THE ENDOGENOUS PROTEIN CONTENT OF RUMINANT PROXIMAL DUODENAL DIGESTA

by

Christopher G. Bartram, B.Sc.

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

University of Nottingham
Faculty of Agricultural Science
Department of Applied Biochemistry and Food Science
Sutton Bonington
Loughborough, Leicestershire
LE12 5RD

May 1987

ABSTRACT

Protein arriving at the ruminant proximal duodenum consists of microbial protein, undegraded feed protein and endogenous protein. In this study, endogenous protein is defined as that fraction of the digesta derived from the animal itself (e.g. enzymes, plasma proteins, sloughed cells and mucus), not including any endogenous protein which may have been incorporated into the microorganisms.

Recent feeding schemes (e.g. ARC 1980, 1984) require an accurate value of the degradability of feed in the rumen. When degradability is determined in vivo failure to account for a quantity of endogenous protein in the proximal duodenal digesta results in an underestimate of the degradability of a ration. Direct estimates of the endogenous protein content of proximal duodenal digesta are therefore required. This thesis describes the development of a method to do this and its application to sheep and cattle.

The approach adopted, based on the concept of isotope dilution, involved a continuous intravenous infusion of L-[4,5-^{3}H]-leucine. This resulted in all body protein becoming labelled. Any label detected in the duodenal digesta must therefore be derived from the animal itself. A comparison of the specific activity of duodenal digesta with that of suitable precursor proteins provided an estimate of the proportion of duodenal digesta of endogenous origin.

Interestingly, the bacterial fraction of duodenal digesta was also labelled. This indicated that bacteria were utilising an endogenous source of leucine and circumstantial evidence suggested that this was derived largely from the rumen epithelium.

The validity of the L-[4,5-^{3}H]-leucine technique was investigated using three sheep fed an essentially protein free diet. Values of endogenous protein flow derived via the proposed technique (2.6 $\pm$ 1.00 g N/day) were compared with those calculated by difference (2.1 $\pm$ 0.92 g N/day).

A possible dietary influence on the endogenous protein content of ruminant proximal duodenal digesta was examined. An estimate of 2.6 ± 0.58 g N/day was derived for three sheep fed a concentrate diet and 2.1 ± 0.22 g N/day for three sheep fed long hay. Thus, contrary to previous suggestions, no significant dietary effect was observed. Possible reasons for this are discussed.

The proposed technique was also applied to derive estimates in cattle. A value of 11.3 ± 1.73 g N/day was obtained for four steers (170 kg) fed silage supplemented with fishmeal.

The continuous intravenous infusion of L-[4,5-^{3}H]-leucine method is the first technique to provide a direct determination of the endogenous protein component of ruminant proximal duodenal digesta which can be applied to any dietary regime and used in sheep and cattle.

ACKNOWLEDGEMENTS

First, I would like to thank Professor Peter J. Buttery for his guidance and encouragement during the preparation of this thesis and for his advice throughout my university education.

In addition, the invaluable surgical expertise of Dr W. Haresign and Ron Walker is gratefully acknowledged, as is the enthusiastic assistance of Dr S.E. Harding with the evaluation of curve fitting routines and general mathematical advice.

I am very grateful to John B. Soar for cannulation of the animals and analytical expertise, Judi Simpson and Chris Freeman for their dedicated care of the animals, Dr Jane Harper and Jim Craigon for statistical advice, and Christine Essex for the excellent preparation of the figures.

Sincere thanks to all members of the Department of Applied Biochemistry and Food Science for their continual co-operation. In particular Dr Janet Dawson, Dr Chris Bruce, Dr Michael Marsden and John Newbold for numerous useful discussions; Christine Palmer, David Bozon and David Howson for their technical assistance, and Dr P.M. Sharpe, Mike Lammiman, Henri, Akho and Ogi for their help during experimental periods.

Many thanks also to Derek Gale, AGRI, and Dr P. Harvey, University of Nottingham, Department of Geology, for analytical assistance.

In addition I would like to express my gratitude to John Whitehead for his care and professionalism in the printing of this thesis.

The funding of the AFRC is gratefully acknowledged.

Finally, I am indebted to Carolyn for her patience, tolerance and understanding, and to my parents for their continual support and encouragement.

CONTENTS

Chapter Two MATERIALS AND METHODS

EXPERIMENTAL PROCEDURES AND RESULTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

Abbreviations

ADAS	Agricultural Development and Advisory Service
AGRI	Animal and Grassland Research Institute
AOAC	Association of Official Analytical Chemists
ARC	Agricultural Research Council
ATP	Adenosine triphosphate
BW	Bodyweight
CTAB	Cetyltrimethyl ammonium bromide
DAPA	Diaminopimelic acid
df	Degrees of freedom
DCP	Digestible crude protein
DM	Dry matter
dpm	Disintegrations per minute
DW	Distilled water
EDTA	Diaminoethanetetra-acetic acid
FR	Fluid rich
IAEA	International Atomic Energy Agency
IU	International units
k corr	Rate constant of corrected digesta specific activity profile
k sal	Rate constant of saliva specific activity profile
k pla	Rate constant of plasma protein specific activity profile
L	Litre
M	Molar
MAD	Modified acid detergent
MAFF	Ministry of Agriculture Fisheries and Food
ME	Metabolisable energy

Abbreviations (continued)

N	Nitrogen
N/A	Not applicable
NAN	Non- ammonia nitrogen
NH_3.N	Ammonia nitrogen
NS	Not significant
OMADR	Organic matter apparently digested in the rumen
OMTDR	Organic matter truly digested in the rumen
PR	Particle rich
Q	Flux
R	Reconstitution factor
RDP	Rumen degradable protein
SED	Standard error of the difference
SEM	Standard error of the mean
SG	Specific gravity
Sp. Activity	Specific activity
SRF	Strained rumen fluid
TCA	Trichloroacetic acid
TD	True digesta
TEA	B-2-Thienyl-D,L-alanine
UDP	Undegradable dietary protein
VFAs	Volatile fatty acids
v/v	volume : volume
w/v	weight : volume
XRFS	X-ray fluorescence spectrophotometry

Chapter One

INTRODUCTION

1.1 Preamble

Ruminants have the ability due to their symbiotic relationship with rumen microorganisms to convert low quality protein sources and non-protein nitrogen compounds in their feed to high quality microbial protein, which is subsequently utilised by the animal. As a result the ruminant can consume dietary constituents unsuitable for inclusion in the rations of non-ruminants and therefore they do not need to compete with humans or non-ruminants for protein.

The design of a feeding scheme which operates with maximum efficiency requires the identification of the requirements of an animal and the matching of those requirements with the specifications of available feedstuffs.

In order to be able to achieve this objective for ruminant species a thorough understanding of rumen dynamics, encompassing biochemical, nutritional and physiological parameters is required. This is in addition to the need for a full understanding of digestive processes in the "stomach" and intestines. This added complexity compared with non-ruminants means it has not yet been possible to feed ruminants to particular amino acid requirements, although this has frequently been suggested as a likely conceptual development (Buttery, 1977; MacRae and Reeds, 1980; Thomas, 1986).

One of the needs to move towards this goal is to be able to define accurately the degradability of a feed in the rumen. To do this requires an accurate method of determining the quantity of endogenous protein in the proximal duodenal digesta. This thesis describes the development of a method to do this and its application to sheep and cattle.

1.2 Nitrogen metabolism in the rumen

1.2.1 Historical perspective

The metabolism of nitrogen in the rumen is a complex interactive process dependent on many factors including the dietary regime and the overall physiological status of the animal. Very significant developments have been made in the subject during the past thirty-five years.

The classical studies of McDonald (1948, 1952, 1954) indicated that dietary protein was modified in the rumen and Loosli et al. (1949) showed that microbial protein was able to meet the requirements of the host. Annison and Lewis (1959) elucidated the main features of the nitrogen cycle in ruminants. The requirement for quantitative data was soon realised and the use of fistulated animals, digesta flow measurements (Faichney, 1975; MacRae, 1974, 1975) and techniques to estimate the size of the microbial component in duodenal digesta were subsequently developed: e.g. Hogan and Weston (1967); Hutton et al. (1971); Smith and McAllan (1970, 1971); Roberts and Miller (1969); Beever et al. (1974).

The application of isotope tracer kinetics, in particular the use of ^{15}N (Nolan, 1975), provided detailed quantitative information on nitrogen metabolism in the ruminant and included estimates of nitrogen recycling.

The development of dynamic computer models (e.g. Black et al., 1981) has provided a conceptual framework within which all the determinants of metabolism and function can be accommodated simultaneously. In order to achieve significant advancement it is necessary to first appreciate the complexity of the animal system. The next logical step is to develop further dynamic models representative of a particular situation and identify additional areas of research of potential importance.

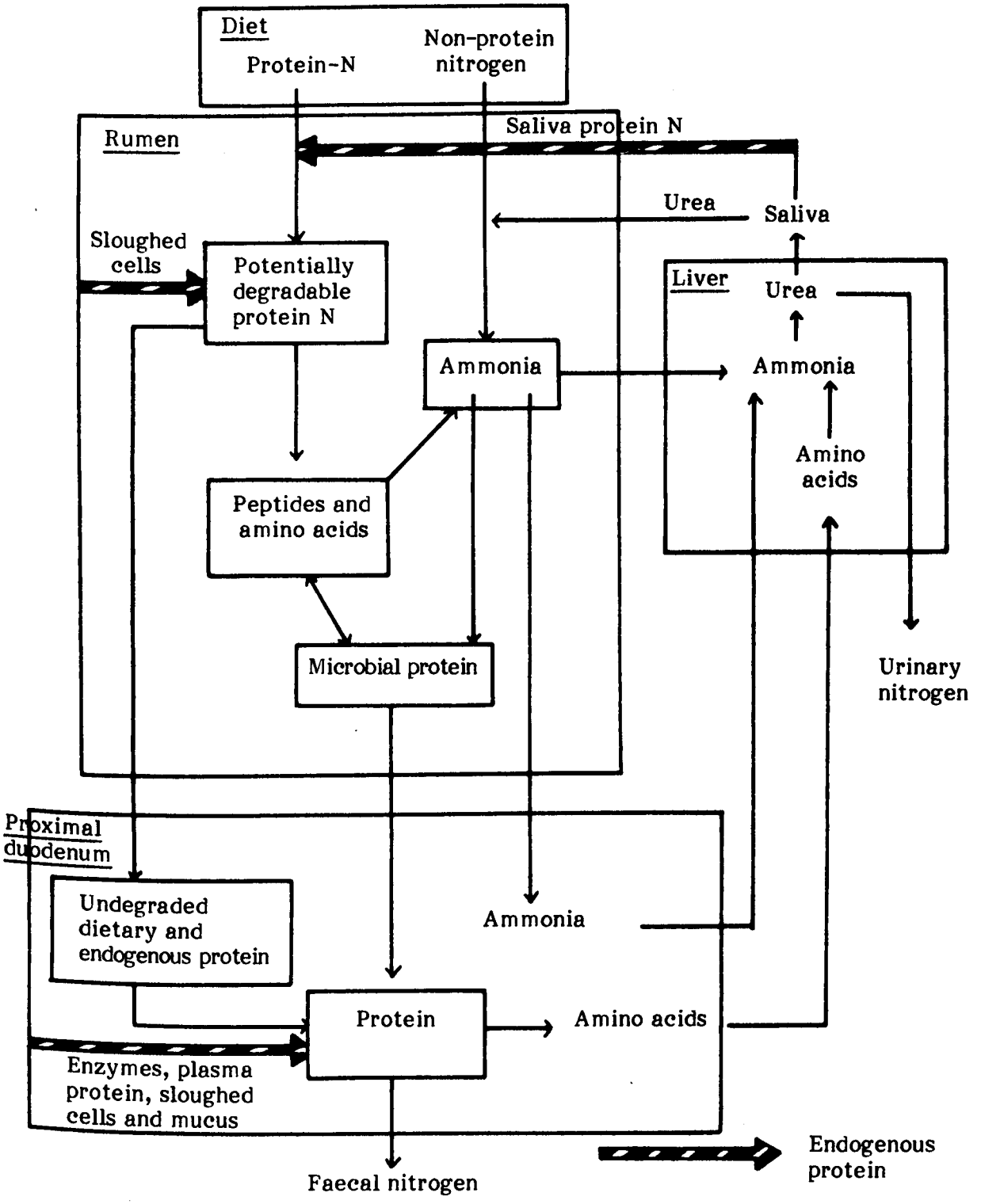
1.2.2 Nitrogen metabolism

A simple scheme of nitrogen metabolism in the ruminant is presented in Fig. 1, indicating the origins of endogenous protein nitrogen inputs arriving at the proximal duodenum.

The normal major fraction of dietary nitrogen is protein but a non-protein nitrogen component including urea, amides, amines, nitrate and nucleic acids may be present in significant quantities in certain conditions. The potentially degradable dietary nitrogen and endogenous nitrogen inputs to the rumen via the saliva and rumen wall are subject to action by bacterial proteases and peptidases resulting in the formation of peptides and free amino acids. The peptides and free amino acids are then available for incorporation into microbial nitrogen, and 20-50% of the nitrogen in bacteria may originate from this pool (Nolan, 1975). Alternatively, peptides and amino acids may be converted to ammonia and could subsequently be reutilised along with ammonia derived directly from the diet to yield microbial nitrogen.

Protein nitrogen flowing from the rumen thus consists of microbial protein, undegraded feed protein and endogenous protein not incorporated into microbial protein. Additional endogenous protein inputs including enzymes, plasma proteins, sloughed cells and mucus occur in the omasum and abomasum. The various endogenous protein inputs to the ruminant digestive tract are considered in detail in the following section.

Fig 1 A simple model of nitrogen metabolism in the ruminant illustrating the origin of endogenous protein inputs



1.3 Endogenous protein nitrogen inputs

1.3.1 Endogenous protein nitrogen inputs to the reticulo-rumen

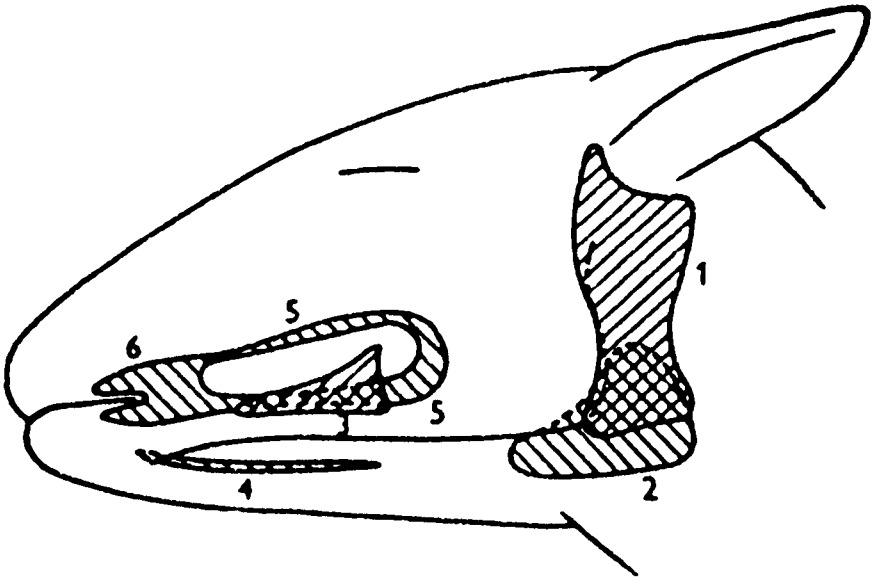
1.3.1.1 Saliva

Ruminant saliva is complex in origin and composition (Phillipson and Mangan, 1959). Many of the most important properties of the salivary glands were discovered during physiological studies in the 19th century. The main paired salivary glands in the sheep are illustrated in Fig. 2 and the details of these glands are given in Table 1. In adult ruminants the parotid and inferior molar; palantine, buccal and pharyngeal, and submaxillary, sublingual and labial glands are characterised as serous, mucous and mixed glands respectively.

As the reticulo-rumen is not a secretory organ the salivary glands are responsible for supplying the fluid input to the rumen, which is necessary both to permit regurgitation and to allow continuous onward flow of digesta down the gut and to encourage rapid mixing and fermentation of the food (Kay, 1966).

The rate of saliva secretion has been considered to be largely determined by stimuli operating on the glands via a number of reflex arcs (Kay, 1966) and has been shown to be influenced to a large extent by the quantity and nature of the food eaten (Balch, 1958; Wilson and Tribe, 1963). Colin (1886) originally demonstrated that dry food stimulated the most rapid flow of parotid saliva and that the volume of saliva added to a given weight of food was greatest for roughage and feeds that require prolonged chewing. The total volume of saliva secreted has been estimated as 6-16 L per day in sheep (Kay, 1960) and 56 L per day (Colin, 1886) -190 L per day

Fig 2 The main paired salivary glands of the sheep (from Kay, 1960)



1. parotid
2. submaxillary
3. inferior molar
4. sublingual
5. buccal
6. labial

The pharyngeal and palantine glands are not shown

Table 1 Characteristics of the salivary glands of sheep (from Kay, 1960)

Glands	Mean weight g	Cell type	Saliva volume L/24 hr	Saliva flow	Saliva type
Both parotid	24	Serous	3-8	Continuous; responds to stimulation of mouth oesophagus and reticulo-rumen	Fluid, isotonic, strongly buffered
Both inferior molar	6	Serous	1-2		
Palatine, buccal and pharyngeal	21	Mucous	2-6	Discontinuous; responds to stimulation of mouth, oesphagus and reticulo-rumen	Very mucous, isotonic or nearly so, strongly buffered
Both submaxillary	18	Mucous and serous	0.4-0.8	Discontinuous; stimulated by feeding; little response to rumination or stimulation of oesophagus or reticulo-rumen. Labial probably similar	Mucous, hypotonic, weakly buffered
Both sublingual	1	Mucous and serous	0.1?		
Labial	11	Mucous and serous	?		

(Bailey, 1961) in cattle. The latter value was reported in cows consuming a grass diet.

Ruminant saliva is isotonic with plasma and has concentrations of volatile fatty acid similar to those seen in the blood (Annison, 1954). Since ruminant salia is devoid of amylase (Palmer, 1916), protease (Wegner et al., 1940) and lipase (Garton, 1958), it is evident that ruminal digestion is completely unaided by the action of salivary enzymes in the adult ruminant (Kay, 1960). The average pH of sheep parotid saliva is 8.09 as secreted (McDougall, 1948) and the major organic constituents of ruminant saliva are protein, urea and volatile fatty acids. The protein component of saliva is now considered in detail.

Saliva contains a number of different proteins (McIntosh, 1975). Lyttleton (1960) determined that saliva collected by suction in cows contained 1.0-2.0 mg protein per ml. Saliva collected using an oesophageal fistula was shown to consist of a mixture of high molecular weight mucin (essentially carbohydrate in nature) and proteins of molecular size 30,000 to 80,000 (see Clarke et al., 1974). Analysis of ovine submaxillary glycoprotein (Gottschalk and Simmonds, 1960) indicated equimolar amounts of N-acetylneuraminic acid and N-acetylgalactosamine, linked to a polypeptide chain in which the amino acids serine, threonine, glycine and proline predominated. N-acetyneuraminic acid is the mucoprotein constituent of saliva that is responsible for its viscous nature (Hungate, 1966). The main physiological function of ovine submaxillary mucoprotein to cover foodstuffs with a lubricant layer of mucus, facilitate swallowing and protect teeth and the cells lining the upper digestive tract against injury, was considered closely related to its high intrinsic viscosity (Pigman and Gottschalk, 1966). Ovine submaxillary gland mucoprotein was found to be susceptible to trypsin (Gottschalk and Fazekas, 1960).

Analysis of the lower molecular weight proteins found in bovine saliva, using polyacrylamide gel electrophoresis (McIntosh et al., 1982), identified eleven protein bands. This analysis formed part of an extensive investigation on the genetics of the susceptibility to bloat in cattle (Clarke et al., 1974) and followed the illustration by Mangan (1959) that the mucoprotein of saliva aspirated from under the tongue in cattle was a powerful foaming agent and may contribute to the stability of foam in the rumen. Further studies have shown that a group of three proteins, referred to as band 4, are present as a major component only in cows of high bloat susceptibility (Clarke et al., 1974). McIntosh (1975) provided evidence that band 4 is affected by diet. Interestingly, it was originally demonstrated in humans and other mammals that salivary proteins can be induced by a change of diet (Squires, 1953). In an attempt to identify characteristic protein bands, band 6 of adult cows and heifers cross-reacted with bovine serum albumin antisera (McIntosh, 1975).

Phillips and Mangan (1959) observed that in the case of submaxillary saliva, protein nitrogen may be present at five times the concentration of urea nitrogen, depending on the type of stimulation. Residual saliva and submaxillary saliva were shown to have a higher concentration of protein nitrogen than parotid saliva and inflation of the rumen was shown to cause an increase in the mucoprotein content of submaxillary saliva. The responses of the submaxillary gland to feeding were shown to vary with the nature and consistency of the food and values of 15 g protein nitrogen per day were predicted to enter the rumen of an adult cow. An investigation on the flow and protein content of parotid saliva (Brightling et al., 1977) provided evidence that when sheep ate freshly provided food there was a greater volume of parotid saliva and its

protein content also increased. Futher studies have concerned the elucidation of the mechanism of this phenomena (Patterson and Titchen, 1979; Patterson and Titchen, 1980; Patterson et al., 1982). The purpose of the increased secretion of protein is obscure. Parotid salivary protein has been shown to include four major fractions with molecular weights ranging from about 25,000 to 150,000 and the proportions of these proteins have been found to change on feeding (Voigt et al., 1980). A recent investigation (Froetschel et al., 1986) on the effects of slaframine, an agent which stimulates the release of gastrointestinal tract secretions without affecting cardiovascular function observed a decrease in salivary protein and N-acetylneuraminic acid concentrations with increased saliva flow rates. Clearly the situation is complex.

A consideration of the functions of ruminant saliva in relation to protein metabolism indicates that salivary nitrogen could make an important contribution to the rumen when the intake of dietary nitrogen was low. The concentration of urea in saliva is affected by the rate of secretion (Somers, 1961a) but on a long-term basis is determined by the concentration of urea in blood. Blood urea in turn depends on the nature of the diet, in particular on the concentration of ammonia in the rumen (Lewis et al., 1957). Phillipson and Mangan (1959) suggested that salivary protein may also play a similar role to urea in that it can be an internal source of nitrogen for the rumen microorganisms and indicated the metabolism of salivary mucoprotein by the rumen microorganisms in rumen liquor during feeding periods. This potential ability to recycle nitrogen is illustrative of a remarkable adaptation by ruminants to seasonal inadequacies which may occur in their diet. The process enables digestible crude protein to be synthesised from otherwise waste urea and also

stimulates microbial fermentation of poor fodder, reduces bulk and retention time of undigested food and thus enhances appetite.

1.3.1.2 Rumen epithelium

a. Physiological considerations

The stratified squamous epithelium of the reticulo-rumen constitutes up to 1% of the body mass of sheep and cattle (Chalupa, 1972) and is extremely metabolically active. The cells of the basal and transitional layers contain large numbers of mitochondria, and high activities of lactate, malate, succinate and butyrate dehydrogenases and carbonic anhydrase are evident (Sakata et al., 1980b). The rate at which the rumen is renewed may be of significance in the productivity of the ruminant since its constant turnover represents a significant loss of energy and protein (Webster, 1980). The keratinised cells of the rumen epithelium possess fine outward projections of the cell membrane and are coated with polysaccharide (Fell and Weekes, 1975). Rumen papillae extend the surface area approximately seven times in domestic cattle and the extent of vascularisation can be compared with that of an intestinal villus (Warner and Flatt, 1965). Goodlad (1981) indicated that the rumen epithelium can alter its tissue mass in a variety of ways, namely by changing the fraction of basal cells that are proliferating, changing the time taken for cell division and adjusting cell transit times. An epithelium with a high mitotic rate and a slow rate of transit will tend to be relatively thick.

Initial considerations suggest that the maturation of the rumen and development of the papillae depends on the nature of the food eaten and this has been confirmed. Goodlad (1981) determined the mean turnover time of the rumen epithelium in sheep, using [^{3}H]-thymidine as 10.9 days

in animals fed a concentrate based diet, 16.5 days in roughage fed animals and 4.3 days in the transition period between the two diets. Estimates of cell turnover in the gastrointestinal wall of sheep using [6-^{3}H]-thymidine (Rowe and James, 1982) suggested that the amount of cell synthesis in the rumen epithelium is affected by the physical form of the diet and may represent approximately 3% of total body cell production.

A consideration of the regulation of growth in the rumen epithelium is an interesting phenomenon with nutritional and pathological consequences. If the rates of cell division and turnover are disturbed, pathological conditions such as hyperkeratosis, parakeratosis and rumenitis may occur (Fell and Weekes, 1975). Hormonal and nutritional factors that influence the intestine and gastric epithelium may also influence the rumen epithelium. At a molecular level the differentiation of cells is poorly understood and mechanisms acting at numerous levels in the metabolism of cells may have a role in this process (Cooper and Lipkin, 1973).

It is thus evident that epithelial proliferation is not absolutely related to a direct action of coarse or fibrous feeds but may depend on a more complicated secondary stimulation. The postulated role of volatile fatty acids acting via a hormonal mechanism (Sakata et al., 1980a) is considered in the discussion (section 6.4).

b. The role of bacteria adherent to the rumen epithelium

Direct observations of the stratified squamous rumen epithelium by electron microscopy (Bauchop et al., 1975) have shown that the highly keratinised distal cells are subject to digestion by adherent bacteria. Cheng et al. (1979) defined an adherent population in the rumen, which was intermittent, occasionally very extensive and taxonomically heterogenous.

The predominant bacterial population of the rumen epithelium, gram positive cocci, were not restricted to the lumenal surface of the epithelial cells, but were also found in the intercellular spaces and even between the superficial cells and the underlying tissue (Dinsdale et al., 1980).

The bacterial flora of the rumen may be considered as three taxonomically and functionally distinct populations: rumen fluid bacteria, feed particle associated bacteria and bacteria adherent to the rumen epithelium. The subpopulations of bacteria in the rumen are illustrated in Fig 3. The rumen fluid and feed particle associated bacteria have been extensively studied but evidence for the existence and function of the adherent epithelial population has only recently been examined. The existence of a distinct population of bacteria adherent to the rumen epithelium suggests that this population has developed in response to specific factors in this ecological niche. The functions of the bacteria adherent to the rumen epithelium include:

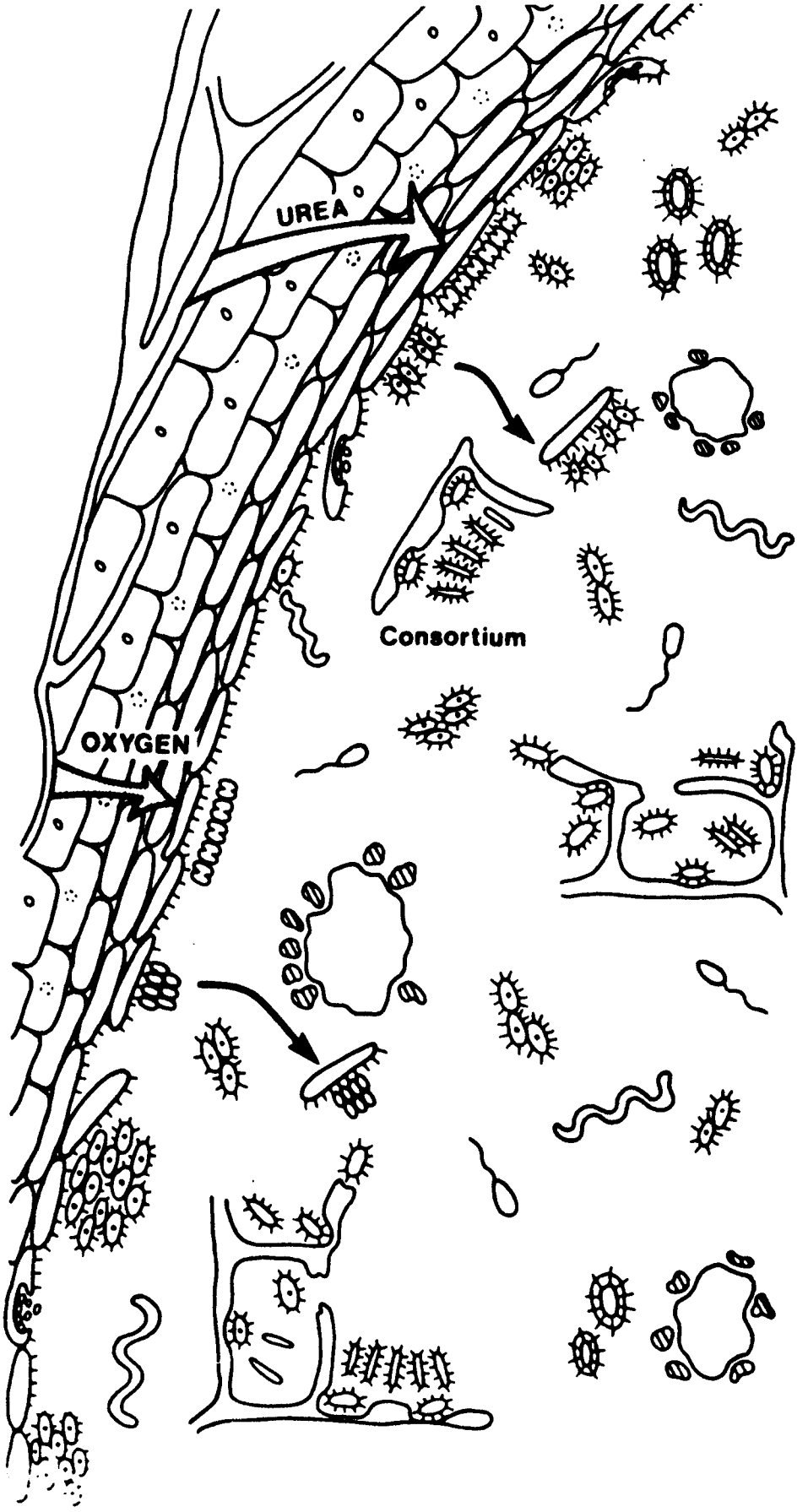
i) Tissue recycling

Electron microscopy of highly keratinised distal cells of the stratified squamous epithelium has shown that the cells colonised by bacteria often occur in 'pits' produced by their own digestive activity and whole distal cells have frequently been seen to be subject to digestion by invading adherent bacteria. Mead and Jones (1981) observed cavities in the rumen epithelium, containing bacteria, as a result of exfoliation of distal epithelial cells. In vitro studies indicated that the epithelial cells were rapidly broken down once they were released into the rumen (Dinsdale et al., 1980).

This process of tissue recycling benefits the host in that dead cells are recycled and the absorptive capacity of the rumen wall is

Fig 3 Diagram showing the subpopulations of bacteria in the rumen

Bacteria occur in the rumen fluid as individual cells and as slime-enclosed microcolonies, they adhere to food particles, and they adhere to the distal cells of the stratified squamous epithelium of the rumen itself (from Cheng et al., 1979)



enhanced facilitating the movement of metabolites. The adherent bacteria derive several advantages from attachment to the tissue surface. For example, they may be protected from digestion by ciliate protozoans or avoid being washed out of the rumen. Also they may gain easy access to substrates, due to continual movement of the rumen wall and the transport of metabolites through the epithelium (McCowan et al., 1978).

ii) Urea metabolism

Cheng et al. (1979) observed that the adherent population produced urease and that the sloughing of heavily colonised distal epithelial cells accounted for the urease activity of rumen fluid. The entry of urea into the rumen via the rumen epithelium has been shown to increase when the rumen ammonia concentration decreases (Kennedy and Milligan, 1978a) and an apparent inverse relationship has been observed between urease activity and rumen ammonia concentration (Chalupa et al., 1970).

Cheng and Wallace (1979) proposed a model based on that of Houpt (1970) to describe the mechanism of the passage of endogenous urea through the rumen wall and the role of ureolytic epithelial bacteria on the urea flux. It was suggested that feedback control is mediated by the effect of rumen ammonia on urease activity. Thus at high rumen ammonia levels, removal of urea by urease from the inner surface of the rumen wall is slower than at low concentrations of rumen ammonia, and therefore the rate of diffusion across the rumen wall is reduced.

The adherent bacteria are thus vital components in the process of recycling of endogenous urea nitrogen and may also control the rate at which it occurs (Wallace et al., 1979).

iii) Oxygen scavenging

The adherent bacteria are envisaged to contribute to the maintenance of fastidious anaerobic conditions for the obligate anaerobes within the rumen. Cheng et al. (1979) identified 34 out of 160 strains of adherent bacteria isolated to date as facultative anaerobes.

iv) Proteolytic activity

This specific bacterial population has been shown to be highly proteolytic and to contribute more than 10% of the total protease activity of rumen fluid (Dinsdale et al., 1980).

The bacteria adherent to the rumen epithelium thus appear to play an important role in the metabolism of the rumen and the ruminant (Cheng et al., 1979).

1.3.2 Endogenous protein nitrogen inputs to the omasum and abomasum

Initial observations on endogenous protein nitrogen inputs to the omasum and abomasum were made in surgically modified animals equipped with abomasal pouches. Masson and Phillipson (1952) noted that the dilution of omasal contents by gastric juice was such that gastric juice was the greater in volume of the two components which formed the abomasal contents. Hill (1960) considered the abomasum to correspond morphologically and functionally to the gastric secretory stomach in other animals. Harrison and Hill (1962) in studies with animals fitted with a gastric pouch in the pyloric region of the abomasum, observed a continuous secretion of a clear viscous fluid, which contained strands of visible mucus and clumps of desquamated epithelial cells. This secretion amounted to

5-6 litres per day in a 50 kg sheep and 30 litres per day in a 400 kg steer (Hill, 1961). The passage of food to the abomasum was continuous and a continuous abomasal secretory activity was observed, dependent on the nature and physical characteristics of the diet and on the frequency of feeding.

Relatively recent work by Harrop (1974) indicated that protein was the major nitrogenous secretory component of both the fundic and pyloric regions of the abomasum, amounting to 0.5-2.8 g of nitrogen per day, derived mainly from the fundic region of the abomasum.

There is a lack of accurate information concerning the overall protein composition of ruminant abomasal secretions. The protein constituents in human gastric juice have been relatively well defined by chemical and electrophoretic means and include: specific gastric proteins such as the proteolytic enzymes; non-proteolytic enzymes; visible and dissolved mucus; blood group substances; various binding and inhibiting factors; and plasma proteins such as albumin, α-2-macroglobulin, transferrin, immunoglobulins and a few α and B-globulins (Schulze and Heremans, 1966; Glass, 1968). Immunological analysis of proteins in in vivo neutralised human gastric juice has provided evidence that gastric juice contains some 'intrinsic' protein in addition to that derived from the serum. Glass (1968) identified a glycoprotein, gastrone, which was identical to a similar protein in saliva and had the mobility of a beta-2-globulin.

Campbell (1961) investigated the passage of plasma albumin into the lower gastrointestinal tract of sheep using intravenously infused ^{131}[I]-labelled albumin and identified the physiological secretion or exudation of plasma albumin into the jejunum. This input was calculated to be approximately 13% of the total nitrogen entering the small intestine.

Recent studies by Dargie and Berry (1979) using ^{125}[I] and ^{51}[Cr]-labelled plasma proteins, as part of studies investigating the pathogenic effect of liver fluke (Fasciola hepatica) in sheep, obtained estimates of albumin lost into the gut per day of 1.4 g on a normal crude protein intake (150 g/day) and 0.9 g on a low-protein diet (60 g/day). Rothschild et al. (1970) indicated that the most important aspect in the regulation of albumin synthesis is nitrogen intake, although synthesised hormones may also play a role.

Titchen (1984) indicated the complexity of the control of gastric secretion in ruminants. Gastrin may be involved in a trophic action on the gastrointestinal mucosal mass which has been demonstrated in other animals (Johnson, 1980).

Mucus enters the gastrointestinal tract prior to the proximal duodenum via saliva, respiratory and nasal sources and in abomasal secretions. Mucus is secreted in a gel-like form with a discernible structure (Florey, 1955) and can be separated from the fluid phase by mechanical means such as filtration (Heatley, 1959). Soluble mucins present in gastrointestinal secretions are almost certainly derived from the gel-like mucus. The rate at which this change of visible mucus into soluble mucins occurs is increased in the presence of certain enzymes abundant in the small intestine. The soluble mucin appears to be relatively resistant to digestion by these enzymes. However, microbial enzymes are effective in degrading it and little nitrogen in soluble mucins derived from mucus is excreted in the faeces (Hecker, 1973).

1.4 The assessment of the protein requirements of ruminants

1.4.1 Development

The measurement of digestible crude protein (DCP) originally formed the basis of the method most widely used to assess the protein requirements of ruminants and the extent to which feeds meet these requirements. The simple concept of the DCP model was that the nitrogen available to the animal was given by the nitrogen intake minus the nitrogen excreted in the faeces. This clearly did not accurately represent the actual situation. It was considered that since equal intakes of DCP from different sources neither promoted the same absorption of amino acids from the small intestine, nor the same production of growth, wool or milk in animals, this simple approach must be abandoned.

The ARC (1965) adopted the available protein system. This was based on the determination of the amount of crude protein of a defined biological value that would have to be absorbed from the digestive tract to meet the calculated requirements of the tissues.

The theoretical limitations of these concepts were evident. The extensive degradation of dietary protein in the rumen and its incorporation into microbial protein was ignored. Also the observation that faecal nitrogen consists largely of undigested microbial protein and the failure to link protein requirement to total energy intake indicated the need for new proposals.

Recent schemes by ARC (1980, 1984) estimate the flow of protein to the duodenum from a constant microbial yield of nitrogen related to dietary energy intake and a variable contribution from dietary protein passing undegraded through the rumen (see section 1.4.2). The ARC (1980)

proposals included a strong recommendation that "the new approach should be regarded as a conceptual framework for future research efforts and a means of focussing attention of those factors for which data are required".

1.4.2 The principle of the ARC (1980, 1984) scheme

In ruminants and other animal species the protein requirements of the tissues are met by the amino acids absorbed from the small intestine. In an ideal system it is necessary to be able to define the amino acid requirement and accurately relate this requirement to the diet. The situation is complex in the ruminant due to the potential microbial manipulation of dietary input. Protein arriving at the small intestine in ruminants consists of microbial protein, dietary protein which has escaped degradation in the rumen and endogenous protein (see section 1.2.2). The degradability of a dietary protein is ideally defined (ARC, 1980) as 1 - (dietary protein entering the duodenum/dietary protein consumed). The undegraded dietary protein thus supplements the microbial and endogenous protein and any representative scheme needs to consider these components.

The principle of the ARC (1980) protein scheme originally proposed by Roy et al. (1977) is summarised below.

The amount of rumen degraded protein (RDP) that is required by the rumen microorganisms for maximal microbial growth from a particular energy input is estimated. This is calculated from the intake of apparently digested organic matter and values taken from the literature of the proportion of apparently digested organic matter that is apparently digested in the rumen and the efficiency of microbial protein synthesis.

The protein supplied to the tissues by the rumen microorganisms is then determined. This is calculated as RDP multiplied by the proportion

of total microbial nitrogen as amino acid nitrogen, the apparent absorption of microbial amino acid nitrogen in the small intestine and the efficiency of utilisation of absorbed microbial amino acid nitrogen.

The tissue protein requirement of the animal for a particular productive process is determined and compared with the protein supplied by the rumen microorganisms. If the protein supplied by the rumen microorganisms is in excess of the tissue requirements the RDP requirement is the minimum protein requirement of the animal and no further calculation is required. If the protein supplied by the rumen microorganisms is less than the tissue requirements the deficit must be provided by protein from undegraded dietary protein (UDP). The UDP requirement is calculated as the protein deficit divided by the product of the efficiency of utilisation of absorbed dietary amino acid nitrogen and the apparent absorbability of dietary amino acid nitrogen in the small intestine. The minimum crude protein needed by the animal is the sum of the RDP and UDP requirements, if the degradability of the dietary protein allowed it to exactly supply the RDP and UDP required.

The formulation of diets thus requires a value for the degradability of the various constituents so that RDP and UDP can be matched to the requirement of the animal. The accurate determination of the degradability of a dietary protein is a major practical problem.

1.4.3 The determination of the degradability of dietary protein

An accurate definition of the problem is not difficult. An ideal expression for the degradability of a diet has been previously defined (section 1.4.2). The ARC (1980) adopted a theoretically technically feasible, but biologically imprecise, oversimplified expression to evaluate

dietary protein degradability (Fig 4, equation a). Inaccuracies which occur when neither endogenous protein nitrogen nor ammonia nitrogen components of duodenal digesta are recognised were accepted. More precise equations to determine degradability are included in Fig 4, b and c. Essentially, the use of the most accurate expression, equation c, has been confounded by lack of quantitative data concerning the actual endogenous protein nitrogen in the duodenal digesta under a particular dietary regime and level of intake.

Even when considered the endogenous protein nitrogen contribution has been assumed to be equal to a constant proportion of dietary protein intake (Satter and Roffler, 1977). Roy et al. (1977) considered it to be equal to the amount of ammonia nitrogen that would need to be absorbed from the rumen to result in equal values for degradation of dietary nitrogen and synthesis of microbial nitrogen. As a result of recent studies elucidating the complex relationship between diet and the urea component of endogenous nitrogen input into the rumen (Kennedy and Milligan, 1980a) and between ammonia absorption and concentration in the rumen (Kempton et al., 1979) it was considered (Kennedy and Milligan, 1980b) that these assumptions are unlikely to be valid for the entire range of diets given to ruminants. Failure to recognise the endogenous component leads to an underestimate of degradability (see section 1.5).

A classification of foods based on a range of degradability estimates was proposed by the ARC (1980) (see Table 2). Clearly this represented only a starting point as individual values were not defined and the range within a classification was quite large. Despite a considerable research effort involving various techniques to determine protein degradation the ARC (1980) concluded that it was not possible to predict

Fig. 4 Equations proposed to determine the degradability of dietary protein

a. $$\text{Degradability} = 1 - \frac{\text{Total duodenal nitrogen flow (g/day)} - \text{Microbial nitrogen flow (g/day)}}{\text{Nitrogen intake (g/day)}}$$

b. $$\text{Degradability} = 1 - \frac{\text{Duodenal protein nitrogen flow (g/day)} - \text{Microbial nitrogen flow (g/day)}}{\text{Nitrogen intake (g/day)}}$$

c. $$\text{Degradability} = 1 - \frac{\text{Duodenal protein nitrogen flow (g/day)} - \text{Microbial nitrogen flow (g/day)} - \text{Endogenous protein nitrogen flow (g/day)}}{\text{Nitrogen intake (g/day)}}$$

Table 2 The proposed classification of feeds based on the extent of protein degradation in the rumen (from ARC, 1980)

Class	Range of degradability	Forages	Cereals	Protein supplements
A	0.71-0.90	Grass hay Legume hay Grass silage (wilted and unwilted) Artificially dried grass (chopped)	Barley	Casein Wheat gluten Groundnut meal Sunflower meal Soya bean meal (unheated) Rapeseed meal Field bean meal Yeast protein
B	0.51-0.70	Grass (fresh or frozen) Legumes (fresh or frozen) Artificially dried grass (ground and pelleted) Artificially dried legume (except sainfoin)(chopped) (Maize silage) (Clover silage)	Maize	Soya bean meal (cooked) Lupin meal Coconut meal
C	0.31-0.50	Artificially dried legume (ground and pelleted)	Milo	Zein Casein (formaldehyde treated) Fish meal
D	0.31	Grass silage (formaldehyde treated) Artificially dried sainfoin (chopped)		

the extent of protein degradation for a particular feed with any degree of precision.

The extent of degradation in the rumen under practical feeding conditions may be considered a function of rate of proteolysis and rumen retention time (Tamminga, 1979). A massive research effort has been directed towards the accurate determination of degradability. It has been estimated using in vivo and in vitro methods.

In vivo studies are expensive, time consuming and tend to be hindered by technical difficulties. Estimates derived from such studies are nevertheless considered the most reliable. However, very often no direct determination of the endogenous protein nitrogen contribution is made. Also, the quantification of the microbial contribution is dependent on the microbial marker and different markers vary (Ling and Buttery, 1978; Siddons et al., 1982) and also show a degree of variability within the same marker (Siddons et al., 1982).

The use of in vitro techniques has been extensively examined and is generally based on the estimate of the proportion of nitrogen which goes into solution after incubation at body temperature for a fixed time. Various solutions have been applied as incubation media: rumen fluid (Little, 1963); diluted NaOH (Lyman et al., 1953); artificial saliva (Wohlt et al., 1973); autoclaved rumen fluid (Wohlt et al., 1973); proteolytic enzymes (Beever et al., 1977; Chamberlain and Thomas, 1979b; Krishnamoorthy et al., 1983) and water at various temperatures (Mertens, 1977). Broderick (1978) attempted to combine values of protein degradation and rumen turnover resulting in estimates of degradation rate.

Mehrez and Orskov (1977) obtained a direct measurement of protein degradation by incubating a sample of the feedstuff in a Dacron

bag directly in the rumen. In a recent review (Williams, 1986) it was concluded that "currently no laboratory method is capable of predicting in vivo degradability with any degree of accuracy".

It would appear fundamental that the precise determination of protein degradation in vivo is a prerequisite to the validation of a rapid in vitro technique. At present, lack of direct information concerning the endogenous protein nitrogen contribution may have a significant effect on in vivo degradability estimates. The potential importance of a significant endogenous protein nitrogen input is considered in the next section.

1.5 The importance of the accurate assessment of the endogenous protein nitrogen content of proximal duodenal digesta

Protein arriving at the proximal duodenum consists of microbial protein, undegraded feed protein and endogenous protein (see section 1.2.2). The potential importance of a significant endogenous protein nitrogen component in duodenal digesta has been widely recognised (see Phillipson, 1964; Siddons, 1985a and below). However, in the absence of a technique for its direct accurate determination it has either been ignored or an assumed or derived value used in dietary degradability calculations. Previous estimates of the endogenous protein nitrogen flow are discussed in section 6.3.

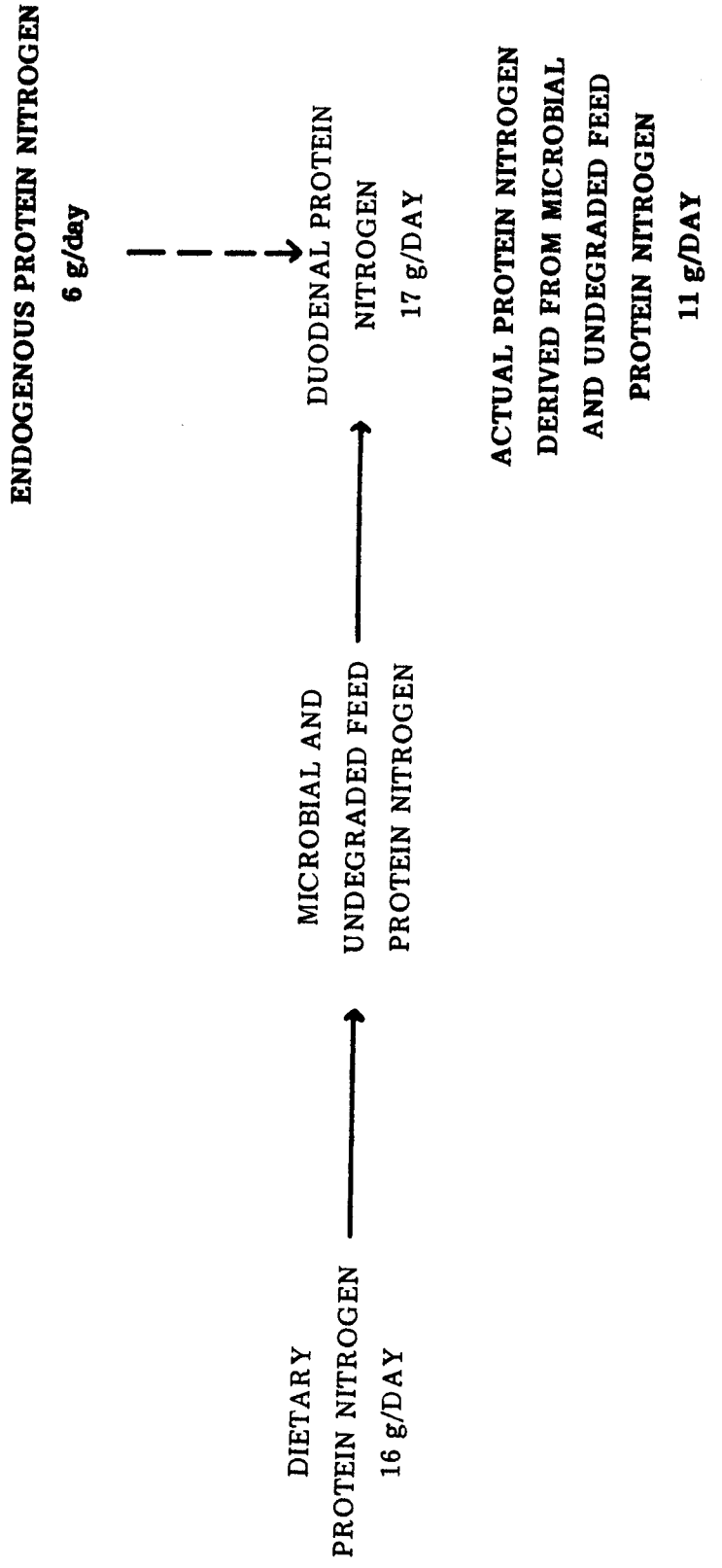
Smith (1979) in discussing new information required for proposed feeding schemes (ARC, 1980), recognised the requirement to equate essential amino acid supply entering the small intestine with demand and the need to predict this from easily measurable properties of the diet. Information on the entry of endogenous protein into the digestive tract and how it varies with diet, level of intake, hormonal status and species was considered necessary for an improved theoretical understanding and greater practical precision.

MacRae and Reeds (1980) considered that in order to make any systematic study of degradability and capture of non-protein nitrogen, especially if the long term aim was a study of the dynamics of rumen fermentation, there was little alternative but to develop methods which could estimate the endogenous nitrogen contribution anterior to the duodenum.

The amount of endogenous protein secretion could alter quite considerably degradability estimates. Evidence is now accruing that the endogenous protein nitrogen flow may be as high as 6 g protein nitrogen per day in sheep (MacRae and Reeds, 1980). This is considerably greater than the 1-2 g protein nitrogen per day originally proposed (Phillipson, 1964). The importance of this greater value is that conventional degradability estimates of lucerne of 45% (ARC, 1980) could in fact be as high as 81% if 6 g of the duodenal protein nitrogen was endogenous protein rather than undegraded dietary nitrogen. As some of the endogenous protein will be degraded to ammonia the actual degradability is probably between 45 and 81%. The overall effect of an underestimate of the degradability of a diet leads to a supply of excess rumen degradable protein and a reduction in the supply of undegraded dietary protein to the small intestine. This could lead to a reduced performance in fast growing beef animals and high yielding dairy cows (Wilson and Strachan, 1980). Excess rumen degradable protein may also be responsible for some of the metabolic disorders associated with early lactation (Webster et al., 1982).

MacRae and Reeds (1980) also indicated a further concept of a significant endogenous protein nitrogen input, that of a futile cycle (Fig 5). If dietary nitrogen intake was 16 g per day and duodenal protein nitrogen flow was 17 g per day, conventional analysis of the data would

Fig 5 The concept of a futile cycle of endogenous protein nitrogen



indicate that there was negligible loss of protein nitrogen anterior to the proximal duodenum. However, if a significant input of endogenous protein was occurring, say 6 g protein nitrogen per day, then the net amount of protein nitrogen derived from the diet (either microbial or undegraded feed protein) was actually 11 g protein nitrogen per day, which is considerably lower than the value assumed.

In order to relate the profile of available amino acids to a particular requirement it is necessary to have some measure of the amino acid requirements of the animal. The futile cycle phenomena may provide a possible explanation as to why researchers who have tried to measure uptake of amino acids in the portal vein have failed to account for all the amino acids which disappear from the small intestine (see MacRae and Reeds, 1980). Absorption may be measured by difference between the duodenum and the ileum or by amino acid uptake in the hepatic portal venous blood. The latter approach is technically difficult but it is clear from the data available that much less amino acid appears in the hepatic portal venous blood than disappears from the small intestine. The difference between the two methods may be due to a significant endogenous protein contribution. Information concerning the endogenous protein input and factors influencing this input are thus required to improve the accuracy of the prediction of absorption of amino acids and hence the requirements of the animal.

1.6 The determination of the endogenous protein content of ruminant proximal duodenal digesta

1.6.1 The problem

As explained previously values for the endogenous protein content of ruminant proximal duodenal digesta are required for animals fed different conventional rations. In this investigation endogenous protein is defined as that fraction of the total protein arriving at the proximal duodenum derived from the animal itself, not including any endogenous protein which may have been incorporated into the microorganisms.

Previous attempts to determine endogenous protein secretion in ruminants have included:

i) The analysis of the protein nitrogen content of gastric secretions in surgically modified animals (Phillipson, 1964; Harrop, 1974).

ii) The determination of total duodenal digesta protein flow and microbial protein flow in animals fed purified diets; hence the endogenous protein component has been determined by difference (see Kaufmann, 1977). As the microbial component is the major fraction of the total duodenal digesta protein a large error is introduced into this calculation.

iii) The development of ^{15}N tracer kinetics in the study of nitrogen metabolism in ruminants has generally resulted in 'indirect' estimates of total endogenous protein secretion (Nolan and MacRae, 1976; Kennedy and Milligan, 1980b; Siddons et al., 1985a). They have as yet failed to account for the fraction metabolised by the rumen microorganisms. An exception is the study of Brandt et al. (1981).

iv) The application of the intragastric infusion technique (Orskov et al., 1979).

v) The examination of the amino acid profiles of the various components of the duodenal digesta (Evans et al., 1975).

Merits and faults of these methods are discussed in detail in section 6.3. In this investigation an alternative method is proposed which attempts to determine directly the endogenous protein content of proximal duodenal digesta in animals fed conventional diets.

1.6.2 The proposed technique

An alternative technique is proposed using the concept of isotope dilution.

The development of isotopic tracer techniques, including the use of labelled amino acids, has enabled major advances to be made in the quantitative study of metabolism. To be of use isotopic tracers must behave physically and chemically exactly as their natural counterparts. The use of modern sensitive equipment for the detection of radioactivity means that the dose can be relatively small in relation to the number of natural atoms and consequently does not influence the system under investigation. Biological systems may be considered biochemically as an assortment of pools or compartments each made up of identical molecules enclosed by physical boundaries. The dynamic equilibrium of body pools which tend to remain constant in size while undergoing replacement by input equal to output is defined as the 'steady-state'. The tracer can be delivered to a compartment of a biological system via a single dose or by continuous infusion.

The theoretical concept of a continuous infusion is considered with reference to Fig 6. During a continuous infusion of an isotope to a biological system serial measurement of the specific activity of a 'precursor' compartment (A) in that system yields a rising curve which approaches a horizontal asymptote. The curve can often be described by a single exponential equation: $y = A(1 - e^{-kt})$ (Waterlow and Stephen, 1967) where,

y = Specific activity

A = Plateau specific activity

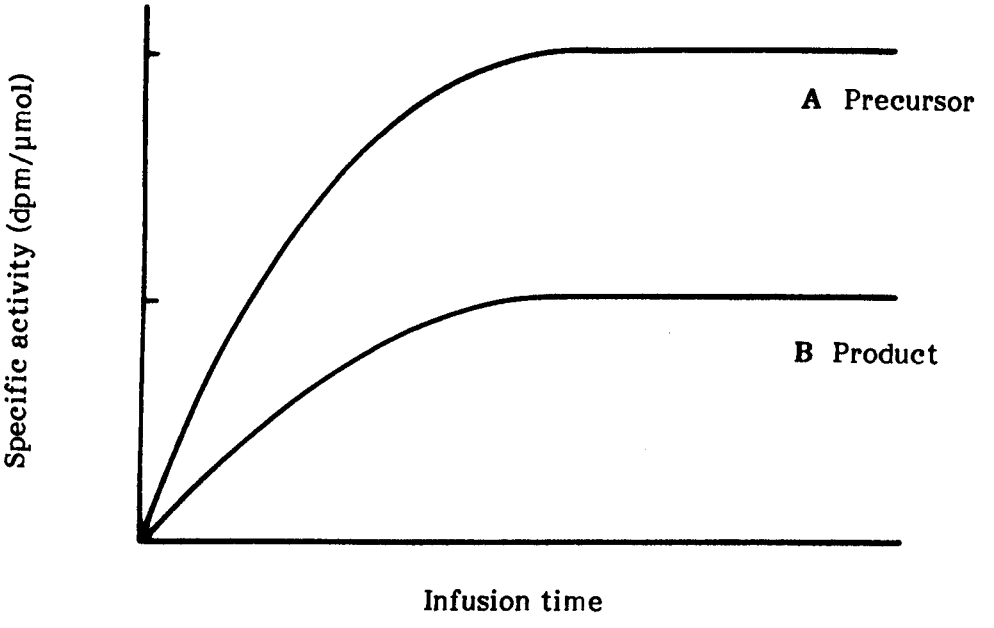
k = Rate constant

t = Time

Serial sampling from a product compartment (B) results in an exponential curve which achieves a lower specific activity than the 'precursor' compartment (A). A comparison of the specific activity profiles of these compartments enables the proportion of input to compartment B which is derived from compartment A to be calculated. This general principle was applied in the present study to determine directly the endogenous protein content of proximal duodenal digesta.

The approach adopted involved a long term intravenous infusion of a labelled amino acid. This resulted in all body proteins becoming labelled. Any label detected in the duodenal digesta must therefore be derived from the animal itself. It was envisaged that a comparison of the specific activity of duodenal digesta with that of a suitable precursor protein would provide an estimate of the proportion of duodenal digesta of endogenous origin. This method does of course require a detailed knowledge of the components of the endogenous secretions and of their changes in specific activity.

Fig 6 The specific activity profile of precursor (A) and product (B) compartments determined during a continuous infusion of a labelled amino acid



$$\frac{\text{Area under curve B}}{\text{Area under curve A}} = \text{Fraction of B derived from A}$$

It was considered possible that microbes arriving at the duodenum may become labelled as a result of the incorporation of any of the label entering the digestive tract. Any label taken up by the bacteria or protozoa can be corrected for following isolation of a sample of the microbial component of the digesta. The shape of the digesta curve corrected for a labelled microbial component (corrected digesta curve) was very important.

The shape of the specific activity profile of the corrected digesta represents a combination of the various endogenous inputs (i.e. enzymes, plasma proteins, sloughed cells and mucus). The specific activity profile of each of these components has a characteristic shape. The identification of a suitable precursor representative of endogenous secretions is not straightforward. In the consideration of an extreme situation endogenous inputs could for example be all enzymatic secretions or all plasma protein type materials. It was envisaged that the identification of a suitable precursor taken to represent either enzymes or plasma proteins must provide a range of estimates for the proportion of duodenal digesta of endogenous origin.

Thus saliva, which could be readily obtained, was considered to have a specific activity profile equivalent to endogenous input such as enzymes (see discussion, section 6.1.2). Plasma protein was considered to have a specific activity profile representative of intestinal secretions such as albumin/globulins (see discussion, section 6.1.2). Rapidly turning over proteins (e.g. enzymes) would be expected to have a higher specific activity than their slower turning over counterparts (e.g. plasma proteins). Therefore a comparison of the specific activity profile of corrected digesta with that of saliva provided a minimum estimate of the proportion of

duodenal digesta of endogenous origin. Similarly, a comparison of the specific activity profile of corrected digesta with that of plasma protein gave a maximum estimate of the proportion of duodenal digesta of endogenous origin.

The difficulty is choosing which precursor to use or indeed the relative proportions of several precursors. Some information on which to make this decision could be obtained by comparison of the shapes of the change in specific activity of the endogenous component of the digesta with that of the likely precursors. This thesis describes a method of doing this.

The tracer used in these studies was L-[4,5-^{3}H]-leucine. L-[4,5-^{3}H]-leucine was chosen as the investigatory amino acid because:

1. Leucine has virtually no metabolic role other than protein synthesis.

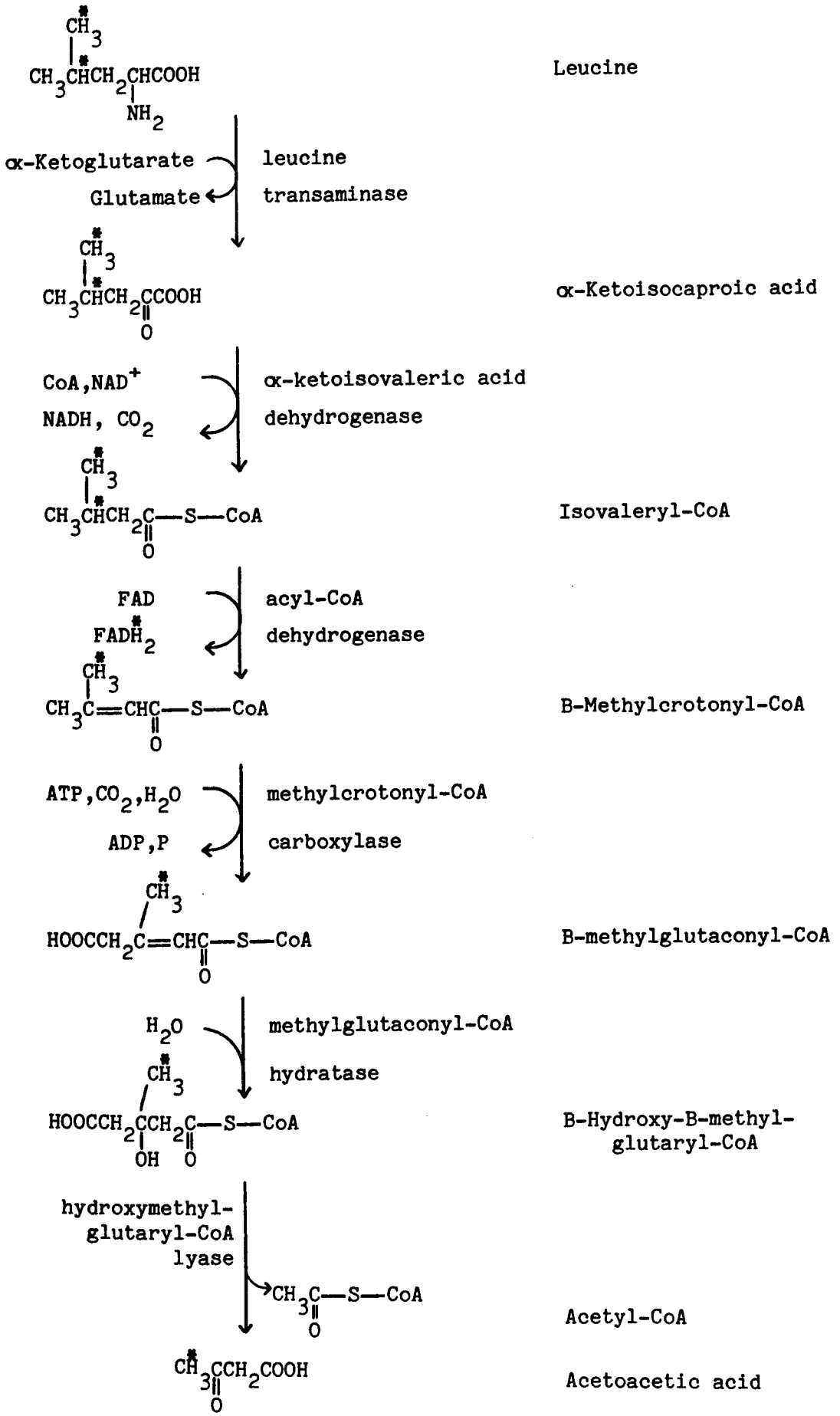
2. The tritium label on the 4,5-position effectively follows the intact carbon skeleton (see Fig 7), since the label is lost during degradation of the molecule and rapidly equilibrates with the water pool.

3. Leucine is a stable amino acid.

1.6.3 Summary of preliminary investigations

The suggestion that the endogenous protein component of ruminant proximal duodenal digesta may ultimately be quantified directly via a continuous intravenous infusion of L-[4,5-^{3}H]-leucine was initially proposed and examined by Peter J. Buttery and John V. Nolan at the University of New England, Armidale, Australia, in 1982. Initial investigations indicated that the technique had considerable potential to

Figure 7 The catabolic pathway of L-leucine



The catabolism of L-leucine and the concomitant release of the tritium label(*), within the rumen. The released label is conceived to rapidly equilibrate with the water pool (from Lehninger, 1975)

enable the endogenous protein component of proximal duodenal digesta to be determined directly.

1.6.4 Thesis objectives and plan

The investigations were designed initially to verify and ultimately to apply the proposed intravenous infusion of L-[4,5-^{3}H]-leucine technique to determine directly the endogenous protein content of ruminant proximal duodenal digesta. The validity of the proposed technique was examined using sheep fed an essentially protein free diet. The method was then applied to investigate any dietary effect on the endogenous protein component of proximal duodenal digesta in sheep fed either hay or a concentrate ration. Estimates were also derived for steers fed a diet of silage supplemented with fishmeal.

The merits and drawbacks of the method are critically examined in the discussion chapter. Potential ways of improving the method are also considered.

Chapter Two

MATERIALS AND METHODS

This section contains details of materials and general methodology common to all experiments. Specific aspects of experimental design and procedure are included in the relevant results section. Except where indicated, all chemicals were obtained from Fisons Scientific Apparatus Plc., Loughborough, Leics., and were of analytical grade wherever appropriate.

Radiochemicals were obtained from Amersham International Plc., Amersham, Bucks, and were used without further purification.

2.1 Experimental diets

Formulation of experimental diets was calculated according to the recommendations detailed in MAFF (1975).

Preparation of experimental diets was undertaken in the Experimental Mill of the University of Nottingham School of Agriculture. Routine analysis of diets was performed in the Analytical Laboratory, Department of Applied Biochemistry and Food Science, according to the methods of the AOAC (1975).

Initial silage analysis was provided by ADAS and later specific toluene corrected dry matter values determined at the Animal and Grassland Research Institute (AGRI), Hurley.

2.2 Experimental animals

All animals used in the study were equipped with a rumen cannula and a proximal duodenal T-piece cannula. At least 3 months elapsed following surgery before introduction to any experimental regimes. During this time the animals were housed in traditional pens and offered maintenance diets. Animals were wormed during this period using an appropriate anthelmintic preparation (sheep-Nemicide, ICI Plc., Macclesfield, Cheshire; steers, Panacur, Hoechst UK Ltd, Milton Keynes).

In order to facilitate adaption and ease of sample collection during the actual experimental period, all animals were routinely handled, accustomed to sampling and maintained for short periods in metabolism crates. Approximately one week before the experimental period animals were gradually adapted to the experimental ration and were then

transferred to metabolism crates. Throughout the experimental period they were maintained under constant light. Water was available ad libitum. Feed was provided via an automatic continuous feeding system, each animal receiving approximately one twelfth of its daily ration at 2 hourly intervals.

2.3 Synopsis of experimental protocol

A general outline of the experimental protocol routinely adopted is given in Fig 8.

Digesta flow markers were intraruminally infused to determine the flow of digesta constituents. L-[4,5-^{3}H]-leucine was intravenously infused to label the body proteins. Samples of rumen fluid, duodenal digesta, saliva and plasma were taken at regular eight hour intervals during the eight day intravenous isotope infusion periods. The amount of label incorporated in various fractions was determined. Measurements of the microbial protein nitrogen flow were determined using DAPA and ^{15}N as markers.

2.4 Cannulation of the jugular veins

A cannula (intravenous cannula set, internal diameter 1.0 mm, Portex Ltd.) was inserted into each jugular vein one day before the start of the isotope infusion. One cannula was used to infuse the isotope and the other to sample the blood. The neck of the animal was shaved around the jugular vein and the area thoroughly washed with the skin sterilant chlorhexidene (0.5% in 70% spirit). A small piece of adhesive tape was fastened to the cannula at the point of entry into the jugular vein. The

Fig. 8 Routine experimental protocol

Pre-experimental		Surgical preparation of animals – rumen and duodenal T-piece cannula
	3 months	Housed in traditional animal pens, maintenance diet, animals wormed and acclimatised to experimental conditions
	1 week	Gradual introduction to experimental diet. Transferred to metabolism crates
	2 weeks	Fed experimental diet via continuous feeders
Experimental period	Day 1	Continuous intraruminal infusion of digesta flow markers commenced – initial administration of primer dose (½ normal daily infusion volume)
	Day 3	Cannulation of the jugular veins
	Day 4	Intravenous infusion of L-[4,5-^{3}H]-leucine commenced (sampling at regular 8 hr intervals of rumen fluid, duodenal digesta, saliva and plasma to day 12)
	Day 6	Intraruminal infusion of [^{15}N]-labelled ammonium sulphate commenced (major steer experiment only)
	Day 7	Urine collection to day 11 (initial sheep experiment only)
	Day 10	12 x 2 hr duodenal digesta samples pooled for digesta flow determination (steer experiments only)
	Day 11	End intraruminal marker infusions. Samples of rumen fluid taken for Cr decay determination
	Day 12	End intravenous infusion of L-[4,5-^{3}H]-leucine

cannula was then secured in place using a suture through the tape and skin. The cannulae were then trained along the back of the animal and firmly secured in position. The infusion cannula was extended with sterile manometer tubes (Letrocath, 150 cm, internal diameter 2 mm, Vygon, France). A peristaltic infusion pump (Miniplus 2, Gilson, France) and proportioning tubing (Chemlab Instruments, England) were used to deliver the infusate via 0.86 mm diameter infusion tubing (Portex UK Ltd, Hythe, Sussex) at a constant rate. All tubing was flushed with chlorhexidene and rinsed with sterile water immediately prior to the infusion. The cannulae were successfully maintained throughout the eight day duration of the experimental period.

2.5 Infusion of L-[4,5-^{3}H]-leucine

The L-[4,5-^{3}H]-leucine and carrier leucine were injected into 550 ml sterile saline bags (Viaflex, Travenol Laboratories Ltd, Norfolk, England) in the sterile environment of a laminar flow cabinet. The carrier leucine solution was autoclaved before administration and was included at a concentration equivalent to that in plasma. The saline bags were routinely prepared and replaced each day and the exact volume delivered determined by the change in weight.

2.6 Sampling of body fluids

All sampling was carried out at regular eight hour intervals throughout the eight day infusion period. After initial processing all samples were stored at -15°C.

2.6.1 Rumen digesta

Strained rumen fluid (SRF) samples were obtained by suction using a sampling device (see Fig 9) and a 60 ml syringe. Initially 40 ml were withdrawn and returned to the rumen in order to flush the sampler. A 50 ml portion was then withdrawn, transferred to a 50 ml polythene centrifuge tube and retained in ice prior to fractionation (see section 2.6.3). The pH of SRF was obtained immediately after collection (Pye Model 78 pH meter, Cambridge).

2.6.2 Duodenal digesta

Duodenal digesta was collected by gravity from the T-piece cannula. The cannulae were inserted in the proximal duodenum on the latero-ventral side of the abdomen. At each sampling interval the first 10 ml of digesta was discarded as being unrepresentative. Approximately 80 ml of digesta were then collected with sheep and 120 ml during steer experiments, the exact volume depending on specific sample requirements. Samples were thoroughly mixed and a 50 ml portion taken for the isolation of the microbial fraction (see section 2.6.3). The remainder of the digesta was frozen and freeze dried.

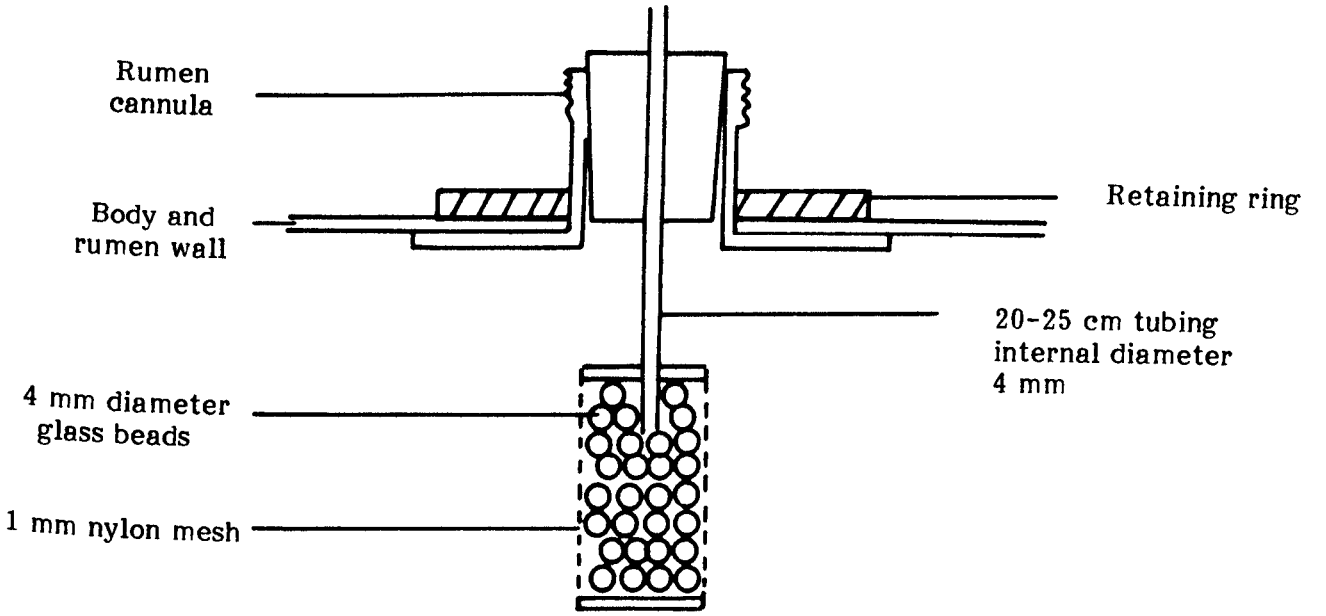
2.6.3 Fractionation of digesta samples

Freshly collected fully mixed duodenal digesta and strained rumen fluid were treated as follows to obtain a microbial fraction, using a modification of the method as described by Mathers and Miller (1980).

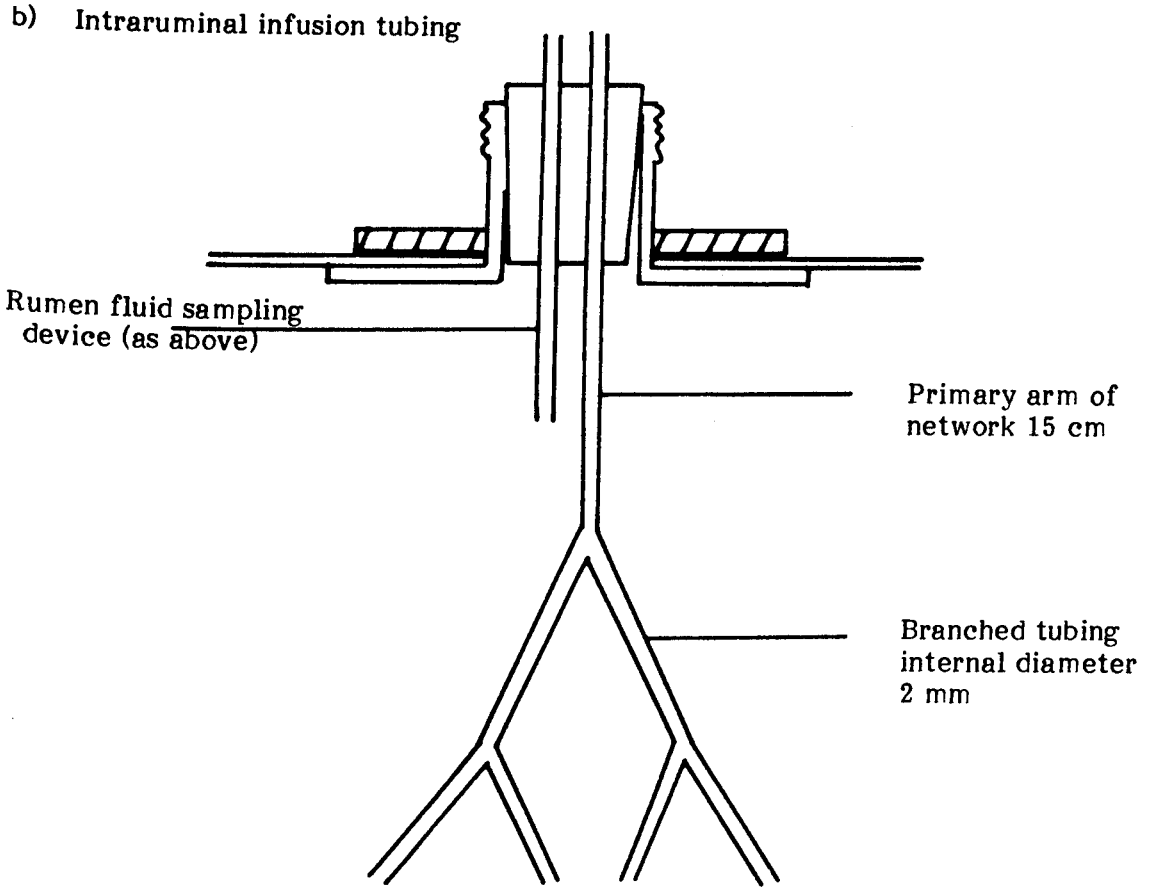
A 50 ml sample was initially centrifuged (MSE Model 18, MSE Ltd, London) to obtain a microbial fraction (1000 g, 3 min, 4°C). The

Fig 9 In situ strained rumen fluid (SRF) sampling device and intraruminal infusion tubing

a) Strained rumen fluid (SRF) sampling device



b) Intraruminal infusion tubing



The tubing was flexible allowing the sampling device and the infusion tubing to move freely within the rumen

Table 3 Composition of McDougall's Buffer (McDougall, 1948)

Salt	g/litre
$NaHCO_3$	9.80
$Na_2HPO_4.12H_2O$	9.30
NaCl	0.47
KCl	0.57
$CaCl_2$ (anhydrous)	0.04
$MgCl_2$ (anhydrous)	0.06

McDougall's Buffer was prepared and stored at 0°C. During the preparation the $CaCl_2$ was dissolved in approximately 50 ml of DW, added to the mixture already in a volume of 900 ml and the volume made up to 1 litre. Immediately before use the buffer was gassed with CO_2 until the pH was 6.8.

supernatant was decanted into a 50 ml polythene centrifuge tube and centrifuged (35,000 g, 30 min, 4°C). A portion of clear supernatant was immediately acidified (0.2 ml of 10M HCl to approximately 25 ml of SRF), frozen and retained at -15°C for ammonia determination and chromium analysis. The pellet was resuspended in McDougall's buffer (McDougall, 1948) (Table 3) and centrifuged again (35,000 g, 30 min, 4°C). The washings were discarded and the bacterial pellet immediately frozen and freeze dried (EF4 Modulyo freeze drier, Edwards High Vacuum, Crawley, Sussex). Routine microscopic examination of the pellet identified a pure bacterial fraction.

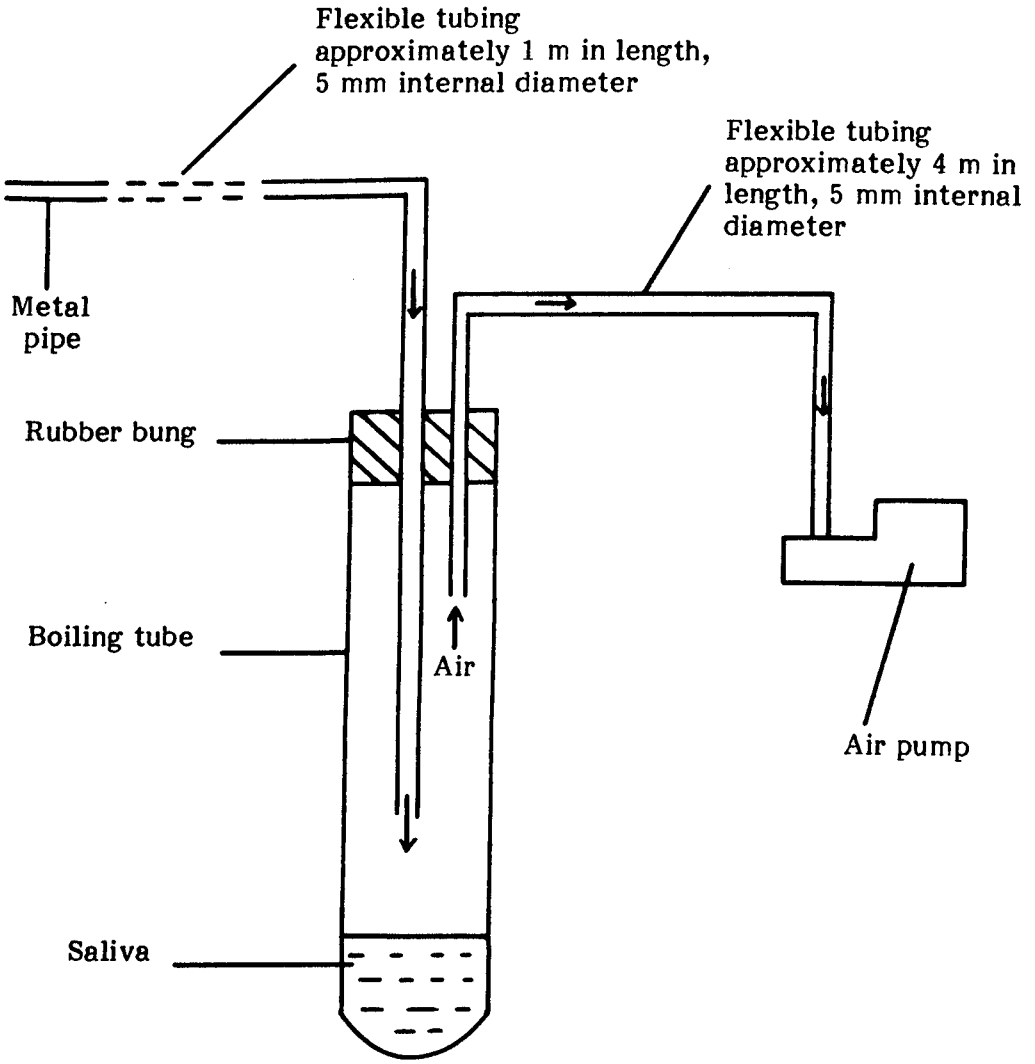
2.6.4 Isolation of protozoal fraction

The initial pellet obtained during fractionation of strained rumen fluid samples was resuspended in 100 ml of McDougall's buffer (pH 6.8, Table 3) and added to a separating funnel incubated in a water bath (39°C). The suspension was deoxygenated and agitated with N_2 gas and then 15 min allowed for separation. The lower protozoal fraction was collected in a polythene centrifuge tube and the remainder discarded. The protozoal fraction was returned to the separation funnel and the procedure repeated twice. Final protozoal fractions were shown to be pure when examined by light microscopy.

2.6.5 Saliva

A saliva sample was collected using a simple suction system (Fig 10) incorporating a metal tube which was placed in the buccal cavity between the lower mandible and the cheek. Approximately 10 ml of saliva

Fig 10 Schematic diagram of saliva collection apparatus



were collected, immediately centrifuged (25,000 g, 4°C, 15 min) to remove feed particles, desquamated cells and bacteria, and frozen.

2.6.6 Plasma

All tubes were rinsed in heparinised saline (37.5 IU/ml). A sterile 10 ml syringe was used to withdraw blood via the sample cannula. An initial 5 ml was withdrawn and discarded. A further 10 ml was withdrawn and the plasma isolated by centrifugation (1000 g, 10 min, MSE Centaur 2, bench centrifuge). The sample cannula was flushed with 10 ml sterile heparinised saline.

2.7 Amino acid analysis

2.7.1 Amino acid analysis of protein fractions

The routine procedure and initial stages of sample preparation are detailed below (see Table 4).

2.7.1.1 Routine procedure

All protein fractions were hydrolysed under reflux at 110°C for 22 hr in 6 MHCl. A quantity of DL-norleucine (Sigma Chemicals Co., Poole, Dorset) was used as an internal standard (see Table 4 and section 2.9). The whole hydrolysate or an appropriate aliquot were rotary evaporated to dryness under reduced pressure at 37°C (Bachi, Rotavapor R110, Switzerland) and the dried residue washed three times with distilled water (approximately 5 ml) to ensure removal of all tritiated water. The final residue was dissolved in lithium loading buffer (pH 2.2) (Table 5), filtered prior to analysis (0.2 µm, cellulose nitrate, Millipore UK, London)

Table 4 Summary of protein hydrolysis procedure and sample preparation for amino acid analysis

Sample	Hydrolysis volume (ml) /weight (g)	Volume of 6 MHCl (ml)	Volume (ml) hydrolysate made up to after filtration	Volume of aliquot (ml)	µmol of DL-norleucine internal standard added	Volume of lithium loading buffer (ml)	Dilution for amino acid analysis	µmol/ml external standard (TEA) in final residue*
Diet	0.3	50	250	20	1	5	-	-
Rumen/ duodenal bacteria	0.1	50	A	A	10	5	x10	0.4
Duodenal digesta	0.2	50	A	A	10	5	x10	0.4
Plasma protein	TCA ppt from 2.0 ml plasma	50	A	A	50	5	x10	0.4
Saliva dialysate	4	50	A	A	0.4	2	x 2	0.4

* See section 2.9

All protein fractions were hydrolysed under reflux at 100°C for 22 hr. DL-norleucine was added as an internal standard. The whole hydrolysate (indicated by 'A') or a smaller aliquot was rotary evaporated (37°C) to dryness. The final residue was dissolved in lithium loading buffer (pH 2.2, Table 5) and where appropriate a quantity of the external standard TEA added to the final sample. The use of TEA in addition to DL-norleucine is described in the text (see section 2.9).

Table 5 Composition of LKB lithium loading buffer, pH 2.2

Constituent	per litre
Citric acid.H_2O	9.6 g
$LiOH.H_2O$	8.4 g
Phenol	1.0 g
Thiodiglycol	20 ml
HCl (SG 1.16)	16 ml

and stored at 1°C. A quantity of an external standard B-2-Thienyl-D,L-alanine (TEA) (Sigma Chemical Co., Poole, Dorset) was added to the final residue (Table 4) (see section 2.9 for comments). Internal and external standards were included at appropriate concentrations to give peak areas on the chromatogram similar to those of the amino acids in the sample.

The amino acid concentrations of all protein fractions were analysed on the LKB 4400 automatic amino acid analyser (LKB Instruments, London). A modified program was developed to allow rapid determination of isoleucine, leucine, norleucine and TEA concentrations where full amino acid profiles were not required (see Appendix 1).

2.7.1.2 Sample preparation

(a) Diets

Appropriate weights were hydrolysed, filtered, made up to a known volume, an aliquot taken and prepared as indicated in Table 4.

(b) Duodenal digesta, rumen and duodenal microbial fractions

A known weight of dry duodenal digesta (approximately 0.4 g) and rumen/duodenal microbial isolates (approximately 100 mg) were hydrolysed (see Table 4). A fraction of the final residue dissolved in Li^+ buffer was used for DAPA analysis. Further dilution of the sample was required for amino acid determination.

(c) Salivary protein

A 5 ml portion of saliva was dialysed (Dialysis tubing, Medicell International, London) for 24 hr against two changes of distilled water (approximately 5 litres). The dialysed saliva was assumed to be devoid of

free amino acids, peptides and salts. A 4.0 ml portion of the dialysate was hydrolysed.

(d) Plasma protein

Plasma proteins were isolated by precipitation with trichloroacetic acid (TCA) (20% w/v). A 2.0 ml sample of plasma was placed in a 50 ml polythene centrifuge tube and 2 ml of TCA (20% w/v) added. The contents were thoroughly mixed and allowed to stand in ice for 30 min. After centrifugation (1000 g, 10 min) the supernatant was discarded and the pellet washed twice in TCA (20% w/v). DL-norleucine (Sigma Chemicals Co., Poole, Dorset) (5 ml of 10 mM) was added to the final pellet, the pellet resuspended and washed into a round bottom flask with 50 ml 6 MHCl.

2.7.1.3 The recovery of L-leucine after acid hydrolysis

The recovery of L-leucine (Sigma Chemicals Co., Poole, Dorset) after acid hydrolysis has been routinely assessed in this laboratory and found to be satisfactory. An exact weight of digesta (0.2 g), containing approximately 30 µmol leucine, was assayed separately in the presence of added leucine (5-10 µmol). The extra leucine content was determined and expressed as a percentage of the leucine added. The mean ($\pm$ SEM, n = 6) recovery of leucine was 100.7 $\pm$ 0.73%.

2.7.2 Analysis of plasma free amino acids by picric acid precipitation

The concentration of plasma free amino acids was determined by precipitation of the protein with picric acid. The picric acid was removed by anion exchange chromatography and analysis then carried out using a split stream automatic analyser.

2.7.2.1 Preparation of anion exchange resin column for removal of picric acid

A suspension of Dowex AG-2X8 resin (Biorad, USA) was prepared in 1M HCl. The resin, supported by glass wool, was poured into a glass chromatograph column sufficient to form a resin bed 50 x 8 mm. The fines were removed and the column washed with approximately 50 ml of deionised water until the pH of the effluent was greater than 4.

2.7.2.2 Sample preparation

A 3.0 ml sample of plasma was placed in a 50 ml polythene centrifuge tube, DL-norleucine (0.4 ml, 1 mM) added as an internal standard, then frozen and freeze dried. Picric acid (10 ml, 1% w/v) was added to the freeze dried residue, the contents thoroughly mixed and allowed to stand at 4°C for 1 hr. The mixture was centrifuged (1000 g, 10 min) and the supernatant poured onto the previously prepared anion exchange column. The mixture was allowed to pass down the column and the effluent collected in a round bottom flask. A further 10 ml of picric acid was added to the centrifuge tube, the pellet resuspended, and the procedure repeated. The column was then washed with 5 ml of 0.02M HCl. The effluent from the column was rotary evaporated (37°C) to dryness and washed three times with distilled water to remove any tritiated water. The washed dry residue was dissolved in 2.0 ml of lithium sample buffer (pH 2.2) (Table 5).

2.7.2.3 The recovery of plasma free leucine

The recovery of L-leucine after plasma protein precipitation with picric acid has been routinely determined in this laboratory and found to be

satisfactory. A volume of 3.0 ml plasma containing approximately 0.35 µmol leucine, was assayed separately and in the presence of added leucine (0.1-0.2 µmol). The extra leucine content was determined and expressed as a percentage of the leucine added. The mean (± SEM, n = 6) recovery of leucine was 101.9 ± 1.49%.

2.7.2.4 Preparative amino acid analysis for the separation and determination of L-[4,5-^{3}H]-leucine specific activity in plasma free amino acid preparation

Radioactively labelled leucine was isolated and collected using a split stream ion exchange chromatographic technique, based on the use of a modified Evans Electroselenium (EEL) Model 294 amino acid analyser (Halstead, Essex) fitted with a Locarte autoloader, timers and column (Locarte Co., London). Details of the system are presented in Fig 11 and Table 6. The peristaltic pump (Miniplus 2, Gilson, France) was adjusted to deliver a known quantity (approximately 80%) of the total flow from the column to the fraction collector (Ultrovac 2070, LKB Instruments, S. Croydon, Surrey). The flow rate from the column was routinely checked and the proportion delivered to the fraction collector determined for each run. A known volume of sample (approximately 0.5 ml) and TEA (0.2 ml, 1 mM) were loaded onto the column via the Locarte autoloaders, and the split stream fraction collected between 80-110 min after loading which represented the elution of counts in the leucine region. Fractions were collected directly into scintillation vials (Hughes and Hughes, Romford, Essex) and approximately 5 ml of a xylene-based scintillant (Optiphase X) added. The corresponding peaks on the recorder chart from the colorimetric reaction with ninhydrin appeared between 100-130 min and were integrated directly.

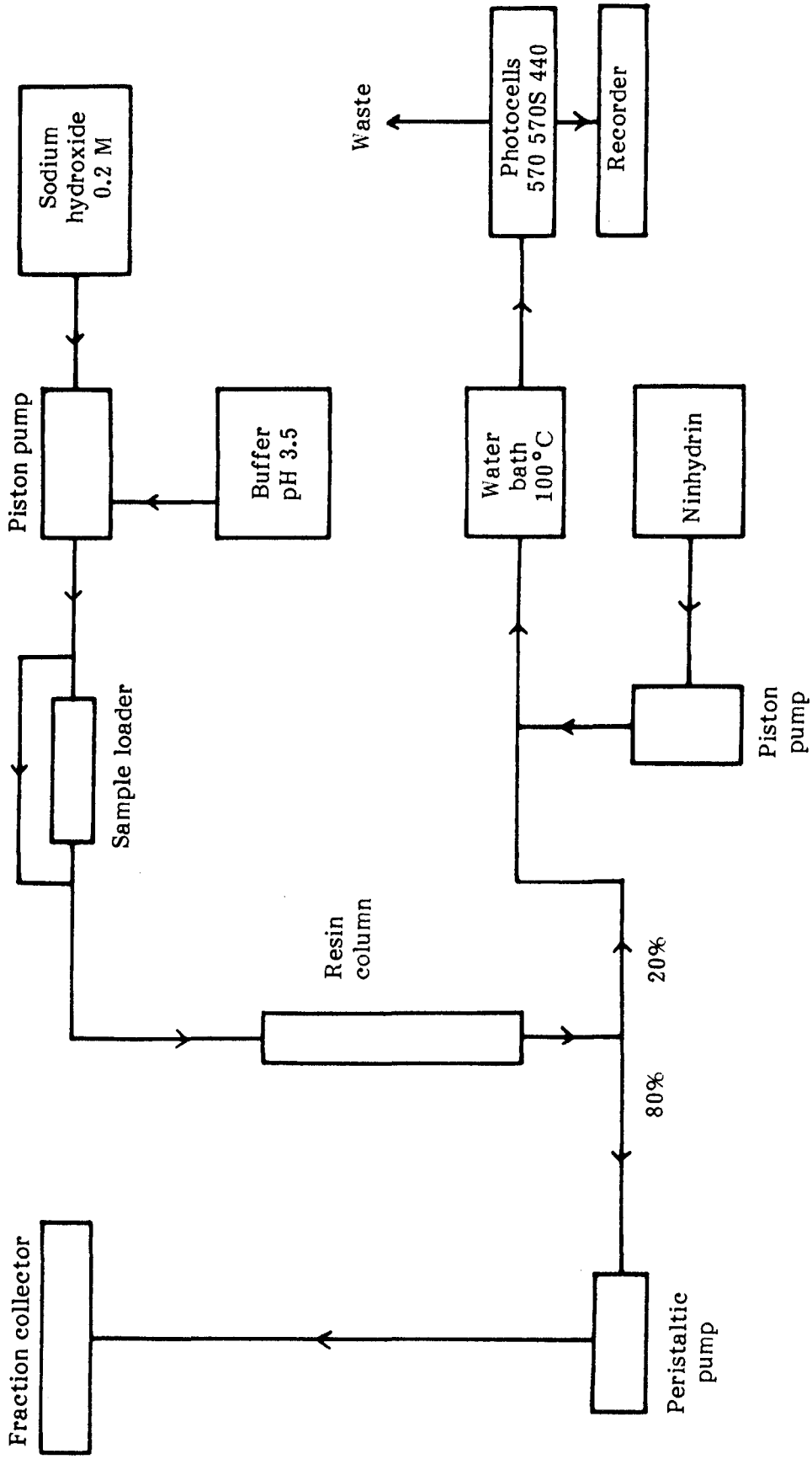


Fig 11 *Schematic diagram of split stream amino acid analyser*

Table 6 Details of split stream amino acid analyser

Model type	EEL Model 294
Resin	Aminex A5 (Biorad, USA)
Column height	200 mm
Column diameter	9 mm
Column temperature	29 °C
Buffer	Lithium 3.65
Buffer run time	110 min
Regneration time	20 min
Equilibration time	60 min
Buffer pressure	12-14 Kp
Ninhydrin pressure	213-220 Kp
Buffer flow rate	0.64 ml/min
Ninhydrin flow rate	0.5 ml/min
Fraction collection time	1.5 min

2.8 Determination of radioactivity

During the present study radioactivity present in the protein fractions was shown for all practical purposes to be associated only with leucine. A hydrolysed sample was counted directly (see below) and also after isolating the leucine fraction of an equivalent volume of the same hydrolysate using preparative amino acid analysis (section 2.7.2.4). The activity associated with the leucine fraction was compared with that of the total hydrolysate. No activity was detected in any other amino acid fraction. The mean ($\pm$ SEM, n = 8) recovery of radioactivity corresponding to leucine was 98.0 $\pm$ 0.78.

Appropriate samples of protein hydrolysates (0.2-1.0 ml) were transferred to scintillation vials, scintillant added (10 : 1 v/v, scintillant : sample), the vials shaken and radioactivity subsequently determined. Radioactivity in all samples was determined by liquid scintillation counting using a Tri-Carb 4640 counter (Packard Instrument Company Inc., USA). Samples were compared against an external standard, automatically corrected for quenching and finally expressed as disintegrations per minute (dpm).

2.9 Determination of specific activity

The specific activity (dpm/µmol) of a sample was determined as the ratio of the radioactivity : leucine content in the final sample. Radioactivity was determined directly as described in the previous section. The leucine concentration was determined using internal and external standards (Table 4). The internal standard DL-norleucine was used to estimate the concentration of leucine in the original sample. The external

standard TEA, added to the final residue, was used to determine the amount of leucine in the sample applied to the preparative column, in order to calculate the specific activity.

$$\text{Specific activity (dpm/}\mu\text{mol)} = \frac{\text{dpm}}{\text{leucine concentration (TEA)}}$$

The leucine content of all protein hydrolysates was analysed using an LKB 4400 automatic amino acid analyser (LKB Instruments, London) (section 2.7.1). Plasma free leucine was analysed on the EEL analyser (section 2.7.2.4).

2.10 Dry matter flow determination

2.10.1 Development of methods

Classical techniques in ruminant nutrition have often employed lignin as a suitably inert internal marker. Dry matter flow is calculated from the lignin intake and the concentration of lignin in the digesta. Recent attempts to determine dry matter flow more precisely concern the use of the dual-phase marker technique (Faichney, 1975). During the period of this investigation considerable developments have been made in this laboratory concerning the method of dry matter flow determination.

2.10.2 Preparation of digesta flow markers

2.10.2.1 Liquid phase marker — Chromium EDTA (Cr-EDTA)

Cr-EDTA was prepared as described by Binnerts et al. (1968). A suspension of 40 g disodium diaminoethanetetra-acetic acid (EDTA) in 600 ml distilled water (DW) was added to a solution of 28.4 g $CrCl_3$ in

400 ml distilled water. The mixture was boiled for 1 hr, allowed to cool and excess EDTA removed by the addition of 8 mls 1 M $CaCl_2$. The pH was adjusted to 7.0 by dropwise addition of 40% (w/v) NaOH, the solution filtered (Whatman No. 1 filter paper), and made up to 1 litre with distilled water.

Chromium was infused at a rate of 250 mg Cr per kg dry matter intake.

2.10.2.2 Solid phasemarkers

(a) Ruthenium phenanthroline

The ruthenium (ii) phenanthroline complex was prepared as described by Tan et al. (1971). Ruthenium chloride xH_20,3g, (Johnson Mathey Chemicals, Royston, Herts), 2.16 g of potassium chloride, 480 ml absolute alcohol and 420 ml 0.2M HCl were added to a 5 litre round bottom flask along with antibumping granules and refluxed for 20 min. The mixture was cooled, the apparatus converted to distilling mode and a distillate volume of 480 ml collected. The flask was again cooled, 8.4 g of 1,10 phenanthroline hydrate and 2.1 g of sodium hypophosphite added, the condenser returned to reflux mode, and the contents further refluxed for 4 hr, swirling occasionally. The final solution was allowed to cool, filtered and made up to 600 ml with distilled water.

Ruthenium was infused at a rate of 20 mg Ru per kg dry matter intake.

(b) Ytterbium acetate

A solution of ytterbium acetate (Rare Earth Products Ltd, Widnes, Cheshire) was prepared as described by Siddons et al. (1985b).

Ytterbium was infused at a rate of 50 mg Yb per kg dry matter intake. The ytterbium acetate was dissolved in distilled water and infused separately from the Cr-EDTA solution.

The digesta flow markers were intraruminally infused for a period of 4-5 days prior to the commencement of the intravenous infusion and sampling regime, in order that equilibrium concentrations be achieved (see Fig 8). A primer dose of half the daily dose was administered at the onset of the intraruminal infusion (Weston and Hogan, 1967).

2.10.3 Analysis of digesta flow markers

2.10.3.1 Determination of chromium concentration in liquid samples

The chromium concentration of infusate solutions, rumen and duodenal fluids and urine was determined directly by atomic absorption spectrophotometry (Varian AA-1275, Japan). The operating conditions of the instrument are outlined in Table 7. Standards were prepared from potassium dichromate over an appropriate range dependent on the concentration of chromium in the samples. Calibration curves were consistently linear.

2.10.3.2 Determination of chromium in oven dry duodenal digesta samples

Chromium in digesta was extracted by a procedure similar to that described by Christian and Coup (1954) modified by Siddons et al. (1985b). Approximately 0.5 g of digesta was ashed in a conical flask at 550°C for 16 hr and 6 ml digestion acid (250 ml orthophosphoric acid (880 g/L), 50 ml manganese sulphate (100 g/L), 250 ml concentrated sulphuric acid per litre) added to the ash. The mixture was heated to boiling and 3 ml potassium

Table 7 Operating conditions of Varian 1275 atomic absorption spectrophotometer

	Chromium	Ytterbium
Wavelength	357.9 nm	398.8 nm
Spectral band pass	0.1 nm	0.5 nm
Fuel	acetylene	acetylene
Support	nitrous oxide	nitrous oxide
Lamp	7 mA	7 mA

bromate solution (45 g/L) added. Heating was continued until the solution turned a deep purple and white fumes evolved after which the mixture was heated for a further 3 min and then allowed to cool. After diluting to 100 ml with distilled water, the chromium content was measured using standards of potassium dichromate, dissolved in digestion acid (60 ml/L).

2.10.3.3 Determination of ruthenium and chromium in solid duodenal digesta by x-ray fluorescence spectrophotometry (XRFS)

(a) Preparation of samples

Freeze dried duodenal digesta was finely ground and individual samples pooled on an equal weight basis to provide four replicates per animal. Portions of pooled digesta (4.0 g) were compressed into self supporting discs using tungsten carbide plattens, in a 32 mm die, using a hydraulic press adjusted to deliver 10 tonnes pressure. The discs were prepared without a briquetting aid. Developments in the application of the dual phase marker technique and increased sample availability subsequently resulted in an improved method of digesta preparation (see section 2.10.3.4 a)

(b) Preparation of standards

Duodenal digesta was collected from the sheep fed the experimental ration prior to the commencement of the infusion. The digesta was frozen, freeze dried and finely ground and a series of 4.0 g portions weighed into plastic bottles to which increment volumes of infusate were added to produce a calibration curve. The standards were frozen, freeze dried, thoroughly mixed and prepared into discs.

(c) Operating conditions of XRFS

The procedure adopted was based on that of Evans et al. (1977) incorporating a Phillips all-vacuum x-ray fluorescence spectrophotometer (XRFS) PW 1400. The operating conditions of the instrument are outlined in Table 8. This analysis was carried out for the author at the Department of Geology, University of Nottingham.

2.10.3.4 Determination of ytterbium concentration in solid digesta

The method adopted is based on that used routinely at the AGRI, Hurley, as described by Siddons et al. (1985b).

(a) Preparation of samples

Steers were sampled for duodenal digesta every 2 hr over a 24 hr period and individual samples bulked on an equal volume basis. The pooled digesta was thoroughly mixed and subsequently fractionated to obtain fluid rich (FR) and particle rich (PR) components. An appropriate portion of the pooled digesta was cetrifuged (1500 g, 10 min) and a particle rich fraction obtained (PR), the resulting supernatant removed, and added back to the original pooled digesta (FR). Samples were frozen and freeze dried.

Triplicate portions (approximately 0.53 g) of finely ground freeze dried fractionated digesta samples were accurately weighed into 50 ml Pyrex glass bottles (Fisons Scientific Apparatus, Plc.) and dried overnight at 100°C. Dry matter was determined and the samples then ashed at 550°C in a muffle furnace for 16 hours. After cooling the ash was extracted with 10 ml of 0.46 M HNO_3 containing 1000 ppm potassium (as potassium chloride) for 24 hr on a rotary shaker (Model G-33, New Brunswick Scientific Co., New Jersey). The mixture was centrifuged

Table 8 Operating conditions of Phillips PW 1400 XRFS for ruthenium and chromium analysis in whole duodenal digesta samples

	Ruthenium	Chromium
Counter	scintillation	scintillation
Counting time (secs)		
Peak	80	40
Background	40 +ve/-ve	40 -ve
Angle (20°C)		
Peak	69.34	16.31
X-ray tube	Chromium	Rhodium
Tube voltage (kv)	80	50
Tube current (mA)	25	60
Collimator (Lithium fluoride 200 crystal)	Fine	Fine

(1000 g, 10 min) and the supernatants analysed using atomic absorption spectrophotometry (see Table 7 for operating conditions) against the standards prepared as indicated in the following section.

Sheep digesta was prepared in exactly the same manner except samples were pooled over a 5 day period and the resultant supernatant from the PR fraction was not added back to the FR digesta.

(b) Preparation of standards

Standards used in the analysis of samples must be matrix matched. Thus for each dietary regime blank digesta was obtained prior to the marker infusion. The blank digesta was fractionated, frozen, freeze dried and ground in an identical manner to the samples.

Quadruplicate samples of blank digesta (approximately 0.53 g) of PR and FR fractions were prepared and appropriate volumes of a ytterbium solution (50 µg/ml) and distilled water added to give a final concentration range of 0-9 µg Yb/ml (see Table 9). Standards were mixed by gently shaking and then dried and extracted exactly as the samples. Attention was directed to ensure sample weights corresponded to standard weights in order to maintain a similar digesta : acid ratio and hence maximise matrix matching.

Infusion solutions were diluted in nitric acid/DW to obtain a final concentration of 0.46 M HNO_3 1000 ppm potassium and were analysed against a set of standards prepared in 0.46 M HNO_3 1000 ppm potassium.

2.10.3.5 Determination of lignin in duodenal digesta and diet

The method was based upon that of Van Soest (1963). Approximately 1 g of air dried sample was weighed into a 600 ml beaker and 100 ml of 1% cetyltrimethyl ammonium bromide (10 g CTAB in 1 L

Table 9 Preparation of ytterbium standards

Approximate weight of blank digesta (g)	Volume ytterbium acetate solution (50 µg Yb/ml) added (ml)	Volume DW added (ml)	Extraction volume of acid (ml)	Final concentration ytterbium (µg/ml)
0.53	-	5	10	-
0.53	0.6	4.4	10	3
0.53	1.2	3.8	10	6
0.53	1.8	3.2	10	9

Appropriate volumes of ytterbium acetate solution (50 µg Yb/ml) were added to approximately 0.53 g of freeze dried blank digesta, mixed, dried, ashed and extracted with acid as described in the text (section 2.10.3.4 a)

of 0.5 M H_2SO_4) solution added. The mixture was gently refluxed for 2 hr, using a round bottom flask as a condenser. The contents of the beaker were then filtered under vacuum through a weighed, sintered Pyrex crucible (porosity No. 1), the residue washed three times with hot distilled water and allowed to air dry. The crucible was then placed in a suitable receptacle and sufficient 26.8 M H_2SO_4 added to cover the residue. The contents were stirred with a glass rod and the acid allowed to slowly perculate throught the sinter for 3 hr, adding acid when required to keep the residue covered. The crucible was then returned to the suction flask, the acid removed, and the residue washed with warm distilled water to remove all traces of acid. Samples were dried overnight at 100°C, cooled in a dessicator, weighed, and ashed at 550°C for a minimum of 4 hr. The crucibles were cooled and reweighed.

Calculation:

$$\text{\% detergent lignin} = \frac{\text{(weight of crucible + residue) - (weight of crucible + ash)}}{\text{Sample weight}} \times 100$$

2.11 Determination of the proportion of microbial protein in duodenal digesta

2.11.1 2,6-Diaminopimelic acid (DAPA) determination

DAPA was routinely used as a microbial marker in all investigations. The technique was based on the original method of Hutton et al. (1971), modified by Ling (1976).

Calculation:

$$\text{Bacterial nitrogen flow (g/day)} = \text{DAPA flow (g/day)} \times \frac{\text{Bacterial N\%}}{\text{Bacterial DAPA\%}}$$

2.11.1.1 Acid hydrolysis

Appropriate weights of rumen microbial and duodenal digesta samples were hydrolysed, rotary evaporated and prepared as indicated in section 2.7.1.

2.11.1.2 DAPA analysis

A simple amino acid analyser was constructed as described by Ling (1976) (see diagram Fig 12 and Table 10, details of analyser system). A constant sample volume was loaded on the column, eluted with pH 3.3 citrate buffer and peak areas obtained from the linear output by weighing the cut out peaks. A series of DAPA standards (0.12-0.36 µmol) (Sigma Chemicals Co., Poole, Dorset) were routinely analysed and the standard curve obtained was consistently linear.

As a routine procedure an exact weight of digesta (0.2 g), containing approximately 0.15 µmol DAPA, was assayed separately and in the presence of added DAPA (0.1-0.3 µmol). The extra DAPA was determined and expressed as a percentage of the DAPA added. The mean ($\pm$ SEM, n = 6) recovery of DAPA was 101.1 $\pm$ 1.24%.

2.11.2 [^{15}N]-labelled diammonium sulphate (^{15}N) determination

For comparative purposes ^{15}N in addition to DAPA was employed as a microbial marker in the major steer investigation.

2.11.2.1 Preparation of infusate

A combined solution of $^{15}(NH_4)_2SO_4$ (7% atoms enrichment ^{15}N; 200 mg ^{15}N/day) prepared using 98% and 5% atom enrichment

Fig 12 Schematic diagram of analyser system used for diaminopimelic acid (DAPA analysis

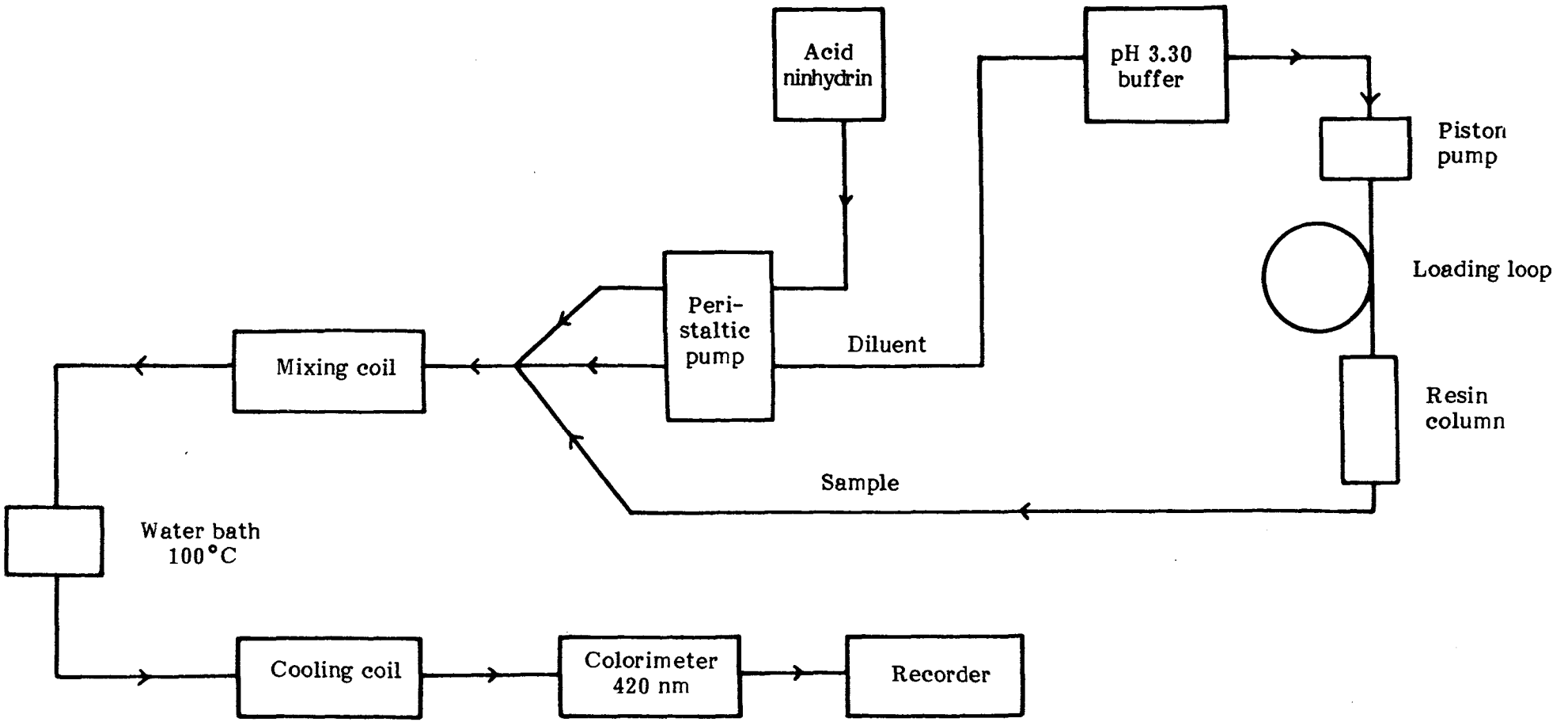


Table 10 Details of analyser system used for diaminopimelic acid (DAPA) analysis

Resin	Aminex A5 (Biorad, USA)
Column height	125 mm
Column diameter	9 mm
Column temperature	50°C
Buffer	Na citrate pH 3.30
Regeneration	0.2 M NaOH
Buffer pressure	80-120 lb/in^2
Buffer flow rate	1.10 ml/min
Diluent and ninhydrin flow rate	1.10 ml/min
Reaction coil	17 m at 100°C
Colorimeter	Vitatron (Fisons) Filter 410 nm
Recorder	Vitatron (Fisons)
Chart speed	0.25 cm/min

Reagent composition

Buffer pH 3.30

Constituent	per litre
Sodium citrate	19.6 g
Polyoxyethylene lauryl ether	0.33 g
Thiodiglycol	5 ml
Caprylic acid	0.1 ml

Acid ninhydrin

Constituent	per litre
Ninhydrin	25 g
Glacial acetic acid	600 ml
Orthophosphoric acid (0.6 M)	400 ml

$^{15}(NH_4)_2SO_4$ and Yb acetate (50 mg Yb per kg dry matter intake) was intra-ruminally infused during days 6 to 11 of the experimental period (see Fig 8). The Yb acetate was dissolved in DW, filtered (0.2 µm, cellulose nitrate, Millipore UK, London), $^{15}(NH_4)_2SO_4$ added and the final solution stored at 1°C. Prior to the infusion samples of 'blank' duodenal digesta and rumen microbes were obtained (see sections 2.6.1-2.6.3) and stored at -15°C.

2.11.2.2 Sample preparation and analysis

Samples for ^{15}N duodenal digesta non ammonia nitrogen (^{15}NAN) enrichment and rumen microbial ^{15}N enrichment determination were prepared according to the methods of the United Nations International Atomic Energy Agency (see IAEA (1975) and Siddons et al. (1985c)). The nitrogen content of the rumen microbial samples was determined using a Carlo Erba NA 1500 (Erba Science UK Ltd) nitrogen analyser and microbial ^{15}N and duodenal digesta ^{15}NAN % enrichments were analysed using a mass spectrometer (VG MM622, VG Isogas Ltd). This analysis was carried out at the AGRI, Hurley.

Calculation:

a. Proportion of microbial N in duodenal NAN $= \frac{\text{\% enrichment duodenal NAN}}{\text{\% enrichment microbial N}}$

b. Microbial N flow (g/day) = a. x total duodenal NAN flow (g/day)

2.12 Organic matter determination

An accurately determined oven dry weight of sample was placed in a pre-weighed Vitreocil crucible (Fisons Scientific Apparatus Plc.) and ashed in a muffle furnace at 550°C for 16 hr. The sample was cooled in a vacuum dessicator and reweighed.

Calculation:

$$\text{Organic matter \%} = 100 - \text{\% Ash} \qquad \text{(AOAC, 1975)}$$

2.13 Modified acid detergent (MAD) fibre determination

Approximately 1 g of air dried sample was weighed into a 600 ml beaker, 100 ml 1% CTAB solution added (10 g CTAB in 1 L of $0.5MH_2SO_4$) and the mixture gently refluxed for 2 hr. The contents of the beaker were filtered under vacuum through a weighed sintered crucible (porosity No. 1), the residue washed three times with hot distilled water and acetone and then sucked dry. The samples were finally dried overnight at 100°C, cooled and reweighed.

Calculation:

$$\text{\% MAD fibre} = \frac{\text{Extracted sample weight (g)}}{\text{Dry sample weight (g)}} \times 100$$

2.14 Nitrogen analysis

2.14.1 Total nitrogen

2.14.1.1 Solids

The nitrogen content of diets, digesta and rumen microbes was determined using the micro-Kjeldahl technique (Tecator Digestion System 20, Bristol).

An accurate amount of a representative sample (0.1-0.5 g) was digested in 10 ml of concentrated sulphuric acid (SG 1.84) with a copper based catalyst (Kjeltab CK, Thompson and Cooper Ltd, Runcorn, Cheshire) at 420°C. The samples were heated for a further 20 min after the formation of a green digest and then removed from the heating mantle. The addition of a small volume of DW (approximately 20 ml) during cooling prevented the digests solidifying. The solutions were then made up to a final volume of 100 ml with DW and subsequently diluted to a final ammonia nitrogen concentration of 5-25 μg NH_3.N per ml.

2.14.1.2 Saliva nitrogen

A 5.0 ml portion of saliva was dialysed as described (section 2.7.12.3), retained in dialysis tubing and digested with 2 ml of concentrated sulphuric acid (as above). The digest was made up to 50 ml.

2.14.1.3 Glycine standards

As a routine procedure two glycine standards (0.1 g) were analysed with each batch of samples digested. The mean ($\pm$ SEM, n = 6) recovery of glycine nitrogen (18.66 g N per 100 g sample) was 99.9 $\pm$ 1.30%.

2.14.2 Determination of ammonia nitrogen content of digests

The ammonia nitrogen content of the digests was determined by the phenol-hypochlorite reaction (Davidson et al., 1970) on a Technicon Autoanalyser (Technicon Instruments Co. Ltd, Chertsey, Surrey). According to the sample dilution an appropriate range of ammonium sulphate standards (5-25 µg NH_3.N per ml) were prepared with an equivalent concentration of acid and catalyst. A consistently linear standard curve was obtained.

2.14.3 Ammonia nitrogen

2.14.3.1 Rumen fluid

Appropriate dilutions of acidified rumen fluid samples, obtained during preparation of the microbial fraction (section 2.6.3), were analysed against ammonium sulphate standards prepared in distilled water, on a Technicon Autoanalyser (see previous section).

2.14.3.2 Duodenal digesta

A representative sample of digesta was accurately weighed (approximately 0.2 g), thoroughly mixed with 5.0 ml of 0.2M HCl, incubated at 4°C for 1 hr and centrifuged (1000 g, 10 min). The supernatant was diluted 1 : 10 with DW and analysed for NH_3.N as above against standards prepared in a 0.02M HCl.

EXPERIMENTAL PROCEDURES AND RESULTS

All data presented were statistically analysed using the computer package called GENSTAT (GENSTAT V, 1984, 4.04B, Lawes Agricultural Trust, Rothamstead Experimental Station).

Three levels of significance were routinely adopted, $p<0.05$, $p<0.01$ and $p<0.001$ in order of increasing statistical significance.

Chapter 3

A COMPARISON OF THE ENDOGENOUS PROTEIN FLOW CALCULATED USING THE INTRAVENOUS INFUSION OF L-[4,5-^{3}H]-LEUCINE TECHNIQUE, WITH VALUES DETERMINED BY DIFFERENCE IN SHEEP FED AN ESSENTIALLY PROTEIN-FREE DIET

3.1 Introduction

Protein nitrogen arriving at the small intestine in ruminants fed conventional rations consists of undegraded feed, microbial and endogenous components. Several techniques have been developed in order to quantify the microbial component (section 1.2.1). If the undegraded feed component is not present then the endogenous protein leucine flow can theoretically be estimated by difference from the duodenal digesta total leucine flow and the duodenal digesta bacterial leucine flow.

The aim of this experiment was to compare the value for the endogenous protein leucine flow derived using the proposed intravenous infusion technique with that obtained by difference when feeding an essentially protein-free diet. (i.e. when the undegraded feed component of the digesta was very small).

3.2 Experimental animals

Three Suffolk cross wethers (A, B, C) approximately 40 kg in weight, each equipped with a 37 mm rumen cannula and an 11 mm proximal duodenal T-piece cannula were used in this experiment. Throughout the experimental period the animals were housed in metabolism crates, within a controlled environment (24 hr light). Water was available ad libitum.

3.3 Experimental diet

The composition of the essentially protein-free diet and the proximate analysis are given in Table 11. The complete ration (800 g freshweight per day) was provided in a peletted form via an automatic feeder which delivered one twelfth of the ration at 2 hr intervals. Previous experiments using ruminants fed an absolute protein-free diet have frequently encountered problems with inappetance due to the unpalatable nature of the diet. In certain instances these difficulties have been overcome by the direct input of diet into the rumen via the rumen fistula (Maeng and Baldwin, 1976). However, it is desirable with isotope tracer kinetic techniques that steady-state conditions exist within the rumen. This could not be achieved with the manual input of feed refusals to the rumen. Thus small amounts of degradable components, barley and grassmeal, were included in the ration. There were no feed refusals throughout the duration of the experiment.

3.4 Experimental procedure

The basic experimental protocol concerning animal management, time-scale of digesta flow marker and isotope infusions and sampling routine is given in Fig 8. Specific details regarding this experiment are considered below.

L-[4,5-^{3}H]-leucine was intravenously infused at a constant rate of 0.5 mCi per day (specific activity 11.8 mCi/mmol) over eight days (see section 2.5). Ruthenium phenanthroline (15 mg/day) and chromium EDTA (130 mg/day) were intraruminally infused as predominant solid and liquid digesta flow markers respectively to determine the flow of digesta

Table 11 Composition and analysis of the 'protein-free' diet

Component	g/kg (as is basis)
Corn starch	270
Chopped barley straw	200
Glucose	200
Barley	100
Grassmeal	60
Nutramol 25 *	50
Urea	40
Mineral and vitamin mix **	50
Vegetable oil	30

* Rumenco, Burton-upon-Trent, Derbyshire

** Sheep mineral and vitamin supplement contained sodium chloride, steamed bone flour, limestone and trace minerals and vitamins: Mg 15 ppm. Se 8 ppm, Vit. A 320,000 ppm, Vit. D3 64,000 ppm, Vit. E 400 ppm (Frank Wright Ltd, Derbyshire).

Analysis

	Actual
Dry matter (g/100 g)	89.3
Dry matter intake (g/day)	714
Organic matter (g/100 g DM)	93.4
Estimated metabolisable energy (MJ/kg DM)+	11.4
Nitrogen content (g/100 g DM)	2.63

\+ Estimated from MAFF (1975)

constituents using the dual phase marker technique (Faichney, 1975) (see section 2.10).

Samples of plasma, saliva, rumen fluid and duodenal digesta were obtained routinely at eight hour intervals during the eight day isotope infusion period and relevant specific activity profiles determined (see sections 2.6-2.9). Urine output was monitored over the last four days in order to calculate chromium absorption. A series of rumen fluid samples were taken over approximately 24 hr following the cessation of the marker infusion for chromium analysis (see section 2.10.3.1).

3.5 Experimental results

3.5.1 Protein bound leucine specific activity profiles

The specific activity of the protein bound leucine fractions was determined (see section 2.9) approximately 12 times during the infusion period. The increase in specific activity of total duodenal digesta, duodenal microbial protein, saliva protein and plasma protein fractions was assumed to be the form of a single exponential curve (section 1.6) described by the equation:

$$y = A(1 - e^{-kt})$$

where y = Specific activity of fraction

A = Plateau specific activity

k = Rate constant

t = Time

Specific activity profiles were derived using a least squares curve fitting procedure (NAG Library, 1983, Routine E04CGF, NAG Mk X Numerical

Algorithm Group, Oxford) using the programs of Dr S.E. Harding on the IBM 3081 mainframe computer at Cambridge via the JANET link. The profile parameters A and k were obtained and the time taken to attain 95% plateau levels, as the theoretical plateaux are only achieved at time infinity, was also calculated, i.e. t (95% plateau) = - ln 0.05/k. The specific activity profiles of the various fractions for the three animals studied in this experiment are included in Appendix 2 (2.1-2.3). Parameters related to the profiles are detailed in Appendix 3. Representative curves are now considered in detail.

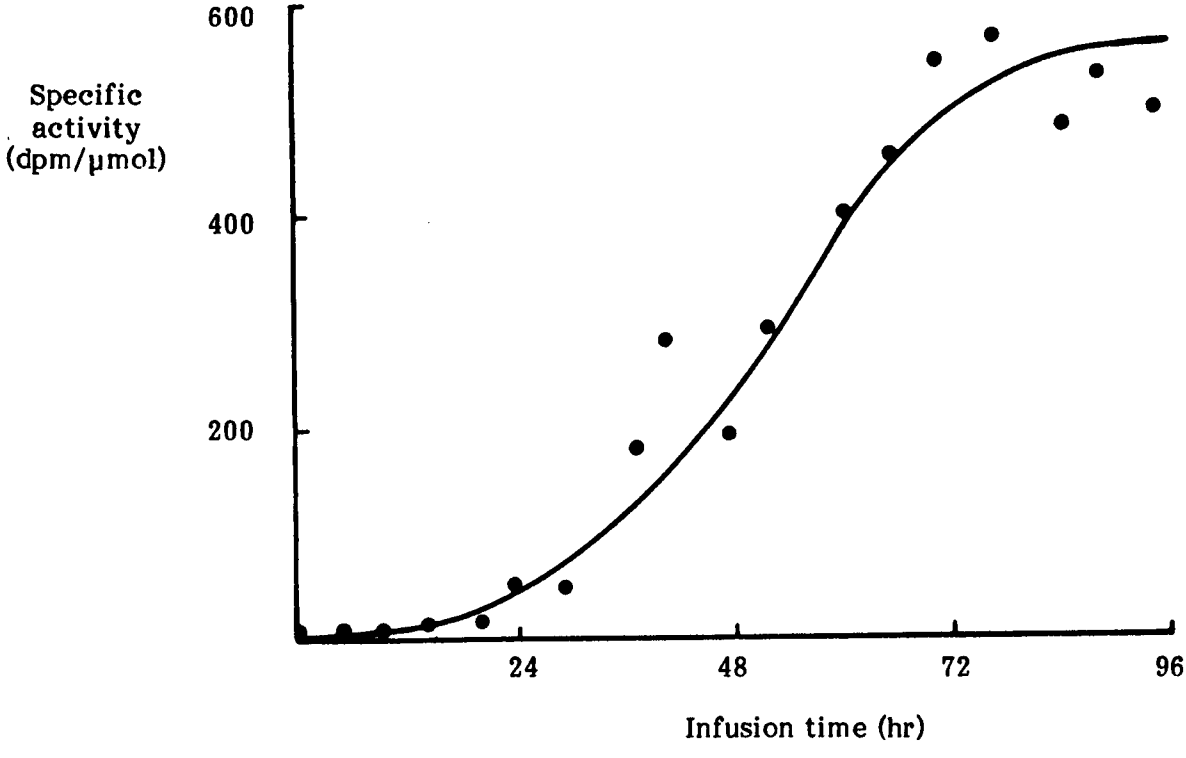
3.5.1.1 Total duodenal digesta

The shape of the total duodenal digesta specific activity curve was similar in all animals studied. In general, there was a steady rise during the initial 100 hr of the infusion after which the value appeared to plateau. From the determined equations for the curves the mean (± SEM, n = 3) time taken to achieve 95% of plateau was 363 ± 145 hr.

3.5.1.2 Rumen bacteria

An interesting observation was that the rumen bacteria were labelled. The specific activity curve was sigmoidal in shape (Fig 13) in animals intravenously infused with labelled leucine. The detection of labelled leucine in the bacteria indicated that they were utilising an endogenous source of leucine. The initial lag phase suggested the incorporation of leucine into bacterial protein had a time delay in it. Bacterial isolates were examined microscopically and found to be pure. As the salivary protein leucine specific activity profile showed no lag phase (see section 3.5.1.5) it was considered possible that the bacteria were obtaining much of their label from the rumen epithelium (see section

Fig 13 The specific activity (dpm/μmol) of rumen bacterial protein leucine in a sheep intravenously infused with L-[4,5-^{3}H]-leucine



6.4 (ii) for further discussion). This was supported by the absence of any detectable activity in the rumen free leucine pool. Possibly the lag phase was due to the time taken for the accessible parts of the rumen epithelium to become labelled.

3.5.1.3 Duodenal bacteria

The shape of the duodenal bacterial protein leucine specific activity curve was similar in all animals studied. A steady increase in specific activity was observed with indications of a plateau towards the end of the infusion. The true plateau (95%) (mean $\pm$ SEM, n = 3) was predicted to occur at 737 $\pm$ 318 hr. The fact that the microbial fraction was labelled meant that to determine the activity in the total digesta due to the endogenous secretions it would be necessary to first accurately determine the amount of label in the digesta due to the microbial fraction and then to subtract this from the total activity (section 3.5.2.1).

3.5.1.4 Rumen protozoa

Rumen protozoa were isolated and found to be labelled. However, limited sample availability precluded the determination of an appropriate specific activity profile.

3.5.1.5 Saliva

The specific activity profile of salivary protein leucine was considered to be similar to that of all the enzymic inputs into the digestive tract flowing from the abomasum (see section 1.6). The plateau in specific activity approached that of the plasma free leucine (see section 3.5.6). The shape of the curve was very similar in all animals studied. The curves approached a plateau region after approximately 60 hr and the true plateau

(95%) (mean ± SEM, n = 3) was predicted to occur at 153 ± 9 hr. Any discrepancy between individual data points and the predicted curve may be due to difficulties in obtaining a homogenous saliva sample.

3.5.1.6 Plasma protein

The specific activity profile of plasma protein leucine was taken to represent the profile of the endogenous leakage of proteins such as albumin and globulins into the digestive tract (see section 1.6). Theoretically the shape of the specific activity profile should be exponential but appeared to be essentially linear over 200 hr. The mean (± SEM, n = 3) predicted (95%) plateau estimate was calculated to occur after 169 ± 91 days.

3.5.2 Calculation of the endogenous protein leucine flow using the intravenous infusion of L-[4,5-^{3}H]-leucine technique

The calculation involved the derivation of a specific activity curve representative of the endogenous input. This curve was then compared with suitable endogenous precursor curves (see section 3.5.2.2) to give an estimate of the proportion of total duodenal digesta of endogenous origin. The need to correct the duodenal digesta for the presence of labelled microbial protein created problems. Basically it was necessary to determine the total [^{3}H]-leucine flow in the digesta per unit time and then to subtract the duodenal microbial [^{3}H]-leucine flow (see section 3.5.2.1). The flow of total duodenal digesta leucine and duodenal microbial leucine were used in this calculation. Duodenal microbial leucine flow was estimated from microbial nitrogen flow. The latter was determined using diaminopimelic acid (DAPA) as a microbial marker (see Table 12). Data required in the determination of the flow of total duodenal digesta leucine and duodenal microbial leucine are included in Table 13.

Table 12 Duodenal digesta protein nitrogen flow (g/day) and bacterial nitrogen flow (g/day) in sheep fed an essentially protein-free diet

	Sheep			Mean[+]	SEM
	A	B	C		
Duodenal digesta dry matter flow (g/day)[++]	421	407	431	420	7.0
Mean ± SEM, n = 4	14.6	30.7	22.7		
Duodenal digesta protein nitrogen (g/100g DM)[+++]	2.96	2.86	2.84	2.89	0.037
Mean ± SEM, n = 4	0.125	0.110	0.042		
Duodenal digesta protein nitrogen flow (g/day)	12.46	11.64	12.24	12.11	0.245
Duodenal digesta DAPA (g/100g DM)	0.189	0.163	0.140	0.164	0.014
Mean ± SEM, n = 6	0.022	0.016	0.012		
Rumen bacteria DAPA (g/100g DM)	0.539	0.514	0.495	0.516	0.0127
Mean ± SEM, n = 6	0.051	0.051	0.032		
Rumen bacteria nitrogen (g/100g DM)	7.58	7.02	6.41	7.00	0.338
Mean ± SEM, n = 4	0.185	0.377	0.133		
DAPA flow (g/day)	0.796	0.663	0.603	0.687	0.057
Rumen bacteria nitrogen (g/100g DM) / Rumen bacteria DAPA (g/100g DM)	14.06	13.66	12.95	13.56	0.325
Bacterial nitrogen flow (g/day)	11.19	9.06	7.81	9.35	0.987
Maximum endogenous protein nitrogen flow (g/day) (not corrected for undegraded feed)	1.27	2.58	4.43	2.76	0.917

[+] Mean ± SEM estimate of 3 sheep
[++] Determined with reference to dietary lignin
[+++] Duodenal digesta NAN

Table 13 The determination of the flow of duodenal digesta leucine (mmol/day) and duodenal bacterial leucine (mmol/day) in sheep fed an essentially protein-free diet

	Sheep			Mean+	SEM
	A	B	C		
Duodenal digesta dry matter flow (g/day)	421	407	431	420	7.0
Mean ± SEM, n = 4	14.6	30.7	22.7		
Duodenal digesta leucine (g/100 g DM)	1.15	1.34	1.08	1.19	0.078
Mean ± SEM, n = 12	0.039	0.039	0.033		
Duodenal digesta leucine flow (g/day)	4.84	5.45	4.66	4.98	0.239
Duodenal digesta leucine flow (mmol/day)	36.89	41.54	35.52	37.98	1.822
Duodenal bacterial protein leucine (g/100 g DM)	2.51	2.21	2.40	2.37	0.088
Mean ± SEM, n = 12	0.072	0.066	0.100		
Rumen bacterial nitrogen (g/100 g DM)	7.58	7.02	6.41	7.00	0.338
Mean ± SEM, n = 4	0.185	0.377	0.133		
Bacterial nitrogen flow (g/day)*	11.19	9.06	7.81	9.35	0.987
Bacterial dry matter flow (g/day)	147.8	129.6	121.7	133.0	7.73
Duodenal bacterial leucine flow (g/day)	3.71	2.87	2.92	3.17	0.272
Duodenal bacterial leucine flow (mmol/day)	28.28	21.88	22.26	24.14	2.073
Maximum endogenous protein leucine flow (g/day) (not corrected for undegraded feed)	1.13	2.58	1.74	1.82	0.420

\+ Mean ± SEM estimate of 3 sheep

* Determined using DAPA as a microbial marker (see Table 12)

The shape of the "digesta-microbial" curve, derived from the total duodenal digesta specific activity profile after correction for the labelled duodenal microbial component, represents the actual endogenous input. The shape of the "digesta-microbial" or corrected digesta curve was similar in all animals studied. Plateau regions were approached after about 100 hr and final predicted 95% plateaux (mean $\pm$ SEM, n = 3) calculated to occur after 403 $\pm$ 154 hr.

The shape of the corrected digesta curve is important. Since the curve represents the actual endogenous input its shape provides an indication as to the relative importance of the various endogenous sources. If the curve resembled the salivary protein leucine specific activity profile then fast turnover proteins, e.g. enzymes would be considered as the major fraction of the endogenous component. Similarly if the corrected curve was similar to the plasma protein specific activity profile then endogenous inputs such as albumin or globulins would be considered more important.

Calculation procedures

Specific activity profiles of the various fractions for sheep B are included in Fig 14. The calculation procedure is outlined below, including data for sheep B.

3.5.2.1 Derivation of corrected digesta specific activity profile

i) The increase in specific activity with time of total duodenal digesta leucine was assumed to be described by a single exponential equation: $y = A(1 - e^{-kt})$ (see Fig 14 a)

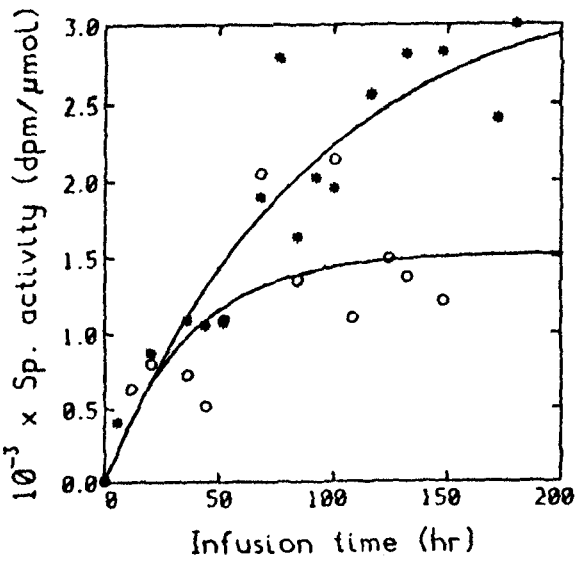
Fig 14 The specific activity (dpm/μmol) of protein leucine in various fractions during a 200 hr continuous intravenous infusion of L-[4,5-^{3}H]-leucine and the derivation of the specific activity profile of corrected digesta in a representative animal (sheep B)

a. The specific activity of protein leucine (dpm/μmol) in total duodenal digesta (*) and duodenal microbes (O)

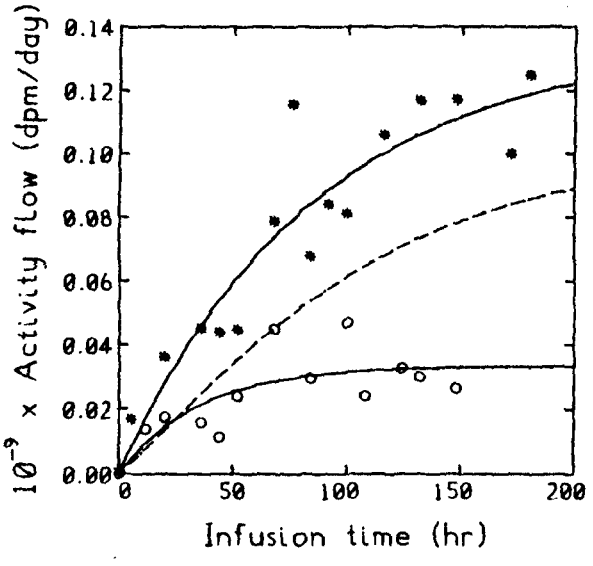
b. The activity flow (dpm/day) of [^{3}H]-leucine in total duodenal digesta (*), duodenal microbes (O) and corrected digesta (- - -)

c. The specific activity of protein leucine (dpm/μmol) in plasma (*), saliva (O) and corrected digesta (- - -)

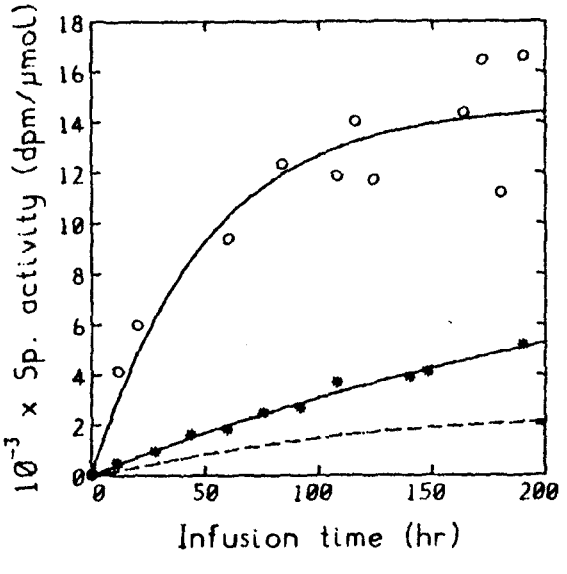
a)


10^{-3} x Sp. activity (dpm/μmol)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
Infusion time (hr)

b)


10^{-9} x Activity flow (dpm/day)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
50
100
150
200
Infusion time (hr)

c)


10^{-3} x Sp. activity (dpm/μmol)
18
16
14
12
10
8
6
4
2
0
0
50
100
150
200
Infusion time (hr)

where y = Specific activity total duodenal digesta

A = Plateau specific activity total duodenal digesta

k = Rate constant

t = Time

This point was discussed in section 1.6. Specific activity profiles were derived using a least squares curve fitting procedure (see section 3.5.1).

ii) The increase in specific activity with time of duodenal microbial protein leucine was also assumed to be the form of a single exponential and curves were derived as indicated above (see Fig 14 a).

iii) The total duodenal digesta leucine and duodenal microbial leucine specific activity profiles were converted to the form activity flow per day (dpm/day) at each point in time (see Fig 14 b), i.e.

Total duodenal digesta $[^3H]$-leucine flow (dpm/day)	=	Total duodenal digesta leucine flow (μmol/day)	x	Total duodenal digesta leucine specific activity (dpm/μmol)
Duodenal microbial $[^3H]$-leucine flow (dpm/day)	=	Duodenal microbial leucine flow (μmol/day)	x	Duodenal microbial leucine specific activity (dpm/μmol)

iv) The activity flow of $[^3H]$-leucine in total duodenal digesta not attributable to the microbial fraction was then determined by subtraction (see Fig 14 b), i.e.

Corrected flow of activity (dpm/day)	=	Total duodenal digesta $[^3H]$-leucine flow (dpm/day)	-	Duodenal microbial $[^3H]$-leucine flow (dpm/day)

v) The specific activity of the corrected fraction was then calculated:

$$\text{Corrected digesta specific activity (dpm/μmol)} = \frac{\text{Corrected flow of activity (dpm/day)}}{\text{Total duodenal digesta leucine flow (μmol/day)}}$$

The area under this fitted curve (see Fig 14 c) was determined by integration between zero and 200 hr. As part of a further calculation (see section 3.5.2.3 b) the curve was considered a single exponential, with a rate constant (k corr). The validity and practicality of this assumption is given in section 6.1.3. The next stage involved the derivation of maximum and minimum endogenous protein leucine inputs by reference to specific activity profiles of the plasma protein and the salivary protein.

3.5.2.2 Derivation of maximum and minimum endogenous protein leucine flows

Saliva and plasma protein leucine were considered suitable precursors to enable a range of endogenous inputs to be calculated (see section 1.6). The increase in specific activity with time of saliva and plasma protein leucine was assumed to be in the form of a single exponential (see section 3.5.1). Specific activity profiles were derived using curve fitting procedures (see Fig 14 c) and the areas under the curves determined by integration between zero and 200 hr. The maximum and minimum proportion of total duodenal digesta leucine of endogenous origin was then calculated:

$$\text{Maximum proportion of total digesta leucine of endogenous origin} = \frac{\text{Area under corrected digesta specific activity curve}}{\text{Area under plasma protein specific activity curve}} = 0.453$$

$$\text{Minimum proportion of total digesta leucine of endogenous origin} = \frac{\text{Area under corrected digesta specific activity curve}}{\text{Area under saliva protein specific activity curve}} = 0.120$$

These values were converted to maximum and minimum endogenous leucine flows:

Maximum endogenous leucine flow (g/day)	=	Maximum proportion of total digesta leucine of endogenous origin	x	Total leucine flow (g/day)
	=	0.453 x 5.45		
	=	2.47 g/day		
Minimum endogenous leucine flow (g/day)	=	Minimum proportion of total digesta leucine of endogenous origin	x	Total leucine flow (g/day)
	=	0.120 x 5.45		
	=	0.65 g/day		

This range of endogenous leucine flow may be converted to endogenous protein nitrogen flow assuming that protein equals 6.25 x nitrogen and a leucine content of endogenous protein. The assessment of the leucine content of endogenous protein and the conversion of endogenous protein leucine to endogenous protein nitrogen is considered in the discussion (section 6.2). The actual endogenous leucine value would be expected to be in this range. The range was large ($0.64 \pm 0.098 - 2.11 \pm 0.326$, mean $\pm$ SEM, n = 3). An approach to define more accurately a definitive value for the endogenous protein component of proximal duodenal digesta was developed. Initial analysis involved a comparison of the shapes of the specific activity curves to determine whether saliva or plasma protein was the more appropriate precursor. An alternative method based on the determination of the proportion of the precursor fractions in the secretion was developed which resulted in one estimate for the endogenous secretion. These methods are considered in the next section. Values of endogenous protein determined using the proposed technique are compared with those calculated by difference in section 3.5.3.

3.5.2.3 The determination of a single value for the endogenous protein leucine flow

The shape of the specific activity profile of the corrected digesta represents that of the endogenous protein secretions (see section 1.6). If enzymic materials are the major endogenous secretion it was proposed that the shape of the saliva and corrected digesta specific activity profiles would be very similar (see section 6.1.2). Similarly, if plasma proteins such as albumin or globulins formed the major component of endogenous secretions then the shape of the specific activity curve for plasma protein is proposed to be similar to the corrected digesta curve (see section 6.1.2).

(a) Comparison of corrected digesta and precursor specific activity profiles

The initial approach was to compare the shape of the specific activity curves visually. The shape of the corrected digesta curve in general resembled more closely that of the saliva curve than that of the plasma protein curve.

Preliminary analysis of the curve shapes involved a comparison of the rate constant of the corrected digesta curve (kcorr) with the rate constant of the saliva (ksal) and plasma protein (kpla) curves. It was considered that if this resulted in a non-significant difference between kcorr and ksal and a significant difference between kcorr and kpla then saliva could be considered as a more appropriate precursor. This precursor would then be used in further experiments to provide an estimate for endogenous protein where conventional diets were fed. The rate constants of the specific activity profiles for individual animals are included in Appendix 3. The rate constants were analysed using an analysis of variance. The variation

in the data due to the difference between animals was accounted for by considering the animals as blocks. The results of this analysis were inconclusive (see Table 14). Although not significantly different at the 5% level the rate constants were significantly different at the 10% level. There was no significant difference ($p>0.1$) between both the saliva and corrected digesta and plasma protein and corrected digesta rate constants. There was a significant difference ($p<0.05$) between the saliva and plasma protein rate constants.

By necessity there were few animals in this experiment and hence the standard errors were large but it is believed that the trends were of considerable interest. The data strongly indicate that neither saliva nor plasma protein were representative of endogenous secretions.

(b) An alternative approach — derivation using linear interpolation

This involved the analysis of the rate constants of the corrected digesta (kcorr), saliva (ksal) and plasma protein (kpla) specific activity profiles and the maximum and minimum endogenous protein leucine estimates. If saliva was entirely representative of the endogenous secretions then the shape of the saliva and corrected curves would be the same and therefore the ratio of the rate constants (kcorr/ksal) would be unity. If plasma protein was entirely representative of the endogenous secretions then the shape of the plasma and corrected curves would be the same, and therefore the ratio of the rate constants (kcorr/kpla) would be unity. The endogenous input is likely to the derived from a combination of the various sources. It was proposed that the determination of the ratio kcorr/k precursor (ksal or kpla) would indicate the relative importance of the precursors. The ratio kcorr/ksal was considered proportional to the minimum endogenous protein leucine estimate and the ratio kcorr/kpla was

Table 14 Comparison of rate constants (/hr) of corrected digesta and precursor specific activity profiles in sheep fed an essentially protein-free diet

	Specific activity curve				Statistical significance of curve differences
	Saliva	Plasma protein	Corrected digesta	SED	
Rate constant (/hr) (mean of 3 animals)	0.0197	0.0015	0.0117	0.00560	$p<0.1$

(See Appendix 3 for individual animal data)

Results of t-test (4 d.f.) for rate constants

Specific activity curve	Saliva	Plasma protein	Corrected digesta
Saliva	-	*	NS
Plasma protein		-	NS
Corrected digesta			-

NS Difference not significant $p>0.1$

* Significantly different $p<0.05$

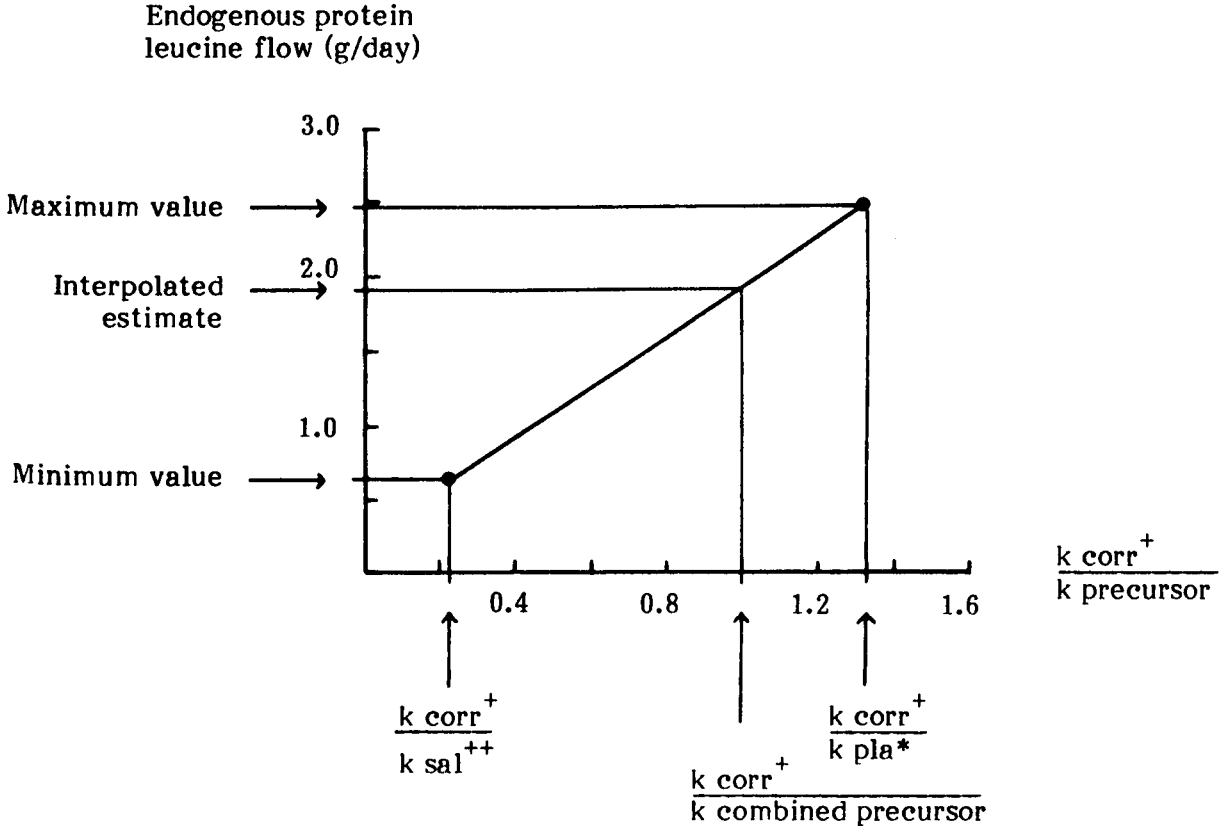
considered proportional to the maximum endogenous protein leucine estimate.

The value of the endogenous protein leucine flow when kcorr/k 'combined precursor' was unity, i.e. when the corrected digesta and endogenous secretion curves were the same shape, was determined by linear interpolation. This value was considered the endogenous protein leucine input for an individual animal under a specific dietary regime (see Fig 15). Extensive analysis indicated that this was an appropriate interpretation (see discussion). This process gives one value for the endogenous leucine flow rather than a range. The approach outlined in section 3.1 is considered in the next section. The maximum, minimum and 'interpolated' estimates of endogenous protein flow determined using the proposed technique were compared with those obtained by difference (total leucine flow - microbial leucine flow).

3.5.3 Results of experiments to compare various methods to calculate endogenous protein flow from [^{3}H]-leucine data with estimates obtained by difference using sheep fed an essentially protein-free diet

Dry matter flow into the duodenum was determined with reference to dietary lignin. Unfortunately analytical difficulties were encountered in the use of the dual phase marker technique using ruthenium and chromium. Principally it was not possible to take enough sample. In a comparison of various methods to determine the endogenous protein flow absolute measurements of dry matter flow are not crucial. Bacterial nitrogen flow was estimated using DAPA as a microbial marker (see Table 12). The aim of this experiment was to calculate the endogenous leucine flow by difference and compare these estimates with the values

Fig 15 Determination of a single estimate of the endogenous protein leucine flow using linear interpolation



$$\frac{k\ corr^{+}}{k\ sal^{++}} = 0.228$$

$$\frac{k\ corr^{+}}{k\ pla^{*}} = 1.33$$

Minimum endogenous protein leucine flow (g/day) = 0.65 g/day

Maximum endogenous protein leucine flow (g/day) = 2.47 g/day

By linear interpolation, value of endogenous protein leucine flow (g/day) when $\frac{k\ corr^{+}}{k\ precursor} = 1.0$ = 1.92 g/day

\+ k corr = Rate constant of corrected digesta specific activity profile

++ k sal = Rate constant of saliva specific activity profile

* k pla = Rate constant of plasma protein specific activity profile

determined using the [^{3}H]-leucine technique. As outlined above it was proposed that this would validate the technique.

From the data in Table 13 the maximum endogenous protein leucine flow may be determined by difference (total duodenal digesta leucine flow - duodenal bacteria leucine flow). Due to the small amount of protein in the ration the maximum endogenous protein leucine flow was corrected for an undegraded feed leucine component (0.44 g/day) estimated from the leucine intake (1.93 g/day) and the predicted degradability of the diet (0.77). The mean estimates of endogenous protein leucine derived using the various [^{3}H]-leucine technique methods and the mean corrected by difference value are included in Table 15. Data for individual animals are included in Appendix 4. The data was analysed using an analysis of variance. The variation in the data due to the difference between animals was accounted for by considering the animals as blocks. The methods were shown to differ ($p<0.05$). Caution must be used because many of the values are confounded, e.g. interpolated determination depends on the saliva and plasma estimates. Also all of the values are dependent on flow parameters. The saliva and plasma protein [^{3}H]-leucine estimates were significantly different ($p<0.05$) from the corrected by difference estimate. The endogenous leucine value derived by interpolation of the [^{3}H]-data was numerically very similar to that derived directly from the flow data (the difference was not significant even at $p>0.1$). This gave some confidence in the proposed techique.

As indicated previously, estimates of endogenous protein leucine flow may be converted to endogenous protein nitrogen flow if the leucine content of endogenous secretions is estimated. This is considered in the discussion (see section 6.2).

Table 15 Comparison of estimates of endogenous protein leucine flow (g/day) determined using the [^{3}H]-leucine technique (various methods) and by difference (corrected) estimate in sheep fed an essentially protein-free diet

	[^{3}H]-leucine technique					Statistical significance of method differences
	Saliva	Plasma protein	Inter-polated	Difference corrected+	SED	
Endogenous protein leucine flow (g/day) mean of 3 animals	0.64	2.11	1.09	1.38	0.299	*

+ Assumes degradability of ration = 0.77

* Significantly different $p<0.05$

(see Appendix 4 for individual animal data)

Results of t-test (6 d.f.) for endogenous protein leucine estimates

Method	Saliva	Plasma protein	Interpolated	Difference corrected
Saliva	-	**	N/A	*
Plasma protein		-	N/A	*
Interpolated			-	NS
Difference corrected				-

** Significantly different $p<0.01$

* Significantly different $p<0.05$

NS Difference not significant $p>0.1$

N/A Not applicable (see text)

In summary, the range of endogenous inputs predicted using the proposed technique encompassed the corrected by difference estimate. Plasma protein as a precursor overestimated the endogenous protein flow and saliva underestimated it. The relevant interpolated endogenous estimate was not significantly different ($p>0.1$) from the corrected by difference estimate. This indicated the validity of the use of the interpolated estimate in further experiments using conventional rations. Ideally more animals should have been used but this was not practical.

There are a number of potential important sources of error in the determination of the endogenous protein flow by difference. These include the estimation of the undegraded feed fraction and the failure to consider a protozoal component of the digesta. It was concluded that the proposed technique provided a suitable method to determine directly the endogenous protein content of proximal duodenal digesta which could be applied to different dietary regimes. The theoretical validity and experimental evaluation of the proposed technique are discussed in sections 6.1 and 6.7, respectively.

3.5.4 Rumen parameters, the flow of digesta constituents and the efficiency of bacterial nitrogen synthesis in sheep fed an essentially protein-free diet

Values determined for rumen volume, liquid phase dilution rate and rumen and duodenal liquid outflow rates are included in Table 16. Data used in the determination of the efficiency of bacterial nitrogen synthesis are given in Table 17. The mean estimate of the efficiency of bacterial nitrogen synthesis (28 g N/kg OMADR) is in agreement with values given by the ARC (1980) for sheep fed a similar ration (29-34 g N/kg OMADR).

Table 16 Rumen volume (L), liquid phase dilution rate (/day) and rumen and duodenal liquid outflow rates (L/day) in sheep fed an essentially protein-free diet

	Sheep			Mean*	SEM
	A	B	C		
Rumen volume (L)	3.5	6.2	4.6	4.8	0.78
Liquid phase dilution rate (/day)	2.79	2.75	1.96	2.50	0.270
Rumen liquid outflow (L/day)	9.6	17.2	9.0	11.9	2.64
Duodenal liquid outflow (L/day)	11.7	15.5	16.2	14.5	1.40

* Mean $\pm$ SEM estimate of 3 sheep

Table 17 The digestion of organic matter in the rumen and the efficiency of bacterial nitrogen synthesis in sheep fed an essentially protein-free diet

	Sheep			Mean*	SEM
	A	B	C		
Dry matter intake (g/day)	714	714	714		
Organic matter intake (g/day)	669	669	669		
Dry matter flow (g/day)	421	407	431	420	7.0
Mean ± SEM, n = 4	14.6	30.7	22.7		
Organic matter flow (g/day)	333	318	343	331	7.3
Mean ± SEM, n = 4	7.7	22.3	22.2		
Organic matter apparently fermented in the rumen (g/day)	336	351	326	338	7.3
Bacterial nitrogen flow (g/day)	11.19	9.06	7.81	9.35	0.987
Bacterial organic matter flow (g/day)**	95.7	77.8	66.7	80.1	8.45
Proportion of organic matter intake apparently digested in the rumen	0.502	0.525	0.487	0.505	0.0111
Proportion of organic matter intake truly digested in the rumen	0.645	0.641	0.587	0.624	0.1870
Efficiency of bacterial nitrogen synthesis (g N/kg OMADR)+	33.3	25.9	23.9	27.7	2.86
Efficiency of bacterial nitrogen synthesis (g N/kg OMTDR)++	25.9	21.2	19.9	22.3	1.82

* Mean ± SEM estimate of 3 sheep

** 117 g bacterial NAN per kg bacterial organic matter (Lindsay and Hogan, 1972)

\+ Organic matter apparently digested in the rumen

++ Organic matter truly digested in the rumen

3.5.5 Amino acid composition of diet, rumen bacteria and duodenal digesta in sheep fed an essentially protein-free diet

The amino acid composition of the essentially protein-free diet, rumen bacteria and duodenal digesta were determined. The amino acid intake (g/day) and the duodenal amino acid flow (g/day) for each animal are given in Table 18. In agreement with previous observations in animals fed low protein diets duodenal amino acid flow was greater than intake (see for example Harrison et al., 1973).

A detailed analysis of the amino acid profiles of rumen bacteria and duodenal digesta for the sheep in this experiment and equivalent profiles from sheep fed different diets studied later in this investigation is included in section 4.5.4.

3.5.6 Estimation of leucine flux through the plasma free pool in sheep fed an essentially protein-free diet

The specific activity of plasma free leucine increased rapidly and approached a plateau within 6 hr. During the 200 hr infusion period the specific activity of the plasma free leucine increased gradually.

Data used in the determination of leucine flux through the plasma free pool are presented in Table 19. From the mean plateau specific activity of plasma free leucine the flux rate of leucine through the plasma free pool was calculated:

$$\text{Flux, Q } (\mu\text{mol/day}) = \frac{\text{Infusion rate (dpm/day)}}{\text{Mean plateau specific activity of plasma free leucine (dpm/}\mu\text{mol)}}$$

Table 18 Amino acid intake (g/day) and amino acid flow at the proximal duodenum (g/day) in sheep fed an essentially protein-free diet

Amino acid	Amino acid intake (g/day)	Amino acid flow at the proximal duodenum (g/day) (each value is a mean of 3 samples)					
		Sheep A		Sheep B		Sheep C	
		Mean	SEM	Mean	SEM	Mean	SEM
Aspartic acid	2.08	8.74	0.814	8.44	0.576	6.91	0.380
Threonine	0.985	4.09	0.371	3.52	0.300	3.16	0.143
Serine	1.20	3.59	0.267	3.83	0.323	3.03	0.152
Glutamic acid	5.10	9.78	0.970	10.02	0.696	8.22	0.474
Proline	1.71	3.62	0.315	3.07	0.513	3.09	0.355
Glycine	1.34	4.18	0.379	3.75	0.194	3.48	0.174
Alanine	1.61	5.35	0.432	5.00	0.294	4.37	0.273
Valine	1.36	3.89	0.191	4.10	0.282	3.52	0.211
Methionine	0.471	1.47	0.211	1.33	0.136	1.25	0.141
Isoleucine	0.978	3.17	-	3.74	0.248	3.08	0.192
Leucine	1.93	5.78	0.370	5.41	0.213	4.80	0.276
Tyrosine	0.821	2.65	0.279	2.84	0.210	2.30	0.147
Phenylalanine	1.24	3.16	0.257	3.22	0.266	2.64	0.152
Lysine	0.593	5.43	0.419	5.29	0.352	4.37	0.215
Histidine	0.464	1.32	0.124	1.16	0.090	1.10	0.080
Arginine	1.41	3.40	0.188	3.05	0.231	2.58	0.142

Table 19 Estimate of leucine flux (mmol/day) through the plasma free pool in sheep fed an essentially protein-free diet

	Sheep			Mean+	SEM
	A	B	C		
Infusion rate (mCi/day)	0.54	0.58	0.50	0.54	0.023
Plasma free leucine specific activity (dpm/μmol) x 10^{-3}	14.1	12.3	10.5	12.3	1.04
Mean ± SEM, n = 7	1.12	0.86	1.61		
Leucine flux (mmol/day)	85.0	104.7	105.7	98.5	6.74

\+ Mean ± SEM estimate of 3 sheep

Chapter 4

INVESTIGATION INTO THE EFFECT OF DIET ON THE ENDOGENOUS PROTEIN LEUCINE FLOW AT THE PROXIMAL DUODENUM IN SHEEP USING THE L-[4,5-^{3}H]-LEUCINE TECHNIQUE

4.1 Introduction

Several recent studies (e.g. Kennedy and Milligan, 1980b; Egan, 1984) have suggested that the endogenous protein flow may be dietary dependent. A number of factors can be considered involved in the regulation of endogenous protein secretion, e.g. dietary fibre, nitrogen intake, type of rumen fermentation and hormonal status of the animal. The overall control mechanism is likely to be an integral combination of these factors.

The aim of this experiment having previously developed and verified a technique to determine directly the endogenous protein flow at the proximal duodenum was to investigate any dietary influence. In order to achieve this diets which were designed to provide an extreme range of endogenous protein flow at the proximal duodenum were used. Animals were fed either a concentrate or a long hay diet.

4.2 Experimental animals

Six Suffolk cross wethers, approximately 40 kg in weight, each equipped with a 37 mm rumen cannula and an 11 mm proximal duodenal T-piece cannula were used in this experiment. Throughout the experimental period the animals were housed in metabolism crates within a controlled environment (24 hr light). Water was available ad libitum.

Animals D, E, F were given the concentrate diet while G, H, K were given hay.

4.3 Experimental diets

The composition, analysis and comparative intakes of the concentrate and hay diets are given in Table 20. The concentrate ration (700 g freshweight per day) was provided in a pelleted form via an automatic feeding system which delivered one twelfth of the daily ration at 2 hr intervals. Attempts to feed the long hay via a moving belt automatic feeding system proved impractical. Therefore, long hay was offered four times a day (4 x 350 g freshweight per day). Total feed intake was determined and was observed to be continuous. The level of feeding was predetermined following a pilot trial and originally estimated using ARC (1980). Hay fed animals had free access to mineral blocks (Super Chelated Rockies, Tithebarn Ltd, Southport). Diets were designed to be approximately isonitrogenous and isoenergetic. Unfortunately in practice actual nitrogen intakes were lower in the hay fed animals.

4.4 Experimental procedure

The routine experimental protocol concerning animal management, time scale of isotope and digesta flow marker infusions and sampling routine is given in Fig 8. Specific details concerning this experiment are given below.

L-[4,5-^{3}H]-leucine was intravenously infused at a rate of 0.25 mCi per day (specific activity 5.9 Ci/mol) throughout the eight day infusion period (see section 2.5). Ytterbium acetate (50 mg Yb per kg dry matter

Table 20 Composition, analysis and comparative intakes of the concentrate and hay diets

Concentrate diet

Component	g/kg (as is basis)
Barley	540
Wheat	240
Extracted soya bean meal	145
Grassmeal	50
Minerals and vitamins*	25

* see Table 11 for composition

Analysis

Dry matter (g/100 g)	89.6
Organic matter (g/100 g DM)	94.9
Estimated metabolisable energy^{+} (MJ/kg DM)	12.7
Nitrogen content (g/100 g DM)	3.01
MAD fibre (g/100 g DM)	5.85

(continued . . .

Table 20 (continued)

Long hay

Analysis

Dry matter (g/100 g)	92.0
Organic matter (g/100 g DM)	94.7
Estimated metabolisable energy+ (MJ/kg DM)	8.4
Nitrogen content (g/100 g DM)	1.16
MAD fibre (g/100 g DM)	33.7

+ determined from MAFF (1975)

Comparative intakes

	Concentrate	Hay
Dry matter (g/day)	627	908
Metabolisable energy (MJ/day)	8.0	7.6
Nitrogen intake (g/day)	18.9	10.5
MAD fibre (g/day)	36.7	306

intake) and chromium EDTA (250 mg Cr per kg dry matter intake) were intraruminally infused as predominant solid and liquid digesta flow markers respectively (see section 2.10). Appropriate samples of duodenal digesta taken during days 7 to 11 of the infusion period were pooled and subsequently fractionated into particle rich and fluid rich components (see section 2.10.3.4). Dry matter flow was determined using the dual phase marker technique (Faichney, 1975) (see Appendix 5 for details of calculation). Samples of plasma, saliva, rumen fluid and duodenal digesta were obtained routinely at eight hour intervals during the eight day isotope infusion period and relevant specific activity profiles determined (see sections 2.6-2.9). A series of rumen fluid samples were taken over approximately 24 hr following the cessation of the marker infusion for chromium analysis (see section 2.10.3.1).

4.5 Experimental results

4.5.1 Protein bound leucine specific activity profiles

The specific activity of the protein bound leucine fractions was determined (see section 2.9) approximately 8 times during the infusion period. The increase in specific activity of the various protein fractions was assumed to be in the form of single exponentials and specific activity profiles were derived as indicated in section 3.5.1. The specific activity profiles of the various fractions for each animal are included in Appendix 2 (2.4-2.9). The profile parameters for each individual animal are given in Appendix 3. The rate constants of the saliva, plasma protein and corrected digesta specific activity curves were compared between diets using an analysis of variance (see Table 21). Representative curves are considered below.

Table 21 Comparison of the rate constants (/hr) of the saliva, plasma protein and corrected digesta specific activity profiles in sheep fed either concentrates or hay

Specific activity profile	DIET		SED	Statistical significance of diet
	Concentrate	Hay		
	Rate constant (/hr)+			
Saliva	0.0328	0.0522	0.01121	NS
Plasma protein	0.0040	0.0028	0.00203	NS*
Corrected digesta	0.0175	0.0198	0.00769	NS

\+ Each value is a mean of 3 animals. See Appendix 3 for individual animal data.

NS Difference not significant ($p > 0.1$)

* See text

4.5.1.1 Total duodenal digesta

The shape of the total duodenal digesta specific activity profile was similar in all animals studied. There was a gradual rise during the initial 100 hr of the infusion after which the specific activity appeared to plateau. The mean (± SEM, n = 3) time taken to achieve 95% of plateau was 253 ± 72.5 hr for the animals fed the concentrate diet and 187 ± 31.5 hr for the animals fed hay.

4.5.1.2 Duodenal bacteria

The shape of the duodenal bacteria specific activity curve was similar in all animals studied. A gradual increase in specific activity was observed with indications of a plateau towards the end of the infusion. The mean (± SEM, n = 3) time taken to achieve 95% of plateau was 483 ± 65.3 hr for the sheep fed the concentrate diet and 273 ± 66.0 hr for those fed hay.

4.5.1.3 Saliva

The shape of the saliva specific activity profile was similar in all animals studied. The curves approached a plateau region after approximately 50 hr. The mean rate constant for each diet is given in Table 21. No significant dietary effect was observed ($p>0.1$). The calculated overall mean (± SEM, n = 6) time taken to reach 95% plateau was 169 ± 83.1 hr.

4.5.1.4 Plasma protein

The plasma protein specific activity curves appeared to be essentially linear over 200 hr. The mean rate constant for each diet is

given in Table 21. No significant dietary effect was observed ($p>0.1$). However, the value for sheep F was vastly different to that of sheep D and E (see Appendix 3). An accurate comparison was difficult with only three animals per diet. The overall mean ($\pm$ SEM, n = 6) predicted 95% plateau estimate, was 1071 $\pm$ 1034 days. Excluding sheep F a realistic estimate of the predicted 95% plateau was obtained (36 $\pm$ 7.4 days).

4.5.1.5 Corrected digesta curve

The shape of the specific activity profile of corrected digesta was similar in all animals studied. The curves approached a plateau region after approximately 75 hr. The mean rate constant for each diet is given in Table 21. No significant dietary effect was observed ($p>0.1$). The mean ($\pm$ SEM, n = 6) time taken to reach 95% plateau was 189 $\pm$ 37.4 hr.

4.5.2 The determination of the endogenous protein leucine flow using the L-[4,5-^{3}H]-leucine technique in sheep fed either concentrates or hay

Minimum, maximum and interpolated estimates of endogenous protein leucine flow were calculated as indicated in section 3.5.2 for each individual animal. Dry matter flow was determined using the dual phase marker technique (Faichney, 1975) (see Appendix 5 for details of calculation). A summary of data used in the determination of bacterial nitrogen flow using DAPA as a microbial marker and the proportion of duodenal digesta of microbial origin is included in Table 22. Data for individual animals is included in Appendix 6. Data required in the calculation of duodenal digesta and duodenal bacterial leucine flows are summarised in Table 23. Data for individual animals are included in Appendix 7.

Table 22 Duodenal digesta protein nitrogen flow (g/day) and bacterial nitrogen flow (g/day) in sheep fed either concentrates or hay

	DIET			
	Concentrate		Hay	
	Mean+	SEM	Mean+	SEM
Duodenal digesta dry matter flow (g/day)	266	37.7	626	51.4
Duodenal digesta protein nitrogen (g/100 g DM)	3.01	0.253	2.72	0.205
Duodenal digesta protein nitrogen flow (g/day)	8.13	1.60	17.20	2.68
Duodenal digesta DAPA (g/100 g DM)	0.145*	0.027	0.071	0.0086
Rumen bacteria DAPA (g/100 g DM)	0.367	0.1056	0.328	0.0268
Rumen bacteria nitrogen (g/100 g DM)	7.60	0.172	7.92	0.248
DAPA flow (g/day)	0.441*	0.085	0.436	0.038
$\frac{\text{Rumen bacteria nitrogen (g/100 g DM)}}{\text{Rumen bacteria DAPA (g/100 g DM)}}$	25.2	7.93	24.6	2.78
Bacterial nitrogen flow (g/day)	7.42*	0.53	10.53	0.292
Proportion of duodenal digesta of microbial origin	0.73	0.043	0.64	0.081

\+ Mean ± SEM estimate of 3 sheep, unless otherwise indicated.

* Value for sheep F omitted in mean determination, see text, i.e. n = 2.

Data for individual animals is included in Appendix 6.

Table 23 The flow of duodenal digesta leucine (mmol/day) and duodenal bacterial leucine (mmol/day) in sheep fed either concentrates or hay

	DIET			
	Concentrate		Hay	
	Mean[+]	SEM	Mean[+]	SEM
Duodenal digesta dry matter flow (g/day)	266	37.7	626	51.4
Duodenal digesta leucine (g/100 g DM)	1.53	0.098	1.36	0.051
Duodenal digesta leucine flow (g/day)	4.14	0.776	8.52	0.869
Duodenal digesta leucine flow (mmol/day)	31.58	5.913	64.97	6.624
Duodenal bacterial protein leucine (g/100 g DM)	2.52	0.217	3.05	0.075
Rumen bacterial nitrogen (g/100 g DM)	7.60	0.172	7.92	0.248
Bacterial nitrogen flow (g/day)[++]	7.42*	0.53	10.53	0.292
Bacterial dry matter flow (g/day)	97.8*	3.2	133.7	4.92
Duodenal bacterial leucine flow (g/day)	2.49*	0.445	4.08	0.205
Duodenal bacterial leucine flow (mmol/day)	18.92*	3.41	31.04	1.558

\+ Mean ± SEM estimate of 3 sheep, unless otherwise indicated

++ Determined using DAPA as a microbial marker (see Table 22)

* Value for sheep F omitted in mean determination, see text, i.e. n = 2.

Data for individual animals is included in Appendix 7.

Further analysis (see section 4.5.3) suggested the estimate of bacterial nitrogen flow for sheep F was unrealistic. This was attributed possibly to the low value for DAPA in duodenal digesta (see Appendix 6). Relevant values for this sheep were therefore omitted in the determination of the mean of certain parameters (see Table 22 and 23). As indicated previously, estimates of endogenous protein leucine were determined for each animal and realistic estimates derived in each case (see below).

Mean estimates of the endogenous protein leucine flow for each diet determined using the proposed technique and the theoretical maximum value (i.e. total protein leucine flow - bacterial leucine flow) are given in Table 24. The theoretical maximum value represents endogenous protein leucine flow plus any undegraded feed leucine. Data for individual animals is included in Appendix 8. The data were analysed using an analysis of variance.

In the sheep fed the concentrate diet the mean maximum endogenous protein leucine flow estimate derived using plasma protein as the precursor approached the mean theoretical maximum. None of the estimates of endogenous protein leucine flow derived using the [^{3}H]-leucine technique were significantly different between diets ($p > 0.1$). This suggested that the endogenous protein leucine flow is not dietary dependent for the rations investigated. The mean theoretical maximum value was significantly greater ($p < 0.05$) in the sheep fed hay than in those fed concentrates. This suggests that there is more undegraded dietary protein in the proximal duodenal digesta of the hay fed animals.

The lack of a significant difference in endogenous protein leucine flow between diets contradicts previous suggestions regarding endogenous protein flow in ruminants (see section 4.1). In this study the definition of

Table 24 Estimates of endogenous protein leucine flow (g/day) determined using the L-[4,5-^{3}H]-leucine technique and the theoretical maximum value in sheep fed either concentrates or hay

	Endogenous protein leucine flow* (g/day)		SED	Statistical significance of diet
	Concentrate diet	Hay diet		
[^{3}H]-leucine technique				
a) Saliva	0.577	0.712	0.122	NS
b) Plasma protein	1.92	2.94	0.689	NS
c) Interpolated	1.11	0.898	0.264	NS
Theoretical maximum++	2.13	4.45	0.774	+

* Each value is a mean of 3 animals (see Appendix 8 for individual animal data)

NS Difference not significant ($p>0.1$)

\+ Significantly different ($p<0.05$)

++ Theoretical maximum = Total protein leucine flow - bacterial leucine flow = Endogenous protein leucine + undegraded feed leucine.

'endogenous protein' excludes the fraction of the endogenous inputs to the rumen which may be incorporated into microbial protein (see section 1.6). The incorporation of endogenous leucine by rumen bacteria has been observed (see section 3.5.1.2).

The suggestion that the endogenous protein leucine flow at the proximal duodenum is not dietary dependent with respect to the dietary regimes investigated is discussed further in section 6.4.

The conversion of values for endogenous protein leucine to endogenous protein nitrogen is very important. As indicated in section 3.5.2.2 this requires a knowledge of the leucine content of endogenous secretions. This is considered in the discussion (section 6.2).

4.5.3 Rumen parameters, the flow of digesta constituents and the efficiency of bacterial nitrogen synthesis in sheep fed either concentrates or hay

Values of rumen pH, rumen ammonia, rumen volume, liquid phase dilution rate and rumen and duodenal liquid outflow rates were analysed using an analysis of variance (see Table 25). Rumen volume and rumen and duodenal liquid outflow were significantly higher ($p<0.01$) in the hay fed animals as was rumen pH ($p<0.05$). The liquid phase dilution rate was also higher in these animals although the difference was not statistically significant ($p>0.05$). Rumen ammonia concentration was significantly higher ($p<0.01$) in the concentrate fed animals.

A comparison of certain flow parameters and the efficiency of bacterial nitrogen synthesis for the concentrate and hay diets using an analysis of variance is included in Table 26. Data used in the

Table 25 Rumen parameters and duodenal liquid outflow rate (L/day) in sheep fed either concentrates or hay

Parameter	DIET		SED	Statistical significance of diet
	Concentrate	Hay		
Rumen pH	5.9	6.6	0.256	*
Rumen ammonia (mM)	28.8	3.2	3.28	**
Rumen volume (L)	2.5	6.6	0.63	**
Liquid phase dilution rate (/day)	1.42	2.03	0.280	NS
Rumen liquid outflow (L/day)	3.3	13.5	1.51	**
Duodenal liquid outflow (L/day)	5.1	15.8	2.31	**

NS Difference not significant ($p > 0.05$)

* Significantly different ($p < 0.05$)

** Significantly different ($p < 0.01$)

Table 26 Duodenal digesta dry matter flow (g/day), organic matter flow (g/day), digestion of organic matter in the rumen, bacterial nitrogen flow (g/day) and the efficiency of bacterial nitrogen synthesis in sheep fed either concentrates or hay

Parameter	DIET		SED	Statistical significance of diet
	Concentrate+	Hay+		
Dry matter intake (g/day)	627	908	-	-
Organic matter intake (g/day)	595	860	-	-
Dry matter flow (g/day)	266	626	63.8	**
Organic matter flow (g/day)	220	526	69.2	*
Organic matter apparently fermented in the rumen (g/day)	375	334	57.3	NS
Bacterial nitrogen flow (g/day)	7.42++	10.53	0.488	**
Proportion of organic matter intake apparently digested in the rumen	0.63	0.39	0.083	*
Proportion of organic matter intake truly digested in the rumen	0.72	0.50	0.070	*
Efficiency of bacterial nitrogen synthesis (g N/kg OMADR)	21.9++	33.0	6.47	NS
Efficiency of bacterial nitrogen synthesis (g N/kg OMTDR)+++	18.4++	25.5	3.38	NS

\+ Each value is a mean of three animals unless otherwise indicated

++ Value for sheep F omitted (see text), i.e. n = 2

+++ 117 g bacterial NAN per kg bacterial organic matter (Lindsay and Hogan, 1972)

Data for individual animals is included in Appendix 9.

* Significantly different ($p<0.05$)

** Significantly different ($p<0.01$)

NS Difference not significant ($p>0.05$)

determination of the efficiency of bacterial nitrogen synthesis for individual animals are given in Appendix 9.

Dry matter flow was significantly higher ($p<0.01$) in the hay fed animals as also was organic matter flow ($p<0.05$). The amount of organic matter apparently fermented in the rumen was not significantly different between the two diets ($p>0.05$) but the proportions of organic matter intake apparently and truly digested in the rumen were higher ($p<0.05$) in the concentrate fed animals. Bacterial organic matter flows were estimated assuming 117 g bacterial NAN per kg bacterial organic matter (Lindsay and Hogan, 1972). Bacterial nitrogen flow was significantly higher in the hay fed sheep ($p<0.01$). The analysis of bacterial nitrogen flow and estimates of the efficiency of bacterial nitrogen synthesis excluded data for sheep F. These values were considered as 'missing values' in the GENSTAT program. Unrealistic estimates were obtained for sheep F, possibly as a result of the low measured concentration of DAPA in duodenal digesta (see Appendix 6). The apparent and true efficiencies of bacterial nitrogen synthesis (g N/kg OMDR) were not significantly different with diet ($p>0.05$). The mean $\pm$ SEM apparent efficiency of bacterial nitrogen synthesis (g N/kg OMADR) for the concentrate and hay diets of 21.9 ± 1.50 ($n = 2$) and 33.0 ± 5.54 ($n = 3$) were similar to the values proposed by the ARC (1984) of 26.1 ± 7.46 ($n = 18$) and 30.0 ± 11.71 ($n = 19$) for sheep fed similar diets.

A comparison of the total digesta nitrogen flow parameters is included in Table 27. Data for individual animals are included in section 6.6. Intake of nitrogen was greater in sheep on the concentrate diet. Digesta total nitrogen flow was significantly higher in the hay fed animals ($p<0.05$). Net losses of total nitrogen thus occurred between mouth and

Table 27 Duodenal digesta total nitrogen, protein nitrogen and ammonia nitrogen flows (g/day) in sheep fed either concentrates or hay

Parameter	DIET		SED	Statistical significance of diet
	Concentrate+	Hay+		
Digesta total nitrogen flow (g/day)	9.4	18.2	3.17	*
Digesta protein nitrogen flow (g/day)	8.1	17.2	3.12	*
Digesta ammonia nitrogen flow (g/day)	1.28	0.96	0.115	*

\+ Each value is a mean of 3 animals.

* Significantly different ($p<0.05$)

Data for individual animals is included in section 6.6

duodenum in the concentrate fed animals compared with net gains in those fed hay. Digesta ammonia nitrogen flows were significantly higher ($p<0.05$) in the concentrate fed animals and protein nitrogen flow significantly higher ($p<0.05$) in the animals fed hay. The determination of the in vivo degradability of the rations is considered in section 6.6.

4.5.4 Amino acid composition of diets, rumen bacteria and duodenal digesta in sheep consuming protein free, concentrate and hay diets

The amino acid composition (g amino acid/100 g total amino acids of the protein-free, concentrate and hay diets is given in Table 28. The amino acid profiles of rumen bacteria and duodenal digesta (g amino acid/100 g total amino acids) are included in Table 29. Each sample was a mean of three determinations.

Data were analysed using an analysis of variance. Data were considered as a split-plot design with animals as the main plot within which rumen and duodenal samples were compared.

For all amino acids, except proline, the diet was not shown to have any effect on the difference between the duodenal digesta and rumen microbial concentrations.

Within a diet there was a close similarity in amino acid composition of rumen bacteria and duodenal digesta; significant differences occurring only with aspartic acid, proline, tyrosine and histidine ($p<0.001$) and serine and lysine ($p<0.01$).

A comparison of the composition of rumen bacteria and duodenal digesta samples between diets indicated a general similarity, significant differences occurring only in the concentrations of aspartic acid, threonine and tyrosine ($p<0.01$) and serine and lysine ($p<0.05$).

Table 28 Amino acid composition (g amino acid/100 g total amino acids) of the essentially protein-free, concentrate and hay diets

Amino acid	Amino acid composition (g amino acid/100 g total amino acids)		
	Protein-free diet	Concentrate diet	Hay diet
Aspartic acid	8.95	7.95	10.49
Threonine	4.23	3.67	5.68
Serine	5.15	4.60	5.17
Glutamic acid	21.88	27.41	12.95
Proline	7.36	9.76	8.20
Glycine	5.76	4.34	6.33
Alanine	9.96	4.30	7.65
Valine	5.82	5.03	6.79
Methionine	2.02	1.21	1.19
Isoleucine	4.20	3.91	4.99
Leucine	8.27	7.56	9.57
Tyrosine	3.52	3.18	3.41
Phenylalanine	5.33	4.99	5.93
Lysine	2.54	4.19	5.73
Histidine	1.99	2.27	1.82
Arginine	6.07	5.64	5.75

Table 29 *Amino acid composition (g amino acid/100 g total amino acids) of rumen bacteria and duodenal digesta in sheep fed essentially protein-free, concentrate and hay diets*

Amino acid	Amino acid composition (g amino acid/100 g total amino acids)						SED for comparing mean samples on same diet(6df)	SED for comparing mean samples on different diets(6df)	Statistical significance of effect		
	Protein-free diet		Concentrate diet		Hay diet				Main effects		Interaction
	Rumen bacteria	Duodenal digesta	Rumen bacteria	Duodenal digesta	Rumen bacteria	Duodenal digesta			diets	samples	diet/samples
Aspartic acid	12.78	12.23	12.00	10.92	12.44	11.68	0.187	0.237	**	***	NS
Threonine	6.14	5.51	6.10	5.83	6.91	6.85	0.476	0.413	**	NS	NS
Serine	5.01	5.36	4.32	4.97	4.24	4.89	0.200	0.217	*	**	NS
Glutamic acid	14.04	14.50	13.22	12.82	13.02	12.45	0.513	0.635	NS	NS	NS
Proline	2.79	4.99	3.78	4.67	3.75	4.42	0.313	0.344	NS	***	*
Glycine	5.58	5.86	5.71	6.23	6.08	6.04	0.259	0.260	NS	NS	NS
Alanine	7.93	7.48	7.37	7.53	7.65	7.34	0.250	0.282	NS	NS	NS
Valine	6.00	5.86	6.22	6.19	6.43	6.47	0.143	0.218	NS	NS	NS
Methionine	1.58	1.99	1.89	1.77	1.70	1.37	0.419	0.404	NS	NS	NS
Isoleucine	5.66	5.13	5.66	5.72	5.71	5.36	0.297	0.320	NS	NS	NS
Leucine	8.38	8.18	10.15	11.18	8.56	9.26	0.430	1.060	NS	NS	NS
Tyrosine	4.71	3.99	4.37	3.64	4.51	4.04	0.151	0.122	**	***	NS
Phenylalanine	4.79	4.65	4.99	5.08	4.94	5.47	0.197	0.265	NS	NS	NS
Lysine	8.21	7.74	7.76	7.25	7.67	6.57	0.257	0.305	*	**	NS
Histidine	1.57	1.82	1.81	2.02	1.67	2.03	0.062	0.090	NS	***	NS
Arginine	4.51	4.66	4.59	4.18	4.74	4.86	0.228	0.216	NS	NS	NS

NS Not significantly different ($p>0.05$) * Significantly different ($p<0.05$)

** Significantly different ($p<0.01$) *** Significantly different ($p<0.001$)

Thus although the amino acid intake (g amino acid/100 g total amino acid) on the protein-free, concentrate and hay diets varied, the amino acid profiles of rumen bacteria and duodenal digesta were similar. This illustrates the buffering action of the bacterial fraction on the composition of protein at the duodenum and the general constant composition of the bacterial fraction between diets.

4.5.5 Estimation of leucine flux through the plasma free pool in sheep fed either concentrates or hay

Leucine flux through the plasma free pool was calculated from the mean plateau specific activity of plasma free leucine as indicated in section 3.5.6. Data used in the calculation are presented in Table 30.

Table 30 Estimate of leucine flux (mmol/day) through the plasma free pool in sheep fed either concentrates or hay

	DIET									
	Concentrate					Hay				
	Sheep D	Sheep E	Sheep F	Mean+	SEM	Sheep G	Sheep H	Sheep K	Mean+	SEM
Infusion rate (mCi/day)	0.23	0.21	0.28	0.24	0.021	0.25	0.23	0.31	0.26	0.024
Plasma free leucine specific activity $(dpm/\mu mol) \times 10^{-3}$	4.9	4.5	7.6	5.7	0.97	7.1	4.8	7.9	6.6	0.93
Mean ± SEM, n = 5	0.46	0.53	0.60			0.37	0.28	0.52		
Leucine flux (mmol/day)	104.2	103.6	81.8	96.5	7.37	78.2	106.4	87.1	90.6	8.32

+ Mean ± SEM estimate of 3 sheep

Chapter 5

THE DETERMINATION OF THE ENDOGENOUS PROTEIN LEUCINE FLOW USING THE INTRAVENOUS INFUSION OF L-[4,5-^{3}H]-LEUCINE TECHNIQUE IN STEERS FED SILAGE SUPPLEMENTED WITH FISHMEAL

5.1 Preliminary investigation

5.1.1 Introduction

Recent trends in agriculture have intensified the need to fully understand nitrogen metabolism in forage fed cattle. An estimate of the endogenous protein content of proximal duodenal digesta is essential if this aim is to be achieved.

A preliminary experiment was performed using a single steer in order to resolve any practical problems associated with the eight day intravenous infusion/sampling routine and to determine the optimum level of isotope infusion.

5.1.2 Experimental animal

A Friesian steer (M), approximately 400 kg in weight and two years of age, equipped with a 37 mm rumen cannula and a 19 mm proximal duodenal T-piece cannula was involved in this experiment. Throughout the experimental period the animal was housed in a metabolism crate, within a controlled environment (24 hr light). Water was available ad libitum.

5.1.3 Experimental diet

The analysis of the silage (E4) and fishmeal supplement (Provimi 66, British White fishmeals Ltd) are given in Table 31. Silage was made on the University Farm from a 24 hr wilted first cut predominantly perennial ryegrass sward. The additive Add-F (BP Nutrition [UK] Ltd, Northwich, Cheshire), 85% formic acid, was applied at an approximate rate of 3 L per tonne. The silage was ensiled in a concrete-walled clamp covered with a black plastic sheet and tyres. Silage for the experimental period was obtained from the clamp in a single batch, in hessian sacks, frozen and stored on the experimental farm. Silage was offered at a rate of 24 g DM silage per kg liveweight of animal and the fishmeal supplement included at a level of 50 g fishmeal per kg DM intake of silage.

A preliminary feeding trial determined the actual amount fed. The actual intake was 45 MJ ME per day, equivalent to the maintenance requirement for a 400 kg steer (MAFF, 1975). The ration was provided via a moving belt automatic feeding system, the animal receiving approximately one twelfth of its daily ration at 2 hr intervals. Feed intake was determined daily. The animals had free access to mineral blocks (Super Chelated Rockies, Tithebarn Ltd, Southport).

5.1.4 Experimental procedure

The routine experimental protocol concerning animal management, time scale of isotope and digesta flow marker infusions and sampling routine is given in Fig 8. Specific details concerning this experiment are detailed below.

Table 31 Analysis of silage and fishmeal supplement

Silage

	Preliminary investigation (E4)	Main investigation (E5)
Dry matter (g/100 g)(toluene corrected)	22.4	23.9
Organic matter (g/100 g DM)	91.1	92.4
Metabolisable energy (MJ/kg DM)	9.7	10.4
pH	3.7	4.1
MAD fibre (g/100 g DM)	37.0	32.3
Total nitrogen content (g/100 g DM)	1.75	2.03

Fishmeal

Dry matter (g/100 g)	90.0
Organic matter (g/100 g DM)	82.7
Estimated metabolisable energy (MJ/kg DM)	11.1
Nitrogen content (g/100 g DM)	11.3

L-[4,5-^{3}H]-leucine was infused at a rate of 1 mCi per day (specific activity 27.6 Ci/mol) (see section 2.5). Ytterbium acetate (50 mg Yb per kg dry matter intake) and Cr EDTA (250 mg Cr per kg dry matter intake) were infused intraruminally (see section 2.10) and dry matter flow calculated using the dual phase marker technique (Faichney, 1975) (see Appendix 5 for details of calculation). Samples of duodenal digesta were collected every 2 hr for 24 hr during day 6 of the infusion period, pooled, temporarily stored at 4°C, thoroughly mixed and subsequently fractionated (see section 2.10.3.4). Samples of plasma, saliva, rumen fluid and duodenal digesta were obtained routinely at eight hour intervals during the eight day isotope infusion period and relevant specific activity profiles determined (see section 2.6-2.9). A series of rumen fluid samples were taken over approximately 24 hr following the cessation of the marker infusion for chromium analysis (see section 2.10.3.1).

The results of this preliminary investigation are included in the main investigation (see section 5.3).

5.2 Main investigation

5.2.1 Introduction

The aim of the experiment was to determine the endogenous protein leucine content of proximal duodenal digesta in young steers fed a silage ration supplemented with fishmeal. For comparative purposes ^{15}N was employed as a microbial marker in addition to DAPA.

5.2.2 Experimental animals

Four Friesian steers (P, R, T, V), approximately 170 kg in weight and 6 months of age, equipped with a 37 mm rumen and a 19 mm proximal duodenal T-piece cannula, were involved in this investigation. Throughout the experimental period the animals were housed in metabolism crates, within a controlled environment (24 hr light). Water was available ad libitum.

5.2.3 Experimental diet

The analysis of the silage (E5) and fishmeal supplement are given in Table 31. Details of silage preparation, storage and feeding regimen were as for the preliminary investigation (section 5.1.3), except that this silage (E5) was a second cut silage. The complete diet provided 42 MJ ME per day allowing for maintenance and a predicted 0.65 kg liveweight gain per day (MAFF, 1975).

5.2.4 Experimental procedure

The experimental procedure for the main investigation was equivalent to that adopted in the preliminary investigation (section 5.1.4). In addition, ^{15}N-labelled diammonium sulphate was infused intraruminally with the ytterbium acetate during days 6 to 11 of the experimental period (see Fig 8 and section 2.11.2) as a microbial marker. Samples of duodenal digesta derived from the fractionated pooled digesta (section 2.10.3.4) and a series of rumen microbial samples were analysed for enrichment (section 2.11.2.2).

5.3 Experimental results

5.3.1 Protein bound leucine specific activity profiles

The specific activity of the protein bound leucine fractions was determined (see section 2.9) approximately 8 times during the infusion period. The increase in specific activity of the various protein fractions was assumed to be in the form of single exponentials and specific activity profiles were derived as previously (see section 3.5.1). The specific activity profiles of the various fractions for each animal are included in Appendix 2 (2.10-2.14). The profile parameters for each individual animal are given in Appendix 3. The mean rate constants of the saliva, plasma protein and corrected digesta curves for all steers are included in Table 32. Representative curves are considered below.

5.3.1.1 Total duodenal digesta

In general there was a gradual rise in the specific activity of total duodenal digesta, the curve approaching a plateau region towards the end of the infusion period. From the determined equation for the curves of steers M, P and T the mean ($\pm$ SEM, n = 3) time taken to achieve 95% of plateau was 388 $\pm$ 56 hr. The curves for steers R and V appeared essentially linear.

5.3.1.2 Duodenal bacteria

In all steers, apart from P which approached a plateau after approximately 100 hr, the duodenal bacterial protein specific activity curve appeared essentially linear over 200 hr.

Table 32 Rate constants (/hr) of saliva, plasma protein and corrected digesta specific activity profiles in steers intravenously infused with L-[4,5-^{3}H]-leucine

Specific activity profile	Rate constant (/hr) (mean of 5 steers)+	SEM
Saliva	0.02088	0.004612
Plasma protein	0.00171	0.000519
Corrected digesta	0.00458	0.001775

+ See Appendix 3 for individual animal data

5.3.1.3 Saliva

The shape of the saliva specific activity profile was similar in all steers. The curves approached a plateau region after approximately 100 hr and the mean (± SEM, n = 5) time taken to achieve 95% of plateau was 17.4 ± 36 hr.

5.3.1.4 Plasma protein

The shape of the plasma protein specific activity profile was similar in all steers. The curves appeared to be essentially linear over 200 hr. The mean (± SEM, n = 4), predicted (95%) plateau estimate was calculated to occur after 68 ± 17.9 days. The value for steer R was not included in this determination (see Appendix 3).

5.3.1.5 Corrected digesta curve

In general, there was a gradual rise in the specific activity of corrected digesta with a plateau region being approached after approximately 150 hr. The mean (± SEM, n = 3) time taken to achieve 95% of plateau for steers M, P and T was was 463 ± 112 hr. The curves for steers R and V were essentially linear.

5.3.2 The determination of the endogenous protein leucine flow using the L-[4,5-^{3}H]-leucine technique in steers fed silage supplemented with fishmeal

Minimum, maximum and interpolated estimates of endogenous protein leucine flow were calculated for each animal as indicated in section 3.5.2. Dry matter flow was determined using the dual phase

marker technique (Faichney, 1975) (see Appendix 5 for details of calculation).

Data used in the determination of bacterial nitrogen flow using DAPA as a microbial marker and the proportion of duodenal digesta of microbial origin are given in Table 33. Estimates of microbial nitrogen flow and the proportion of duodenal digesta of microbial origin determined using ^{15}N are also included in Table 33.

Data used in the calculation of duodenal digesta leucine flow and duodenal microbial leucine flow are given in Table 34. Duodenal microbial leucine flows determined using DAPA or ^{15}N were not significantly different ($p>0.1$) when analysed using a paired t-test. The ^{15}N values were used in the calculation of the endogenous protein leucine flow as previous comparative studies (e.g. Siddons et al., 1982) have suggested ^{15}N as the preferred microbial marker.

Estimates of the endogenous protein leucine flow derived using the proposed technique and the theoretical maximum value (total protein leucine flow − microbial leucine flow) are given in Table 35. The theoretical maximum value represents endogenous protein leucine plus any undegraded dietary leucine. The mean ($\pm$ SEM, n = 4) interpolated estimate of endogenous protein leucine flow in 170 kg steers fed silage supplemented with fishmeal was 4.81 $\pm$ 0.743 g per day. The conversion of values for endogenous protein leucine to endogenous protein nitrogen is considered in the discussion (see section 6.2).

Table 33 Duodenal digesta protein nitrogen flow (g/day) and microbial nitrogen flow (g/day) (estimated using ^{15}N and DAPA) in steers fed silage supplemented with fishmeal

Parameter		STEER M	P	R	T	V	Mean+	SEM
Duodenal digesta dry matter flow (g/day)		3128	1616	2209	2286	2259	2093	159.6
Duodenal digesta protein nitrogen flow (g/day)		91.3	51.4	68.9	68.6	73.4	65.6	4.85
Rumen bacteria DAPA (g/100 g DM)		0.403	0.415	0.312	0.300	0.361	0.347	0.0262
Mean ± SEM, n = 4		0.0030	0.0100	0.0092	0.0104	0.0559		
Rumen bacteria nitrogen (g/100 g DM)		8.28	8.36	8.18	7.97	7.87	8.10	0.109
Mean ± SEM, n = 8		0.329	0.085	0.130	0.131	0.082		
DAPA flow (g/day)		2.20	1.19	1.78	1.37	1.56	1.48	0.127
Rumen bacteria nitrogen (g/100 g DM) / Rumen bacteria DAPA (g/100 g DM)		20.5	20.1	26.2	26.6	21.8	23.7	1.61
Microbial nitrogen flow (g/day)*	^{15}N	-	32.5	46.7	39.0	44.8	40.8	3.20
	DAPA	45.3	23.9	46.7	36.4	34.1	35.3	4.68
Proportion of duodenal digesta of microbial origin*	^{15}N	-	0.632	0.677	0.569	0.610	0.622	0.0225
	DAPA	0.495	0.465	0.677	0.531	0.465	0.534	0.0501

\+ Mean ± SEM 4 steers, main investigation

* Estimates based on ^{15}N or DAPA as the microbial marker

Table 34 The flow of duodenal digesta leucine (mmol/day) and duodenal microbial leucine flow (mmol/day) (estimated using ^{15}N and DAPA) in steers fed silage supplemented with fishmeal

Parameter		STEER M	P	R	T	V	Mean+	SEM
Duodenal digesta leucine flow (mmol/day)		340.9	202.0	286.2	278.8	285.8	263.2	20.47
Duodenal bacterial protein leucine (g/100 g DM) Mean ± SEM, n = 8		2.90 0.340	3.13 0.060	3.12 0.112	3.49 0.069	3.59 0.085	3.33	0.122
Rumen bacterial nitrogen (g/100 g DM) Mean ± SEM, N = 8		8.26 0.329	8.36 0.085	8.18 0.130	7.97 0.131	7.87 0.082	8.10	0.109
Microbial nitrogen flow (g/day)*	^{15}N	-	32.5	46.7	39.0	44.8	40.8	3.20
	DAPA	45.1	23.9	46.7	36.4	34.1	35.3	4.68
Microbial DM flow (g/day)*	^{15}N	-	389	571	489	569	505	43.0
	DAPA	546	286	571	457	433	437	58.6
Duodenal microbial leucine flow (mmol/day)*	^{15}N	-	92.7	135.8	130.2	155.8	128.6	13.18
	DAPA	120.7	68.2	135.8	121.5	118.6	111.0	14.76

\+ Mean ± SEM 4 steers, main investigation

* Estimates based on ^{15}N or DAPA as the microbial marker

Table 35 Estimates of endogenous protein leucine flow (g/day) determined using the intravenous infusion of L-[4,5-^{3}H]-leucine technique and the theoretical maximum value in steers fed silage supplemented with fishmeal

	Endogenous protein leucine flow (g/day) L-[4,5-^{3}H]-leucine technique			
Steer	Saliva	Plasma protein	Interpolated	Theoretical maximum +
M	4.06	17.81	6.46	28.89*
P	1.65	6.17	4.31	14.34
R	3.14	12.84	4.71	19.73
T	2.65	8.50	3.34	19.50
V	2.15	7.79	6.86	17.06
Mean++	2.40	8.83	4.81	17.66
SEM	0.321	1.424	0.743	1.260

\+ Total protein leucine flow - microbial leucine flow (based on ^{15}N) = Endogenous protein leucine + undegraded feed leucine

++ Mean ± SEM 4 steers (170 kg), main investigation

* Based on microbial leucine flow determined using DAPA

5.3.3 Rumen parameters, the flow of digesta constituents and the efficiency of microbial protein synthesis in steers fed silage supplemented with fishmeal

Values of rumen and duodenal pH, rumen ammonia, rumen volume, liquid phase dilution rate and rumen and duodenal liquid outflow are given in Table 36. The unrealistic high value for the rumen volume of steer R may be attributed to sampling difficulties.

Data used in the calculation of the efficiency of microbial protein synthesis are given in Table 37. The mean (± SEM, n = 4) efficiency of microbial protein synthesis (g N/kg OMADR) was 20.3 ± 2.08, determined using ^{15}N as the microbial marker. This estimate is lower than the values proposed by the ARC (1984) of 26.8 for cattle consuming all grass silage diets and 36.0 for cattle offered grass silage plus concentrates. The value is in close agreement with recent estimates for a similar diet, e.g. 17 g N/kg OMADR for formic acid treated silage supplemented with barley (Chamberlain et al., 1986) and 23 g N/kg OMADR for formic acid treated silage supplemented with barley and soya (Rooke et al., 1985).

The flow of duodenal digesta nitrogen constituents and the determination of the in vivo degradability of the ration are considered in section 6.6.

5.3.4 The amino acid composition of diet, rumen bacteria and duodenal digesta in steers fed silage supplemented with fishmeal

The amino acid composition (g amino acid/100 g total amino acids) of the diet, rumen bacteria and duodenal digesta are included in Table 38.

Table 36 Rumen parameters, duodenal pH and duodenal liquid outflow rate (L/day) in steers fed silage supplemented with fishmeal

Parameter	STEER					Mean+	SEM
	M	P	R	T	V		
Rumen pH Mean ± SEM, n = 7	6.63 0.109	7.03 0.081	7.01 0.101	6.99 0.066	6.89 0.074	6.98	0.031
Duodenal pH Mean ± SEM, n = 5	-	6.06 0.103	6.06 0.093	5.90 0.071	5.87 0.116	5.97	0.051
Rumen ammonia (mM) Mean ± SEM, n = 8	2.3 0.61	4.5 0.48	6.8 0.56	5.3 0.81	4.7 0.73	5.33	0.52
Rumen volume (L)	55	41	*	60	39	47	6.7
Liquid phase dilution rate (/day)	1.66	1.84	0.82	2.30	2.02	1.75	0.323
Rumen liquid outflow (L/day)	92	75	141	138	78	108	18.2
Duodenal liquid outflow (L/day)	104	53	76	77	86	73	7.0

+ Mean ± SEM 4 steers, main investigation

* Unrealistic value obtained (see text)

Table 37 *The digestion of organic matter in the rumen and the efficiency of microbial nitrogen synthesis (based on ^{15}N or DAPA) in steers fed silage supplemented with fishmeal*

Parameter		STEER					Mean+	SEM
		M	P	R	T	V		
Dry matter intake (kg/day)		4.50	3.78	4.49	3.80	4.14	4.05	0.168
Organic matter intake (kg/day)		4.08	3.48	4.12	3.50	3.81	3.73	0.151
Dry matter flow (g/day)		3128	1616	2209	2286	2259	2093	159.6
Organic matter flow (g/day)		2700	1270	1723	1877	1848	1680	140.5
Organic matter apparently fermented in the rumen (g/day)		1380	2210	2397	1623	1962	2048	167.4
Microbial nitrogen flow (g/day)*	^{15}N	-	32.5	46.7	39.0	44.8	40.8	3.20
	DAPA	45.1	23.9	46.7	36.4	34.1	35.3	4.68
Microbial organic matter flow (g/day)*	^{15}N	-	339	498	426	496	440	37.5
	DAPA	476	249	498	399	378	381	51.2
Proportion of organic matter apparently digested in the rumen		0.338	0.635	0.582	0.464	0.515	0.549	0.0375
Proportion of organic matter truly digested in the rumen*	^{15}N	-	0.733	0.703	0.585	0.645	0.667	0.0327
	DAPA	0.455	0.707	0.703	0.578	0.614	0.651	0.0823
Efficiency of microbial nitrogen synthesis (g N/kg OMADR)*	^{15}N	-	14.7	19.5	24.0	22.8	20.3	2.08
	DAPA	32.7	10.8	19.5	22.4	17.4	17.5	2.47
Efficiency of microbial nitrogen synthesis (g N/kg OMTDR)*	^{15}N	-	12.8	16.1	19.0	18.2	16.5	1.38
	DAPA	24.3	9.7	16.1	18.0	14.6	14.6	1.78

\+ Mean ± SEM 4 steers, main investigation

* Estimates based on ^{15}N or DAPA as the microbial marker

Table 38 Amino acid composition (g amino acid/100 g total amino acids) of the diet, rumen bacteria and duodenal digesta in steers fed silage supplemented with fishmeal

Amino acid	Amino acid composition (g amino acid/100 g total amino acids)				
	Diet	Rumen bacteria		Duodenal digesta	
		Mean	SEM	Mean	SEM
Aspartic acid	6.83	12.38	0.159	11.54	0.309
Threonine	4.34	6.40	0.131	6.18	0.209
Serine	3.41	3.99	0.155	4.73	0.175
Glutamic acid	10.22	12.65	0.118	12.97	0.276
Proline	4.05	3.46	0.201	4.58	0.207
Glycine	8.50	6.01	0.052	7.76	0.471
Alanine	17.99	7.24	0.174	7.31	0.219
Valine	8.23	7.01	0.167	6.63	0.483
Methionine	1.76	1.89	0.073	2.06	0.098
Isoleucine	6.30	5.73	0.049	5.51	0.106
Leucine	11.18	8.38	0.067	9.19	0.257
Tyrosine	2.39	4.98	0.087	4.54	0.125
Phenylalanine	5.91	5.49	0.080	5.76	0.207
Lysine	4.20	8.03	0.065	7.23	0.212
Histidine	1.64	1.66	0.050	2.13	0.053
Arginine	3.05	4.76	0.097	4.95	0.130

+ Mean $\pm$ SEM, 4 steers, main investigation

Thomas et al. (1980) determined the passage of amino acids to the duodenum in sheep given silage and silage plus barley. The mixture of amino acids passing to the duodenum and absorbed in the small intestine was low in methionine (15-20 g per kg total amino acids) and lysine (60-76 g per kg total amino acids) in these animals compared with sheep given hay and hay and cereal diets (see Chamberlain and Thomas, 1979a). Similar values were recorded for steers in this study. Caution should be used in the detailed interpretation of this data as no precautions were taken to prevent oxidation of methionine in the amino acid preparation.

Kelly and Thomas (1975) suggested that methionine was limiting for tissue synthesis in sheep given a diet of formic acid treated silage and indicated that a limited supply of methionine and lysine to the tissues may be an important factor contributing to the low nutritive value of silage nitrogen for dairy cows. Chamberlain et al. (1986) observed that in cows fed silage diets the supply of methionine and lysine to the duodenum was low and that these amino acids may be limiting for milk production.

5.3.5 Estimation of leucine flux through the plasma free pool in steers fed silage supplemented with fishmeal

Leucine flux through the plasma free pool was calculated from the mean plateau specific activity of plasma free leucine as indicated in section 3.5.6. Data used in the calculation are presented in Table 39.

Table 39 Estimate of leucine flux (mmol/day) through the plasma free pool in steers fed silage supplemented with fishmeal

	STEER					Mean+	SEM
	M	P	R	T	V		
Infusion rate (mCi/day)	0.92	1.02	0.99	0.90	0.89	0.95	0.032
Plasma free leucine specific activity $(dpm/\mu mol) \times 10^{-3}$	3.9	6.9	6.1	4.7	7.7	6.4	0.64
Mean ± SEM, n = 5	0.28	0.45	0.61	0.49	1.10		
Leucine flux (mmol/day)	523.7	328.2	360.3	425.1	256.6	342.6	35.0

+ Mean ± SEM 4 steers, main investigation

Chapter Six

DISCUSSION

6.1 Theoretical evaluation of the continuous intravenous infusion of L-[4,5-^{3}H]-leucine technique to determine directly the endogenous protein content of ruminant proximal duodenal digesta

6.1.1 The L-[4,5-^{3}H]-leucine technique — a summary

The proposed technique is an apparently novel approach. A fundamental problem with previous research in this area has been the inability to isolate the endogenous component of the duodenal digesta in the presence of microbial and undegraded feed protein. Using the proposed technique which involves a long term intravenous infusion of [^{3}H]-leucine, body proteins become labelled. Any radioactivity in duodenal digesta might therefore have been expected to be derived from endogenous sources. Unfortunately, the microbial fraction also became labelled (see section 3.5.1.2). The activity in the total duodenal digesta was corrected for this (see section 3.5.2.1). As a first approximation the major sources of endogenous protein could be considered as coming from the plasma or from secreted enzymes. The extent of fermentation of endogenous protein inputs to the rumen and the possible importance of undegraded sloughed cells are considered later in section 6.1.4. As described in section 3.5.2.2 a comparison of the specific acitivity of the duodenal digesta with either that of the saliva or blood plasma proteins enables two possible estimates of the endogenous secretions in the digesta to be obtained. The difference between these estimates was relatively large. A method for calculating one value was developed and its validity explored.

6.1.2 The precursor concept

The plasma protein fraction was considered representative of the endogenous inputs to the digestive tract such as albumin/globulins.

There is little information in the literature concerning the origin of the protein material in the salivary glands of ruminants. Limited information is available for non-ruminants.

Langstroth *et al.* (1938) identified in the submaxillary saliva of cats a protein material originating within the secretory cells. These authors observed that the glandular membrane did not normally permit the passage of large molecules from the tissue fluids into the secretion in appreciable amounts. They suggested that the mechanism responsible for the secretion of protein material consisted of a chemical reaction or chain of reactions, resulting in the transformation of granular material to a form readily carried out of the cells by the flow of water and that this mechanism was of the same nature as that operating in the pancreas, i.e. in modern terms *de novo* synthesis of proteins.

Castle *et al.* (1972) studied the steps in the synthesis of the secretory granules within the acinar cells of the rabbit parotid gland using a pulse-labelling technique and again observed the *de novo* synthesis of protein (amylase). The intracellular processing of secretory protein was similar to that in the pancreas, except that the rate was slower and the storage more prolonged. Glass (1968) identified an intrinsic glycoprotein, gastrone, in human *in vivo* neutralised gastric juice which was identical to a similar protein in saliva (see section 1.3.2).

Some evidence is available to indicate that salivary protein is not completely synthesised *de novo*. Holman (1959) identified serum proteins,

particularly albumin and gamma globulin in human saliva. Ellison (1967) identified in human saliva 'intrinsic' secretory materials and serum proteins. Also, studies in this laboratory using sodium dodecyl sulphate polyacrylamide gel electrophoresis (Laemmli, 1970) suggested that proteins synthesised de novo and plasma proteins were present in ruminant saliva. Similar conclusions were drawn by McIntosh (1975) The major question in the present context however, is how much of the protein in saliva is synthesised de novo and how much is derived from plasma protein.

During the present study the plateau specific activity of saliva protein was achieved relatively quickly (see section 3.5.1.5) suggesting that the majority of the protein was being synthesised de novo. Since there was little evidence of a slow increase in the specific acitivity of saliva protein it was concluded that the specific acitivity of salivary protein would be similar to that of enzymes secreted in the gastrointestinal tract.

Examination of the specific acitivity curves of the corrected digesta (i.e. total digesta - microbes) indicated that the endogenous component of the digesta was not composed entirely of a fraction synthesised at the same rate as either that expected for the enzymes or blood protein. After detailed consideration it was concluded that the endogenous fraction was composed of at least two components, one of which was considered to be similar to digestive enzymes and another which was considered to be similar to plasma protein. This concept is examined further below.

6.1.3 The validity of the interpolated estimate

A method was proposed (see section 3.5.2.3) to derive a single value for the endogenous protein content of proximal duodenal digesta.

Using the saliva specific activity profile gave low values for the endogenous protein flow while the specific activity profile of plasma protein gave high values. These two values were considered the extreme possibilities. Studies with purified diets suggested that the true value was somewhere between (see section 3.5.3). A method of determining this value from the shape of the corrected digesta, saliva and plasma protein specific activity profiles was developed (see section 3.5.2.3)

The corrected digesta specific activity profile is derived from the total duodenal digesta and duodenal microbial protein specific activity curves (see section 3.5.2.1). The specific activity profiles of total duodenal digesta and duodenal microbial protein were assumed to be single exponentials. This was necessary as the limited number of data points precluded any further detailed analysis of the curves. Any errors introduced as a result were considered of minimal significance (see below). Although the consideration of the corrected digesta curve as a single exponential (see section 3.5.2.1) is strictly an approximation, any error introduced by this procedure was again considered insignificant because of the similarity of exponential curves wih varying numbers of components (see Isenberg et al., 1973). For a particular precursor the ratio k corrected digesta/k precursor (see section 3.5.2.3) was proportional to the endogenous leucine estimate derived using that precursor. A linear relationship between endogenous protein leucine flow and the ratio of the rate constants (k corrected digesta/k precursor) was considered an appropriate interpretation.

It is accepted that there are many assumptions involved in the determination of the endogenous protein content of proximal duodenal digesta using the proposed technique. Nevertheless, estimates derived

using the [^{3}H]-leucine method agreed closely with those calculated by difference in sheep fed an essentially protein-free diet (see section 3.5.3).

In attempting to determine the precision required in estimates of endogenous protein secretions the effect of variation in the value on calculating in vivo protein degradability is given in section 6.7.3. This should be considered in conjunction with the sensitivity analysis to be described in section 6.7.2.1. In the latter case the effect of errors in the measurement of the components used to calculate the endogenous protein secretion are considered.

6.1.4 The degradation of endogenous protein entering the rumen and the importance of a significant undegraded sloughed cell endogenous component

Endogenous protein inputs to the rumen include sloughed cells and saliva (see section 1.3).

Sloughed cells originate from the mouth, buccal cavity, oesophagus, rumen-reticulum, omasum and abomasum. Evidence for the direct incorporation of amino acids from the rumen epithelium by the rumen microbes has been obtained in this study (see section 3.5.1.2). The extent of this incorporation is difficult to predict. Estimates obtained from the literature vary.

Orskov (1986) indicated that it is likely that most of the nitrogen (protein) in the epithelial cells of the buccal cavity, oesophagus and rumen would be degraded to ammonia in the conventionally fed animal.

Dinsdale et al. (1980) obtained epithelial cells from sheep maintained by intragastric infusion. The rate of digestion of these cells in rumen fluid was estimated in vitro. After 48 hours, 43% of the dry matter of epithelial cells and their remnants had been lost.

Evidence for the degradation of 'sloughed cells' in the entire digestive tract has been obtained. Mason (1969) identified no cells of animal origin in the faeces of sheep fed high fibre rations. This suggested that the majority of shed intestinal cells must have been autolysed rapidly or been degraded by microbial attack. Mason and Milne (1971) observed that the degradation of bacterial mucopolypeptide synthesised in the rumen occurred only in the caecum and colon.

The fact that proximal duodenal digesta may contain an undegraded sloughed cell endogenous component must also be considered. In the preparation of a microbial fraction, any sloughed cells are removed with the feed during the initial centrifugation stage (see section 2.6.3). Thus if sloughed cells are present in proximal duodenal digesta then a sloughed cell component is included in the 'corrected digesta' curve (i.e. total duodenal digesta - microbial fraction, see section 3.5.2.1 for derivation). However, no precursor is readily available which can be considered representative of a sloughed cell fraction. Since the fractional synthetic rate of the rumen epithelium is 0.26-1.5/day (Davis et al., 1981), the predicted time for an endogenous component derived from the epithelium to achieve plateau might be expected to be longer than that of salivary protein but shorter than that of blood protein (fractional synthetic rate of albumin estimated to be 0.028-0.035/day, Campbell et al. (1961); Dargie and Berry (1979)). If a precursor representative of sloughed cells were available a third estimate of endogenous protein flow, in addition to those derived using either saliva or plasma protein as appropriate precursors would be possible. This might provide an additional point on the regression line relating endogenous protein flow to the ratio of the rate constants (k corrected digesta/ k precursor).

There is little definitive information on the extent of degradation of saliva protein in the rumen.

Phillipson and Mangan (1959) observed that salivary mucoprotein was readily metabolised in the rumen. Hogan (1975) suggested that the nitrogen components of saliva were hydrolysed and deaminated almost as rapidly as casein by rumen microorganisms in vivo. Hashimoto et al. (1963) indicated that a proteolytic enzyme from Streptomyces griseus at low concentrations was found to cause extensive degradation of bovine submaxillary mucin and produce considerable amounts of free amino acid and dialysable sialic acid in the form of glycopeptides.

However, Nugent et al. (1983) considered that bovine submaxillary mucin was degraded very slowly in the rumen or escaped degradation and was available for digestion and absorption in the small intestine. The present technique accommodates the possibility that salivary protein escapes rumen degradation, since its specific activity has been measured directly.

6.2 The determination of endogenous protein nitrogen flow from leucine data

The endogenous protein fraction of proximal duodenal digesta probably consists of several components (see section 1.3). The amino acid composition of this fraction is extremely difficult to determine. If the leucine content of endogenous protein was known estimates of endogenous protein nitrogen could be derived easily from the leucine flow data.

The endogenous protein fraction can be considered to consist of enzymes, plasma proteins, undegraded sloughed epithelial cells and mucus. The problem is essentially to estimate the relative proportions of these components and their leucine content.

Orskov (1986) determined the amino acid composition of rumen and abomasal fluids in steers maintained using the intragastric infusion technique (see section 6.3). In the absence of a rumen microflora and undegraded dietary protein these values were considered to represent the amino acid composition of endogenous protein. A value of 7.2 g leucine per 100 g protein was reported for the rumen fluid and 3.6 g leucine per 100 g protein for the abomasal fluid. The value for the abomasal fluid remains difficult to explain as the leucine content of neither bovine pepsinogen nor the plasma protein components of abomasal secretions are this low (see below). The leucine content of mucus may be important. It has been reported to be around 2 g leucine per 100 g protein (see Harding, 1987).

In this study a value of 6.8 g leucine per 100 g endogenous protein was applied in the conversion of endogenous protein leucine flow to endogenous protein nitrogen flow (see below). This figure is the leucine content of whole-body protein in sheep (Reeds and Lobley, 1980). A similar value has been obtained in this laboratory for young steers (J. Newbold, personal communication). The justification for the use of this value is now considered.

An alternative approach often adopted by other workers (see section 6.3) would be to assume that the leucine content of endogenous secretions was equivalent to either the leucine content of enzymes (pepsin) or plasma proteins (albumin). Values reported for the leucine content of bovine stomach mucosa pepsinogen include 6.95% (see Fasman (1976), Handbook of Biochemistry and Molecular Biology) and 6.68% (Chow and Kassell, 1968). The leucine content of bovine serum albumin is 12.3% (see Phelps and Putnam, 1960).

However, as indicated previously, it has been shown in this investigation that endogenous secretions are not exclusively enzymic or

plasma proteins as the specific acitivity profile of corrected digesta could not be considered to exclusively resemble that of either saliva or plasma protein (see section 3.5.2.3 a). The determination of the interpolated estimate of endogenous protein flow involves the assessment of the proportions of the enzymic and plasma protein components in the endogenous fraction.

In theory, a more accurate assessment of endogenous protein nitrogen flow may be made if the leucine content of the enzymic and plasma protein components were known, i.e.

$$\text{Leucine content of endogenous secretions (\%)} = \left[\left(1 - \frac{a}{b}\right) \times X\right] + \left[\left(1 - \frac{c}{b}\right) \times Y\right]$$

where a (g/day) = interpolated endogenous protein leucine estimate (g/day) − minimum endogenous protein leucine estimate (g/day)

b (g/day) = maximum endogenous protein leucine estimate (g/day) − minimum endogenous protein leucine estimate (g/day)

c (g/day) = maximum endogenous protein leucine estimate (g/day) − interpolated endogenous protein leucine estimate (g/day)

X = leucine content of enzymic secretions (%)

Y = leucine content of plasma protein secretions (%)

Thus,

$$\text{Endogenous protein nitrogen flow (g/day)} = \text{Endogenous protein leucine flow (g/day)} \times \frac{100}{\text{Leucine content of endogenous secretion (\%)}} \times 6.25^{*}$$

* protein = N x 6.25

As indicated above, the leucine content of bovine pepsinogen is known (approximately 6.8%, mean of 2 estimates). However, it is difficult to predict the proportions of the different plasma protein constituents (e.g.

albumin and alpha, beta and gamma globulins) entering the digestive tract. Also the leucine content of each component may vary (see Phelps and Putnam, 1960). Possibly of greater importance is that using this approach no account is taken of the leucine content of undegraded sloughed cells or mucus.

Supportive evidence for the use of 6.8% as the leucine content of endogenous protein was derived using data from the initial experiment in this study. Sheep were fed an essentially protein-free diet. The maximum endogenous protein nitrogen flow was calculated by difference using a traditional approach from the total protein nitrogen flow and the bacterial nitrogen flow (see Table 12).

Since digesta total nitrogen flow (g/day) and microbial nitrogen flow (g/day) both include a microbial nucleic acid nitrogen component, this fraction is accounted for in the derivation of the endogenous protein nitrogen flow by difference. The maximum endogenous protein nitrogen value was corrected for the very small undegraded feed nitrogen component (0.70 g/day) estimated from the theoretical protein nitrogen intake and the predicted degradability of the diet (0.77). This corrected by difference value was then compared with estimates of endogenous protein nitrogen derived using the [^{3}H]-leucine technique (the latter calculated from endogenous protein leucine estimates assuming that leucine is 6.8% of endogenous protein and protein = N x 6.25).

This is therefore a less direct comparison than that based on leucine flow outlined in section 3.5.3. The mean estimates of endogenous protein nitrogen flow derived using the various [^{3}H]-leucine technique methods and the mean corrected by difference value are included in Table 40. Data for individual animals are given in Appendix 10. The

Table 40 Comparison of estimates of endogenous protein nitrogen flow (g/day) determined using the [^{3}H]-leucine technique (various methods) and by difference (corrected) estimate in sheep fed an essentially protein-free diet

	[^{3}H]-leucine technique[+]					Statistical significance of method differences
	Saliva	Plasma protein	Inter-polated	Difference corrected[++]	SED	
Endogenous protein nitrogen flow (g/day) mean of 3 animals	1.50	4.96	2.55	2.06	0.850	*

[+] Assumes endogenous protein = 6.8% leucine (see text) and protein = N x 6.25

[++] Assumes degradability of ration = 0.77

* Significantly different $p<0.05$

(see Appendix 10 for individual animal data)

Results of t-test (6 d.f.) for endogenous protein nitrogen estimates

Method	Saliva	Plasma protein	Interpolated	Difference corrected
Saliva	-	**	N/A	NS
Plasma protein		-	N/A	*
Interpolated			-	NS
Difference corrected				-

* Significantly different $p<0.05$

** Significantly different $p<0.01$

NS Difference not significant $p>0.1$

N/A Not applicable (see text)

data were analysed using an analysis of variance. The variation in the data due to the differences between animals was accounted for by considering the animals as blocks. The methods were shown to differ ($p<0.05$). The [^{3}H]-leucine technique plasma protein endogenous protein nitrogen estimate was significantly different from the corrected by difference estimate ($p<0.05$). The saliva and interpolated estimates were not significantly different from the corrected by difference estimate ($p>0.1$). A comparison of either the saliva and interpolated estimates or the plasma protein and interpolated estimates was not applicable (see section 3.5.3). The results of this analysis were similar to that based on leucine flow (see section 3.5.3). This suggests that a value of 6.8% as the leucine content of endogenous secretions is applicable. A further interesting calculation is possible.

Using the corrected by difference estimates of endogenous protein leucine flow and endogenous protein nitrogen flow an estimate of the leucine content of the endogenous fraction can be made:

Mean 'by difference' estimate of endogenous protein leucine flow (corrected for undegraded feed) = 1.38 g leucine/day (see Table 15)

Mean 'by difference' estimate of endogenous protein nitrogen flow (corrected for undegraded feed) = 2.06 g nitrogen/day (see Table 40)

Leucine = 10.67% N

Therefore, endogenous leucine nitrogen flow (g/day) = 1.38 x 0.1067 = 0.147 g/day

Therefore, % leucine nitrogen in endogenous nitrogen = $\frac{\text{Endogenous leucine N flow}}{\text{Endogenous N flow}}$

$= \frac{0.147}{2.06} = 7.15\%$

Table 41 Estimates of endogenous protein nitrogen flow (g/day) at the proximal duodenum in sheep and cattle derived using the intravenous infusion of L-[4,5-^{3}H]-leucine technique

Diet/Animal	Endogenous protein nitrogen flow (g/day)+	Mean	SEM
Essentially protein-free			
Sheep A	1.28		
Sheep B	4.52	2.55	0.998
Sheep C	1.86		
Concentrate			
Sheep D	3.48		
Sheep E	2.81	2.60	0.581
Sheep F	1.50		
Long hay			
Sheep G	1.75		
Sheep H	2.09	2.11	0.217
Sheep K	2.50		
Silage + fishmeal			
Steer M	15.17		
Steer P	10.13		
Steer R	11.07	11.32	1.73
Steer T	7.85		
Steer V	16.12		

+ Assumes endogenous protein = 6.8% leucine (see text) and protein = N x 6.25

Thus on a nitrogen basis leucine can be considered to be 7.15% of the endogenous secretions. To accurately convert this value into a by weight basis would of course require detailed information on the total amino acid composition of endogenous secretions. Since leucine can be considered a typical amino acid in terms of its percentage nitrogen content it may therefore be suggested that the leucine content of most proteins would be similar when calculated either on a weight basis or on a nitrogen basis. A definitive conclusion from this comparison concerning the leucine content of endogenous secretions is however also complicated by assumptions made in the determination of the undegraded feed component of the digesta.

Thus considering the information available at the present time a value of 6.8% (leucine content of whole body protein, Reeds and Lobley, 1980) was considered appropriate as the leucine content of endogenous protein. This value was used in the conversion of endogenous protein leucine flow to endogenous protein nitrogen flow. Estimates of endogenous protein nitrogen flow (g/day) using the proposed technique for all animals in this study are given in Table 41.

6.3 Previous estimates of endogenous protein secretion in ruminants

In this investigation endogenous protein is defined as that fraction of the duodenal digesta derived from the animal, excluding any endogenous nitrogenous material incorporated into bacteria and protozoa.

Previously various attempts have been made to predict endogenous protein secretion in ruminants. Estimates of endogenous protein secretion and details of the dietary regimes and techniques used are included in Table 42.

Table 42 Estimates of endogenous protein nitrogen secretion in ruminants

Animal	Diet	Technique	Endogenous protein nitrogen secretion (g N/day)	Endogenous protein nitrogen secretion (mg N/kg $BW^{0.75}$ per day	Reference	Comments
Sheep	-	Abomasal pouch	1-2	-	Phillipson (1964)	No allowance for undegraded endogenous flow from the rumen
Sheep	Chaffed meadow hay & flaked maize	Abomasal pouch	0.5-2.8	29-161		——— " ———
Sheep	Lucerne cubes	[^{15}N]-'digesta-diversion'	6.0	-	Nolan & MacRae, (1976)	No allowance for microbial incorporation of endogenous secretions
Sheep	Agrostis festuca	[^{14}C]-'dual infusion'	3.0	139-172	MacRae et al. (1979)	Indirect approach
Sheep	Heather	——— " ———	2.3	107-132	——— " ———	——— " ———
Sheep	Hay	[^{15}N]-'digesta-diversion'	6.6	-	Nolan et al. (unpublished) (see MacRae & Reeds (1980)	No allowance for microbial incorporation of endogenous secretions
Sheep	Silage	——— " ———	4.6	-	——— " ———	——— " ———
Sheep	Brome grass pellets	[^{15}N]-'dual infusion'	8.1-10.8 (1.3 g saliva; 6 g rumen epithelium	358-477	Kennedy & Milligan (1980b)	——— " ———
Dairy cows	Purified diet/urea	[^{15}N]-infusion	20 g	-	Brandt et al. (1981)	Novel approach, needs verification. Difficult to apply.
Dairy cows	Semi-synthetic	'by-difference'	15% of total N	-	Kaufmann & Hagemeister (unpublished) see Kaufmann (1977)	Indirect approach, an approximation
Steers	Intragastric infusion		5.0-5.2 (flow from rumen)	57-65	Orskov & McLeod (1982)	No allowance for microbial incorporation of endogenous secretions
Steers	——— " ———		4.9-6.6 (flow from rumen)	74-98	Orskov & McLeod (1983)	——— " ———
			7.9-9.6 (flow from abomasum)	116-142	——— " ———	——— " ———
Dairy cows	——— " ———		7.0-12.1 (flow from rumen)	38-67	Orskov et al. (1986)	——— " ———
Young steers	——— " ———		5.8 (flow from rumen	90	——— " ———	——— " ———
Young steers	——— " ———		13.5 (flow from abomasum	195	——— " ———	——— " ———
Sheep	——— " ———		1.20 (flow from rumen	75	——— " ———	——— " ———
Sheep	——— " ———		2.63 (flow from abomasum	163	——— " ———	——— " ———
Steers	Straw, tapioca and supplement	Amino acid profile	0.1-1.6	3-51	Cockburn and Williams (1984)	Indirect approach, incorporated amino acid profile bovine pepsinogen
Sheep		Isotope dual labelling	15-29 (includes input to small intestine	-	Egan & Smith (unpublished) (see Egan et al., 1984)	Detailed information unavailable
Sheep	Silage	[^{15}N]-'dual infusion' and digesta diversion	3.8	202	Siddons et al. (1985a)	No allowance for microbial incorporation of endogenous secretions
Sheep	Hay	——— " ———	5.9	314	——— " ———	——— " ———
Sheep	Essentially protein free	Long term intravenous infusion of L-[4,5-^{3}H]-leucine	2.6	159	This study	Direct estimate of endogenous protein nitrogen arriving at proximal duodenum. Assumes endogenous protein = 6.8% leucine, protein = N x 6.25
Sheep	Concentrate	——— " ———	2.6	153	——— " ———	——— " ———
Sheep	Hay	——— " ———	2.1	117	——— " ———	——— " ———
Mature steer	Silage + fishmeal	——— " ———	15.2	169	——— " ———	——— " ———
Young steers	——— " ———	——— " ———	11.3	241	——— " ———	——— " ———

Phillipson (1964) using surgically modified sheep estimated the endogenous protein nitrogen flow to be 1-2 g per day. These values were derived from data on the nitrogen content and volume of secreted gastric juices. Harrop (1974) using sheep with abomasal pouches obtained similar values. Despite the low level of sophistication involved in their determination the values obtained agree closely with the estimates derived in this investigation.

The accurate determination of duodenal digesta flow and the development of isotope tracer techniques has enabled quantitative estimates of nitrogen metabolism in the forestomachs and abomasum to be obtained. This information has included values for the total endogenous nitrogen input prior to the duodenum.

Nolan and MacRae (1976) derived an estimate of 6 g total endogenous protein nitrogen per day. This estimate was obtained, as described below, as part of a series of experiments using isotope tracer techniques (see Nolan and Leng, 1974), designed to develop a model to describe nitrogen metabolism in sheep given lucerne cubes (Nolan, 1975; Nolan et al., 1976).

Two sheep were given lucerne cubes (16 g N/day). The animals were each prepared with a rumen cannula and a re-entrant cannula at the duodenum and ileum. Both sheep received a 36 hr intraruminal infusion of [^{15}N]-ammonium sulphate. The ^{15}N-enriched duodenal digesta was removed from the animals throughout this period and was replaced with equal amounts of previously collected non-labelled digesta. This procedure prevented the digestion of ^{15}N-microbial protein in the small intestine and any subsequent return of ^{15}N-label to the rumen thus making calculations easier. Over a 24 hr period inputs to the rumen (feed plus endogenous urea and endogenous protein) must equal nitrogen outputs from the rumen

(ammonia absorption plus duodenal flow). Feed nitrogen intake (16 g/day) and duodenal digesta protein nitrogen flow (17 g/day) were readily determined. The endogenous urea component in the equation (1.5-2.0 g/day) was derived from an earlier investigation. This involved sheep fed a similar diet intravenously infused with [^{14}C]-urea (see Nolan et al., 1973). Ammonia absorption was determined from a series of calculations as follows:

1. From the enrichments of rumen bacteria and duodenal digesta protein nitrogen the proportion of duodenal digesta of microbial origin was estimated as 40%.

2. From the enrichments of rumen ammonia and rumen bacteria it was calculated that rumen bacteria derive 60% of their nitrogen from rumen ammonia. Thus approximately 5 g of bacterial nitrogen was considered to be derived from rumen ammonia.

3. From the infusion rate of ^{15}N the ammonia production rate was detemined as 12 g per day.

4. The ammonia absorbed was therefore 7 g per day. Substitution of appropriate values in the nitrogen balance equation resulted in an estimate of an endogenous protein nitrogen component of 6 g per day, i.e.

$$\text{Feed} + \text{endogenous urea} + \text{endogenous protein} = \begin{matrix}\text{ammonia} \\ \text{absorption}\end{matrix} + \begin{matrix}\text{duodenal} \\ \text{flow}\end{matrix}$$

$$16 + 2 + \text{endogenous protein} = 7 + 17$$

This estimate of endogenous protein does not represent endogenous protein arriving at the proximal duodenum as defined in this

study. No account is taken of any incorporation of endogenous protein by the rumen microbes.

MacRae et al. (1979) in an experiment with sheep fed either indigenous hill herbage (Agriostis festuca) or heather, infused [^{14}C]-urea intravenously and [^{14}C]-bicarbonate intraruminally. The addition of non-ammonia nitrogen (NAN) was determined from the nitrogen intake and the flow of NAN at the proximal duodenum. An estimate of urea entering the rumen bicarbonate (CO_2) pool was calculated as the product of the rumen bicarbonate production rate and the percentage transfer of plasma urea to rumen bicarbonate. Endogenous protein was calculated as the non-urea addition. The values were considered a minimum amount of endogenous protein, because if any direct absorption of ammonia from the rumen occurred then more endogenous protein would be needed to balance the equation.

The data of Nolan et al. (unpublished) included in Table 42, for sheep fed either hay or silage were obtained from a repeat of the 'digesta-diversion' lucerne experiment (Nolan and MacRae, 1976). Six sheep were studied on each diet. The estimates of endogenous protein nitrogen secretion (4.6 g N/day - silage, 6.6 g N/day - hay) were considerably higher than both the values originally proposed by Phillipson (1964) and the values determined in this investigation.

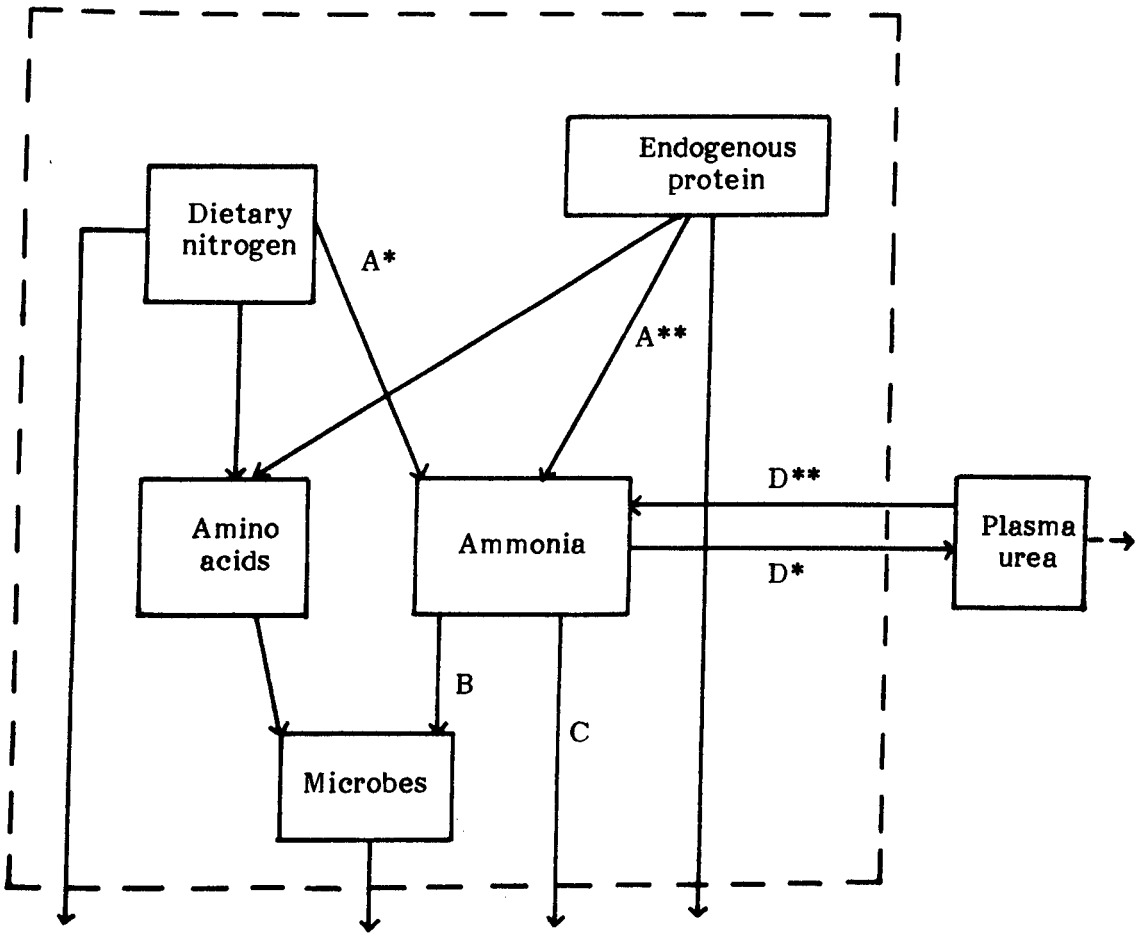
Kennedy and Milligan (1980b) proposed an alternative method based on ^{15}N tracer kinetics to derive an estimate of the input of endogenous protein into the forestomachs of sheep. The approach was again based on the equation that nitrogen input to the rumen is equivalent to nitrogen output (i.e. Feed + endogenous protein + endogenous urea = ammonia absorption + duodenal flow). The calculation used data obtained

following an intravenous infusion of ^{15}N-urea and an intraruminal infusion of ^{15}N-ammonium chloride (see Kennedy and Milligan, 1978b). Again it must be noted that this approach does not account for incorporation of endogenous protein by the rumen microbes.

From the enrichment in blood urea and rumen ammonia, the input, output and interchange of nitrogen between pools was calculated (see Fig 16). A value was determined for the input of nitrogen into the rumen ammonia pool not derived from plasma urea, i.e. derived from food nitrogen and endogenous protein (A). From values of the relative enrichments of microbial nitrogen and rumen ammonia and the flow of microbial protein from the abomasum, the amount of nitrogen removed from the ammonia pool by incorporation of ammonia into microbes leaving the abomasum was determined (B). The subtraction of this quantity plus the amount of ammonia leaving the abomasum (C) from the amount of ammonia not derived from endogenous urea sources (A) gave a value for the net loss of ammonia to blood urea prior to the duodenum (D).

Endogenous protein nitrogen input to the forestomachs and abomasum was calculated as: Total nitrogen leaving the abomasum plus the net loss of rumen ammonia to blood urea prior to the duodenum (D) minus feed nitrogen. This value underestimated endogenous protein input to the forestomachs by the amount of fixation of rumen ammonia in body tissues. From a determined salivary secretion rate and an estimated protein content of saliva (Hogan, 1975), 1.3 g of the total 8.1-10.8 g of endogenous protein nitrogen was attributed to salivary protein nitrogen entering the duodenum. Thus approximately 6 g endogenous protein nitrogen per day was considered to be derived from the sloughing of the rumen epithelium, assuming an input of 1-2 g due to gastric secretion in the abomasum (Phillipson, 1964).

Fig 16 The determination of endogenous protein secretion to the forestomachs and abomasum using ^{15}N as proposed by Kennedy and Milligan (1980b)



A = A* + A** = Rumen ammonia not derived from plasma urea.

D = D* - D** = Net loss of rumen ammonia to plasma urea = A - B - C

Endogenous protein secretion to the forestomachs and abomasum = Total N leaving the abomasum + D - Feed N

+ This does not account for incorporation of endogenous protein by rumen microbes (see text)

Siddons et al. (1985a) derived a model for nitrogen transactions occurring both within and between the digestive tract and the tissues of sheep fed either silage or dried grass. Intraruminal and intravenous isotope infusions and the digesta-diversion technique were employed. The rations were isoenergetic but contrasting in relation to nitrogen supply. A net loss between the mouth and duodenum of 4.0 g nitrogen per day with the silage fed animals compared with a net gain of 5.5 g per day with the dried grass fed animals, suggested that more of the silage nitrogen was degraded in the rumen. An allowance of 1.5 g endogenous protein nitrogen (Harrop, 1974) altered the degradability values of the silage and dried grass diets from 0.56 and 0.20 to 0.71 and 0.43 respectively. The importance of endogenous inputs to the forestomachs was recognised. From data on dietary nitrogen intake, nitrogen flow to the duodenum and nitrogen loss by absorption it was calculated that endogenous protein nitrogen entering the forestomachs amounted to 3.8 and 5.9 g per day on the silage and dried grass diets, respectively. It was noted that if the extent of fermentation of this input was low, i.e. if all endogenous protein nitrogen entering the forestomachs passed undegraded to the duodenum, the estimated degradability would be 0.85 for both the silage and dried grass.

The values for endogenous protein secretion derived using isotope tracer techniques based on the nitrogen balance equation are larger than both the original estimates made by Phillipson (1964) and the values determined in this investigation. This was predicted to occur as using these techniques, as stressed previously, no account is taken of the extent of fermentation of endogenous inputs to the rumen. The degradation of endogenous protein inputs entering the rumen was discussed in section 6.1.4. If extensive fermentation of endogenous protein inputs to the rumen

does occur values of endogenous protein derived based on the 'nitrogen balance' equation must overestimate the endogenous protein content of proximal duodenal digesta. A further error is involved as the estimates are derived indirectly by subtraction. These potential inaccuracies illustrate the requirement for a direct determination of the endogenous protein content of proximal duodenal digesta.

Brandt et al. (1981) derived an estimate of the endogenous protein input in cows fed a semi-synthetic ration with urea as the only source of nitrogen.

Thus in the absence of undegraded feed, digesta was considered to consist of an endogenous protein nitrogen fraction (x) and a microbial protein nitrogen fraction (y). Therefore, (x) + (y) = 1.0. ^{15}N was intraruminally infused for 12 days and (x) calculated from enrichment values in digesta, microbes and 'endogenous components'. It was assumed that ^{15}N endogenous enrichment was exemplified by the enrichment determined in milk-N. An average value of 3.6 g endogenous protein nitrogen per kg DM passing to the duodenum was determined (approximately 20 g/day), equivalent to about 11-16% of the protein nitrogen reaching the duodenum. Fundamentally this technique is similar to that used in this investigation. This technique was a novel attempt to solve the problem. However, several difficulties in this approach became evident. The crucial assumption that endogenous enrichment was exemplified by the the enrichment determined in milk-N was not verified. Also the technique is confined to lactating animals. Although a correction factor is proposed to account for an undegraded feed fraction in animals fed conventional rations this is likely to involve a considerable error term. The lack of further data obtained using this method may be attributed to these difficulties.

Kaufmann and Hagemeister (unpublished data, see Kaufmann 1977) suggested that endogenous secretions of the abomasum, small intestine, gall bladder and pancreas, determined using semi-synthetic rations and re-entrant cannulae amounted to 15% of the nitrogen in the intestines.

The development and application of the 'intragastric infusion technique' by Orskov and fellow workers of the Rowett Research Institute (see Orskov et al., 1979) has enabled the investigation of aspects of nitrogen metabolism in ruminants previously considered impossible to quantify.

The technique involves the maintenance of animals using an intraruminal infusion of volatile fatty acids and an intraabomasal infusion of protein and other essential nutrients. Nitrogen flow is uncomplicated by the activity of microorganisms in the forestomachs (Orskov and McLeod, 1982). The mitotic index of the rumen epithelium of infused animals was similar to that in conventionally fed animals (Dinsdale et al., 1980), as was their growth rate and nitrogen retention (Orskov et al., 1979). The technique has been used to predict the flow of protein nitrogen from the rumen uncomplicated by dietary and microbial contributions. This value represents endogenous protein derived from sloughed rumen epithelial tissue and epithelial abrasion from the respiratory tract, mouth and oesophagus. Estimates of 5.0-5.2 g protein nitrogen per day were estimated to flow from the rumen in steers (Orskov and McLeod, 1982). Orskov and McLeod (1983) determined a flow of 4.9-6.6 g protein nitrogen per day from the rumen and 7.6-9.6 g protein nitrogen per day from the abomasum in steers. Thus about two-thirds of the endogenous nitrogen in the abomasum was considered derived from the rumen. Additional similar

estimates recently determined in cattle (Orskov et al., 1986), using the intragastric infusion technique are also included in Table 42.

The absence of a rumen microbial population in the animals maintained using the intragastric infusion technique means that the values derived using this approach could be considered as maximum estimates of endogenous protein as no degradation of endogenous protein inputs to the rumen occurs. However, Orskov et al. (1986) suggest that the abomasal secretion of nitrogen may be greater in the normally fed animal and therefore the secretion of nitrogen in an animal maintained via the intragastric infusion technique may be a minimal one. In animals fed conventional rations some degradation of 'ruminal' endogenous protein is likely (see section 6.1.4). The abomasal non-ammonia nitrogen (NAN) outflow minus the rumen NAN outflow equals the endogenous NAN addition from the omasum and abomasum. In four young steers (240-315 kg) (see Orskov et al., 1986) this was approximately 105 mg NAN/kg $BW^{0.75}$. It can therefore be predicted that for the steers in the present investigation, weighing 170 kg, approximately 5 g of endogenous protein nitrogen was derived from the omasum and abomasum. A further 6 g must therefore have originated from undegraded endogenous protein nitrogen secretions into the rumen.

Evans et al. (1975) proposed the 'amino acid profile technique' to provide simultaneous estimates of the proportions of bacteria, protozoa, feed and endogenous components in duodenal digesta. A computer program simulated the mixing of the known amino acid profiles of individual digesta constituents in different proportions in order to produce a profile similar to that in composite duodenal digesta. A modified simpler method of calculation based on multiple regression analysis was proposed by Cottle and Nolan (1982).

A major limitation with this approach is that least squares analyses of profiles are very sensitive to changes in the concentration of individual amino acids. The amino acid profiles of bacteria and protozoa can be readily predicted. The composition of undegraded feed protein leaving the rumen was estimated from the diet. However, the proportion of each amino acid resistant to degradation appears to vary widely between diets (see McMeniman et al., 1976; Tamminga et al., 1979). Also there still exists the problem of determining the amino acid composition of the endogenous fractions. If endogenous protein inputs to the rumen are extensively degraded the amino acid composition of endogenous secretions to the omasum and abomasum are always important. Offer et al. (1978) considered the endogenous profile to be most closely represented by the profile of pepsinogen while Cottle and Nolan (1982) used the amino acid profile of albumin.

Cockburn and Williams (1984) using the amino acid profile procedure derived estimates of 0.1-1.6 g N per day, in steers of approximately 100 kg in weight, incorporating the amino acid profile of porcine pepsin as representative of endogenous secretions. The amino acid composition of the endogenous secretions was considered in section 6.2.

Egan et al. (1984) predicted that 4-13 g endogenous protein nitrogen passed into the gastrointestinal tract prior to the proximal duodenum in a 50 kg sheep per day. Using radioisotope double-labelling methods in surgically prepared sheep they estimated the total contribution of endogenous protein nitrogen to amino acid absorbed from the small intestine to be 15-29 g per day. Details of the exact procedure adopted were not reported. Values were greater on high protein diets and when more undigested fibre flowed to the duodenum.

As previously indicated (see section 1.5) the need for an accurate determination of the endogenous protein content of proximal duodenal digesta has been widely recognised for a long time. Advances have clearly been limited by the lack of an appropriate technique. This problem is not a new one. Strictly, it may be considered that only after a suitable technique to determine directly what is required has been developed can the significance of the findings be considered.

Some of the previous techniques adopted, e.g. many of the ^{15}N studies, have derived estimates indirectly. This could possibly lead to incorrect conclusions and questionable application of the data. The revolutionary technique developed by Orskov and fellow researchers at the Rowett has opened up many new fields. However, a direct estimate of endogenous protein arriving at the proximal duodenum in animals fed conventional rations is not possible.

The method proposed in this investigation involving a continuous intravenous infusion of L-[4,5-^{3}H]-leucine and the principle of isotope dilution is thought to be the first technique to provide a direct determination of the endogenous protein component of proximal duodenal digesta in ruminants which can be applied to any dietary regime and used in sheep and cattle.

Values derived using the proposed technique can be used directly to improve the accuracy of degradability estimates which are essential for more precise ration formulation. This will lead to an overall improvement in the efficiency of animal production.

The drawbacks of the [^{3}H]- technique are considered in relation to the degree of accuracy required later in the discussion (see section 6.7).

6.4 The effect of diet on the endogenous protein content of ruminant proximal duodenal digesta

The endogenous protein content of proximal duodenal digesta was determined in sheep fed either concentrates or hay (see Chapter 4). The diets were formulated to provide a theoretical maximum range of endogenous protein secretions. Previous work, for example Kennedy and Milligan (1980b) has suggested higher values of endogenous protein secretion were more likely in sheep fed hay than in sheep fed concentrates, possibly as a result of increased abrasion of epithelial cells. However, no significant differences ($p>0.1$) were observed between the diets in this study (see Table 24, section 4.5.2).

The probable influence of diet on the various endogenous protein inputs is now considered, and an explanation of the apparent lack of a dietary effect proposed.

i) Rumen epithelium

Previous hypotheses (see below) predicted that the quantity of sloughed cells entering the rumen was related to the level of dietary fibre and level of intake. This was attributed to increased abrasion and digesta flow rate in forage fed animals. Kennedy and Milligan (1980b), using their technique outlined in section 6.3 derived higher values for the endogenous protein content of digesta (as defined by them) at higher levels of feeding. Studies on pigs (Bergner et al., 1983; Low and Rainbird, 1983) reported an increased endogenous secretion with an increasing amount of bulk passing through the stomach.

Cell turnover in the rumen epithelium has been studied directly. Rowe and James (1982) indicated that the amount of cell synthesis in the

rumen epithelium was affected by the physical form of the diet. Higher values of cell turnover were observed in sheep fed high fibre diets. However, Goodlad (1981) noted that the mitotic index of the ruminal epithelium increased rapidly when the diet was changed from roughage to concentrates. After several days the mitotic index declined to a new level, but was still higher than that observed initially. This suggested a self-regulatory mechanism of the rumen epithelium which decreased the rate of production of new cells after a new tissue mass was reached. The mean turnover time of the rumen epithelium was estimated to be approximately 16.5 days when sheep were fed the roughage diet, 10.9 days when fed the concentrate diet and 4.3 days when the sheep were in transition between the diets. Concentrate diets are widely associated with hyperplasia of the rumen epithelium and with hyperkeratosis and parakeratosis (Fell and Weekes, 1975). Dinsdale et al. (1980) examined the effect of diet on the mitotic index of the rumen epithelial cells. It was higher in sheep fed barley than in hay fed animals (see Table 43). These authors concluded that it was not clear whether this reflected a faster rate of turnover of the rumen epithelium in barley fed animals. The effect of diet on the turnover of the rumen epithelium is clearly not simple.

Sakata and Tamate (1978) studied the regulatory mechanism of cell proliferation in the rumen epithelium. The mitotic activity of the rumen papillae was estimated histologically under various nutritional conditions. It was observed that this was dietary dependent, depressed by fasting, increased by subsequent refeeding and extremely stimulated by intermittent feeding. Such observations led to a hypothesis that an increase in the rate of intraruminal production of volatile fatty acids (VFAs) promoted proliferation of epithelial cells in the organ. In sheep

Table 43 The effect of diet on the mitotic index of the rumen basal epithelial cells (from Dinsdale et al., 1980)

Diet	Number of animals	Number of determinations	Mitotic index*
Barley	4	28	1.00 ± 0.26
Hay	4	28	0.51 ± 0.19

* The mitotic index was calculated as the number of cells undergoing mitosis per 100 basal cells ± standard deviation

given butyrate over a short period of time, the mitotic index increased rapidly after administration and then tended to decline. In sheep given butyrate slowly, mitotic indices did not show significant fluctuations. The stimulatory effect seemed to be transient. This also suggested that the rumen epithelium may have a conservation mechanism to keep its mass constant.

Sakata and Tamate (1979) considered that the mitogenic effect of propionate and acetate on the rumen epithelial cells, although apparent, was weaker than that of butyrate. In practice, an increase in feed intake and the proportion of concentrate in the diet, results in a higher proportion of butyrate, rapid production of VFAs and a lower pH. These three conditions in the rumen may stimulate rumen epithelial cell proliferation.

Although butyric acid produces the maximum stimulatory effect on the rumen epithelium in vivo, in vitro studies indicated that butyric acid generally inhibited mammalian cell proliferation. This apparent contradiction strongly suggested a hormonal influence. Insulin and glucagon were known to be mediated by butyric and propionic acids (Manns and Boda, 1967). Insulin is one of the essential factors for mammalian cell proliferation in vitro and was thus considered a likely mediator in VFA-stimulated epithelial cell proliferation. This was initially supported by the observation that cell proliferation of the oral epithelium is depressed in alloxan treated diabetic rats (Hamilton and Blackwood, 1977).

Sakata et al. (1980a) found that insulin stimulated epithelial cell proliferation in the adult sheep rumen. It was proposed that the stimulatory effect of intraruminal administration of butyrate and propionate (and possible acetate) is mediated at least partly by insulin released by the acids. Glucagon could be another mediator. The

gastrointestinal hormones pancreozymine, secretin and tetragastrin may also stimulate the rumen epithelial cell proliferation via an increase in plasma insulin. Neural mediation is another possible mechanism. Cooper and Lipkin (1973) indicated that in humans development of the epithelial cells is influenced by blood supply, hormones, stress, diurnal variation and mitotic inhibitory substances.

Goodlad (1981) found no significant relationship between the relative molar proportions of the different VFAs and the mitotic index. It was recognised that VFA proportions do not necessarily provide accurate estimations of VFA production rates. The low rumen pH associated with concentrate feeding and the resultant increase in the passage of VFAs through the rumen epithelium was implicated as part of the regulatory mechanism.

Endogenous protein input to the rumen from the rumen epithelium is therefore probably dietary dependent. A quantitative estimate of the extent of any changes in this endogenous protein input due to diet is clearly difficult to predict as numerous factors may be involved. In terms of the accurate prediction of endogenous protein arriving at the proximal duodenum the extent of incorporation of endogenous protein input to the rumen by the rumen microbes must be considered (see section 6.1.4). If this was extensive, as far as endogenous protein arriving at the proximal duodenum is concerned, the effect of diet on the input of endogenous protein to the rumen from the rumen epithelium may be of minimal significance.

ii) Salivary protein

The salivary glands are numerous, heterogenous in nature (see section 1.3.1.1) and secrete different types of saliva which vary in their protein content (Phillipson and Mangan, 1959).

A change in the protein composition of saliva has been observed on different diets (McIntosh, 1975). Somers (1961b) observed a linear relationship between saliva urea nitrogen and nitrogen intake, although total saliva nitrogen was not a simple function of nitrogen intake. Doyle et al. (1982) showed that the non-urea-N increased from 1.25 to 4.38 g protein/day (N x 6.25) as the quantity of the dietary roughage increased. Voigt et al. (1980) observed that when sheep ate freshly provided food the flow and protein content of the parotid saliva increased, but could be decreased by a B-adrenergic blocking agent. Patterson et al. (1982) observed a 9-42 fold increase in the protein content of parotid saliva of sheep during feeding compared with that at rest. This suggested that the increase in flow and protein content of parotid saliva during feeding was attributable to sympathetic stimulation, mediated by the ingestion of food.

Estimates of the protein nitrogen content of saliva were made in this study for the sheep fed the concentrate and hay diets (see Table 44). Similar values were obtained for each diet. These estimates resembled those of Kennedy and Milligan (1980b) (see section 6.3).

Attempts to assess the relative importance of the saliva and rumen epithelial endogenous protein inputs to the rumen microbes may be made using information obtained in this study. A lag phase was observed in the specific activity profile of rumen bacteria in animals intravenously infused with L-[4,5-^{3}H]-leucine (see section 3.5.1.2). This lag phase which occurred during the first twenty four hours of the infusion was attributed to the development and migration of the rumen epithelial cells and their subsequent digestion by the adherent bacteria. Since no lag phase was evident in the saliva specific activity profile (see section 3.5.1.5) this implied that salivary protein was not the major endogenous protein

Table 44 Estimates of the saliva protein nitrogen input to the rumen (g/day) in sheep fed either concentrates or hay

Diet/sheep	Saliva protein nitrogen* (μg N/ml saliva)	Estimated saliva protein nitrogen input to the rumen (g/day)**
Concentrate		
Sheep D	137.3 ± 1.60 (3)	
Sheep E	194.1 ± 9.75 (2)	1.1 - 2.9
Sheep F	220.3 ± 6.45 (2)	
Hay		
Sheep G	158.6 ± 23.1 (2)	
Sheep H	216 (1)	1.2 - 3.1
Sheep K	215 (1)	

* Mean ± SEM, number of samples in parentheses

** Assumes saliva secretion rate of 6-16 L/day (Kay, 1960)
Mean value for the three sheep for saliva protein nitrogen input to the rumen (g/day).

(Note; protein nitrogen not total nitrogen)

contribution to the rumen bacteria. Therefore, the major contribution to the endogenous protein input to the rumen bacteria was considered to be from the rumen epithelium. This could also imply that salivary protein is resistant to degradation and reincorporation by the rumen bacteria. The degradation of salivary protein in the rumen was discussed in section 6.1.4. The lag phase was absent in all bacterial protein leucine specific activity profiles when sheep were intraruminally infused with L-[4,5-^{3}H]-leucine (Marsden, 1984). Therefore the lag phase was not attributable to free amino acid/peptide uptake from the rumen contents by the microorganisms.

Interestingly, a lag phase was not clearly evident in the specific activity profile of duodenal bacteria. No explanation of this was available. The consideration of the duodenal bacterial specific activity profile as a single exponential in the derivation of the endogenous protein flow (see section 3.5.2.1) was therefore considered an acceptable approximation. The importance of the accurate assessment of the microbial fraction in the calculation of the endogenous protein flow is examined in section 6.7.2.

iii) Enzymes

Hill (1960) supported earlier observations that there were differences in the volume of gastric juice secreted in ruminants after different foodstuffs were fed. The secretory response after eating hay was less than after eating concentrates. The frequent passage of digesta from the reticulo-rumen to the abomasum was considered the most important stimulus of gastric secretion. The potential role of VFAs was recognised as was the reflex control of gastric secretion via sensory nerve endings in the reticulo-rumen. Van Bruchem and Van t'Klooster (1980) reported that proteins are the main determinants of abomasal secretion of acid in sheep.

The amino acid composition was considered to be important. Thus under physiological conditions, minimal variation may be expected in abomasal secretion as microbial protein forms the major component of duodenal digesta and is relatively constant in amino acid composition (see section 4.5.4). However, the quantity of amino acids may also be important. Twombly Snook (1965) noted that in rats dietary protein influenced proteolysis by regulating the synthesis, secretion and inactivation of proteolytic enzymes. Variations in the response of intestinal enzyme activity to the source of dietary nitrogen were ascribed to differences in the rate of digestion, absorption and amino acid composition of the diet.

iv) Plasma protein

Rothschild (1970) suggested that nitrogen uptake was the most important aspect of the regulation of total albumin production. A short fast was observed to decrease the amount of albumin synthesised. A hormonal mechanism was considered to be involved. Nevertheless this gave no direct indication as to the amount of albumin secreted into the gut. Later studies, for example Dargie and Berry (1979), determined directly the passage of plasma albumin into the gut in sheep (see section 1.3.2). The value determined (0.9 g/day) on a low protein diet (60 g crude protein/day) was lower than that (1.4 g/day) on a conventional protein diet (150 g crude protein/day).

Any attempt to predict from physiological considerations the effect of diet on the endogenous protein content of ruminant proximal duodenal digesta is very difficult. The diet may influence all the components of the endogenous secretions. An accurate assessment of changes in plasma protein and enzymic secretions with diet remain speculative. Also accurate quantitative information on the extent of

microbial reincorporation of endogenous inputs to the rumen is not available (see section 6.1.4).

The results of this investigation suggest that the endogenous component of ruminant proximal duodenal digesta is unaltered by diet (see section 4.5.2). An explanation of this phenomena is now considered.

As suggested previously (Hill, 1960) plasma protein and enzymic secretions may be lower in the hay fed animals than in the concentrate fed animals. If so, in order for the overall endogenous protein flow to be similar regardless of diet, the undegraded endogenous flow from the rumen must be higher in the hay fed animals. This could occur as a result of an increase in the flow of salivary protein. Alternatively, a 'threshold' may have been reached in the extent of incorporation of endogenous protein from the rumen epithelium by the rumen microbes. Several different combinations exist, all highly speculative, dependent on the relative importance of the various endogenous components, the turnover of the rumen epithelium and the extent of the microbial reincoporation of the endogenous protein inputs to the rumen.

It was considered interesting to attempt to determine whether or not the rumen bacteria in the hay fed animals were reincorporating the endogenous inputs to the rumen to the same extent as those in the concentrate fed animals. Attempts were made to investigate this by detailed examination of the specific activity profiles of the bacterial fraction for the different diets. Although the plateau specific activity in the hay fed animals was lower and was achieved earlier than in the concentrate fed animals, the quantity of sloughed cells entering the rumen or actually degraded are impossible to define from the specific activity curves.

Orskov et al. (1986) in studies using animals maintained via the intragastric infusion technique, observed that changes in the rumen osmotic pressure, while affecting liquid flow, had no consistent effect on nitrogen flow from the rumen. It was suggested the epithelial shedding is therefore controlled by factors other than liquid flow.

Clearly, the situation is complex. It may be sufficient to accept that as far as practical application is concerned no significant dietary variation in the endogenous protein content of ruminant proximal duodenal digesta occurs. However, from a physiological point of view it is interesting to speculate as to why no significant dietary variation was apparent. Such information may be considered essential in the prediction of endogenous protein flow in animals fed diets other than those studied in this experiment.

6.5 Estimation of total body protein synthesis

The expression for leucine flux through the plasma free pool was defined in section 3.5.6 (i.e. Flux, Q = Infusion rate/Specific activity at plateau of plasma free leucine). This equation expresses the fact that at plateau the amount of isotope entering the metabolic pool by the infusion must equal the amount leaving for protein synthesis or for amino acid oxidation. It is normally assumed that any label which enters the whole-body protein pool remains there and is not released back into the metabolic pool during the course of the infusion.

An additional equation enables the rate of protein synthesis, Z, and breakdown, B, to be calculated, if the rates of amino acid oxidation, E and uptake from the intestines, I are known:

$$Q = Z + E = I + B$$

Leucine flux was investigated for all animals in this investigation (see respective results chapters). The amino acid 'intake', I, can be estimated from the duodenal digesta leucine flow and an estimate of the true absorbability (0.85; ARC, 1984). Protein breakdown can be determined by difference. Under 'steady-state' conditions it is assumed that protein breakdown equals protein synthesis.

The calculation can be extended to provide estimates of total body protein synthesis if it is assumed that leucine is a fixed proportion of body protein:

$$\text{Protein synthesis (g/day)} = \frac{\text{Flux (mmol/day)}}{\text{mmol leucine per g protein*}}$$

* leucine = 6.8% body protein (Reeds and Lobley, 1980)

Values of 15-20 g/kg $BW^{0.75}$ per day were expected for animals at maintenance (see Lobley et al., 1980; Bryant and Smith, 1982). All estimates of protein synthesis derived in this study were exceedingly low. This may be attributed to the recycling of label during the relatively long 200 hr infusion period. Previous estimates were derived following much shorter infusion periods (8 hr).

Bryant and Smith (1982) using [^{3}H]-tyrosine as the tracer amino acid reported higher values of protein synthesis in sheep fed hay and concentrates than in sheep fed barley straw. Although much lower overall estimates were obtained in this study a similar dietary trend was observed in the sheep fed concentrates or hay.

6.6 The application of endogenous protein estimates in the determination of the degradability of a diet, and an illustration of their importance in ration formulation

Estimates of the in vivo degradability of the concentrate and hay diets in each sheep and data required in their determination are included in

Table 45 Degradability estimates of the concentrate and hay rations in sheep

Parameter		DIET					
		Concentrate			Hay		
		Sheep D	Sheep E	Sheep F	Sheep G	Sheep H	Sheep K
Nitrogen intake (g/day)		18.9	18.9	18.9	9.8	11.0	10.8
Digesta total nitrogen flow (g/day)		9.88	11.90	6.48	14.63	16.29	23.59
Digesta protein nitrogen flow (g/day)		8.66	10.60	5.14	13.64	15.53	22.44
Digesta ammonia nitrogen flow (g/day)		1.22	1.30	1.33	0.99	1.76	1.14
Bacterial nitrogen flow (g/day)		6.89	7.95	3.28	9.95	10.85	10.80
Endogenous protein nitrogen flow (g/day)		3.48	2.81	1.50	1.75	2.09	2.50
Degradability estimate (%)*	a	84	79	83	52	51	++
	b	91	86	90	62	58	++
	c	+(109)	+(101)	98	80	77	15

* Degradability estimates were calculated using the following equations: a, b and c, given in Fig 4.

a. $$\text{Degradability} = 1 - \frac{\text{Total duodenal nitrogen flow (g/day)} - \text{Microbial nitrogen flow (g/day)}}{\text{Nitrogen intake (g/day)}}$$

b. $$\text{Degradability} = 1 - \frac{\text{Duodenal protein nitrogen flow (g/day)} - \text{Microbial nitrogen flow (g/day)}}{\text{Nitrogen intake (g/day)}}$$

c. $$\text{Degradability} = 1 - \frac{\text{Duodenal protein nitrogen flow (g/day)} - \text{Microbial nitrogen flow (g/day)} - \text{Endogenous protein nitrogen flow (g/day)}}{\text{Nitrogen intake (g/day)}}$$

\+ Calculated degradability in excess of 100%
++ Calculated degradability less than 0

Table 46 Degradability estimates of the silage supplemented with fishmeal ration in steers

Parameter		STEER M	P	R	T	V
Dry matter intake (kg/day)		4.50	3.78	4.49	3.80	4.14
Nitrogen intake (g/day)		99.3	92.6	110.3	93.0	99.9
Digesta total nitrogen flow (g/day)		97.6	54.8	75.8	74.1	78.4
Digesta ammonia nitrogen flow (g/day)		6.3	3.4	6.9	5.5	5.0
Digesta protein nitrogen flow (g/day)		91.3	51.4	68.9	68.6	73.4
Microbial nitrogen flow (g/day)		45.1	32.5	46.7	39.0	44.8
Endogenous protein nitrogen flow (g/day)		15.2	10.1	11.1	7.9	16.1
Degradability estimate (%)*	a	47	76	74	62	66
	b	54	80	80	68	71
	c	69	91	90	77	88

* Degradability estimates were calculated using the following equations: a, b and c, given in Fig 4.

a. $$\text{Degradability} = 1 - \frac{\text{Total duodenal nitrogen flow (g/day)} - \text{Microbial nitrogen flow (g/day)}}{\text{Nitrogen intake (g/day)}}$$

b. $$\text{Degradability} = 1 - \frac{\text{Duodenal protein nitrogen flow (g/day)} - \text{Microbial nitrogen flow (g/day)}}{\text{Nitrogen intake (g/day)}}$$

c. $$\text{Degradability} = 1 - \frac{\text{Duodenal protein nitrogen flow (g/day)} - \text{Microbial nitrogen flow (g/day)} - \text{Endogenous protein nitrogen flow (g/day)}}{\text{Nitrogen intake (g/day)}}$$

Table 47 An illustration of the effect of a change in the degradability of the silage and compound feed components of the ration on RDP and UDP supply in the dairy cow (600 kg cow giving 30 kg milk per day at 3.5% butterfat and 8.7% solids not fat, with no increase or decrease in liveweight) (adapted from Wilson and Strachan, 1980)

			RDP (g/day)	UDP (g/day)
		Requirements:	1644	680
Component	kg/day as fed			
Ration in theory				
Silage	37	(83% degradability)	1114	230
Barley	2		158	24
Ensiled brewers grains	5		158	68
16% compound	6.5	(65% degradability)	665	358
			2095	680
		Surplus/deficit:	+ 451	-
Actual ration				
Silage	37	(93% degradability)	1250	94
Barley	2		158	24
Ensiled brewers grains	5		158	68
16% compound	6.5	(75% degradability)	767	256
			2333	442
		Surplus/deficit:	+ 689	-238*

* The deficit of about 240 g UDP is theoretically equivalent to the protein requirement for approximately 4 kg milk

Table 45. Similar data for steers fed silage supplemented with fishmeal are included in Table 46. The degradability of a ration was calculated using the equations outlined in section 1.4.3. It is evident that failure to consider an endogenous component in the calculation of the degradability of a ration results in an overall underestimate of the degradability of a ration by at least 10%.

The importance of this in accurate ration formulation is illustrated in Table 47. An example is given to illustrate the effect of an increase in the degradability of the silage and the compound feed components of the ration by 10% on the protein supplied to a 600 kg dairy cow, giving 30 kg milk per day of 3.5% butterfat and 8.7% solids not fat, with no increase or decrease in liveweight (adapted from Wilson and Strachan, 1980). The overall degradability of the ration was increased by about 8% and the undegradable protein supply decreased by about 240 g per day. This represents the protein required for about 4 kg milk. This assumes that no allowance was made for an endogenous component in the original calculation of the degradability of the silage.

6.7 Experimental evaluation of the continuous intravenous infusion of L-[4,5-^{3}H]-leucine technique

6.7.1 Verification of the L-[4,5-^{3}H]-leucine technique — an assessment of the approach adopted

The aim of the initial experiment with sheep fed an essentially protein free diet was to compare the value for endogenous leucine derived by difference with the value calculated using the proposed technique (see section 3.1).

The interpolated estimate of endogenous leucine flow was very similar to that derived by difference. The failure to consider a protozoal fraction and the assumed degradability of the ration could explain why the estimates were not exactly the same. These aspects are considered below.

Diaminopimelic acid (DAPA) was used in the determination of the flow of bacterial protein. A protozoal component of proximal duodenal digesta was therefore not accounted for. This potential inaccuracy would lead to an overestimate of the endogenous component of duodenal digesta using the difference method. In addition, the estimates of endogenous protein flow calculated using the proposed technique would also theoretically be overestimated since a protozoal component is included in the corrected digesta curve.

The quantitative significance of a protozoal fraction remains questionable. Weller and Pilgrim (1974) fed sheep on maintenance rations of lucerne and wheat hay and concluded that the flow of protozoal nitrogen was of minimal significance. These observations differed from those of Harrison et al. (1979). These authors suggested that protozoal protein may be an important nutritional component of the total protein entering the duodenum of sheep fed semi-purified diets.

However, in the author's opinion any inaccuracy in the microbial protein flow as a result of the failure to account for a protozoal component is not important in the derivation of the endogenous protein flow estimate using the proposed technique. The importance of an accurate estimate of microbial flow is considered in section 6.7.2.1.

The inclusion of a small amount of protein in the ration meant that it was necessary to correct the endogenous estimate obtained by difference (Total digesta leucine - bacterial leucine) for an undegraded

feed component. This introduced a further error into the by difference estimate of endogenous protein flow. The fraction of undegraded feed leucine was estimated from the leucine intake and a theoretical degradability of the ration. For each animal the corrected endogenous protein leucine by difference estimate was numerically only marginally greater than the estimate derived using the proposed technique (see Appendix 4). This could have occurred due to an overestimate of the degradability of the ration, but this is unlikely, as a theoretical calculation of degradability would be expected to underestimate it (see section 1.4.3). Alternatively, an underestimate of the endogenous leucine flow using the proposed technique is possible. However, the small numerical differences between the 'difference corrected' and interpolated estimates were not considered of any importance. Minimal differences are inevitable considering the large number of components in each calculation. Ideally more animals should have been used but this was not practical at the time.

6.7.2 An examination of potential sources of error in the determination of endogenous protein nitrogen flow using the proposed technique

6.7.2.1 The derivation of the interpolated estimate of endogenous protein leucine flow

As numerous parameters are involved in the derivation of the interpolated estimate of endogenous protein leucine flow a sensitivity analysis was used to define the relative importance of the various components in the calculation.

Inaccuracies in the specific acitivity values of individual data points may occur due to experimental error or biological variation.

Providing there are sufficient data points the importance of these random individual errors may be considered minimal in the fitting of the curve.

In the sensitivity analysis it was therefore decided to alter the curve parameters (plateau estimates (A) and rate constants (k)) fitted to the original data points, rather than the individual data points themselves.

A computer program was used in which either A or k of each of the four experimentally determined specific activity profiles (total duodenal digesta, duodenal bacteria, plasma protein and salivary protein) was altered in turn and the effect on the calculated endogenous protein flow monitored. This was carried out on data from a representative animal. The evaluation of any inaccuracy in the predicted plateau (A) and rate constant (k) values was not straightforward. Initially it was decided to increase and decrease by 2% the parameters A and k of the experimentally determined specific acitivity profiles. The percentage change in the maximum, minimum and interpolated endogenous protein estimates was determined. The results are presented in Table 48 (a). The maximum change in the maximum and minimum endogenous protein estimate (approximately 4%) occurred as a result of a 2% alteration of the plateau estimate (A) of total duodenal digesta. The maximum change in the interpolated estimate (approximately 6%) occurred as a result of a change in the rate constant (k) of total duodenal digesta.

The effect of a 5% alteration in the plateau (A) and rate constant (k) values of the total duodenal digesta curve was therefore also examined. This resulted in approximately a maximum 8% change in the maximum and minimum endogenous protein estimates. A reduction in the original value of k for total duodenal digesta by 5% resulted in an increase in the interpolated endogenous protein estimate by approximately 22%. This

Table 48 *Determination of parameters of major importance in the calculation of the endogenous* protein flow using the intravenous infusion of L-[4,5-^{3}H]-leucine

a. Specific acitivity profile parameters

Specific activity profile	Parameter[$]	Alteration (%)	Percentage alteration in the various estimates of endogenous protein flow		
			Minimum	Maximum	Interpolated
Total duodenal digesta	A	+2	+2.6	+2.9	- 1.0
——— " ———	" "	-2	-3.9	-2.9	+ 1.0
——— " ———	k	+2	+1.3	+1.4	- 5.5
——— " ———	" "	-2	-2.0	-1.2	+ 5.3
Duodenal bacteria	A	+2	+1.3	-1.0	+ 2.9
——— " ———	" "	-2	+1.0	+1.0	- 2.7
——— " ———	k	+2	*	*	+ 3.8
——— " ———	" "	-2	*	*	- 3.5
Saliva	A	+2	-2.0	*	*
——— " ———	" "	-2	+2.0	*	*
——— " ———	k	+2	-1.0	*	*
——— " ———	" "	-2	+1.0	*	*
Plasma protein	A	+2	*	-2.1	- 1.8
——— " ———	" "	-2	*	+1.9	+ 1.8
——— " ———	k	+2	*	-1.6	*
——— " ———	" "	-2	*	+1.6	*
Total duodenal digesta	A	+5	+7.1	+7.6	- 1.0
——— " ———	" "	-5	-7.8	-7.6	+ 2.9
——— " ———	k	+5	+3.9	+3.6	-13.3
——— " ———	" "	-5	-3.9	-3.8	+21.5

$ A = Plateau estimate, k = rate constant

* No alteration, or alteration less than 1%

Table 48 (continued)

b. Flow data

Component	Alteration (%)	Percentage alteration in the various estimates of endogenous protein flow		
		Minimum	Maximum	Interpolated
Duodenal bacteria leucine	+ 5	-2.6	-2.6	+ 7.7
——— " ———	- 5	+2.6	+2.4	- 6.0
——— " ———	+10	-5.2	-5.0	+18.6
——— " ———	-10	-4.6	+5.0	-10.4
Total duodenal digesta leucine and duodenal bacteria leucine	+ 5	+5.2	+5.0	+ 4.9
——— " ———	- 5	-5.2	-5.0	- 5.1
——— " ———	+10	+4.6	+4.5	+30.1
——— " ———	-10	-6.5	-6.6	-17.7

indicated the sensitivity of the interpolated estimate to a change in the rate constant of total duodenal digesta.

The sensitivity of the maximum, minimum and interpolated estimates of endogenous protein flow to changes in microbial flow and also microbial and total duodenal digesta flow rates was also examined (see Table 48 (b)).

The introduction of a 5% change in the duodenal microbial flow resulted in a change of less than 3% in the maximum and minimum estimates of endogenous protein flow and an alteration of less than 8% in the interpolated estimate. A 5% change in the duodenal microbial and total duodenal digesta flows altered the maximum, minimum and interpolated estimates of endogenous protein flow by approximately 5%.

The effect of a larger alteration was also examined. An increase or decrease in the duodenal microbial flow by 10% resulted in a maximum 5% change in the maximum and minimum endogenous protein estimates and a 19% change in the interpolated estimate. An increase or decrease in the duodenal microbial flow and the total duodenal digesta flow by 10% resulted in a 7% change in the maximum and minimum endogenous protein estimates and an increase of up to 30% in the interpolated estimate.

The accurate assessment of dry matter flow has always presented difficulties. The dual phase marker approach (Faichney, 1975; see Appendix 5) is probably the most accurate technique available. Errors in flow measurements are difficult to predict. An accuracy of only $\pm$ 10% may be considered a generous estimate.

The accurate determination of microbial flow remains somewhat imprecise. Methodological differences clearly exist (see Ling and Buttery, 1978; Siddons et al., 1982). The advantages and disadvantages of the

various techniques to determine microbial flow (see section 1.2.1) have been extensively reviewed in the literature (see for example Smith, 1975; Tamminga, 1978; Stern and Hoover, 1979). Since ^{15}N accounts for a protozoal component it is considered to be a more appropriate microbial marker than DAPA. The importance of the use of different microbial markers, in practice, on the interpolated estimate of endogenous protein flow are examined below.

In the main steer investigation (see section 5.2) DAPA and ^{15}N were used simultaneously as microbial markers. Athough no statistically significant differences ($p>0.1$) were observed between markers (see section 5.3.2) ^{15}N in general, as expected, provided a higher value for microbial nitrogen flow than DAPA. The interpolated estimates of endogenous protein leucine flow derived using ^{15}N and DAPA are included in Table 49. These estimates were not significantly different ($p>0.1$) analysed using a paired t-test. A significant correlation ($p<0.05$) between the two methods was observed which accounted for 91% of the variance. The reduced importance of an absolute estimate of microbial flow in the determination of endogenous protein flow using the proposed technique is therefore apparent.

6.7.2.2 The conversion of endogenous protein leucine values to endogenous protein nitrogen

The examination of the effect on the final endogenous protein nitrogen flow estimate of the substitution of an alternative value for the leucine content of endogenous protein is important. In the present calculation any change in the figure for the leucine content of endogenous secretions from 6.8% (see section 6.2) would have a direct influence on the estimate of endogenous protein nitrogen flow. Evidence that this figure is

Table 49 Estimates of endogenous protein leucine flow (g/day) in steers fed silage supplemented with fishmeal, determined using the intravenous infusion of L-[4,5-^{3}H]-leucine technique using ^{15}N or DAPA as a microbial marker

Microbial marker	Interpolated estimate of endogenous protein leucine flow (g/day)				Mean+	SEM
	Steer P	Steer R	Steer T	Steer V		
^{15}N	4.31	4.71	3.34	6.86	4.81	0.74
DAPA	3.68	4.71	3.49	8.31	5.05	1.12

+ Mean estimate of 4 steers. Mean estimates of endogenous protein leucine flow determined using ^{15}N or DAPA were not significantly different $p>0.1$ (SED = 0.432), analysed using a paired t-test

$$DAPA = (1.46^{(a)} \pm 0.255 \times {}^{15}N) - 1.99 \pm 1.27$$

(a) Significant $p<0.05$ (2 df) and accounts for 91% of the variance

appropriate was discussed in section 6.2. The influence of error in the endogenous protein nitrogen estimate on the in vivo degradability estimate of the ration is examined in section 6.7.3.

6.7.2.3 Conclusion

The error involved in the proposed estimate of endogenous protein nitrogen flow for an individual animal is difficult to predict. A major error in the technique involves the evaluation of the rate constant for the specific activity profiles. These errors can be considered to be randomised between animals fed the same diet. Even so, the variation between animals fed the same diet was large (see Table 41). Part of this error is undoubtedly due to animal variation. A large between animal variation in endogenous protein secretion was also reported by Harrop (1974) and Orskov (1986).

6.7.3 The degree of accuracy required

The importance of the precise determination of endogenous protein nitrogen flow when the values are applied in the accurate assessment of the degradability of a ration must be considered.

The effect of a change in the proposed value of endogenous protein nitrogen on the final degradability estimate was examined. This was carried out using data from the main steer investigation. The results are given in Table 50. A $\pm$ 20% change in the endogenous protein nitrogen resulted in only an approximate $\pm$ 3% alteration in the degradability estimate. Inaccuracies in the degradability estimate of this magnitude are at present probably of minimal importance in the majority of ration formulations. A 40% change in the endogenous protein nitrogen estimate,

Table 50 The effect of an alteration in the proposed value of endogenous protein nitrogen on degradability estimates in steers fed silage supplemented with fishmeal

Values used in degradability determination[$]					
Total nitrogen intake (g/day)	Digesta protein nitrogen flow (g/day)	Microbial nitrogen flow (g/day)	Endogenous protein nitrogen flow (g/day)	Alteration in endogenous protein nitrogen flow (%)	Degradability estimate* (%)
99.0	65.6	40.8	11.3	0	86.4
———	"	———	13.6	+20	88.7
———	"	———	9.0	-20	84.0
———	"	———	15.8	+40	90.9
———	"	———	6.8	-40	81.8

$ Mean estimate of 4 steers, main investigation, see Table 46 .

* Degradability =

$$1 - \frac{\text{Duodenal protein nitrogen flow (g/day)} - \text{Microbial nitrogen flow (g/day)} - \text{Endogenous protein nitrogen flow (g/day)}}{\text{Nitrogen intake (g/day)}}$$

although considered highly unlikely, resulted in only an approximate ± 5% alteration in the degradability estimate. Clearly, a large error in the endogenous protein nitrogen estimate results in a relatively small change in the degradability estimate.

6.8 The prediction of the endogenous protein content of proximal duodenal digesta

The overall objective of this study was to predict directly the endogenous protein content of proximal duodenal digesta in sheep and cattle fed various diets. This was achieved. The evaluation of a simple approach to predict endogenous protein nitrogen flow was considered necessary. The fact that no significant dietary effect was observed for the rations investigated suggested that endogenous protein flow was not directly related to any dietary parameters. The proposal that overall 'total-body metabolism' was involved (i.e. possibly a hormonally mediated homeostatic mechanism) suggested that endogenous protein secretion may be related to metabolic body weight. This was investigated using 0.66, 0.73 and 0.75 as the exponents. A similar percentage of the variance was accounted for in each case (77-79). The best regression was achieved using kg bodyweight$^{0.66}$ (see Fig 17). The proposed equation to determine the endogenous protein nitrogen flow (g/day) is therefore:

$$\text{Endogenous protein nitrogen flow (g/day)} = 0.378 \text{ kg Bodyweight}^{0.66} - 1.70$$

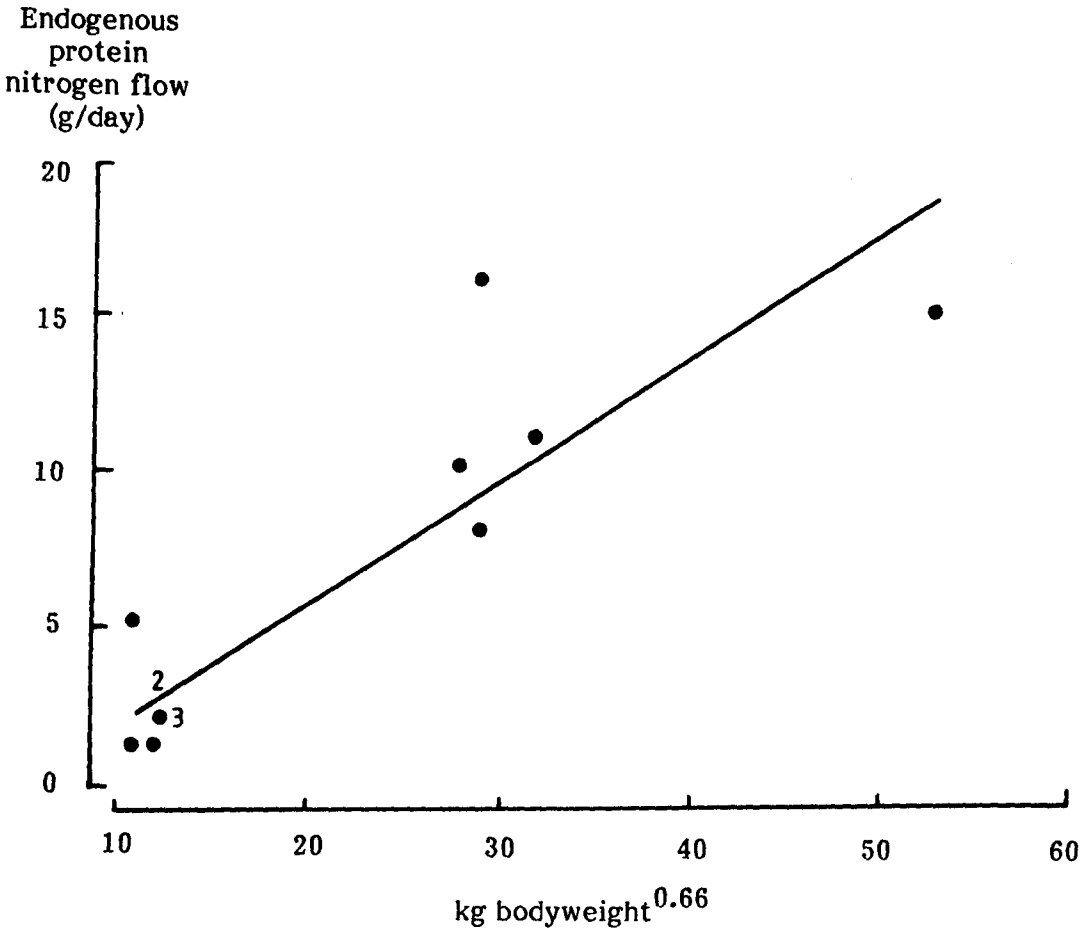
Thus for a 40 kg sheep, predicted endogenous protein nitrogen flow is 2.6 g, and for a 200 kg steer, 11 g. This equation must be treated with caution due to the lack of data for endogenous protein nitrogen over a wide range of animal weights. A valid criticism is that the equation is

Fig 17 The relationship between endogenous protein nitrogen flow (g/day) and kg bodyweight$^{0.66}$ with a regression line fitted

Endogenous protein nitrogen flow (g/day) =

$$(0.378^{(a)} \pm 0.0536 \times \text{kg bodyweight}^{0.66}) - 1.70 \pm 1.25$$

(a) Significant $p<0.001$ (12 df) and accounts for 79% of the variance


Endogenous protein nitrogen flow (g/day)
20
15
10
5
0
2
3
10
20
30
40
50
60
kg bodyweight 0.66

based essentially on three points. An assessment of the sheep and steer data independently indicated a minimal relationship between the variables. This was expected due to the similar weights of the animals used, the relatively large between animal variation and the small number of animals studied.

6.9 Further development and application of the proposed technique

The intravenous infusion of L-[4,5-^{3}H]-leucine technique provides a method to determine directly the endogenous protein content of ruminant proximal duodenal digesta. It can be applied to any dietary regime. Potential ways of improving the accuracy of the estimate, if considered necessary (see below), are now discussed and possible further applications of the technique proposed.

Ideally more animals should have been used in the initial study and the use of a totally protein free diet may be considered more appropriate. Practical limitations prevented the implementation of these improvements.

The conversion of endogenous protein leucine flow to endogenous protein nitrogen flow is a fundamental problem. Methods of improving the accuracy of this include the analysis of the corrected digesta specific activity curve into all its individual components, i.e. an ultimate refinement of the approach outlined in section 6.2. This would necessitate the availability of specific activity profiles representative of all the endogenous components (enzymes, plasma protein, sloughed cells and mucus). The leucine content of these components would also be required in addition to an accurate quantitative assessment of the extent of degradation of sloughed epithelial cells. Although an estimate of the latter

should be readily obtainable using samples from animals maintained using the intragastric infusion technique the feasibility of the remainder of the above proposals is at present probably unrealistic.

An evaluation of the mechanism of control of the different endogenous secretions may provide invaluable information. This is likely to involve an intricate combination of numerous factors.

It is interesting that the technique provides estimates of endogenous protein nitrogen which are in close agreement with values derived using early, relatively unsophisticated approaches. In realistic terms it is considered that the values derived are sufficiently accurate at present to enable the precision of degradability estimates to be increased to a required level. However, it may be necessary to evaluate further the accuracy of the general prediction equation proposed in the previous section.

Further applications of the technique can be considered assuming unlimited resources. A knowledge of whether the endogenous protein component of proximal duodenal digesta varies with level of feed intake may be important. A direct assessment in the dairy cow would also be of interest. In addition, as the passage of plasma albumin into the digestive tract increases during periods of parasitic infection (see Cottle and Nolan, 1982), the determination of endogenous protein flow in affected animals would provide information concerning the protein/energy metabolism in the host.

BIBLIOGRAPHY

AGRICULTURAL RESEARCH COUNCIL (ARC) (1965) The Nutrient Requirements of Farm Livestock. No 2 Ruminants. HMSO; London.

AGRICULTURAL RESEARCH COUNCIL (ARC) (1980) The Nutrient Requirements of Ruminant Livestock. Commonwealth Agricultural Bureaux; Farnham Royal.

AGRICULTURAL RESEARCH COUNCIL (ARC) (1984) The Nutrient Requirements of Ruminant Livestock, Supplement No 1. Commonwealth Agricultural Bureaux; Farnham Royal.

ANNISON, E.F. (1954) Studies on the volatile fatty acids of sheep blood with special reference to formic acid. Biochem. J., **58**, 670-680.

ANNISON, E.F. & LEWIS, D. (1959) Nitrogen Metabolism. In Metabolism in the Rumen, pp 92-120. Methuen, London.

AOAC (ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS) (1975) Official Methods of Analysis, 12th Edition. Ed. W. Horowitz. AOAC; Washington DC, USA.

BAILEY, C.B. (1961) Saliva secretion and its relation to feeding in cattle. The rate of secretion of mixed saliva in the cow during eating, with an estimate of the magnitude of the total daily secretion of mixed saliva. Br. J. Nutr., **15**, 443-451.

BALCH, C.C. (1958) Observations on the act of eating in cattle. Br. J. Nutr., **15**, 371-382.

BAUCHOP, T., CLARKE, R.T.J. & NEWBROOK, J.C. (1975) Scanning electron microscope study of bacteria associated with the rumen epithelium of sheep. Appl. Microbiol., **30**, 668-675.

BEEVER, D.E., HARRISON, D.G., THOMPSON, D.J., CONNELL, S.B. & OSBOURN, D.F. (1974) Method for the estimation of dietary and microbial protein in duodenal digesta of ruminants. Br. J. Nutr., **32**, 99-112.

BEEVER, D.E., THOMPSON, D.J., CAMMELL, S.D. & HARRISON, O.G. (1977) The digestion by sheep of silages made with and without the addition of formaldehyde. J. agric. Sci., **88**, 61-70.

BERGNER, H., BERGNER, U. & SIMON, O. (1983) Measurement of ^{15}N amino acid excretion and endogenous N-secretion in ^{15}N and ^{14}C labelled pigs. In Protein Metabolism and Nutrition. Proceedings of the Fourth International Symposium, Vol. 2, pp 340-342. INRA; Paris.

BINNERTS, W.T., VAN'T KLOOSTER, A. Th. & FRENS, A.M. (1968) Soluble chromium indicator measured by atomic absorption in digesta experiments. Vet. Rec., p 470.

BLACK, J.L., BEEVER, D.E., FAICHNEY, G.J., HOWARTH, B.R. & GRAHAM, N.M. (1981) Simulation of the effects of rumen function on the flow of nutrients from the stomach of sheep; Part 1 - Description of a computer program. Agric. Systems, **6**, 195-219.

BRANDT, M., ROHR, K. & LEBZEIN, P. (1981) Estimation of postruminally secreted protein-N in duodenal digesta of dairy cows by means of 15-N. In The Fourth International Symposium on Amino Acids. Serack; Poland.

BRIGHTLING, P., PATTERSON, J. & TITCHEN, D.A. (1977) Changes in the protein concentration in parotid salivary secretion of sheep when fed. Procs. Aust. Physiol. Pharm. Soc., **8**, 141P.

BRODERICK, G.A. (1978) In vitro procedures for estimating rates of ruminal protein degradation and proportions of protein escaping the rumen undegraded. J. Nutr., **108**, 181-190.

BRYANT, D.T.W. & SMITH, R.W. (1982) Protein synthesis in muscle of mature sheep. J. agric. Sci., **98**, 639-643.

BUTTERY, P.J. (1977) Aspects of the biochemistry of rumen fermentation and their implication in ruminant productivity. In Recent Advances in Animal Nutrition pp 2-24. Eds W. Haresign and D. Lewis. Butterworths; London.

CAMPBELL, R.M., CUTHBERTSON, D.P., MACKIE, W., MacFARLANE, A.S., PHILLIPSON, A.T. & SUDSANEH, S. (1961) The passage of plasma albumin into the intestine of sheep. J. Physiol., **158**, 113-131.

CASTLE, J.D., JAMIESON, J.D. & PALADE, G.E. (1972) Radioautographic analysis of the secretory process in the parotid acinar cell of the rabbit. J. Cell Biol., **53**, 290-311.

CHALUPA, W. (1972) Metabolic aspects of NPN utilization in ruminant animals. Fedn Proc., **31**, 1152-1164.

CHALUPA, W., CLARKE, J., OPILGER, P. & LAVKER, R. (1970) Ammonia metabolism in rumen bacteria and mucosa from sheep fed soy protein or urea. J. Nutr., **100**, 161-169.

CHAMBERLAIN, D.G. & THOMAS, P.C. (1979a) Ruminal nitrogen metabolism and the passage of amino acids to the duodenum of sheep receiving diets containing hay and concentrates in various proportions. J. Sci. Fd. Agric., **30**, 677-686.

CHAMBERLAIN, D.G. & THOMAS, P.C. (1979b) Prospective laboratory methods for estimating the susceptibility of feed proteins to microbial breakdown in the rumen. Proc. Nutr. Soc., **38**, 138A.

CHAMBERLAIN, D.G., THOMAS, P.C. & QUIG, J. (1986) Utilization of silage nitrogen in sheep and cows : amino acid composition of duodenal digesta and rumen microbes. Grass Forage Sci., **41**, 31-38.

CHENG, K.J., McCOWAN, R.P. & COSTERTON, J.W. (1979) Adherent epithelial bacteria in ruminants and their roles in the digestive tract. Am. J. Clin. Nutr., **32**, 139-148.

CHENG, K.J. & WALLACE, R.J. (1979) The mechanism of passage of endogenous urea through the rumen wall and the role of the ureolytic epithelial bacteria in the urea flux. Br. J. Nutr., **42**, 553-557.

CHOW, R.B. & KASSELL, B. (1978) Bovine pepsinogen and pepsin. 1. Isolation purification and some properties of the pepsinogen. J. Biol. Chem., **243**, 1718-1724.

CHRISTIAN, K.R. & COUP, M.R. (1954) Measurement of feed intake by grazing cattle and sheep. VI. The determination of chromic oxide in faeces. N.Z. J. Sci. Tech., **36**A, 328-330.

CLARKE, R.T.J., JONES, W.T. & REID, C.S.W. (1974) Bloat in cattle. XLI. Proteins of saliva as possible genetic markers in breeding programmes to reduce bloat susceptibility in cattle. J. Agric. Res., **17**, 411-415.

COCKBURN, J.E. & WILLIAMS, A.P. (1984) The simultaneous estimation of the amounts of protozoal, bacterial and dietary nitrogen entering the duodenum of steers. Br. J. Nutr., **51**, 111-132.

COLIN, M.G. (1886) Traite de Physiologie Comparee des Animaux; 3rd Edition, pp 646-677. (Bailliere de Fils, Paris).

COOPER, M. & LIPKIN, M. (1973) Growth and development of normal and diseased gastrointestinal cells. Digestion, **8**, 191-200.

COTTLE, D.J.S. & NOLAN, J.V. (1982) The use of amino acid profiles to measure microbial protein outflow from the rumen. Proc. Aust. Soc. Anim. Prod., **14**, 588-591.

DARGIE, J.D. & BERRY, C.I. (1979) The hypoalbuminaemia of ovine fascoliasis : the influence of protein intake on the albumin metabolism of infected and pair-fed control sheep. Int. J. Parasitol., **9**, 17-25.

DAVIDSON, J., MATHIESON, J. & BOYN, A.W. (1970) The use of automation in determining nitrogen by the Kjeldahl method with final calculations by computer. Analyst, **95**, 181-193.

DAVIS, S.R., BARRY, T.N. & HUGHSON, G.A. (1981) Protein synthesis in tissues of growing lambs. Br. J. Nutr., **46**, 409-419.

DINSDALE, D., CHENG, K.J., WALLACE, R.J. & GOODLAD, R.A. (1980) Digestion of epithelial tissue of the rumen wall by adherent bacteria in infused and conventionally fed sheep. Appl. Environ. Microbiol., **39**, 1059-1066.

DOYLE, P.T., EGAN, J.K. & THALEN, A.J. (1982) Parotid saliva of sheep. 1. Effects of level of intake and type of roughage. Aust, J. Agric. Res., **33**, 573-584.

EGAN, A.R., BODA, K. & VARADY, J. (1984) Regulation of nitrogen metabolism and recycling. In Control of Digestion and Metabolism in Ruminants, pp 386-402. Eds L.P. Milligan, W.L. Grovum and A. Dobson. Prentice Hill; New Jersey, USA.

ELLISON, S.A. (1967) Proteins and glycoproteins of saliva. In Handbook of Physiology, Section 6; Alimentary Canal, Vol. 2, pp 553-559. American Physiological Society; Washington DC.

EVANS, R.A., AXFORD, R.F.E. & OFFER, N.W. (1975) A method for estimating the quantities of microbial and dietary protein flowing in the duodenal digesta of ruminants. Proc. Nutr. Soc., **34**, 65A.

EVANS, C.D., MacRAE, J.C. & WILSON, S. (1977) Determination of ruthenium and chromium by X-ray fluorescence spectrometry and the use of inert ruthenium (ii) phenanthroline as a solid phase marker in sheep digestion studies. J. agric. Sci., **89**, 17-22.

FAICHNEY, G.J. (1975) The use of markers to partition digestion within the gastro-intestinal tract of ruminants. In Digestion and Metabolism in the Ruminant, pp 277-291. Eds I.W. McDonald and A.C.I. Warner. The University of New England Publishing Unit; Armidale, Australia.

FASMAN, G.D. (1976) Handbook of Biochemistry and Molecular Biology, 3rd Edition (Ed.). Proteins, Vol. 3, p 509. CRC Press, Ohio.

FELL, B.F. & WEEKES, T.E.C. (1975) Food intake as a mediator of adaptation in the ruminal epithelium. In Digestion and Metabolism in the Ruminant, pp 101-118. Eds I.W. McDonald and A.C.I. Warner. The University of New England Publishing Unit; Armidale, Australia.

FLOREY, H.W. (1955) Mucin and the protection of the body. Proc. R. Soc. Lond. B., **143**, 147-158.

FROETSCHEL, M.A., CROOM, W.J., HOGLER, W.M., TATE Jnr, L.P. & BROQUIST, H.P. (1986) Effects of slaframine on ruminant digestive function : resting salivary flow and composition in cattle. J. Anim. Sci., **62**, 1404-1411.

GARTON, G.H., HOBSON, P.N. & LOUGH, A.K. (1958) Lipolysis in the rumen. Nature, **182**, 1511-1512.

GLASS, G.B. (1968) Protein in gastric secretions. In The Physiology of Gastric Secretion, pp 456-477. Eds L.S. Semb and J. Myren. The Williams and Wilkins Co.; Baltimore.

GOODLAD, R.A. (1981) Some effects of diet on the mitotic index and the cell cycle of the rumen epithelium of sheep. Q. J. Expt. Physiol., **66**, 487-499.

GOTTSCHALK, A. & FAZEKAS, DE St. GROTH, S. (1960) Studies in muco-proteins. 3. The accessibility to trypsin of the susceptible bonds in ovine submaxillary gland mucoprotein. Biochim. biophys Acta., **43**, 513-519.

GOTTSCHALK, A. & SIMMONDS, D.H. (1960) Studies on mucoproteins. 2. Analysis of the protein moiety of ovine submaxillary gland mucoprotein. Biochim. biophys Acta., **42**, 141-146.

HAMILTON, A.I. & BLACKWOOD, H.J.J. (1977) Insulin deficiency and cell proliferation in oral mucosal epithelium of the rat. J. Anat., **124**, 757-763.

HARDING, S.E. (1987) Int. J. Biol. Macromol. (in press).

HARRISON, D.G., BEEVER, D.E., THOMSON, D.J. & OSBOURN, D.F. (1973) The influence of diet upon the quantity and types of amino acids entering and leaving the small intestine of sheep. J. agric. Sci., **81**, 391-401.

HARRISON, D.G., BEEVER, D.E. & OSBOURN, D.F. (1979) The contribution of protozoa to the protein entering the duodenum of sheep. Br. J. Nutr., **41**, 521-527.

HARRISON, F.A. & HILL, K.J. (1962) Digestive secretions and the flow of digesta along the duodenum of the sheep. J. Physiol., **162**, 225-243.

HARROP, D.J.F. (1974) Nitrogen metabolism in the ovine stomach. 4. Nitrogenous components of the abomasal secretions. J. agric. Sci., **83**, 249-257.

HASHIMOTO, Y., TSUIKI, S., NISIZAWA, K. & PIGMAN, W. (1963) Action of proteolytic enzymes on purified bovine submaxillary mucin. Ann. N.Y. Acad. Sci., **106**, 233-246.

HEATLEY, N.G. (1959) Some experiments on partially purified gastrointestinal mucosubstance. Gastroenterology, **37**, 304-312.

HECKER, J.F. (1973) The fate of soluble mucin in the gastro-intestinal tract of sheep. J. agric. Sci., **80**, 63-69.

HILL, K.J. (1960) Abomasal secretion in the sheep. J. Physiol., **154**, 115-132.

HILL, K.J. (1961) The abomasum. Vet. Rev. Annotations, **7**, 83-106.

HOGAN, J.P. (1975) Protein and amino acid nutrition in the high producing dairy cow. Quantitative aspects of nitrogen utilisation in ruminants. J. Dairy Sci., **58**, 1164-1177.

HOGAN, J.P. & WESTON, R.H. (1967) Quantitative aspects of microbial protein synthesis in the rumen. In Physiology of Digestion and Metabolism in the Rumen, pp 474-485. Ed. A.T. Phillipson. Oriel Press; Newcastle-upon-Tyne.

HOLMAN, H., NICKEL Jr., W.F. & SLEISENGER, M.H. (1959) Hypoproteinemia antedating intestinal lesions and possibly due to excessive serum protein loss into the intestine. Am. J. Med., **27**, 963-975.

HOUPT, T.R. (1970) Transfer of urea and ammonia to the rumen. In Physiology of Digestion and Metabolism in the Rumen, pp 475-485. Ed. A.T. Phillipson. Oriel Press; Newcastle-upon-Tyne.

HUNGATE, R.E. (1966) The Rumen and its Microbes, p 168. Academic Press, New York and London.

HUTTON, K., BAILEY, F.J. & ANNISON, E.F. (1971) Measurement of the bacterial nitrogen entering the duodenum of the ruminant using DAPA as a marker. Br. J. Nutr., **25**, 165-173.

IAEA (INTERNATIONAL ATOMIC ENERGY AGENCY) (1979) laboratory training manual on the use of nuclear techniques in animal research. In Technical Reports Series No. 193. IAEA; Vienna.

ISENBERG, I., DYSON, R.D. & HENSON, R. (1973) Studies on the analysis of fluorescence decay data by the method of moments. Biophys. J., **13**, 1090-1115.

JOHNSON, L.R. (1980) Effect of gastrointestinal hormones on growth of gastrointestinal tissue. In Gastrointestinal Hormones, pp 507-527. Ed. G.B. Jerzy Glass. Raven Press; New York.

KAUFMANN, W. (1977) Economic and other considerations governing decisions on the advisability of incorporating additional and new sources of protein and non protein nitrogen into the diet of beef cattle. In Protein and Non Protein for Ruminants, pp 101-120. Pergamon Press; Oxford.

KAY, R.N.B. (1960) Rate of flow and composition of various salivary secretions in sheep and calves. J. Physiol., **150**, 515-537.

KAY, R.N.B. (1966) The influence of saliva on digestion in ruminants. Wld Rev. Nutr. Diet, **6**, 292-325.

KELLY, N.C. & THOMAS, P.C. (1975) The effect of intravenous infusions of methionine on the voluntary intake of grass silage by sheep. J. Br. Grassld Soc., **30**, 237-239.

KEMPTON, T.J., NOLAN, J.V. & LENG, R.A. (1979) Protein nutrition of growing lambs. 2. Effect on nitrogen digestion of supplementing a low-protein cellulosic diet with either urea, casein or formaldehyde treated casein. Br. J. Nutr., **42**, 303-315.

KENNEDY, P.M. & MILLIGAN, L.P. (1978a) Transfer of urea from the blood to the rumen of sheep. Br. J. Nutr., **40**, 149-154.

KENNEDY, P.M. & MILLIGAN, L.P (1978b) Effects of cold exposure on digestion, microbial synthesis and nitrogen transformations in sheep. Br. J. Nutr., **39**, 105-117.

KENNEDY, P.M. & MILLIGAN, L.P (1980a) The degradation of and utilization of endogenous urea in the gastrointestinal tract of ruminants : A review. Can. J. Anim. Sci., **60**, 205-221.

KENNEDY, P.M. & MILLIGAN, L.P. (1980b) Input of endogenous protein into the forestomachs of sheep. Can. J. Anim. Sci., **60**, 1029-1032.

KRISHNAMOURTHY, U., SNIFFEN, C.J., STERN, M.D. & VAN SOEST, P.J. (1983) Evaluation of a mathematical model of rumen digestion and an in vitro simulation of rumen proteolysis to estimate the rumen undegraded nitrogen content of feedstuffs. Br. J. Nutr., **50**, 555-568.

LAEMMELI, U.K. (1970) Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate. Nature, **227**, 680-685.

LANGSTROTH, G.O., McRAE, D.R. & STRAVRAKY, G.W. (1938) The secretion of protein material in the parasympathetic submaxillary saliva. Proc. Roy. Soc. Lond. B., **125**, 335-347.

LEHNINGER, A.L. (1975) Biochemistry, p 571. Worth; New York.

LEWIS, D., HILL, K.J. & ANNISON, E.F. (1957) Studies on the portal blood of sheep. 1. Absorption of ammonia from the rumen of sheep. Biochem. J., **66**, 587-592.

LINDSAY, J.R. & HOGAN, J.P. (1972) Digestion of two legumes and rumen bacterial growth in defaunated sheep. Aust. J. Agric. Res., **23**, 321-330.

LING, J.R. (1976) Protein Metabolism in the Rumen. Ph.D Thesis, Nottingham University.

LING, J.R. & BUTTERY, P.J. (1978) The simultaneous use of RNA, ^{35}S,2,6-diaminopimelic acid and 2-aminoethylphosphonic acid as markers of microbial nitrogen entering the duodenum of sheep. Br. J. Nutr., **39**, 165-179.

LITTLE, C.O., BURROUGHS, W. & WOODS, W. (1963) Nutritional significance of soluble nitrogen in dietary proteins for ruminants. J. Anim. Sci., **22**, 358-363.

LOBLEY, G.E., MILNE, V., LOVIE, J.M., REEDS, P.J. & PENNIE, K. (1980) Whole body and tissue protein synthesis in cattle. Br. J. Nutr., **43**, 491-501.

LOOSLI, J.K., WILLIAMS, H.H., THOMAS, W.E., FERRIS, F.H. & MAYNARD, L.A. (1949) Synthesis of amino acids in the rumen. Science, **110**, 144-145.

LOW, A.G. & RAINBIRD, A.L. (1983) Effect of dietary fibre (guar gum) on endogenous nitrogen secretion in the jejunum of pigs. In Protein Metabolism and Nutrition. Proceedings of the Fourth International Symposium, Vol. 2., pp 343-346. INRA; Paris.

LYMAN, C.M., CHANG, W.Y. & COUCH, J.R. (1953) Evaluation of protein quality in cottonseed meals by chick growth and by chemical index method. J. Nutr., **49**, 679-690.

LYTTLETON, J.W. (1960) Bloat in cattle. VIII. The proteins of bovine saliva. N.Z. J. Agric. Res., **3**, 63-68.

McDONALD, I.W. (1952) The role of ammonia in ruminal digestion of protein. Biochem. J., **51**, 86-90.

McDONALD, I.W. (1954) The extent of conversion of food protein to microbial protein in the rumen of the sheep. Biochem. J., **56**, 120-125.

McDOUGALL, E.I. (1948) The composition and output of sheep saliva. Biochem. J., **43**, 99-109.

McINTOSH, J.T. (1975) The components of bovine saliva in relation to bloat. Proc. N.Z. Soc. Anim. Prod., **35**, 29-34.

McINTOSH, J.T., McLAREN, R.D. & COCKREM, F.R.M. (1982) Genetics of the susceptibility to bloat in cattle. 3. Quantification of protein bands from bovine saliva separated by polyacrylamide gel electrophoresis. N.Z. J. Agric. Res., **25**, 191-198.

McMENIMAN, N.P., BEN-GHADALIA, D. & ARMSTRONG, D.G. (1976) Nitrogen-energy interactions in rumen fermentation. In Protein Metabolism and Nutrition (European Association for Animal Production, Publication number 16), pp 217-229. Eds D.J.A. Cole, K. Boorman, P.J. Buttery, D. Lewis, R.J. Neale and H. Swan. Butterworths; London.

MEAD, R.J. & JONES, G.A. (1981) Isolation and presumptive identification of adherent epithelium bacteria ("Epimural" bacteria) from the ovine rumen wall. Appl. Environ. Microbiol., **41**, 1020-1028.

MEHREZ, A.Z. & ORSKOV, E.R. (1977) A study of the artificial fibre bag technique for determining the digestibility of feeds in the rumen. J. agric. Sci., **88**, 645-650.

MERTENS, D.R. (1977) Importance and measurement of protein insolubility in ruminant diets. In Proceedings 1977 Georgia Nutrition Conference for the Feed Industry, p 30.

NOLAN, J.V. (1975) Quantitative models of nitrogen metabolism in sheep. In Digestion and Metabolism in the Ruminant, pp 416-431. Eds I.W. McDonald and A.C.I. Warner. The University of New England Publishing Unit; Armidale, Australia.

NOLAN, J.V. & LENG, R.A. (1974) Symposium on "Tracer Techniques in Nutrition". Isotope techniques for studying the dynamics of nitrogen metabolism in ruminants. Proc. Nutr. Soc., **33**, 1-8.

NOLAN, J.V. & MacRAE, J.C. (1976) Absorption and recycling of nitrogenous compounds in the digestive tract of sheep. Proc. Nutr. Soc., 110A.

NOLAN, J.V., NORTON, B.W. & LENG, R.A. (1973) Nitrogen cycling in sheep. Proc. Nutr. Soc., **32**, 93-98.

NOLAN, J.V., NORTON, B.W. & LENG, R.A. (1976) Futher studies of the dynamics of nitrogen metabolism in sheep. Br. J. Nutr., **35**, 127-148.

NUGENT, J.H.A., JONES, W.T., JORDAN, D.J. & MANGAN, J.L. (1983) Rates of proteolysis in the rumen of the soluble proteins casein, fraction 1 (18S) leaf protein, bovine serum albumin and bovine submaxillary mucoprotein. Br. J. Nutr., **50**, 357-368.

OFFER, N.W., AXFORD, R.F.E. & EVANS, R.A. (1978) The effect of dietary energy source on nitrogen metabolism in the rumen of sheep. Br. J. Nutr., **40**, 35-44.

ORSKOV, E.R., GRUBB, D.A., WENHAM, G. & CORRIGALL, W. (1979) The sustenance of growing and fattening ruminants by intragastric infusion of volatile fatty acid and protein. Br. J. Nutr., **41**, 553-558.

ORSKOV, E.R. & MacLEOD, N.A. (1982) The flow of N from the rumen of cows and steers maintained by intraruminal infusion of volatile fatty acids. Proc. Nutr. Soc., **41**, 76A.

ORSKOV, E.R. & MacLEOD, N.A. (1983) Flow of endogenous nitrogen from the rumen and abomasum of cattle given protein-free nutrients. Proc. Nutr. Soc., **42**, 61A.

ORSKOV, E.R., MacLEOD, N.A. & KYLE, D.J. (1986) Flow of nitrogen from the rumen and abomasum in cattle and sheep given protein free nutrients. Br. J. Nutr., **56**, 241-248.

PALMER, C.C. (1916) The diastase in the saliva of the ox. Am. J. Physiol., **41**, 483-491.

PATTERSON, J., BRIGHTLING, P. & TITCHEN, D.A. (1982) Beta-adrenergic effects on composition of parotid salivary secretion of sheep on feeding. Q. Jl. Expt. Physiol., **67**, 57-67.

PATTERSON, J. & TITCHEN, D.A. (1979) Sympathetic and B-adrenergic stimulation of parotid salivary secretion in sheep. Q. Jl. Expt. Physiol., **64**, 175-184.

PATTERSON, J. & TITCHEN, D.A. (1980) B-adrenergic stimulation of parotid salivary secretion in sheep. J. Physiol., **298**, 44P.

PHELPS, R.A. & PUTNAM, F.W. (1960) Chemical composition and molecular parameters of purified plasma proteins. In The Plasma Proteins, pp 143-178. Ed. F.W. Putnam. Academic Press; London.

PHILLIPSON, A.T. (1964) The digestion and absorption of nitrogenous compounds in the ruminant. In Mammalian Protein Metabolism, Vol. 1, pp 71-103. Eds H.N. Munro and J.B. Allison. Academic Press; New York.

PHILLIPSON, A.T. & MANGAN, J.L. (1959) Bloat in cattle. XVI. Bovine saliva : the chemical composition of the parotid, submaxillary and residual secretions. N.Z. J. Agric. Res., **2**, 990-1001.

PIGMAN, W. & GOTTSCHALK, A. (1966) Submaxillary gland glycoproteins. In Glycoproteins, their Composition and Function, pp 434-445. Ed. A. Gottschalk. Biochim. biophys. Acta., Library Vol. 5. Elsevier; Amsterdam.

REEDS, P.J. & LOBLEY, G.E.C. (1980) Symposium on 'Nitrogen Metabolism'. Protein synthesis : are there real species differences? Proc. Nutr. Soc., **39**, 43-52.

ROBERTS, S.A. & MILLER, E.L. (1969) An estimate of bacterial protein synthesis in sheep on a constant feeding regime. Proc. Nutr. Soc., **28**, 32A.

ROOKE, J.A., GREIFE, H.A. & ARMSTRONG, D.G. (1985) The digestion by cattle of silage containing diets fed at two dry matter intakes. 1. Digestion of organic matter and nitrogen. Br. J. Nutr., **53**, 691-708.

ROTHSCHILD, M.A., ORATZ, M. & SCHREIBE, S.S. (1970) Albumin metabolism. In Plasma Protein Metabolism, pp 199-205. Eds M.A. Rothschild and I. Waldmann. Academic Press; London.

ROWE, J.B. & JAMES, S. (1982) Measurement of cell turnover in the gastrointestinal wall of sheep using single injections of [6-^{3}H] thymidine. Proc. Nutr. Soc., **42**, 2A.

ROY, J.H.B., BALCH, C.C., MILLER, E.L., ORSKOV, E.R. & SMITH, R.H. (1977) Calculation of the N-requirement for ruminants from nitrgen metabolism studies. In Protein Metabolism and Nutrition. Proceedings of the Second International Symposium, pp 126-129. Wageningen.

SAKATA, T., HIKOSAKA, K., SHIOMURA, Y. & TAMATE, H. (1980a) Stimulatory effect of insulin on ruminal epithelium cell mitosis in adult sheep. Br. J. Nutr., **44**, 325-331.

SAKATA, T., HIKOSAKA, K., SHIOMURA, Y. & TAMATE, H. (1980b) The stimulatory effect of butyrate on epithelial cell proliferation in the rumen of the sheep and its mediation by insulin : differences between in vivo and in vitro studies. In Cell Proliferation in the Gastrointestinal Tract, pp 123-137. Eds D.R. Appleton, J.P. Sunter and A.J. Watson. Pitman Medical; UK.

SAKATA, T. & TAMATE, H. (1978) Rumen epithelial cell proliferation accelerated by rapid increase in intraruminal butyrate. J. Dairy Sci., **61**, 1109-1113.

SAKATA, T. & TAMATE, H. (1979) Rumen epithelium cell proliferation accelerated by propionate and acetate. J. Dairy Sci., **62**, 49-52.

SATTER, L.D. & ROFFLER, R.E. (1977) Influence of nitrogen and carbohydrate inputs on rumen fermentation. In Recent Advances in Animal Nutrition pp 25-49. Eds W. Haresign and D. Lewis. Butterworths; London.

SCHULTZE, H.E. & HEREMANS, J.F. (1966) The proteins of digestive secretions. 1. The proteins of gastric juice. In Molecular Biology of Human Proteins with special reference to Plasma Proteins, Vol. 1, pp 773-789. Elsevier; Amsterdam.

SIDDONS, R.C., BEEVER, D.E. & NOLAN, J.V. (1982) A comparison of methods for the estimation of microbial nitrogen in duodenal digesta of sheep. Br. J. Nutr., **48**, 377-389.

SIDDONS, R.C., NOLAN, J.V., BEEVER, D.E. & MacRAE, J.C. (1985a) Nitrogen digestion and metabolism in sheep consuming diets containing contrasting forms and levels of nitrogen. Br. J. Nutr., **54**, 175-187.

SIDDONS, R.C., PARADINE, J., BEEVER, D.E. & CORNELL, P.R. (1985b) Ytterbium acetate as a particulate-phase digesta-flow marker. Br. J. Nutr., **54**, 509-519.

SIDDONS, R.C., PARADINE, J., GALE D.L. & EVANS, R.T. (1985c) Estimation of the degradability of dietary protein in the sheep rumen by in vivo and in vitro procedures. Br. J. Nutr., **54**, 545-561.

SMITH, R.H. (1975) Nitrogen metabolism in the rumen and the composition and nutritive value of nitrogen compounds entering the duodenum. In Digestion and Metabolism in the Ruminant. pp 399-415. Eds I.W. McDonald and A.C.I. Warner. University of New England Publishing Unit; Armidale, Australia.

SMITH, R.H. (1979) What new information is required by those proposing schemes for the feeding of ruminants? In Protein Metabolism in the Ruminant, pp 11.1-11.12. Ed. P.J. Buttery. ARC; London.

SMITH, R.H. & McALLAN, A.B. (1970) Nucleic acid metabolism in the ruminant. Formation of microbial nucleic acids in the rumen in relation to the digestion of food nitrogen, and the fate of dietary nucleic acids. Br. J. Nutr., **24**, 545-556.

SMITH, R.H. & McALLAN, A.B. (1971) Nucleic acid metabolism in the ruminant. 3. Amounts of nucleic acids and total and ammonia nitrogen in digesta from the rumen, duodenum and ileum of calves. Br. J. Nutr., **25**, 181-190.

SOMERS, M. (1961a) Factors influencing the secretion of nitrogen in sheep saliva. 4. The influence of injected urea on the quantitative recovery of urea in the parotid saliva and urinary excretion of sheep. Aust. J. Expt. Biol. Med. Sci., **39**, 145-156.

SOMERS, M. (1961b) Factors influencing the secretion of nitrogen in sheep saliva. 2. The influence of nitrogen uptake upon blood urea nitrogen and upon the total nitrogen and urea nitrogen in the parotid saliva of sheep. Aust. J. Expt. Biol. Med. Sci., **39**, 123-132.

SQUIRES, B.T. (1953) Human salivary amylase secretion in relation to diet. J. Physiol., **119**, 153-156.

STERN, M.D. & HOOVER, W.H. (1979) Methods for determining and factors affecting rumen microbial protein synthesis : A review. J. Anim. Sci., **49**, 1590-1603.

TAMMINGA, S. (1978) Measurement of microbial protein synthesis in the rumen. In Ruminant Digestion and Feed Evaluation. pp 5.1-5.13. Eds D.F. Osbourn, D.E. Beever and D.J. Thomson. ARC; London.

TAMMINGA, S. (1979) Protein degradation in the forestomachs of ruminants. J. Anim. Sci., **49**, 1615-1630.

TAN, T.N., WESTON, R.H. & HOGAN , J.P. (1971) Use of ^{103}Ru-labelled tris (1,10-phenanthroline) ruthenium (II) chloride as a marker in digestion studies with sheep. Int. J. Appl. Radiat. Isotopes, **22**, 301-308.

THOMAS, P.C. (1986) Future ideas on dairy cow nutrition. The Feed Compounder, **6**, 7-12.

THOMAS, P.C., CHAMBERLAIN, D.G., KELLY, N.C. & WATT, M.K. (1980) The nutritive value of silages : digestion of nitrogenous constituents in sheep receiving diets of grass silage and grass silage and barley. Br. J. Nutr., **43**, 469-479.

TITCHEN, D.A. (1984) Gastrointestinal peptide hormone distribution, release and action in ruminants. In Control of Digestion and Metabolism in Ruminants, pp 227-248. Eds L.P. Milligan, W.L. Grovum and A. Dobson. Prentice Hall; New Jersey, USA.

TWOOMBLY SNOOK, J. (1965) Effect of diet on intestinal proteolysis. Fedn Proc., **24**, 941-945.

VAN BRUCHEM, J. & VAN T'KLOOSTER, A.Th. (1980) Effect of protein on abomasal secretion of acid in sheep. Br. J. Nutr., **44**, 307-312.

VAN SOEST, P.J. (1963) Use of detergents in the analysis of fibrous feeds. 2. A rapid method for the determination of fibre and lignin. J. Ass. Off. Agric. Chem., **46**, 829-835.

VOIGT, P., PATTERSON, J. & TITCHEN, D.A. (1980) Observations on the secretion and some characteristics of protein in parotid saliva of sheep. Procs. Aust. Physiol. Pharm. Soc., **11**, 38P.

WALLACE, R.J., CHENG, K.J., DINSDALE, D. & ORSKOV, E.R. (1979) An independent microbial flora of the epithelium and its role in the ecomicrobiology of the rumen. Nature, **279**, 424-426.

WARNER, R.G. & FLATT, W.P. (1965) Anatomical development of the ruminant stomach. In Physiology of Digestion in the Ruminant, pp 24-38. Ed. R.W. Dougherty. Butterworths; Washington DC, USA.

WATERLOW, J.C. & STEPHEN, J.M.L. (1967) The measurement of total lysine turnover in the rat by intravenous infusion of L-(U-^{14}C)-lysine. Clin. Sci., **33**, 489-506.

WEBSTER, A.J.F. (1980) Energy costs of digestion and metabolism in the gut. In Digestive Physiology and Metabolism in Ruminants, pp 468-484. Eds Y. Ruckesbusch and P. Thivend. MTP Press Ltd; Lancaster, England.

WEBSTER, A.J.F., SIMMON, S.I.P. & KITCHERSIDE, M.A. (1982) Forage protein and the performance and health of the dairy cow. In Forage Protein in Ruminant Animal Production, pp 89-95. Occasional publication (Number 6) of the British Society of Animal Production.

WEGNER, M.I., BOOTH, A.N., BOHSTEDT, G. & HART, E.B. (1940) The in vitro conversion of inorganic nitrogen to protein by microorganisms from the cow's rumen. J. Dairy Sci., **23**, 1123-1129.

WELLER, R.A. & PILGRIM, A.F. (1974) Passage of protozoa and volatile fatty acids from the rumen of sheep and from a continuous in vitro fermentation system. Br. J. Nutr., **32**, 341-351.

WESTON, R.H. & HOGAN, J.P. (1967) The digestion of chopped and ground roughages by sheep. 1. The movement of digesta through the stomach. Aust. J. agric. Res., **18**, 789-801.

WILLIAMS, A.P. (1986) Laboratory methods of determining protein degradability in ruminants. Res. Devel. Agric., **3**, 1-8.

WILSON, A.D. & TRIBE, D.E. (1963) Effect of diet on the secretion of parotid saliva by sheep. 1. Daily secretion of saliva by caged sheep. Aust. J. Agric. Res., **14**, 670-679.

WILSON, P.N. & STRACHAN, P.J. (1980) The contribution of undegraded protein to the protein requirements of dairy cows. In Recent Advances in Animal Nutrition, pp 99-118. Ed. W. Haresign. Butterworths; London.

WOHLT, J.F., SNIFFEN, C.J. & HOOVER, W.H. (1973) Measurement of protein solubility in common feedstuffs. J. Dairy Sci., **56**, 1052-1057.

Appendix 1 Details of automatic amino acid analyser (LKB 4400) programs

a) Full lithium citrate system

Column	250 x 4.5 mm
Resin	Aminex A9 (Biorad, California, USA)

Buffers	Li molarity	pH	
A (equilibration)	0.2	2.8	(contains 1.5% Propan-2-ol)
B	0.3	3.0	
C	0.6	3.02	
D	1.0	3.45	
E	1.6	3.3	
Li OH (regeneration)	0.3		

Column temperature	35°C —— 60°C
Flow - Buffer	20 ml/hr
- Ninhydrin	20 ml/hr
Total cycle time	4.5 hr

Absorption measured at 570 and 440 nm

570 nm output integrated with SpectraPhysics SP 4270 (SpectraPhysics, California, USA)

b) Modified (TEA) system

As above but elution with 50 : 50 mixture of buffers C and D, column temperature 35°C.

Total cycle time 2 hr.

APPENDIX 2

The specific activity (dpm/μmol) of protein leucine in various fractions during a 200 hr continuous intravenous infusion of L-[4,5-^{3}H]-leucine for all animals studied in the investigation

2.1 Sheep A

a) (*) Total duodenal digesta

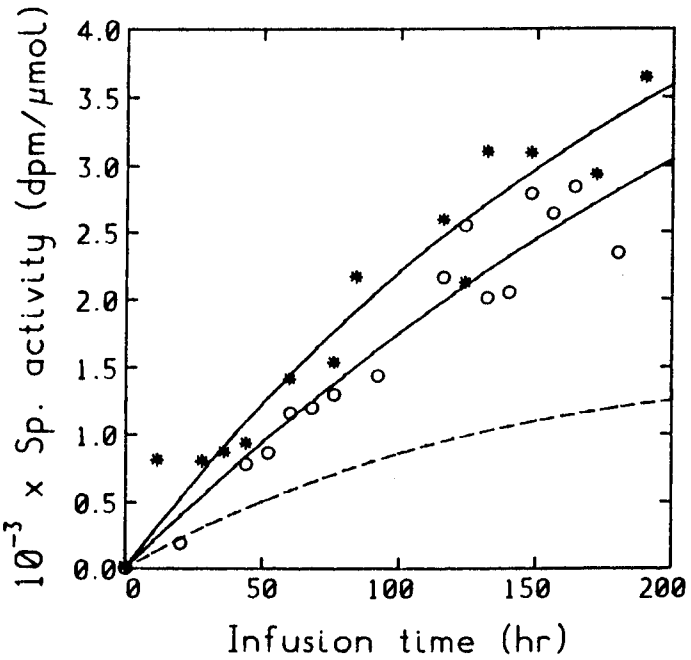
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

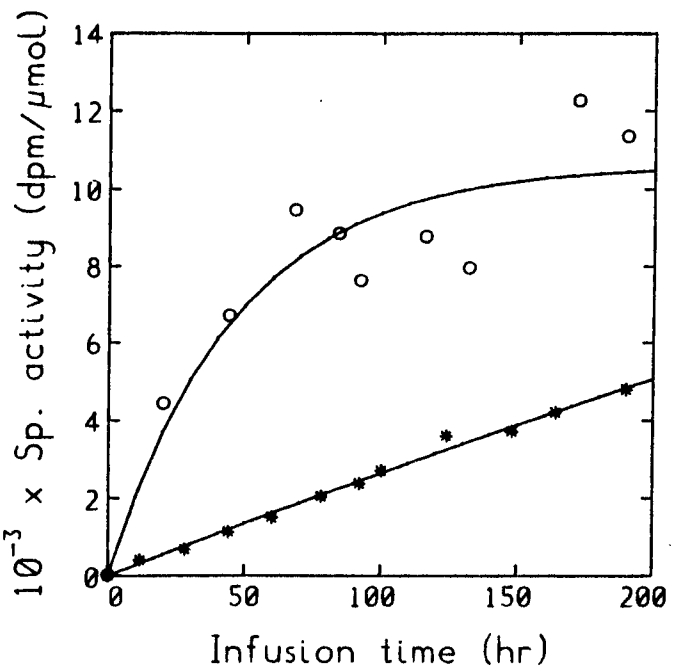
b) (*) Plasma protein

(O) Saliva

a)


10^{-3} x Sp. activity (dpm/μmol)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
Infusion time (hr)

b)


10^{-3} x Sp. activity (dpm/μmol)
14
12
10
8
6
4
2
0
0
50
100
150
200
Infusion time (hr)

2.2 Sheep B

a) (*) Total duodenal digesta

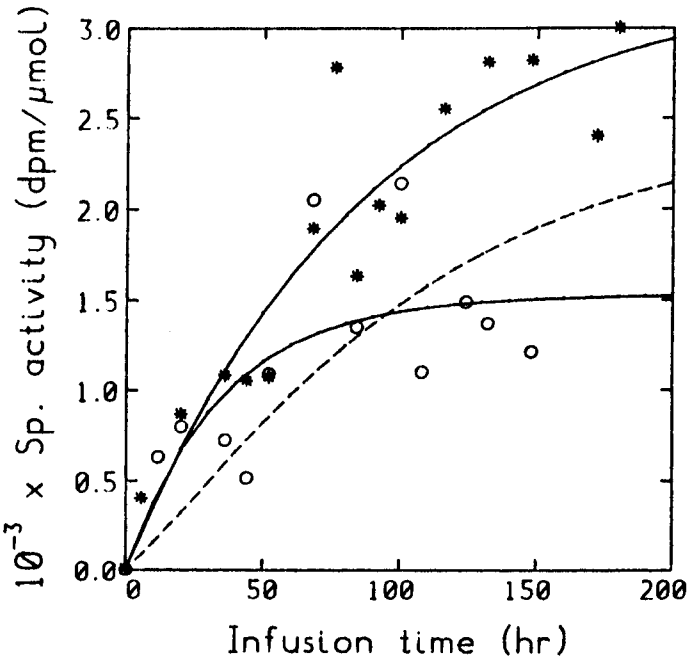
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

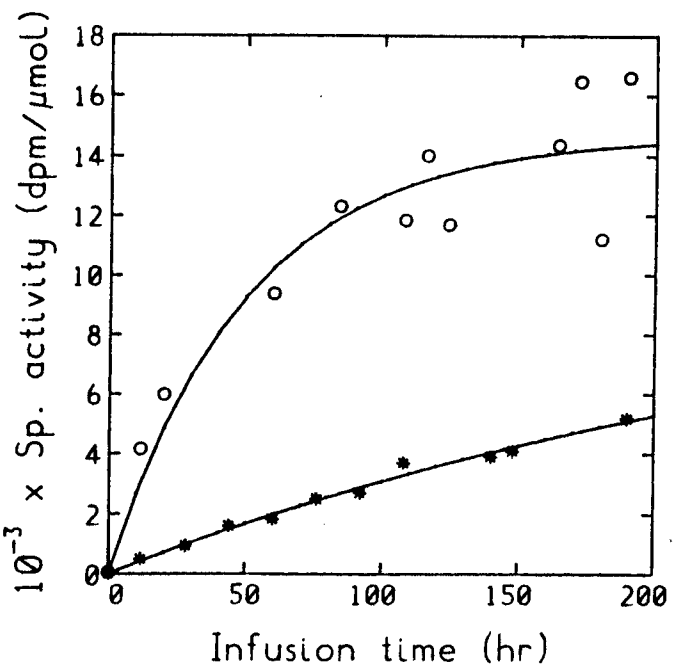
b) (*) Plasma protein

(O) Saliva

a)


10^{-3} x Sp. activity (dpm/μmol)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
Infusion time (hr)

b)


10^{-3} x Sp. activity (dpm/μmol)
18
16
14
12
10
8
6
4
2
0
0
50
100
150
200
Infusion time (hr)

2.3 Sheep C

a) (*) Total duodenal digesta

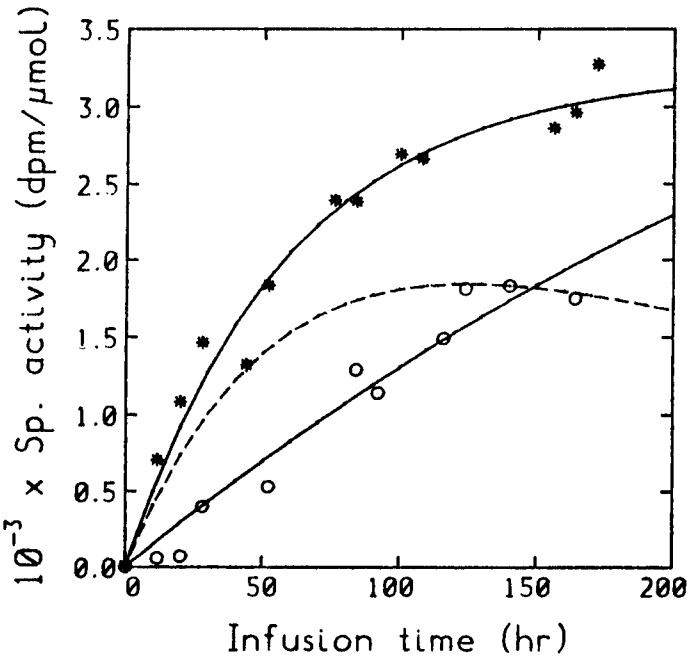
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

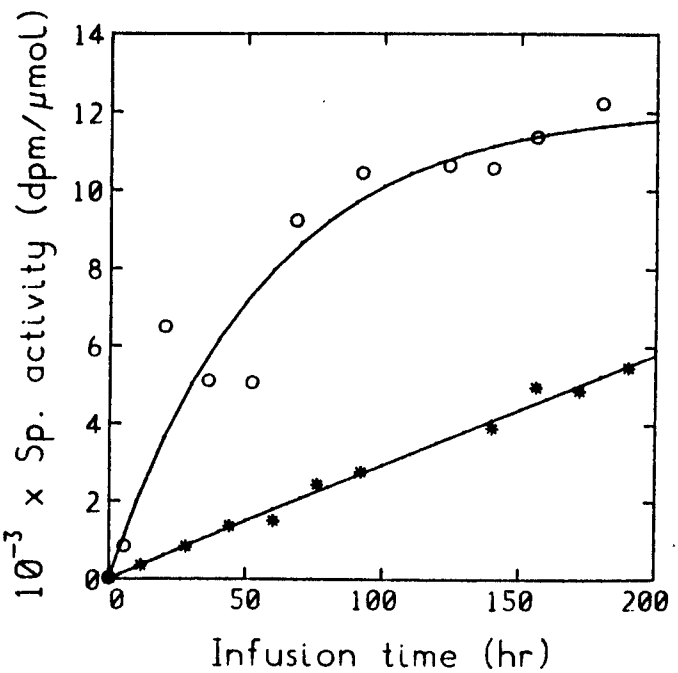
b) (*) Plasma protein

(O) Saliva

a)


10^{-3} x Sp. activity (dpm/μmol)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
Infusion time (hr)

b)


10^{-3} x Sp. activity (dpm/μmol)
14
12
10
8
6
4
2
0
0
50
100
150
200
Infusion time (hr)

2.4 Sheep D

a) (*) Total duodenal digesta

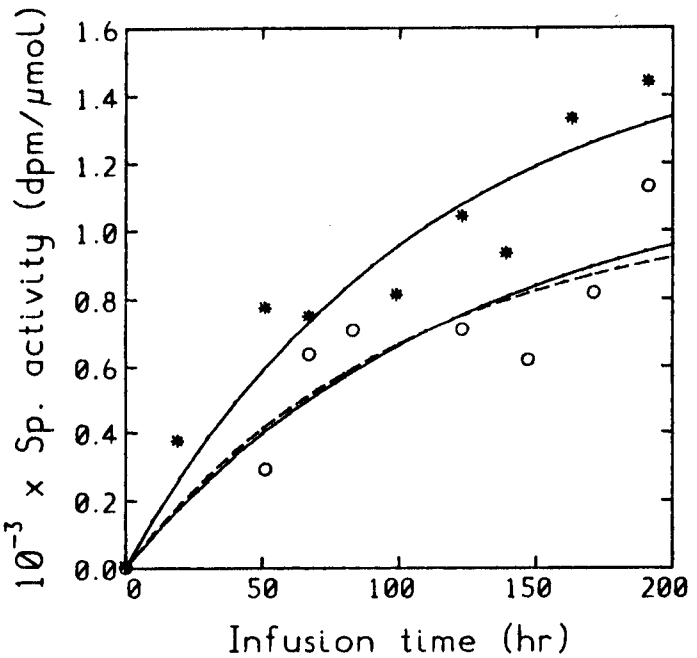
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

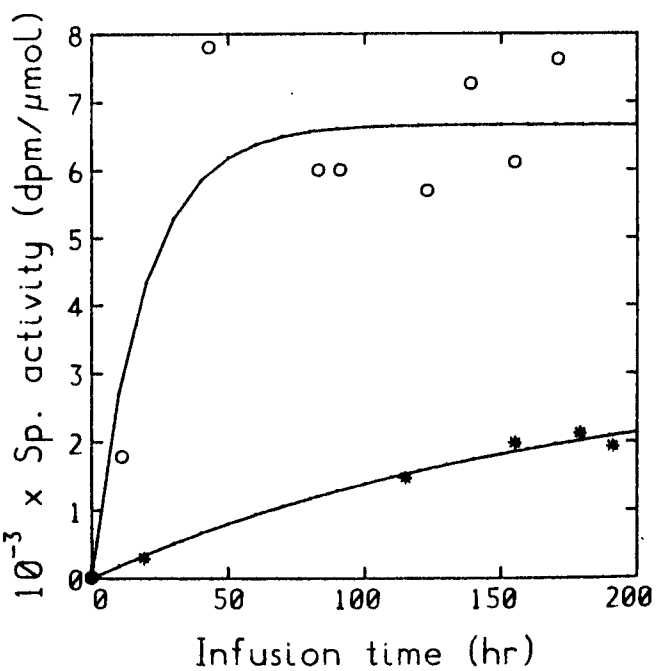
b) (*) Plasma protein

(O) Saliva

a)


10^{-3} x Sp. activity (dpm/μmol)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
Infusion time (hr)

b)


10^{-3} x Sp. activity (dpm/μmol)
8
7
6
5
4
3
2
1
0
0
50
100
150
200
Infusion time (hr)

2.5 Sheep E

a) (*) Total duodenal digesta

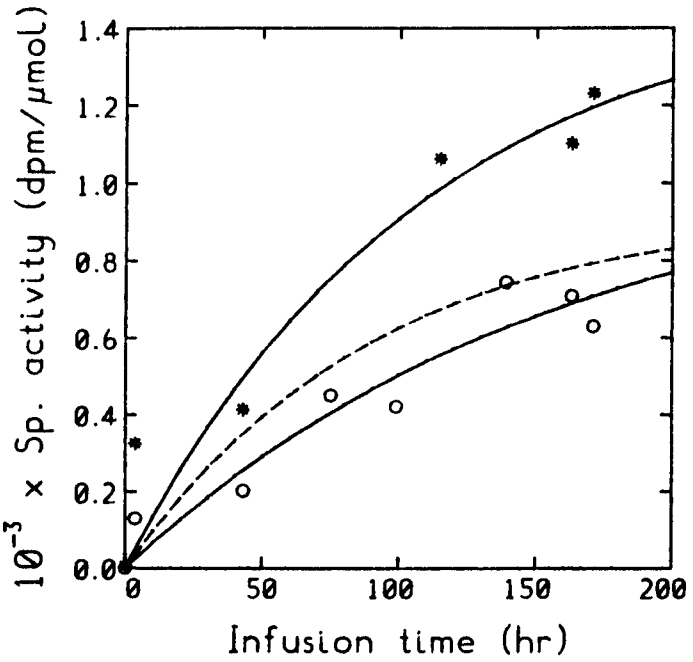
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

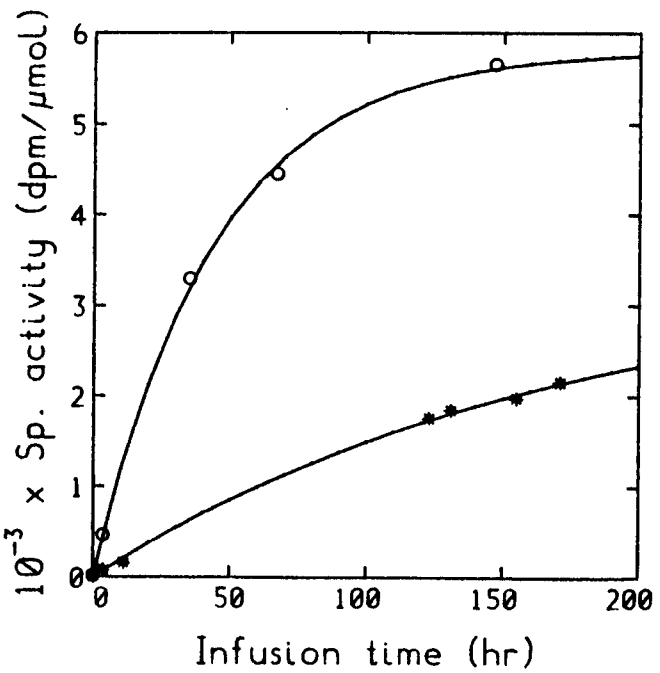
b) (*) Plasma protein

(O) Saliva

a)


10^{-3} x Sp. activity (dpm/μmol)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
Infusion time (hr)

b)


10^{-3} x Sp. activity (dpm/μmol)
6
5
4
3
2
1
0
0
50
100
150
200
Infusion time (hr)

2.6 Sheep F

a) (*) Total duodenal digesta

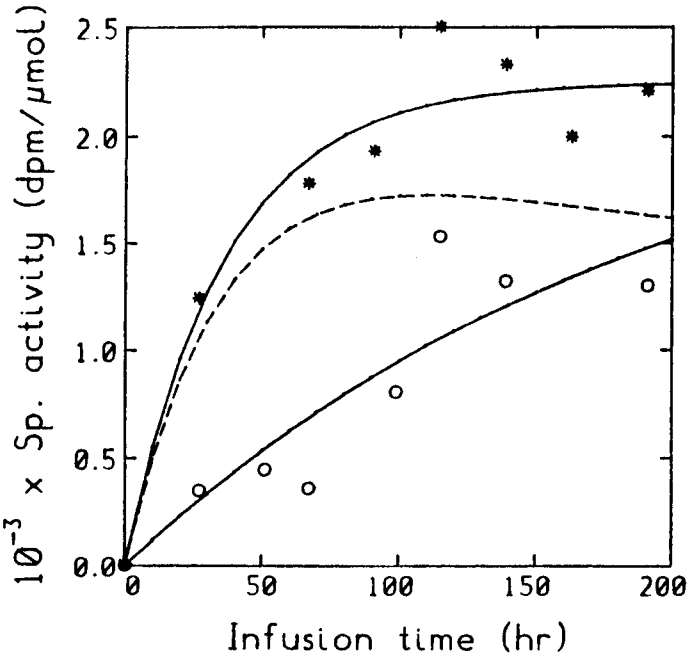
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

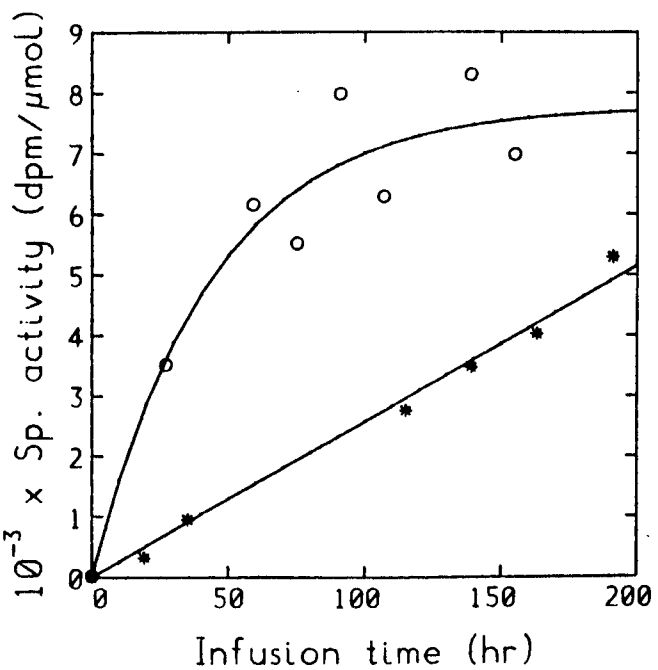
b) (*) Plasma protein

(O) Saliva

a)


10^{-3} x Sp. activity (dpm/μmol)
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
Infusion time (hr)

b)


10^{-3} x Sp. activity (dpm/μmol)
9
8
7
6
5
4
3
2
1
0
0
50
100
150
200
Infusion time (hr)

2.7 Sheep G

a) (*) Total duodenal digesta

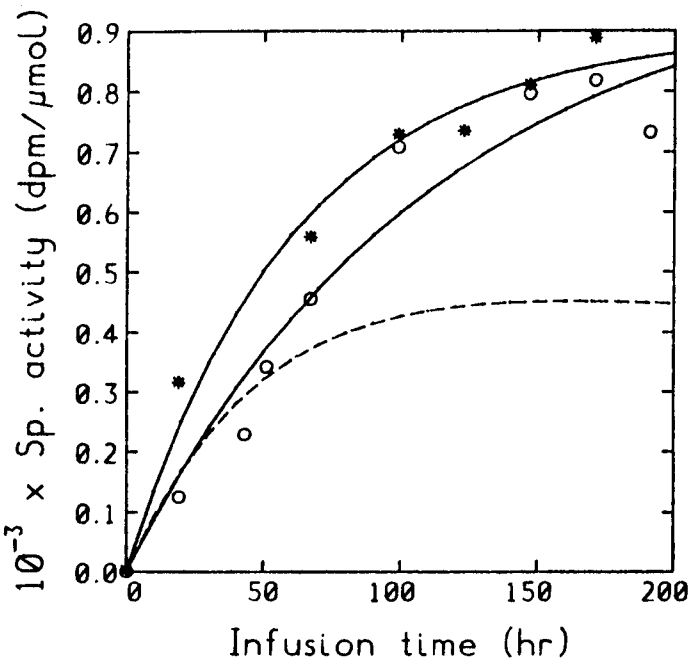
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

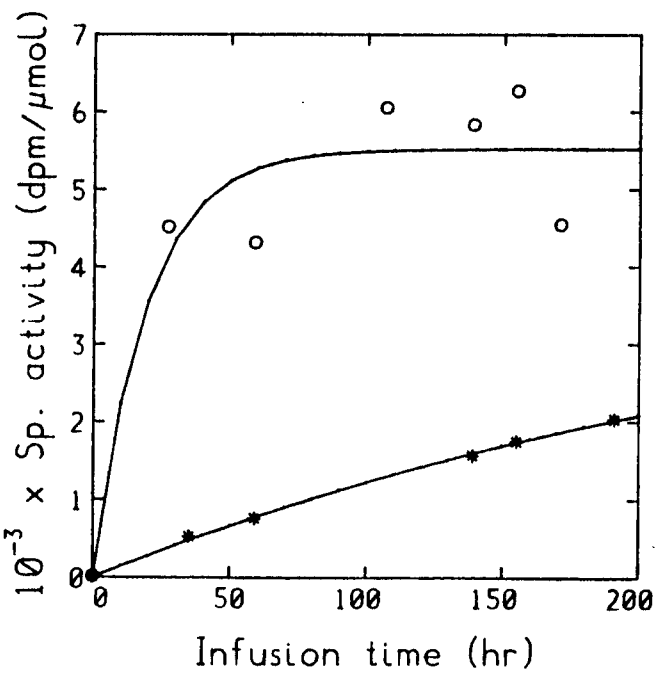
b) (*) Plasma protein

(O) Saliva

a)


10^{-3} x Sp. activity (dpm/μmol)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
50
100
150
200
Infusion time (hr)

b)


10^{-3} x Sp. activity (dpm/μmol)
0
1
2
3
4
5
6
7
0
50
100
150
200
Infusion time (hr)

2.8 Sheep H

a) (*) Total duodenal digesta

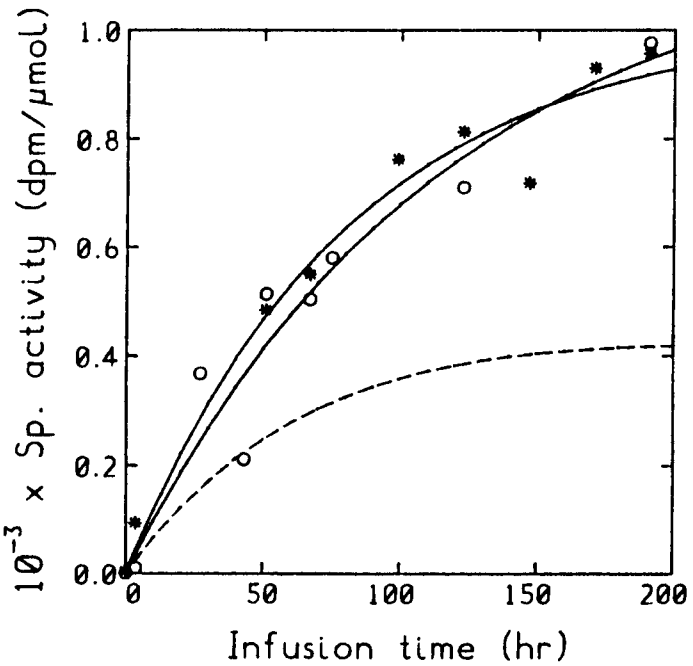
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

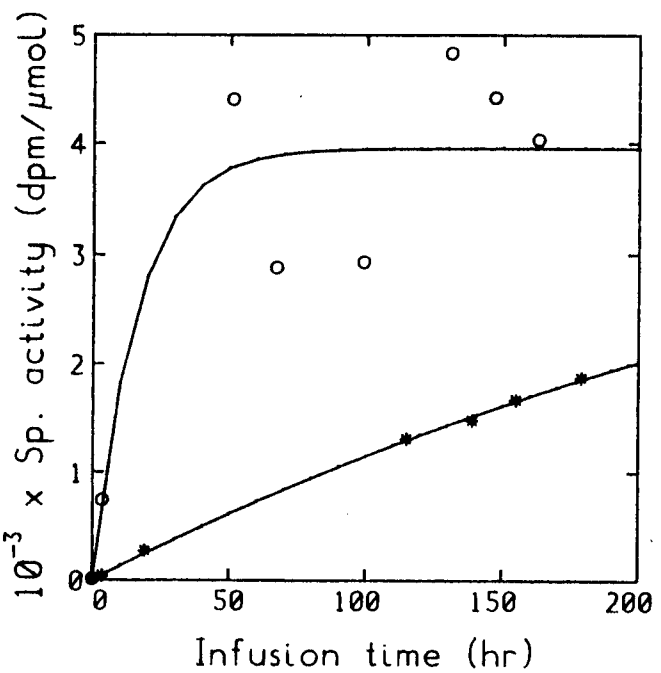
b) (*) Plasma protein

(O) Saliva

a)


1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
10^{-3} x Sp. activity (dpm/μmol)
Infusion time (hr)

b)


5
4
3
2
1
0
0
50
100
150
200
10^{-3} x Sp. activity (dpm/μmol)
Infusion time (hr)

2.9 Sheep K

a) (*) Total duodenal digesta

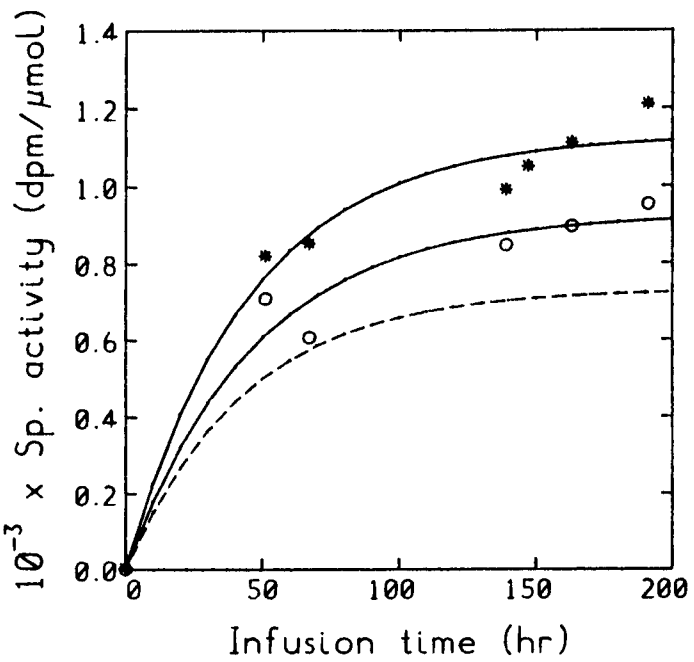
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

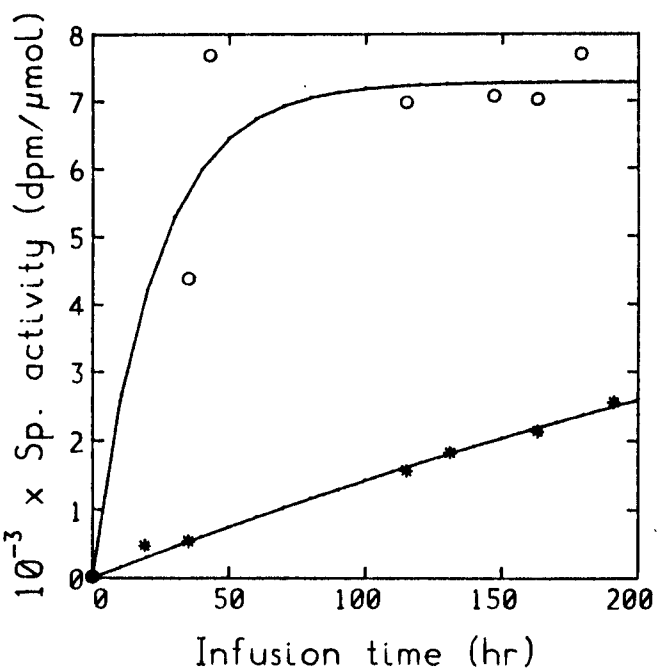
b) (*) Plasma protein

(O) Saliva

a)


10^{-3} x Sp. activity (dpm/μmol)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
Infusion time (hr)

b)


10^{-3} x Sp. activity (dpm/μmol)
8
7
6
5
4
3
2
1
0
0
50
100
150
200
Infusion time (hr)

2.10 Steer M

a) (*) Total duodenal digesta

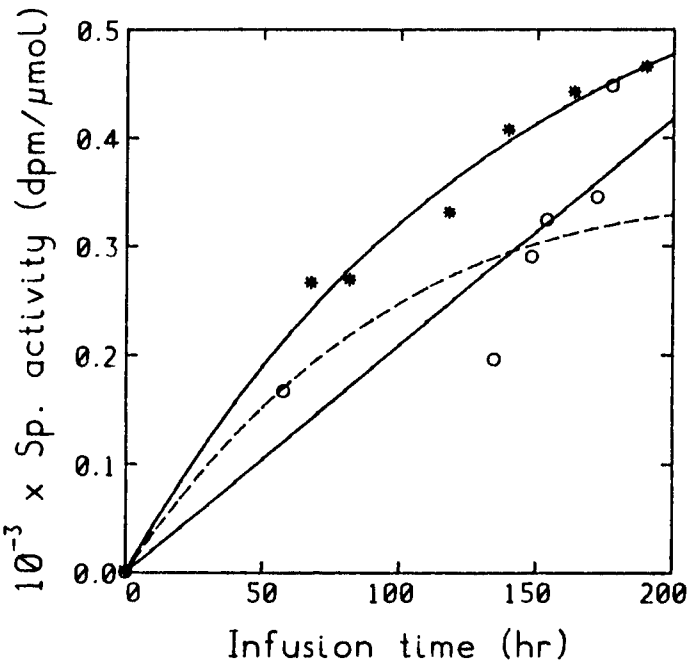
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

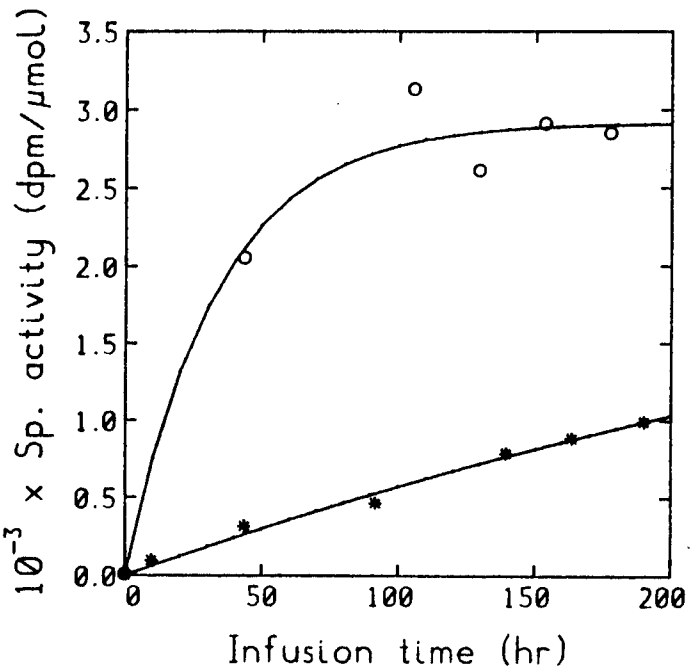
b) (*) Plasma protein

(O) Saliva

a)


0.5
0.4
0.3
0.2
0.1
0.0
10^{-3} x Sp. activity (dpm/μmol)
0
50
100
150
200
Infusion time (hr)

b)


3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
10^{-3} x Sp. activity (dpm/μmol)
0
50
100
150
200
Infusion time (hr)

2.11 Steer P

a) (*) Total duodenal digesta

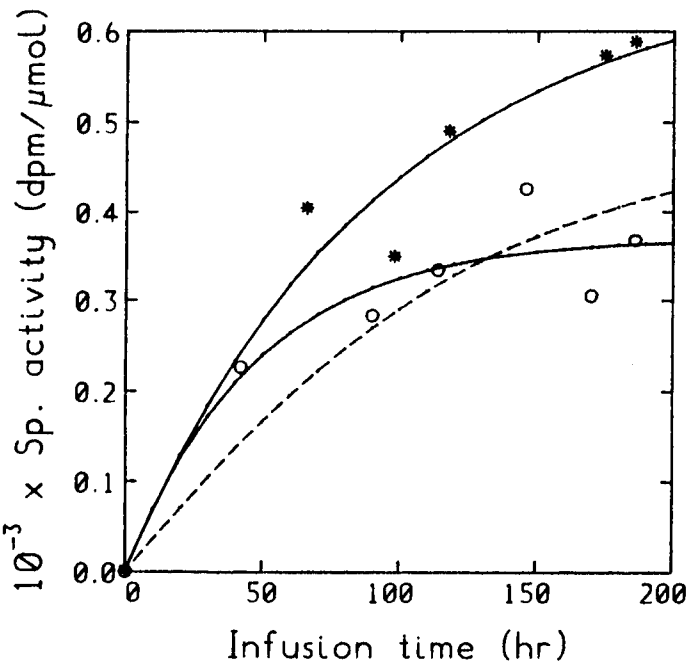
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

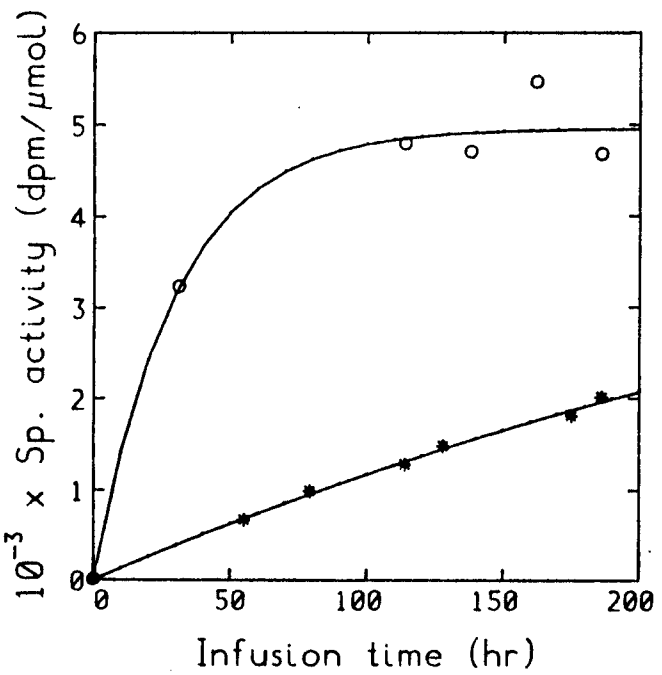
b) (*) Plasma protein

(O) Saliva

a)


10^{-3} x Sp. activity (dpm/μmol)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
50
100
150
200
Infusion time (hr)

b)


10^{-3} x Sp. activity (dpm/μmol)
0
1
2
3
4
5
6
0
50
100
150
200
Infusion time (hr)

2.12 Steer R

a) (*) Total duodenal digesta

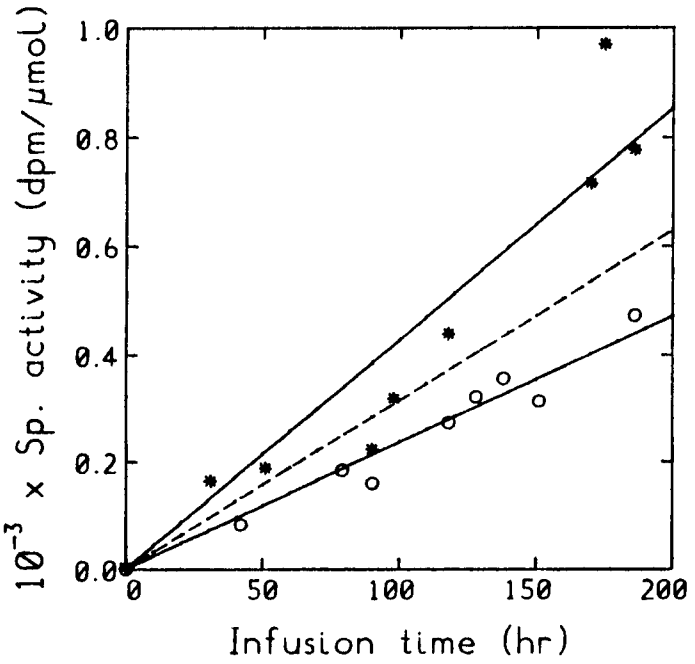
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

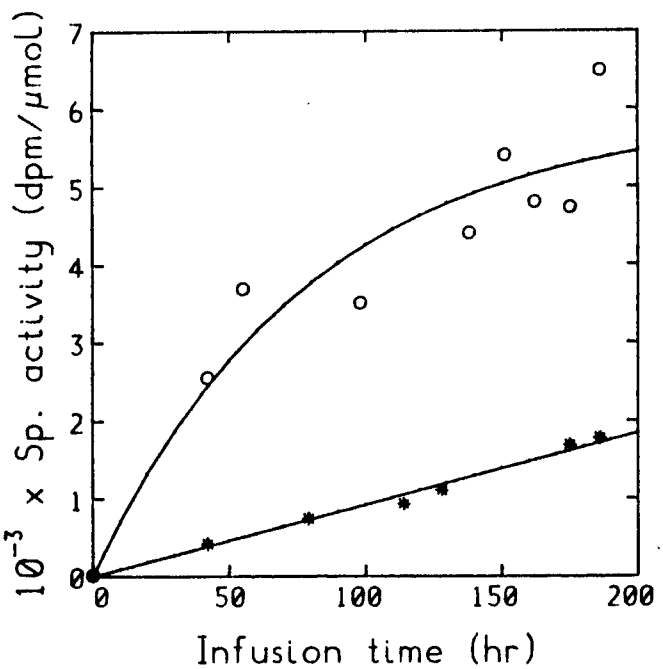
b) (*) Plasma protein

(O) Saliva

a)


1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
10^{-3} x Sp. activity (dpm/μmol)
Infusion time (hr)

b)


7
6
5
4
3
2
1
0
0
50
100
150
200
10^{-3} x Sp. activity (dpm/μmol)
Infusion time (hr)

2.13 Steer T

a) (*) Total duodenal digesta

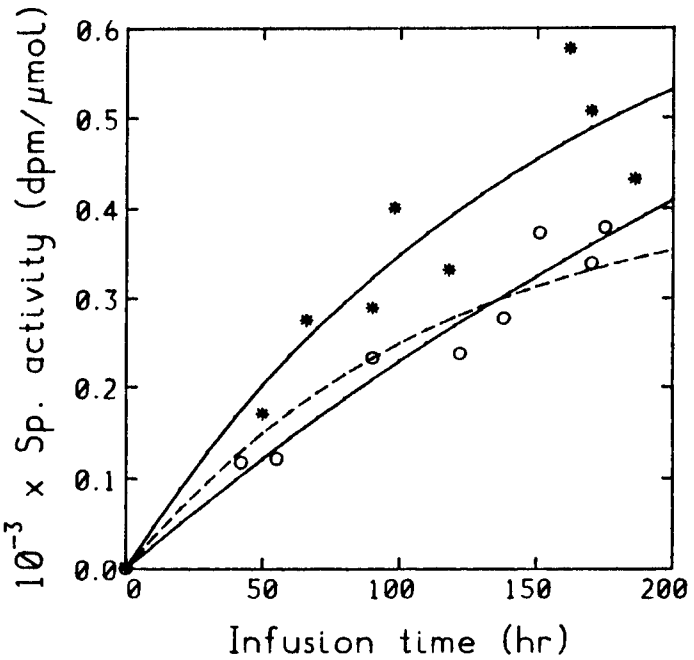
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

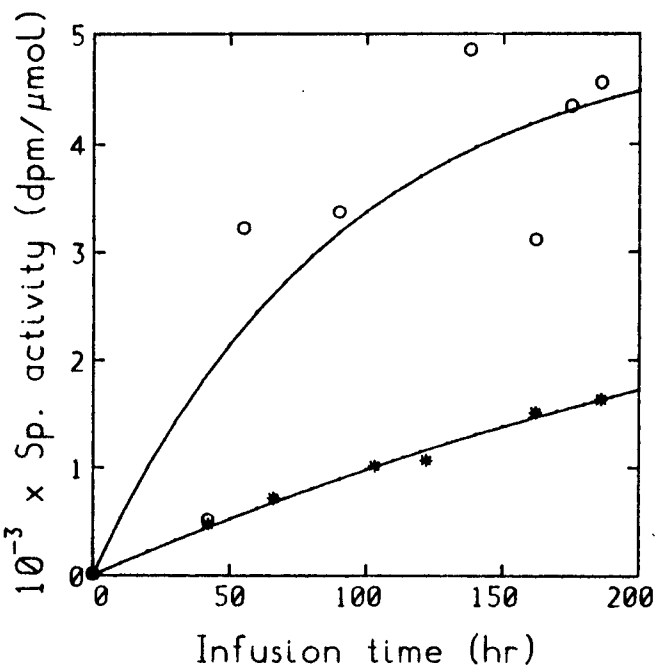
b) (*) Plasma protein

(O) Saliva

a)


10^{-3} x Sp. activity (dpm/μmol)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
50
100
150
200
Infusion time (hr)

b)


10^{-3} x Sp. activity (dpm/μmol)
0
1
2
3
4
5
0
50
100
150
200
Infusion time (hr)

2.14 Steer V

a) (*) Total duodenal digesta

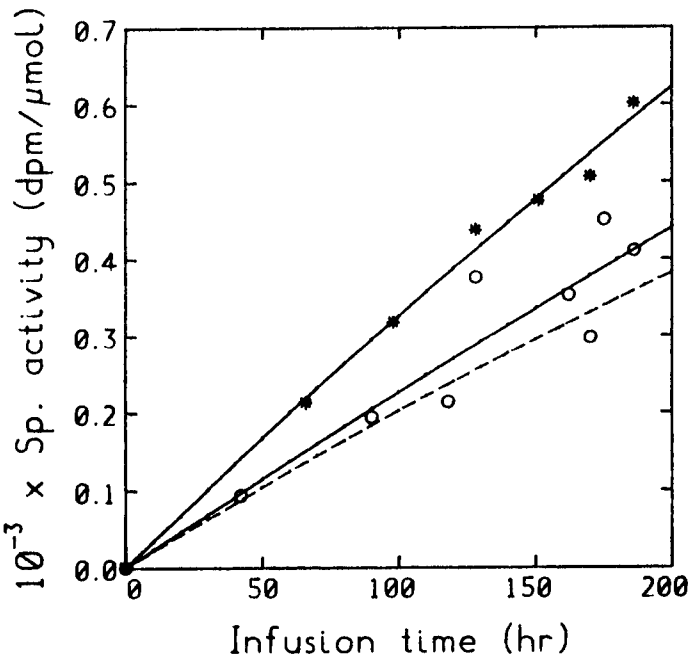
(O) Duodenal microbes

(- - -) Corrected duodenal digesta

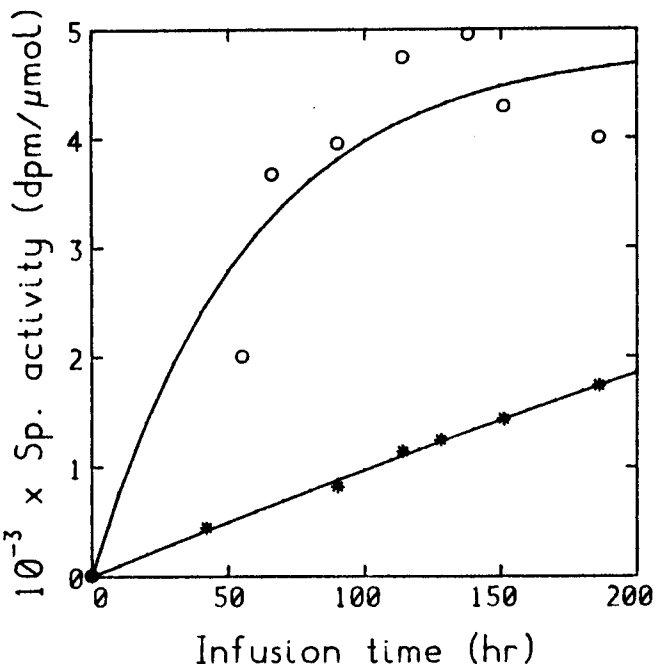
b) (*) Plasma protein

(O) Saliva

a)


10^{-3} x Sp. activity (dpm/μmol)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
50
100
150
200
Infusion time (hr)

b)


10^{-3} x Sp. activity (dpm/μmol)
0
1
2
3
4
5
0
50
100
150
200
Infusion time (hr)

Appendix 3 Parameters relating to the specific activity profile of protein leucine in various fractions during a 200 hr continuous intravenous infusion of L-[4,5-^{3}H]-leucine for all animals studied in the investigation

a) Rate constants (k)

Animal	Rate constant of specific activity profile (/hr)				
	Duodenal digesta	Duodenal microbial protein	Corrected digesta	Saliva	Plasma protein
A	0.00461	0.00302	0.00708	0.02150	0.00085
B	0.01148	0.02846	0.00455	0.01997	0.00342
C	0.01676	0.00269	0.02359	0.01776	0.00037
D	0.00912	0.00804	0.00959	0.05248	0.00617
E	0.00930	0.00628	0.01056	0.02288	0.00575
F	0.02771	0.00500	0.03225	0.02304	0.00002
G	0.01635	0.00905	0.02085	0.05180	0.00349
H	0.01227	0.00865	0.01571	0.06178	0.00283
K	0.02221	0.02119	0.02275	0.04316	0.00207
M	0.00725	0.00004	0.00906	0.02986	0.00211
P	0.01063	0.02080	0.00437	0.03376	0.00271
R	0.00001	0.00002	0.00017	0.01263	0.00003
T	0.00638	0.00233	0.00807	0.01104	0.00268
V	0.00096	0.00053	0.00122	0.01713	0.00103

Appendix 3 (continued)

b) Predicted time of 95% plateau (hr)

Animal	Predicted time of 95% plateau (hr)[+]				
	Duodenal digesta	Duodenal microbial protein	Corrected digesta	Saliva	Plasma protein
A	650	992	423	139	3524
B	261	105	658	150	553
C	179	1114	127	169	8097
D	329	373	312	57	486
E	322	477	284	131	521
F	108	599	93	130	++
G	183	331	144	578	858
H	244	346	191	49	1059
K	135	141	108	69	1447
M	413	++	331	100	1420
P	282	144	686	89	1105
R	++	++	++	237	++
T	470	1286	371	271	1118
V	3121	5652	2456	175	2909

++ Predicted time of 95% plateau $= \frac{\ln 0.05}{-k}$ (hr)

++ Value in excess of 15,000 hrs

Appendix 3 (continued)

c) Plateau specific activity (dpm/μmol) x 10^{-3}

Animal	Plateau specific activity (dpm/μmol) x 10^{-3}				
	Duodenal digesta	Duodenal microbial protein	Corrected digesta	Saliva	Plasma protein
A	5.96	6.72	1.70	10.62	32.4
B	3.27	1.53	4.03	14.67	10.7
C	3.23	5.52	2.00	12.15	81.1
D	1.60	1.20	1.08	6.66	3.01
E	1.50	1.08	0.96	5.81	3.42
F	2.25	2.40	1.83	7.78	1084
G	0.90	1.01	0.49	5.52	4.14
H	1.02	1.17	0.46	3.95	4.67
K	1.13	0.93	0.73	7.28	7.64
M	0.62	48.9	0.42	2.92	3.01
P	0.67	0.37	0.85	4.96	4.95
R	467	96.5	18.3	5.93	325
T	0.74	1.10	0.44	5.04	4.16
V	3.6	4.37	1.77	4.85	9.94

Appendix 4 Estimates of endogenous protein leucine flow (g/day) determined using the [^{3}H]-leucine technique and by difference estimates based on leucine flow data in sheep fed an essentially protein-free diet

	Method				
	Intravenous infusion of [^{3}H]-leucine technique			Difference	
	Saliva	Plasma protein	Interpolated	Maximum	Corrected[+]
Endogenous protein leucine flow (g/day)					
Sheep A	0.46	1.46	0.55	1.13	0.69
B	0.65	2.47	1.92	2.58	2.14
C	0.80	2.40	0.79	1.74	1.30
mean	0.64	2.11	1.09	1.82	1.38
SEM	0.098	0.326	0.422	0.420	0.420

[+] Assumes degradability of ration = 0.77

Appendix 5 The determination of duodenal digesta flow using the dual phase marker technique (Faichney, 1975)

Given that equilibrium has been achieved and is maintained by a continuous infusion of the two digesta flow markers, the concentrations of these markers (expressed as a fraction of the daily dose per unit of digesta) in digesta flowing past any sampling point must be equal.

If sub-samples of digesta are prepared by centrifugation of a sample of digesta so that centrifuged digesta (PR) and fluid rich digesta (FR) contain different proportions of liquid and particle matter, the true digesta (TD) can be reconstituted by combining PR and FR mathematically, so that the concentrations of the two markers are the same.

Therefore, if x = a quantity of dry digesta (FR)

y = a quantity of dry digesta (PR) which when added to or removed from x reconstitutes true digesta (TD)

$Cr_{FR}, Cr_{PR}, Cr_{TD}$ = concentrations of the solute marker (fraction of daily dose per kg DM)

$Yb_{FR}, Yb_{PR}, Yb_{TD}$ = concentrations of the particulate marker (fraction of daily dose per kg DM)

Then $$x.Cr_{FR} + y.Cr_{PR} = x.Yb_{FR} + y.Yb_{PR}$$

and $$\frac{x.Yb_{FR} + y.Yb_{PR}}{x.Cr_{FR} + y.Cr_{PR}} = 1$$

$$\frac{y}{x} = \frac{Yb_{FR} - Cr_{FR}}{Cr_{PR} - Yb_{PR}} = R \qquad (1)$$

where R is the reconstitution factor, i.e. the number of units of PR that must be added to (or removed from) one unit of FR to obtain TD.

$$\text{Then } Yb_{TD} = \frac{Yb_{FR} + R.Yb_{PR}}{1 + R} \quad (2)$$

$$\text{And similarly } Cr_{TD} = \frac{Cr_{FR} + R.Cr_{PR}}{1 + R} \quad (2)$$

$$\text{DM flow of TD (kg/day)} = \frac{1}{Yb_{TD}} \text{ or } \frac{1}{Cr_{TD}} \quad (3)$$

The flow of any constituent of true digesta can be calculated by substituting its concentration for that of the marker in equation 2 and multiplying by the flow calculated in equation 3.

Worked example

Marker concentration (fraction of daily dose, per kg DM)

	FR	PR
Yb	0.427	0.505
Cr	0.472	0.223

(Original raw data was corrected for 5% chromium absorption. This was based on observations in this laboratory which supported those in the literature, see Weston and Hogan, 1967).

$$R = \frac{0.427 - 0.472}{0.223 - 0.505} = 0.158$$

$$Yb_{TD} = \frac{0.427 + (0.158 \times 0.505)}{1 + 0.158}$$

$$= 0.437$$

$$\text{Dm flow (kg/day)} = \frac{1}{Yb_{TD}} = \frac{1}{0.437} = 2.286 \text{ kg/day}$$

Appendix 6 Duodenal digesta protein nitrogen flow (g/day) and bacterial nitrogen flow (g/day) in sheep fed either concentrates or hay

	DIET					
	Concentrate			Hay		
	Sheep D	Sheep E	Sheep F	Sheep G	Sheep H	Sheep K
Duodenal digesta dry matter flow (g/day)	306	302	191	539	621	717
Duodenal digesta protein nitrogen (g/100 g DM) Mean ± SEM, n = 4	2.83 0.173	3.51 0.333	2.69 0.323	2.53 0.102	2.50 0.276	3.13 0.106
Duodenal digesta protein nitrogen flow (g/day)	8.66	10.60	5.14	13.64	15.53	22.44
Duodenal digesta DAPA (g/100 g DM) Mean ± SEM, n = 5	0.172 0.0171	0.118 0.0196	0.043 0.0072	0.087 0.0036	0.058 0.0050	0.067 0.0030
Rumen bacteria DAPA (g/100 g DM) Mean ± SEM, n = 6	0.556 0.0548	0.353 0.0400	0.191 0.0104	0.370 0.0084	0.278 0.0286	0.335 0.0318
Rumen bacteria nitrogen (g/100 g DM) Mean ± SEM, n = 6	7.28 0.588	7.87 0.341	7.65 0.384	7.85 0.221	8.38 0.333	7.53 0.258
DAPA flow (g/day)	0.526	0.356	0.082	0.469	0.360	0.480
$\frac{\text{Rumen bacteria nitrogen (g/100 g DM)}}{\text{Rumen bacteria DAPA (g/100 g DM)}}$	13.1	22.3	40.1	21.2	30.1	32.5
Bacterial nitrogen flow (g/day)	6.89	7.95	3.28	9.95	10.85	10.80
Proportion of duodenal digesta of microbial origin	0.79	0.76	0.65	0.74	0.70	0.48

Appendix 7 The flow of duodenal digesta leucine (mmol/day) and duodenal bacterial leucine (mmol/day) in sheep fed either concentrates or hay

	DIET					
	Concentrate			Hay		
	Sheep D	Sheep E	Sheep F	Sheep G	Sheep H	Sheep K
Duodenal digesta dry matter flow (g/day)	306	302	191	539	621	717
Duodenal digesta leucine (g/100 g DM) Mean ± SEM, n = 7	1.52 0.059	1.71 0.108	1.37 0.097	1.39 0.066	1.26 0.041	1.43 0.046
Duodenal digesta leucine flow (g/day)	4.65	5.16	2.62	7.49	7.83	10.25
Duodenal digesta leucine flow (mmol/day)	35.44	39.33	19.97	57.09	59.68	78.13
Duodenal bacterial protein leucine (g/100 g DM) Mean ± SEM, n = 7	2.15 0.126	2.90 0.077	2.50 0.189	2.91 0.085	3.17 0.207	3.06 0.055
Rumen bacterial nitrogen (g/100 g DM) Mean ± SEM, n = 6	7.28 0.588	7.87 0.341	7.65 0.384	7.85 0.221	8.38 0.333	7.53 0.258
Bacterial nitrogen flow (g/day)	6.89	7.95	3.28	9.95	10.85	10.80
Bacterial dry matter flow (g/day)	94.6	101.0	43.0	127.0	130.8	143.3
Duodenal bacterial leucine flow (g/day)	2.04	2.93	1.08	3.69	4.15	4.39
Duodenal bacterial leucine flow (mmol/day)	15.51	22.33	8.20	28.11	31.60	33.42

Appendix 8 Estimates of endogenous protein leucine flow (g/day) determined using the L-[4,5-^{3}H]-leucine technique and the theoretical maximum value in sheep fed either concentrates or hay

Diet	Sheep	Endogenous protein leucine flow (g/day) [^{3}H]-leucine technique			
		Saliva	Plasma protein	Interpo-lated	Theoretical maximum*
Concentrate					
	D	0.464	2.17	1.48	2.61
	E	0.630	2.07	1.20	2.23
	F	0.638	1.51	0.638	1.54
	Mean	0.577	1.92	1.11	2.13
	SEM	0.0567	0.205	0.248	0.313
Hay					
	G	0.549	2.36	0.745	3.80
	H	0.672	2.21	0.889	3.68
	K	0.915	4.26	1.06	5.86
	Mean	0.712	2.94	0.898	4.45
	SEM	0.1075	0.660	0.0910	0.708

* Total protein leucine flow - bacterial leucine flow = Endogenous protein leucine + undegraded feed leucine

Appendix 9 Duodenal digesta dry matter flow (g/day), organic matter flow (g/day), digestion of organic matter in the rumen, bacterial nitrogen flow (g/day) and the efficiency of bacterial nitrogen synthesis in sheep fed either concentrates or hay

	DIET					
	Concentrate			Hay		
	Sheep D	Sheep E	Sheep F	Sheep G	Sheep H	Sheep K
Dry matter intake (g/day)	627	627	627	846	946	931
Organic matter intake (g/day)	595	595	595	802	897	880
Dry matter flow (g/day)	306	302	191	539	621	717
Organic matter flow (g/day)	257	255	148	432	511	635
Organic matter apparently fermented in the rumen (g/day)	338	340	447	370	386	245
Bacterial nitrogen flow (g/day)	6.89	7.95	3.28	9.95	10.85	10.80
Bacterial organic matter flow (g/day)*	59	68	28	86	93	92
Proportion of organic matter intake apparently digested in the rumen	0.568	0.571	0.751	0.461	0.430	0.278
Proportion of organic matter intake truly digested in the rumen	0.667	0.686	0.797	0.568	0.534	0.383
Efficiency of bacterial nitrogen synthesis (g N/kg OMADR)	20.4	23.4	7.3	26.9	28.1	44.1
Efficiency of bacterial nitrogen synthesis (g N/kg OMTDR)	17.4	19.5	6.9	21.8	22.7	32.1

* 117 g bacterial NAN per kg bacterial organic matter (Lindsay and Hogan, 1972)

Appendix 10 Estimates of endogenous protein nitrogen flow (g/day) determined using the [^{3}H]-leucine technique and by difference estimates based on nitrogen flow data in sheep fed an essentially protein-free diet

	Method				
	Intravenous infusion of [^{3}H]-leucine technique[+]			Difference	
	Saliva	Plasma protein	Interpolated	Maximum	Corrected[++]
Endogenous protein nitrogen flow (g/day)					
Sheep A	1.09	3.42	1.28	1.27	0.57
B	1.54	5.80	4.52	2.58	1.88
C	1.88	5.65	1.86	4.43	3.73
mean	1.50	4.96	2.55	2.76	2.06
SEM	0.228	0.770	0.998	0.917	0.917

[+] Assumes endogenous protein = 6.8% leucine and protein = N x 6.25

[++] Assumes degradability of ration = 0.77