OBSERVATIONS ON THE RUMINAL PROTEIN DEGRADATION PRODUCTS AND THE ABSORPTION OF RUMINALLY DERIVED FREE AND PEPTIDE-BOUND AMINO ACIDS VIA OVINE FORESTOMACH EPITHELIA IN VITRO.

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(ABSTRACT)

Production of ammonia N, α-amino N, and peptide N was investigated following in vitro ruminal incubation of solvent soybean meal (SBM), dehydrated alfalfa, corn gluten feed, fish meal, distillers dried grains with solubles (DDG), cotton seed meal, brewers fried grains, meat and bone meal, blood meal, prolac, and casein (CAS). The influence of milling procedures on the production of ammonia N, α-amino N, and peptide N was also evaluated using different batches of soybean meals and distillers dried grains with solubles. The concentrations of peptide N and ammonia N measured in the cell free media at 0, 2, 4, 6, and 8 h were increased linearly ($P < 0.001$) with time. The mean concentrations of α-amino N were lower ($P < 0.05$) than the mean concentrations of peptide N and ammonia N. Production of peptide N, α-amino N, and ammonia N were varied ($P < 0.05$, time *x* protein) between proteins and between batches. Irrespective of the protein used, the amino acid composition of peptides (<3,000 MW) that appeared at 8 h had specific patterns suggesting differential utilization of peptides by ruminal microorganisms. Cell-free supernatants obtained following incubation (8 h) of SBM, CAS, and DDG were used as mucosal substrates in parabiotic chambers to quantify absorption of free and peptide-bound amino acids via ruminal and omasal epithelia of sheep. Serosal appearance of amino acids in peptide form was nearly three times higher ($P < 0.001$) than free amino acids. On tissue dry weight basis, serosal appearance of amino acids was greater ($P < 0.01$) across omasal than via ruminal tissues. There was a greater serosal appearance of amino acids from CAS than from SBM. Total, total essential (EAA), total nonessential (NEAA), and individual amino acid appearance in serosal fluids varied ($P < 0.05$, amino acid form *x* protein source) among SBM, CAS, and DDG. Collectively, these results indicate that the forestomach epithelia of sheep possess the potential to absorb ruminally derived peptides (relatively large amounts) and free amino acids (relatively small amounts). Also, the ruminal microbial degradation of

dietary proteins may influence the amounts and types of free and peptide-bound amino acids absorbed via forestomach.
(**Key words:** Ammonia, Amino acid, Peptide, Protein, Rumen, Omasum, Microorganisms)

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TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENTS iv

LIST OF TABLES viii

LIST OF FIGURES xi

CHAPTER I. INTRODUCTION 1

CHAPTER II. REVIEW OF LITERATURE 3

THE SIGNIFICANCE OF THE RUMINANT FORESTOMACH 3

PROTEIN METABOLISM IN RUMINANTS: CLASSICAL CONCEPTS AND NEW PERSPECTIVES 7

Dietary Protein Degradation in the Reticulorumen 7

Factors Influencing Microbial Protein Breakdown 12

Methods to Predict Ruminal Protein Degradation 14

Accumulation of Products Following Digestion of Proteins in the Rumen 16

Mechanisms of Peptide Accumulation in the Forestomach 21

Alternative Sources of Peptides and Amino Acids Added to the Ruminal Digesta 24

Duodenal Flow of Ruminal Protein Degradation Products 26

ABSORPTION OF PROTEIN DIGESTION PRODUCTS ACROSS THE RUMINANT FORESTOMACH 26

Ammonia Absorption 26

Amino Acid Transport 27

Evidence on Intact Peptide Absorption 30

Mechanisms of Peptide Absorption 30

Nutritional Importance of Peptide Transport 31

A DESCRIPTION ON PROTEIN INGREDIENTS USED IN THIS STUDY 33

CHAPTER III. OBJECTIVES 37

CHAPTER IV. OBSERVATIONS ON RUMINAL PROTEIN 38

DEGRADATION PRODUCTS FOLLOWING IN VITRO INCUBATION WITH MIXED MICROORGANISMS
ABSTRACT 38
INTRODUCTION 39
MATERIALS AND METHODS 39
Preparation of Inoculant Enriched with Mixed Ruminal Microorganisms 39
In vitro Incubation of Dietary Proteins 40
Chemical Analysis 42
Statistical Analysis 42
RESULTS 43
Changes in Ammonia N Concentration with Time 43
Changes in α-Amino N Concentration with Time 44
Changes in Peptide N Concentration with Time 45
The Amino Acid Composition of Ruminal Protein Degradation Products 46
DISCUSSION 48
IMPLICATIONS 55
CHAPTER V. ABSORPTION OF RUMINALLY DERIVED PEPTIDES AND FREE AMINO ACIDS VIA ISOLATED OVINE RUMINAL AND OMASAL EPITHELIA 68
ABSTRACT 68
INTRODUCTION 69
MATERIALS AND METHODS 69
In vitro Incubation of Dietary Proteins 70
Animals, Feeding and Tissue Collection 70
Buffer Preparation 71
Uptake Measurement 71
Statistical Analysis 72
RESULTS AND DISCUSSION 73

Mucosal Concentrations 73
Serosal Appearances 74
IMPLICATIONS 79
CHAPTER VI. EPILOGUE 83
LITERATURE CITED 87
APPENDIX-A
Preparation of Mixed Ruminal Microbial Culture for Incubation Experiments 111
APPENDIX-B
Procedure for Amino Acid Uptake Experiment Using Parabiotic Chambers 114
APPENDIX-C
Statistical Analysis Examples 118
VITA 125

LIST OF TABLES

Table

2.1. RUMINAL DEGRADABILITY ESTIMATES OF SOME SELECTED FEED PROTEINS 9

4.1. COMPOSITION OF THE BUFFER AND THE NUTRIENT MEDIUM USED FOR INCUBATION EXPERIMENTS 61

4.2. CRUDE PROTEIN % OF THE PROTEIN SOURCES USED IN THE INCUBATION EXPERIMENTS 62

4.3 FREE AMINO ACID, PEPTIDE (TOTAL) AMINO ACID, AND PEPTIDE (< 3,000 MW) AMINO ACID CONCENTRATIONS IN EXTRACELLULAR MEDIA FOLLOWING IN VITRO RUMINAL INCUBATION (8H) OF DEHYDRATED ALFALFA (DA), SOYBEAN MEAL (SBM), CORN GLUTEN FEED (CGF), FISH MEAL (FM) AND DISTILLER DRIED GRAIN WITH SOLUBLES (DDG; EXPERIMENT 1) 63

4.4. FREE AMINO ACID, PEPTIDE (TOTAL) AMINO ACID, AND PEPTIDE (< 3,000 MW) AMINO ACID CONCENTRATIONS IN EXTRACELLULAR MEDIA FOLLOWING IN VITRO RUMINAL INCUBATION (8H) OF COTTON SEED MEAL (CSM), BREWERS DRIED GRAINS (BDG), PROLAC (PRL), BLOOD MEAL (BLM) AND MEAT AND BONE MEAL (MBM; EXPERIMENT 2) 64

4.5. FREE AMINO ACID, PEPTIDE (TOTAL) AMINO ACID, AND PEPTIDE (< 3,000 MW) AMINO ACID CONCENTRATIONS IN EXTRACELLULAR MEDIA FOLLOWING IN VITRO RUMINAL INCUBATION (8H) OF EXPELLER SOYBEAN MEAL (ESB) AND DIFFERENT BATCHES OF SOLVENT SOYBEAN MEALS 65

(SSR1, SSR2, SSB AND SSS; EXPERIMENT 3)

4.6. FREE AMINO ACID, PEPTIDE (TOTAL) AMINO ACID, AND PEPTIDE (< 3,000 MW) AMINO ACID CONCENTRATIONS IN EXTRACELLULAR MEDIA FOLLOWING IN VITRO RUMINAL INCUBATION (8H) OF DIFFERENT BATCHES OF DISTILLERS DRIED GRAINS WITH SOLUBLES (DGR1, DGR2, DGR3, DGB AND DGS; EXPERIMENT 4) 66

4.7. CONCENTRATIONS OF FREE AMINO ACIDS, PEPTIDE (TOTAL) AMINO ACIDS, AND PEPTIDE (< 3,000 MW) AMINO ACIDS PERSISTED IN THE EXTRACELLULAR MEDIA FOLLOWING IN VITRO RUMINAL INCUBATION CASEIN (EXPERIMENT 5) 67

5.1. THE INITIAL CONCENTRATIONS OF FREE, PEPTIDE (TOTAL) AND PEPTIDE (< 3,000 MW) AMINO ACIDS AMONG MUCOSAL SUBSTRATES PREPARED FROM SOYBEAN MEAL (SBM), CASEIN (CAS), AND DISTILLERS DRIED GRAINS WITH SOLUBLES (DDG) 80

5.2. MAIN EFFECT MEANS OF PROTEIN SOURCE, AMINO ACID FORM, AND TISSUE TYPE ON SEROSAL APPEARANCE OF AMINO ACIDS FOLLOWING 240 MIN OF INCUBATION WITH SUBSTRATES PREPARED FROM SOYBEAN MEAL (SBM), CASEIN (CAS), AND DISTILLERS DRIED GRAINS WITH SOLUBLES (DDG) 81

5.3. SEROSAL APPEARANCE OF FREE, PEPTIDE (TOTAL), AND PEPTIDE (< 3,000 MW) AMINO ACIDS VIA RUMINAL AND OMASAL EPITHELIA INCUBATED FOR 240 MIN WITH SUBSTRATES PREPARED FROM SOYBEAN MEAL (SBM), CASEIN, AND DISTILLERS DRIED GRAINS WITH SOLUBLES (DDG) 82

LIST OF FIGURES

Figure

2.1. THE DIGESTIVE SYSTEM OF A GOAT SHOWING (A) ESOPHAGUS, (B) RETICULUM, (C) RUMEN, (D) OMASUM, (E) ABOMASUM, (F) SMALL INTESTINE, AND (G) LARGE INTESTINE 6

2.2. MAJOR PATHWAYS OF N METABOLISM IN THE RUMEN 8

2.3. A SCHEMATIC REPRESENTATION OF THE PROTEIN UTILIZATION BY RUMINAL BACTERIA 22

4.1. CHANGES IN CONCENTRATION (MG/L) OF (A) AMMONIA N (B) α-AMINO N, AND (C) PEPTIDE N IN THE EXTRACELLULAR MEDIA DURING IN VITRO RUMINAL INCUBATION OF SOYBEAN MEAL (SBM), FISH MEAL (FM), DEHYDRATED ALFALFA (DA), DISTILLERS DRIED GRAINS WITH SOLUBLE MEAL (DDG), AND CORN GLUTEN FEED (CGF; EXPERIMENT 1) 56

4.2. CHANGES IN CONCENTRATION (MG/L) OF (A) AMMONIA N, (B) α-AMINO N, AND (C) PEPTIDE N IN THE EXTRACELLULAR MEDIUM DURING IN VITRO RUMINAL INCUBATION OF COTTON SEED MEAL (CSM), BREWERS DRIED GRAINS (BDG), PROLAC (PRL), BLOOD MEAL (BLM), AND MEAT AND BONE MEAL (MBM; EXPERIMENT 2) 57

4.3. CHANGES IN CONCENTRATION (MG/L) OF (A) AMMONIA N, (B) α-AMINO N, AND (C) PEPTIDE N IN THE EXTRACELLULAR MEDIUM DURING IN VITRO RUMINAL INCUBATION OF EXPELLER EXTRACTED SOYBEAN MEAL (ESB) AND DIFFERENT BATCHES OF SOLVENT EXTRACTED SOYBEAN MEALS (SSR1, SSR2, SSB AND SSS; EXPERIMENT 3) 58

4.4. CHANGES IN CONCENTRATION (MG/L) OF (A) AMMONIA N, (B) α-AMINO N, AND (C) PEPTIDE N IN THE EXTRACELLULAR MEDIUM DURING IN VITRO RUMINAL INCUBATION OF DIFFERENT BATCHES OF DISTILLERS DRIED GRAINS WITH SOLUBLE MEALS (DGR1, DGR2, DGR3, DGB AND DGS; EXPERIMENT 4) 59

4.5. CHANGES IN THE CONCENTRATIONS (MG/L) OF AMMONIA N, α-AMINO AMINO N AND PEPTIDE N IN THE EXTRACELLULAR MEDIUM DURING IN VITRO RUMINAL INCUBATION OF CASEIN (EXPERIMENT 5) 60

Chapter 1

INTRODUCTION

The complicated gut anatomy, coupled with the massive intervention of microorganisms in the digestive process, have largely delayed the complete understanding of amino acid nutrition in ruminants. Sequential breakdown of dietary proteins into peptides, amino acids and ammonia due to the microbial activity in the rumen was recognized from very early studies on ruminant protein metabolism (Annison, 1956). It is generally assumed that this process of microbial protein degradation proceeds very rapidly until ammonia is formed, hence an accumulation of intermediates (peptides and amino acids) does not occur to any significant level in the rumen. Thus, ruminant nutritionists frequently discuss the fate of dietary protein N in terms of its conversion to ammonia, incorporation into microbial proteins, postruminal digestion of microbial and undegraded dietary proteins and subsequent absorption of amino acids in the intestine (NRC, 1985).

The existence of an alternative mode of amino acid absorption in ruminants has been strongly demonstrated through continuous efforts of this laboratory. Peptides as a major form of amino acid absorption in ruminants was suggested when they constituted a high proportion (79%) of the total amino acids appearing in the portal circulation of steers (Koeln et al., 1993). The forestomach as a major site of peptide absorption in ruminants was hypothesized when a large net flux (approximately 77%) of peptide-bound amino acids was observed in the plasma of non-mesenteric drained viscera of both wethers and steers (Webb et al., 1993). Using different techniques, the ability of ruminal and omasal epithelia to absorb both free and peptide amino acids (Matthews and Webb, 1995), and some understanding on the specific mechanisms involved in this process was also revealed (Matthews, et al., 1996b; Mc Collum, 1996; Pan et al., 1997). However, the information on the magnitude and nutritional significance of peptide and amino acid absorption across the ruminant forestomach is not very well understood.

If peptides and amino acids are to be absorbed from the forestomach, they must be present in the ruminal digesta. The measurement of peptides in the ruminal fluid has

not been of wide interest until recently. Early observations of very low ruminal concentrations of free amino acids (Annison, 1956), and the small contribution of free amino acids absorbed by the rumen (Leibholz, 1971a) are the basis for the belief that amino acid absorption from the rumen is not important. However, an accumulation of peptides (Chen et al., 1987a) and amino acids (Leibholz, 1969) in the ruminal fluid following feeding of protein diets have been reported. Accumulation of specific peptides due to the resistance to ruminal microbial degradation was also revealed (Chen et al., 1987c; Wallace et al., 1990a). However, the amounts and precise patterns of accumulation of peptides and amino acids appears to vary with different studies. A combination of analytical and animal variations could partly be responsible for the above. But the differences in diet appear to play a major role on the accumulation of these protein degradation products in the rumen.

Chapter II

REVIEW OF LITERATURE

The Significance of the Ruminant Forestomach

The presence of a complex stomach in ruminants marks one of higher stages of evolutionary development in mammals. The ruminant stomach consists of four major compartments (rumen, reticulum, omasum, and abomasum) that have similar embryonic origins as the stomach of nonruminants (Figure 2.1). However, the last compartment (abomasum) is considered the only structure that is analogous anatomically and functionally to the glandular stomach of monogastric species. The rumen, reticulum, and omasum are assumed to be outgrowths of the conventional mammalian stomach, and are collectively known as the 'forestomach' in ruminants.

The ruminant forestomach possesses a variety of functional properties; to serve as an organ of storage and delayed passage of ingested feed can be considered one of its basic functions. The large ruminal capacity and the longer retention time of feeds in the forestomach are two main attributes to achieve this activity. Reticulorumen volumes of around 60 to 100 L are quite common in cattle (Church, 1960), and the contents of the forestomach can account for approximately 15 to 20% of the total body weight of ruminants (Giesecke and VanGylswyk, 1975). The mean retention time of fluid in cattle, sheep, and goats fed forage diets is approximately 10h. The particles are retained for a longer time which varies among species, and is approximately 28h for cattle and 20h for sheep and goats (Lechner et al., 1991). Due to large ruminal capacity and a selective retention of particles within the rumen, ruminants are capable of retaining feeds for a longer time in their forestomach to facilitate rumination, fermentation, and absorption of nutrients.

The forestomach harbors a diverse microbial population of bacteria, protozoa, and fungi (Orpin and Joblin, 1988). The bacteria are the dominant microbial group in the rumen (approximately 10^{10}/mL) and are absolutely essential for the normal ruminal function. The numbers of protozoa are much less (approximately 10^{6}/mL). But protozoa can account for around 40 to 60% of the total microbial mass in the rumen due to their comparatively larger size (Leng and Nolan, 1984). Fungi can account for about 8% of

the microbial mass in the rumen of animals fed lignified fiber diets (Citron et al., 1987), but their numbers were quite low when diets rich in concentrates were given (Fonty et al., 1987).

The reticulorumen provides a favorable environment for microbial growth and survival. Nutrients are regularly supplied mainly via ingested feeds. Some nutrients are also added with saliva and by diffusion through the ruminal epithelium. The condition in the rumen is highly anaerobic with a redox potential of between –300 and –350mV. On average, the ruminal contents are usually between 85 to 93% moisture due to the dilution of feeds with saliva secreted during feeding. Typical quantities of saliva produced per day are around 150 L in cattle and 10 L in sheep (McDonald et al., 1982). The anaerobic and moist conditions favor the survival and growth of a broad category of microbes. Due to the buffering capacity of saliva and rapid absorption of VFA, electrolytes, and ammonia through the ruminal wall, pH is maintained mostly between 5.5 to 7. Temperature is near optimum (39 to 41^0C) for many enzyme activities, which is controlled mainly by the animal's homeothermic mechanisms and partly due to the heat generated during fermentation. The rhythmic ruminal contractions help to bring microorganisms in contact with freshly ingested or ruminated feeds. End products of microbial fermentation are continuously removed by absorption and passage out of the stomach thus preventing the chances of growth inhibition (Church, 1960).

The breakdown of feed constituents into simple compounds by the microorganisms in the reticulorumen has been recognized as a major function of the forestomach. The ability of the ruminant to digest β-linked cell wall carbohydrates enabled them to consume a wide range of feeds of plant origin. The carbohydrates are digested to yield pyruvate, lactate, VFA (acetic, propionic, and butyric) and CO_2 (Van Soest, 1982). Proteins are broken down to peptides, amino acids, and ammonia (Annison, 1956). Dietary lipids are hydrolyzed to free fatty acids and glycerol (Hoffman, 1973).

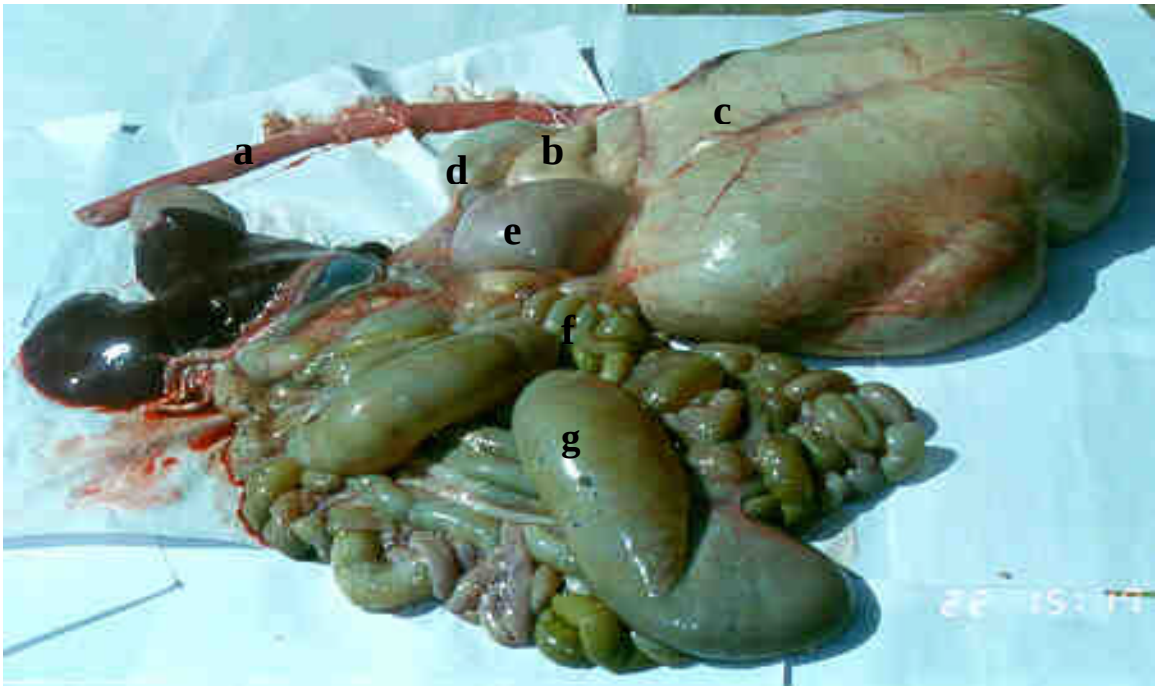
The forestomach is also involved in the synthesis of several compounds. Synthesis of microbial proteins from feed proteins and nonprotein N is a major product of ruminant N metabolism (Leng and Nolan, 1984). The microbes in the rumen can synthesize all essential amino acids when supplied with a source of ammonia and carbon

skeleton (Loosly, 1949). The ruminal microorganisms can also synthesize essentially all of the B-complex vitamins and vitamin K (Church and Pond, 1988). Additionally, unsaturated fatty acids are hydrogenated to yield saturated fats in the rumen (Jenkins, 1993).

The ruminant forestomach may also serve as an important site of nutrient absorption. All three compartments are lined with a stratified, squamous, nonglandular epithelium that exhibits transport ability (Steven and Marshall, 1970). The mucosal surface of the rumen contains numerous papillae, which may serve as organs of absorption. The omasum consists of a large number of laminae of different orders and sizes. The small particles of digesta are slowly passed through the interspaces between adjacent laminae, thus allowing water and other nutrients (VFA, Na^+ and Cl^-) to be absorbed before digesta reaches the abomasum (Englehardt and Hauffe, 1975). The ions (Na^+, H^+) responsible for active transport of nutrients (Webb and Matthews, 1994) and transporter proteins such as Na^+/K^+ ATPase and Na^+/H^+ exchanger are reported to exist in the forestomach epithelium (Martens and Gabel, 1988). The osmotic gradient established by the active transport of Na^+ across the granulosa strata (Gabel et al., 1993) is considered to be important for the non-mediated absorption of nutrients. The multi-layered epithelial cell structure and comparatively "loose" tight junction of the granulosa strata (Fell and Weekes, 1975) would be some useful anatomical features for a possible paracellular absorption of nutrients (Matthews and Webb, 1995).

Microorganisms in the rumen are known to modify several toxic compounds in to harmless substances. Those toxic compounds are found in a variety of feeds; examples for such anti-nutritional compounds include mimosine in *Leucaena leucocephala* (Singh, 1990), saponins in raw soybean (Liener, 1969), HCN in cassava (Singh, 1990) and gossipol in cottonseed meal (Kornegay et al., 1961). Feeding excessive amounts of such feeds to monogastric species and preruminants leads to detrimental effects. But ruminants can frequently prevent ill effects from those compounds due to the detoxification by the microorganisms in the rumen.

Figure 2.1. The digestive system of a goat showing (a) esophagus, (b) reticulum, (c) rumen, (d) omasum, (e) abomasum, (f) small intestine, and (g) large intestine.



Protein Metabolism in Ruminants: Classical Concepts and New Perspectives

The complexity in ruminant N metabolism is evident by many studies on their protein nutrition. The complicated stomach anatomy of ruminants and the heavy interrelationship found between the microorganisms and host animal in the digestive process appear to contribute mainly to this complexity. The major pathways of N metabolism in the rumen have been recognized for many years. A schematic representation of the major N pathways in the rumen is illustrated in Figure 2.2. Further details on these metabolic pathways and quantitative understanding of each pool are still gathering.

Proteins are the main nitrogenous materials in most ruminant diets. Non protein nitrogen (NPN) in the form of peptides, free amino acids, amides, amines, nucleotides, urea, uric acids and nitrates can occur in varying proportions. Usually the true protein content accounts for 75 to 85% of the total N in most of the forage plants and seeds (Lindberg, 1985). However, the NPN fraction can contribute to over half of the total N in some feeds such as legume forages and ensiled feeds (Reid, 1994). In general, a mixed concentrate-forage diet fed to ruminants contains approximately 85 to 95% of the dietary nitrogen in true protein form (Satter and Roffler, 1975).

Dietary Protein Degradation in the Reticulorumen. Feed proteins are degraded at varying rates and to varying extents due to the microbial activity in the rumen, the first major change that occurs when these are consumed by ruminants. The extent of protein degradation influences the N needs of ruminal microorganisms as well as the amino acid requirements of ruminants, and thus becomes an important parameter when determining the protein value of a feed (Madsen and Hvelplund, 1985). Presented in Table 2.1 are ruminal protein degradation values estimated after compiling several previous measurements by Satter (1986) and by NRC (1989) for some selected feeds. The protein degradation in the rumen varies widely between feeds, within feeds, and with different chemical or physical treatments (ARC, 1980).

Table2.2. Major Pathways of N Metabolism in Ruminants (Classical Concepts).

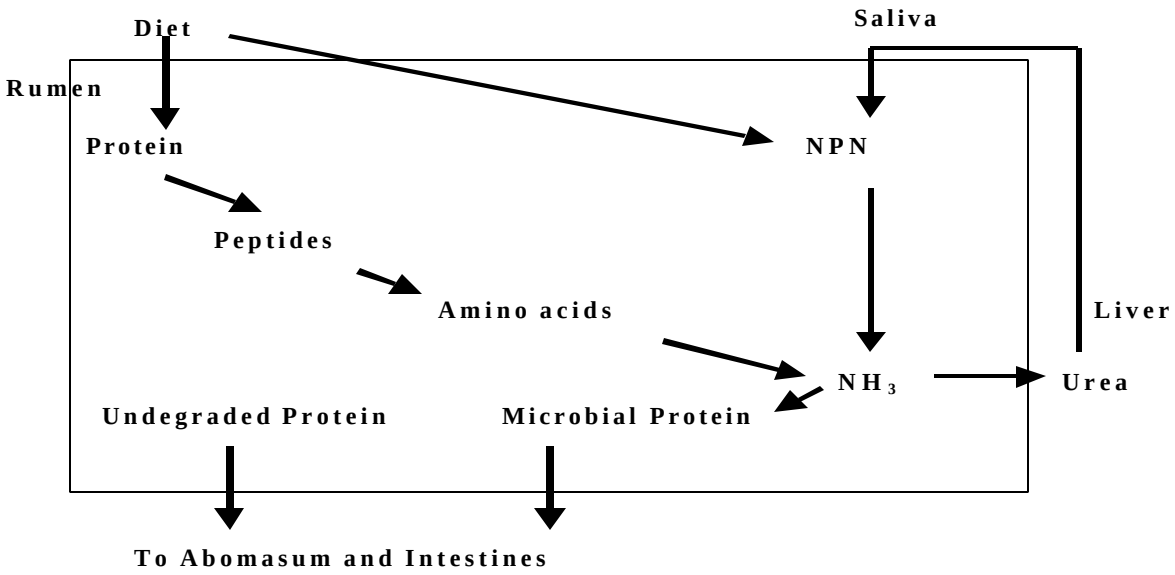


Table 2.1. Ruminal Degradability Estimates of Some Selected Feed Proteins.

Feed	Satter (1986)	NRC (1989)
	---------- %	----------
Alfalfa dehydrated	44	41
Blood meal	32	18
Brewers dried grains	47	51
Casein	N.D.[a]	81
Corn gluten feed	80	75
Cotton seed meal	59	57
Distillers dried grains with soluble	N.D.	53
Fish meal	20	40
Meat and bone meal	40	51
Soybean meal	73	65

[a.] Not Determined

Degradation of proteins in the rumen consists of several steps. An association between microorganisms and substrate should occur at the beginning. This association may involve either the adsorption of a soluble protein to the bacterial cell surface or of the adhesion of bacteria to an insoluble substrate or the ingestion of a particulate substrate by protozoa. Proteolytic cleavage of the protein to peptides, followed by hydrolysis of peptides to amino acids may occur in the next steps. Both peptides and amino acids can be transported into the microbial cell and either protein synthesis or deamination may take place. Deamination will result in the production of ammonia, VFA, CO_2, and methane. (Wallace, 1994). The ammonia can be used for assimilation and resynthesis of microbial proteins. When the production rate of ammonia in the rumen exceeds the capacity of ammonia-utilizing species, large quantities can accumulate. Excess ammonia is absorbed across the reticulorumen or is passed to the lower gut for absorption and is converted to urea in the liver. Some of the urea is recycled back to the rumen via blood or saliva but a significant proportion may be lost in the urine (Russell et al., 1991).

All the enzymes that convert proteins to ammonia in the rumen are assumed to be microbial in origin. This assumption is well supported by the observations of Brock and coworkers (1982) who suggested that enzyme activities were predominantly associated with the small particle phase, rather than the fluid phase. Bacteria, protozoa and to a lesser extent anaerobic fungi, can all carry out proteolysis, peptidolysis and deamination in the rumen (Broderick et al., 1991).

Bacteria are generally regarded as being mainly responsible for degrading dietary protein. The surface area presented to the ruminal fluid by bacteria is four times that of protozoa, and as the metabolic rate is related to surface area, bacteria are metabolically more important (Buttery, 1976). Between 30 to 50% of the bacteria isolated from ruminal fluid have proteolytic activity towards extracellular protein (Prins et al., 1983), and a mixed population is necessary to account for the degradation activity found in the rumen (Wallace and Brammall, 1985). Numerous bacterial species are involved in the protein breakdown. Most attention has been focused on three species considered to be the major proteolytic organisms, namely *Bacteroides ruminicola*, *Bacteroides amylophilus* and *Bacteroides fibrisolvens*. Several other ruminal bacteria are also

reported to be proteolytic. These include the species of *Clostridium, Eubacterium, Strptococus, and Selenomonas* (Wallace and Brammall, 1985; Wallace, 1994).

Protozoa are actively engaged in the hydrolysis of proteins in the rumen. Several species of ruminal protozoa including *Ophrysocolex spp., Entodinium* and *Eudiplodinium medium* have been identified as being proteolytic (Williams and Coleman, 1992). When the protein digesting enzyme activities of ruminal protozoa and bacteria were compared, protozoa exhibited a higher aminopeptidase and trypsin-like activity (Forsberg et al., 1984). The reduced ruminal ammonia concentrations frequently observed in defaunated animals also suggest that protozoa can greatly influence the ruminal N metabolism (Leng and Nolan, 1984). But, protozoa poorly degrade soluble proteins in the diet. The specific activity of protozoa accounted for only one-tenth of the activity in the breakdown of azocasein (Brock et al., 1982). Protozoa seem to be mainly involved on the degradation of bacterial cells and insoluble feed proteins (Hino and Russell, 1986). By converting bacterial protein into protozoal protein, and with the selective retention of protozoa in the rumen (Viera, 1986), they can serve as a continuous source of N within the forestomach following death and lysis.

Studies on the protein degradation ability of ruminal fungi are scanty. Several ruminal fungi species were reported to have a distinctive extracellular metaloprotease that has a trypsin-like protease activity (Wallace and Joblin, 1985). But the studies of Brock et al., (1982) suggest that fungal protease activity is low in the rumen. Michael and coworkers (1993) evaluated the proteolytic and peptidase activities of seven of the most common strains of ruminal fungi in vitro. Proteolytic activity was detected only in one strain (i.e. *Piromyces sp.*). All the strains exhibited aminopeptidase activity but carboxypeptidase activity was not found in any strain. Their study further concluded that the contribution of ruminal fungi is relatively insignificant in comparison to the total proteolytic and peptidase activity in the rumen. Thus, the influence of fungi on protein degradation in the rumen appears to be minor.

The proteolytic activity in the rumen, the numbers of proteolytic species and the predominant proteolytic species present all appear to be influenced by diet. Switching cows from hay-concentrate diet to one containing fresh alfalfa caused a nine-fold increase in proteolytic activity (Nugent and Mangan, 1981). *Bacteroides ruminicola* has been

identified as the predominant species of proteolytic bacterium found in the rumen of cattle and sheep under variety of dietary conditions (Wallace and Brammall, 1985). *Bacteroides amylophilus* can also be a very active proteolytic organism particularly with the ingestion of high starchy diets (Blackburn and Hobson, 1962). Changing the protein in the diet from casein to less readily degraded ovalbumin can stimulate the growth of *B. fibrisolvens*, a bacterial species with a higher proteolytic activity (Cotta and Hespell, 1986).

Factors Influencing Microbial Protein Breakdown. The degradation of dietary protein in the rumen is influenced by a number of factors. Solubility of the protein, which is usually measured in artificial saliva at body temperature, seems to be an important factor influencing degradability. Soluble proteins tend to be more readily or completely degraded than insoluble proteins. A good correlation was obtained between the solubility and the rate of protein breakdown in the rumen for several feeds (Henderickx and Martin, 1963). Because ruminal microorganisms and extracellular enzymes must come in contact with feeds through a water to feed interaction, it can be expected that soluble proteins are frequently degraded at faster rates than insoluble ones (Nocek and Russell, 1988). However, the opposite was observed with certain proteins including soluble proteins such as albumin which is hydrolyzed slowly while some insoluble proteins, such as hide powder, are degraded rapidly (Wallace, 1983). By demonstrating that soluble and insoluble proteins of soybean meal were hydrolyzed at almost identical rates, Mahadevan et al. (1980) showed that solubility or insolubility of a protein is not by itself an indication of the protein's resistance or susceptibility to hydrolysis by rumen microbial proteases. Therefore, the solubility and the rate of ruminal degradation are not correlated universally with all the proteins and feeding conditions.

The extent of protein degradation is influenced by the retention time of protein in the rumen which may vary with its particle size and intake (Church, 1960). With increasing intake, the proportion of insoluble N degraded in the rumen decreases, presumably due to a decreased rumen retention time (Lechner-Doll et al., 1991). Ruminal retention time of proteins varies not only between feeds but also between animals (Balch and Campling, 1965). High producing ruminants consuming large

quantities of feed are likely to have a smaller fraction of dietary protein degraded in the rumen than animals consuming low or moderate amounts (Satter, 1986).

The primary, secondary, and tertiary structures of the protein molecule can have a great influence on its accessibility by proteolytic enzymes and thus can affect ruminal degradability. When proteins are extensively crosslinked with disulfide bonds, their ruminal degradability is slow due to the poor accessibility by enzymes (Nugent and Mangan, 1978). Casein, which has essentially a linear secondary and tertiary structure (having no disulfide bonds), is very sensitive to degradation (Mangan, 1972). Bovine serum albumin (BSA) has a complex tertiary structure with 6% cysteine, disulfide bonds and possesses a greater resistance to degradation. When different proteins were subjected to treatment with mercaptoethanol or performic acid to cleave the disulfide bonds, no difference was found in their rates of degradation (Mahadevan et al., 1980). The cyclic feature of ovalbumin can greatly reduce the rate of proteolysis even though this protein is more soluble in ruminal fluid (Mangan, 1972).

Feed processing and storage can have a marked influence on the breakdown of protein in the rumen. During processing and storage, feeds may be exposed to heat that can alter the nature of the protein. By-product feeds are frequently dried during processing and ensiled feeds may be exposed to elevated temperatures during storage. During different feed processing methods such as pelleting, extrusion and steam rolling, a sufficient heat to alter protein is usually generated. Heat used in the drying process of fish protein can induce formation of S-S cross-linking from sulfhydryl oxidation (Opstvedt et al., 1984) and can lower rate of ruminal proteolysis (Chen et al., 1987b). Mehrez and coworkers (1980) have studied the effect of processing methods of fishmeal on the rate and extent of ruminal degradation. They suggested that the most important factor was the length of storage prior to processing, which is presumably correlated to the extent of hydrolysis or denaturation of the fish protein. Also, the addition of formalin as a preservative in the storage of fish prior to processing, drying method, and the addition of antioxidants are all factors that may affect degradability of protein in the rumen. Studying the effect of heat treatment on ruminal protein degradation in cottonseed meal, Broderick and Craig (1980) concluded that the heat treatment decreases ruminal degradation partly by blocking reactive sites for microbial proteolytic enzymes and partly

by reducing protein solubility. Therefore, the ruminally degradable N content may vary between and within feeds depending on the method of processing and storage conditions.

Methods to Predict Ruminal Protein Degradation. A considerable amount of effort has been made in the investigation of methods to measure ruminal protein degradation. These methods can be broadly classified as in vivo, in situ, and in vitro techniques. More details on each of these procedures can be found in several reviews (Johnson, 1966; Lindberg, 1985; Nocek, 1988).

The in vivo procedures are designed to measure the amounts of total and microbial proteins reaching the duodenum or abomasum using cannulated animals. The microbial proteins are determined using specific markers such as diamino pimelic acid (DAPA), RNA, ^{35}S, ^{32}P, and ^{15}N (Tamminga, 1979). The undegraded dietary fraction is estimated by the difference between total and microbial proteins. The undegraded dietary fraction also includes proteins added by endogenous sources and partially degraded proteins in the rumen. Therefore, Clark and coworkers (1992) proposed that the more accurate terminology for this N fraction would be nonammonia nonmicrobial N (NANMN). To overcome these limitations, Hogan and Weston (1970) suggested a method to predict the endogenous protein flow using equations and subtract from NANMN fraction. The in vivo measurements of ruminal protein degradation are expensive, time consuming, labor intensive, and subject to error due to inaccurate estimation of endogenous proteins as well as in differentiation of feed and microbial proteins using markers (Stern et al., 1994).

The in situ procedures have been commonly used to predict ruminal degradation. This technique involves suspending dacron polyester or nylon bags containing feeds in the rumen of cannulated animals and measuring N disappearance at various time intervals. The in situ methods provide an opportunity to use digestive process in the rumen of a live animal similar to what occurs under in vivo conditions. The popularity of this method also lies in its relative simplicity, low cost and its ability to measure the rate of N disappearance in the rumen (Orskov et al., 1980). The assumption of a constant flow rate is an inherent weakness of this procedure. The estimates of protein degradation by in situ methods may also depend on several factors. These include porosity of the bags, particle size of feed samples, ratio of sample weight to bag surface area, bag

placement in the rumen, and the colonization of bacteria in the feed residues (Weakly et al., 1983). The use of standardized procedures has been recommended to overcome the discrepancies that might occur when using this technique (Lindberg, 1985; Nocek, 1988).

The necessity to maintain surgically prepared animals imposes a severe restriction on the use of both in vivo and in situ techniques for routine determination of ruminant degradability of large numbers of feed samples. Hence, many in vitro procedures have been devised to estimate ruminal degradability under laboratory conditions. Ability to quantify end products of dietary protein degradation in the rumen before these products flow to the duodenum or are absorbed by the gastrointestinal tract is an important advantage of in vitro techniques. Also, the use of markers may not be required and the complications that might arise due to the addition of endogenous N can be eliminated. Reduction of cost and time would also be added advantages of in vitro procedures (Chamberlain and Thomas, 1979).

The development of continuous culture fermenters provide an opportunity to study ruminal N degradation by more closely simulating the ruminal environment in a laboratory (Czerkuski and Breckenridge, 1977). In these systems, solid feeds can be added continuously at variable rates and the turnover of solids and fluid in vessels may be varied independently. Reliable procedures are required for differentiation of effluent digesta into microbial and dietary N fractions. A good correlation was reported between the continuous culture fermentation and in vivo measurement of ruminal degradability (Lindberg, 1985). In comparison to other in vitro methods, continuous culture fermentation techniques are more expensive, elaborate, and not suitable when a large number of samples are to be analyzed.

Nitrogen solubility has been used to predict ruminal degradability because of the high correlation observed between the two parameters in some purified proteins (Hendrix and Martin, 1963). Several solvents such as Burrough's mineral buffer (Burrough et al., 1950), McDougal's mineral buffer (Crooker et al., 1978), and Durand's buffer (Lindberg, 1985) have been used to estimate solubility. But a poor correlation between N solubility and in vivo protein degradation has been frequently reported (Stern and Satter, 1984). Thus, the solubility is not synonymous with degradability as previously proposed.

The use of proteolytic enzymes to estimate dietary protein degradation in the rumen was attempted. Proteases from bacteria, fungi and plants were used (Krishnamoorthy et al., 1982; Poos-Floyd et al., 1985) and variable responses were reported. The most suitable protease preparation for predicting feed protein degradation appears to be the one that is prepared from mixed ruminal microorganisms (Mahadevan et al., 1987). However, the extraction of protease from ruminal microbes is very tedious.

The measurement of ammonia production during in vitro incubation of feeds with ruminal fluid has been used to study protein degradation (Chamberlain and Thomas, 1979). But the accumulation of ammonia during incubation of proteins occurs as a net effect of ruminal protein degradation and microbial utilization for protein synthesis. Also, the concentration is greatly influenced by the amount and nature of the fermentable carbohydrates available. Consequently an underestimation of the true degradability is usually the result (Broderick, 1982). To overcome these limitations, a modified in vitro procedure to inhibit microbial protein synthesis by adding hydrazine or chloramphenicol was suggested (Broderick, 1987).

An alternative in vitro method based on measurement of ammonia concentration and gas (CO_2 & CH_4) production during incubation of feeds with ruminal fluid was proposed by Raab et al. (1983). Starch was added in graded amounts and the gas production and NH_3 concentration were measured. Ammonia released at zero gas production was extrapolated and this point was considered as zero microbial growth. The requirement of a large number of incubations for more accurate estimation of ruminal protein degradation would be the major drawback in this procedure. Several other modified in vitro procedures have also been proposed (Broderick and Clayton, 1992; Mahadevan et al., 1979). Thus, the methods to measure ruminal protein degradation are numerous and each method has its own merits and limitations. The specific objective of the researcher and the availability of resources would be two major determinants in the choice of a method.

Accumulation of Products Following Digestion of Proteins in the Rumen. Ammonia is considered to be a major end product of fermentation of nitrogenous compounds by the microorganisms in the rumen. The concentration of ammonia in the ruminal fluid can vary from 0 to 130 mg/dL for a wide variety of dietary conditions

(Hungate, 1966). Satter and Roffler (1974) indicated that the mean ruminal ammonia concentration ranged from .8 to 56 mg/dL ruminal fluid increasing with percent of dietary N level. Ammonia is produced in the rumen by the metabolism of proteins as well as NPN compounds in the diets and from those added endogenously (Leng and Nolan, 1984). Incorporation into microbial proteins would be the primary route of ammonia loss from the rumen. Studies with ^{15}N indicate that 50 to 75 % of the microbial N in the rumen of animals fed common diets is derived from the ruminal ammonia pool (Oldham, 1981). Ammonia is an essential nutrient for several species of ruminal bacteria namely, *Bacteroides amylophilus, Bacteroides succinogen, Eubacterium ruminantium, Methanobacterium ruminantium, Ruminicocus albus,* and *Ruminicocus flavefaciens* (Hungate, 1966). Synthesis of amino acids from ammonia by the ruminal microorganisms requires the use of carbon skeleton and energy. Carbon from a wide variety of sources (e.g. Carbohydrates, VFA) could be used, but specific carbon skeletons may be required for the synthesis of some amino acids (Tillman and Sidhu, 1969). There has been considerable controversy over the optimum concentration of ammonia in the rumen to sustain maximum microbial yields. The reported values range from 1 mM (Schaefer et al., 1980) to 15.8 mM (Allen and Miller, 1976) and may depend on the dietary situation and animal variations. When the production rate of ammonia exceeds the capacity of ammonia utilizing species, large quantities can accumulate in the rumen. The excess ammonia is mainly absorbed across the rumen wall or may pass out to the duodenum for absorption.

Reported values of the free amino acid concentration in the rumen are frequently low (Annison, 1956; Wright and Hungate, 1967). This observation was hypothetically explained as due to the rapid deamination by microorganisms (Chalupa, 1976). Leibholz (1969) found that free amino acid concentration was dependent on the dietary protein intake and may exceed free amino acid concentration in the blood plasma. In a recent review of literature, Matthews and coworkers (1996) indicated that the reported values of the concentration of free amino acids in the strained ruminal fluid were .12 to 1.5 mg/dL prefeeding and .72 to 6 mg/dL postfeeding. Increased amino acid concentrations observed postfeeding implies that the utilization of free amino acids can also be rate limiting during ruminal protein degradation. The appearance of peak amino acid

concentration was apparently different in different studies, most likely due to dietary and animal variations.

It was usually assumed that all amino acids are catabolized equally during ruminal fermentation (Burroughs et al., 1975). But substantial evidence to indicate different rates of degradation of individual amino acids and interactions among certain amino acids during microbial catabolism were reported (Chalupa, 1976; Tamminga, 1979). Nonessential amino acids are usually degraded very rapidly in the rumen. Essential amino acids are broken down at different rates depending on the amino acid. Methionine and valine are more stable while arginine and threonine are very susceptible to degradation (Chalupa, 1976). Therefore, depending on the diet and animal variations, specific amino acids may potentially be available in the rumen.

Until recently, many nutritionists have neglected the magnitude and the nutritional significance of peptides produced following the protein hydrolysis in the rumen. In an early study, Winter et al. (1964) observed that tungstic acid precipitated as much as seven times more soluble N from ruminal fluid than trichloroacetic acid. Several years later, Chen et al. (1987a) noted that tungstic acid but not trichloroacetic acid, precipitates peptides in addition to the proteins. Referring to the above observations, Russell et al., (1991) hypothesized that the peptides could represent a sizable proportion of the soluble N fraction in the ruminal digesta. Mangan (1972) also noted a large increase in nonammonia, NPN when casein was infused into the rumen, but whether it was due to the peptides was not certain. When Chen and Russell (1990) incubated casein and soybean proteins with mixed ruminal bacteria, the proteins were degraded rapidly (.68 to .72 mg $N \cdot L^{-1} \cdot min^{-1}$, respectively). But the rate of ammonia production was nearly 10-fold lower (.08 and .1 mg $N \cdot L^{-1} \cdot min^{-1}$, respectively), and the microbial proteins were increased by only about 3%. Consequently, as much as 80% of the degraded protein N could not be accounted by considering only ammonia and microbial protein production. The fraction of non-ammonia, non-protein N (NAN-NPN) that accumulated was large. This NAN-NPN reacted more strongly with ninhydrin following acid hydrolysis suggesting that peptides were the main component of this fraction. Peptide accumulation following ruminal protein degradation has also been demonstrated in a number of studies (Chen et al., 1987b; Broderick and Wallace, 1988;

Broderick et al., 1990). Compiling previously reported measurements on the concentration of peptide N in ruminal fluid, Matthews and coworkers (1996) indicated that the values ranged from 1.5 to 6 mg/dL prefeeding, and 10 to 27 mg/dL postfeeding. All these studies have clearly indicated the presence of increased peptide concentrations in the rumen following protein meals, but the total concentrations, rates of production, and time required for the decrease of peptide concentration to a prefeeding level varied among studies.

The diet can have a major influence on the accumulation of peptides in the rumen. The peptide accumulation has been suggested to occur when diets containing rapidly degraded proteins are supplemented (Chen et al., 1987b). Sheep fed a diet containing casein (a rapidly degraded protein) resulted in an accumulation of peptides in the rumen, but no accumulation was observed when either urea or ovalbumin replaced casein (Broderick and Wallace, 1988). Replacing half of the soybean meal in the diet of lactating dairy cows with slowly degraded proteins (either extruded soybean meal or fish meal) resulted in a lower concentration of peptide N in the ruminal fluid (Chen et al., 1987b). They suggested that peptides accumulate in the rumen during the proteolysis of rapidly degraded proteins due to the saturation of ruminal bacterial proteinases with substrates, but that peptide uptake by bacteria would exceed the rate of release from more slowly degraded proteins.

Williams and Cockburn (1991) refuted the concept that the peptide accumulation in the rumen is highly correlated with the degradability and the solubility of the proteins. They fed steers, a diet of straw and tapioca supplemented with either urea, casein, formaldehyde treated casein, decorticated groundnut meal, soybean meal, maize gluten meal, or fish meal to determine the effect of degradability on ruminal amino acid and peptide concentrations. The peptide N concentrations at 1 h after feeding of straw supplemented urea, casein, formaldehyde treated casein, decorticated groundnut meal, soybean meal, maize gluten meal, and fish meal were 2.4, 160, 87, 76.5, 21, 136, and 90 mg/L, respectively. They concluded that peptides were accumulated postfeeding, but the peptide N concentrations were poorly correlated with the degradability and solubility of the proteins.

Differences that may occur in the level and frequency of feeding can also influence the production of peptides in the rumen. Chen et al. (1987b) fed diets (12 times/d) supplemented with soybean meal to provide 14.5, 17.1, and 20.6% CP to lactating cows. When the protein content of the diet was increased from 14.5 to 17.1%, the ruminal peptide N concentration also increased (from 106 to 154 mg/L). However, the ruminal peptide concentration was not further increased with an additional increase in protein level, indicating that the protease activity in the rumen was saturated with substrates. Greater concentrations of ruminal peptides were also noted as the frequency of feeding was decreased from 12 times/d to once a day.

Persistence of specific peptides resistant to further hydrolysis in the rumen is a relatively new concept. The size of the peptide has been suggested to influence their susceptibility to ruminal degradation (Cooper and Ling, 1985). Pittman and Bryant (1964) observed that ruminal bacteria utilized large oligopeptides more rapidly than the small peptides. Hence, the small peptides can frequently accumulate in the extracellular ruminal fluid. Wallace et al. (1990b) have also indicated that the peptides containing three or more amino acids are hydrolyzed and utilized more rapidly than the dipeptides. Using di- to pentapeptides of alanine and glycine, Wallace et al. (1990a) have further studied the influence of peptide size on the rate of disappearance in the rumen. Ala_2, among alanine peptides, and $Gly_{5,}$ among glycine peptides, were slowly hydrolyzed suggesting that the peptide size and the amino acid composition can have an interaction effect on the rate of degradation of peptides.

The amino acid composition and the structure of the peptide substrates have been considered to be important determinants of their susceptibility to microbial degradation. Chen et al., (1987c) separated tripticase (pancreatic digest of casein containing mostly peptides) into alcohol soluble and insoluble fractions using 90% isopropyl alcohol. The alcohol soluble fraction had an abundance of peptides composed of hydrophobic amino acids (leucine, tryptopan, tyrosine, phenylalanine, proline, and valine). The insoluble fraction contained peptides with a large proportion of hydrophilic amino acids (arginine, aspartic acid, glutamic acid, and lysine). When these two fractions were incubated with mixed ruminal bacteria in vitro, hydrophilic peptides were metabolized more rapidly than the hydrophobic peptides (39 vs 18 mg of NH_3 per g of bacterial protein per h). Yang

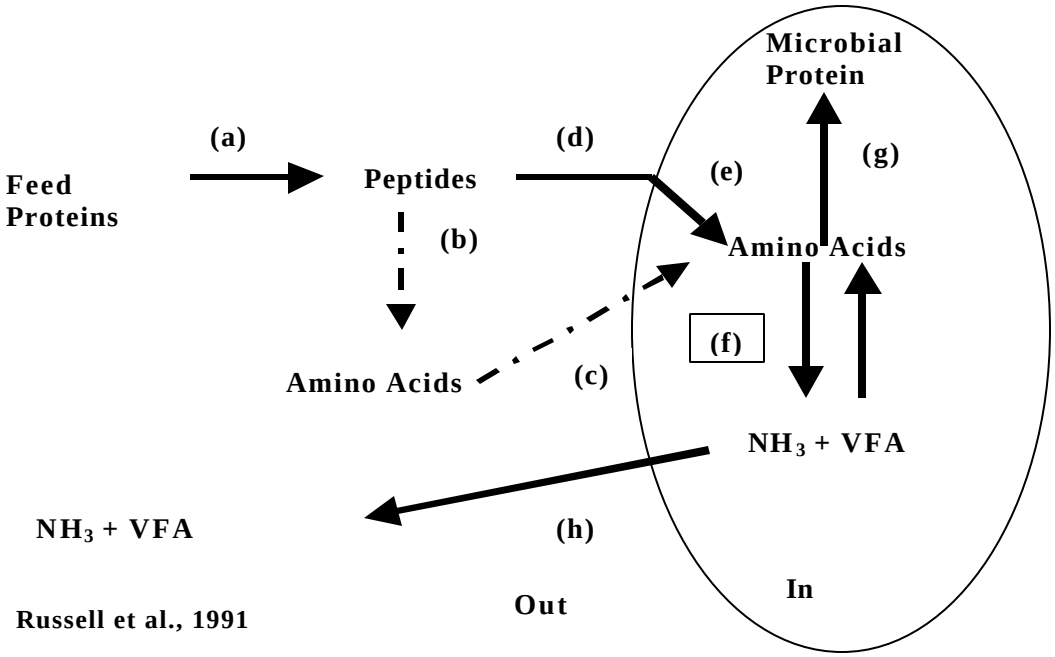
and Russell (1992) incubated enzymatic digests of casein and gelatin with an inoculant of mixed ruminal bacteria and measured the persistence of peptides in the incubation medium. The results showed that ruminal bacteria were unable to degrade much of the peptides from enzymatic digests of casein and gelatin, even when the incubation period was as long as 96 h. The peptides resistant to microbial degradation contained a large amount of proline. Therefore, they hypothesized that proline-containing peptides might be degraded at a slower rate than other peptides in the rumen.

Some selectivity may occur during the metabolism of peptides by ruminal microorganisms and the resistant peptides could specifically persist in the ruminal fluid. Peptides of different size, structure, and amino acid composition are broken down at different rates in the rumen. The structural differences of proteins and variations in feed processing conditions may greatly influence the persistence of specific peptides in the ruminal fluid.

Mechanisms of Peptide Accumulation in the Ruminant Forestomach. The mechanisms involved in the accumulation of peptides during ruminal protein degradation are being investigated. Figure 2.3 illustrates a scheme that has been proposed to explain further details of the utilization of proteins by ruminal bacteria (Russell et al., 1991). According to this scheme, the protein utilization by ruminal microorganisms is a multistep process involving proteolysis, peptide hydrolysis, the uptake of peptides or amino acids into the microbial cells and either fermentation or microbial protein synthesis. As the protein utilization by ruminal microorganisms occurs in several distinctive steps to yield a number of intermediate products with different degradation characteristics, accumulation of intermediate products during dietary protein degradation might be possible.

Proteolysis of dietary proteins will result in the production of a variety of peptides in the rumen. Proteases are mainly associated with the cell surface of bacteria (Kopency and Wallace, 1982) and the hydrolysis of proteins to peptides usually occurs extracellularly.

Figure 2.3. A schematic representation of the protein utilization by ruminal bacteria showing (a) proteolysis, (b) extracellular peptide hydrolysis, (c) amino acid transport, (d) peptide transport, (e) intracellular peptide hydrolysis, (f) amino acid fermentation, (g) microbial protein synthesis, and (h) diffusion of NH_3 and VFA.



Peptides of a transportable size are produced by some extracellular peptidase activity in the next step (Russell et al., 1991). When soy protein hydrolysate was incubated with mixed ruminal bacteria, high molecular weight peptides (> 3,000 MW) disappeared rapidly resulting in an accumulation of smaller peptides (500 to 1,000 MW) in the extracellular medium (Depardon et al., 1995). The microorganisms in the rumen may either transport these smaller peptides directly into their cells or hydrolyze them further to amino acids extracellularly. As the ruminal bacteria have transport systems for the uptake of both peptides (Chen et al., 1987a) and free amino acids (Russell et al., 1988), protein utilization can proceed in either route.

The majority of bacteria in the rumen are gram-negative, and their outer membrane could act as a selective barrier to many substances. Porins, which serve as channels to allow nutrient passage through the outer membrane have a maximum size exclusion limit of around 600 (Nikaido, 1979) to 750 Dalton (Westlake and Mackie, 1990). Also, the peptide transporters in the cytoplasmic membrane of both gram-negative and positive bacteria are reported to have a size exclusion of approximately five amino acids (Higgins and Gibbson, 1986). Thus, peptides smaller than five amino acids are usually transportable. However, *Bacteroides ruminicola* are reported to take up peptides containing 16 amino acids (Pittman et al., 1967).

Experiments comparing the rates of utilization of free versus peptide amino acids by ruminal microorganisms indicate a rapid use of peptides. When Prins et al., (1979) added free and peptide-bound forms of glycine, methionine, valine, and histidine to a medium containing mixed ruminal microorganisms, peptide forms disappeared rapidly. Also, the ammonia production by mixed ruminal bacteria was rapid when peptides rather than the corresponding free amino acids were incubated (Chen et al., 1987a). The rapid utilization of peptides was suggested as due to the dominance of peptide uptake pathways in comparison to those of free amino acids. This hypothesis is further confirmed as the peptidase activities were found mostly membrane-bound or intracellular (Broderick et al., 1988). Therefore, peptide uptake is the preferred method of transport followed by either membrane or intracellular hydrolysis of peptides. As amino acid catabolism depends on intracellular enzymes and cofactors, the amino acid deamination must occur intracellularly (Hino and Russell, 1985).

Accumulation of peptides in the extracellular ruminal fluid suggests that either extracellular peptidase activity or peptide uptake into the bacterial cells could be the rate limiting steps in the overall degradation of some feed proteins (Russell et al. 1991). Ruminal bacteria could utilize only a small fraction of peptides regardless of the concentration used suggesting that some peptides were specifically resistant to further degradation in the rumen (Cotta and Russell, 1982). Accumulation of dialanine (Wallace et al., 1989), hydrophobic (Chen et al., 1987c) and proline containing (Yang and Russell, 1992) peptides in the rumen indicates that the size, structure and the amino acid composition of the peptides can have a great influence on the peptide utilization by the microorganisms in the rumen. Therefore, specific peptides could persist in the rumen depending on the nature of the protein fed to the animal.

Alternative Sources of Peptides and Amino Acids Added to the Ruminal Digesta. Peptides and amino acids can be produced in the rumen from sources other than feed proteins. These alternative sources include the microbial proteins that are recycled within the reticulorumen and the endogenous proteins added into the forestomach. A significant contribution to the ruminal NAN pool can be made by these sources.

A substantial proportion of N is recycled within the forestomach of ruminants due to microbial cell lysis. In a study using intraruminal injection of (^{15}N) ammonia sulfate to sheep fed alfalfa chaff diet, Nolan and Leng (1972) noted that the amount of ammonia recycled within the rumen accounted for 30% of the total ammonia flux. Extensive recycling of N in the rumen has also been reported in several other studies (Demeyer and Van Nevel, 1979; Cottle, 1980). A portion of this N recycling may be attributed to the ammonia absorption from the forestomach and its return as urea. But according to Nolan and Leng (1972), the proportion of N recycled this way could account for only one-third of the total N recycling; the remainder being recycled due to the turnover of microbial proteins within the rumen. When (^{15}N) ammonia was infused into the rumen of sheep fed hay based diets, as much as 50% of the microbial mass was turned over before N passed to the lower gut (Nolan and Stachiw, 1979).

The turnover of microbial proteins in the rumen can occur due to a number of reasons. Predation of bacteria by ruminal protozoa is considered to be a major factor (Williams and Coleman, 1992), and around 40% of bacteria in the rumen were shown to

be engulfed (Abe and Kandatsu, 1969). Coleman and Sanford (1979) calculated that with a protozoal concentration of 10^6 cells/mL, 2.4 to 45 g of bacteria are engulfed daily in the rumen. Bacterial protein turnover can occur even in the absence of protozoa. Autolysis of bacteria, particularly during starvation has often been reported (Russell et al., 1983). Bacteriolytic microorganisms similar to mycoplasma (Hungate, 1966), anaeroplasma (Robinson and Hungate, 1973) and bacteriophages (Klieve and Bauchop, 1988) can also cause bacterial lysis in the rumen. Protozoa may also lyse within the rumen. Observations on high ruminal protozoal mass (Williams and Coleman, 1992), their rapid lysis under in vitro conditions (Coleman, 1985) and selective retention in the forestomach region (Leng, 1982) suggest that protozoa can also contribute to a significant proportion of the microbial proteins recycled in the rumen. Additionally, dietary manipulations such as salt feeding can also have an influence on microbial protein turnover in the rumen (Wells and Russell, 1996).

Endogenous sources other than urea can also add significant amounts of protein N to the ruminal digesta. The endogenous protein N (EPN) includes mucoproteins from saliva and cellular debris from sloughing and abrasion of gastrointestinal tract epithelial cells. These endogenous proteins are also reported to degrade in the rumen (Hogan, 1975) and would contribute to the ruminal NAN pool. In cattle fed forage diets, the mean flow of EPN from the rumen and abomasum was estimated to be 6.2 and 23 g/d, respectively (Hart and Leibholz, 1990). These values corresponded to 2.2 and 6.3 g/kg DM intakes, respectively. The flow of EPN was increased as the DM intake increased. Estimates by Baily and Balch (1961) suggest that the flow of saliva in cattle fed forage diets was 90 L/d contributing 5.4 g proteins per day. Their calculations further indicate that an amount of nitrogen equivalent to approximately 10% dietary N is recycled via saliva. According to Orskov and McLeod (1982), about 5g of EPN was added daily from the rumen of cows and steers due to the sloughing off and abrasions of epithelial cells. The flow of gastric juice secreted in cattle was found to be 30 L/d contributing between 8 to 19g endogenous protein N (Hill, 1961). Summarizing previous data, Egan et al. (1986) suggest that between 3 to 12g of endogenous proteins are usually added daily before digesta reaches the duodenum.

Duodenal Flow of Ruminal Protein Degradation Products. Nitrogenous compounds reaching the small intestine of ruminants are generally assumed to consist of microbial and undegraded dietary proteins, nucleic acids of microbial origin, constituents of bacterial cell walls, ammonia, and endogenous N sources (Armstrong et al., 1977). Peptides and amino acids that could arise due to the microbial activity in the rumen have usually been ignored. Therefore, very limited information is available on the duodenal flow of these products in ruminants. Recently, Chen et al. (1987b) studied the effect of dietary proteins on the peptide flow from the rumen of lactating dairy cows. When the cows were fed a forage diet with a soybean meal supplement, the estimated peptide flow from the rumen was over 200 g/d. Their calculations suggest that at this rate of flow, peptides escaping ruminal degradation could have accounted for more than 25% of the milk protein synthesized by the cows. Even if the soybean was heat-treated to decrease its solubility, a substantial amount of peptides (100 g/d) was reported to flow out of the rumen. However, the calculations of Broderick and Wallace (1988) indicate that the free amino acids and peptides can accounts for only 1.2 to 2.4% of the total NAN flow to the duodenum. Variation in diets, animals, and the analytical techniques used, can be the reasons for above discrepancy in the duodenal flux of peptides and amino acids. The effect of diet on the amount and composition of peptides and free amino acids flowing out of the rumen is yet to be known.

Absorption of Ruminal Protein Digestion Products Across the Ruminant Forestomach

Ammonia Absorption. Ammonia absorption is one pathway of N disappearance from the rumen. McDonald (1948) first demonstrated the absorption of ammonia from the rumen of sheep. A few years later, Lewis (1957) observed that the portal blood ammonia concentration increased in a curvilinear function with ruminal ammonia concentration. At higher ruminal concentrations, ammonia absorption across the rumen is increased, strictly by passive diffusion processes. The data of Hogan (1961) suggested that the ammonia absorption through ruminal wall was dependent on the concentration gradient at pH 6.5, but the losses were negligible at pH 4.5. Bloomfield et al. (1963) also reported a reduction in ammonia absorption across ruminal forestomach with decreasing ruminal pH. The dependency of the rate of ammonia absorption on ruminal pH is

dictated by the behavior of ammonia as a weak base. In solution, ammonia exists in a state of equilibrium between un-ionized (NH_3) and ionized (NH_4^+) forms, and the extent of ionization is highly pH dependent. The pK_a for this equilibrium is near 9. Therefore, the proportion of free NH_3 vs NH_4^+ increases at higher pH. The ammonium ion (NH_4^+) is less readily absorbed through the rumen wall than the un-ionized (NH_3) form (Visek, 1968). Consequently, increased rates of absorption occur at high pH due to the increases in concentration of free NH_3. Thus, the ruminal concentrations and pH play an important role in the absorption of ammonia across forestomach epithelium.

Amino Acid Transport. According to the current knowledge, free amino acids are the major form in which nitrogen is absorbed to meet the tissue needs of ruminants (NRC, 1985). The small intestine, specifically the more distal region (i.e. ileum) is considered to be the principal site of amino acid absorption in ruminants (Webb and Matthews, 1994). Data is accumulating to suggest with a reasonable degree of certainty that ruminant forestomach is a site of amino acid absorption. But the magnitude and the mechanisms of this process are yet to be understood.

Using both in vitro and in vivo procedures, Cook et al. (1965) demonstrated the ability of the ruminant forestomach epithelial tissue to absorb free amino acids. Sections of forestomach tissues (rumen, reticulum, and omasum) obtained from two goats were folded into sacs and suspended in a saline bath which was gassed with oxygen. Solutions of glycine in saline (perfusate) were placed in each sac. The disappearance of glycine from the perfusate and the appearance of glycine in the bath were determined over a period of 2 to 3 h. They were able to show that the glycine was transferred from perfusate to bath in all the tissues. In their in vivo experiments, absorption of amino acids across the rumen was demonstrated using two catheterized goats and a steer. When the goats were either forced fed ground shelled corn (450 g) or when glycine (15 g) was infused into the rumen, the blood glycine concentration in the ruminal vein increased markedly and remained elevated for a considerable period of time. In the goat that was infused with glycine into the rumen, the blood glycine concentration in the ruminal vein exceeded that of the jagular vein by 49 μmole/dL at 1 h after feeding. A marked difference was still evident after 2 h but was no longer after 3 h. Similarly, when a mixture of amino acids (serine, threonine, methionine, aspartic acid, asparagine,

glutamine, isoleucine and leucine) was placed in the rumen of a steer, the concentrations of these amino acids in the ruminal vein increased to different levels. Thus individual amino acids appear to be absorbed across the rumen in a different manner. However, their study was criticized, as the initial concentrations of amino acids used may not occur under normal physiological conditions in the rumen.

Subsequently, Leibholz (1971a) determined the relative rates of amino acid absorption across ruminal epithelium by placing a mixture of amino acids in the washed rumen of sheep. The amounts of individual amino acids lost from the rumen over a 4-h period ranged from almost zero to 50% of the initial concentrations placed in the rumen. However, her calculations of amino acid absorption across the rumen accounted for only 6% of the total N absorbed by the rumen. This estimate of N absorption was based on her assumptions that amino acids and ammonia are usually present in the rumen in 1 : 8, and the absorption occurs at 1 : 2 ratios. In another parallel study, Leibholz (1971b) further suggested that the amount of amino acids lost from the rumen depended on their concentration found in the ruminal fluid. Thus, Leibholz's estimates of amino acid absorption across the rumen may be subject to considerable variation depending on dietary and animal conditions.

The specific mechanisms involved in the absorption of free amino acids across the forestomach epithelium is not very well known. When Leibholz (1971b) investigated the absorption of histidine across ruminal epithelial tissues (in vitro), the transport process was clearly dependent on the substrate concentration used and was not saturable at concentrations between 30 to 600 μmoles. However, a greater uptake of L-histidine than D-histidine across ruminal epithelium suggested that the absorption was associated with factors other than simple diffusion. Also, the increases in histidine uptake in the presence of glucose or VFA and the inhibition in histidine uptake in the presence of 2,4, dinitrophenol and iodoacetate implied that energy was required for this process. Further, the transfer of L-histidine across the ruminal epithelium was reduced by 10 to 50% in the presence of other amino acids suggesting that a common mediated transport mechanism could be involved in the absorption of these amino acids.

Matthews and Webb (1995) studied the absorption of methionine and methionylglycine via ruminal and omasal epithelia of sheep using parabiotic chambers.

Serosal appearance and tissue accumulation of ^{35}S-methionine and ^{35}S-methionylglycine was quantified over a period of 240 min. The quantity of methionine transferred was linearly dependent on time, greater across omasal than ruminal epithelia, and was greater than methionylglycine. Transepithelial passage of methionine was non-saturable between substrate concentrations of 0.375 to 12 m*M*. Hence they assumed that the absorption of methionine across ruminal and omasal epithelia occurred by non-mediated mechanisms.

McCollum (1996) investigated the absorption of lysine across ruminal and omasal epithelia of wethers using parabiotic chambers. The appearance of lysine (using ^{3}H-L-lysine as a representative marker) in the serosal buffers increased linearly with time for both tissues during 60-min incubation. When the absorption was expressed on a tissue dry weight basis, more lysine absorption was observed in omasal tissues than in ruminal tissues. The tissues responded differently to increases in the mucosal concentrations of lysine. The serosal appearance of lysine across ruminal epithelia increased proportionally as the lysine concentration increased in the mucosal buffers between 0.09 to 3 m*M*. Because the lysine uptake was not saturable across ruminal tissues, she postulated that this occurred through nonmediated processes. But the appearance of lysine in the serosal buffers of omasal tissues increased proportionately up to a substrate concentration of 1.5 m*M* and then plateaued suggesting a mediated absorption of lysine across omasal epithelium.

The potential for mediated absorption of lysine in omasal epithelium was evaluated further by studying the functional expression of ovine omasal mRNA in *Xenopus laevis* oocytes (Matthews et al., 1996b). Compared to water injected oocytes, RNA injected oocytes displayed a greater ability to absorb lysine from both Na^{+}-free and Na^{+}-containing buffers. The linear rate of lysine uptake by RNA-injected oocytes was approximately two times greater than that demonstrated by water-injected oocytes. Within RNA fractions, the amount of induced lysine absorption did not differ between buffers. Based on the above observations the authors indicate that the induced uptake of lysine occurred by Na^{+} independent processes. In order to verify if this RNA-induced uptake was the result of an increase in mediated absorption ability, the uptake of 0.05 mM lysine by water- and RNA-injected oocytes was evaluated in the presence of leucine, glutamate or cystein. The absorption of lysine by oocytes was completely inhibited in the

presence of leucine but was not affected by glutamate displaying characteristic $b^{o,+}$-like mediated transport activity.

Evidence on Intact Peptide Absorption Across the Forestomach. Transmembrane transport of peptides is a phenomenon widely distributed throughout the nature and is present in animals including man, bacteria, fungi, yeast and in growing seeds (Matthews, 1991). Also, it was demonstrated to occur in several organs of the mammalian body including intestine, liver, kidney and brain (Leibach and Ganapathy, 1996). Evidence of the absorption of peptides across the gastrointestinal tract of ruminants have been revealed in a number of recent studies (Koeln and Webb, 1982; Seal and Parker, 1991; Backwell et al., 1995).

The first indication on the possibility of peptide absorption across ruminant forestomach surfaced when DiRienzo (1990) quantified the fluxes of free and peptide amino acids across the nonmesenteric-drained viscera of steers and wethers fed a hay and concentrate (30:70) based diet. Flux of peptide amino acids across the nonmesenteric-drained viscera accounted for 77% of the total portal drained viscera in both steers and wethers. The ability of ruminal and omasal epithelial tissues to transport peptides was demonstrated subsequently using different in vitro procedures (Matthews and Webb, 1995; McCollum, 1996; Pan, 1996). Matthews and Webb (1995) used ruminal and omasal epithelia collected from sheep to study absorption of L-carnosine and L-methionylglycine. The tissues collected were placed in parabiotic chambers with mucosal surface exposed to a buffered solution (pH 6.0) containing varying concentrations of the dipeptides. The serosal surface was exposed to a buffer (pH 7.0) with no peptides. The chambers were maintained at 39^0C with O_2 supply throughout the experiment period. Serosal samples obtained at regular intervals for 240 min were analyzed for carnosine and methionylglycine. The quantity of both peptides transferred was linearly increased during 240-min incubation. With increasing mucosal concentrations of dipeptides, a linear increase in the serosal appearance of peptides was noted. When peptide uptake was expressed on tissue dry weight basis, omasal epithelia exhibited a greater ability to absorb peptides than ruminal epithelia.

Mechanisms of Peptide Absorption. Proton-dependent dipeptide transport activity in sheep omasal epithelium was demonstrated in a recent study conducted in this

laboratory (Matthews et al., 1996b). When size-fractionated poly (A+) RNA isolated from omasal epithelial tissues of sheep were injected into defolliculated *Xenopus laevis* oocytes, an increased rate of glycyl-L-sarcosine absorption was recorded by the oocytes. The dependency of glycyl-L-sarcosine absorption on the presence of a pH gradient was also noted in this study. Thus, carrier-mediated absorption can be an important mechanism of peptide transport across the forestomach region of ruminants.

Pan (1996) revealed further details on the carrier-mediated peptide transport activity in sheep omasal epithelium. Poly (A^+) RNA isolated from sheep omasal epithelium was injected into *Xenopus laevis* oocytes and the peptide transport capability was measured by impaling oocytes with a microelectrode to monitor membrane potential. The study concluded that several di-, tri-, and tetrapeptides with different amino acid composition could serve as substrates for the mediated peptide transport activity in sheep omasum. Because the RNA injected oocytes did not respond to some di-, tri-, and tetrapeptides used in this study, the author suggests that substrate specificity may be present in the absorption of peptides across ruminant forestomach.

The interactions of L-methionylglycine, glycyl-L-Leucine, L-carnosine, and methionylglycyl-L-methionyl-L-methionine when glycyl-L-sarcosine was absorbed across sheep omasal epithelial tissues were investigated using parabiotic chambers (McCollum, 1996). The accumulation of glycyl-L-sarcosine in epithelial tissues was inhibited by the other peptides used in this experiment suggesting a common mediated transport mechanism involved in peptide absorption across omasal epithelium. Serosal appearance of glycyl-L-sarcosine was stimulated by these peptides. Therefore, she postulated that this increased uptake of glycyl-L-sarcosine might be due to paracellular absorption. The necessary components for paracellular absorption of peptides are known to present in the forestomach of ruminants (Matthews et al., 1996). Thus, paracellular absorption can be an important mechanism of peptide transport across the forestomach in ruminants.

The Nutritional Importance of Peptide Transport. The existence of peptide transport mechanisms in the body can offer a variety of advantages. One important proposition about the fate of absorbed peptides is their ability to serve as sources of amino acids for peripheral tissue utilization. Direct and indirect evidence on the

utilization of peptides by body tissues and organs can be found in several reviews (Krzysik and Adibi, 1977; Webb et al., 1993; Matthews et al., 1996). Using in vitro cell culture studies, Pan et al., (1996) were able to demonstrate that the methionine containing peptides can be efficiently used as sources of methionine for protein accretion and cell proliferation of ovine skeletal muscles and bovine mammary epithelial cells. The cultured cells were able to utilize methionine-containing peptides with responses ranging from 29 to 123% of the response of free methionine. Also, methionine-containing peptides were able to promote the synthesis of secreted proteins as effectively as free methionine by cultured mammary epithelial cells (Wang et al., 1996). The above studies emphasized that the peptides can be directly used at the cellular level as sources of amino acids for protein accretion by peripheral tissues, and for milk protein synthesis.

Rapid absorption of peptides in comparison to the free amino acids has been frequently reported (Adibi and Phillips, 1968; Matthews et al., 1968; Lis et al., 1971). The influence that the composition and structure of peptides might have on this process can not be overlooked. But, when partially hydrolyzed proteins (containing mostly small peptides) were used to replace free amino acids in test diets, the absorptive advantages of peptides could be very clearly noticed. A greater rate and extent of amino acid absorption from partially hydrolyzed proteins than the corresponding mixtures of free amino acids was commonly observed (Silk et al., 1980; Hara et al., 1984).

Use of peptides has gained a wide interest in clinical nutrition. Because certain amino acids (e.g. tyrosine, cystine) are known to be sparingly soluble in free form but are very soluble when provided as peptides (Grimble and Silk, 1989). Also, amino acids such as glutamine and tryptophan can be relatively unstable in solution, but are very stable when present as peptides (Hemmarkvist et al., 1988). Therefore, provision of those amino acids in peptide form may help to overcome insolubility and instability problems. The other benefits of dietary supplementation of peptides may include conservation of metabolic energy, steady appearance of amino acids in the blood and prevention of diarrhea due to lower osmotic load (Daniel et al., 1994; Ganapathy et al., 1994). Thus, there can be a variety of advantages in the use of peptides for the nutritionists and particularly in future ruminant feeding schemes, more emphasis will be placed on the form of amino acids to be supplemented.

A Description on the Protein Ingredients Used in this Study.

A general description on the protein ingredients used for in vitro ruminal incubation experiments is presented below.

Dehydrated Alfalfa Meal. The leaves of alfalfa are harvested at an early stage of maturity and are artificially dried and ground to make alfalfa meal. It is a fairly good source of protein (17.4% CP) with a respectable amino acid balance (NRC, 1988). But the protein quantity and the quality can vary considerably according to the age of the plant, soil fertility, variety and processing conditions. It is relatively high in crude fiber (24%), and constitutes a fair amount of tannins, which may depress protein digestibility (Millic et al., 1972). Due to the lower protein digestibility, and the presence of saponins that impairs growth (Leamaster and Cheek, 1979), its use is limited in swine and poultry diets. However, dehydrated alfalfa meal is used as a supplement to provide vitamins, xanthophylls and unidentified growth factors in the diets of monogastric species.

Blood Meal. This is a by-product of the animal slaughter and meat processing industry. The coagulated blood is dried by either spray drying or flash drying procedures, and is ground to make blood meal. It is extremely high in protein (over 80% CP), but the digestibility of the protein is lower than most other animal protein feeds. Blood meal is a rich source of lysine and leucine, but the concentration of isoleucine is very low (NRC, 1988). Also, the mineral levels are quite low in blood meal with the exception of iron. Because of its high concentration of available lysine, blood meal is ideal for incorporation into cereal grain-based diets. Its use in diets is restricted due to low palatability, poor digestibility, and due to the imbalanced amino acid composition (Miller, 1990).

Brewers' Dried Grains. Brewers' grains consist of the insoluble residue that remains after most of the starches and sugars have been removed from barley and possibly other grains (maize and rice) in the brewing process. The wet brewers' grains contain about 70 to 75% moisture, and are sometimes given to cattle, sheep and horses in this form. Brewers' grains are dried to facilitate handling, transport, and storage. The dried brewers' grains usually contain more than 90% dry matter of which between 20 to 27% is crude protein depending on the amount and nature of additives used (Morrison,

1950). The protein is particularly low in lysine and tryptophan. High fiber contents and the low energy values for monogastric species limit its use in their diets. Brewers' dried grains are extensively used in dairy diets primarily as a source of protein and secondarily as a source of energy (Ensminger and Olentine, 1978).

Corn Gluten Feed. During wet milling manufacture of cornstarch or syrup, most of the starch, gluten, and germ are extracted, and the remains of the shelled corn are used as the corn gluten feed. Basically this feed contains hulls, evaporated steep water, and germ meal. The protein content of corn gluten feed is relatively high (24% CP), but the amino acids are not very well balanced. It is usually low in lysine and tryptopan (Holden, 1990). The calcium and phosphorus levels of corn gluten feed are generally high but the availability of the phosphorus is low (Burnell et al., 1989). Corn gluten feed is chiefly used as a protein supplement in dairy cow diets.

Cotton Seed Meal. Dehulled, oil-extracted cottonseeds are ground to a meal with a certain amount of ground cottonseed hulls to make cottonseed meal. The extraction of oil involves either expeller (screw press) or solvent procedures. Cottonseed meal is one of the most popular protein supplements for cattle and sheep (Morrison, 1950). The crude protein content usually varies from 36 to 41% depending on the amount of hulls added and the processing method. The protein quality can vary according to the different processing conditions. The meal is generally low in lysine, calcium, and carotene. It is a good source of phosphorus. Raw cottonseeds contain appreciable amounts of free gossypol, which can be toxic to nonruminants. Most of the free gossypol is destroyed due to the heat during processing. It is relatively non-toxic and is palatable to ruminants (Tanksley, 1990).

Distillers Dried Grains with Solubles. This product is obtained after the removal of ethyl alcohol by distillation from the yeast-fermented grains, and by condensing and drying at least three-fourths of the solids of the resultant whole stillage. The composition of the feed may be influenced by the raw materials used, as well as processing procedures. The crude protein content of this feed is approximately 27% (NRC, 1988). Distillers dried grains with solubles have been successfully used in dairy, beef, and sheep diets primarily as a source of protein. Due to the relatively high crude fiber levels (4.4 to 12.1%) and high fat contents (8.4 to 9.8%) the feed is also valued as an energy source for

ruminants. As a whole, distillers dried grains with solubles are a good source of vitamin E, water soluble vitamins and phosphorous (Newland and Mahan, 1990).

Fish Meal. As a by-product of the fisheries industry, fish meal is produced using dried, ground whole fish or fish cuttings, with or without the extraction of part of the oil. Several types of fish meals are commercially available depending on the type of fish (Herring, Menhaden, Sardine etc.) used. The protein content in all the fish meals usually ranges between 55 to 70% (Morrison, 1950). Fish meal is a rich source of essential amino acids, including lysine, methionine, and tryptophan. Therefore, it is a useful supplement to cereal-based diets. Also, it is a good source of B-vitamins and minerals such as calcium and phosphorus. The feeding value of fish meals can vary according to the method of drying, the type of raw material used and the partial decomposition before processing. Presence of high levels of fish oil in the meal can impart a fishy taste to eggs, meat, and milk, and can lead to rancidity during storage. The high price limits the use of this valuable feed in animal diets (McDonald et al., 1982).

Meat and Bone Meal. In meat processing plants, a fair amount of usable animal tissues and bones are discarded. Those tissues and bones are dried and ground to make a valuable protein meal. The crude protein content in meat and bone meal usually ranges between 45 to 50% (Ensminger and Olentine, 1978). The protein of this meal is of fairly good quality with a high concentration of lysine. The protein quality may differ according to processing and storage conditions. Because of its rich bone content, meat and bone meal is a very good source of minerals particularly calcium, phosphorus, and magnesium. It is also high in B complex vitamins (McDonald et al., 1982).

Soybean Meal. This byproduct feed is reputed as the most popularly used protein ingredient in animal diets. Soybean meal is produced during the extraction of oil from soybeans by solvent or mechanical methods. The meal is toasted and ground during processing. The protein content of soybean meals is generally standardized by dilution with soybean hulls. The solvent extracted soybean meals usually contain between 44 to 50% crude protein (NRC, 1988). The expeller process tends to extract less oil than the solvent process. Consequently, the expeller-extracted soybean meal contains a lower amount of protein (41 to 44%) and a higher amount of fat (4 to 5%) than that of solvent extracted soybean meals. The popularity of soybean meals in animal diet formulation is

attributed to several factors including widespread availability, palatability, and high protein and energy contents. Soybeans have a number of stimulatory (e.g. genistein) and inhibitory (e.g. antitrypsin factor, saponins) substances. Most of these substances are usually destroyed due to the heat during processing (Church and Pond, 1988).

Casein. This is a generic term for a group of phosphoproteins, which constituted to about 85% of the total milk protein. In milk, casein exists as its calcium salt (viz. Calcium casienate) in distinct globular particles (micelles) of <10 to 780 mμ. Each micelle contains four recognized components (α_s, β, κ and γ-casein), which differ in their electrical charge. Commercial casein is obtained from fat-free skim milk by precipitation with acids, ammonium sulfate and enzymes (rennin), and by sedimentation using high speed centrifugation (Brunner, 1977). Purified casein has been used in many ruminal studies (Russell et al., 1983; Broderick and Wallace, 1988) as a reference protein.

Chapter III

OBJECTIVES

The overall objective of the research presented in this dissertation was to demonstrate the potential of forestomach tissues of sheep to absorb peptides and free amino acids produced due to the microbial degradation of proteins in the rumen. Specific objectives included:

1) To estimate peptide, free amino acid, and ammonia productions in the extracellular medium when different dietary proteins are incubated (in vitro) with mixed ruminal microorganisms.
2) To observe the influence of milling differences of a protein on peptide, amino acid, and ammonia production following ruminal microbial degradation.
3) To determine the compositions of free and peptide-bound amino acids persisting in the extracellular medium following ruminal microbial degradation of different proteins and among different batches of a protein.
4) To investigate the ability of ruminal and omasal epithelia of sheep to absorb ruminally-produced peptides and free amino acids by in vitro methods.
5) To observe whether dietary proteins influence this latter process.

Chapter IV

OBSERVATIONS ON RUMINAL PROTEIN DEGRADATION PRODUCTS FOLLOWING IN VITRO INCUBATION WITH MIXED MICROORGANISMS

ABSTRACT

The influence of proteins and milling procedures on the production of peptide, α-amino, and ammonia-N was investigated following incubation of proteins using a mixed microbial culture prepared from ruminal contents of lactating cows. The proteins evaluated were solvent soybean meal, dehydrated alfalfa, corn gluten feed (CGF), fish meal, distillers dried grains with soluble (DDG), cotton seed meal (CSM), brewers dried grains, meat and bone meal, blood meal, prolac and casein. Soybean meals and DDG obtained from different mills and at different times from the same mill were also compared in separate experiments. The concentrations of peptide N, α-amino N, and ammonia N appearing in the cell free media were measured at 0, 2, 4, 6 and 8 h of incubation. The amino acid profiles of free and peptide-bound fractions at 8 h were measured. The concentrations of peptide N and ammonia N were increased ($P < 0.001$) with time. The concentrations of α-amino N were lower ($P < 0.05$) than the concentrations of peptide N and ammonia N. There were time *x* protein interactions ($P < 0.05$) between proteins and between batches for peptide, α-amino, and ammonia-N. Different proteins and batches had amino acid *x* protein interactions ($P < 0.05$) for concentrations of total essential, total nonessential, total and individual amino acids (8 h) in free and peptide-bound fractions. Low molecular weight (< 3,000 MW) peptide amino acids contributed between 30 (CSM) and 55% (CGF) of the total peptide amino acids at 8 h. Irrespective of the protein used, specific patterns in the amino acid contents of peptides (< 3,000 MW) were noted; methionine and histidine contents were very low, while proline, glycine and alanine contents were high in this fraction. During protein degradation in the rumen, peptides can accumulate in the ruminal fluid and the microorganisms can exhibit a differential utilization of peptides.

(**Key words** : Cow, Rumen, Microorganisms, Protein, Amino acid, Peptide)

Introduction

Dietary proteins consumed by ruminants may be degraded extensively by microbial activity in the rumen. Ruminal microorganisms degrade feed proteins through a series of steps and synthesize microbial proteins or yield energy by fermentation (Russell et al., 1991). Peptides, amino acids, and ammonia are produced as intermediates or end products during this process (Annison, 1956). Early investigations often indicated low concentrations of peptides and amino acids in the ruminal fluid which was believed to be because of the rapid degradation of these by ruminal microorganisms (Wright and Hungate, 1967; Mangan, 1972).

Evidence indicates that peptides (Chen et al., 1987a; Broderick et al., 1990) and, to a lesser extent, free amino acids (Broderick and Kang, 1980) can accumulate in ruminal fluid for a considerable time post feeding. It also appears that some peptides (Chen et al., 1987c; Yang and Russell, 1990) and free amino acids (Chalupa, 1976) are particularly resistant to ruminal microbial degradation. Evidence continues to accumulate suggesting that peptides and free amino acids resulting from the microbial activity in the rumen can be an important source of N for ruminants (Webb and Matthews, 1994). Therefore, carefully studying the factors governing peptide and amino acid production in the rumen will be of benefit in planning future ruminant feed formulations. The present study assumes that the variations among proteins and processing conditions can influence the amounts and the types of protein degradation products accumulating in the rumen. Hence, a series of experiments were conducted to make quantitative and qualitative estimates of peptide, free amino acid, and ammonia production in ruminal fluid during incubation (in vitro) of a variety of proteins and to investigate how the differences of milling may affect this process.

Materials and Methods

Unless noted otherwise, all chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Preparation of the Inoculant Enriched with Mixed Ruminal Microorganisms. Ruminal contents were obtained from two ruminally cannulated lactating Holstein cows fed a diet consisting of: 337 g alfalfa hay, 225 g barley silage, 247 g high moisture corn, 90 g dried brewers grain, 67 g whole cotton seeds, 22 g soybean meal, and 12 g

minerals/kg DM. Whole ruminal contents were collected from the bottom of the rumen approximately 2 h after feeding. Strained ruminal fluid (SRF) was obtained by squeezing the ruminal contents through eight layers of cheesecloth. To extract some of the particle-associated organisms, the remaining solid residue was washed four times with a total volume of warm (39^0C) buffer (Table 4.1) equal to the original volume of SRF. The SRF plus buffer extract were mixed and filtered through eight layers of cheesecloth. Ruminal contents collected from two cows were processed in a like manner and equal volumes were mixed together. This SRF plus buffer extract was then centrifuged at 5,000 *x* g for 30 min to harvest mixed ruminal microorganisms (Luchini et. al., 1996). The pellets obtained after centrifugation were blended (30 s) with a volume of nutrient medium (Table 4.1) equal to the original volume of SRF. The harvested, mixed ruminal microorganisms were then transferred to a 4L bottle and allowed to incubate at 39^0C in a water bath under CO_2 for 6 h (Preincubation; Luchini et. al., 1996). The necessity of having a preincubation period was noted in preliminary investigations. The bottle containing the inoculant was shaken manually at regular intervals during preincubation to prevent sedimentation. The composition of the buffer and the nutrient medium used are presented in the Table 4.1. Mercaptoethanol was added (0.16 mL/L) to the nutrient medium to serve as a reducing agent during incubation of proteins (Broderick, 1987). The pH of the buffer and the nutrient medium was adjusted to 6.9 and was saturated with CO_2. The temperature was maintained at 39^0C using a constant temperature bath.

*In vitro Incubation of Dietary Proteins. P*roteins were collected from three major feed manufacturers located around the Virginia Polytechnic Institute and State University, namely, Roanoke City Mill, Roanoke, VA (RCM), Big Spring Mill, Elliston, VA (BSM), and Southern States Mill, Richmond, VA (SSM). A total of 18 protein sources (one kg each) were collected from the above feed manufacturers. Air dried samples were ground to pass through a 1 mm screen using a Cyclotech mill and subsamples of 250 g were obtained. The crude protein contents of the protein sources were analyzed (AOAC, 1990) and are shown in Table 4.2. Five proteins were used in each incubation experiment (except in Exp. 5 where only casein was used). The protein sources used in each experiment were as follows:

Exp. 1: Soybean meal (solvent, RCM), fish meal (menhaden, SSM), dehydrated alfalfa (RCM), corn gluten feed (RCM), and distillers dried grains with soluble (RCM),
Exp. 2: Cottonseed meal (SSM), brewers dried grain (SSM), blood meal (SSM), meat and bone meal (SSM), and prolac (BSM) which contained 42% meat and bone meal, 40% hydrolyzed feather meal, and 14% fish meal,
Exp. 3: Four solvent-extracted SBM samples and one expeller-extracted SBM sample (ESB) were compared. Samples of solvent soybean meal included batches from different mills and batches from the same mill processed at different times. These were solvent soybean meal of Big Spring Mill (SSB), Southern States Mill (SSS) and Roanoke City Mill batches 1 and 2 (SSR1 and SSR2, respectively),
Exp. 4: Different batches of distillers dried grain with solubles were compared (DGR1, DGR2, DGR3, DGB, DGS). The DGR1, DGR2, and DGR3 represented three different batches obtained from the same mill (RCM). DGB and DGS represented distillers dried grains with soluble samples of Big Spring Mill and Southern States Mill, respectively, and
Exp. 5: Only casein (United States Biochemical Corporation) was compared with a control treatment (no added protein source).

Incubation was performed in plastic tubes (50 mL) sealed with rubber stoppers that were fitted with Bunsen gas release valves as described by Tilley and Terry (1963). The incubation time periods, amounts of proteins, and inoculant added to the tubes were all decided based on preliminary experiments. Duplicate tubes from five protein treatments and the control (blank tubes with all components except proteins added) were allowed to incubate 0, 2, 4, 6, and 8 h in a constant temperature bath (39^0C). The proteins equal to 0.5 mg N/mL of inoculant were placed in each tube before the incubation started. Incubations began by dispensing 20 mL of the inoculant into each tube using an Oxford bottle top dispenser. The space above the liquid in each tube was flushed with CO_2. The tubes were then sealed with rubber stoppers and stirred on a vortex mixer. This point was considered as time zero. To confirm viable ruminal microbial activity during the study period, the numbers of active protozoa surviving in the media were estimated (by microscopic examination) in some randomly selected tubes at the beginning and end of incubation. The incubation was arrested by adding 5 mL of

25% trichloroacetic acid (TCA) into each tube and stirring on a vortex mixer at each time point. The tubes were then held on ice (4^0C) for 60 min and centrifuged (27,000 *x* g, 25 min, 4^0C) to harvest a cell free supernatant containing soluble components including ammonia, amino acids, and peptides produced as a result of the degradation of proteins. Ten milliliters of the supernatant from each tube were drawn and stored at -20^0C until analyzed. Incubations were conducted on four separate runs for each study (except in Exp. 5) using the same procedures and cows. For Exp. 5, incubations were performed in one run using eight replicate tubes for each point of time of casein and control treatments.

Chemical Analysis. The concentration of ammonia N in each supernatant was measured by the indophenol reaction as described by Chaney and Marbach (1962). Total and α-amino N concentrations were analyzed using the ninhydrin color reaction in hydrolyzed and nonhydrolyzed (6N HCl, 24 h, 110^0C) samples as described by Broderick and Kang (1980). The concentration of peptide N was calculated as the difference between hydrolyzed and nonhydrolyzed samples. The amino acid composition of free and peptide bound fractions that persisted in the ruminal fluid at the end of incubations (8 h) were determined by HPLC. The samples were analyzed using two procedures: without filtration or with a filtration step using a Centricon-3-microconcentrator of 3,000 MW cut-off filter (Amicon, Beverly, MA). The filtrates obtained after centrifugation (2,700 *x* g, 2 h) by both methods were divided into two parts: one for the determination of free amino acids, the other for the determination of total amino acids after acid hydrolysis (vaporized HCl at 112^0C for 24 h). The individual amino acid concentrations were determined using a Pico Tag Amino Acid Analysis System (Waters Millipore Corp., Milford, MA). Peptide amino acid concentrations were calculated as the difference between hydrolyzed and nonhydrolyzed samples.

Statistical Analysis. Data were analyzed as a completely randomized design. The effects of protein sources, time, and protein sources x time on peptide N, α-amino N, and ammonia N concentrations were evaluated by analysis of variance using the GLM procedure of SAS (1988). The differences among protein sources on the mean concentration of peptide N, α-amino N, and ammonia N concentrations at each time interval were compared using the Tukey's Student Range Test. The statistical significance of differences among individual amino acid concentrations between the

protein treatments was assessed using the GLM procedure of SAS (1988), followed by Duncan's Multiple Range test. Orthogonal contrast was used to compare free vs peptides and peptides (total) vs peptides (< 3,000 MW) for essential (EAA), nonessential (NEAA), total, and individual amino acids.

Results

Changes in Ammonia N Concentrations With Time. Figure 4.1A shows the changes in concentration of ammonia N in the extracellular media during incubation of five proteins (DA, SBM, DDG, FM and CGF) used in Exp.1. When the N concentrations of ruminal protein degradation products obtained at 0, 2, 4, 6 and 8 h of incubations were pooled for each protein, ammonia N accounted for between 52 (CGF) to 82% (FM) of the total N. The ammonia N concentration increased linearly ($P < 0.01$) with time in all proteins, and there was a time *x* protein interaction ($P < 0.01$). The mean ammonia N concentration varied from 5.82 ± 3.22 at 0 h to 171.44 ± 11.28 mg/L at 8h. At 6 and 8 h of incubation, FM had the highest ($P < 0.05$) ammonia N concentrations.

The proteins used in Exp. 2 (CSM, BDG, BLM, PRL and MBM) also showed a linear increase ($P < 0.05$) in the concentration of ammonia N with time (Figure 4.2A). The mean ammonia N concentrations varied from 3.65 ± 2.22 at 0 h to 276.73 ± 14.30 mg/L at 8h. There was also a time *x* protein interaction ($P < 0.05$). The treatments of MBM and PRL had the highest ($P < 0.05$) ammonia production at 8 h. The contribution of ammonia N to the total estimated ruminal degradation products of Exp. 2 was between 78 (MBM) and 86% (CSM).

Presented in the Figure 4.3A are the changes in ammonia N concentration during incubation of different batches of SBM used in Exp.3. Ammonia N contributed between 80 (ESB) and 83% (SSB) of the total ruminal protein degradation products of Exp. 3. The different batches of SBM had a time *x* protein interaction ($P < 0.05$) in ammonia N concentrations. The changes in ammonia N concentration with time among different SBM were linear ($P < 0.001$) for SSR2, SSB and SSS and (or) quadratic ($P < 0.05$) for SSR1 and ESB. The ammonia N concentration of ESB at 2, 4, 6 and 8 h were lower ($P < 0.05$) than the solvent extracted meals of SSR2, SSB and SSS.

Data presented in the Figure 4.4A show the changes in ammonia N concentration following incubation of different batches of DDG (Exp. 4). Ammonia N

accounted for between 65 (DGR3) to 71% (DGR1) of the total ruminal protein degradation products of Exp. 4. The different batches of DDG also showed a time *x* protein interaction ($P < 0.05$) in ammonia N concentration. The pattern of ammonia N production was linear ($P < 0.001$) for DGR1, DGR2 and DGR3, and (or) quadratic ($P < 0.001$) for DGB and DGS. The treatment of DGB had higher ($P < 0.05$) ammonia N production than the other treatments at 2, 4, 6 and 8 h of incubation.

Very high ammonia N production (from 7.28 ± at 0 h to 1,121.86 ± mg/L at 8 h) was observed during the incubation of casein (Figure 4.5). Ammonia N accounted for 78% of total N of estimated protein degradation products in comparison to 8% for α-amino N and 14% for peptide N in casein. The ammonia N concentration of casein increased quadratically ($P < 0.001$) with time.

Changes in a -Amino N Concentrations With Time. Presented in the Figure 4.1B are the changes in α-amino N concentrations following incubation of different proteins in Exp.1. The concentrations of α-amino N were much lower than ammonia N concentrations contributing between 4 (FM) to 11% (CGF) of the total N in protein degradation products. The mean α-amino N concentrations obtained by averaging 40 observations of each protein incubated to 0, 2, 4, 6 and 8 h varied between 3.88 ±1.47 (SBM) and 23.02 ± 3.15 (CGF) mg/L. There was a time *x* protein interaction ($P < 0.05$) primarily because of the fact that there was a decrease in α- amino N concentration with CGF and DA during the first 4 and 6 h. In contrast, concentration of α-amino N were influenced little by time for DDG, SBM and FM. Corn gluten feed showed the highest ($P < 0.05$) α-amino N concentrations across all times in Exp. 1.

Relatively low concentrations of α-amino N were consistently observed in the incubation of different proteins (Figure 4.2B), different SBM (Figure 4.3B) and different DDG (Figure 4.4B) used in the experiments of 2, 3, and 4, respectively. The mean concentrations of α-amino N obtained by averaging 200 observations of Exp. 1, Exp2, and Exp. 3 were 7.99 ± 1.11, 5.96 ± 0.57 and 1.23 ± 0.49 mg/L, respectively. Also, variable patterns in the concentration of in α-amino N with time were noted among proteins. Increases in α-amino N concentration after a lag period of 2 to 4 h were observed with all the proteins used in Exp. 2 (Figure 4.2B). The α-amino N

concentration tended to increase after a lag period (2 to 4 h) with all the SBM treatments (Figure 4.3B). The α-amino N concentration in all the DDG treatments (Exp. 4) declined with time ($P < 0.05$), and were near zero after 8 h of incubation (Figure 4.4B). Incubation of casein (Exp. 5) showed a quadratic ($P < 0.001$) increase in α-amino N concentration with time, and the concentration varied from 4.57 ± 2.08 at 0 h to 108 ± 7.63 mg/L at 8 h (Figure 4.5).

Changes in Peptide N Concentrations With Time. The data for peptide N concentration following incubation of proteins in the Exp.1 are presented in Figure 4.1C. The mean concentrations of peptide N obtained by averaging all 40 observations for each protein incubated to 0, 2, 4, 6 and 8 h varied from 44.16 ± 3.05 (SBM) to 103.64 ± 5.27 (CGF) mg/L. Peptide N contributed a considerable proportion, representing 14 (FM) to 36% (CGF) of the total ruminal protein degradation products of Exp. 1. There were differences in peptide N production among proteins as indicated by a time *x* protein interaction ($P < 0.01$). Corn gluten feed had the highest peptide N concentration across all time intervals. The initial concentration of peptide N in CGF was fairly high which showed a declining trend before leveling off at about 4 h. The peptide N concentration increased linearly ($P < 0.05$) during the incubation of other proteins in Exp. 1.

The contribution of peptide N to the total estimated ruminal protein degradation products of Exp. 2 was between 11 (BLM) to 17% (MBM). The mean concentration of peptide N (22.06 mg/L) obtained by averaging all 200 observations in the Exp. 2 was lower ($P < 0.05$) than the ammonia N concentration (137.24 mg/L) and was higher ($P < 0.05$) than the α-amino N concentration (7.99 mg/L). All the proteins used in the Exp. 2 showed a linear ($P < 0.05$) increase in peptide production (Figure 4.2B). Also, there was a time *x* protein interaction ($P < 0.05$) in peptide N concentration. The treatments of MBM and PRL had a comparatively higher ($P < 0.05$) peptide N concentration than the other treatments across all the times.

When different batches of SBM were incubated, Peptide N accounted for 13% of the total N in the estimated protein degradation products in comparison to 5% for α-amino N, and 82% for ammonia N. The accumulation of peptide N during incubation of different SBM (Exp. 3) was obvious (Figure 4.3C). While there were some differences ($P < 0.05$) among SBM samples, all responded generally the same with a linear ($P <$

0.01) increase in peptide N concentration with time. Incubation of different batches of DDG also showed a linear ($P < 0.05$) increase of peptide N concentration with time (Figure 4.4C). Peptide N contributed to 28% of the total N in protein degradation products in comparison to 3% for α-amino N, and 69% for ammonia N during the incubation of different batches of DDG. A higher ($P < 0.05$) average peptide N production than the other DDG treatments was observed with DGR1.

An accumulation of peptide N (from 7.86 at 0 h to 194 mg/L at 8 h) was observed during the incubation of casein (Figure 5). The peptide N concentration of casein increased quadratically ($P < 0.001$) with time.

The Amino Acid Composition of Ruminal Protein Degradation Products. The concentrations of amino acids present in the extracellular media following incubation (8 h) of DA, SBM, FM, CGF and DDG (Exp. 1) are given in Table 4.3. The data include 16 amino acids that were present in free, peptide (total) and peptide (< 3,000 MW) bound fractions. Asparagine, cystine, glutamine, and tryptophan are not included in the data, as these amino acids are known to be severely affected during hydrolysis (Blackburn, 1968; Wallace et al., 1993). The mean concentration of EAA in the free form was greater ($P < 0.002$) than the concentration of EAA in peptide form. The mean concentration of NEAA was greater ($P < 0.01$) for peptides than for free amino acids. The concentrations of peptide-bound aspartate, glycine, proline, serine and tyrosine were higher ($P < 0.001$) than their concentrations in the free form. Conversely, the concentrations of arginine, histidine, methionine and threonine were higher ($P < 0.001$) in the free form than in the peptide-bound form. The low molecular weight peptide (< 3,000 MW) amino acids contributed between 34% (SBM) and 55% (CGF) of the total peptide-bound amino acids. The concentrations of EAA, NEAA and total amino acids were greater ($P < 0.001$) for peptides (total) than for peptides (< 3,000 MW). The mean concentrations of arginine, histidine, methionine and threonine did not differ ($P > .05$) between the two peptide fractions. Variations ($P < 0.01$) among protein sources were observed in EAA, NEAA, and total amino acids within free, peptide (total), and peptide (< 3,000 MW) amino acid fractions. Corn gluten feed had the highest ($P < 0.05$) amino acid concentrations for EAA, NEAA and total amino acids in both free and peptide forms. Some similarity across treatments was observed in the appearance of certain amino acids within the low

molecular weight (< 3,000 MW) peptide fraction. All proteins of Exp. 1 had a zero concentration of peptide-bound methionine following 8-h incubation. Also, the concentration of histidine was very low (between 0 to 4% of total amino acids). The concentrations of peptide-bound (< 3,000 MW) glutamate, proline, glycine, and alanine tended to be high among all the proteins. Glutamate, proline, glycine, and alanine in combination contributed between 46 (DA) to 55% (FM) of the total peptide-bound (< 3,000 MW) amino acids.

The variations in amino acid composition in the cell free media following the incubation of proteins in the Exp. 2 are shown in the Table 4.4. The mean concentrations of EAA and total amino acids were lower ($P < 0.01$) for peptides than for amino acids in the free form. The opposite was true for NEAA. The concentrations of peptide-bound aspartic acid, glutamate, proline, glycine, and serine were higher ($P < 0.01$) than their concentrations in the free form. The low molecular weight (< 3,000 MW) peptide-bound amino acids contributed between 30 (CSM) and 48% (MBM) of the total peptide-bound amino acids. The concentrations of EAA, NEAA, and total amino acids were lower ($P < 0.001$) for peptides (< 3,000 MW) than for peptides (total). Differences ($P < 0.01$) among protein treatments were found in the EAA, NEAA and total amino acid concentrations of free and peptide-bound fractions. In the low molecular weight (< 3,000 MW) peptides, the concentration of histidine was very low (< 1% of total amino acids) among all the treatments. Glutamate, proline, glycine and alanine in combination contributed between 44 (CSM) to 67% (MBM) of the total peptide-bound (< 3,000 MW) amino acids.

The mean concentrations of EAA ($P < .001$), NEAA ($P < 0.07$), and total amino acids ($P < 0.001$) were lower for peptides than for free amino acid fraction during the incubation of different SBM (Table 4.5). The concentrations of EAA ($P < 0.005$) and total amino acids ($P < 0.001$) were lower ($P < 0.001$) for peptides (< 3,000 MW) than for peptides (total). Variations ($P < 0.01$) among different SBM sources were noted for peptide (total) bound amino acids. The low molecular weight peptides accounted for 16 (SSR1) to 33% (SSB) of the total peptide bound amino acids. The treatments of SSR1, SSR2, SSS and ESB had a zero concentration of peptide-bound (< 3,000 MW) methionine after 8 h of incubation. The concentrations of peptide-bound (< 3,000 MW)

arginine, histidine and lysine were also low among all SBM treatments. Proline, glycine and alanine in combination contributed between 71 (SSB) and 88% (SSR2) of the total peptide-bound (< 3,000 MW) amino acids of SBM treatments.

Table 4.6 shows the amino acid composition data of different DDG treatments (Exp. 5). The mean concentrations of EAA, NEAA, and total amino acids were greater ($P < 0.001$) for peptide than for free amino acid fraction. Variations among different DDG treatments were found in the concentration of peptide bound EAA, NEAA, total, and individual amino acids. The low molecular weight (< 3,000 MW) peptides contributed between 29 (DGR3) to 40% (DGB) of the total peptide-bound amino acids. The mean concentrations of EAA, NEAA, and total amino acids were greater ($P < 0.001$) for peptide (total) than for peptide (< 3,000 MW). Variations were also noted among different DDG sources in the concentrations of peptide-bound (< 3,000 MW) amino acids. The concentration of peptide-bound (< 3,000 MW) methionine was zero (DGR3, DGB and DGS) or very low (DGR1 and DGR2). Alanine, glutamate, glycine and proline contributed to a large proportion (between 79 to 84% of the total amino acids) in the peptides (< 3,000 MW) of all DDG treatments.

The composition of free and peptide-bound amino acids persisted in the media following incubation (8 h) of casein is presented in the Table 4.7. Relatively higher concentrations of EAA, NEAA, and total amino acids were observed in the free ($P < 0.001$) than in the peptide-bound fractions. The concentrations of aspartic acid, glycine, proline, serine, and threonine were higher ($P < 0.001$) in the peptide fractions than in the free amino acid fraction. The low molecular weight (< 3,000 MW) peptides accounted for 48% of the total peptide-bound amino acids in casein. The patterns of amino acid appearance in the peptide (< 3,000 MW) fraction were similar to those observed in the experiments 1, 2, 3 and 4. Zero concentrations of histidine, methionine, and tyrosine were found in the peptide (3,000 MW) fraction while glutamate, proline, glycine, and alanine accounted for over 70% of the total amino acids in this fraction.

Discussion

A broad objective of the present series of experiments was to understand the patterns and the possible mechanisms involved in the production of peptide N, α-amino N, and ammonia N during dietary protein degradation in the rumen. There are several

reasons why results from this type of study would be of value. These include the following: (i) the amount of feed proteins that can be converted to peptides, free amino acids, and ammonia in the rumen appears to be quite considerable (Annison, 1956; Russell et al., 1991), (ii) all of these products have been shown to contribute directly to the ruminal microbial protein synthesis (Nolan, 1975; Argyle and Baldwin, 1989), (iii) there is a wide interest in controlling ruminal protein fermentation at peptide and free amino acid levels (Broderick et al., 1991), and (iv) peptides and free amino acids resulting from microbial activity in the rumen have been suggested as important substrates for direct absorption via the ruminant forestomach and intestine (Webb and Matthews, 1994). But, time course data on the appearance and metabolism of ruminal protein degradation products are somewhat limited and are often confusing due to the differences in substrates, experimental procedures, and animals or microorganisms used.

The production of peptide N, α-amino N, and ammonia N was measured in this study by incubating (in vitro) a variety of common dietary proteins with a mixed ruminal microbial culture and measuring the N concentrations associated with each fraction in the cell free media at regular time intervals. Time course data on ruminal degradation products using purified proteins or synthetic peptides are available (Broderick and Wallace, 1988; Wallace et al., 1993). But ruminal microorganisms usually do not encounter such substrates under normal feeding conditions. Therefore, protein ingredients commonly used in animal diet formulations were selected for these experiments. Also, the substrates consisted of different proteins (Exp.1 and Exp. 2) as well as the different batches of the same protein (Exp. 3 and Exp. 4).

Previous measurements of ruminal protein degradation products carried out by in vivo procedures are available (Annison, 1956; Leibholz, 1969). As soon as they are produced, the ruminal protein degradation products can leave the rumen with the digesta flow to the duodenum (Chen et al., 1987b) or can be absorbed across the forestomach epithelia (Leibholz, 1971). Therefore, measurements of peptide N, α-amino N, and ammonia N concentrations in ruminal fluid in vivo, may not provide the actual patterns of these compounds produced during ruminal protein degradation. In the present study, a mixed microbial culture prepared from the ruminal contents obtained from lactating dairy cows was used to simulate the microbial activity in the rumen. The amount of protein

added (0.5 mg N / mL of inoculation medium) to each incubation tube was decided upon based on the preliminary results (data not shown) and this level reasonably compares with the level of protein usually present in the ruminal fluid following dietary protein supplementation (Waldo, 1967). Additionally, (i) nutrients and other conditions required for a normal ruminal microbial activity (Johnson, 1963) were maintained throughout incubations, (ii) incubations were performed for a relatively short time (8 h) to prevent inhibition of microbial activity due to the accumulation of end products, and (iii) the presence of active protozoa during incubations was confirmed by microscopic examinations of protozoal numbers surviving in the media. Therefore, the catabolic and anabolic processes of the ruminal microorganisms can be expected to be at least somewhat similar to what would occur under in vivo conditions during the incubation period studied.

Peptides, amino acids, and ammonia are produced in the medium not only due to the degradation of feed proteins, but also from the microbial proteins recycled during ruminal incubations. The amounts of N recycled from microbial proteins could be considerable both under in vitro and in vivo conditions. With respect to the above, Leng (1973) estimated that approximately 30% of the bacterial proteins produced are usually degraded within the rumen in intact animals. To help account for the effects due to recycling of microbial proteins, and the residual N compounds of the inoculant, references were made using control treatments (with no added proteins). Therefore, the measurements of peptide N, α-amino N, and ammonia N should reasonably represent the effect due to dietary protein addition.

The specific objective of the experiments 1 and 2 was to observe the production of peptide N, α-amino N, and ammonia N during the incubation of different dietary proteins. A total of ten different proteins were evaluated in Exp. 1 (SBM, FM, DA, CGF and DDG) and in Exp.2 (CSM, BDG, PRL, BLM and MBM). The objective of the next two experiments was to investigate how different batches of SBM (Exp. 3) and DDG (Exp.4) influence the production of peptide N, α-amino N, and ammonia N during incubation. Four solvent-extracted SBM samples obtained from three mills (SSR1, SSR2, SSB and SSS) and one expeller-extracted SBM sample (ESB) were evaluated in the Exp. 3. The SSR1 and SSR2 were obtained at two different times from Roanoke City

Mills. Three DDG samples obtained from Roanoke City Mills (DGR1, DGR2 and DGR3) and a sample each from Big Spring Mill (DGB) and Southern States Mill (DGS) were tested in the Exp. 4. The objective of the Exp. 5 was to investigate the production of peptide N, α-amino N and ammonia N during incubation of the purified protein, casein.

A large accumulation of ammonia N was observed in all the experiments. This agrees well with previous measurements on ammonia N (Annison, 1956), which recognized ammonia as the major end product of protein catabolism in the rumen. The protein utilization by ruminal microorganisms is known to occur in several distinct steps including solubilization, proteolysis, peptide hydrolysis, transport of peptide and amino acids into bacteria, fermentation, and microbial protein synthesis (Russell et al., 1991). Accumulation of ammonia N during incubations indicates that the ruminal protein fermentation occurs extensively and the ammonia is produced in excess of the microbe's capacity to utilize it for protein synthesis.

The concentrations of α-amino N were frequently low and the pattern of α-amino N appearance varied widely among proteins. Other workers also reported similar concentrations of α-amino N in the extracellular ruminal fluid (Wallace, 1979; Broderick and Wallace, 1988). The low extracellular concentrations of α-amino acids suggest that the degradation of peptides to free amino acids mostly occur intracellularly. Rapid uptakes of amino acids by ruminal microorganisms and/or rapid deamination could also be possible reasons for low extracellular concentrations of free amino acids. When Broderick and Craig (1989) incubated casein and bovine serum albumin with mixed ruminal microorganisms, the intracellular free amino acid concentrations started to increase prior to the appearance of extracellular free amino acids. This observation suggests that peptide uptake followed by intracellular hydrolysis may be the major route of amino acid absorption by ruminal microorganisms. The enhanced growth responses observed when amino acids were supplied as peptides in comparison to the free forms (Argyle and Baldwin, 1989) further substantiates the thought that peptides are the preferred substrates for absorption by ruminal microorganisms. Thus, low concentrations of free amino acids can frequently appear in the extracellular ruminal fluid.

The peptide N was produced to a substantial level and there was a continuous build up in the concentration of peptide N during incubations. Accumulation of peptide N observed throughout the present series of experiments is in agreement with some previous observations on ruminal protein degradation products (Russell et al., 1983; Chen et al., 1987a). The above observations strongly indicate that the ruminal microorganisms degrade dietary proteins extracellularly to peptides, and that the subsequent steps in the degradation of proteins can occur at relatively slower rates than the rate of proteolysis. Therefore, the extracellular hydrolysis of peptides and/or peptide transport into the microbial cells could be rate limiting steps during protein utilization by ruminal microorganisms (Chen et al., 1987a). Also, it was reported that ruminal microorganisms can saturate their growth responses to peptides and amino acids at low concentrations such as 10 mg/L (Argyle and Baldwin, 1989), and some peptides are particularly resistant to further degradation in the rumen (Yang and Russell, 1992). As a result, ruminal microorganisms may not be utilizing large quantities of peptides produced due to proteolysis. Hence, the peptides can accumulate in the extracellular ruminal fluid during the degradation of dietary proteins in the rumen.

Accumulation of total peptide-bound amino acids was always greater than the low molecular weight (< 3,000 MW) peptide amino acids. This observation suggests that some of the accumulated peptides are of large molecular weight (>3,000 MW). The accumulation of larger peptides indicates that the size of those peptides may have influenced their further degradation in the rumen. As the peptide transport systems of ruminal bacteria are known to transport peptides below 5 to 16 amino acid residues (Russell et al., 1991), peptides of transportable size should be produced by extracellular peptidase activity. Therefore, the accumulation of large molecular weight peptides in the medium could have been due to the lack of extracellular peptidase activities to degrade them into a transportable size.

Substantial proportions of low molecular weight (< 3,000 MW) peptides were also present in the extracellular medium. If the average molecular weight of an amino acid of a peptide is assumed to equal 137 Da (Chen et al., 1987a), then this fraction should contain peptides smaller than 22 amino acid residues and are mostly of transportable size. Thus, the factors other than the size would have been more important

on the resistance of these peptides to the uptake by ruminal microorganisms. Presence of some common patterns observed in the amino acid profiles of this peptide fraction throughout all the incubation experiments indicate that the amino acid composition and the structure of low molecular weight peptides can influence their uptake by ruminal microorganisms. Some previous studies (Chen et al., 1987c; Broderick et al., 1988) also proposed the presence of such patterns in the peptides present in the ruminal fluid. When Chen and coworkers incubated tripticase (pancreatic digest of casein containing mostly peptides) with mixed ruminal bacteria, peptides containing hydrophilic amino acids (arginine, aspartic acid, glutamate and lysine) were metabolized more rapidly than those containing hydrophobic amino acids (leucine, tryptophan, tyrosine, phenylalanine, proline and valine). When the enzymatic digest of casein and gelatin were incubated, mixed ruminal bacteria were unable to utilize all of the peptides even when the incubation period was as long as 96 h (Yang and Russell, 1992). Those peptides that persisted in the media contained a large proportion of proline. All above observations imply that the ruminal microorganisms can have preferences or resistance in the utilization of certain peptides and the composition and structure of the peptides appear to be important determinants of their susceptibility or resistance to microbial degradation. The results of the present study support the idea that ruminal microorganisms prefer methionine and possibly histidine and tyrosine containing peptides. Consequently, peptides containing those amino acids can be frequently lacking in the extracellular peptide (< 3,000 MW) fraction of the ruminal digesta. Alternatively, the presence of relatively high proportions of glutamate, proline, glycine and alanine containing peptides were noted irrespective of the protein used. High glutamate contents could probably be due to the presence of high concentration of this amino acid in the dietary proteins used (Jurgens, 1993). However, proline, glycine and alanine contents are generally not found in very high concentrations among the feed proteins used. The resistance of proline (Yang and Russell, 1992) and glycine (Broderick et al., 1988) containing peptides to further degradation by ruminal microorganisms were demonstrated previously using synthetic peptides. Therefore, high proline, glycine, and alanine contents in the low molecular weight (< 3,000 MW) peptide fraction indicate that the peptides containing those amino acids could be resistant to further degradation by ruminal microorganisms

due to lack of uptake mechanisms and extracellular peptidase activities. Consequently, those resistant peptides could frequently accumulate in the ruminal fluid.

The differences observed in the production of peptide N, α-amino N, and ammonia N among proteins (Exp. 1 and Exp. 2) indicate that the variations among proteins can influence the multi-step process of ruminal protein metabolism. The differences in amino acid composition and the structure of the proteins may have a major influence at one or more of the above steps to produce different types and amounts of ruminal protein degradation products. Variations in the production of peptide N, α-amino N, and ammonia N were also observed among the samples of different batches of the same protein (Exp. 3 and Exp. 4). The amino acid composition data also reveals that the concentrations of individual amino acids present in the extracellular peptide and free amino acid fractions varies among proteins (Exp. 1 and Exp. 2) and among different batches of the same protein (Exp. 3 and Exp. 4). The differences in primary (amino acid sequence), secondary, and tertiary structures (folding and disulfide bridges) and differences in solubility due to variations in starting materials and processing conditions of the proteins could be the reasons for the above variations. When Yoon et al. (1995) estimated ruminal degradability of menhaden fish meal, the degradability varied considerably among samples depending on the raw material used, and/or the processing conditions. The heat used during processing may change the nature of proteins by inducing disulfide bonds and by losing amino acids (Opstredt et al., 1984). Also, the length of storage prior to processing could have an influence on the rate and extent of ruminal degradation of some feed proteins (Mehrez et al., 1980). It can be assumed that the differences may have occurred among different batches of SBM and DDG proteins used in experiments 3 and 4 due to the differences in raw materials, storage and processing conditions. Additionally, the microbial activities occurring during the processing of some byproduct feeds such as DDG and CGF may also have an effect on their ruminal degradability. Therefore, those differences in the starting material and the changes that occur in the nature of the protein due to the differences in storage and processing conditions appear to influence the ruminal production of peptide N, α-amino N, and ammonia N.

Implications

The data presented in this study demonstrated that peptides can accumulate during the ruminal degradation of dietary proteins, and there is differential utilization of peptides by microorganisms in the rumen. If future research prove that the ruminally produced peptides can serve as a source of absorbed amino acids for ruminants then the present findings may be useful in planing dietary supplementation strategies to increase their efficiency of protein utilization.

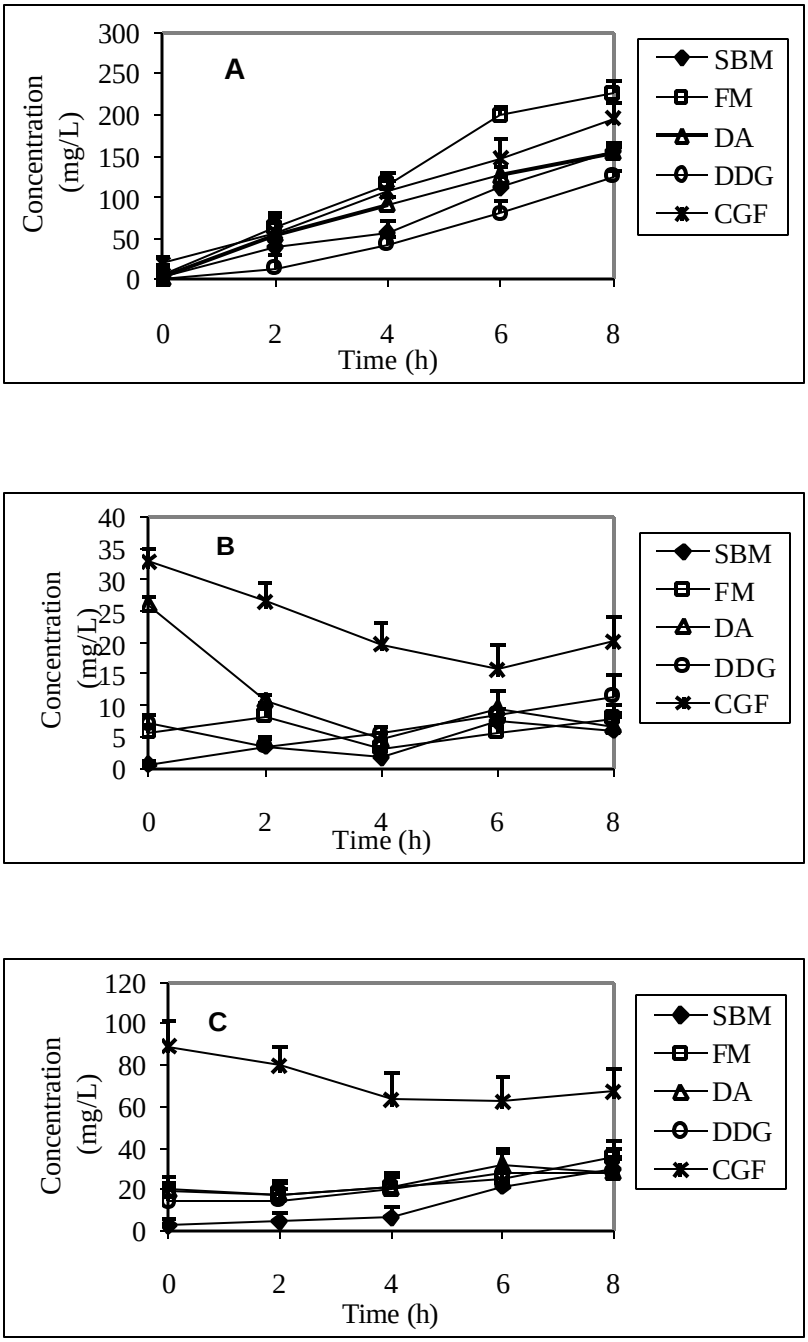


Figure 4.1. Changes in concentrations (mg/L) of (A) ammonia N, (B) α- amino N, and (C) peptide N in the extracellular medium during in vitro ruminal incubation of soybean meal (SBM), fish meal (FM), dehydrated alfalfa (DA), distillers dried grains with solubles (DDG), and corn gluten feed (CGF; experiment 1).

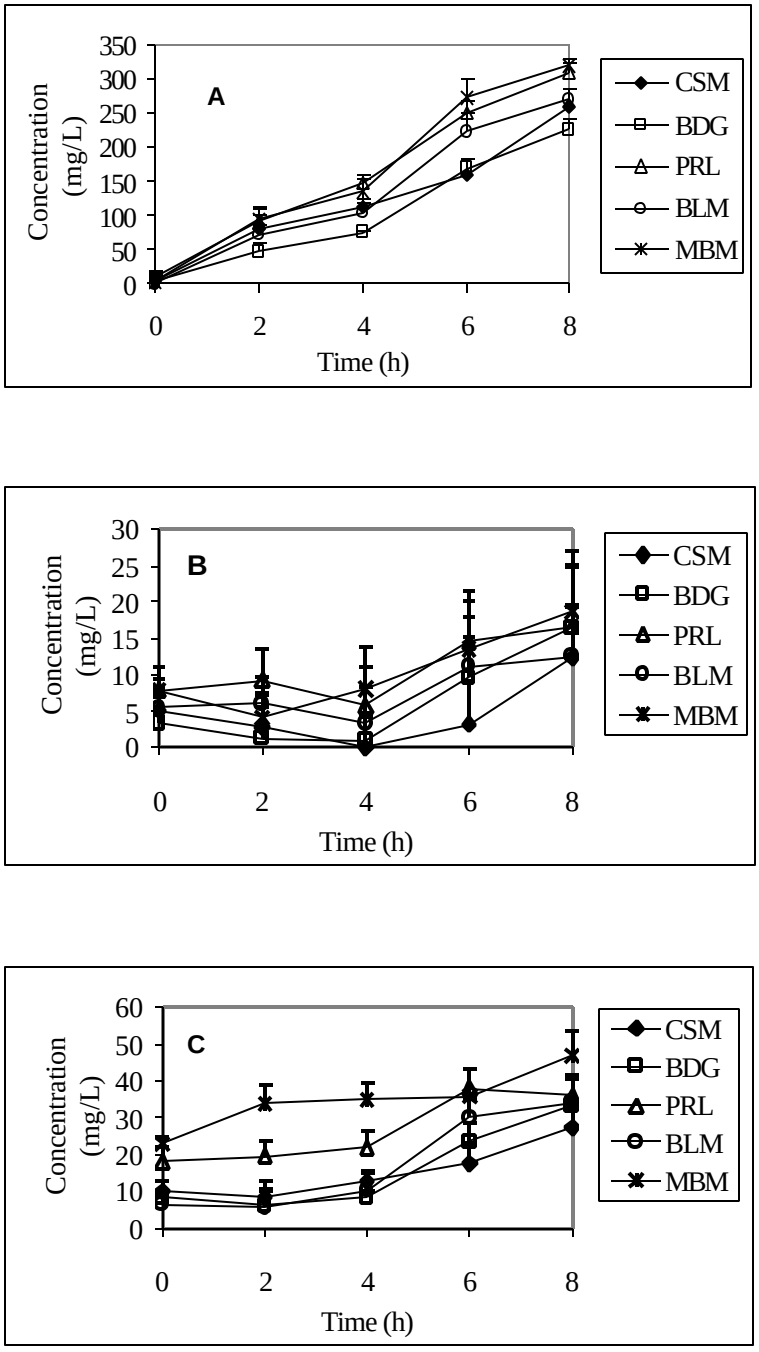


Figure 4.2. Changes in concentration (mg/L) of (A) ammonia N, (B) α-amino N, and (C) peptide N in the extracellular medium during in vitro ruminal incubation of cotton seed meal (CSM), brewers dried grains (BDG), prolac (PRL), blood meal (BLM), and meat and bone meal (MBM; experiment 2).

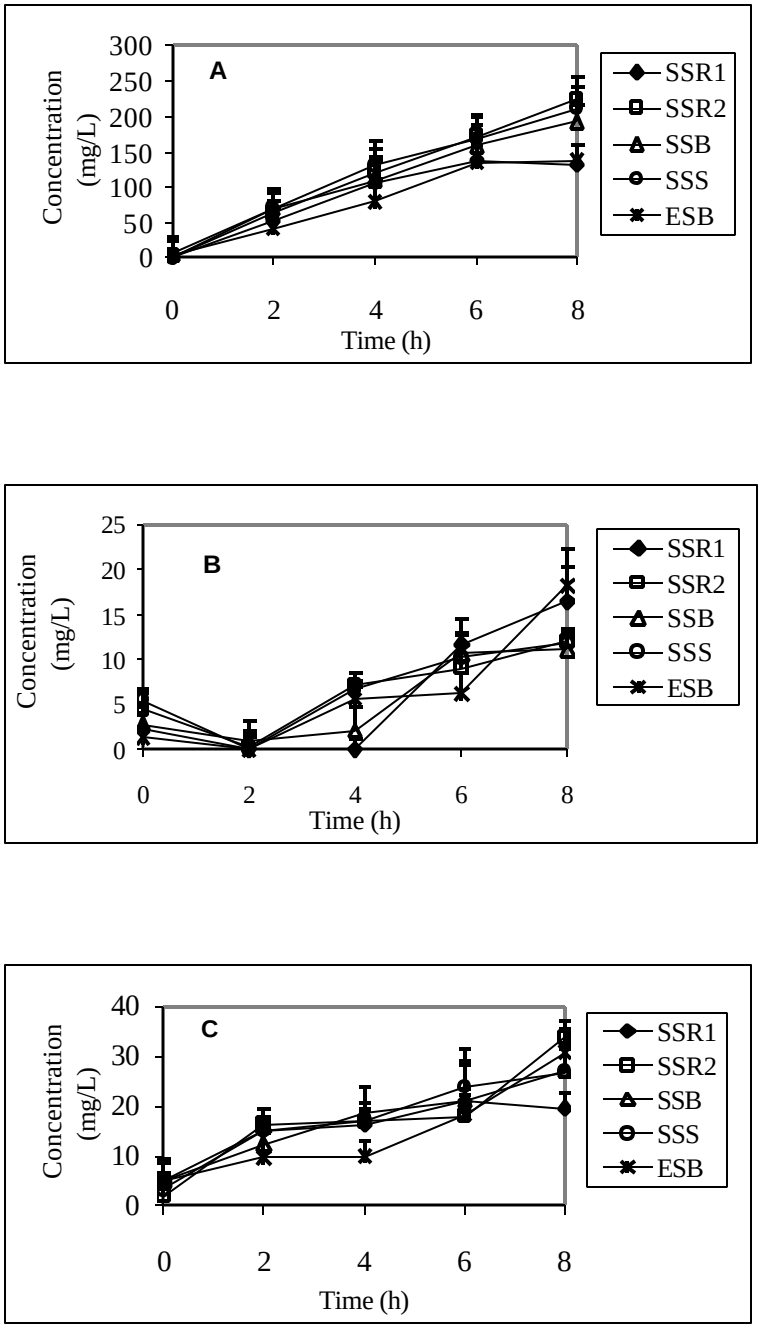


Figure 4.3. Changes in concentration (mg/L) of (A) ammonia N, (B) α-amino N, and (C) peptide N in the extracellular medium during in vitro ruminal incubation of expeller soybean meal (ESB) and solvent soybean meals (SSR1, SSR2, SSB, and SSS) collected from different mills and batches (experiment 3).

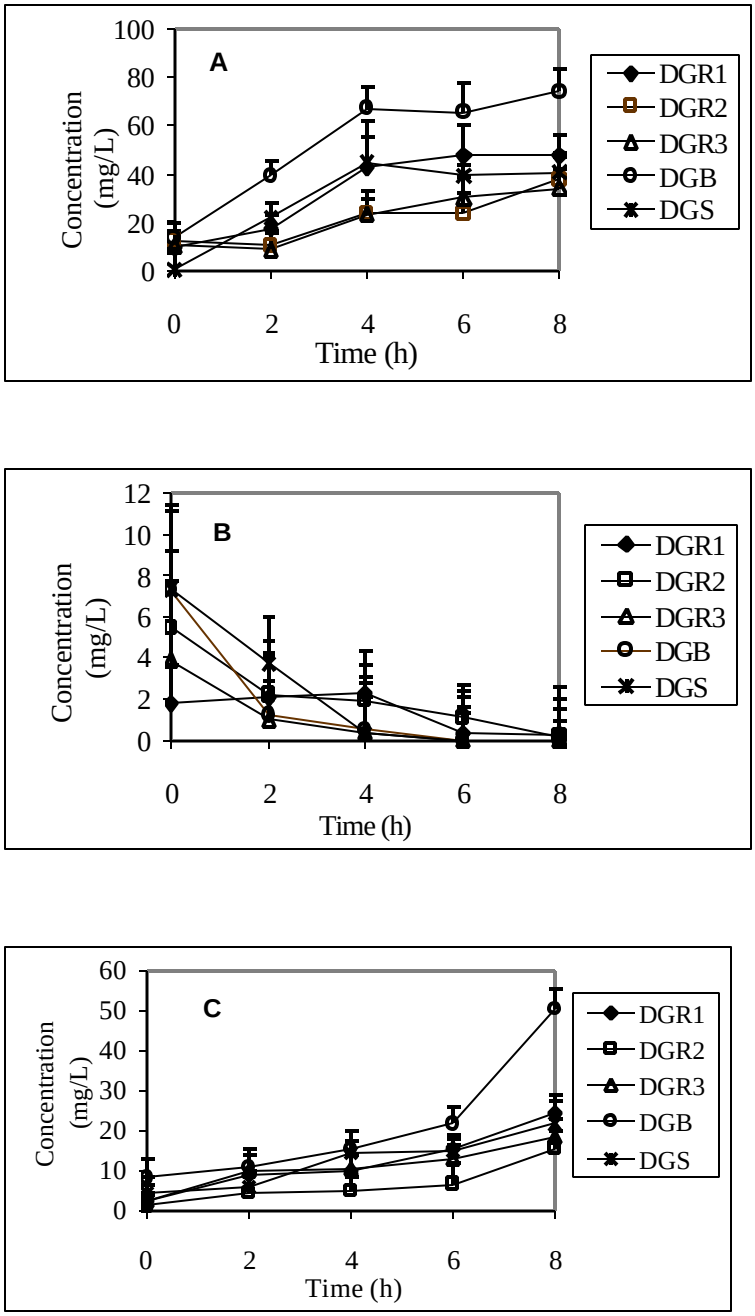


Figure 4.4. Changes in concentration (mg/L) of (A) ammonia N, (B) α-amino N, and (C) peptide N in the extracellular medium during in vitro ruminal incubation of distillers dried grains with solubles (DGR1, DGR2, DGR3, DGB, and DGS) collected from different mills and batches.

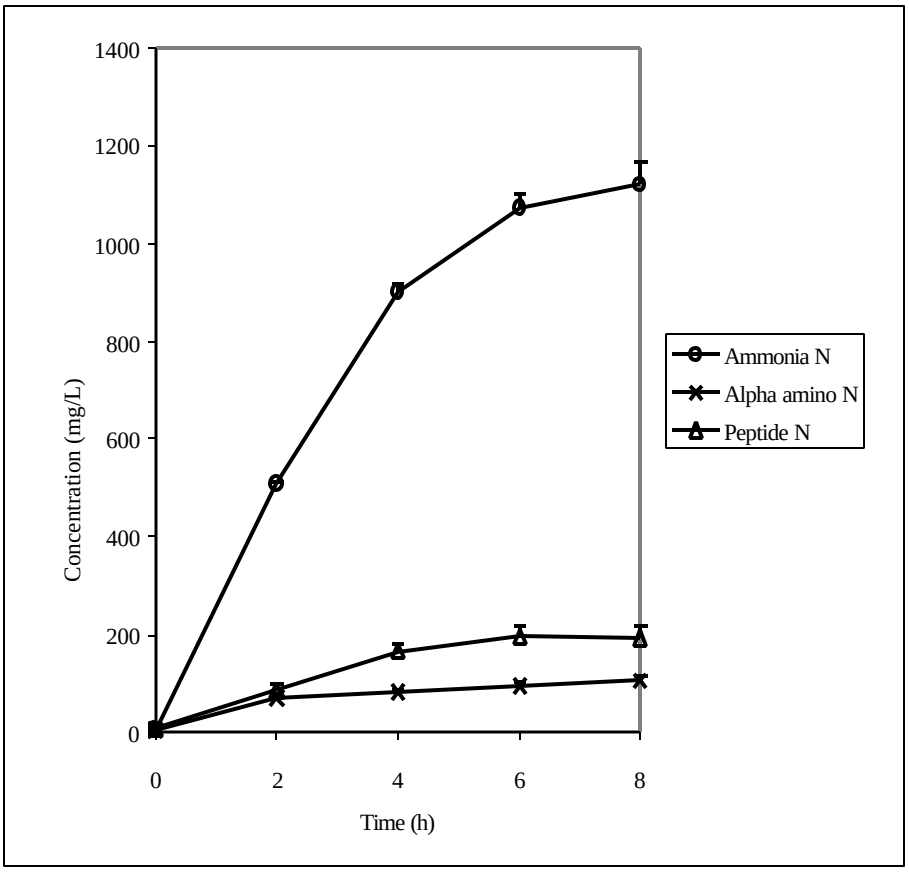


Figure 4.5. Changes in the concentrations (mg/L) of ammonia N, alpha amino N and peptide N in the extracellular medium during in vitro ruminal incubation of casein (experiment 5).

Table 4.1. Composition of the buffer and the nutrient medium used for incubation experiments.[a]

Constituent	Amounts (g/L)	
	Buffer	Nutrient Medium
Cellulose	-	4.00
Starch	-	1.00
KH_2PO_4	0.60	0.60
$Na_2HPO_4.7H_2O$	1.20	1.20
$NaHCO_3$	3.50	3.50
KCl	4.00	4.00
NaCl	4.00	4.00
$MgSO_4$	0.15	0.15
$CuSO_4.5H_2O$	-	0.002
$MnSO_4.5H_2O$	-	0.0004
$ZnSO_4.7H_2O$	-	0.0001
$FeSO_4.7H_2O$	-	0.075
$CoCl_2.6H_2O$	-	0.002
$CaCl_2$	0.55	0.55

[a] modified from Loper et al., 1966.

Table 4.2. Crude protein % of the protein sources used in the incubation experiments.

Protein source	Mill / Batch[a]	Crude Protein (%)
1. Soybean meal (solvent)	RCM #1	50.24
2. Soybean meal (solvent)	RCM # 2	51.51
3. Soybean meal (solvent)	BSM	48.69
4. Soybean meal (solvent)	SSM	50.90
5. Soybean meal (expeller)	RCM	48.37
6. Fish meal (Menhaden)	SSM	66.38
7. Dehydrated alfalfa	RCM	17.85
8. Corn Gluten Feed	RCM	23.91
9. Distillers dried grains with solubles	RCM # 1	28.01
10. Distillers dried grains with solubles	RCM # 2	28.61
11. Distillers dried grains with solubles	RCM # 3	29.46
12. Distillers dried grains with solubles	BSM	28.10
13. Distillers dried grains with solubles	SSM	25.27
14. Cotton seed meal	SSM	46.16
15. Brewers dried grain	SSM	30.39
16. Meat and bone meal	SSM	59.81
17. Prolac	BSM	61.79
18. Blood meal	SSM	99.42
19. Casein	USBC	93.03

[a] RCM = Roanoke City Mill, BSM = Big Spring Mill, SSM = Southern States Mill, USBC = United States Biochemical Corporation

Table 4.3 Free amino acid, peptide (total) amino acid, and peptide (< 3,000 MW) amino acid concentrations in extracellular media following in vitro ruminal incubation (8h) of dehydrated alfalfa (DA), soybean meal (SBM), corn gluten feed (CGF), fish meal (FM) and distiller dried grain with solubles (DDG; experiment 1).

	Free amino acids							Peptide (total) amino acids							Peptide (< 3,000 MW) amino acids							Probability[e]	
Amino acid	DA	SBM	CGF	FM	DDG	Mean	S.E.	DA	SBM	CGF	FM	DDG	Mean	S.E	DA	SBM	CGF	FM	DDG	Mean	S.E.	1 vs 2, 3	2 vs 3
	mg/L																						
Arginine	4.44^{b}	5.12^{b}	4.86^{b}	26.40^{a}	4.38^{b}	9.04	1.57	2.26^{b}	2.16^{b}	6.07^{a}	0^{c}	4.08^{ab}	2.91	1.20	1.20^{cd}	0.88^{d}	3.87^{a}	2.83^{b}	1.90^{c}	2.13	0.24	0.001	NS
Histidine	3.87^{ab}	3.97^{ab}	2.97^{ab}	6.64^{a}	2.52^{b}	3.99	0.76	0.64^{c}	0^{d}	6.46^{a}	0^{d}	2.55^{b}	1.93	0.93	0.03^{c}	0^{c}	4.02^{a}	0^{c}	1.56^{b}	1.12	0.89	0.001	NS
Isoleucine	2.19^{b}	3.17^{ab}	6.03^{a}	1.08^{b}	1.86^{b}	2.87	0.74	3.34^{b}	2.51^{b}	8.95^{a}	3.01^{b}	3.50^{b}	4.26	0.49	1.88^{b}	1.06^{b}	5.55^{a}	1.12^{b}	1.23^{b}	2.17	0.36	NS	0.001
Leucine	1.91^{b}	3.14^{b}	10.06^{a}	1.25^{b}	1.81^{b}	3.64	1.17	3.47^{b}	3.03^{b}	11.71^{a}	4.36^{b}	4.41^{b}	5.40	0.58	1.66^{b}	0.89^{b}	7.31^{a}	1.58^{b}	1.45^{b}	2.58	0.42	NS	0.001
Lysine	6.54	6.32	5.33	6.84	5.87	6.18	0.41	7.74 ^{bc}	5.99^{c}	12.69^{a}	7.79^{bc}	9.30^{b}	8.70	0.51	4.36^{b}	2.71^{c}	6.32^{a}	2.59^{c}	4.34^{b}	4.06	0.36	NS	0.001
Methionine	5.56^{a}	1.80^{b}	4.38^{ab}	3.21^{ab}	3.15^{ab}	3.62	0.55	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0.001	NS
Phenylalanine	1.55^{b}	2.95^{ab}	4.26^{a}	0.33^{b}	2.68^{ab}	2.35	0.73	2.01^{b}	2.21^{b}	4.82^{a}	2.95^{b}	2.74^{b}	2.95	0.22	1.11^{b}	0.73 ^{b}	2.78^{a}	1.21^{b}	0.80^{b}	1.33	0.17	NS	0.001
Threonine	2.23^{b}	1.67^{b}	4.19^{a}	1.66^{b}	1.82^{b}	2.31	0.26	5.25 ^{bc}	3.56^{c}	12.79^{a}	4.48^{bc}	6.10^{b}	6.44	0.63	2.88^{b}	1.17^{c}	7.89^{a}	0.98^{c}	2.35^{b}	3.06	0.47	0.001	0.001
Valine	3.11^{b}	4.41^{b}	9.02^{a}	1.79^{b}	2.73^{b}	4.21	1.05	4.38^{b}	2.88^{b}	12.74^{a}	3.70^{b}	4.72^{b}	5.68	0.68	2.45^{b}	1.17^{b}	8.15^{a}	1.14^{b}	1.74^{b}	2.93	0.53	NS	0.001
Total essential	31.40^{b}	32.56^{b}	51.10^{a}	49.20^{a}	26.82^{b}	38.22	4.11	29.08^{bc}	22.34^{c}	76.24^{a}	26.28^{b}	37.39^{b}	38.27	3.82	15.57^{b}	8.61^{c}	45.88^{a}	11.46^{c}	15.37^{b}	19.38	2.56	0.002	0.001
Alanine	5.50^{b}	6.70^{b}	14.21^{a}	3.63^{b}	6.25^{b}	7.26	1.62	7.11^{b}	5.88^{b}	20.23^{a}	8.43^{b}	10.50^{b}	10.43	0.92	2.81^{bc}	1.49^{c}	11.77^{a}	2.37^{bc}	3.58^{b}	4.40	0.68	NS	0.001
Aspartic acid	0.15^{b}	0.32^{b}	0.93^{a}	0.22^{b}	0.22^{b}	0.37	0.06	2.67^{b}	2.30^{b}	7.71^{a}	2.13^{b}	2.99^{b}	3.56	0.53	1.74	1.16	2.11	.86	1.16	1.41	0.22	0.001	0.001
Glutamic acid	4.70^{b}	12.76^{a}	18.12^{a}	7.55^{ab}	12.14^{a}	11.05	1.46	7.64^{b}	7.84^{b}	36.16^{a}	8.17^{b}	13.14^{b}	14.59	2.48	3.95^{b}	1.52^{b}	14.80^{a}	2.18^{b}	2.94^{b}	5.08	1.37	NS	0.001
Glycine	3.38^{ab}	3.55^{ab}	6.68^{a}	2.29^{b}	4.10^{ab}	4.00	0.82	5.12^{b}	3.66^{b}	15.51^{a}	6.69^{b}	6.91^{b}	7.58	0.79	3.10^{b}	1.65^{c}	9.62^{a}	3.73^{b}	3.05^{b}	4.23	0.59	0.001	0.001
Proline	2.52^{b}	1.85^{b}	4.82^{a}	2.40^{b}	3.07^{b}	2.93	0.25	4.70^{bc}	3.60^{c}	20.72^{a}	5.79^{bc}	7.99^{b}	8.56	1.24	2.75^{bc}	1.84^{c}	14.52^{a}	3.18^{bc}	4.27^{b}	5.31	0.97	0.001	0.001
Serine	0.97^{b}	0.82^{b}	1.93^{a}	0.85^{b}	0.84^{b}	1.08	0.12	5.59^{b}	4.63^{b}	12.06^{a}	5.65^{b}	6.98^{b}	6.98	0.50	2.01^{b}	1.02^{c}	5.77^{a}	1.56^{bc}	1.80^{bc}	2.43	0.33	0.001	0.001
Tyrosine	1.96^{ab}	2.19^{a}	2.86^{a}	1.20^{b}	2.33^{a}	2.11	0.33	0.39	0.47	1.11	0.43	0.69	0.62	0.21	0	0	0.47	0.05	0	0.11	0.15	0.001	0.01
Total nonessential	19.17^{b}	28.19^{b}	49.54^{a}	18.13^{b}	28.96^{b}	28.80	4.39	33.22^{c}	28.37^{c}	113.49^{a}	37.30^{ab}	49.19^{b}	52.31	6.22	16.36^{b}	8.68^{c}	59.07^{a}	13.93^{b}	16.80^{b}	22.97	3.91	0.01	0.001
Total	50.57^{a}	60.75^{a}	100.64^{b}	67.33^{a}	55.78^{a}	67.01	8.13	62.30^{bc}	50.71^{c}	189.73^{a}	63.58^{bc}	86.58^{b}	90.58	9.84	31.93^{b}	17.29^{c}	104.95^{a}	25.39^{bc}	32.17^{b}	42.34	6.28	NS	0.001

[a,b,c,d] Within a row and within a category, means lacking a common superscript letter differ (P<.01).

[e] 1= free amino acids, 2 = peptide (total) amino acids, and 3 = peptide (<3,000 MW) amino acids.

Table 4.4 Free amino acid, peptide (total) amino acid, and peptide (< 3,000 MW) amino acid concentrations in extracellular media following in vitro ruminal incubation (8h) of cotton seed meal (CSM), brewers dried grains (BDG prolac (PRL), blood meal (BLM) and meat and bone meal (MBM; experiment 2).

	Free amino acids							Peptide (total) amino acids							Peptide (< 3,000 MW) amino acids							Probability[e]	
Amino acid	CSM	BDG	PRL	BLM	MBM	Mean	S.E.	CSM	BDG	PRL	BLM	MBM	Mean	S.E.	CSM	BDG	PRL	BLM	MBM	Mean	S.E.	1 vs 2.3	2,vs 3
	mg/L																						
Arginine	9.75^{ab}	8.62^{b}	14.78^{a}	8.53^{b}	11.54^{ab}	10.64	0.42	2.11^{b}	1.86^{b}	2.67^{ab}	1.89^{b}	3.01^{a}	2.31^{b}	0.15	0.59^{b}	0.62^{b}	1.57^{a}	0.28^{b}	1.68^{a}	0.95	0.13	0.001	0.001
Histidine	2.66	2.77	3.02	3.23	2.82	2.90	0.10	1.19	1.10	1.39	1.19	1.55	1.28	0.07	0.34	0.52	0.45	0.16	0.66	0.43	0.06	0.001	0.001
Isoleucine	1.10	1.14	1.18	1.14	1.18	1.15	0.03	2.49	2.60	2.73	2.40	2.82	2.61	0.10	0.83^{b}	1.04^{a}	1.15^{a}	0.80^{b}	1.03^{a}	0.97	0.04	0.001	0.001
Leucine	1.62	1.53	1.54	1.56	1.59	1.57	0.04	3.08^{b}	3.28^{b}	4.08^{a}	3.40^{b}	4.44^{a}	3.65	0.15	0.53c	0.84^{bc}	1.54^{a}	0.93^{b}	1.59^{a}	1.08	0.09	0.001	0.001
Lysine	9.02^{b}	11.08^{a}	9.93^{ab}	10.16^{ab}	9.57^{ab}	9.95	0.22	6.20	7.52	7.34	6.75	7.45	7.05	0.28	2.37	3.18	3.82	2.49	2.97	2.97	0.22	0.001	0.001
Methionine	1.68	0.72	0.73	0.64	0.70	.90	0.17	0	0.64	0.63	0.63	0.74	0.53	0.13	0.83	0	0.11	0.06	0.15	0.23	0.17	0.001	0.001
Phenylalanine	0.25	0.50	0.41	0.51	0.20	.37	0.06	2.71	2.57	2.89	2.32	2.88	2.67	0.11	0.89^{b}	0.83^{b}	1.29^{a}	0.80^{b}	1.32^{a}	1.03	0.07	NS	0.001
Threonine	1.97^{b}	2.72^{a}	2.05^{b}	1.83^{b}	1.94^{b}	2.10	0.07	3.69	3.80	3.86	3.54	4.13	3.81	0.11	0.72^{b}	0.93^{ab}	1.19^{a}	0.91^{ab}	1.21^{a}	0.99	0.06	0.001	0.001
Valine	2.08^{b}	1.98^{b}	2.32^{ab}	2.42^{a}	2.23^{ab}	2.21	0.05	2.69^{b}	3.13^{ab}	3.33^{a}	2.57^{b}	3.57^{a}	3.06	0.14	0.54^{b}	0.93^{a}	1.10^{a}	0.51^{b}	1.19^{a}	0.85	0.06	0.001	0.001
Total essential	30.13^{b}	31.06^{b}	35.96^{a}	30.00^{b}	31.77^{b}	31.78	0.63	24.15^{b}	26.50^{ab}	28.92^{ab}	24.68^{b}	30.57^{a}	26.97	0.81	7.66^{b}	8.90^{b}	12.22^{a}	6.94^{b}	11.78^{a}	9.50	0.58	0.001	0.001
Alanine	3.78^{c}	4.64^{b}	4.98^{b}	4.67^{b}	5.73^{a}	4.76	0.17	7.38^{c}	8.47^{b}	8.90^{b}	7.40^{c}	10.36^{a}	8.50	0.25	2.06^{d}	2.81^{c}	3.47^{b}	2.45^{cd}	4.38^{a}	3.04	0.19	NS	0.001
Aspartic acid	0.08	0.11	0	0	0	0.04	0.02	1.80^{b}	2.07^{b}	2.33^{ab}	1.92^{b}	2.74^{a}	2.17	0.09	0.76^{b}	1.03^{b}	1.41^{a}	0.99^{b}	1.52^{a}	1.14	0.06	0.001	0.001
Glutamic acid	9.67^{a}	7.39^{b}	7.17^{b}	8.32^{ab}	7.57^{b}	8.02	0.30	7.90^{ab}	7.91^{ab}	8.29^{ab}	7.53^{b}	9.87^{a}	8.30	0.31	0.88^{b}	2.84^{ab}	3.97^{a}	2.76^{ab}	4.81^{a}	3.05	0.41	0.001	0.001
Glycine	2.02	2.03	2.45	2.04	2.59	2.23	0.06	4.45^{c}	5.17^{c}	7.77^{b}	4.48^{c}	11.42^{a}	6.66	0.49	2.29^{c}	2.80^{c}	5.15^{b}	2.23^{c}	7.70^{a}	4.03	0.38	0.001	0.001
Proline	3.13^{c}	3.23^{c}	3.80^{b}	3.23^{c}	4.15^{a}	3.51	0.11	3.28^{c}	3.64^{c}	6.05^{b}	3.10^{c}	8.75^{a}	4.97	0.39	1.48^{c}	1.79^{c}	4.16^{b}	1.49^{c}	6.25^{a}	3.03	0.34	0.001	0.001
Serine	1.00	1.16	1.15	1.08	1.17	1.11	0.03	4.94^{c}	5.41^{b}	6.00^{a}	4.97^{c}	6.10^{a}	5.48	0.14	1.04^{b}	1.35^{b}	1.96^{a}	1.24^{b}	2.04^{a}	1.53	0.08	0.001	0.001
Tyrosine	1.11	1.37	1.28	1.15	1.18	1.22	0.03	1.28	1.05	1.09	1.10	1.36	1.18	0.13	0.04	0	0.14	0.02	0.21	0.08	0.05	0.001	0.04
Total nonessential	20.80	19.93	20.83	20.50	22.39	20.89	0.49	31.04^{c}	33.72^{c}	40.44^{b}	30.50^{c}	50.60^{a}	37.26	1.51	8.55^{d}	12.61^{c}	20.25^{b}	11.19^{cd}	26.91^{a}	15.90	1.27	0.001	0.001
Total	50.93^{b}	50.98^{b}	56.78^{a}	50.50^{b}	54.16^{ab}	52.67	1.01	55.01^{c}	60.22^{c}	69.36^{b}	55.18^{c}	81.18^{a}	64.19	2.13	16.20^{c}	21.52^{c}	32.47^{b}	18.12^{c}	38.69^{a}	25.40	1.73	0.001	0.001

[a,b,c,d] Within a row and within a category, means lacking a common superscript letter differ (P<.01).

[e] 1= free amino acids, 2 = peptide (total) amino acids, and 3 = peptide (<3,000 MW) amino acids.

Table 4.5. Free amino acid, peptide (total) amino acid, and peptide (< 3,000 MW) amino acid concentrations in extracellular media following in vitro ruminal incubation (8h) of expeller soybean meal (ESB) and different batches of solvent soybean meals (SSR1, SSR2, SSB and SSS; experiment 3).

	Free amino acids							Peptide (total) amino acids							Peptide (< 3,000 MW) amino acids							Probability[e]	
Amino acid	ESB	SSR1	SSR2	SSB	SSS	Mean	S.E.	ESB	SSR1	SSR2	SSB	SSS	Mean	S.E.	ESB	SSR1	SSR2	SSB	SSS	Mean	S.E.	1 vs 2, 3	2 vs 3
	mg/L																						
Arginine	6.85	6.86	7.26	7.54	7.06	7.11	0.21	1.36^{b}	1.23^{b}	1.40^{b}	2.75^{a}	1.77^{ab}	1.70	0.22	0	0	0	1.17	0	0.23	0.30	0.001	0.001
Histidine	2.65	2.55	3.15	2.76	2.62	2.75	0.19	0.66^{ab}	1.37^{a}	0.31^{b}	0.77^{ab}	0.89^{ab}	0.80	0.19	0.02	0.24	0	0.20	0.03	0.10	0.15	0.001	0.001
Isoleucine	1.04	0.94	0.92	0.66	0.67	0.85	0.10	2.03^{ab}	1.91^{b}	1.93^{b}	2.64^{a}	2.26^{ab}	2.15	0.13	0.47	0.45	0.57	1.14	0.72	0.67	0.11	0.001	0.001
Leucine	1.31	1.29	1.27	1.16	1.12	1.23	0.08	2.50^{ab}	2.38^{b}	2.40^{b}	3.60^{a}	2.81^{ab}	2.74	0.22	0.39	0.32	0.54	1.67	0.57	0.70	0.26	0 .001	NS
Lysine	9.20	8.92	9.42	9.46	9.24	9.25	0.53	3.32	2.06	3.10	3.39	2.41	2.86	0.42	0	0	0	0.53	0	0.11	0.43	0.001	0.001
Methionine	0.56	0.93	0.53	0.54	0.59	0.63	0.12	0.20	0.20	0.31	0.36	0.29	0.27	0.05	0	0	0.08	0.26	0.11	0.09	0.09	0.001	0.001
Phenylalanine	0	0.04	0.18	0.15	0.18	0.11	0.05	1.90^{b}	1.89^{b}	1.83^{b}	2.63^{a}	2.03^{ab}	2.05	0.14	0.44	0.29	0.36	0.83	0.19	0.42	0.15	0.005	0.08
Threonine	1.36	1.40	1.43	1.50	1.35	1.41	0.05	2.73^{ab}	2.52^{b}	2.59^{b}	3.71^{a}	2.98^{ab}	2.90	0.21	1.03	0.79	0.91	1.63	1.02	1.08	0.20	NS	0.01
Valine	1.81	1.76	1.65	1.55	1.56	1.67	0.13	1.93	1.85	1.97	2.74	2.27	2.15	0.17	0.10	0.05	0.24	0.90	0.24	0.31	0.19	NS	0.01
Total essential	24.76	24.69	25.82	25.30	24.38	24.99	0.95	16.62^{bc}	15.32^{c}	15.84^{bc}	22.59^{a}	17.72^{b}	17.63	0.37	2.45	2.15	2.69	8.32	2.88	3.70	1.48	0.001	0.005
Alanine	2.30	2.53	2.57	2.45	2.33	2.43	0.10	5.00	4.57	4.80	5.88	5.51	5.15	0.29	1.01	0.81	1.10	1.51	1.10	1.11	0.14	0.001	0.001
Aspartic acid	.09	0.10	0.06	0.05	0.12	0.08	0.03	0.88	.80	0.89	1.45	0.77	0.96	0.12	0	0.03	0.01	0.17	0	0.04	0.05	0.001	0.001
Glutamic acid	7.55	8.38	7.68	7.04	7.79	7.69	0.50	2.93^{b}	2.48^{b}	3.75^{ab}	5.21^{a}	3.18^{ab}	3.51	0.43	0	0	0	0	0	0	0.47	0.001	0.001
Glycine	1.25	1.22	1.29	1.27	1.26	1.26	0.06	2.64^{ab}	2.43^{b}	2.56^{ab}	3.49^{a}	2.90^{ab}	2.80	0.18	0.86	0.88	0.96	1.58	1.04	1.06	0.15	0.001	0.001
Proline	1.85	1.81	1.83	1.93	1.79	1.84	0.06	2.46^{ab}	2.24^{b}	2.26^{b}	3.32^{a}	2.94^{ab}	2.64	0.17	1.26	0.77	1.15	2.06	1.30	1.31	0.19	0.001	0.001
Serine	0.67	0.86	0.85	0.88	0.76	0.80	0.04	3.09^{ab}	2.80^{b}	2.90^{b}	4.33^{a}	3.28^{ab}	3.28	0.24	0.46	0.36	0.42	1.39	0.59	0.64	0.21	0.001	0.001
Tyrosine	0.83	0.95	0.97	0.89	0.79	0.89	0.05	0.94	1.18	0.83	1.52	1.19	1.13	0.12	0.04	0.05	0	0.57	0.05	0.14	0.12	0.001	0.008
Total nonessential	14.53	15.85	15.25	14.49	14.84	14.99	0.72	17.94^{b}	16.49^{b}	17.98^{b}	25.20^{a}	19.77^{ab}	19.47	1.37	3.64	2.89	3.64	7.27	4.08	4.30	1.18	0.07	NS
Total	39.30	40.54	41.06	39.80	39.22	39.98	1.52	34.55	31.88	33.81	47.78	37.49	37.10	1.71	6.08	5.04	6.33	15.60	6.96	8.00	2.60	0.001	0.001

[a,b,c,d] Within a row and within a category, means lacking a common superscript letter differ (P<.01).
[e] 1 = free amino acids, 2 = peptide (total) amino acids, and 3 = peptide (<3,000 MW) amino acids.

Table 4.6. Free amino acid, peptide (total) amino acid, and peptide (< 3,000 MW) amino acid concentrations in extracellular media following in vitro ruminal incubation (8h) of different batches of distillers dried grains with solubles (DGR1, DGR2, DGR3, DGB and DGS; experiment 4).

	Free amino acids							Peptide (total) amino acids							Peptide (< 3,000 MW) amino acids							Probability[e]	
Amino acid	DGR1	DGR2	DGR3	DGB	DGS	Mean	S.E	DGR1	DGR2	DGR3	DGB	DGS	Mean	S.E.	DGR1	DGR2	DGR3	DGB	DGS	Mean	S.E	1 vs 2, 3	2 vs 3
	---------- mg/L ----------																						
Arginine	3.64	3.96	4.27	4.15	3.52	3.91	0.16	3.97^{a}	2.07^{b}	2.56^{b}	2.97^{ab}	2.94^{ab}	2.90	0.24	1.46	0.73	0.37	1.08	1.16	0.96	0.12	0.001	0.003
Histidine	1.51	1.51	1.54	1.55	1.40	1.50	0.07	1.82^{a}	0.75^{b}	0.92^{b}	2.21^{a}	1.66^{a}	1.47	0.12	0.92^{a}	0.26^{b}	0.09^{b}	1.14^{a}	0.69^{a}	0.62	0.09	0.001	0.001
Isoleucine	0.42	0.46	0.27	0 .33	0.43	0.38	0.06	3.03^{ab}	2.21^{b}	2.60^{b}	3.37^{a}	2.54^{b}	2.75	0.13	0.98^{b}	0.87^{b}	1.13^{b}	1.60^{a}	0.83^{b}	1.08	0.07	0.001	0.001
Leucine	0.89	0.99	1.36	0.86	0.84	0.99	0.08	4.53^{a}	2.56^{b}	3.09^{b}	4.87^{a}	3.20^{b}	3.65	0.26	1.18	0.62	0.49	2.02	0.81	1.03	0.12	0.001	0.001
Lysine	2.67	2.89	3.18	3.22	2.42	2.87	0.20	5.62	4.24	4.51	4.93	4.39	4.74	0.34	2.15^{a}	1.82^{ab}	1.41^{b}	1.93^{ab}	1.98^{ab}	1.86	0.15	0.001	0.001
Methionine	2.73	3.43	3.79	2.77	2.40	3.02	0.43	0.39^{b}	9.00^{a}	0^{b}	0.50^{b}	0.02^{b}	1.98	0.19	0.62	0.19	0	0	0	0.16	0.20	0.001	0.001
Phenylalanine	0	0.24	0.20	0	0	0.09	0.03	2.74	1.57	2.05	2.67	2.16	2.24	0.19	0.86^{ab}	0.47^{b}	0.66^{b}	1.10^{a}	0.82^{ab}	0.78	0.07	0.003	0.004
Threonine	1.27	1.32	1.32	1.39	1.15	1.29	0.07	5.25^{a}	3.42^{c}	3.83^{bc}	4.86^{ab}	4.10^{bc}	4.29	0.17	1.81^{a}	1.32^{b}	1.28^{b}	2.16^{a}	1.57^{ab}	1.63	0.08	NS	0.001
Valine	1.79	1.93	2.42	1.95	2.20	2.06	0.26	3.73^{a}	2.20^{b}	2.38^{b}	3.62^{ab}	2.46^{b}	2.88	0.22	0.92	0.32	0	1.11	0.04	0.48	0.27	NS	0.001
Total essential	14.91	16.71	18.36	16.23	14.35	16.11	1.01	31.09^{a}	28.02^{a}	21.93^{b}	30.00^{a}	23.46^{b}	26.90	1.45	10.91^{ab}	6.61^{c}	5.41^{c}	12.13^{a}	7.90^{bc}	8.59	0.72	0.001	0.05
Alanine	3.65^{ab}	5.19^{a}	5.09^{a}	3.18^{b}	4.40^{ab}	4.30	0.38	7.80	6.42	6.76	7.68	7.31	7.19	0.15	3.42	2.94	3.14	3.68	3.78	3.39	0.09	0.001	0.001
Aspartic acid	1.16	1.22	1.22	1.47	1.27	1.27	0.05	3.97^{a}	2.15^{b}	1.55^{b}	3.41^{a}	3.23^{a}	2.86	0.24	0.65	0	0	0.27	0.04	0.19	0.13	0.001	0.001
Glutamic acid	5.39^{b}	6.16^{b}	6.06^{b}	7.65^{a}	5.40^{b}	6.13	0.24	11.18^{a}	7.89^{b}	5.15^{b}	9.18^{a}	7.73^{b}	8.22	0.52	2.94^{a}	1.11^{ab}	0^{b}	1.43^{ab}	1.27^{ab}	1.35	0.41	0.001	0.001
Glycine	0.93	1.01	1.05	1.11	1.00	1.02	0.04	4.75^{a}	4.10^{ab}	3.63^{b}	4.78^{a}	3.90^{b}	4.23	0.15	1.98^{b}	2.23^{ab}	1.63^{b}	2.53^{a}	1.73^{b}	2.02	0.08	0.001	0.001
Proline	1.51	1.43	1.53	1.61	1.32	1.48	0.07	6.07^{a}	4.13^{b}	4.64^{b}	7.49^{a}	5.15^{ab}	5.50	0.28	3.29^{b}	2.83^{b}	2.85^{b}	5.31^{a}	3.15^{b}	3.48	0.18	0.001	0.001
Serine	0.42	0.69	0.78	0.72	0.43	0.61	0.05	6.21^{a}	4.15^{b}	4.25^{b}	5.40^{ab}	4.67^{b}	4.93	0.19	1.65^{a}	1.20^{ab}	0.82^{b}	2.03^{a}	1.57^{a}	1.45	0.10	0.001	0.001
Tyrosine	0.68	0.62	0.61	0.55	0.38	0.57	0.06	2.63^{a}	1.49^{b}	2.13^{ab}	2.46^{a}	1.97^{ab}	2.14	0.14	0.79^{b}	0.48^{b}	0.59^{b}	1.20^{a}	0.75^{b}	0.76	0.07	0.001	0.001
Total nonessential	13.75^{b}	16.32^{a}	16.34^{a}	16.29^{a}	14.19^{b}	15.38	0.35	42.61^{a}	30.33^{c}	28.10^{c}	40.38^{ab}	33.95^{bc}	35.07	1.39	14.72^{ab}	10.80^{b}	9.03^{b}	16.45^{a}	12.29^{b}	12.66	0.77	0.001	0.001
Total	28.66^{b}	33.03^{ab}	34.70^{a}	32.51^{ab}	28.54^{b}	31.49	1.12	73.70^{a}	58.35^{b}	50.03^{b}	70.38^{a}	57.41^{b}	61.97	1.52	25.64^{a}	17.40^{b}	14.44^{c}	28.57^{a}	20.19^{b}	21.25	0.73	0.001	0.001

[a,b,c,d] Within a row and within a category, means lacking a common superscript letter differ (P<.01).

[e] 1= free amino acids, 2 = peptide (total) amino acids, and 3 = peptide (<3,000 MW) amino acids.

Table 4.7. Concentrations of free amino acids, peptide (total) amino acids, and peptide (<3,000 MW) amino acids persisted in the extracellular media following in vitro ruminal incubation (8 h) of casein (experiment 5).

Amino acids	Free AA		Peptide (total) AA		Peptide (<3,000 MW) AA		Probability[a]	
	Mean	SE	Mean	SE	Mean	SE	1 vs 2,3	2 vs 3
	----------mg/L----------							
Arginine	2.76	0.09	2.90	0.18	1.26	0.11	0.001	0.001
Histidine	13.18	0.59	-	-	-	-	0.001	NS
Isoleucine	15.36	0.77	5.57	0.56	2.48	0.59	0.001	0.001
Leucine	25.17	1.29	6.53	0.87	2.38	0.88	0.001	0.001
Lysine	12.09	1.28	10.20	0.84	4.35	1.18	0.001	0.001
Methionine	0.72	0.16	0.19	0.26	-	-	0.02	NS
Phenylalanine	15.04	1.06	4.13	0.53	0.89	0.69	0.001	0.001
Threonine	0.98	0.05	5.74	0.47	3.27	0.31	0.001	0.001
Valine	16.95	0.70	4.70	0.61	1.87	0.45	0001	0.001
Total essential	102.25	5.70	33.32	4.58	7.15	3.71	0.001	0.001
Alanine	7.64	1.30	5.88	0.39	2.63	0.24	0.001	0.002
Aspartate	1.35	0.07	3.30	0.37	2.85	0.42	0.001	NS
Glutamate	20.85	1.56	11.80	1.91	10.86	1.99	0.001	NS
Glycine	2.03	0.22	6.58	1.46	4.72	0.32	0.001	0.001
Proline	1.19	0.06	6.83	0.36	4.50	0.26	0.001	0.001
Serine	0.83	0.04	4.69	0.31	2.39	0.18	0.001	0.001
Tyrosine	13.37	0.95	-	-	-	-	0.001	0.05
Total nonessential	47.26	3.85	33.37	3.83	24.82	2.85	0.001	0.008
Total	149.51	9.45	66.69	8.23	31.97	5.85	0.001	0.001

[a] 1= free amino acids, 2 = peptide (total) amino acids, and 3 = peptide (<3,000 MW) amino acids.

Chapter V

ABSORPTION OF RUMINALLY DERIVED PEPTIDES AND FREE AMINO ACIDS VIA ISOLATED OVINE RUMINAL AND OMASAL EPITHELIA

ABSTRACT

Absorption of free and peptide-bound amino acids via ovine ruminal and omasal epithelia was quantified using parabiotic chambers. Substrates consisted of cell-free supernatants obtained following in vitro incubation (8 h) of either soybean meal (SBM), casein (CAS), or distillers dried grains with solubles (DDG) in a buffered ruminal inoculum. Amino acid concentrations in mucosal (0 h) and serosal (240 min) buffers were measured by HPLC with and without filtration (through 3,000 MW filters). The mucosal concentrations of total peptide amino acids in DDG, CAS and SBM were 178, 224 and 267 mg/L, respectively. Peptides (< 3,000 MW) contributed between 15 (SBM) and 38% (DDG) of total peptide amino acids in mucosal fluid. The total free amino acid concentrations of DDG, SBM and CAS in mucosal fluids (0 h) were 14, 28 and 113 mg/L, respectively. Total essential amino acids (EAA) consisted of 44, 46 and 54% of total amino acids, respectively for peptide (< 3,000 MW), peptide (total) and free amino acids. Serosal appearances of total amino acids were 391, 519 and 683 $\mu g \cdot L^{-1} \cdot mg^{-1}$ dry tissue for SBM, DDG and CAS, respectively. Total amino acid appearances in serosal fluids were 296, 444, and 853 $\mu g \cdot L^{-1} \cdot mg^{-1}$ dry tissue for free amino acids, peptides (< 3,000 MW) and peptides (total), respectively. Serosal appearances of total amino acids via ruminal and omasal tissues were 286 and 776 $\mu g \cdot L^{-1} \cdot mg^{-1}$ dry tissue, respectively. Total, EAA, total nonessential (NEAA) and individual amino acid appearances in serosal fluids were varied ($P < 0.05$, amino acid form × protein source) among SBM, DDG and CAS. The absorption of ruminally derived amino acids across ovine forestomach epithelia are greater for peptides than for free amino acids, and greater via omasal than via ruminal tissues. Dietary protein used for ruminal microbial degradation may influence the amounts and types of free and peptide-bound amino acids absorbed via forestomach epithelia.

Key Words: Rumen, Omasum, Amino acids, Peptides, Absorption

Introduction

During the degradation of proteins to ammonia in the rumen, peptides and amino acids are produced as two inevitable products (Broderick and Wallace, 1988). Evidence indicates that nutritionally significant quantities of peptides (Chen et al., 1987a) and amino acids (Leibholz, 1969) can persist in the extracellular ruminal fluid for a period of time post feeding. In our previous experiments, the accumulation of peptides (large amounts) and free amino acids (small amounts) was demonstrated (in vitro) following ruminal microbial fermentation of a variety of commonly fed dietary proteins (Chapter IV). Though a substantial amount of these protein degradation products can accumulate in the rumen, their exact fate is not clearly understood.

The duodenal flow of peptides and amino acids has been investigated (Broderick and Wallace, 1988 Chen et al., 1987b). A considerable disagreement exists in the duodenal flow of peptides and free amino acids estimated by different workers. The ability of the ruminant forestomach epithelial tissues to absorb free and peptide bound amino acids (Matthews and Webb, 1995) and some understanding on the mechanisms involved in these processes (Matthews et al., 1996a) have also been demonstrated using radio labeled and synthetic substrates. These workers emphasize that the transport of peptides through the forestomach region could be an important route of their absorption. If significant amounts of ruminally-produced peptides are transported across the forestomach region, fundamental concepts of the protein nutrition of ruminants may need to be altered. Therefore, the present study was designed to investigate the absorption potential of ruminally-produced peptides and free amino acids via isolated ovine ruminal and omasal epithelial tissues, and to determine the influence that different dietary proteins may exert on this process.

Materials and Methods

Unless noted otherwise, all chemicals and reagents were prepared from Sigma Chemical Company (St. Louis, MO). The Virginia Tech Animal Care Committee approved protocol was used in the care and management of animals. Collection of tissues and measurement of uptake were described previously (Matthews and Webb, 1995). A previous experiment conducted in this laboratory confirmed the ability of

experimental buffers to maintain tissue metabolism throughout a 240-min incubation period in parabiotic chambers (Matthews and Webb, 1995).

In vitro Incubation of Dietary Proteins. Three proteins, soybean meal (solvent; SBM), casein (CAS) and distillers dried grains with solubles (DDG), were selected for in vitro ruminal incubation. The cell-free filtrates obtained following in vitro incubation of the above proteins with a ruminal inoculant were used as mucosal substrates for the uptake experiment. Preparation of buffer, inoculant medium and the incubation of proteins were all done similar to those described previously during the ruminal incubation experiment (Chapter IV) with some modifications. Incubation was performed in 250-ml plastic centrifuge bottles sealed with rubber stoppers fitted with Bunsen valves. Duplicate tubes containing protein plus inoculant were allowed to incubate (39^0C) for 8 h. At the end of incubation, the tubes were immediately centrifuged (27,000 x g, 25 min, 4^0C) to remove cellular and other insoluble matter. The supernatants containing peptides and free amino acids produced due to the degradation of proteins were collected and were filtered further through tissue culture filter units (0.2μ, NALGENE, Rochester, NY). The filtrates were stored immediately at -20^0C. To obtain sufficient substrates, three incubations were conducted on separate days. Filtrates obtained from different days were composited for each protein, the pH was adjusted to 6.9 and were stored at -20^0C to be used in the uptake experiment. The above filtrates (warmed to 39^0C) were used as mucosal fluids of the parabiotic chambers to quantify serosal appearance of peptides and amino acids across ruminal and omasal epithelia.

Animals, Feeding and Tissue Collection. Ruminal and omasal tissues were obtained from two Dorset × Suffolk × Finn wethers (Mean BW 43.18 kg). Amino acid uptake was quantified in two runs (on two separate days), and tissues used on a day were collected from one wether. Wethers were housed with continuous access to water and fed a mixed diet containing 50% ground shelled corn, 30% orchardgrass hay, 13.3% soybean meal, 5% molasses, 0.5% limestone, 0.5% trace mineral salt and 0.42% defluorinated rock phosphate (as-fed basis). Decoquinate (Cocci Control Crumbles Medicated, Southern States, Inc., Richmond, VA) to supply 0.5 mg day^{-1} $sheep^{-1}$ were also added to the diet. The animals had been previously injected with vitamin A (500,000 IU), vitamin D (75,000 IU), vitamin E (3.7 IU kg –1), and Se (55 $\mu g.kg^{-1}$). At

the time of tissue collection, the selected wether was stunned with a captive-bolt pistol (Super Cash Mark 2, Accles and Shelvoke LTD., Birmingham, England), and exsanguinated. The abdominal cavity was opened and the stomach was removed quickly. The reticulorumen was opened along the dorsal surface and digesta was removed by rinsing with tap water. The omasum was opened through the omasal orifice and the digesta was removed. Ruminal and omasal tissues were placed in 0.85% NaCl and immediately transported to the laboratory. All rinsing solutions and buffers used were maintained at 39^0C. Ruminal epithelial tissue was stripped from the underlying muscle layer by careful dissection and then cut into pieces (≈ 4 cm x 4 cm). Omasal epithelial tissue was prepared by peeling apart the opposing surfaces of individual plies. To remove adhering digesta particles, the tissues were washed with gentle agitation first in two baths of 0.85% NaCl then in four baths of Krebs Ringer Phosphate (KRP) buffer (pH 7.4, Umbreit et al., 1964). The tissues were finally held in oxygenated KRP buffer (pH 7.4).

Buffer Preparation. Krebs Ringer Phosphate buffer (pH 7.4) was used for tissue preparation. This buffer was prepared on the day before the actual uptake measurement and was stored at -4^0C overnight. The buffer was then warmed to 39^0C in a water bath and gassed with 95% O_2 / 5% CO_2 for 1h before use. The KRP buffer used in the serosal chambers of the parabiotic units contained 10 mM D-glucose and enough D-mannitol to equalize osmolarity with the substrate used in the corresponding mucosal chamber. Buffers used for uptake measurements were refrigerated (4^0C) overnight in 40-mL sealed tubes. Two hours before the beginning of uptake measurements, these buffers were aspirated into 20-mL syringes (Sherwood Medical, St. Louis, MO) that were capped with 25-gauge needles (Becton Dickinson and Co., Rutherford, NJ), inserted into neoprene stoppers and placed into a 39^0C water bath.

Uptake Measurement. Parabiotic units consisting of two L-shaped glass chambers of equal volume separated by a neoprene o-ring and held together by a clamp were used. Epithelial tissues were mounted between the two chambers of the parabiotic units. The chambers were designated as mucosal or serosal with reference to tissue orientation. The area of exposed tissue was 1.77 cm^2. Uptake measurements were initiated by loading the appropriate substrates and buffers followed by inserting

gassing/sampling devices into mucosal and serosal chambers of each parabiotic unit (Matthews and Webb, 1995). Mucosal chambers were filled with 15 mL of KRP buffer (pH 6.9) as a control, or with a cell-free supernatant obtained following incubation of soybean meal (SBM), casein (CAS) and distillers dried grains with solubles (DDG). Serosal chambers were filled with 15 mL of KRP buffer (pH 7.4) containing 10 mM D-glucose and enough D-mannitol to equalize osmolarity with the substrate used in the corresponding mucosal chambers. For each of the two runs, 10 parabiotic units were prepared for ruminal and ten for omasal tissues. For each tissue, three replicates of each protein and a control were prepared. All chambers were gassed with 95% O_2 / 5% CO_2 at a similar rate using polypropylene tubing. The mucosal buffer was sampled at 0 min and the serosal buffer was sampled at 240 min. Sampling was performed by attaching a 1 mL syringe to the luer stub adapter of the sampling line of each chamber and withdrawing .6 mL of buffer. After 240 min, tissues were removed and the area exposed to the buffer was excised, blotted with absorbent paper, dried (100^0C, 24h), and the dry weight was recorded. Amount of free and peptide amino acids appearing in the serosal buffer were quantified by HPLC using two procedures: without filtration or with a filtration step using a Centricon-3-microconcentrator of 3,000 MW cut-off filter (Amicon, Beverly, MA). The filtrates obtained after centrifugation (2,700 *x* g, 2 h) by both methods were divided into two parts, one for the determination of free amino acids by immediate analysis, the other for the determination of total amino acids after acid hydrolysis (6N HCl at 112^0C for 24 h). The individual amino acid concentrations were determined using a Pico tag Amino Acid Analysis System (Waters Millipore Corp., Milford, MA). Peptide amino acid concentrations were calculated as the difference between hydrolyzed and nonhydrolyzed samples. The serosal appearance was expressed as $\mu g \cdot L^{-1} \cdot mg^{-1}$ dry tissue.

Statistical Analysis. The data were analyzed using the GLM procedure of SAS (1988). Split-plot designs were used to analyze both mucosal concentrations and serosal appearances. Animals (Runs) as main plots, and amino acid forms, tissue types and protein sources as sub plots were used in $2 \times 3 \times 2 \times 3$ factorial combinations. Mucosal concentration data were evaluated for the effect of animal, amino acid form, tissue type, protein source, and amino acid form $\times$ protein source. Orthogonal contrasts were used to partition the effects of amino acid form and protein source on mucosal concentration.

Serosal appearance data were evaluated for the effect of animal, amino acid form, tissue type, protein source, amino acid form × protein source, amino acid form × tissue type, protein source × tissue type and amino acid form × tissue type × protein source. Orthogonal contrasts were used to partition the effect of amino acid form, tissue type and protein source on serosal appearance. Student's t test was employed to evaluate whether serosal appearances (as a fraction of initial mucosal concentrations) differed from zero.

Results and Discussion

This experiment was designed to investigate the potential of ovine ruminal and omasal epithelia to absorb free and peptide amino acids that are produced due to protein degradation in the rumen. Cell-free supernatants obtained following in vitro ruminal incubation of SBM, CAS, and DDG were used as mucosal substrates to simulate ruminal fluid conditions with regard to free and peptide amino acid production during degradation of these proteins in the rumen. The SBM was selected because of its common inclusion in ruminant diets. The CAS and DDG were selected because of the relatively higher peptide and free amino acid productions observed in previous incubation experiments.

Mucosal Concentrations. Table 5.1 shows the initial concentrations of free and peptide amino acids among mucosal substrates. The mean mucosal concentrations of EAA, NEAA, and total amino acids were greater ($P < 0.001$) for peptides than for free amino acids. Total amino acid concentrations of peptides (total) were two (SBM) to 13 (DDG) times higher ($P < 0.001$) than the total free amino acid concentrations. Low molecular weight (< 3,000 MW) peptides accounted for 15 (SBM) to 38% (DDG) of the total peptide-bound amino acids. Greater concentrations ($P < 0.05$) of low molecular weight (< 3,000 MW) peptides than free amino acids were observed with SBM and DDG.

The specific protein used for ruminal incubation influences the concentration of a particular amino acid present in the ruminal fluid either in free or peptide form. Mucosal concentrations of total, EAA, NEAA, and individual amino acids varied ($P< 0.01$, amino acid form x protein interaction) among protein sources. Free amino acid concentrations for total, EAA and NEAA were highest ($P < 0.001$) in CAS. Free amino acid concentrations of SBM were greater ($P < 0.05$) than DDG for total, EAA, and NEAA. The SBM exhibited the highest ($P < 0.05$) peptide (total) amino acid concentration for

total, EAA and NEAA. The highest ($P < 0.05$) concentrations of peptide (< 3,000 MW) bound NEAA and total amino acids were found in DDG. The ratio of free amino acid concentrations of EAA : NEAA ranged from 1:1 (SBM) to 1.7:1 (DDG). Ratios of EAA and NEAA varied from 1:1.7(SBM) to 1.5:1 (CAS) for peptides (< 3,000 MW).

Thus, in this in vitro system, the concentration of peptide amino acids was greater than the concentration of free amino acids. These concentrations are well within the concentrations of free (7.2 to 60 mg/L) and peptide-bound (100 to 270 mg/L) amino N reported to exist in the ruminal fluid of sheep and cows post feeding (Matthews et al., 1996a). These observations emphasize that peptide accumulation in ruminal fluid can exceed that of free amino acids following the degradation of proteins by microorganisms in the rumen. As would be expected, the variations observed in the amino acid concentrations among the different mucosal substrates indicate that dietary proteins can influence the quantities of free and peptide amino acid produced in the rumen.

Serosal Appearances. The main effect means and the individual treatment effects on the serosal appearance of free and peptide amino acids via ruminal and omasal epithelia are presented in the Tables 5.2 and 5.3, respectively. Serosal appearances are expressed as $\mu g \cdot L^{-1}$mg dry tissue^{-1} assuming that the surface area is different between equal cross sections of ruminal and omasal epithelia and the mass of dry tissue is more related to surface area than is cross sectional area (Stevens and Stetler, 1966). Matthews and Webb (1995) also suggested that uptake expressed on a tissue dry weight basis is an appropriate way of comparing translocation of free and peptide amino acids via ruminal and omasal epithelia. The average tissue dry weights of ruminal and omasal epithelia exposed to the buffers in the parabiotic chambers were 54.55 ± 4.71 and 20.82 ± 0.83 mg, respectively. To account for residual free and peptide-bound amino acids of tissue origin that may be transferred to serosal buffers from tissues directly, the serosal appearance data for SBM, CAS, and DDG were corrected using controls that had only KRP buffers as mucosal fluids. It is reasonable to assume then that, with the correction for the blank, serosal appearance data of SBM, CAS, and DDG represent free and peptide amino acids that were translocated from mucosal fluids.

Serosal appearances of EAA, NEAA, and total amino acids were greater ($P < 0.001$) for peptides than for free amino acids (Table 5.2). The serosal appearance of amino acids in peptide form was nearly three times higher ($P < 0.001$) than free amino acids. The uptake of aspartic acid, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, and valine were higher ($P < 0.05$) for peptides contributing 80% of the total peptide bound amino acid appearance. Conversely, the serosal appearances of alanine, glutamic acid, glycine, and tyrosine were higher ($P < 0.001$) in the free form than the peptide forms. In peptide form, the EAA accounted for about 82% of the serosal appearance of amino acids. In contrast, only 24% of serosal appearance was EAA in the free form. Low molecular weight (< 3,000 MW) peptides accounted for a little more than half of the total peptide amino acids that appeared in the serosal fluid.

These results strongly support some previous research conducted in this laboratory, which suggest that the forestomach can be an important site of peptide-bound (relatively large) and free (relatively small) amino acid absorption in ruminants (Webb et al., 1993; Matthews et al., 1996a). Those findings are in consistent with the observations of Seal and Parker (1996), who reported a greater net appearance of peptide amino acids than free amino acids across portal drained viscera than across mesenteric drained viscera.

Serosal appearance of amino acids was greater ($P < 0.01$) across omasal than across ruminal tissues (Table 5.2). The serosal appearance of total free amino acids across omasal tissue was about 1.9 times greater than the total free amino acid uptake across ruminal tissue. Meanwhile, peptide amino acid uptake was 2.7 (total) to 3.6 (< 3,000 MW) times greater across omasal tissues than via ruminal tissues. This accounts for the amino acid form x tissue type interaction ($P < 0.001$) that was observed (Table 5.3). These observations support the concept that omasal epithelium has a greater ability to translocate both free and peptide-bound amino acids than does the ruminal epithelial tissues on a tissue dry weight basis. It appears also that omasal epithelium may have a greater ability to translocate peptides than ruminal epithelium. Using carnosine, methionine and methionylglycine, Matthews and Webb (1995) previously showed that ovine omasal epithelium has a greater capacity to absorb free and peptide-bound amino acids than does ruminal epithelium. This hypothesis is also supported by the

comparatively higher metabolic activity (Engelhardt and Hauffe, 1975), large numbers of branching cells in the stratum basale (Steven and Marshall, 1970), greater potential electrolyte flux (Martens and Gabel, 1988), and a greater blood supply (Engelhardt and Hales, 1977) reported for the omasum. However, the actual amounts of total free and peptide-bound amino acids absorbed via the rumen or the omasum under in vivo conditions will also be influenced greatly by the retention time of protein digesta and the overall surface area presented by the two organs.

When serosal appearance of amino acids were calculated as fractions of initial mucosal concentrations, glutamic acid and tyrosine (free forms) had values greater than 1. Simultaneously, the serosal appearance of peptide-bound glutamic acid and tyrosine were negligible though in combine they have contributed to >15% of the total amino acids in mucosal fluids. The above observation possibly indicate that the peptides have undergone hydrolysis during the passage through ruminal and omasal epithelia and the corresponding free amino acids were released in to the serosal fluids. A considerable hydrolysis of methionylglycine dipeptides during the transepithelial passage through ruminal and omasal epithelia was reported using a similar in vitro system (Matthews and Webb, 1995).

Translocation of free and peptide bound amino acids had not occurred in the same proportions to their initial mucosal concentrations. Arginine, glycine, and tyrosine showed a relatively high translocation (> 50% of the initial concentrations) though the initial mucosal concentrations of these amino acids were very low (<10% of total free amino acids). Meanwhile, the appearance of aspartic acid, histidine, isoleucine and methionine were very low (<10%) or negligible though substantial amounts of these free amino acids were present in the mucosal substrates. Meanwhile, the appearance of peptide-bound arginine, isoleucine, and methionine was quite high (> 50% of initial mucosal concentrations). Serosal appearance of peptide-bound aspartic acid and glutamic acid were very low (<5% of initial mucosal concentrations) though these two amino acids accounted for >25% of the total peptide-bound amino acids in the mucosal fluids. These observations indicate that some selectivity may be present in the absorption of both free and peptide amino acids via ruminal and omasal epithelia.

Multiple mechanisms could have been involved in the transport of free and peptide amino acids via ruminal and omasal epithelia. In the first place, diffusion appeared to play a major role in the absorption of peptides. The mucosal concentration data of the present study along with previous observations (Broderick and Wallace, 1988), suggest greater concentrations of peptides than free amino acids in ruminal fluid following protein degradation. Greater serosal appearance of peptide amino acids observed across ruminal and omasal tissues probably reflects the concentration effect of the mucosal substrates. The greater osmotic driving force created by relatively higher peptide concentrations in mucosal substrates may have favored more peptide amino acid absorption for transport via ruminal and omasal epithelial tissues by facilitated or simple diffusion mechanisms. The present observations on selective absorption of peptide amino acids indicate that processes other than simple diffusion may have been involved in their transport. Therefore, facilitated diffusion could be a major mechanism involved in the transport of peptides in the present study.

Carrier-mediated active absorption could also have been involved in the absorption of peptide-bound amino acids via ruminal and omasal epithelia. Presence of messenger RNA that encode for proteins capable of H^+ dependent dipeptide transport activity has been demonstrated in the omasal epithelia of sheep (Matthews et al., 1996b). Also, the existence of a peptide transporter protein (Pep T1) in omasum and rumen of sheep and cows was detected using a probe developed to detect $Poly(A)^+$ RNA transcripts (Chen et al., 1999). The other conditions (acidic pH levels to develop proton gradients, H^+ and Na^+ ions, Na+/H+ exchangers and Na+/K+ ATPase) essential for carrier mediated active absorption of peptides are also reported to present in the forestomach region of the ruminants (Matthews et al., 1996a). The conditions favoring carrier-mediated active absorption of peptides (H^+ and Na^+ ions and proton gradient) were maintained in the experimental buffers. Therefore, carrier-mediated active absorption would have been involved in the translocation of some peptide amino acids particularly those which were present in low concentration in the mucosal fluid.

Paracellular absorption may also have been involved in the transport of peptides via ruminal and omasal tissues. Paracellular transport has been suggested as a possible mechanism of peptide absorption via forestomach epithelia (McCollum, 1997) whose

tight junctions are considered to be relatively loose than the enterocytes (fell and Weeks, 1975). Presence of high luminal concentrations and carrier-mediated mechanisms were recognized as two prerequisites for the paracellular absorption of nutrients (Madara and Pappenheimer, 1987). Relatively high concentrations of peptides in mucosal fluids and the possible involvement of carrier-mediated mechanisms observed in the present study support that paracellular absorption could be an important mechanism of peptide transport via forestomach epithelia of ruminants.

Also, several mechanisms appeared to involve in the absorption of free amino acids. Non-saturable absorption of free amino acids was reported in some previous uptake studies (Leibholz, 1971; Matthews and Webb, 1995) to suggest that diffusion may be involved in the transport of free amino acids via the forestomach. Carrier-mediated facilitative transport was proposed in the transport of lysine and arginine across ruminal tissues (Fejes et al., 1991). McCollum (1996) also demonstrated saturable uptake of lysine via omasal tissues. But it was not certain if this occurred by carrier-mediated active or facilitative transport. The present observations on relatively low concentrations of free amino acids in the mucosal substrates suggest that simple and facilitated diffusion mechanisms are of minor importance in the absorption of free amino acids via those forestomach epithelial tissues. The present observations on low mucosal concentrations along with selective absorption of free amino acids strongly support that carrier-mediated active absorption could be involved to a greater in their transport.

Serosal appearances of amino acids were influenced by protein source (Table 5.2). There was a greater serosal appearance of amino acids from CAS than from SBM. Differences between SBM and DDG were not significant. Uptake of lysine ($P < 0.09$), methionine ($P < 0.001$), and glycine ($P < 0.06$) were greater for CAS than for SBM. The above variations found in the serosal appearance of free and peptide-bound amino acids among different mucosal treatments suggest that different proteins used for ruminal incubation can influence amino acid absorption via the forestomach. Different proteins used for in vitro ruminal incubations have created mucosal substrates of variable free and peptide-bound amino acid concentrations, which appeared to influence absorption via forestomach epithelial tissues. Therefore, dietary protein modifications in the rumen

seems to influence greatly on the absorption of free and peptide-bound amino acids via the ruminant forestomach.

In summary, the results of this study provide evidences that peptides and free amino acids resulting from the microbial degradation of proteins in the rumen can be absorbed intact via ruminal and omasal epithelia. The absorption of peptides via forestomach epithelia can usually exceed free amino acids. On tissue dry weight basis, omasal epithelia could exhibit a greater capacity to translocate free and peptide-bound amino acids than does ruminal epithelia. The dietary protein used for ruminal microbial fermentation can manipulate the absorption of free and peptide-bound amino acids via forestomach epithelia.

Implications

The results of this study indicate that the peptides and free amino acids produced following the degradation of proteins by the microorganisms in the rumen can be absorbed via ruminant forestomach. With more understanding on the magnitude and the methods of manipulating this route of amino acid absorption, the information may be incorporated in models that predict N supply to the ruminants. Also, supplementation of amino acids via forestomach of ruminants would be of prime concern in future dietary formulation efforts if the importance of this route of amino acid absorption were clearly understood.

Table 5.1. The initial concentrations of free, peptide (total) and peptide (<3,000 MW) amino acids among mucosal substrates prepared from soybean meal (SBM), casein (CAS), and distillers dried grains with solubles (DDG).

Amino acid	Free amino acids				Peptide (total) amino acids				Peptide (<3,000 MW) amino acids				SE	Significance of contrast[a]			
	SBM	CAS	DDG	Mean	SBM	CAS	DDG	Mean	SBM	CAS	DDG	Mean		1 vs 2	1 vs 3	4 vs 5,6	5 vs 6
	mg/L																
Arginine	2.23	1.70	2.22	2.05	13.73	10.22	6.12	10.03	1.32	2.55	1.90	1.92	0.44	0.035	0.001	0.001	0.001
Histidine	2.71	2.54	2.39	2.55	4.02	3.30	2.80	3.37	0.00	1.53	1.47	1.00	0.19	NS	NS	NS	0.001
Isoleucine	1.32	10.97	0.11	4.13	13.13	12.73	7.69	11.19	1.44	3.09	2.37	2.30	0.50	0.001	0.001	0.001	0.001
Leucine	1.55	12.22	0.51	4.76	21.73	17.69	11.69	17.03	2.26	3.61	3.18	3.01	0.73	0.001	0.001	0.001	0.001
Lysine	0.90	11.16	0.60	4.22	19.48	25.81	13.82	19.70	3.28	6.66	4.15	4.70	0.83	0.001	0.001	0.001	0.001
Methionine	1.24	1.91	1.46	1.54	5.61	5.99	1.16	4.25	1.98	3.70	3.32	3.00	0.10	0.001	NS	0.001	0.001
Phenylalanine	1.16	7.23	0.00	2.80	15.14	12.94	8.14	12.07	1.71	2.47	2.18	2.12	0.51	0.001	0.001	0.001	0.001
Threonine	0.79	2.71	0.92	1.47	14.96	13.57	11.65	13.39	1.78	2.85	3.50	2.71	0.54	0.072	NS	0.001	0.001
Valine	2.18	10.11	0.53	4.27	13.09	11.00	8.94	11.01	1.43	2.22	3.03	2.23	0.46	0.001	0.001	0.001	0.001
Total essential	14.08	60.55	8.74	27.79	120.89	113.25	72.01	102.04	15.20	28.68	25.10	22.99	3.91	0.001	0.001	0.001	0.001
Alanine	4.94	10.91	1.58	5.81	15.81	13.17	14.39	14.46	2.44	2.39	5.73	3.52	0.53	0.001	NS	0.001	0.001
Aspartic acid	0.00	6.31	0.04	2.12	35.27	30.06	22.64	29.32	6.58	5.30	8.17	6.68	1.22	NS	0.001	0.001	0.001
Glutamic acid	1.96	18.01	1.29	7.09	43.94	26.39	28.09	32.80	8.32	1.95	11.34	7.20	1.38	0.005	0.001	0.001	0.001
Glycine	3.05	5.04	0.63	2.91	12.54	12.10	10.75	11.80	2.39	3.77	4.61	3.59	0.41	0.001	0.008	0.001	0.001
Proline	2.38	3.04	0.66	2.03	12.66	9.81	12.37	11.62	2.83	3.12	7.28	4.41	0.43	0.006	0.001	0.001	0.001
Serine	0.14	1.21	0.30	0.55	15.61	12.06	11.67	13.11	2.47	2.58	3.34	2.80	0.56	0.026	0.007	0.001	0.001
Tyrosine	1.35	8.19	0.55	3.36	10.58	7.11	6.05	7.91	0.85	0.00	1.64	0.83	0.43	NS	0.001	0.002	0.001
Total nonessential	13.82	52.71	5.05	23.87	146.41	110.70	105.96	121.02	25.88	19.11	42.11	29.03	4.75	0.001	0.001	0.001	0.001
Total	27.90	113.26	13.79	51.66	267.30	223.95	177.97	223.06	41.08	47.79	67.21	52.02	8.55	0.001	0.001	0.001	0.001

[a]1 = SBM, 2 = CAS, 3 = DDG, 4 = Free amino acids, 5 = peptides (total) amino acids, 6 = peptides (< 3,000 MW) amino acids.

Table 5.2. Main effect means of protein source, amino acid form, and tissue type on serosal appearance of amino acids following 240 min of incubation with substrates prepared from soybean meal (SBM), casein (CAS), and distillers dried grains with solubles (DDG).

Amino acid	Protein source			Amino acid Form			Tissue		SE	Significance of contrast			
	SBM	CAS	DDG	Free	Peptide (total)	Peptide (<3,000MW)	Ruminal	Omasal		SBM vs CAS	SBM vs DDG	Free vs Peptide	Ruminal vs Omasal
	----------μg·L^{-1}mg dry tissue^{-1}----------												
Arginine	32.97	35.83	40.50	35.35	32.56	41.39	18.67	54.19	3.13	NS	NS	NS	0.001
Histidine	12.00	14.02	13.06	0.75	36.85	1.48	9.67	16.39	2.10	NS	NS	0.001	0.003
Isoleucine	16.81	26.22	20.20	0.38	32.21	30.63	10.99	31.16	4.00	NS	NS	0.001	0.001
Leucine	30.12	31.77	35.12	6.99	64.26	25.76	21.05	43.62	4.30	NS	NS	0.001	0.001
Lysine	15.41	27.16	26.73	12.08	43.58	13.64	16.57	29.63	14.29	0.09	NS	NS	0.10
Methionine	101.30	319.57	149.99	0.99	321.91	247.97	70.03	310.55	38.88	0.001	NS	0.001	0.001
Phenylalanine	14.55	17.39	19.01	0.00	51.05	0.00	13.81	20.22	3.36	NS	NS	0.001	0.05
Threonine	16.69	19.03	20.44	7.57	40.11	8.47	10.55	26.88	2.79	NS	NS	0.001	0.001
Valine	21.02	28.68	26.11	6.16	51.28	18.38	16.82	33.72	3.19	0.10	NS	0.001	0.001
Total essential	260.87	519.67	351.26	70.27	673.81	387.71	188.16	566.33	48.95	0.001	NS	0.001	0.001
Alanine	21.11	27.54	25.02	38.96	26.75	7.96	16.72	32.39	3.41	NS	NS	0.001	0.001
Aspartic acid	11.99	14.04	17.05	0.19	38.04	4.86	9.57	19.15	12.49	NS	NS	NS	0.10
Glutamic acid	22.22	23.61	26.20	72.03	0.00	0.00	20.00	28.02	5.80	NS	NS	0.001	NS
Glycine	20.03	29.47	31.02	33.23	23.79	23.50	17.64	36.04	2.88	0.06	NS	0.001	0.03
Proline	20.62	25.75	27.46	11.60	50.14	12.09	15.22	34.00	3.15	NS	NS	0.001	0.001
Serine	10.85	9.86	17.98	2.99	27.14	8.30	5.85	19.94	2.81	NS	NS	0.05	0.01
Tyrosine	23.31	32.76	23.10	66.51	12.66	0.00	12.75	40.03	8.17	NS	NS	0.001	NS
Total nonessential	130.13	163.03	167.84	225.51	178.78	56.72	97.75	209.57	25.66	NS	NS	0.001	0.01
Total	391.00	682.70	519.10	295.78	852.59	444.43	285.91	775.90	58.47	0.001	NS	0.001	0.001

Table 5.3. Serosal appearance of free, peptide (total), and peptide (< 3,000 MW) amino acids via ruminal and omasal epithelia incubated for 240 min with substrates prepared from soybean meal (SBM), casein, and distillers dried grains wit solubles (DDG).

Amino acid	SBM						CAS						DDG						Interactions		
	Free		Peptide (total)		Peptide<3,000 MW		Free		Peptide (total)		Peptide<3,000 MW		Free		Peptide (total)		Peptide<3,000 MW		Protein x Form	Protein x tissue	Form x Tissue
	Ruminal	Omasal	Ruminal	Omasal	Ruminal	Omasal	Ruminal	Omasal	Ruminal	Omasal	Ruminal	Omasal	Ruminal	Omasal	Ruminal	Omasal	Ruminal	Omasal			
	---------- μgL^{-1} mg dry $tissue^{-1}$ ----------																				
Arginine	7.6	32.5	11.0	58.9	36.4	51.4	29.9	63.7	8.3	40.1	15.6	57.4	28.8	49.7	14.6	62.5	16.0	71.5	NS	NS	NS
Histidine	0.0	0.0	28.8	38.0	0.0	5.2	1.3	3.2	24.9	51.1	3.7	0.0	0.0	0.0	28.4	50.0	0.0	0.0	NS	NS	0.001
Isoleucine	0.0	0.0	21.3	34.3	9.6	35.7	2.3	0.0	21.6	48.3	8.4	76.8	0.0	0.0	23.0	44.8	12.9	40.5	NS	NS	0.04
Leucine	2.2	0.0	49.2	76.2	17.2	35.9	7.2	4.9	39.6	87.7	11.3	40.0	14.7	13.0	35.4	97.4	12.7	37.5	NS	NS	0.004
Lysine	6.7	14.6	32.1	20.3	8.5	10.4	8.1	21.6	42.7	65.2	6.4	18.9	7.3	14.2	25.0	76.2	12.3	25.4	NS	NS	NS
Methionine	0.0	0.0	58.2	233.7	16.4	299.5	0.0	0.0	121.1	991.7	118.9	635.7	0.0	5.9	136.5	340.3	129.1	288.1	NS	0.04	0.01
Phenylalanine	0.0	0.0	41.2	46.1	0.0	0.0	0.0	0.0	40.3	64.0	0.0	0.0	0.0	0.0	42.8	71.9	0.0	0.0	NS	NS	0.03
Threonine	5.1	12.0	25.6	53.4	3.0	1.1	8.9	4.8	13.8	64.7	3.5	18.4	10.7	4.0	18.8	64.4	5.7	19.1	NS	NS	0.001
Valine	3.2	1.6	37.4	59.5	5.5	18.9	8.9	15.7	36.1	69.4	9.3	32.6	1.4	6.1	29.9	75.4	19.7	24.3	NS	NS	0.07
Total essential	24.8	60.7	304.8	620.4	96.6	458.1	66.6	113.9	398.4	1482.2	177.1	879.8	62.9	92.9	354.4	882.9	208.4	506.4	0.02	0.01	0.001
Alanine	27.2	47.1	18.7	26.5	3.4	3.7	36.9	63.7	16.9	35.6	0.0	12.2	23.6	35.3	17.2	45.6	6.6	21.9	NS	NS	0.001
Aspartic acid	0.0	0.0	25.2	42.3	1.5	3.1	1.1	0.0	27.1	52.9	3.1	0.0	0.0	0.0	22.8	58.0	5.4	16.1	NS	NS	NS
Glutamic acid	58.8	74.5	0.0	0.0	0.0	0.0	57.4	84.3	0	0	0.0	0.0	63.8	93.3	0.0	0.0	0.0	0.0	NS	NS	0.06
Glycine	26.1	37.5	5.1	15.5	12.8	23.2	31.4	51.5	12.7	39.0	12.9	29.2	23.0	29.8	16.2	54.3	18.5	44.4	0.06	NS	0.001
Proline	8.7	16.2	29.1	54.7	6.2	8.9	12.2	15.9	30.3	76.2	2.2	17.7	9.6	7.0	29.5	81.0	9.2	28.4	NS	NS	0.002
Serine	0.8	1.6	11.0	38.5	4.0	9.1	8.8	4.7	4.0	40.7	0.0	0.0	2.2	0.0	9.9	60.2	12.0	23.7	NS	NS	0.001
Tyrosine	22.8	106.9	10.2	0.0	0.0	0.0	23.5	125.8	20.2	27.0	0.0	0.0	28.7	91.4	9.3	9.2	0.0	0.0	NS	NS	0.001
Total nonessential	144.4	283.8	99.3	177.5	27.9	48.0	171.3	345.9	111.2	271.4	18.2	59.1	150.9	256.8	104.9	308.3	51.7	134.5	NS	NS	0.001
Total	169.2	344.5	404.1	797.9	124.5	506.1	237.9	459.8	509.6	1753.6	195.3	938.9	213.8	349.7	459.3	1191.2	260.1	640.9	0.02	0.01	0.001

Chapter VI

EPILOGUE

The present study provides further evidence to suggest that peptides and free amino acids resulting from the microbial digestion of proteins in the rumen are potentially absorbed via ruminal and omasal epithelia, and that the absorption of peptides exceeds that of free amino acids. Results from this study further suggest that the amounts and types of free and peptide amino acids absorbed via the forestomach of ruminants may be influenced by dietary modifications. These results are complementary to previous research conducted in this laboratory (Webb et al., 1993; Matthews et al., 1996a), which provided substantial evidence for the presence of mechanisms involved in the absorption of free and peptide amino acids via the ruminant forestomach. Yet, several questions need to be resolved before this information is practically used in feeding strategies to improve protein utilization of ruminants. These questions include: (1) to what extent are free and peptide-bound amino acids absorbed via the forestomach, (2) how do the diet and other factors influence this process, (3) will increasing the amount of peptide and free amino acid absorption via the forestomach achieve a higher efficiency in nutrient utilization, and (4) what dietary modifications can be used with greater economic advantage to achieve a higher forestomach absorption of peptides and free amino acids.

Thus, future studies should be directed to understand the magnitude and the nutritional significance of peptide and free amino acid absorption across forestomach epithelia. To confirm the present findings and to gain more knowledge on the relative amounts of free and peptide bound amino acids absorbed via the reticulorumen and omasum, use of in vivo techniques will be most appropriate. Measurement of peptide and free amino acid fluxes in the non-mesenteric drained viscera of catheterized ruminants (of different species and stages of production) receiving a variety of diets could provide a better understanding of the magnitude of forestomach absorption process with dietary and animal conditions. Use of radiolabeled substrates in the diet or infused into the rumen will also be helpful.

Also, a thorough understanding of the mechanisms involved in forestomach absorption of free and peptide amino acids and means of regulating these events is of fundamental importance. In vitro studies designed to measure serosal absorption of amino acids using radiolabeled or fluorescent markers, and cloning experiments to determine the nucleic acid sequences of peptide and amino acid transporters in the forestomach epithelia will be useful to understand these mechanisms.

The studies on the nutritional significance of peptide absorption in ruminants are also very exciting. Experiments conducted in this laboratory (Pan et al., 1996; Wang et al., 1996) and elsewhere (Backwell et al., 1994) have demonstrated that the small molecular weight peptides can be efficiently utilized as sources of amino acids for the synthesis of milk and muscle proteins. Long-term experiments designed to measure productivity (milk production, body weight gain etc.,) of animals, whose amino acid requirements are met by supplying either peptide or free forms, could provide further understanding of the nutritional significance of peptide absorption. The ability of ruminants to absorb peptides for tissue protein utilization would allow us to consider them as a more efficient group of animals than we thought before.

The traditional N balance schemes for ruminants (NRC, 1985; Sniffen et al., 1992) have been based on several empirical equations to predict N inputs, production of ammonia and microbial protein in the rumen, and availability of free amino acids in the small intestine from microbial and undegraded dietary proteins. No allowance is made in any of these schemes for the amino acids (free and peptide-bound) that can be available from direct absorption through forestomach epithelia. If future research confirms significant amino acid absorption across the forestomach, then this will become an integral component in subsequent diet formulation efforts for ruminants.

Controlling the rate and extent of dietary protein degradation in the rumen is a major consideration among ruminant nutritionists. Because, the degradation of dietary protein in the rumen is often considered to be an inefficient process, particularly when a higher level of production is sought. High quality protein supplements are frequently fed under these circumstances to meet additional requirements of the animal. But, a substantial amount of protein can be lost due to microbial fermentation as ammonia. This ammonia is mostly diffused into the blood and is excreted as urea. Several techniques

have been proposed to control ruminal protein degradation, which include physical, chemical and biological means to protect protein from microbial attack (Broderick et al., 1991). If future research confirms a substantial absorption of peptides and free amino acids via the forestomach, then the need for 'by passing' proteins from ruminal degradation would become a less important activity. The investigations on controlling the intermediate steps of ruminal protein degradation (e.g. peptide hydrolysis, peptide uptake by bacteria and deamination) may be more useful in this context. The use of ruminally-protected peptides and free amino acids (peptide and amino acid analogues, acetylated peptides etc.) may be the preferred substrates for incorporation with ruminant diets. These protected peptides or amino acids can be included to provide the most limiting amino acids of host animals. Inclusion of compounds such as ionophores, which cause peptide accumulation in the rumen (Whestone et al., 1981) and reduce the deamination activity (Newbold et al., 1990) may also be beneficial. There appears to be an unexplored potential for new microbial feed additives to inhibit peptidolytic or deaminative activity of indigenous ruminal microorganisms. Genetic engineering and molecular biological techniques can also provide important contributions to manipulate ruminant N metabolism. There is much speculation on genetic manipulation of ruminant microorganisms while the technique might be applied for the alteration of genes of animals and in the manufacture of feeds. A recent study (Madeira et al., 1995) also demonstrated a selective means of inhibiting the growth of peptide-degrading bacteria. This novel approach (smugglin) allows inhibition of bacterial growth using peptide mimetics that transport via the peptide permeases. All of the above methods offer potential but require additional research before practical use will be realized. The potential of using such methods will ultimately depend on how they influence host animal nutrition.

The current feeding strategies to maximize production from ruminants has often resulted in overfeeding of high quality proteins, the most costly ingredient in the diet. The forestomach absorption of peptides and free amino acids can be very useful in finding a suitable solution to achieve efficient N utilization by ruminants. The success of attempts to control amino acid fermentation in the rumen or supplementation of limiting amino acids in protected forms may be enhanced by the ability of forestomach epithelia to

absorb peptides and free amino acids. Adoption of such strategies in maximizing the efficiency of N utilization by ruminants and minimizing wastage can have important nutritional and environmental consequences.

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APPENDIX A

PREPARATION OF MIXED RUMINAL MICROBIAL CULTURE FOR INCUBATION EXPERIMENTS

Procedure for the Collection of Whole Ruminal Contents

Modified from Tilley and Terry (1963).

1. Ruminally cannulated cows should be full fed common diet at least 7 days before first extraction of ruminal contents.
2. Withhold feed and water from animals 2 h prior to the collection of ruminal contents.
3. Allow animals to calm down and be cooperative.
4. Open the cannula. Insert hand (with a shoulder length glove) deep into the rumen through the cannula and mix ruminal contents thoroughly.
5. Collect whole ruminal contents carefully by hand, paying attention to get a representative sample from various locations of the rumen.
6. Quickly transfer ruminal contents into preheated (38^0 to 39^0C) Styrofoam coolers. Always keep the containers tightly closed except during transferring of ruminal contents.
7. Amount of whole ruminal contents required would be approximately double the amount of ruminal fluid needed for incubation. Close the cannula after a sufficient quantity of ruminal contents is collected.
8. Immediately transfer ruminal contents to the laboratory.
9. Ruminal contents of individual animals will be collected and processed separately.
10. Following rules are to be adhered during the collection of ruminal contents-
    (a) Use of same animals when collecting ruminal contents at different days.
    (b) Collecting ruminal contents at a standard time and by same technique each day.
    (c) Collecting and transporting ruminal contents quickly to prevent temperature shock.

Preparation of Buffer and Nutrient Medium

1. Buffer and nutrient medium should be prepared once for all runs prior to collection of ruminal fluid and refrigerated until used.
2. Use each constituent according to the proportions given in the table below (Modified from Loper et al., 1966).
3. Place these constituents (except $CaCl_2$, cellulose and starch) into a liter beaker. Add about 500 mL of deionized water and mix until completely dissolved. Then add $CaCl_2$, cellulose and starch and mix until completely dissolved.
4. Transfer this solution to a liter volumetric flask and dilute to volume with deionized water.
5. Transfer the solution back into the beaker. Measure the pH. Send CO_2 to bubble through the solution until pH 6.8 to 7.0 is reached.

6. Transfer to a storage container and store at 4^0C until use.

Constituents used for the preparation of buffer and nutrient medium.

Constituent	Amounts (g/L)	
	Buffer	Nutrient Medium
Cellulose	_	4
Starch	_	1
KCl	4	4
NaCl	4	4
KH_2PO_4	0.60	0.60
$Na_2HPO_4.7H_2O$	1.20	1.20
$NaHCO_3$	3.50	3.50
$MgSO_4$	0.15	0.15
Ca_2Cl_2	0.55	0.55
$CuSO_4.5H_2O$	_	0.002
$FeSO_4.7H_2O$	_	0.075
$MnSO_4.5H_2O$	_	0.004
$ZnSO_4.7H_2O$	_	0.001
$CoCl_2.6H_2O$	_	0.002

Processing of Whole Ruminal Contents in the Laboratory

The objective of this step is to eliminate residual feed particles from the whole ruminal contents while retaining a mixed ruminal microbial population consisting protozoa, fluid associated bacteria and particle associated bacteria.

1. Put whole ruminal contents into a large plastic funnel (lined with four layers of surgical gauze) placed on a measuring cylinder.
2. Squeeze ruminal fluid through surgical gauze, measure the volume and immediately transfer to a large (about 4 L) bottle placed in a water bath (39^0C), supplied with CO_2.
3. Collect the residue separately into another container.
4. Repeat the steps (1), (2) and (3) above if necessary until a sufficient volume of rumen fluid is obtained. The amount of rumen fluid required will be equal to a portion of one fifth of the total inoculant medium needed for incubation.
5. Measure the weight of the total residue.

6. Add an equal weight of buffer solution (warmed to 39^0C) to the residue. Supply CO_2 for 10 to15 s. Close the container and shake well to resuspend the material. Squeeze the liquid through cheese cloth into the measuring cylinder.
7. Repeat step (6) above three more times with the residue. Discard the residue. Immediately transfer the washed suspension to the bottle containing the rumen fluid and shake the bottle well.
8. Add buffer to the above solution, if required until the ratio of ruminal fluid : buffer reaches 1 : 4.
9. Transfer the ruminal fluid-buffer mixture into 250-mL centrifugation bottles. Centrifuge the bottles at 5,000 X g for 30 min. at 4^0C. Discard the supernatant and collect sediments into a blender.
10. Mix sediments with 750 mL of nutrient medium and blend for 30 sec.
11. Transfer the above mixture into a large (4L) bottle and add nutrient medium until the volume reaches the amount of original ruminal fluid-buffer used.
12. Leave this medium at 39^0C in a constant temperature bath for 6 h with a CO_2 supply.

Incubation of Feed Samples

1. Use air dried substrates (1 kg) ground to approximately 0.5 mm particle size using a Cyclotech mill.
2. Weigh triplicate samples from a particular substrate (equal to 0.35 g DM) into labeled 50 mL tubes and stored at 38^0 C until required.
3. Tubes should be fitted with stoppers equipped with Bunsen valves.
4. Pipette 35 mL of rumen fluid-buffer mixture into each of the incubation tubes.
5. Direct CO_2 in the space above the liquid of each tube for 15 s before tightly stoppering. Mix contents gently by a vortex mixer.
6. Incubate tubes in a water bath (at 39^0C). Run separate samples for 30, 60, 90 and 120 min of incubation.
7. Include triplicate blank tubes for each time interval that were treated in the same manner as with other tubes. A blank contains only ruminal fluid and mineral solution in the 1 : 4 ratio, with no substrate added.
8. Shake the contents in the tubes manually at 30, 60, 90 and 120 min of incubation.
9. After incubation, prepare the samples for the analysis of NH_3, alpha amino and peptide-N. The samples for NH_3-N assay are prepared by adding two drops of conc. H_2SO_4 to each 10 mL of incubation mixture and vortexing before storage at -20C. The samples for alpha amino and peptide-N assays are prepared by adding 2 mL of 25% TCA to 8 mL of incubation mixture, vortexing and allowing to stand at 4^0C overnight before storage at -20^0C.

APPENDIX B

Procedure for Amino Acid Uptake Experiment Using Parabiotic Chambers

1. Tissues are collected from one sheep for each run. A healthy and strong animal must be selected for tissue collection.
2. Stun the animal with a captive bolt gun and record the time.
3. Open the abdominal cavity and remove the rumen and omasum. The rumen is opened along the dorsal surface while the omasum is opened through the omasal orifice. Ruminal tissues will be collected from all the compartments.
4. Wash the tissues by immersion in tap water (39^0C) with gentle agitation.
5. Repeat until all the digesta is removed.
6. Immerse the washed tissues in 0.85% saline (39^0C) and immediately transfer the tissues to the laboratory.
7. Remove the ruminal epithelium from the underline muscle layer by careful dissection, cut the ruminal epithelium into sections 4cm *x* 4cm, and thoroughly clean by rinsing in succession; first twice with saline solution, then four times with buffer solution. The omasal epithelium is prepared by peeling apart opposing surfaces of the individual plies, followed by washing in the saline and buffer solutions.
8. Place the tissues in the holding buffer.
9. Mount the tissues between Ussing chamber halves. Place the o-ring on the serosal side and mount the tissue on the chamber before placing mucosal side on top. Clamp the chambers tightly and place a lead weight over the clamp.
10. Load 15 mL of mucosal fluid into the mucosal chamber then add 15 mL of serosal fluid into the serosal chamber. Use the supernatants of incubated feed samples as the mucosal fluid and KRP buffer (pH 7.4, osmolarity adjusted) as the serosal fluid. In the control treatment, use KRP buffer (pH 6.9) as the mucosal fluid (instead of supernatant).
11. Place the unit in the water bath (39^0C).
12. Insert the gassing+sampling device into each chamber and begin gassing. It is important to make sure that O_2 supply is not too strong and does not cause the fluids to bubble out of the chambers. Mark time zero as soon as gassing + sampling device is attached to the first chamber.
13. As soon as the gassing + sampling device is attached to the chambers, draw out .6 mL of sample by inserting 1-mL syringe into the luer stub of the sampling device from both chambers. Transfer the sample into labeled 1.5-mL centrifuge tubes to be stored at -20^0C for future analysis.
14. Repeat the above steps 10, 11, 12 and 13 to set up all the other chamber units.
15. Do sampling at 30, 60, 120 and 240 min. after zero time.
16. At the end of sampling, stop O_2 supply, disconnect gassing + sampling devices and take chambers out of the water bath. Collect mucosal and serosal fluid into a container. Disconnect two halves of each chamber and collect tissues separately into a tray.

17. Excise a circle of each tissue with #15 cork bore. Gently blot the tissue samples and place them in labeled aluminum pans of known weight.
18. Dry tissues in an oven (100^0C) for 24 h and record the dry weight of tissues + pan. Calculate the dry weight of tissues.

Buffers and Reagents

1. Mucosal fluid: supernatants of incubated proteins or KRP buffer (pH6.9, as the control treatment).
2. Serosal fluid : KRP buffer (pH 7.4, osmolarity adjusted) + glucose
3. Holding buffer : KRP buffer (pH7.4)
4. Saline (0.85%)

Preparation of Buffers and Reagents

KRP Buffer

Chemical	**Amount (g/L)**
NaCl	7.0128
Na_2HPO_4	2.2146
KCl	0.3727
$MgSO_4.7H_2O$	0.1445
$CaCl_2$	0.2886

Mix the above chemicals in order with about 950 mL of deionized water in a liter beaker. Stir until dissolved completely. Check pH and adjust to either 7.4 or 6.9 by adding 0.1N HCl. Transfer into a liter volumetric flask and bring to the volume by adding more deionized water.
For KRP buffer used as the mucosal fluid, add glucose 1.8020 g / L.

Saline 0.85%

Weigh 8.5 g NaCl into a liter volumetric flask, add deionized water to volume and mix.

Mucosal Fluid

Mucosal substrates are obtained by incubating proteins to obtain a peak peptide production. At the end of incubation, centrifuge tubes at 27,000 *x* g for 25 min. Collect supernatant and filter first through glass wool then through tissue culture filter units (.2 µ) and store at -20^0C.

Adjusting Osmolarity

Check osmolarity of the mucosal and serosal fluids. Calculate the mOsm difference;

mOsm difference = mOsm of mucosal fluid - mOsm of serosal fluid

Add mannitol to equalize osmolarity.

Amount (g) of mannitol to be added for each 100 mL of sample = 0.01822 x mOsm difference

Labeling (For six parabiotic units)

Chamber	Mucosal fluid	Tissue
A	Soybean meal	Omasal
B	Fish meal	Omasal
C	KRP (pH6.9)	Omasal
D	Soybean meal	Ruminal
E	Fish meal	Ruminal
F	KRP (pH6.9)	Ruminal

Color: Serosal - green; Mucosal - blue

*Six serosal syringes (20 mL)

Labeled: (green) AS, BS, CS, DS, ES, and FS

Loaded: 15 mL of KRP buffer (pH 7.4) + glucose (osmolarity adjusted)

to place in each appropriate serosal chamber.

*Six mucosal syringes (20 mL)

Labeled: (blue) AM, BM, CM, DM, EM and FM

Loaded: 15 mL of supernatants of incubated proteins or KRP buffer (pH6.9)

to place in each appropriate mucosal chamber.

*12 screw-top containers (20 mL)

Labeled: Mucosal (blue) AM, BM, CM, DM, EM and FM

Serosal (green) AS, BS, CS, DS, ES and FS

loaded with appropriate serosal and mucosal fluids in the night before.

*Six weighing pans

Labeled: A, B, C, D, E and F

*60 sampling syringes (1 mL)

Labeled: AM1, AM2, AM3, AM4, AM5, AS1, AS2, AS3, AS4, AS5
BM1, BM2, BM3, BM4, BM5 BS1, BS2, BS3, BS4, BS5
CM1, CM2, CM3, CM4, CM5, CS1, CS2, CS3, CS4, CS5
DM1, DM2, DM3, DM4, DM5, DS1, DS2, DS3, DS4, DS5
EM1, EM2, EM3, EM4, EM5, ES1, ES2, ES3, ES4, ES5
FM1, FM2, FM3, FM4, FM5, FS1, FS2, FS3, FS4, FS5

*60 centrifuge tubes (1.5 mL)

Labeled: as above correspond to each sampling syringe.

to store samples (-20^{0}C) for later analysis.

Day before the Experiment

1. Place aluminum weighing pans in drying oven (100^{0}C) for at least 12 h.
2. Set water baths to exactly 39^{0}C and check temperature.
3. Check O_2 and sampling devices.
4. Adjust osmolarity of serosal buffer by adding mannitol.
5. Pipette 15 mL of serosal fluid into appropriately labeled screw-top containers.
6. Pipette 15 mL of mucosal fluid into appropriately labeled screw-top containers.
7. Keep both serosal and mucosal fluids refrigerated.
8. Keep things ready for tissue collection and preparation:
   sheep, two large containers for saline and buffer, scissors, sharp knives, bolt gun with cartridges, a hack saw, trash bags, a big tray, seven beakers (liter capacity), saline and buffers, cork bore (#15), a glass tray, racks, needles and rubber stoppers.

Day of Experiment

1. Start water bath (39^{0}C) and warm saline.
2. Aerate KRP buffer, while stirring for at least one hour before use. Warm KRP buffer in water bath (39^{0}C).
3. Warm the serosal and mucosal fluids (placed in screw-top containers) to room temperature.
4. Transfer serosal and mucosal fluids into appropriate syringes using needles attached to long pieces of tubing. Make sure to shake the tubes to resuspend all buffer particulate before aspirating. Leave a small air pocket within the loaded syringes to allow for mixing. Top the syringes with needles that are attached to rubber stoppers. Load the syringes in racks and place in water bath.
5. Connect sampling + gassing devices.
6. Set up the tissue washing area in the lab with two beakers of saline and five beakers of oxygenated, warm KRP buffer (pH 7.4).

APPENDIX C

Statistical Analysis Examples

Example C.1. ANALYSIS OF VARIANCE FOR COMPARISON OF AMMONIA N, α-AMINO N, AND PEPTIDE N CONCENTRATIONS IN INCUBATION EXPERIMENT 1.

General Linear Model Procedure

Dependant variable: Ammonia N

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Model	29	971047.86	33484.41	24.25	0.0001
Error	210	290012.16	1381.01		
Corrected total	239	1261060.02			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	733830.55	183457.64	132.84	0.0001
Protein	5	174147.41	34829.48	25.22	0.0001
Time * Protein	20	63069.90	3153.49	2.28	0.002
Contrast					
Time Linear	1	730836.80	730836.80	529.20	0.0001
Time Quadratic	1	790.97	790.97	0.57	0.45

Dependant variable: α-AMINO N

Model	29	16088.60	554.78	15.53	0.0001
Error	210	7503.51	35.73		
Corrected total	239	23592.11			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	900.27	225.07	6.30	0.0001
Protein	5	11649.29	2329.86	65.21	0.0001
Time * Protein	20	3539.04	176.95	4.95	0.0001
Contrast					
Time Linear	1	282.16	282.16	7.90	0.0054

Dependant variable: Peptide N

Model	29	124872.87	305.96	9.69	0.0001
Error	210	93342.38	444.49		
Corrected total	239	218215.26			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	5333.44	333.36	3.00	0.0195
Protein	5	109294.24	21858.85	49.18	0.0001
Time * Protein	20	10245.19	512.26	1.15	0.2992
Contrast					
Time Linear	1	3276.34	3276.34	7.37	0.0072
Time Quadratic	1	1208.01	1208.01	2.72	0.1007

Example C.2. ANALYSIS OF VARIANCE FOR COMPARISON OF AMMONIA N, α-AMINO N, AND PEPTIDE N CONCENTRATIONS IN INCUBATION EXPERIMENT 2.

General Linear Model Procedure

Dependant variable: Ammonia N

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Model	29	2317464.59	79912.57	38.69	0.0001
Error	210	433792.10	2065.68		
Corrected total	239	2751256.69			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	2050843.41	512710.85	248.20	0.0001
Protein	5	174115.24	34823.05	16.86	0.0001
Time * Protein	20	92505.94	4625.30	2.24	0.0025
Contrast					
Time Linear	1	2017966.04	2017966.04	976.90	0.0001
Time Quadratic	1	7669.09	7669.09	3.71	0.0554

Dependant variable: α-AMINO N

Model	29	8734.91	301.20	1.22	0.2105
Error	210	51726.95	246.32		
Corrected total	239	60461.86			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	4910.79	1127.70	4.98	0.0007
Protein	5	2727.21	545.44	2.21	0.0541
Time * Protein	20	1096.91	54.85	0.22	0.9999
Contrast					
Time Linear	1	2744.77	2744.77	11.14	0.0010
Time Quadratic	1	1911.06	1911.06	7.76	0.0058

Dependant variable: Peptide N

Model	29	39890.97	1375.55	7.38	0.0001
Error	210	39115.52	186.26		
Corrected total	239	79006.49			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	17186.90	4296.73	23.07	0.0001
Protein	5	19121.49	3824.30	20.53	0.0001
Time * Protein	20	3582.58	179.13	0.96	0.5102
Contrast					
Time Linear	1	15424.78	15424.78	82.81	0.0001

Example C.3. ANALYSIS OF VARIANCE FOR COMPARISON OF AMMONIA N, α-AMINO N, AND PEPTIDE N CONCENTRATIONS IN INCUBATION EXPERIMENT 3.

General Linear Model Procedure

Dependant variable: Ammonia N

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Model	29	1025287.90	35354.75	5.82	0.0001
Error	210	1275240.02	6072.57		
Corrected total	239	2300527.92			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	884570.51	221142.63	36.42	0.0001
Protein	5	92476.32	18495.26	3.05	0.0112
Time * Protein	20	48241.07	2412.05	0.40	0.9910
Contrast					
Time Linear	1	872624.19	872624.19	143.70	0.0001
Time Quadratic	1	10791.88	10791.88	1.78	0.1839

Dependant variable: α-AMINO N

Model	29	8310.47	286.57	8.05	0.0001
Error	210	7475.36	35.60		
Corrected total	239	15785.83			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	6426.81	1606.70	45.14	0.0001
Protein	5	434.59	86.92	2.44	0.0355
Time * Protein	20	1449.07	72.45	2.04	0.0071
Contrast					
Time Linear	1	4696.38	4696.38	131.93	0.0001
Time Quadratic	1	1280.97	1280.97	35.99	0.0001

Dependant variable: Peptide N

Model	29	18662.16	643.52	4.12	0.0001
Error	210	32761.21	156.01		
Corrected total	239	51423.37			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	12726.65	3181.66	20.39	0.0001
Protein	5	3896.44	779.29	5.00	0.0002
Time * Protein	20	2039.07	101.95	0.65	0.8679
Contrast					
Time Linear	1	12269.80	12269.80	78.65	0.0001
Time Quadratic	1	1.73	1.73	0.01	0.9162

Example C.4. ANALYSIS OF VARIANCE FOR COMPARISON OF AMMONIA N, α-AMINO N, AND PEPTIDE N CONCENTRATIONS IN INCUBATION EXPERIMENT 4.

General Linear Model Procedure

Dependant variable: Ammonia N

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Model	29	108515.78	3741.92	5.65	0.0001
Error	210	139170.84	662.72		
Corrected total	239	247686.62			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	66556.07	16639.02	25.11	0.0001
Protein	5	27611.95	5522.39	8.33	0.0001
Time * Protein	20	14347.77	717.39	1.08	0.3697
Contrast					
Time Linear	1	60313.39	60313.39	91.01	0.0001
Time Quadratic	1	3325.84	3325.84	5.02	0.0261

Dependant variable: α-AMINO N

Model	29	2067.91	71.31	1.68	0.0205
Error	210	8903.45	42.40		
Corrected total	239	10971.36			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	1192.75	298.19	7.03	0.0001
Protein	5	239.60	47.92	1.13	0.3454
Time * Protein	20	635.55	31.78	0.75	0.7712
Contrast					
Time Linear	1	1119.99	1119.93	26.42	0.0001
Time Quadratic	1	52.04	52.04	1.23	0.2692

Dependant variable: Peptide N

Model	29	25793.66	889.44	6.05	0.0001
Error	210	30884.66	147.07		
Corrected total	239	56678.32			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	7504.20	1876.05	12.76	0.0001
Protein	5	9317.80	1863.56	12.67	0.0001
Time * Protein	20	8971.66	448.58	3.05	0.0001
Contrast					
Time Linear	1	6896.44	6896.44	46.89	0.0001
Time Quadratic	1	286.53	286.53	1.95	0.1643

Example C.5. ANALYSIS OF VARIANCE FOR COMPARISON OF AMMONIA N, α-AMINO N, AND PEPTIDE N CONCENTRATIONS IN INCUBATION EXPERIMENT 5.

General Linear Model Procedure

Dependant variable: Ammonia N

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Model	9	14569487	1618832	586.01	0.0001
Error	70	193374	2762		
Corrected total	79	14762861			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	4920653.9	1230163.5	445.31	0.0001
Protein	1	7327810.2	7327810.2	2652.62	0.0001
Time * Protein	4	2321023.3	580255.8	210.05	0.0001
Contrast					
Time Linear	1	7327810.2	7327810.2	2652.62	0.0001

Dependant variable: α-AMINO N

Model	9	163054.28	18117.14	107.21	0.0001
Error	70	11828.75	168.98		
Corrected total	79	174883.03			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	24882.43	6220.61	36.81	0.0001
Protein	1	111054.92	111054.92	657.20	0.0001
Time * Protein	4	27116.93	6779.23	40.12	0.0001
Contrast					
Time Linear	1	111054.92	111054.92	657.20	0.0001

Dependant variable: Peptide N

Model	9	461361.56	51262.40		
Error	70	99209.43	1417.28		
Corrected total	79	560571.00			
Tests of hypothesis using the type III MS for Time *x* Protein (Source) as an error term					
Time	4	135998.55	33999.64	23.99	0.0001
Protein	1	234342.85	234342.85	165.35	0.0001
Time * Protein	4	91020.17	22755.04	16.06	0.0001
Contrast					
Time Linear	1	234342.85	234342.85	165.35	0.0001

Example C.6. ANALYSIS OF VARIANCE FOR COMPARISON OF MUCOSAL CONCENTRATIONS OF FREE AND PEPTIDE-BOUND TOTAL AMINO ACIDS (AMINO ACID UPTAKE EXPERIMENT).

General Linear Model Procedure

Dependant variable: Total amino acids

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Model	9	804116.17	89346.24	219.88	0.0001
Error	98	39820.76	406.33		
Corrected total	107	8043936.93			

R^2	CV	Root MSE
0.952815	18.83136	20.15773

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Protein	2	29764.68	14882.34	36.63	0.0001
AA Form	2	685332.31	342666.15	843.31	0.0001
Tissue	1	921.61	921.61	2.27	0.1353
Protein * AA Form	4	88097.57	22024.39	54.20	0.0001
Contrast					
Protein 1 vs. Protein 2	1	4566.33	4566.33	11.24	0.0011
Protein 2 vs. Protein 3	1	10750.67	10750.67	26.46	0.0001
AA Form 1 vs. AA Form 2	1	165869.84	165869.84	408.21	0.0001
AA Form 2 vs. AA Form 3	1	519462.46	519462.46	1278.41	0.0001
Tissue 1 vs. Tissue 2	1	921.61	921.61	2.27	0.1353

Example C.7. ANALYSIS OF VARIANCE FOR COMPARISON OF SEROSAL APPEARANCE OF FREE AND PEPTIDE-BOUND TOTAL AMINO ACIDS VIA RUMINAL AND OMASAL EPITHELIA (AMINO ACID UPTAKE EXPERIMENT).

General Linear Model Procedure

Dependant variable: Total amino acids

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Model	18	26767301.22	1487072.29	9.96	0.0001
Error	89	13284778.50	149267.17		
Corrected total	107	40052079.72			

R^2	CV	Root MSE
0.6683	65.3849	386.35

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Animal	1	65911.95	65911.95	0.44	0.5081
Protein	2	3552533.52	1776266.76	11.90	0.0001
AA Form	2	7171072.72	358536.36	24.02	0.0001
Tissue	1	7755603.82	7755603.82	51.96	0.0001
Protein * AA Form	4	1826442.75	456610.69	3.06	0.0207
Protein * Tissue	2	2170694.19	1085347.09	7.27	0.0012
AA Form * Tissue	2	2741344.97	1370672.49	9.18	0.0002
Protein * AA Form * Tissue	4	1483697.31	370924.33	2.48	0.0492
Contrast					
Protein 1 vs. Protein 2	1	3367605.05	3367605.05	22.56	0.0001
Protein 2 vs. Protein 3	1	297169.10	297169.10	1.99	0.1617
AA Form 1 vs. AA Form 2	1	4482729.43	4482729.43	30.03	0.0001
AA Form 2 vs. AA Form 3	1	2688343.29	2688343.29	18.01	0.0001
Tissue 1 vs. Tissue 2	1	7755603.82	7755603.82	51.96	0.0001

VITA

Vajira Parakrama Jayawardena, son of late Daya and Somalatha Jayawardena, was born on January 25, 1962 in Kandy, Sri Lanka. He received his secondary education from Vidyartha College, Kandy and graduated in 1980. He received his B.Sc. (Agriculture) degree from the University of Peradeniya in December 1985. He started his career as an Assistant Lecturer at the University of Peradeniya in 1986 and obtained a Masters degree in Animal Science from the same University in 1992. He was promoted to a Senior Lecturer in 1994. His doctoral studies were initiated at Virginia Tech in October 1994 with the financial support received from the John Lee Pratt Animal Nutrition Program. He married Monica Fernando in 1991, and is a father of two children (Nathashi and Nimesha). He is a member of Sri Lanka Association of Animal Production.