and all intakes decreased rapidly for two weeks. However, this same silage was again offered to three of these sheep after six months of good nutrition. Two of the sheep maintained an intake of greater than 600g DM/day over a period of several weeks. The intake of the third sheep, and of an additional sheep decreased, but in the first case was restored by duodenal methionine infusion, and in the second by daily supplementation with formaldehyde-treated casein, given into the rumen. These results, together with the significant differences between animals in voluntary intake in Expt. 2, suggest that the previous nutritional history of individual animals, with respect to protein status, will have a marked effect on the response of the sheep to low methionine availability.

(2) Availability of other amino acids: During the discussion of results in this chapter, reference has frequently been made to the effect of the availability of other amino acids on methionine requirement. The balance in the utilisation of methionine for tissue protein synthesis, or extra-protein functions has been shown to change with increasing intake of amino acids and methionine for monogastrics (Grau and Kamei, 195 ; Finkelstein and Mudd, 1967; Shannon et al., 1972; Aguilar et al., 1974). While few data on methionine metabolism in ruminants are available, a similar situation appears to exist. Hence a point of intersection in a response curve, may simply indicate a change in the function for which methionine is being utilised. In this respect, different requirements have been predicted from different parameters in the same experiment (Tao et al., 1974).

The "response curves" (Figs. 4.3 - 4.7), for N balance, plasma methionine and percentage oxidation of methionine to CO_2, with increasing levels of duodenal methionine per unit of metabolic body weight, show that these parameters did respond to increased methionine availability. However, no clearcut points of intersection were detected. In particular, response in the percentage of methionine oxidised to CO_2 appeared to be linear. A similar result for rats was reported by Aguilar et al. (1972). Aguilar et al. (1974) suggested that some feature of the metabolism of methionine affected oxidation, and thus prevented the presence of a clearly defined point of intersection.

The level and type of production response should also be considered as a criterion with which to define requirement. These points are emphasised by the wide range of methionine requirements reported in the literature, from 105 - 125 mg methionine entering the duodenum per unit of metabolic live-weight per day (Wakeling et al., 1970), to 207 mg supplemental methionine per unit of metabolic live-weight per day (Reis et al., 1973). Thus, the methionine requirement of sheep appears to be affected by a variety of factors which should be defined for any estimate of requirement.

GENERAL DISCUSSION

The amino acid status of sheep fed silage appears to be an important factor in regulating voluntary intake. Thus, Expt. 1 was undertaken to compare the effects of formalin-treatment of silage, and I/P methionine infusion, on the digestion and utilisation of silage by sheep. In Expt. 2, two alternative methods of increasing duodenal amino acid flow were studied. In addition, each of the treatments was supplemented with duodenal methionine, to compare the utilisation of methionine at varying levels of duodenal amino acid flow.

The main differences in chemical composition between silage and herbage, are the low water soluble carbohydrate content, and high proportion of NPN in the total N fraction. These differences will combine to limit the flow of amino acids into the duodenum, since readily available energy is required for efficient utilisation of NPN by rumen micro-organisms (Belasco, 1956; Bloomfield et al., 1958; Henderickx and Martin, 1963). The three treatments; formalin-treatment of silage (Expt. 1), formaldehyde-treated casein and energy supplements (Expt. 2), all increased the flow of amino acids into the duodenum, to a maximum of 167g/day. A comparison of the data from both experiments indicated that amino acid intake accounted for 67% of the variation in duodenal amino acid flow. Hence, the degree of amino acid degradation during ensiling, appears to be a key factor in determining its efficiency of utilisation. Formalin-treatment of silage prevented the degradation of protein and amino acids in the silage stack (Barry and Fennessy, 1972).

A comparison of the DM intake required to supply 100 g amino acids/day, entering the duodenum, on silage, compared to other diets, indicated the need for a relatively high intake of silage to maintain this flow. In Expt. 2, intake of the control silage decreased with

time. This decrease was prevented by both the formaldehyde-treated casein and energy supplements. The formaldehyde-treated casein was largely protected from degradation in the rumen, but appeared to be digested in the intestines. Supplementation with energy also increased the flow of amino acids into the duodenum, by increasing the conversion of NPN to protein within the rumen. Hence, the amino acid status of the sheep was increased by these two treatments, and intake was maintained. Thus, the utilisation of silages with a low amino acid content may be improved by supplementation with "protected-protein" or a source of readily available energy.

The decrease in intake in Expt. 2 was reversed by duodenal supplementation with methionine, suggesting that silage-fed sheep may be in an amino acid imbalance with respect to methionine. N balance was significantly increased by duodenal methionine and total plasma amino acid concentration was decreased. Thus, the availability of methionine appeared to limit tissue protein synthesis. However, N balance at the same level of methionine availability was higher in Expt. 1, compared to Expt. 2. This suggests that a higher proportion of the available methionine was used for tissue protein synthesis in Expt. 1. Since intake was maintained on all treatments in Expt. 1, but decreased on the control silage in Expt. 2, the susceptibility of sheep to methionine imbalance appears to depend on the rate of tissue protein synthesis in the animal. Intake was also partially maintained by the two supplements in Expt. 2, and hence methionine imbalance appears to be more severe at lower levels of duodenal amino acids.

Hence, the particular problem of silage, in having low amino acid, and readily available energy contents, appears to limit the flow of amino acids into the duodenum. Low availability of amino acids to the

tissues, may then result in a state of methionine imbalance, which in turn may limit intake. The susceptibility of sheep to methionine imbalance, depends on the protein status of individual animals.

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APPENDICES

APPENDIX 1

Silage DM intakes - Expt. 2.

A. Mean daily intake during seven day balance period (gDM/day).

Treatment	Sheep numbers					
	No methionine			+ methionine		
	821	1157	1303	821	1157	1303
Control	443	366	637	409	597	675
+ protein	648	608	696	688	663	701
+ energy	611	629	652	594	667	681

B. Mean daily intake during three day collection of duodenal digesta samples (gDM/day).

Treatment	Sheep numbers					
	No methionine			+ methionine		
	821	1157	1303	821	1157	1303
Control	335	293	610	515	559	688
+ protein	610	641	701	660	654	701
+ energy	461	587	679	572	678	691

APPENDIX 2

Analyses of Variance - Expt. 2

Source:

Replicates	-	Rep.
Treatment	-	Trtmt.
Methionine	-	Met.
Treatment x Met Interaction	-	T x M
Animals	-	Ans.
Error	-	Err.

Mean Square - MS

Significance - Sig.

**	-	$p < 0.01$
*	-	$p < 0.05$
NS	-	Not significant

	Rep.	Trtmt.	Met.	T x M	Ans.	Err.
Silage DM Intake						
MS	7016.99	45453.50	35823.22	9188.47	30045.50	2662.81
Sig.	NS	**	*	NS	**	
N Intake						
MS	3.28	155.74	22.22	2.84	22.55	3.03
Sig.	NS	**	*	NS	*	
AA Intake						
MS	85.2	5612.4	555.6	102.7	453.7	38.0
Sig.	NS	**	**	NS	**	
Total rumen VFA concentration						
MS	93.46	205.02	-	-	238.64	3.37
Sig.	**	**	-	-	**	
Molar proportion of acetate						
MS	2.55	29.30	-	-	11.42	18.46
Sig.	NS	NS	-	-	NS	
Molar proportion of propionate						
MS	5.35	0.22	-	-	7.66	7.66
Sig.	NS	NS	-	-	NS	
Molar proportion of butyrate						
MS	0.66	9.91	-	-	1.05	4.20
Sig.	NS	NS	-	-	NS	
NH_3-N concentration in rumen						
MS	28.69	1.93	-	-	82.34	1.69
Sig.	**	NS	-	-	**	

	Rep.	Trtmt.	Met.	T x M	Ans.	Err.
% OM digested in stomach						
MS	32.06	0.72	37.56	68.06	142.72	54.39
Sig.	NS	NS	NS	NS	NS	
% energy digested in stomach						
MS	19.90	0.87	120.12	118.40	261.31	45.49
Sig.	NS	NS	NS	NS	NS	
% N digested in stomach						
MS	309.93	915.54	54.08	387.92	1494.29	175.60
Sig.	NS	*	NS	NS	*	
Non-amino acid-N entering the duodenum						
MS	0.3	9.1	9.3	1.8	3.6	0.72
Sig.	NS	**	**	NS	NS	
Non-amino acid-N intake						
MS	0.23	0.70	1.30	0.37	1.23	0.13
Sig.	NS	*	**	NS	**	
Ratio of duodenal NAA-N to NAA-N intake						
MS	0.02	0.82	0.23	0.25	0.23	0.07
Sig.	NS	**	NS	NS	NS	
Amino acids entering the duodenum						
MS	531.85	5978.77	1265.05	73.83	570.37	212.78
Sig.	NS	**	NS	NS	NS	
Ratio of duodenal AA to AA intake						
MS	0.52	0.20	0.002	0.08	0.23	0.013
Sig.	NS	**	NS	*	**	
Ratio of duodenal AA to N intake						
MS	0.62	3.38	0.00	0.88	5.18	0.67
Sig.	NS	NS	NS	NS	*	
N entering the duodenum						
MS	11.29	97.15	70.41	3.97	4.31	5.07
Sig.	NS	**	**	NS	NS	
AA as % total N entering the duodenum						
MS	6.67	664.73	91.58	4.00	161.34	10.55
Sig.	NS	**	*	NS	**	

	Rep.	Trtmt.	Met.	T x M	Ans.	Err.
Duodenal amino acids (expressed as a % of the total)						
Arg						
MS	0.062	0.318	0.014	0.040	0.004	0.024
Sig.	NS	**	NS	NS	NS	
His						
MS	0.174	0.051	0.161	0.091	0.027	0.112
Sig.	NS	NS	NS	NS	NS	
Ile						
MS	0.346	0.151	0.245	0.045	0.016	0.043
Sig.	*	NS	NS	NS	NS	
Leu						
MS	0.109	0.361	0.000	0.117	0.073	0.267
Sig.	NS	NS	NS	NS	NS	
Lys						
MS	0.196	0.111	0.094	0.011	0.389	0.044
Sig.	NS	NS	NS	NS	*	
Met						
MS	0.082	0.027	0.014	0.001	0.004	0.011
Sig.	*	NS	NS	NS	NS	
Phe						
MS	0.142	0.641	0.014	0.027	0.394	0.053
Sig.	NS	**	NS	NS	*	
Thr						
MS	0.202	1.684	0.347	0.007	0.144	0.148
Sig.	NS	**	NS	NS	NS	
Val						
MS	0.813	0.017	0.005	0.012	0.091	0.044
Sig.	**	NS	NS	NS	NS	
Ala						
MS	0.651	7.104	0.005	0.245	0.517	0.096
Sig.	*	**	NS	NS	*	
Asp						
MS	0.351	8.376	0.180	0.027	0.336	0.261
Sig.	NS	**	NS	NS	NS	
Glu						
MS	0.317	50.616	0.027	0.136	0.517	0.228
Sig.	NS	**	NS	NS	NS	

	Rep.	Trtmt.	Met.	T x M	Ans.	Err.
Gly						
MS	0.046	7.835	0.094	0.011	1.732	0.199
Sig.	NS	**	NS	NS	*	
Pro						
MS	0.598	12.494	1.125	0.012	1.941	0.169
Sig.	NS	**	*	NS	**	
Ser						
MS	0.202	0.096	0.142	0.069	0.037	0.008
Sig.	**	**	**	*	NS	
Tyrosine						
MS	0.602	0.204	0.109	0.261	0.121	0.137
Sig.	NS	NS	NS	NS	NS	
% DM intake digested in intestines						
MS	45.59	23.53	85.83	216.25	464.8	56.8
Sig.	NS	NS	NS	NS	*	
% OM intake digested in intestines						
MS	36.75	64.15	83.20	79.75	783.65	19.85
Sig.	NS	NS	NS	NS	**	
% energy intake digested in intestines						
MS	22.93	55.89	284.78	73.62	269.39	60.61
Sig.	NS	NS	NS	NS	NS	
% N intake digested in intestines						
MS	117.42	463.75	70.47	163.31	544.22	340.70
Sig.	NS	NS	NS	NS	NS	
% duodenal N digested in intestines						
MS	15.40	78.50	58.64	54.69	89.45	28.77
Sig.	NS	NS	NS	NS	NS	
Apparent digestibility of DM						
MS	0.620	24.712	9.680	2.565	2.007	5.362
Sig.	NS	NS	NS	NS	NS	
Apparent digestibility of OM						
MS	3.407	23.924	3.309	0.211	0.091	2.037
Sig.	NS	**	NS	NS	NS	
Apparent digestibility of energy						
MS	2.08	14.39	10.89	2.44	0.77	3.92
Sig.	NS	NS	NS	NS	NS	

	Rep.	Trtmt.	Met.	T x M	Ans.	Err.
Apparent digestibility of N						
MS	0.367	126.34	16.63	3.57	1.24	9.36
Sig.	NS	**	NS	NS	NS	
N balance						
MS	7.46	18.93	25.92	0.46	0.68	2.67
Sig.	NS	*	**	NS	NS	
Met available for absorption						
MS	0.53	4.01	22.98	0.06	0.40	0.19
Sig.	NS	**	**	NS	NS	
% Met in total duodenal AA						
MS	0.599	0.643	21.56	1.55	0.14	0.14
Sig.	NS	NS	**	**	NS	
Plasma met concentration						
MS	147.83	10.45	1521.68	10.38	91.93	20.40
Sig.	*	NS	**	NS	NS	
Total plasma amino acid concentration						
MS	52751.53	39103.69	1144947.36	15996.77	89478.80	25968.51
Sig.	NS	NS	**	NS	NS	
% met in total plasma amino acids						
MS	0.862	0.124	9.976	0.041	0.644	0.109
Sig.	*	NS	**	NS	*	
% individual amino acids in total plasma amino acids						
Taurine						
MS	3.68	3.00	20.89	5.62	3.29	1.45
Sig.	NS	NS	**	NS	NS	-
Thr						
MS	0.73	5.48	16.44	0.76	0.17	0.25
Sig.	NS	**	**	NS	NS	-
Ser						
MS	4.75	1.18	21.78	2.77	2.89	0.38
Sig.	**	NS	**	*	*	-
Pro						
MS	0.66	32.49	0.16	1.64	0.37	0.75
Sig.	NS	**	NS	NS	NS	-
Glu						
MS	7.72	4.04	17.80	3.47	11.49	9.49
Sig.	NS	NS	NS	NS	NS	-

	Rep.	Trtmt.	Met.	T x M	Ans.	Err.
Citrulline						
MS	1.34	0.37	21.34	0.44	6.25	0.92
Sig.	NS	NS	**	NS	*	
Gly						
MS	25.20	201.71	273.00	9.00	49.35	13.60
Sig.	NS	**	**	NS	NS	
Ala						
MS	13.17	7.22	18.61	3.47	5.47	1.56
Sig.	*	NS	*	NS	NS	
Val						
MS	0.48	29.27	8.82	0.85	2.29	0.50
Sig.	NS	**	**	NS	NS	
Cys						
MS	1.37	0.06	0.84	0.64	0.12	0.06
Sig.	**	NS	**	*	NS	
Cystathionine						
MS	0.09	0.02	0.97	0.01	0.04	0.01
Sig.	NS	NS	**	NS	NS	
Ile						
MS	0.50	0.63	0.03	0.04	0.30	0.03
Sig.	**	**	NS	NS	**	
Leu						
MS	0.40	3.91	3.29	0.08	1.01	0.04
Sig.	**	**	**	NS	**	
Tyr						
MS	0.50	3.62	5.23	0.12	0.76	0.29
Sig.	NS	**	**	NS	NS	
Phe						
MS	0.13	0.66	4.81	0.10	0.07	0.17
Sig.	NS	NS	**	NS	NS	
Urinary-N						
MS	1.74	28.36	11.68	2.79	3.47	0.78
Sig.	NS	**	**	NS	NS	
Plasma urea concentration						
MS	4.84	23.22	2.83	0.28	7.00	5.29
Sig.	NS	NS	NS	NS	NS	

APPENDIX 3

SA of plasma methionine – Expt. 1

A. Untreated

		Rep. 1	Rep. 2	Rep. 3
Time (h):	0	0	0	0
	0.08	4,093	1,917	441
	0.25	9,799	–	–
	0.50	–	4,168	3,087
	1	15,668	7,373	5,451
	1.5	14,455	–	6,080
	2	14,230	–	8,903
	2.5	17,433	9,516	–
	3	16,792	9,877	–
	3.5	14,013	7,852	6,227
	4	14,651	11,466	6,598
	5	–	–	–
	6	–	10,077	6,752
	7	7,543	7,613	9,197
	8	–	9,967	8,755
	9	–	11,132	9,373
	10	11,938	12,335	–
	11	16,310	–	8,718
	12	16,545	–	7,281
Plateau value (dpm/μmole)		15,957	11,164	8,665
Infusion rate (dpm/h)		6,899,040	6,068,040	4,755,240
~~Turnover~~ Rate constant (h^{-1})		–	1.14	0.85

The turnover constant for Rep. 1 was not calculated, owing to the low correlation coefficient (0.54).

B. Treated

Time (h):	Rep. 1	Rep. 2	Rep. 3
0	0	0	0
0.08	–	848	161
0.25	–	4,204	1,219
0.5	6,147	5,890	2,112
1	7,348	6,617	3,759
1.5	9,330	7,665	–
2	13,443	9,286	–
2.5	10,255	11,071	6,797
3	16,479	–	4,240
3.5	–	–	6,081
4	16,520	7,928	–
5	18,008	–	6,676
6	–	7,921	–
7	11,613	7,679	9,223
8	–	–	6,459
9	10,283	–	6,987
10	11,764	6,591	–
11	–	–	7,789
12	14,303	–	8,562
14	10,884	–	–
18	11,958	–	–
19	18,900	–	–
20	12,756	–	–
22	14,206	–	–
24	19,327	–	–
Plateau value (dpm/μmole)	11,769	7,845	7,092
Infusion rate (dpm/h)	6,206,110	5,230,500	5,127,390
~~Turnover~~ Rate constant (h^{-1})	0.84	2.74	0.77

C. Untreated + met

	Rep. 1	Rep. 2	Rep. 3
Time (h): 0	0	0	0
0.08	-	332	777
0.25	4,540	1,537	-
0.5	-	4,141	-
1	9,159	4,523	-
1.5	-	5,187	3,886
2	-	5,260	4,594
2.5	11,105	6,653	4,140
3	10,317	-	3,142
3.5	10,280	-	4,422
4	11,725	-	-
5	10,451	10,294	-
6	11,471	6,664	5,378
7	11,800	5,784	-
8	11,999	7,982	6,801
9	9,514	6,058	6,125
10	-	9,188	4,996
11	9,179	-	5,521
12	-	6,021	3,797
Plateau value (dpm/μmole)	10,793	6,527	5,436
Infusion rate (dpm/h)	7,354,750	5,614,620	4,309,440
~~Turnover~~ Rate constant (h^{-1})	-	0.79	0.82

The turnover constant for Rep. 1 was not calculated, owing to the limited number of data available.

APPENDIX 4

Plasma amino acid concentrations (as a % of total plasma amino acids) after 1 day of methionine infusion - Expt. 2

	Control	+ Protein	+ Energy
Taurine	Tr.	Tr.	Tr.
Thr	2.8	4.4	2.5
Ser	6.3	6.4	6.1
Pro	3.3	5.4	3.4
Glu	4.9	5.4	4.4
Citrulline	5.3	5.6	5.3
Gly	49.4	36.7	51.7
Ala	10.1	9.6	9.6
Val	5.3	8.9	5.5
Cystathionine	0.5	Tr.	Tr.
Met	1.8	2.1	1.7
Ile	2.4	3.4	2.6
Leu	3.2	5.0	2.7
Tyr	2.8	4.5	2.7
Phe	2.0	2.6	1.7
Total (μmole/l)	1449	1424	1957