ABSORPTION OF CARNOSINE AND METHIONYLGLYCINE BY SHEEP RUMINAL AND OMASAL EPITHELIA

by

James C. Matthews

Thesis submitted to the Graduate Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Animal Science

APPROVED:

K. E. Webb, Jr., CHAIRMAN

J. H. Herbein, Jr.

E. T. Kornegay

October, 1991

Blacksburg, Virginia

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(ABSTRACT)

Carnosine and methionylglycine (using ^{35}S-methionylglycine as a representative marker) absorption and transfer across ruminal and omasal epithelia collected from four (carnosine) and seven (methionylglycine) sheep were studied using parabiotic chambers that were repeatedly sampled over a 240-min incubation. The quantity of dipeptide absorbed or transferred was linearly ($P < .01$) dependent on substrate concentration. Carnosine was transferred intact across both tissues. More carnosine was absorbed ($P < .02$) and transferred ($P < .01$) by the omasal epithelia. Methionylglycine was transferred intact across both tissues, but less ($P < .01$) remained intact in serosal buffer after 240 min incubation with omasal epithelium than with ruminal epithelium. The amount of methionylglycine that accumulated in each tissue was similar. Methionylglycine accumulation in tissues plus transfer after 240 min was greater ($P < .01$) for omasal tissue. The ability of sheep ruminal and omasal epithelia to absorb and transfer carnosine and methionylglycine in parabiotic units was demonstrated. Dipeptide translocation across forestomach epithelial tissues, which has not been reported previously, may be an important route for supplying dietary amino acids to the ruminant.

ACKNOWLEDGEMENTS

The author would like to express his sincere appreciation to Dr. K. E. Webb, Jr. for his patience, guidance, friendship, assistance and cooperation throughout this study and in the preparation of this manuscript.

His sincere appreciation also is expressed to the other members of his committee, Dr. J. H. Herbein, Jr. for his encouragement, comments and suggestions throughout this study and committee service, and Dr. E. T. Kornegay for his encouragement, support and committee service.

I would like to also extended my sincere thanks to Donald Shaw, Kristin Lee, Dr. Patrick Brown, Hans Swinkles and Wei Zhou for their friendship, encouragement, technical discussions and support during this project.

The author also would like to recognize Dr. D. R. Notter for his assistance in the statistical analyses, Werner Fischer of Novabiochem for technical advise for the synthesis of Fmoc-^{35}S-methionine, and Charles Van Wandalon and Bill Welch of Milligen/Biosearch Corporation for their laboratory technique and technical advise for the synthesis of L-^{35}S-methionylglycine.

The author offers his greatest appreciation to his wife and friend, Elizabeth Matthews, without whose encouragement and support he could have never begun, nor continued, his academic career.

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Chapter I

Introduction

The ability of the reticulorumen to absorb VFA, electrolytes, ammonia and water has been characterized and well summarized (Church, 1975; Van Soest, 1987). The omasum has been reported to absorb VFA (Barcroft et al., 1944; Johnston et al., 1961; McSweeney, 1988), electrolytes and ammonia (Oyaert and Bouckaert, 1961; Engelhardt and Hauffe, 1975), and water (Gray et al., 1954; Holtenius and Bjornhag, 1989). However, the ability of the rumen and the omasum to absorb amino acids has not been clearly defined.

Historically, the potential for significant absorption of amino acids from the forestomach has been regarded as minor and, therefore, ignored. This view prevailed throughout early forestomach studies of amino acid-N uptake which demonstrated that at least two of the domestic ruminant species (goats and sheep) possessed the ability to absorb amino acid-N from their forestomachs (Demaux et al., 1961; Cook et al., 1965; Chand et al., 1968; Larzarov and Ivanov, 1970; Leibholz, 1971a, 1971b; Bird and Moir, 1972; Goshtasbpour-Parsi et al., 1974). The work of Leibholz (1971a) particularly affected this opinion because her calculations indicated that only "6% of the total N absorption from the rumen" could have been in the form of amino acids. Her results, coupled with the known rapid metabolism of amino acids by rumen microbes, led nutritionists to generally view amino acid absorption from the ruminant forestomach as minor. Consequently, the fate of dietary amino acids has been discussed in terms of conversion to ammonia N, incorporation into microbial protein, absorption by the intestine and excretion (NRC, 1985b).

The fundamental understanding that peptides may be absorbed in large quantities from the ruminant gastrointestinal tract was indicated by the observation that the primary form of amino acids in the portal blood of calves was as peptides (79%) (Koeln and Webb, 1982). Subsequent research (Schlagheck and Webb, 1984) estimated that the majority of

these peptides were less than 300 Da, implying that the primary forms of amino acids in portal blood of calves were probably di- and tripeptides. Additional research (Read, 1988) has shown that sheep have the ability to absorb peptides from the intestines, as indicated by the measured appearance of peptides in mesenteric blood. The most recent report from this laboratory (Seal and Parker, 1991), however, suggests that the major portion of peptides in the plasma of steers and sheep are larger than 1,500 Da (pentapeptides and larger).

Recent research has challenged the assumption that the forestomach does not contribute in a nutritionally significant manner to the provisioning of amino acids for the ruminant. The mesenteric amino acid flux of sheep and calves of both free amino acid and peptide fractions has been quantified (DiRienzo, 1990). This research indicates that the flux of free amino acids across the stomach region is minimal, thereby supporting the work and conclusions of earlier researchers (Annison, 1956; Leibholz, 1971a) that the net absorption of amino acids from the forestomach of ruminants is not quantitatively important. Of greater significance, both quantitatively and theoretically, are the results which indicate that the flux of peptide amino acids accounted for approximately 77% of the total, portal-drained, amino acid flux in both calves and sheep.

The implication of the above research is that peptide absorption from the gastrointestinal tract is a nutritionally important physiological phenomenon and, therefore, challenges two classic tenets of amino acid provisioning of the ruminant. First, this research suggests that the forestomach is an important site of amino acid absorption. Second, peptide amino acids appear to be the quantitatively important form of amino acid absorption in the ruminant. The purpose of the present research was to investigate the potential for peptide absorption by both ruminal and omasal epithelial tissues of the forestomach.

Chapter II

Review of Literature

Morphology of the Ruminant Forestomach

Organization of the Ruminant Forestomach Epithelium. The epithelial tissues of the ruminant forestomach are unique in their structure and function as compared to other epithelial tissues. Forestomach epithelium lacks the extremely thick, keratinized layer and the glandular secretive ability of protective skin epithelium. This tissue also lacks goblet cells which generate the highly absorptive, acidic microenvironment of intestinal epithelium. However, as a result of the arrangement of its keratinized, squamous epithelial cells into multiple layers (Lavker et al., 1969), the ruminant forestomach epithelium is capable of absorbing large quantities of nutrients from the mechanically abrasive environment of the forestomach lumen.

The ultrastructure of this unusual epithelium has been studied extensively using electron microscopy (Hyden and Sperber, 1965; Lavker et al., 1969; Henrikson, 1970; Steven and Marshall, 1970; Henrikson and Stacy, 1971). There appears to be a consensus among these researchers that the forestomach epithelium is composed of four strata of epithelial cells that are organized in the same spatial order in the rumen, reticulum and omasum. Starting proximal to the basement membrane (adjacent to the connective tissue of the forestomach musculature), the four strata of epithelial cells have been classified as the basale, spinosum, granulosum and corneum. Excellent structural descriptions of the cells composing these four strata have been provided (Laver et al., 1969; Steven and Marshall, 1970).

A requisite feature of these cells is their ability to change both morphologically and histologically as they age. As a given epithelial cell matures, it

is pushed through the strata basale, spinosum, granulosa, and finally terminates its differential migration in the stratum corneum by sloughing off into the rumen lumen. Irregularly spaced filaments of hemidesmosomes serve to attach the basale stratum cells to the basement membrane, whereas desmosomes connect the cells of the basale, spinosum and granulosum strata. These junctions increase in number and structural complexity as cells migrate towards the lumen, ultimately resulting in the fusion of the outer granulosa cells as they approach the corneum layer (Steven and Marshall, 1970). Once the cells enter the stratum corneum, however, desmosomes cease to function. Consequently, the cells of the corneum form an interdigitated layer, held together by interlocking processes of their membranes.

The size of the intercellular spaces within ruminant forestomach strata also have been characterized (Steven and Marshall, 1970). The lumen-facing corneal cells have gaps of 300 to 600 angstroms. The granulosa cell stratum is divided into two regions with the outer granulosa cells having no gaps between them and the inner layer cells having intercellular spaces of 300 to 6,500 angstroms (6,500 being most distal to the lumen). Spinosal cells have gaps of 6,500 angstroms to 1 micron between them. Basale cells have the largest intercellular spaces of the epithelial strata with gaps of 1 to 2 microns existing between those cells most proximal to the lumen, whereas the plasma membranes of the most distal cells present gaps of approximately 1 micron to the basement membrane.

The basement membrane separates the basale epithelial layer from the musculature of the forestomach wall and has been characterized as being a continuous structure that is 250 to 400 microns thick (Hyden and Sperber, 1965).

Potential for Trans-eptihelial Nutrient Passage. As cells migrate toward the luminal surface, there appears to be a reduction in their metabolic potential. Most

researchers reviewed, who employed electron microscopy techniques, observed relatively high numbers of Golgi vesicles, ribosomes, endoplasmic reticulular structures, and mitochondria in the basale stratum cells. A relatively lower number of these cell oraganelles were observed in the spinosum stratum, and essentially none of these intracellular structures were seen in the ganulosum or corneum strata. These observations suggest that the metabolic processes requiring the synthesis of proteins and energy, including energy-dependent transport of solutes, probably do not occur in the granulosa and cornea cells.

Histological studies, however, suggest that the granulosa cells are metabolically active. The presence of carbonic anhydrase in all but the corneum stratum of the forestomach epithelium in calves and cows has been demonstrated (Lauwers and Boedts, 1974). A relatively high number of mitochondria in granulosa cells has been reported (Lavker et al., 1969). Additionally, a thorough histological examination of the ruminal epithelium revealed intense to moderate activity of intracellular dehydrogenases involved in the glycolytic and Krebs cycles in the "transitional zone" between granulosum and corneum strata (de Lahunta, 1965). The presence of these enzymes suggests that granulosa cells, as well as spinosa and basale cells, have the ability to perform energy-demanding metabolic functions, including active transport.

There is some dispute regarding the existence of, and the cellular structures responsible for, the junction of granulosum strata cells (Fell and Weekes, 1975). Granulosa cells have been reported to be completely fused by the formation of zonulae occludentes (desmosomes) just prior to their migration into the stratum corneum (Steven and Marshall, 1970). Thus, these researchers suggest that a physical barrier to the passage of solutes exists at the beginning of the stratum

granulosum. Henrikson (1970), however, failed to observe the presence of zonulae occludentes. Instead, he reported the presence of maculae occludentes, resulting in "punctuated" membrane fusion and suggested that such an interrupted physical barrier would be permeable to solutes. In later work, this laboratory (Henrikson and Stacey, 1971) injected electron-opaque substances into various strata of the epithelium to confirm that solutes could pass through the intercellular spaces of the inner epithelium. They also observed that these same marker substances had failed to pass from the outer corneum into the inner epithelial strata. Therefore, they concluded that a barrier to solute passage across the forestomach epithelium existed and that this barrier was within the outer layer of keratinized epithelial cells (stratum corneum). Their observations of "labyrinth intercellular spaces, thick glycocalyx and natural physical properties" of these keratinized cells, and the calculation of an osmotic permeability coefficient led them to conclude that diffusion of metabolites through intercellular spaces of the outer corneum stratum would be unlikely in the rumen.

Basale stratum cells possess large numbers of mitochondria suggesting that the amount of metabolic energy available for cellular activity is high. These cells also possess both fuzzy-coated micropinocytes (Lavker et al., 1969) which have a selective regulatory protein transport ability (Goldstein et al., 1979), and smooth-coated micropinocytes which are involved in non-specific nutrient transport (Fawcett, 1981). The presence of the above cellular mechanisms suggests that the potential for trans-epithelial nutrient passage is large.

Structure of Forestomach Papillae. Careful scanning of forestomach epithelium with an electron microscope has revealed that the ruminant forestomach (when considered as a whole organ) displays a "complete sequence of transitional

forms" of papillae with many variations of the characteristic papillae existing within each of the forestomach compartments (Scott and Gardner, 1973). Keeping this commonness in mind, each forestomach compartment has papillae that are, superficially, typical to that compartment. The ruminal papillae vary the most in their magnitude of size, but all are essentially tongue-shaped and possess granular and eroded surfaces (Scott and Gardner, 1973). The largest and greatest density of rumen papillae are found in the cranial and ventral sacs of the rumen (Scott and Gardner, 1973; Van Soest, 1987).

Reticular and omasal papillae are characteristically conical in form and posses tips that are distinctly bifid (reticular) and(or) multifid (omasal), and which are more heavily keratinized than the ruminal papillae (Scott and Gardner, 1973). Like the ruminal papillae, the size of the reticular and omasal papillae vary. In the omasum, the largest papillae are arrayed on the area of the laminae most proximal to the reticulum and descend in size, distally, towards the abomasum. The magnitude of this size differential has been described as ranging from 4.5 mm (height) x 2.5 mm (base diameter) to .5 mm x .5 mm (Brownlee and Elliot, 1960). Additionally, these researchers noted that the larger, more proximally located, papillae were present in two to four times the numbers (per surface area) of the smaller, more distally located papillae.

Embryological studies have shown that the omasal laminae are formed by the invagination of epithelial folds, while the conical papillae are formed by an initial invagination of connective tissue followed by a subsequent invagination of the epithelium (Lubis and O'Shea, 1978).

Relationship Between Papillae Structure and Function. Several researchers have suggested that the structure of forestomach papillae and their constitutive

epithelial cells inherently provide unique absorptive functions. The area of heaviest rumen papillae density, also being the region containing papillae of the greatest size, possesses the greatest ability to absorb VFA (Aafres, 1967). In contrast, the work of Brownlee and Elliot (1960) has been interpreted (Scott and Gardner, 1973) as showing that the smaller, and less dense, more distally located omasal papillae are responsible for water and electrolyte absorption, primarily because of their relatively thinner keratin layer as compared to the larger papillae of the omasum. However, an examination of the Brownlee and Elliot (1960) study by the present author failed to extract the supporting data for this statement.

The relative size of papillae and the thickness of their epithelial cells have been suggested to contribute to the absorptive capacity of ruminal epithelial tissue for amino acids (Leibholz, 1971b). Both the size of the papillae and the thickness of their epithelial cell layers were reduced in the starved sheep of this study. Significantly, the epithelial tissue of starved sheep had twice the absorptive capacity for amino acids as compared with the ruminal epithelial tissue of non-starved sheep. This researcher suggests that a thinner coating of epithelia cells over the papillae might present less of a transfusional barrier to amino acid absorption.

The 114-d weight gain of feedlot lambs has been correlated with reticulorumen weight, papillary length, and papillary density (Sinclair and Kunkel, 1959). Weight gains during the final 28 d were additionally correlated with the percentage of "mucosa" of the total reticulorumen tissue weight and with the pigment of epithelium. Individually, each of these reports suggest that the size, density, and epithelial cell mass of the papillae afford them certain absorptive abilities. An interpretation of these reports as to the collective functioning of the forestomach papillae is harder to make.

Vasculature of the Ruminant Forestomach. The arterial supply of the forestomach compartments has been succinctly outlined (Van Soest, 1987) and the venous drainage has been described in detail (Heath, 1968). Blood flow in the forestomach of adult sheep has been characterized (Engelhardt and Hales, 1977). Of the total mean capillary blood flow (7.7 mL min^{-1} · kg body wt^{-1}), 95% was associated with the epithelium and 5% with the musculature, despite the fact that the epithelium of these animals weighed only 18% more than the muscle tissue. The total blood flow of the omasum amounted to nearly 20% of the reticulorumen, even though the total wet weight of omasal tissue was only 11% of the reticulorumen. This relatively greater proportion of omasal epithelial tissue blood flow may be, in part, because of the relatively low proportion of musculature in the omasal laminae as compared to the ruminal stomach wall.

Although the blood flow per 100 g of wet epithelia was not "strikingly different" among the forestomach compartments, the omasal laminar capillary blood flow per unit area was approximately one-third that of the ventral rumen and two-thirds that of the dorsal rumen tissue in sheep (Engelhardt and Hales, 1977). Therefore, these researchers estimated that the omasum accounts for one-third of the ruminant forestomach absorptive area. Accordingly, the absorptive rates for "water and most solutes" in the three forestomach compartments were determined to be similar. It would be interesting to determine how these same blood flow and absorptive rates compare in cattle, given that their omasa are generally recognized as constituting a greater proportion of the forestomach than either the goat or sheep (Stevens and Stettler, 1966; McSweeney, 1988).

Structures important for the exchange of nutrients between the forestomach epithelium and its vasculature network have been observed in the goat (Schnorr,

1971). Scanning of blood vessels with an electron microscope revealed the existence of both pinocytic vesicles and "pores" (fenestrations) in the endothelium of the subepithelial capillaries. These structures are known to facilitate the transfer of substrates from absorptive tissues, including nutritional and regulatory peptides (Goldstein et al., 1979) . These structures were found in the vasculature of the rumen, reticulum and omasum.

Whereas no differences between the rumen, reticulum and omasum epithelial ultrastructures were reported by Hyden and Sperber (1965), it has been observed that local variations in tissue thickness exist and that the branching cells of the stratum basale are more numerous in the omasum than in either the rumen or reticulum (Steven and Marshall, 1970). Evidence has been reviewed (Engelhardt and Hauffe, 1975) that shows that omasal epithelia have greater Ca^{++}- and Mg^{++}-dependent ATPase activity than do ruminal epithelia. Collectively, these facts suggest that the omasal epithelium might possess an inherently greater capacity for rapid translocation of nutrients that are dependent on carrier-mediated transport systems, than does the rumen epithelium.

Models for Trans-epithelial Nutrient Passage. Nutrient transfer models based on the above characterization of the forestomach epithelium and its related structures have been proposed. One model hypothesizes that there is relatively little resistance to the passage of solutes through the forestomach epithelial cells until reaching the basale stratum (Hyden and Sperber, 1965). At this point, the highly interdigitilized arrangement of the basale cells and the presence of desmosomes causes a restriction in the rate of solute diffusion.

Another model, however, suggests that the region of the granulosa stratum proximal to the lumen is where solute flow is restricted, because of the abundance

of desmosomes (Keynes, 1969). This model hypothesizes that the presence of desmosomes necessitates the concommitant presence of Na^+/K^+ ATPases in order to pump Na^+ into the intercellular spaces, thereby creating an osmotic gradient which water would then follow and, presumably, carry soluble nutrients with it. This theory is supported by the observation that corneal cells lack mitochondria and cell nuclei and, therefore, would be incapable of the active transport of solutes. He also argues that the basale stratum cells, which do posses high numbers of mitochondria and which are nucleated, would not be capable of active transport due to the "lack of asymmetry in their connexions with their neighbors," structures suspected as being essential for trans-epithelium transport. The proposed site (granulosa stratum proximal to the lumen) for Na^+/K^+ ATPase by Keynes (1969) has been supported by several researchers (Henrikson, 1970; Fell and Weekes, 1975) and confirmed with a histochemical evaluation (Henrikson and Stacy, 1971).

A third model for water-soluble nutrient passage through the forestomach epithelium has been postulated (Henrikson, 1970) and appears to be plausible based on the anatomical and biochemical evidence presented and discussed above. The primary feature of this model is the movement of Na^+ through the epithelial cell layers and incorporates theories for Na^+ transfer by amphibian skin (Farquhar and Palade, 1963) and for osmotic gradients (Diamond and Bossert, 1967). That is, Na^+ diffuses across the corneum strata and moves intracellularly through the first layers of the proximal granulosa cells, by way of maculae occlundentes. Subsequently, Na^+ is actively transported from the cytosol of the granulosa cells, out through the cell membrane, and into the intercellular spaces by Na^+/K^+ ATPases. This process causes the intercellular regions of the granulosum strata to become hyperosmotic, resulting in water (and soluble nutrients) to be drawn into this region.

As the flow of water and solutes proceeds towards the basement membrane, the intercellular fluid of granulosa cells becomes isosmotic with the intracellular fluid.

A general model for paracellular solute flow across tissues that contain cells connected to each other by zonulae occludentes has been postulated (Pappenheimer, 1989). Because zonulae occludentes can "leak" when subjected to high osmotic gradients, tissues dependent on the integrity of zonulae occludentes to form a barrier to solute passage are capable of displaying differential rates of solute passage and, hence, transport of nutrients. This fact may be of particular importance for the hydrophilic (polar) compounds (such as amino acids and small peptides) whose proportion of enterocyte uptake by diffusion is considered to be less than that of active transport (Matthews, 1991). Hence, these compounds which appear to be normally dependent on carrier-mediated transport could be absorbed by paracellular passage through the cells composing the epithelial strata and into the serosal blood if the integrity of the zonulae occludentes is impaired.

A paracellular pathway for the absorption of small oligopeptides (less than 1,900 Da) is claimed to exist in intestinal enterocytes of hamsters (Atisook and Madara, 1991). During experiments, the desmosomes adjacent to functioning Na^+/glucose cotransporters were shown to be dilated when the transporters were active. Of particular relevance to the potential for the development of such transport conditions in ruminant forestomach epithelia was the demonstrated dependence on the presence of a Na^+ gradient in order for paracellular passage to occur. As discussed below, the maintenance of a Na^+ gradient is thought to be the essential prerequisite for ruminant forestomach solute transfer.

The important feature of the proposed models for trans-epithelium nutrient transport is the dependency on Na^+ to generate an osmotic gradient, down which

water and nutrients flow. The osmotic permeability coefficient of a given forestomach lumen is dependent on a difference existing between the osmotic pressure in the lumen of the forestomach and the bloodstream. This osmotic differential is dependent on the tonicity of the lumen liquor, which can very greatly (Keynes, 1969). The Na^+ is the primary ion responsible for generating the osmotic gradient in ruminant forestomachs (Fell and Weekes, 1975). Because the typical luminal sodium concentration is 80 to 110 mmol/L and the serosal Na^+ concentration is approximately 150 mmol/L (Steven and Marshall, 1970), Na^+ must be actively transported into the intercellular spaces of the non-corneum strata of the epithelium. Therefore, the efficiency of nutrient transfer (whether by diffusion or active transport) from the forestomach into the serosal fluid is absolutely dependent upon the cohesiveness of the keratinized, granulosa cell layer. This solute barrier ensures that the osmotic potential difference across the forestomach epithelium is maintained by preventing the backflow of Na^+ into the lumen (Fell and Weekes, 1975).

If Na^+ was able to freely flow back into the lumen, then loss of the electrical potential gradient would result. Without this electric gradient, Na^+ transport would not be possible and would result in the loss of the intercellular osmotic gradient in the spinosum and basale strata. Consequently, less nutrient transfer from the forestomach lumen into serosal fluid would occur.

A summary of the morphological literature presented suggests that several differences exist between ruminal and omasal epithelial tissues and their supporting structures. As compared to the rumen, the epithelium of the omasum is reported to be more keratinized (Scott and Gardner, 1973), thicker (Steven and Marshall, 1970), and to posses greater Ca^{++}- and Mg^{++}-ATPase activity (Schnorr, 1971).

Structurally, the omasal papillae are reported to be more conical in form than the tongue-shaped ruminal papillae (Scott and Gardner, 1973). Additionally, the omasum is reputed to have a proportionately greater blood flow (blood flow/wet weight of organ) than the rumen (Engelhardt and Hales, 1977).

How these structural differences affect the potential for forestomach peptide absorption is not known. However, when discussing the potential for peptide absorption by ruminal and omasal epithelial tissues it seems logical to consider the amount of substrate that is available for uptake.

Ruminant Forestomach Peptide Concentration

Factors Affecting Forestomach Peptide Concentration.

It has been stated that "under practical feeding conditions" the extent to which protein breakdown occurs in the rumen is dependent on the rate of proteolysis and rumen retention time (Tamminga, 1979). Generally it is agreed that the ruminant digestive tract tissues proximal to the forestomach, and the epithelia of the forestomach, do not secrete proteolytic enzymes. The microbial enzymes of the microflora, however, are thought to be responsible for protein interconversions in the rumen (Wallace and Cotta, 1988). Consequently, the concentration of peptides in forestomach liquors appears to be dependent upon the interaction between feedstuffs and microbes possessing proteolytic enzymes.

In order for peptides to be absorbed from the forestomach, they must first be produced in quantities which exceed their rate of utilization by the microbial population. Whereas it is likely that many proteins are not hydrolyzed to peptides by microbes at rates which exceed their use (Broderick et al., 1988), research data has been summarized to indicate that certain feed proteins, casein, soybean meal

(SBM), and heat-treated SBM, are hydrolyzed in the rumen faster than they are metabolized by microbes, thereby resulting in the "significant" accumulation of peptides in the rumen (Wallace and Cotta, 1988).

Proteins have different rates of forestomach degradation which are ultimately dependent on the types of proteases possessed by the resident microbes. A hierarchy of protein degradation has been proposed (Lewis, 1962) based upon the rapidity that proteins were degraded to ammonia during in vivo rumen fermentation trials: casein, gelatin, ground nut protein > soy protein, wheat gluten > bovine albumin, zein. A composite protein breakdown hierarchy that is based upon the appearance of peptide N, and that has been compiled from the data of other researchers (Mangan, 1972; Nugent et al., 1983; Sniffen et al., 1985; Broderick and Craig, 1989), suggests that the relative rate of ruminal protein breakdown is: casein > plant leaf protein > SBM > bovine serum albumin, ovalbumin. It appears, therefore, that the relative peptide appearance potential parallels the suggested protein degradation rates.

The half-lives of casein and ovalbumin in bovine rumen fluid have been characterized as being approximately 19.5 and 175 min, respectively. These degradation rates did not change when fermented together with mixed bacteria incubations (Mangan, 1972). These results suggest a time range for ruminal protein degradation and that protein degradation is specific in the ruminal environment. This same laboratory, however, has shown that the important feed proteins, casein and ribulose-1,5-biphosphate carboxylase (the primary, soluble plant leaf protein), competitively inhibit the proteolytic rates of each other when fermented together in rumen fluid (Nugent et al., 1983). Increasing the feed intake of dairy cattle also has

been shown to cause a decrease in dietary protein degradation (Tamminga et al., 1979).

Significant quantities of peptides have been observed in rumen fluid collected from lactating dairy cows that had been fed blended diets containing 14, 17, or 20% protein levels. Forty percent of the dietary protein was supplied from corn silage, hay silage, and barley, and 60% was supplied from SBM that had been either solvent extracted, autoclaved, or combined with extruded SBM or fish meal (Sniffen et al., 1985). These researchers concluded from their study that the type and amount of protein in a diet will greatly effect the amount of peptides appearing in rumen fluid. It has been demonstrated that diets can be formulated to account for expected, variable protein solubility and that ammonia, plasma urea, and urinary N are all positively correlated with the solubility of protein feedstuffs (Wohlt et al., 1976). These observations indicate that the interaction of feed proteins among themselves can competitively inhibit their breakdown rates by the forestomach microflora. They also suggest that it may be possible to formulate diets that will result in a specific, predetermined, forestomach peptide concentration.

As discussed above, the degradation of proteins in the ruminant animal, and hence the peptide profile, is fundamentally dependent on the proteolytic activity of rumen microbes. Under most nutritional circumstances the acidic pH of the rumen promotes extensive dietary protein breakdown by the proteolytic activity of microbes (Tamminga, 1979). Although ciliate protozoa are known to posses proteolytic activity, their activity is substantially lower than that of other microbes (Brock et al., 1982). Moreover, the primary role of rumen protozoa is that of metabolizing bacterial protein, rather than to hydrolyze dietary proteins (Wallace and Cotta, 1988). In contrast, Neocallimastix frontalis is a fungus that has been

isolated from sheep rumen and that exhibits proteolytic activities comparable to the most active proteolytic rumen bacteria (Wallace and Joblin, 1985). Of the three dominant bacterial populations of the rumen, comprising mixed rumen microbes, Bacteroides ruminicola is reported to be the primary proteolytic bacterium (Wallace, 1988) and to produce the most ammonia from peptide substrates (Bladen et al., 1961).

Of particular relevance is the fact that B. ruminicola obtains its N from ammonia and peptides, therein "efficiently utilizing the N from fractions containing 4 or more moles of total N per mole of alpha-amino N, but not efficiently utilizing N in fractions containing less than 4 moles of total N per mole of alpha-amino N (that is, not small peptides or free AA)" (Pittman and Byant, 1964). Further research has revealed that this oligopeptide utilization was restricted to peptides smaller than 2,000 Da and that the peptide amino acids were incorporated into the cell protein, and not deaminated for energy use (Pittman et al., 1967). Subsequently, it has been demonstrated that this organism preferentially absorbs two times the amount of hydrophilic peptides as it does hydrophobic ones (Chen et al., 1987c). This fact suggests that a relatively greater proportion of the hydrophobic peptides from feedstuffs will accumulate in the rumen liquor.

The above research must be analyzed with the knowledge that most of the in vitro, microbial protein degradation studies have used microbial flora obtained from strained rumen fluid (SRF). Because of this collection technique, the resulting mixed cultures would not have included proteolytic microbes associated with the strained-out solids. The proteolytic activity of rumen solids has been reported to be 1.5 times greater than the SRF from mixed diets and 2.5 to 6 times greater than SRF from a hay diet (Wallace and Cotta, 1988). These facts suggest that B. ruminicola

and other proteolytic microbial species collectively exert a considerable influence on the profile of forestomach peptide concentrations.

Because the extent of microbial feedstuff degradation is dependent on the forestomach liquor turnover time, the forestomach peptide concentration is affected by the rate of digesta flow through the forestomach. This rate is known to be affected by both the type of feed fed and by the frequency of feeding. For example, increasing the amount of long roughage fed resulted in an increased digesta flow that was four times the magnitude observed after increasing the amount of ground or pelleted concentrates (Tamminga et al., 1979). Additionally, feeding cows the same ration once a day, versus twelve times a day, has resulted in less accumulation of ammonia, soluble protein and peptide N in the rumen (Chen et al., 1987a). Collectively, these reports indicate that the potential for forestomach peptide accumulation can be altered by the feeding regimen employed.

Reported Forestomach Peptide Concentration. There appears to be a paucity of experiments that have attempted to measure the amount of peptide N in the ruminant forestomach. Table 2.1 contains data compiled from several experiments that can be reasonably compared using the same parameters. The purpose of this table is to summarize the range of peptide concentrations that have been measured in the ruminant forestomach and to infer a preliminary understanding of what forestomach peptide concentrations might be expected, depending on the diet fed.

The values reported represent several diets and production states for sheep and cattle. Given the variability of experimental conditions employed by the researchers, who were collecting rumen fluid for in vitro peptide production rate

Table 2.1. REPORTED VALUES OF RUMINAL PEPTIDE-N CONCENTRATIONS FROM STRAINED RUMEN FLUID

Protein supplement	Species	CP,%	Peptide-N[d] (g/L)	Source
casein	sheep	15.0[e]	.10 (1), .08 (2)	Annison et al., 1956
casein	sheep	15.6[f]	.27[g](1), .09 (2)	Broderick and Wallace, 1988
SBM[a]	lact. cows	17.8[h]	.22 (2), .05 (16)	Chen et al., 1987a
SBM-ESB[b]	"	17.8[h]	.16 (2), .06 (16)	"
SBM-FM[c]	"	17.8[h]	.15 (2), .05 (16)	"
---	dry cow	16.0[i]	.19 (1), .06 (4)	Chen et al., 1987b

[a]Soybean meal.

[b]Extruded soybean meal.

[c]Fish meal.

[d]Values in brackets are the time (h) of rumen fluid collection after feeding. The first time listed is the time of maximal peptide-N concentration reported by the researcher(s).

[e]Diet consisted of .60 kg alkali-washed straw (total N .2 to .3%), .15 kg starch, .10 kg sucrose, .20 kg molasses, .50 kg water and .10 kg casein.

[f]Diet (as-fed) consisted of .67 kg ryegrass and .33 kg concentrate (34% casein, DM basis), divided over two feedings.

[g]Values were calculated from reported mmol/L concentrations using the unit ratio 1 g N/13.6 mmol peptide, reported by these researchers.

[h]Diets (DM) contained 41.8% corn silage, 8.8% haylage, barley (at least 20%) and mineral mix. The portion of barley added depended on the CP percentage supplied by the protein supplements: 17.3% SBM (18.0), 17.9% SBM-ESB (9.5, 12.0), 18.1% SBM-FM (7.6, 6.3).

[i]Diet contained 5 kg timothy hay and 5 kg commercial concentrate, divided between two feedings.

studies, these data should not be considered to definitively represent typical in vivo rumen peptide concentrations. However, several general observations seem reasonable and suggest that (1) rumen peptide concentrations peak within 1 to 2 h after feeding, (2) the type of protein supplement fed can affect the post-feeding ruminal peptide concentration and (3) the basal peptide N concentrations are approximately .05 to .06 g/L.

The first two observations are supported by the results of in vitro studies (discussed earlier) which show that common rumen microbes have the ability to rapidly degrade both casein, plant leaf and soybean proteins and that the proteolysis of a given protein may be slowed if it is combined with other proteins. Additionally, Mangan (1972) has reported a maximal increase in casein peptide N 30 min after ruminal infusion and a subsequent decrease to basal levels after 60 min, in steers that had been fed a mixed herbage diet of chaffed hay, grass meal, beet pulp, and various brassicae.

The accuracy of the third observation is harder to evaluate as, conventionally, concentrations of nutrients are expressed as millimolar units when addressing substrate availability. In order to calculate specific mmol/L values for the data in Table 2.1, the ratios of mmol/g N of a given protein (or the mixture of proteins) is needed. Accordingly, Broderick and Wallace (1988) used the conversion ratio of 13.6 mmol peptide/g N for casein to originally report their (Table 1) values as 3.8 mmol/L (1 h) and 1.24 mmol/L (2 h). They then calculated that the average peptide derived from their enzymatic hydrolysis of casein was composed of four amino acid residues. They also have reported an "overall mean" ruminal peptide concentration of 2.95 and 3.09 mmol/L for cows fed alfalfa (60% of DM) based diets (Broderick and Craig, 1989).

If these values are typical of other ruminants, and represent the mixture of peptides likely to be found in ruminant forestomachs, then they suggest that typical rumen peptide concentrations are similar to those thought to exist in the small intestine of mammals after feeding. Whereas a typical peptide concentration range has not been defined for these tissues, the peptide concentration of the mammalian small intestine is generally considered to be greater than the reported range of .05 to 5 mmol/L for amino acids (Matthews, 1991).

The flow of peptide N from the rumen has been estimated to range from 14 to 34 g N/d, depending on the diet, and, at this level, was of a large enough magnitude to account for 25% of the synthesized milk protein (Chen et al., 1987a). Broderick and Wallace (1988) have calculated that if the values of Chen et al. (1987b) were put on parity with their own data, then they would represent two to three times more peptide N available to leave the rumen than did their results. One possible explanation for the discrepancy in these data is that the former researchers fed principally SBM while the latter fed casein. As previously discussed, and as indicated by Table 2.1, casein is known to be more rapidly degraded in the rumen than is SBM. Hence, the peptide N from casein would have been made more quickly available for uptake and utilization by the microbes than from SBM. This may explain the observed relatively lower peptide N levels reported by Broderick and Wallace (1988) and exemplifies the rationale for "protecting" highly soluble proteins from ruminal degradation. Alternatively, or simultaneously, ruminal epithelium absorption of the rapidly produced casein peptide N may have occurred. This concept is important because it suggests that it may not be necessary to protect proteins in order for the ruminant animal to absorb high quality amino acids from dietary protein.

While these speculations on peptide concentration, size and potential for ruminal absorption can only be confirmed with additional studies, they do suggest that the ruminant forestomach possesses apparently similar amounts and sizes of peptides as are found in the lower gastrointestinal tract lumen of other mammals. Therefore, teleologically, it seems reasonable to suggest that the same peptide absorption mechanisms known to be responsible for the absorption of di- and tri-peptides in the small intestine of other mammals may also exist in the forestomach epithelium of ruminants.

Biological Mechanisms of Peptide Absorption

The absorption of peptides from the ruminant forestomach has not been studied. However, the mechanisms thought to be responsible for the absorption of peptides from mammalian intestinal and renal epithelial tissues have been studied and are reviewed below. Several models for the absorption of peptides by these epithelial cells have been proposed. Because it is likely that the physiological phenomenon of peptide absorption occurs by a combination of events, any model must account for luminal, intercellular, and cytosolic mechanisms that may exist. Peptides subjected to luminal hydrolysis are transported as free amino acids after cleavage, whereas peptides resistant to luminal hydrolysis are transported across the enterocytes in the intact state and, subsequent to transport, hydrolyzed by cytosolic peptidases. The combination of these separate and uncoordinated (as currently understood) processes have been suggested by Matthews (1975) and Ganapathy and Leibach (1982) as being the most viable model due to its relative simplicity and because it "...accounts for all the distinctive features of peptide absorption." The model also has the strength that it allows for a high level of cytosolic peptidase

activity which is necessary to maintain a downhill gradient for any diffusion of peptides that might occur.

Characteristics of Carrier-mediated Peptide Transport. Research to characterize the uptake of intact peptides by cell membrane permeases has indicated that peptides are transported as zwitterions, that transport is not Na^+ dependent, that transport requires an energizing or driving force (most commonly H^+ which are symported with peptides into the cell), that transport is an energy-requiring process that ultimately requires the expenditure of ATP, and that the presence of an acidic microenvironment appears to be necessary for most peptide transport.

While it is now generally accepted that peptide transport does not occur coupled with Na^+, early studies employing whole intestinal tissue (Rubino et al., 1971; Shoaf et al., 1980) showed that the removal of Na^+ from the incubation media resulted in the reduction of peptide uptake. The use of hydrolysis-resistant peptides and the development of brush border membrane vesicle (BBMV) techniques have enhanced the characterization of peptide transport by eliminating the confounding effects of peptide hydrolysis by cytosolic or brush border (if papain-treated) peptidases on whole tissue studies. The preponderance of experiments employing hydrolysis-resistant peptides for intestinal tissue preparations and BBMV (especially) have shown that peptide transport is not Na^+-dependent and that amino acid and peptide translocation across cellular membranes occurs by unique and separate permeases (Ganapathy and Leibach, 1982, 1985; Hoshi, 1985; Burston and Matthews, 1987).

The stimulation of peptide transport by an inwardly directed H^+ gradient (suggesting H^+-coupled transport) was first demonstrated in intestinal epithelial

BBMV by Ganapathy and Leibach (1983) and later in renal BBMV (Ganapathy et al., 1984; Takuwa et al., 1985). The results of these investigations indicated that extracellular pH gradients stimulate peptide uptake, that an inward directed proton flow increases peptide uptake even in the absence of Na^+, and that peptide transport is an electrogenic process dependent upon a transmembrane electrical potential. This last conclusion is supported by research conducted by Boyd and Ward (1982) which showed that an increase in intracellular peptide concentration was accompanied by an increase in membrane depolarization. The observation is consistent with the hypothesized H^+/peptide cotransporter model which states that as positively charged protons cross a relatively negatively charged membrane region, the difference in electrical charges are reduced (depolarized).

The evidence is conflicting as to whether peptide transport is actually energized, or driven, by a H^+ gradient. This is due to the lack of evidence for the "overshoot phenomenon," that is, the ability of a H^+ gradient to drive the uphill transport of peptides from the relatively low concentration (extravesicular) region into the higher concentration (intercellular) region. This ability, or lack of it, appears to be species specific and not universal to all mediated transport systems for peptides. The overshoot phenomenon has been demonstrated in rabbit (Ganapathy and Leibach, 1983; Hoshi, 1985), rat (Said et al., 1988; Iseki et al., 1989) and guinea pig (Himukai et al., 1983) using intestinal BBMV. Uptake studies characterizing dipeptide transport in rabbit, rat and human intestinal "mucosa" (Rajendran et al., 1987) and tripeptide transport in human jejunal tissue (Wilson et al., 1989) indicate that these tissues lack the ability to concentrate peptides against a concentration gradient in the presence of a H^+ gradient. Additionally, glutathionine (GSH) transport in rabbit intestinal BBMV has been characterized as being pH-dependent

with optimal transport reported at a pH value of 7.5 and as being energized by mono- and di-cations, especially Ca^{++}, but not H^+ (Vincenzini et al., 1989).

These conflicting data seem to suggest the existence of at least two classes of carrier-mediated peptide transporters, both of which are dependent upon transmembrane electrogenic potential: one class that is energized by a H^+ gradient and a second class that is energized by cations other than H^+ or Na^+. Based on an interpretation of the results of Rubino et al. (1971) and from evidence reported by Ganapathy et al. (1985) and their own cephalosporin uptake experiments, Hori and colleagues (Inui et al., 1988; Kato et al., 1989) have proposed the existence of multiple dipeptide systems in rabbit BBMV. They hypothesize the existence of both an acidic pH-preferring class (uptake driven by an inward H^+) and a neutral pH-preferring class (no inward H^+ gradient) of peptide transporters.

A transporter for the tripeptide GSH has been characterized in rabbit intestinal tissue using BBMV (Vincenzini et al., 1988, 1989). This transporter is unique due to its ability to be maximally stimulated by divalent cations (especially Ca^{++}) and the inability of other peptides to competitively inhibit GSH uptake.

Peptide Transporter Structure. Attempts have been made to characterize the chemical structure of peptide transporters with particular emphasis placed on the chemical groups responsible for substrate binding. Using photo-affinity labeling techniques, Kramer et al. (1988) have identified a 127,000 Da putative binding-protein constituent of the carnosine and glycylproline transporter in rabbit intestinal BBMV that is H^+-dependent. This group has additionally reported (Kramer et al., 1990) that "...only histidine and tyrosine residues are essential for the activity of the H^+-dependent transport system for peptides..." in rabbit enterocyte brush border membranes. In the same tissue, Kato et al. (1989) also suggest that histidine

residues in the transporter are essential for H^+-coupled peptide transport due to their role as peptide binding sites under acidic conditions (pH 6.5). These researchers also report that their investigations suggest that thiol and sulfhydryl groups on the transporter are not essential for peptide binding.

In contrast, maximal peptide transport in rabbit renal cortex BBMV requires the reduction of constitutive dithiol groups present on the transporter at or near the peptide binding site (Miyamoto et al., 1986, 1989). These authors suggest that an interchange between dithiol and sulfhydryl groups may catalyze and(or) regulate the functioning of the renal peptide transporter.

While the actual isolation of peptide transporters has not been reported, the transporter responsible for glycylproline and glycylsarcosine transport has recently been expression cloned in Xenopus lavevis oocytes (Miyamoto et al., 1991). These researchers observed a threefold increase in the ability of oocytes to uptake these peptides, but not the constitutive AA (glycine or sarcosine), after micro-injection of rabbit intestinal mucosal cell poly(A)$^+$ mRNA. This uptake was determined to be H^+-dependent.

It is now well established that peptide transport does not occur by Na^+-coupled transport. Instead, there is a large body of evidence indicating that peptides are cotransported with one type of symporter that is electrogenic in nature, that requires a protonmotive force, and that cotransports two H^+ for every peptide translocated into epithelial cells. It would seem that conditions within the gastrointestinal tract of ruminants are highly favorable to the existence of this type of peptide transporter. The digesta within the ruminant stomach is always at least slightly acidic in nature, although the omasum is less acidic than the rumen (Prins et al., 1972). The size of this proton-motor force is dependent on the diet of the

animal. Additionally, it is not unusual for the pH of digesta in the small intestine of the ruminant to remain acidic throughout the first one-half to two-thirds of the length of the small intestine (Ben-Ghedalia et al., 1974).

Evidence also continues to accumulate for the existence of a second peptide transporter that is pH dependent but that is not energized by H^+ or Na^+. Additionally, the GSH tripeptide transporter appears to represent a third type of transporter that apparently transports only GSH. The physiological significance of these transport types is unknown, but one may speculate that the H^+ energized transporter would function and be found more proximally located in the intestinal tract than the hypothesized pH-neutral transporter or the GSH transporter. In addition, one may also speculate that the proposed GSH transporter has complete substrate specificity for GSH because of the important antioxidant function of GSH in membranes.

Models for Intact Peptide Transport. Hypotheses have been proposed for models of peptide transport that include the integrated but separate functioning of H^+/peptide symporters and Na^+/H^+ exchangers (Ganapathy and Leibach, 1985; Hoshi, 1985), possibly ATP-driven H^+ transporters (Hoshi, 1986) and Na^+/K^+ ATPases. In these models, an existing membrane potential would drive two protons (Hoshi, 1986) across the brush border membrane with one peptide. As the intracellular pH drops, the Na^+/H^+ exchanger would be stimulated to exchange an intracellular H^+ for an extracellular Na^+. Thus, both the intracellular pH and the transmembrane electrical potential would be restored to basal levels. Na^+/K^+ ATPases of the basolateral membrane would then pump the transported Na^+ out of the cell, thereby reestablishing the high extracellular Na^+ gradient, at the cost of ATP hydrolysis.

The above discussion, at least for the H^+/peptide transporter, requires that a pH gradient exist across the apical border of intestinal enterocytes separating the lumen from the cell cytosol in order for peptide transport to occur. Additionally, enterocytes require the biological mechanisms necessary to maintain normal cytosolic pH when protons are transported into the cell along with the peptides and to regenerate the inwardly-directed pH gradient. The presence of a pH gradient of 1 log magnitude was predicted by Hogben et al. (1959) and has been measured by Lucas (1983) in rat proximal jejunal tissue. The presence of Na^+/H^+ exchangers that function to pump H^+ out of the cell in exchange for Na^+ in the presence of a high intravesicular proton concentration have been shown to exist in intestinal brush border membranes in the rat (Murer et al., 1976), rabbit (Knickelbein et al., 1983), and human (Kikuchi et al., 1988). That peptide uptake stimulated Na^+ uptake, but that Na^+ failed to stimulate peptide uptake, provides evidence for the coordinated functioning of the H^+/peptide symporter and the H^+/Na^+ antiporter (Himukai et al., 1983; Cheeseman and Devlin, 1985).

Models for intact peptide absorption that do not require transporters have been proposed. While simple diffusion of peptides across epithelial membranes, principally dependent on the extracellular concentration of the peptide, is generally thought to represent none or only a very small proportion of the total peptide uptake by enterocytes, the ability of the small intestine epithelium to absorb small peptides and other hydrophilic compounds through intercellular junctions has been suggested (Pappenheimer, 1988). The important features of this model for nutrient transport is that Na^+-coupled solute transport results in high concentrations of the transported solute immediately posterior to the zonulae occludentes (intercellular junctions), and thereby creates the force for osmotic flow (Madara and

Pappenheimer, 1987). The subsequent increased flow of luminal liquor through the zonulae occludentes would result in the "dragging" of its dissolved nutrients along with it into the paracellular spaces. The Na^+/glucose transporter-activated paracellular absorption of an oligopeptide (1,900 Da) has been reported in hamster small intestine (Atisook and Madara, 1991). These authors note that the existence of paracellular transport phenomena in the small intestine might help explain the observed meal-related, protein-sparing effect of glucose, as well as offer an alternative to the currently accepted theory of carrier-mediated peptide transport in the mammalian small intestine -- a theory that requires carriers to recognize a multitude of possible peptide substrate combinations by relatively few carrier types, and(or) that would require the existence of many different transporters.

Continued molecular research that isolates peptide transporters from their resident membranes, followed by their placement into artificial membranes, will allow the precise characterization and functioning of these permeases to be described. Molecular genetic analysis may allow the precise classification of multiple, genetically distinct, peptide transport families, as have been described for bacteria (Hiles et al., 1987). Additionally, it is likely that this research will reveal gene sequences that encode for functional groups of peptide permeases and their associated energy-supplying proteins, as has been observed in the extrons that encode for permeases responsible for the transport of nascent peptides out of the endoplasmic reticulum and into the cell lumen (Deverson et al., 1990; Trowsdale et al., 1990; Simon and Blobel, 1991).

The benefit of such basic research is obvious. From the fundamental knowledge of how peptide transporters function comes the potential to manipulate these systems. Recent research has suggested that the forestomach of the ruminant

animal is capable of absorbing peptides in quantities that are nutritionally significant (DiRienzo, 1990). In order that a molecular examination for specific peptide uptake mechanisms in the ruminant forestomach epithelium is justified, evidence that the forestomach epithelial tissues are capable of peptide absorption is required.

Chapter III

ABSORPTION OF CARNOSINE AND METHIONYLGLYCINE BY SHEEP RUMINAL AND OMASAL EPITHELIA[1]

J. C. Matthews

Virginia Polytechnic Institute and State University[2], Blacksburg 24060

ABSTRACT

Carnosine and methionylglycine (using ^{35}S-methionylglycine as a representative marker) absorption and transfer across ruminal and omasal epithelia collected from four (carnosine) and seven (methionylglycine) sheep were studied using parabiotic chambers that were repeatedly sampled over a 240-min incubation. The quantity of dipeptide absorbed or transferred was linearly ($P < .01$) dependent on substrate concentration. Carnosine was transferred intact across both tissues. More carnosine was absorbed ($P < .02$) and transferred ($P < .01$) by omasal epithelia. Methionylglycine was transferred intact across both tissues, but less ($P < .01$) remained intact in serosal buffer after 240 min incubation with omasal epithelium than with ruminal epithelium. The amount of methionylglycine that accumulated in each tissue was similar. Methionylglycine accumulation in tissues plus transfer after 240 min was greater ($P < .01$) for omasal tissue. The ability of sheep ruminal and omasal epithelia to absorb and transfer carnosine and methionylglycine in parabiotic units was demonstrated. Dipeptide translocation

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[1]Supported in part by a Grant-in-Aid of Research from Sigma Xi, The Scientific Research Society.

[2]Dept. of Anim. Sci.

across forestomach epithelial tissues, which has not been reported previously, may be an important route for supplying dietary amino acids to the ruminant.

Key Words: Sheep, Rumen, Omasum, Peptides, Absorption, Transfer

Introduction

Historically, the potential for significant absorption of amino acids from the ruminant forestomach has been regarded as minor. It generally is agreed that there is rapid metabolism of amino acids by rumen microbes. Consequently, the fate of dietary amino acids has been discussed in terms of their conversion to ammonia N, incorporation into microbial protein, absorption by the intestine and excretion (NRC, 1985b).

Recent research has challenged this understanding (DiRienzo, 1990). Whereas the flux of free amino acids across the stomach region was determined to be nominal, thereby supporting the results and conclusions of earlier research (Annison, 1956; Leibholz, 1971a), the flux of peptide amino acids across the stomach region accounted for approximately 77% of the total portal-drained viscera amino acid flux in both calves and sheep. The purpose of the present study was to investigate the potential for peptide absorption and translocation by ruminal and omasal epithelia.

Materials and Methods

Animals, Feeding and Tissue Collection. Tissues were collected from three Hampshire and one Suffolk ewe lamb (avg wt 69.1 kg) to determine carnosine uptake. Tissues from seven Hampshire ewe lambs (avg wt 38.3 kg) were used to determine methionylglycine uptake. All lambs were fed a mixed diet containing 50% ground, shelled corn, 30% orchardgrass hay, 13.3% soybean meal, 5% molasses, .5% trace mineral salt, .42% defluorinated rock phosphate, and .5%

limestone (as-fed basis). Additionally, the diet fed to lambs used in the methionylglycine uptake study contained 17.5 mg decoquinate[3]·kg^{-1}. Lambs were housed and fed as a group with continuous access to water. The quantity of diet fed was sufficient to produce .23 kg body weight gain·head^{-1}·d^{-1} (NRC, 1985a). The lambs were injected with vitamin A (500,000 IU), vitamin D (75,000 IU), vitamin E (3.7 IU·kg^{-1}), and Se (55 ug·kg^{-1}) approximately 18 wk (carnosine study) and 2 wk (methionylglycine study) before the first lamb was removed for tissue collection. The lambs were weighed at 2-wk intervals, and the amount of diet fed was adjusted to maintain rate of growth.

On any given day, tissues were collected from only one lamb. At the time of tissue collection, the heaviest lamb in the group was selected, weighed, stunned with a captive-bolt pistol[4], and exsanguinated. The abdominal cavity was opened quickly and the stomach was removed. The rumen was opened along the dorsal surface and the digesta was removed from the rumen and reticulum by rinsing with tap water. The stomach was then placed in .85% NaCl and transported to the laboratory. All rinsing solutions and buffers used in the preparation and incubation of tissues were maintained at 39°C.

In the laboratory, the ventral sac of the rumen was removed and washed with gentle agitation, successively, in five baths containing .85% NaCl to remove adhering digesta particles. The tissue then was placed in Krebs Ringer Phosphate buffer (pH 7.4) which had been gassed with O_2. Omasal plies were removed and treated in a like manner.

Ruminal epithelium was stripped from the underlying muscle layer by careful dissection. Omasal epithelium was prepared by pealing apart the opposing surfaces

[3]Cocci-Control [ix] Crumbles Medicated, Southern States, Inc., Richmond, VA.
[4]Super Cash, Mark 2, Accles and Shelvoke LTD., Birmingham 6, England.

of individual plies. Epithelial tissues then were cut into sections measuring approximately 4 cm x 4 cm.

Buffer Preparation. Krebs Ringer Phosphate (KRP) buffer (Umbreit et al., 1964), pH 7.4, was used for tissue preparation and as the initial buffer in both chambers of the parabiotic units. After preparation, the KRP buffer (pH 7.4) was held overnight at 39°C in a waterbath and gassed with O_2 for 1 h before use. For uptake measurements, the initial buffer in the mucosal chamber was replaced with 15 mL KRP buffer (pH 6.0) containing an appropriate concentration of peptide. The initial buffer in the serosal chamber was replaced with 15 mL KRP buffer (pH 7.4) containing 10 mmol/L glucose and enough mannitol[5] to equalize osmolarity across the chambers. Buffers used for uptake measurements were refrigerated overnight in individual, 40-mL sealed tubes. Two hours prior to the beginning of the experiment, these buffers were aspirated into 20-mL syringes[6], capped with a 25-gauge needle[7] inserted into a neoprene stopper, and placed into a 39°C waterbath.

Uptake Measurement. Parabiotic units consisted of two L-shaped glass chambers of equal volume separated by a neoprene O-ring and held together by a clamp. Squares of epithelial tissue 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. After tissues were mounted, the mucosal and serosal chambers were loaded with 10 mL of KRP buffer (pH 7.4) and the parabiotic units were placed into a waterbath at 39°C until uptake measurements began. The total elapsed time between stunning of the sheep until beginning uptake measurements was approximately 25 to 30 min.

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[5]Sigma Chemical Corporation, St. Louis, MO.
[6]Sherwood Medical, St. Louis, MO.
[7]Becton Dickinson and Company, Rutherford, NJ.

Uptake measurements were initiated by replacing the initial buffer with appropriate uptake buffers. This point was considered to be time zero. A gassing/sampling apparatus, consisting of two lengths of polypropylene tubing[8] inserted through a rubber stopper, was inserted into each chamber. 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. All chambers were gassed at a similar rate by passing O_2 through the other polypropylene tubing. After the last sample was taken, tissues were removed and the area exposed to buffers was excised, blotted with paper, and weighed.

Carnosine Uptake. For each of the four animals studied, five parabiotic units were prepared for ruminal and omasal epithelial tissues, simultaneously. The mucosal chamber of each parabiotic unit was loaded with KRP buffer (pH 6.0) containing either 0, 6, 12, 24, or 96 mmol/L of L-carnosine (N-beta-alanyl-L-histidine)[9]. Samples were collected from both chambers of each parabiotic unit after 15, 30, 60, 120, 180, and 240 min of incubation and filtered[10]. Filtrate aliquots (.5 mL) were mixed 1:1 with 2 mmol/L alpha-amino butyric acid (internal standard) in .1 N HCl and analyzed[11] for carnosine and histidine concentrations. Beta-alanine was not detectable by the methods employed. The dry weight of tissues was calculated after drying at 100°C for 24 h.

Methionylglycine Uptake. For each of the seven animals studied, six parabiotic units were prepared for ruminal and omasal epithelial tissues, simultaneously. The mucosal chamber of each parabiotic unit was loaded with KRP

[8]Atlantic Tubing Co., Patterson, NJ.
[9]Sigma Chemical Corporation, St. Louis, MO.
[10]Nylon syringe-tip filter, .45 microns, Sunbrokers, Inc., Willington, NC.
[11]PicoTag amino acid analyses system, Waters Div. of Millipore, Milliford Corp., Milford, MA.

buffer (pH 6.0) containing L-^{35}S-methionylglycine and enough methionylglycine[12] to make the final concentration .375, .75, 1.5, 3, 6, or 12 mmol/L. The total radioactivity in each mucosal chamber was the same within an animal but the amount of radioactivity varied between animals (4.13 to 8.02 uCi). The specific activity for each mucosal buffer of each animal was calculated. Samples were collected from both chambers of the parabiotic units after 5, 10, 15, 30, 45, 60, 120, 180 and 240 min of incubation. Sample aliquots of .5 mL were mixed with 5 mL of scintillation fluid[13] and ^{35}S content quantified by liquid scintillation counting[14] after a minimum of 4 h equilibriation time. Recorded cpm were converted to dpm using a standard quench curve.

The amount of methionylglycine appearing in serosal chambers and remaining in mucosal chambers was calculated to be the product of dpm quantified, buffer volume, methionylglycine concentration in the time-zero mucosal buffer, and the specific activity of the time-zero mucosal buffer.

After the last sample was taken (240 min), the remaining buffers were aspirated and stored at -20°C. Cold (2°C) KCl (150 mmol/L) was then added to each chamber (10 mL) to stop tissue metabolism before removal. The area of tissue that was exposed to the buffers (1.77 cm^2) was excised, weighed, and digested in 1.2 mL distilled water and 2 mL Scintigest[15] for 24 h at 50°C in a waterbath. After digestion, 15 mL scintillation fluid[16] were added. The amount of ^{35}S contained in the tissue was quantified after 20 h of equilibriation. The concentration of methionylglycine in the digested tissue samples was calculated by dividing the tissue

[12]Sigma Chemical Corporation, St. Louis, MO.
[13]Scintiverse BD, Fisher Scientific Products Corp., Pittsburgh, PA.
[14]Beckman LS 5000TA Scintillation Counter, Beckman Instruments, Fullerton, CA.
[15]Fisher Scientific Products Corp., Pittsburgh, PA.
[16]Scintiverse BOA, Fisher Scientific Products Corp., Pittsburgh, PA.

dpm by the specific activity of the mucosal chamber. Because tissues from the parabiotic chambers were digested for the quantification of ^{35}S, separate samples were used to estimate the dry matter content of epithelial tissue. Replicate samples of both the ruminal and serosal tissues were incubated in KRP (pH 7.4) at 39°C for 240 min, simultaneously with the uptake measurement. These tissue were blotted, weighed, and dried at 100°C for 24 h.

Characterization of the distribution of ^{35}S among different compounds was accomplished using thin layer chromatography (TLC). Serosal chamber samples from the 12 mmol/L parabiotic units obtained at 240 min were removed from the freezer and vacuum evaporated in a cold finger immersed in liquid nitrogen. The concentrated samples were spotted on TLC plates[17] and developed in methanol:chloroform (22.5:7.5, v/v). Reference standards of methionine[18] and methionylglycine[12] were chromatographed simultaneously. Following chromatography, the TLC plates were dried, sprayed with a solution containing .5% ninhydrin (w/v) in ethanol, and placed in an oven at 100°C for 7 min. Areas within each lane on the TLC plate that corresponded to the migration of the reference standards (methionine and methionylglycine), as well as an area encompassing the lane from approximately the origin up to the methionine area were identified. The TLC coating from each of these three areas was scraped from the plate and placed into scintillation vials containing .5 mL of .1 N HCl. Scintillation fluid[13] (5 mL) was added and ^{35}S content was quantified. The sum of ^{35}S activity detected in the three areas was calculated and the relative proportions of ^{35}S activity present in each area was calculated by dividing the ^{35}S activity found in an area by the total ^{35}S activity.

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[17] Silica F_{254}, 150 microns, 60 angstroms, Universal Scientific, Inc., Atlanta, GA.
[18] Sigma Chemical Corporation, St. Louis, MO.

Synthesis of L-^{35}S-Methionylglycine. L-^{35}S-methionylglycine was synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis techniques (Fields and Noble, 1990). N-Fmoc-L-^{35}S-methionine was synthesized using a modified procedure of Atherton and Sheppard (1989). L-^{35}S-methionine[19] (5 mCi) was added with brisk stirring to 1 mL of 8.15% Na_2CO_3 containing 4.43 mg of methionine[18]. Then 1 ml of 9-fluorenylmethyl succinimidyl carbonate[20] (.033 mol/L in 1,2-dimethoxyethane) was added over the course of 1 h. This acylation reaction was allowed to proceed for 19 h. The reaction mixture was filtered[21], neutralized with HCl to pH 7.0 and vacuum evaporated. Ethyl acetate was added (1.5 mL) and the organic layer was washed with .1 N HCl (four times), then with distilled water saturated with NaCl (three times), and dried by adding $MgSO_4$. The organic layer was then filtered[21] and vacuum evaporated. N-Fmoc-L-^{35}S-methionine/N-Fmoc-L-methionine was crystallized out of solution with cold (2°C), re-distilled petroleum ether, and the ether was removed by vacuum evaporation.

N-Fmoc-Gly-Resin[22] (16.6 mg) was added to a silanized reaction vessel equipped with a Teflon-lined screw cap, sintered glass frit, and stopcock for washing according to the procedure of Atherton and Sheppard (1989). Deprotection of the resin compound was performed by adding 200 uL of 20% piperidine[23] in N,N-dimethylformamide[24] (DMF), shaking[25] for 20 min and then washing with .7 mL of DMF (ten times). This deprotection procedure was repeated once.

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[19]Product No. SJ.235, Amersham Corporation, Arlington Heights, ILL.
[20]Calbiochem Corporation, LaJolla, CA.
[21]Teflon syringe-tip filter, .5 micron, Millipore, Bedford, MA.
[22]Bachem Inc., Torrance, CA.
[23]Fisher Scientific Products Corp., Pittsburgh, PA.
[24]Fisher Scientific Products Corp., Pittsburgh, PA.
[25]Model 4651 Aliquot Mixer, Miles Inc., Diagnostics Division, Elkhart, IN.

N,N'-diisopropylcarbodiimide[26] in DMF (1 mol/L, .5 mL) was added to synthesized N-Fmoc-L-^{35}S-methionine/N-Fmoc-L-methionine (2.9 mg), N-Fmoc-L-methionine[27] (3.0 mg), and 1-hydroxybenzotriazole[28] (67.6 mg). Ester activation was allowed to proceed for 15 min. This reaction mixture was added to the deprotected resin in the reaction vessel and peptide bond formation (acylation) was allowed to proceed for 1.75 h during constant shaking[25]. The resulting N-Fmoc-L-^{35}S-methionylglycine-resin compound was deprotected as described earlier, washed with dichloromethane[29] (three times) and methanol (three times), and vacuum evaporated in the reaction vessel for 4 h.

Cleavage of the peptide from the resin was by Reagent K as described by King et al. (1989). The filtrate was vacuum evaporated for 3 h, and the recovered peptide was stored at -20°C.

Purification of the peptide was performed by HPLC using a reverse phase C_{18} column[30] with a linear gradient (100:0 to 0:100) formed from .1% trifluoroacetic acid[31] and .1% trifluoroacetic acid: acetronitrile[32] (50:50), pumped at a rate of 1 mL/min during 75 min. During elution of radioactively labeled peptides, 1-min fractions were collected[33] from the column and the radioactivity in each was measured[14]. Four fractions that bracketed the 8.7 min elution time of the peptide (as determined by a reference standard) were dried by vacuum evaporation and stored at -20°C until use.

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[26]Sigma Chemical Corporation, St. Louis, MO.
[27]Bachem Inc., Torrance, CA.
[28]Sigma Chemical Corporation, St. Louis, MO.
[29]Sigma Chemical Corporation, St. Louis, MO.
[30]Reverse phase column, .9 X 30 cm, Waters Div. of Millipore, Milliford Corp., Milford, MA.
[31]Fisher Scientific Products Corp., Pittsburgh, PA.
[32]Fisher Scientific Products Corp., Pittsburgh, PA.
[33]Model 328 Fraction Collector, Instrument Specialties Company, Lincoln, NEB.

Statistical Analyses. A split-plot design was used. The effects of animal, substrate concentration, tissue, and their interactions, on mucosal disappearance and serosal appearance of carnosine and methionylglycine were evaluated by analysis of variance using the general linear models procedure of SAS (1988) after natural log transformation of the data. Because of the repeated sampling of each parabiotic unit, the effect of time on the above factors was evaluated by the repeated measures option of SAS (1988). Orthogonal polynomial contrasts were used to partition the effects of concentration and time. Because of the unequal spacing of concentrations used in the carnosine uptake study, and missing observations in the methionylglycine study, contrast coefficients were generated by the Matrix function of SAS (1985).

Differences between tissue means for the proportion of methionylglycine found in the serosal buffer were evaluated by the two-sample T test procedure of SAS (1988) after arcsine transformation of the data. Differences between tissues of tissue-accumulated methionylglycine were evaluated by analysis of variance using the general linear models procedure of SAS (1988).

Results and Discussion

A pH gradient was established initially by loading the mucosal and serosal chambers with pH 6.0 and pH 7.4 KRP buffers, respectively. This serosal-directed proton flow was created because proton gradients have been reported to drive the active transport of peptides by brush border membranes of intestinal (Ganapathy et al., 1984; Hoshi, 1986) and kidney tubule (Takuwa et al., 1985) epithelial tissues. In preliminary experiments, pH measurements were taken throughout 240 min of incubation. Only slight changes in the pH of chamber buffers were observed, indicating that the desired pH gradient was maintained.

Because the amount and rate of nutrient absorption is very likely a function of tissue surface area, it would be desirable to express absorption on a surface area basis, especially when surface areas vary between tissues. But because no meaningful measure of surface area is available and because it is thought that surface area is quite different between equal cross-sections of ruminal and omasal epithelia, it was decided to express all dipeptide measurements on a dry tissue basis. Accordingly, it is assumed that mass of dry tissue is more related to surface area than is cross-sectional area and, therefore, is a better basis for comparing ruminal and omasal epithelia uptake of dipeptides.

Disappearance of carnosine and methionylglycine from mucosal buffer and their appearance in serosal buffer was quantified in these studies. For both peptides, the amount appearing in serosal buffer was relatively low (.8 to 2.1%) when expressed as a proportion of disappearance from mucosal buffer. It is not known whether there actually was a substantial amount of peptide unaccounted for or whether disappearance may have been overestimated. Because there was no evidence to indicate that carnosine is metabolized by the tissues studied (see below), we think that disappearance was overestimated. The reason for this is not apparent. The recovery of both peptides in serosal buffer when expressed as a percent of substrate present was similar to recovery of transported nutrients in other in vitro systems (Cook et al., 1965; Leibholz, 1971b)

Carnosine was selected to investigate the potential for dipeptide transport by ruminal and omasal epithelial tissues because it is not readily hydrolyzed by tissues, therefore, allowing the evaluation of peptide transport without the confounding effects of peptide hydrolysis. The disappearance of carnosine from mucosal buffers (Table 3.1) increased quadratically ($P < .01$). Disappearance increased until about

120 min of incubation and then appeared to stop. This pattern can be seen in the individual means and suggests that ruminal and omasal tissues were equally affected, although the specific time of carnosine disappearance arrestment varies. This conclusion is supported by the fact that a time x tissue interaction was not apparent.

Whereas the cause of this reduction in the rate of carnosine disappearance is not known, it does not appear to be because the tissues were saturated with substrate. If tissue saturation had occurred, then the quantity of carnosine that disappeared at the time of arrestment should have been the same regardless of substrate concentration in the chambers. Clearly, the data show that the quantity of carnosine that had disappeared at the time that carnosine disappearance stopped was different between mucosal chamber buffers of different initial concentrations.

Carnosine disappearance from the mucosal buffers increased linearly ($P < .01$) as concentration increased. The ability of the epithelial tissues to remove carnosine certainly was not exceeded within the range of concentrations studied. More carnosine disappeared ($P < .02$) from omasal tissue chambers than did from ruminal chambers. The omasal epithelia, therefore, displayed a greater ability to absorb carnosine.

The appearance of carnosine in the serosal chamber of the parabiotic units (Table 3.2) increased linearly as length of time increased ($P < .01$) and as the initial concentration increased ($P < .02$). More carnosine ($P < .01$) appeared in the serosal chambers of the omasal tissue compared with serosal chambers of the ruminal tissue. An interaction ($P < .06$) existed between tissue type and concentration and appears to reflect the markedly greater ability of omasal tissue to transfer carnosine.

The disappearance of carnosine from mucosal chambers and its subsequent appearance in serosal chambers of parabiotic units loaded with ruminal and omasal epithelial tissues indicates that carnosine was absorbed and transferred by both forestomach epithelia. Omasal tissue clearly displayed a greater ability to absorb (Table 3.1) and transfer (Table 3.2) carnosine than did ruminal tissue.

The use of HPLC analytical techniques to quantify the amount of carnosine present in the chamber buffers allowed the simultaneous quantification of L-histidine (a constituent amino acid of carnosine) for all samples. Histidine was not detected in either mucosal or serosal buffers, indicating that ruminal and omasal epithelial tissues absorbed and transferred intact carnosine without hydrolysis.

Diffusion appears to be the biological mechanism responsible for carnosine passage because of the linear relationship between quantity of carnosine absorbed (Table 3.1) and transferred (Table 3.2) and substrate concentration. There was no evidence for saturation of the trans-epithelial passage of carnosine, despite the use of relatively high carnosine concentrations in these uptake studies. If carrier-mediated transport had been responsible for carnosine passage through the tissues, then a disproportionately greater amount of carnosine would have been absorbed or transferred at lower concentrations, compared to the amount transferred at higher concentrations.

Because of the high carnosine concentrations used, the possibility existed that the presence of peptide carriers in ruminal and omasal tissues may have been saturated (even at the lowest, 6 mmol/L, level) and, therefore, were undetectable. To examine peptide absorption at more physiological concentrations, and to study the potential for methionylglycine uptake by forestomach epithelia, L-^{35}S-methionylglycine was used as a representative marker for the absorption and

transfer of methionylglycine. The use of this radiolabeled peptide allowed a more sensitive measurement of peptide passage than by HPLC analysis.

Methionylglycine concentrations were selected that bracketed the reported 1 to 4 mmol/L peptide concentrations in dairy cattle forestomach liquor (Broderick and Wallace, 1988; Broderick and Craig, 1989). Additionally, samples were collected earlier in the incubation in order to better define the initial events of methionylglycine passage through epithelial tissues.

The disappearance of methionylglycine from mucosal chambers increased linearly ($P < .01$) as time passed and as concentration increased (Table 3.3). More methionylglycine ($P < .01$) disappeared from omasal tissue chambers, as compared to ruminal tissue chambers. The time x tissue interaction ($P < .01$) seems to be the result of the relatively greater rate of methionylglycine absorption by omasal tissue.

Appearance of methionylglycine in the serosal chambers increased linearly ($P < .01$) with time and concentration (Table 3.4). There was a difference ($P < .02$) in how the substrate concentration influenced the quantity of methionylglycine that appeared in the serosal chambers. This interaction appears to reflect the greater appearance of methionylglycine in omasal tissue serosal buffers, as compared to serosal buffers of ruminal tissue.

These results indicate that methionylglycine was absorbed and transferred by epithelial tissues from both the rumen and omasum. The linearity of the substrate concentration effect on the methionylglycine disappearance (Table 3.3) and appearance (Table 3.4) suggests that diffusion was the mechanism by which methionylglycine absorption and transfer occurred. Methionylglycine was apparently absorbed and transferred in greater quantities by omasal tissue. It

appears then that omasal epithelial tissue has a greater ability to absorb and transfer both of the dipeptides studied than does ruminal epithelial tissue.

The ability of ruminal and omasal tissues to translocate methionylglycine intact was compared after 240 min incubation in parabiotic units loaded with 12 mmol/L methionylglycine (Table 3.5). Separation by TLC showed that approximately 36% of ^{35}S was associated with methionylglycine in the serosal buffer of ruminal epithelium. A much smaller ($p < .01$) proportion of the radiolabel, 4.6%, was associated with the dipeptide in serosal buffer from omasal epithelium. The proportion of ^{35}S associated with methionine was nearly twice as great in serosal buffer from omasal (47.9%) as compared with ruminal (28.6%) epithelium, but the difference was not statistically significant. A relatively large amount of ^{35}S activity was recovered in an unidentified fraction on the TLC plates. This fraction was located on the plates between the origin and methionine, and accounted for 35.3% and 47.5% of the ^{35}S activity recovered in serosal buffer of ruminal and omasal epithelia, respectively. No ^{35}S was detected on the plates beyond the point of methionylglycine migration.

These results indicate that intact methionylglycine absorption and transfer occurred. Additionally, the proportion of the ^{35}S isotope identified with methionylglycine in ruminal tissue chambers was more than in omasal tissue chambers, possibly indicating that omasal tissue has a greater ability to hydrolyze methionylglycine. The large proportion of ^{35}S label in the methionine and pre-methionine fractions of both tissues suggests that significant amounts of methionylglycine were hydrolyzed and further metabolized either during, before, or after transport. Accordingly, the fate of methionylglycine was different between tissue types and suggests that the mechanisms and events responsible for the

appearance and metabolism of ^{35}S-methionylglycine in the serosal buffers may differ between ruminal and omasal tissues. The high proportion of ^{35}S in association with methionylglycine in the serosal fluid is not consistent with previous observations from this laboratory where the net flux of free amino acids across the non-mesenteric drained viscera was nominal (DiRienzo, 1990).

The amount of methionylglycine that accumulated in the experimental tissues was determined after 240 min of incubation (Table 3.6). Tissue accumulation of methionylglycine increased linearly ($P < .01$) as concentration increased. Methionylglycine accumulation in ruminal tissue appeared to be greater, but did not differ statistically from that omasal tissue. These results suggest that passage of ^{35}S (representing methionylglycine) through the two tissues did not differ. Accordingly, the difference in the ability of ruminal and omasal tissues to absorb and transfer dipeptides may be the result of events affecting the initial absorption process.

The quantity of methionylglycine that accumulated in tissues (Table 3.6) was added to the quantity of methionylglycine that appeared in the serosal buffer after 240 min (Table 3.4) and the resulting data (Table 3.7) represent the total quantity of methionylglycine that was either absorbed, or absorbed and subsequently translocated. As the concentration of methionylglycine increased, the amount of methionylglycine increased linearly ($P < .01$). The tissue x concentration interaction ($P < .01$) probably represents the greater ($P < .01$) transfer of methionylglycine by omasal tissue in response to increased substrate concentration. Thus, omasal tissue appears to have a greater ability to transfer methionylglycine than does ruminal tissue.

The results from the methionylglycine study indicate that ruminal and omasal

epithelia have the ability to absorb and transfer intact methionylglycine. Additionally, omasal epithelial tissue displays a greater ability to absorb and transfer methionylglycine than does ruminal epithelial tissue. These conclusions parallel the results from the carnosine study. The observation that omasal epithelia apparently possesses a greater methionylglycine hydrolytic ability suggests that the amount of peptidase activity and rate of dipeptide metabolism between epithelia of the forestomach may differ. Accordingly, it is important to recognize that the methionylglycine appearance (Table 3.4, 3.7) and tissue accumulation (Table 3.6) should be evaluated with the knowledge that proportionately more of the ^{35}S label quantified in ruminal parabiotic units was representative of methionylglycine than that of the ^{35}S quantified in the omasal parabiotic units.

The lack of evidence for carrier-mediated transport of the dipeptides was surprising from a teleological perspective. A pH (proton) gradient was established across the tissues. Such mucosal to serosal directed proton gradients have been used to demonstrate the presence of carrier-mediated dipeptide transport in intestinal and renal (Ganapathy et al., 1983) epithelial brush border membranes. Because omasal liquor is essentially acidic (Prins et al., 1972), and because ruminal liquor can develop pH levels of 5.5 or less (Whitelaw, 1970), it was thought that H^+-dependent peptide transporters might exist in forestomach epithelial tissues. However, the fact that increasing the dipeptide concentrations always resulted in a linear increase in the amount of carnosine and methionylglycine absorbed and transferred, suggests that the phenomenon of H^+-dependent, carrier-mediated transport does not occur in ruminal or omasal epithelia. Consequently, the establishment and maintenance of a mucosal to serosal proton gradient probably was not necessary.

Instead, diffusion appears to have been the mechanism responsible for the passage of the dipeptides studied. The lack of evidence suggesting that the absorption and transfer processes became saturated, plus the clear dependence of the disappearance and appearance of both dipeptides on their initial concentrations, support this conclusion.

Diffusion of nutrients through the forestomach epithelium has been suggested to be dependent on an osmotic gradient generated by active transport of Na^+ (Keynes, 1969; Henrikson, 1970). A compatible model for paracellular solute flow between cells connected by zonulae occludentes (as are the granulosa cells of forestomach epithelia) has been hypothesized (Pappenheimer, 1989). This diffusional pathway may be particularly important for the absorption of dipeptides (small, hydrophilic molecules) by forestomach epithelia, as has been reported in intestinal enterocytes (Atisook and Madara, 1991).

Results from these two peptide uptake studies indicate that both carnosine and methionylglycine can be absorbed into and translocated across ruminal and omasal epithelial tissues. By identifying ruminal and omasal epithelia as being capable of peptide passage, these studies confirm the potential for peptide forestomach passage, as suggested by the determination of large quantities of peptides in the non-mesenteric fraction of portal blood (DiRienzo, 1990). The demonstration that epithelial tissues from the rumen and omasum are capable of absorbing and(or) transferring dipeptides has not been reported before to our knowledge.

A difference in the ability of ruminal and omasal epithelial tissues to hydrolyze methionylglycine was observed. That carnosine was not hydrolyzed during transfer indicates that these tissues may hydrolyze and(or) metabolize

dipeptides to differing degrees. This evidence for differential tissue metabolism of substrate suggests that the structure of the dipeptide substrate may influence its rate and quantity of transfer from the forestomach lumen.

Implications

Results from this study suggest that epithelial tissues of the ruminant forestomach are capable of dipeptide absorption from the forestomach liquor and transfer into forestomach venous vasculature. If the magnitude of these processes is determined to be nutritionally important, or if the mechanisms responsible for peptide transfer can be manipulated, then the practice of supplementing the amino acid needs of ruminants with peptide amino acids may become an integral component of ruminant diet formulation.

TABLE 3.1. MEAN CARNOSINE DISAPPEARANCE FROM MUCOSAL BUFFER[a]

Tissue	Time	Concentration,[b] mmol/L 6.0	12.0	24.0	96.0	Mean[g]	Mean[hi]
	min	nanomol·mg dry tissue^{-1}					
Rumen							
	15	49	76	151	625	225	
	30	49	116	186	851	301	
	60	74	97	202	1,147	380	
	120	109	193	237	1,109	412	
	180	86	144	286	1,119	409	
	240	85	161	288	1,299	458	
	Mean[ce]	75	131	225	1,025		
Omasum							
	15	122	258	368	1,873	655	440
	30	202	241	660	2,666	942	621
	60	219	424	683	2,655	995	688
	120	225	347	794	5,205	1,643	1,027
	180	246	532	900	4,439	1,529	969
	240	300	434	1,018	4,967	1,680	1,069
	Mean[ce]	219	373	737	3,634		
	Mean[df]	147	252	481	2,330		

[a]Data were transformed using the natural log function before analysis. Non-transformed means presented in the table are the means of four animals and represent the quantity of carnosine that disappeared from the mucosal chamber of the parabiotic units.

[b]Values are the initial carnosine concentrations in the mucosal chamber of the parabiotic units.

[c]Mean disappearance across time and within tissue.

[d]Mean disappearance across time and tissues.

[e]Tissues differed ($P < .02$).

[f]Linear concentration effect ($P < .01$).

[g]Mean disappearance across concentrations and within time.

[h]Mean disappearance across concentrations and tissues.

[i]Quadratic time effect ($P < .01$).

TABLE 3.2. MEAN CARNOSINE APPEARANCE IN SEROSAL BUFFER[a]

Tissue	Time	Concentration,[b] mmol/L 6.0	12.0	24.0	96.0	Mean[h]	Mean[ij]
	min	nanomol·mg dry tissue^{-1}					
Rumen							
	15	.16	1.27	.33	.57	.52	
	30	.14	.19	.34	1.45	.46	
	60	.09	.50	.50	2.68	.88	
	120	.46	.50	.92	4.40	1.56	
	180	.90	.90	2.08	7.48	2.63	
	240	.59	.33	1.88	9.80	3.11	
	Mean[cef]	.13	.61	.97	4.40		
Omasum							
	15	2.32	2.05	3.70	4.67	3.19	1.86
	30	3.40	2.97	2.52	8.97	4.47	2.45
	60	4.48	3.25	5.53	20.13	8.35	4.61
	120	6.92	4.45	13.47	52.62	19.37	10.46
	180	8.08	5.55	19.08	65.50	24.55	13.59
	240	10.38	7.98	22.23	101.43	35.50	19.31
	Mean[cef]	5.93	4.38	11.09	42.22		
	Mean[dg]	3.03	2.50	6.03	23.31		

[a]Data were transformed using the natural log formation before analysis. Non-transformed means presented in the table are the means of four animals and represent the quantity of carnosine that appeared in the serosal chamber of the parabiotic units.

[b]Values are the initial carnosine concentrations in the mucosal chamber of the parabiotic units.

[c]Mean appearance across time and within tissue.

[d]Mean appearance across time and across tissues.

[e]Tissues differed ($P < .01$).

[f]Tissue x concentration interaction ($P < .06$).

[g]Linear concentration effect ($P < .02$).

[h]Mean appearance across concentrations and within tissue.

[i]Mean appearance across concentrations and across times.

[j]Linear time effect ($P < .01$).

TABLE 3.3. MEAN METHIONYLGLYCINE DISAPPEARANCE FROM MUCOSAL BUFFER[a]

Tissue	Time	Concentration,[b] mmol/L .375	.75	1.5	3.0	6.0	12.0	Mean[gi]	Mean[hj]
	min	nanomol·mg dry tissue^{-1}							
Rumen									
	5	5	9	19	34	125	134	49	
	10	9	18	36	58	154	234	79	
	15	14	24	55	83	215	356	125	
	30	18	34	70	118	317	466	170	
	45	26	43	91	145	352	594	209	
	60	28	54	109	178	425	747	257	
	120	30	61	123	201	474	820	285	
	180	35	70	141	228	554	937	328	
	240	40	79	153	255	604	1,039	362	
	Mean[ce]	25	46	92	144	356	578		
Omasum									
	5	13	10	28	58	82	170	60	54
	10	26	33	93	173	231	490	174	127
	15	39	57	150	303	434	848	305	215
	30	48	84	207	415	643	1,237	439	305
	45	76	104	254	505	829	1,603	562	385
	60	77	134	315	626	994	2,040	698	477
	120	87	157	371	722	1,203	2,310	808	547
	180	97	179	417	829	1,362	2,648	922	625
	240	111	204	484	941	1,516	2,965	1,037	699
	Mean[ce]	64	107	258	508	810	1,590		
	Mean[df]	44	77	175	326	583	1,084		

[a]Data were transformed using the natural log function before analysis. Non-transformed means presented in the table are the means from seven animals and represent the quantity of methionylglycine that disappeared from the mucosal chamber of the parabiotic units, using ^{35}S-methionylglycine as a representative marker.

[b]Values are the initial methionylglycine concentrations in the mucosal chamber of the parabiotic units.

[c]Mean disappearance across time and within tissue.

[d]Mean disappearance across time and across tissues.

[e]Tissues differed ($P < .01$).

[f]Linear concentration effect ($P < .01$).

[g]Mean disappearance across concentrations and within tissue.

[h]Mean disappearance across concentrations and across tissues.

[i]Time x tissue interaction ($P < .01$).

[j]Linear time effect ($P < .01$).

TABLE 3.4. MEAN METHIONYLGLYCINE APPEARANCE IN SEROSAL BUFFER[a]

Tissue	Time	Concentration,[b] mmol/L .375	.75	1.5	3.0	6.0	12.0	Mean[h]	Mean[ij]
	min	nanomol·mg dry tissue^{-1}							
Rumen									
	5	0	.01	.02	.02	.06	.10	.03	
	10	.01	.04	.04	.07	.12	.23	.08	
	15	.02	.07	.06	.11	.23	.44	.15	
	30	.04	.15	.12	.25	.49	1.01	.34	
	45	.06	.25	.24	.42	.82	1.55	.70	
	60	.08	.31	.28	.57	1.08	2.17	.75	
	120	.18	.62	.58	1.19	2.34	4.48	1.56	
	180	.26	.94	.88	1.80	3.68	6.86	2.40	
	240	.37	1.27	1.23	2.49	5.21	9.82	3.40	
	Mean[ceg]	.24	.64	.58	.90	1.74	2.63		
Omasum									
	5	.08	.23	.39	.48	.25	1.57	.38	.36
	10	.18	.08	.75	.95	.59	3.73	1.05	.74
	15	.28	.15	1.19	1.52	.97	5.75	1.64	.90
	30	.62	.36	2.28	4.43	2.13	12.44	3.71	2.03
	45	.91	.57	3.58	6.94	3.64	18.75	5.73	3.21
	60	1.21	.78	4.76	6.07	5.39	26.43	7.44	4.09
	120	2.45	2.97	7.96	12.61	11.40	54.37	15.70	8.63
	180	3.59	2.56	14.45	21.18	17.39	82.99	23.69	13.05
	240	4.76	3.54	19.22	28.05	22.97	109.53	31.34	17.37
	Mean[ceg]	1.56	1.23	6.33	9.14	7.13	35.06		
	Mean[df]	.90	.93	3.46	5.02	4.44	18.85		

[a]Data were transformed using the natural log function before analysis. Non-transformed means presented in the table are the means of seven animals and represent the quantity of methionylglycine that appeared in the serosal chamber of the parabiotic units, using ^{35}S-methionylglycine as a representative marker.

[b]Values represent the initial methionylglycine concentration in the mucosal chamber of the parabiotic units.

[c]Mean appearance across time and within tissue.

[d]Mean appearance across time and across tissues.

[e]Tissues differed ($P < .01$).

[f]Linear concentration effect ($P < .01$).

[g]Tissue x concentration interaction ($P < .02$).

[h]Mean appearance across concentrations and within tissue.

[i]Mean appearance across concentrations and across times.

[j]Linear time effect ($P < .01$).

TABLE 3.5. ^{35}S DISTRIBUTION AMONG DIFFERING COMPONENTS IN 240 MIN SEROSAL BUFFER OF 12 MMOL/L PARABIOTIC UNITS[a]

Tissue	Item	Fraction[b]		
		Met-Gly[c]	Met	Pre-Met
		---------- % ----------		
Rumen		44.4	12.2	43.7
		45.2	30.6	24.2
		61.8	30.9	7.3
		11.0	44.5	44.5
		18.0	25.0	57.0
	Mean	36.1	28.6	35.3
	SEM	9.4	5.2	8.8
Omasum		4.8	44.6	50.5
		3.2	18.4	78.4
		3.8	62.3	34.0
		7.3	34.1	58.5
		4.0	80.0	16.0
	Mean	4.6	47.9	47.5
	SEM	.7	10.7	10.6

[a]Data were transformed using the arcsine function before analysis. Non-transformed proportions of TLC fractions from five animals are presented in the table and represent the distribution of ^{35}S label found in one of three of the serosal chamber buffer compounds, after 240 min incubation in the 12 mmol/L parabiotic units.

[b]Met-Gly = methionylglycine, Met = methionine, Pre-Met = area of the serosal buffer sample lane from approximately the origin to the methionine area.

[c]The proportion of ^{35}S differed between tissues ($P < .01$).

TABLE 3.6. MEAN METHIONYLGLYCINE ACCUMULATION IN TISSUE[a]

Tissue	Concentration,[b] mmol/L						
	.375	.75	1.5	3.0	6.0	12.0	Mean[e]
	nanomol·mg^{-1} dry tissue						
Rumen	.82	1.68	2.40	4.00	8.48	8.2	4.26
Omasum	1.23	1.20	2.23	3.14	5.53	6.3	3.27
Mean[cd]	1.03	1.44	2.32	3.57	7.01	7.27	

[a]Data were transformed using the natural log function before analysis. Non-transformed means presented in the table are the means of seven animals and represent the quantity of methionylglycine that accumulated in the tissues after 240 min of incubation, assuming all ^{35}S was in the form of L-^{35}S-methionylglycine.

[b]Values are the initial methionylglycine concentrations in the mucosal chamber of the parabiotic units.

[c]Mean methionylglycine accumulation across tissues.

[d]Linear concentration effect ($P < .01$).

[e]Mean methionylglycine accumulation across concentrations.

TABLE 3.7. MEAN METHIONYLGLYCINE ACCUMULATION IN TISSUE PLUS APPEARANCE IN SEROSAL BUFFER[a]

	Concentration,[b] mmol/L						
Tissue	.375	.75	1.5	3.0	6.0	12.0	Mean[efg]
	nanomol·mg^{-1} dry tissue						
Rumen	1.19	2.95	3.63	6.49	13.69	18.01	7.66
Omasum	5.98	4.73	21.45	31.20	28.50	115.87	34.62
Mean[cdg]	3.59	3.84	12.54	18.84	21.10	66.94	

[a]Data were transformed using the natural log function before analysis. Non-transformed means presented in the table are the means of seven animals and represent the quantity of methionylglycine that accumulated in the tissues after 240 min of incubation and that appeared in the serosal side of the parabiotic units, assuming that all ^{35}S was present in the form of L-^{35}S-methionylglycine. These values represent the total quantity of peptides accounted for outside of the mucosal chambers.

[b]Values are the initial methionylglycine concentrations in the mucosal chamber of the parabiotic units.

[c]Mean methionylglycine tissue accumulation plus serosal buffer appearance across tissues.

[d]Linear concentration effect ($P < .01$).

[e]Mean methionylglycine tissue accumulation plus serosal buffer appearance across concentrations.

[f]Tissues differed ($P < .01$).

[g]Tissue x concentration interaction ($P < .01$).

Chapter IV

Epilogue

The results of the present study indicate that sheep ruminal and omasal epithelial tissues have the potential to transfer peptides from forestomach liquor into serosal vasculature. By revealing two potential sites for peptide uptake, this study supports the observation that the stomach region is capable of peptide absorption (DiRienzo, 1990). The exploitation of this fundamental knowledge will depend on understanding the physiological mechanisms responsible for the observed peptide passage across the ruminant forestomach epithelia.

It is concluded from the present study that the absorption and transfer of dipeptides across ruminal and omasal epithelial tissues are not carrier-mediated events. Diffusion therefore, appears to be the mechanism(s) for dipeptide passage. There are several possible methods by which dipeptides could conceivably diffuse through the forestomach epithelia. The idea that peptides diffuse through membrane-spanning protein channels is appealing because of the low cellular energy cost implied, but passage by this mechanism would require peptides to pass through the many cellular membranes of forestomach epithelial tissue. The idea that some part of peptide transfer might occur by paracellular absorption merits consideration because it provides for a mechanistic force (the osmotic pressure-induced flow of forestomach liquor) that appears to be capable of carrying absorbed peptides through epithelial strata without requiring diffusion or active transport through each constiuent membrane.

Results from intestinal paracellular transport studies have indicated that the functioning of the Na^+/glucose cotransporter activates a paracellular osmotic

gradient (Atisook and Madara, 1991). It is speculation whether the low concentrations of glucose typically found in the forestomach are sufficient to initiate this mode of nutrient transfer. However, the active transport of another, more abundant, substrate might produce the same effect. If this paracellular transport phenomenon does exist in the ruminant forestomach, an understanding of its regulation might allow its manipulation, perhaps with feed additives or diet formulation, to result in greater nutrient transfer.

Because of the layered cell structure of ruminant forestomach epithelia, the absorption of peptides might well occur by a combination of uptake mechanisms. Peptide passage might be initiated with the translocation of peptides into the paracellular spaces of the epithelial cell matrix, either by carrier-mediated active transport through the membranes of the granulosa cells or by diffusion through the zonulae occludentes of adjacent granulosa cells. Once across this solute barrier, peptides would then be carried in the osmotic pressure-induced flow of forestomach liquor as it moved through the intracellular network until reaching the basement membrane. Final entry of the peptide into the bloodstream would appear to be facilitated by 1 angstrom gaps between the basale cells adjoining the basement membrane and fenestrations of the venous vasculature.

Although the above translocation of intact peptides is hypothetical, it does allow for the relatively energetically inexpensive passage of peptides through the many cells of the forestomach epithelium. It does not seem reasonable that peptide translocation would occur by active transport across each cell of the forestomach epithelium because of the high cellular energy costs. The potential for peptides to be absorbed from the forestomach lumen in the intact state and then to be hydrolyzed to free amino acids is accommodated by this model. However,

hydrolysis of peptides after absorption would not explain the large quantity of peptides that were measured in the non-mesenteric portal blood draining the stomach region (DiRienzo, 1990).

Results from the present study indicate that omasal epithelial tissue has a greater ability to absorb and transfer dipeptides than ruminal epithelial tissue. Madara and Pappenheimer (1987) have suggested that the solvent drag-generated absorption contributes more to the overall uptake of a substrate in areas of high concentration, as compared to regions of low concentration. In this regard, the omasum with its exposure to higher nutrient concentrations (based on DM determinations), apparently greater dipeptide cleavage ability (results of present study), and higher ratio of epithelium to organ volume (Engelhardt and Hales, 1977), might well be expected to possess and maintain a larger peptide gradient, as compared to the rumen.

The rumen, however, appears to have the greater potential to generate peptides through its larger microbial population (Giesecke et al., 1975). Additionally, the mass of the rumen epithelium is twice that of the omasum (Stevens and Stettler, 1966). Therefore, it may be argued that the ruminal environment provides the greatest potential for manipulation of the peptide transfer phenomenon. The observation from the current study that less hydrolysis of methionylglycine occurred in ruminal tissues, than in omasal tissues, supports this argument and suggests that forestomach absorption of intact peptides may be inherently greater through ruminal epithelium.

For both epithelia, the percentage of methionylglycine that disappeared from the mucosal buffers (Table 3.3) to that available for absorption was large (27 to 31%) compared to the percentage recovered (.32 to 1.42%) in tissues and serosal

buffers (Table 3.7). As discussed in Chapter III, this difference is thought to be the result of overestimating the amount of peptide that disappeared from mucosal buffers. Accordingly, the cause for this error will need to be corrected before any further studies are performed with this experimental system. Consequently, modifications of the parabiotic chambers may be necessary. Specifically, the potential for peptide adsorption to the components of the parabiotic units would be reduced by silanizing the chamber halves and replacing the polypropylene tubing of the gassing and sampling apparatuses with Teflon tubing.

The important implication from this study is that approximately .8 to 1.5% of dipeptides present in the forestomach liquor of ruminants could potentially pass into the bloodstream. Because dipeptide passage appears to be dependent on substrate concentration, the quantity of dipeptides crossing the forestomach epithelia might be increased if their concentration could be maintained or increased. Therefore, the use of feed additives to manipulate the microbial population such that the dipeptide concentration of forestomach liquor is increased, should increase the potential for dipeptide absorption.

One possible candidate is the ionophore, monensin, which has been shown to inhibit the growth of certain proteolytic, Gram-positive bacteria resulting in the accumulation of peptide-N in mixed bacteria cultures (Chen and Russell, 1991). Conceivably, the feeding of this metal/proton antiporter would result in a greater serosal-directed diffusion gradient and, consequently, increased uptake of peptides.

Future studies.

The significance of the present study depends on the quantity of peptides that are typically absorbed. The study data displayed fairly constant within-animal variation, but large between-animal variation. Therefore, in general, more animals

will need to be tested in order that a larger data base is accumulated to allow a more confident evaluation of the potential for peptide passage across forestomach epithelia.

Future parabiotic chamber studies should evaluate the simultaneous transfer of peptides and their constituent amino acids across ruminal and omasal epithelial tissues. The results from such studies would allow the amount of amino acids absorbed and transferred as peptides and free amino acids to be compared. Consequently, the relative nutritional significance of trans-epithelial tissue peptide passage, as compared to amino acid passage, would be indicated.

Future epithelial tissue metabolism trials should be conducted to investigate the fate of absorbed peptides. The cellular processes responsible for the disappearance of peptides from the mucosal chamber without their concomitant appearance in the serosal chambers should be studied. The addition of butyrate to uptake buffers may affect the rate of peptide metabolism by supplying the tissues with a more preferential source of energy than glucose.

Future metabolic inhibition studies should be conducted to further evaluate the mechanism(s) of peptide absorption and transfer. The use of compounds known to inhibit Na^+/K^+ ATPase-dependent transport processes would indicate whether peptide passage is dependent on energy-requiring mechanisms.

Future canulation studies that measure the efficacy of compounds such as monensin to create an increased forestomach peptide concentration and that determine the site of subsequent peptide passage would be appropriate.

The fundamental discovery of this research is that ruminal and omasal epithelial tissues are capable of absorbing and transferring dipeptides. These tissues appear to differ in their ability to absorb and transfer intact carnosine and

methionylglycine. The practical importance of this research is that an alternative route for amino acid supplementation in the ruminal animal has been identified. Further knowledge of the cellular mechanisms responsible for peptide passage across forestomach epithelia might facilitate their manipulation. This knowledge would be especially pertinent because of the current development and use of somatotropic agents capable of promoting increased protein synthesis, provided that all synthesis-limiting amino acids are available.

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APPENDIX

STATISTICAL ANALYSIS EXAMPLES

EXAMPLE OF ANALYSIS OF VARIANCE FOR COMPARISON OF METHIONYLGLYCINE TRANSFER BY RUMINAL AND OMASAL EPITHELIA AFTER NATURAL LOG TRANSFORMATION

General Linear Model Procedure

Dependent variable: Methionylglycine (nanomol/mg dry tissue)

Source	DF	Sum of squares	Mean square	F value	PR > F
Model	187	3161.2963	16.9053	212.94	.0001
Error	545	43.2684	.0794		r^2
Corrected total	732	3204.5647			.9865

CV	SD	Met-Gly Mean
-79.6425	.2818	-.3538

Source	DF	Sum of squares	Mean square	F value	PR > F
Anim	6	16.14445	2.6908	33.89	.0001
Tissue	1	626.4320	626.4320	7,890.40	.0001
Conc	5	811.0520	162.2104	2,043.17	.0001
Anim*Tissue	6	97.6424	16.2737	204.98	.0001
Anim*Conc	30	108.7902	3.6263	45.68	.0001
Tissue*Conc	5	62.9061	12.5812	158.47	.0001
Anim*Tissue*Conc	30	109.8946	3.6631	46.14	.0001
Time	8	1,286.6918	160.8365	2,025.86	.0001
Anim*Time	48	16.1489	.3364	4.24	.0001
Tissue*Time	8	1.0148	.1269	1.60	.1224
Time*Conc	40	2.1594	.0540	.68	.9340

Tests of hypothesis using the Type III MS for Anim*Tissue*Conc as an error term.

Source	DF	Sum of squares	Mean square	F value	PR > F
Anim	6	16.1445	2.6908	.73	.6257
Tissue	1	626.4320	626.4320	171.01	.0001
Conc	5	811.0520	162.2104	44.28	.0001
Anim*Tissue	6	97.6424	16.2737	4.44	.0025
Anim*Conc	30	108.7902	3.6263	.99	.5109
Tissue*Conc	5	62.9061	12.5812	3.43	.0143

Tests of hypothesis using the Type III MS for Anim*Tissue as an error term.

Tissue	1	626.4320	626.4320	38.49	.0008
Contrast					
Linear Conc	1	508.7443	508.7443	31.26	.0014
Quadratic Conc	1	186.7100	186.7100	11.47	.0147

EXAMPLE OF REPEATED MEASURES ANALYSIS OF VARIANCE FOR COMPARISON OF THE EFFECT OF TIME ON METHIONYLGLYCINE TRANSFER BY RUMINAL AND OMASAL EPITHELIA AFTER NATURAL LOG TRANSFORMATION

Repeated Measures Procedure

Univariate Tests for Hypotheses for Within Subject Effects.

Source	DF	Sum of squares	Mean square	F value	Adjusted P > F G-G[a]	H-F[b]
Time	8	820.9804	102.6225	2,890.91	.0001	.0001
Time*Anim	48	15.9130	.3315	9.34	.0001	.0001
Time*Tissue	8	.1351	.0169	.48	.6558	.8714
Time*Conc	40	2.4610	.0615	1.73	.1023	.0121
Time*Anim*Tissue	40	7.7074	.1927	5.43	.0001	.0001
Time*Anim*Conc	224	16.6604	.0744	2.10	.0093	.0001
Time*Tissue*Conc	40	1.8787	.0470	1.32	.2503	.1255
Error	120	4.4260	.0355			

[a] Greenhouse-Geisser epsilon = .2938.

[b] Huynh-Feldt epsilon = 1.5131.

EXAMPLE OF REPEATED MEASURES ANALYSIS OF VARIANCE OF CONTRAST VARIABLES FOR THE EFFECT OF TIME ON METHIONYLGLYCINE TRANSFER BY RUMINAL AND OMASAL EPITHELIA AFTER NATURAL LOG TRANSFORMATION

Repeated Measures Procedure

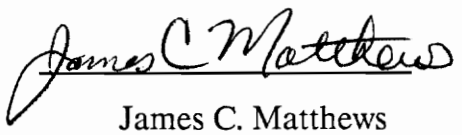
Analysis of Variance of Contrast Variables.

Source	DF	Sum of squares	Mean square	F value	Pr > F
Linear contrast					
Mean	1	818.7127	818.7127	9,188.74	.0001
Anim	6	11.9830	1.9972	22.42	.0001
Tissue	1	.0755	.0756	.85	.3717
Conc	5	.7888	.1578	1.77	.1796
Anim*Tissue	5	6.7901	1.3580	15.24	.0001
Anim*Conc	28	6.4249	.2295	2.58	.0288
Tissue*Conc	5	.4762	.0952	1.07	.4157
Error	15	1.3365	.0891		
Quadratic contrast					
Mean	1	2.0502	2.0502	25.95	.0001
Anim	6	1.9150	.3192	4.04	.0131
Tissue	1	.0055	.0055	.07	.7960
Conc	5	.3224	.0645	.82	.5566
Anim*Tissue	5	.3193	.0638	.81	.5615
Anim*Conc	28	3.1075	.1110	1.40	.2476
Tissue*Conc	5	.4755	.0951	1.20	.3542
Error	15	1.1850			

VITA

James Clyde Matthews, son of Ann and Griffith Matthews, was born May 26, 1958, in Princeton, New Jersey. He married Elizabeth A. Lehman on September 7, 1981, in East Waterboro, Maine. He was graduated from Rutgers, The State University of New Jersey, with a Bachelor of Science degree in Animal Science in December, 1988. He initiated his program of graduate study at Virginia Tech in September, 1989.

He is a member of The American Society of Animal Science and Phi Sigma Biological Honor Society.



James C. Matthews