LYSINE AND GLYCYL-*L*-SARCOSINE ABSORPTION ACROSS OVINE FORESTOMACH EPITHELIUM *IN VITRO*

by

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(ABSTRACT)

Lysine absorption by ruminal and omasal epithelia was studied using parabiotic chambers that were sampled for 60-min. Lysine appearance in serosal buffers and the accumulation of lysine in tissues increased linearly ($P < .001$) with time. Lysine appearance in serosal buffers of ruminal tissue increased proportionally as the concentration of lysine increased in mucosal buffers. However, lysine appearance in serosal buffers of omasal tissue increased proportionally to a substrate concentration of 1.5 m*M*, then plateaued. Total absorption (tissue accumulation plus serosal appearance) increased linearly for ruminal tissue; however, for omasal tissue, total absorption increased linearly to 1.5 m*M* ($P < .001$), then plateaued. Using omasal epithelium, glycyl-*L*-sarcosine (Gly-Sar; .1 m*M*) absorption was studied during co-incubation with glycine and peptide substrates (each at 5 m*M*). Accumulation of Gly-Sar in omasal epithelium was greatest ($P < .05$) when Gly-Sar was present alone. Glycine inhibited ($P < .05$) Gly-

Sar accumulation by 20%, whereas peptide substrates inhibited ($P < .05$) Gly-Sar accumulation by 60 to 85%. The absorption of Gly-Sar (.1 m*M*) alone or during co-incubation with either 10 m*M* butyric acid, or a mixture of VFA was also studied. Accumulation of Gly-Sar in tissue was greatest ($P < .05$) when Gly-Sar was present alone; butyric acid and VFA inhibited ($P < .05$) Gly-Sar accumulation by 50 to 84%. These results suggest absorption of amino acids and peptides by the omasum, and also suggest the mechanism involves mediated as well as possibly paracellular transport.

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INTRODUCTION

Absorption of nutrients from the forestomach of the ruminant has long been studied to determine to what extent nutrients are transported and absorbed through the epithelia to enter the blood supply. Amino acids, peptides, and VFA are among the components resulting from the activity of the microbial population of the forestomach that become available for absorption at this site (Annison, 1956; Broderick and Wallace, 1988). While the study of VFA absorption has involved the efforts of many and led to great expanses of information, the study of transport and absorption of protein components, other than ammonia, from the forestomach has only recently begun to attract the attention of the scientific public. This newly created curiosity has been partially due to the uncovering of clues suggesting that amino acids and small peptides (two to eight amino acids) may be absorbed from this portion of the gastrointestinal tract (DiRienzo, 1990; Koeln et al., 1993; Matthews and Webb, 1995). Free amino acids and peptide-bound amino acids were measured across the gastrointestinal tract and liver of steers, with peptide-bound amino acids accounting for the greatest concentration of amino acids in the arterial blood (Koeln et al., 1993). Specifically, a large net flux of peptide-bound amino acids was observed across the nonmesenteric-drained viscera of calves, suggesting origination from the forestomach and abomasum (DiRienzo, 1990). The ability

of the rumen and omasum to absorb methionine, carnosine, and methionylglycine was determined, but a specific transport mechanism was not evident from this study (Matthews and Webb, 1995). The purposes of the present studies were to determine the mechanisms for amino acid and peptide transport and absorption in the forestomach. Peptide substrates and VFA were utilized to ascertain whether competitive or generic transport systems exist for peptide absorption in the forestomach.

Chapter II

LITERATURE REVIEW

Understanding absorption of nutrients is obviously of great importance to nutritionists. The mechanisms for transport and absorption of various nutrients have been studied for many years to elucidate the specific requirements of these mechanisms. Whether mechanisms require energy, particular molecular size or charge, etc. lead to the dependence of absorption of nutrients on the specific mechanistic requirements. This very reason is why all possible absorptive processes need to be thoroughly understood for nutritionists to utilize these processes for administration of drugs, additives, etc. Currently the accepted mechanisms of nutrient absorption include passive diffusion, facilitated diffusion, and active transport. However, these may not be the only methods for nutrient absorption.

Amino Acid and Peptide Absorption

As a result of microbial degradation in the rumen, amino acids and peptides are made available for absorption by the forestomach (Annison, 1956; Broderick and Wallace, 1988). However, the idea that protein absorption occurs in this portion of the gastrointestinal tract (GIT) has only recently begun to receive acceptance within the scientific community. Measurements of amino acid appearance in the portal blood suggested that up to 79% of the amino acids found were associated with peptides (Koeln et al., 1993). Further investigation revealed that a large net flux of peptide-bound amino

acids was associated with the drainage from the nonmesenteric viscera (DiRienzo, 1990). This nonmesenteric drainage originates from the rumen, reticulum, omasum, abomasum, duodenum, and spleen (Webb et al., 1992).

Peptides and amino acids are available for absorption within the forestomach (Matthews et al., 1996a), but do mechanisms exist in this portion of the GIT to remove these substrates? The ability of the rumen and omasum to absorb *L*-methionine, *L*-carnosine, and *L*-methionylglycine has been determined, although a characterized transport mechanism has yet to be defined (Matthews and Webb, 1995). The omasal epithelium possesses mRNA that encodes for a protein(s) capable of mediating the transport of glycyl-*L*-sarcosine (Matthews et al., 1996b). The mechanisms for the absorption of the protein components thus far observed have not been clearly defined. Absorption of amino acids and peptides occurs in the small intestine using carrier-mediated transport systems, but it is not clear whether this type of transport system is facilitating the absorption of these products in the forestomach.

Epithelial Cell Layers of the Forestomach

Epithelial cells are those cells found lining the membranes of the GIT. A cross section of the rumen wall reveals the layers of these cells which are described from the basal surface toward the luminal surface as stratum basale, stratum spinosum, stratum granulosum, and stratum corneum (Steven and Marshall, 1970; Van Soest, 1994). The stratum basale is arranged against the basement membrane and the cells of the basal layer

are most active in transporting Na^+ into the intercellular spaces (Steven and Marshall, 1970), in other words, here the Na^+/K^+ pump is found. The cell layers, along with the junctional complex, form a barrier to prevent passage of molecules and ions which cannot pass through the cells transcellularly. Intercellular spaces exist between adjacent cells and these spaces are sealed by the junctional complexes, particularly the tight junctions (Darnell et al., 1990). The intercellular spaces are largest in the stratum basale and diminish in size toward the stratum granulosum at which point the tight junctions completely seal off the spaces (Steven and Marshall, 1970). The lateral surface of epithelial cells is composed from the luminal surface toward the basal membrane of tight junctions, belt and spot desmosomes, a gap junction, and a hemidesmosome (Darnell et al., 1990). For many years it was supposed that the components of the complex were bound together and to the cell simply to provide integrity, later, possibilities of cellular communication were given as another purpose of the complex (Cereijido, 1995). Today, the individual components are more clearly understood, however more purposes and components are surely yet to be determined.

Tight junctions, also called zonulae occludens, are found between cells in the area corresponding to the stratum granulosum (Keynes, 1969; Steven and Marshall, 1970; Van Soest, 1994) and apparently serve to seal off the luminal contents from flowing past the basolateral surface of the cells and into the capillaries. In the ruminant animal, the seal created by the tight junctions also keeps the larger microorganisms and bacteria out of the

extracellular spaces between cells. The tight junctions are thought to be one of the key elements necessary for paracellular absorption. The desmosomes bind cells together and do indeed provide for structural integrity in epithelial cells. Belt desmosomes are found directly under tight junctions and form a belt around the cell and provide cell to cell contact. Gap junctions are found the closest toward the basal lamina and allow cells to exchange small molecules. These junctions "help to integrate the metabolic activities of cells in the tissue by assuring that they share a common pool of metabolites" (Darnell et al., 1990).

As indicated previously, these components of epithelial cells are found throughout the GIT, however some of the components may have evolved differently due to necessity along the tract. For instance, the tight junctions of the omasum are thought to be more "leaky" than those of the rumen and reticulum (Gabel, et al., 1993). Also, the cells of the stratum basale are more numerous in the omasum, and cardia, and esophageal groove than in the other portions of the rumen (Steven and Marshall, 1970).

Paracellular Absorption

For the past fifteen years, scientists have been researching the possibility of a fairly nonselective, low energetic process that allows solutes to be absorbed between certain cells instead of entering cells transcellularly. These findings have focused on the absorption occurring in the intestine and the transport phenomena has been described as

paracellular absorption. Paracellular movement of molecules occurs between adjacent cells.

Paracellular absorption was first described by Pappenheimer and Madara (1987) although previous research had suggested that the tight junctions were penetrable by lanthanum and barium (Madara and Trier, 1982). Using hamster small intestinal epithelia *in vitro* and *in vivo*, dilatations were induced between absorptive cells in the presence of glucose and amino acids. After perfusion with 25 m*M* glucose, dilatations were measurable from <.1 to .5 μm in width. Madara and Pappenheimer (1987) proposed that the Na^+-coupled glucose cotransporter led to an increased permeability of the tight junctions and expansion of the lateral spaces providing optimal conditions for transport of luminal nutrients in bulk by solvent drag. They proposed that the presence of glucose at high luminal concentrations is responsible for creating a concentration gradient to encourage solvent drag and because glucose is transported transcellularly on a carrier-mediated system, this may initiate the expansion of the tight junctions. Also, once glucose and Na^+ are inside the cell, the glucose moves into the basolateral extracellular space, thus establishing the osmotic gradient for solvent drag (Ballard et al, 1995). Active transport of glucose is saturated after 10 to 20 m*M*, but after a meal the concentration of glucose in the duodenum is closer to 100 to 300 m*M* (Madara and Pappenheimer, 1987), and at this point, the greater proportion of absorption of hydrophilic nutrients is by solvent drag and may include many molecules found in the luminal contents, including

some peptides. Once the concentration of glucose is reduced, active transport takes over to remove the rest of the nutrients from the lumen. Estimates of the total ingestion of sugars exceed the capacity for active transcellular transport and would suggest that paracellular absorption of these sugars is an important physiological alternative (Ballard et al., 1995).

Components of Paracellular Absorption

Tight Junctions. The most important component for paracellular absorption is the presence of the tight junction. The paracellular pathway is restricted by the junctional complex and nothing can reach the lateral, intercellular spaces without the removal of this restriction. The "tightness" of the tight junctions has been correlated to the number of junctional strands and the transepithelial resistance (TER). Freeze-fracture fixation is a procedure used for the visualization of tight junctions. A normal tight junction freeze-fracture shows a "flat network of anastomosing filaments grouped in a narrow belt, surrounding the cell on the basolateral side near the luminal surface" (Cereijido, 1995). During the examiniation of freeze-fracture replicas of rat ileal epithelial, the penetration of lanthanum was observed between goblet cells which had visibly less junctional strands with less depth than absorptive cells (Madara and Trier, 1982). This led to the conclusion that a greater number of tight junctional strands is correlated with a decreased ability of paracellular penetration by ions. Quantifying TER is the method for measuring tight junctional resistance and therefore permeability. Usually the presence of a substrate

will alter the TER, with increases in TER suggesting a “tighter” tight junction, with little passive leakage, and decreases in TER suggesting expansion of the tight junctions and permeability of the space. Transepithelial resistance is measured by comparing the transcellular resistance, or sum of the resistance of the luminal membrane and the basal membrane, to the transepithelial resistance (Fromter and Diamond, 1972).

Na^+-Coupled, Mediated Transport. Because the tight junctions are such an integral part of the paracellular absorption process, the mechanics of tight junction regulation are therefore also controlling regulators of the paracellular absorption phenomenon. As discussed previously, the presence of glucose resulted in the increased permeability of tight junctions and the expansion of the junctions allowing for nutrient absorption (Madara and Pappenheimer, 1987). Glucose probably is not the controlling factor as much as is a Na^+-coupled, or Na^+ related, mediated transport system for any particular nutrient which may be indicted in the initiation of the tight junction expansion. After activation of apical Na^+-cotransporters, TER decreases and tight junction permeability increases. In studies with octapeptides synthesized from D amino acids (Pappenheimer et al., 1994), glucose (Turner and Madara, 1995), and a Pz-peptide (4-phenylazobenzyloxycarbonyl-*L*-Pro-*L*-Leu-Gly-*L*-Pro-*D*-Arg; Yen and Lee, 1995), each of the substrates facilitated their own paracellular transport by stimulating Na^+ entry into epithelial cells. It has been suggested that, as a result of Na^+-solute cotransport, or Na^+ entry into the cell as a result of a transport process, cell swelling occurs which alters the

cytoskeleton to the point of increasing permeability (Turner and Madara, 1995). In other studies, the presence of glucose resulted in observable dilatations of the tight junction and the penetration of a hemepeptide (MW 1,900; Madara and Pappenheimer, 1987; Atisook and Madara, 1991).

The transport of Na^+ is associated with the Na^+/K^+ ATPase located on the basolateral membrane of epithelial cells. Sodium enters across the apical membrane as a result of being carried down its concentration gradient in association with a Na^+ -coupled transport system, or Na^+ exchanger, which is bringing in another nutrient (Gabel et al., 1993). Once inside the cell, Na^+ disrupts the well established electrical potential so Na^+ is pumped out of the cell across the membrane via the Na^+/K^+ pump at the expense of one ATP. With the addition of amiloride, a Na^+ channel blocker, Pz-peptide penetration was abolished in the lower intestine, but not in the upper intestine. With the addition of an inhibitor of the Na^+/H^+ exchanger, Pz-peptide penetration was abolished in the upper intestine, but not the lower intestine (Yen and Lee, 1995). Knowing that the Na^+ channel is concentrated in the lower intestine and that the Na^+/H^+ exchanger is concentrated in the upper intestine, the specificity of these inhibitors suggest that Na^+ plays a significant role in the paracellular transport of nutrients. Glucose and some amino acids are transported on Na^+-coupled, mediated transport systems while peptides are transported on H^+-coupled, mediated transport systems. However, the peptide/H^+ -cotransporter ultimately results in an exchange of H^+ for Na^+ . Once the peptide has been transported

across the membrane, a Na^+/H^+ -exchanger must remove the H^+ that was carried into the cell and, as a result, a Na^+ ion enters the cell. Because Na^+ is involved in the transport of these molecules, it seems reasonable to conclude that the presence of Na^+ inside the cell, versus the movement of the nutrient itself, may result in the increased permeability of the junctional complex (Yen and Lee, 1995). It is still unclear if this is indeed the cause of the tight junction expansion, and if this is the mechanism for the expansion, it is unclear how Na^+ activates this process.

If the process of removing Na^+ from the cell is involved in the expansion of the tight junctions, this proposal is also energetically efficient because only an indirect cost of ATP will be associated with paracellular absorption. A single ATP is required to remove two Na^+ ions from the cell after it is transported into the cell with a nutrient. If the turnover of a Na^+ -cotransporter, or a Na^+ exchanger, initiates expansion of the tight junctions, then the nutrients can be absorbed through solvent drag, and actually require no additional direct energy cost. Such an efficient pathway of nutrient absorption obviously benefits the animal.

High Luminal Concentration of a Nutrient. The other related component of permeability regulation is high luminal concentration of the nutrient being absorbed. Dilatations observed in the small intestine were visible after the perfusion of glucose at 20 to 25 m*M* concentrations (Madara and Pappenheimer, 1987; Atisook and Madara, 1991). When octapeptides were consumed along with a 5% glucose solution (~ 275 m*M*;

Pappenheimer et al., 1994), the peptides were absorbed. However, a Pz-peptide was able to penetrate the tight junction and be absorbed at only 1 to 5 m*M* concentrations (Yen and Lee, 1994). But even these relatively low concentrations are much higher than physiologically present in the lumen. The necessity for a high luminal concentration is based on the need for an osmotic gradient that will result in solvent drag once the tight junctions expand. Water movement across the tight junction is critical to create the initial flow of luminal contents toward the mucosa. Once the movement of water begins, the nutrients move down their concentration gradients and are, as a result of solvent drag, absorbed (Turner and Madara, 1995). Because it is hard to separate the effects of glucose and Na^+ transport from the effect of osmotic water flow, it is difficult to determine if changes in the tight junction occur prior to or as a result of liquid flow.

Nutrients Absorbed Through Paracellular Absorption

Now that some of the mechanisms of paracellular permeability have been presented, what kind of molecules take advantage of the expansion of the tight junction in epithelial cells? Tight junctions are selectively permeable to cations over anions and there is apparently a limitation on the molecular size of a solute that can be channeled between the cells (Madara and Trier, 1982; Atisook and Madara, 1991; Ballard et al., 1995). Dense La^{3+} deposits were observed in the intercellular spaces of villus goblet cells and less between adjacent absorptive cells (Madara and Trier, 1982). Horseradish peroxidase (MW 40,000) was excluded in both glucose-exposed and glucose-unexposed intestinal

tissues, however a heme-conjugated peptide tracer (MW 1,900) penetrated tight junctions (Atisook and Madara, 1991). Logically, even an apparently nonselective process of absorption such as the paracellular pathway must limit the transport of some molecules or all contents of the lumen could be found in the blood. While tight junctions may not discriminate against different types of nutrients, as do transport mechanisms, they can not allow just any molecule to pass between the cells, therefore charge and molecular size probably limit some solutes. Paracellular absorption has been implicated in the absorption of several types of nutrients including ions, fatty acids, sugars, and peptides.

Ions. Of the total transepithelial flux of Ca^{2+} measured with the addition of vitamin D_3, two-thirds of the absorption was associated with paracellular versus transcellular absorption (Karbach, 1992). With the influx of Ca^{2+}, the creation of an osmotic gradient causes water to move from the lumen into the intercellular space and drives the water out the basal end of the membrane, carrying the solute with it (Karbach, 1992). Both Na^+ and Cl^- fluxes result in transepithelial potential differences which support the idea of active transport processes, but also suggests that some paracellular transport of the ions is occurring in the sheep reticulum (Gabel et al., 1993). When short chain fatty acids (SCFA) were in the buffer, stimulated Na^+ transport was observed, while movement of Cl^- was not changed (Gabel et al., 1993).

Short Chain Fatty Acids. In sheep abomasal mucosa, SCFA reduced the transepithelial potential difference thus increasing paracellular permeability

(Tempelmann, 1993). In yet another study, high (21 m*M*) luminal concentrations of SCFA were absorbed across the abomasal epithelium, intracellularly their charges raised the osmolarity and resulted in cellular swelling, which disrupts the cytoskeleton; the consequence was an increased permeability in the paracellular pathway (Bodeker et al., 1994).

Glucose. As previously described, in the presence of glucose, dilatations of the tight junction were observed (Madara and Pappenheimer, 1987) and a heme-conjugated peptide (MW 1,900) leaked into those dilatations (Atisook and Madara, 1991). However, one study refutes the suggestion that glucose stimulates or enhances paracellular transport. Six carbon sugars, *L*-glucose, *L*-mannose, and *L*-galactose were administered orally and only a small fraction (< 10%) was absorbed via the paracellular pathway. Also, the addition of *D*-glucose did not enhance the absorption of any of the other sugars suggesting that glucose does not enhance the uptake of other nutrients via paracellular absorption (Schwartz et al., 1995).

Peptides. Paracellular absorption of peptides has been described in intestinal segments and Caco-2 monolayers (cultured cell line). Naturally occurring polypeptides are rapidly hydrolyzed by extracellular peptidases, so octapeptides were synthesized from D-amino acids that would not be recognized by peptidases or transport proteins in order to study paracellular absorption (Pappenheimer et al., 1994). Thirty to fifty percent of the D-octapeptides ingested with 5% glucose were absorbed from the GIT of

rats. Because the peptides could not be degraded by peptidases or recognized by transport proteins, the absorption had to occur through the paracellular pathway. It is suggested that glucose facilitated the absorption of peptides by triggering contraction of the perijunctional actin after transcellular transport, dilating tight junctions and permitting absorption by solvent drag (Pappenheimer et al., 1994). Pz-peptide is degraded intracellularly by collengenase in the descending colon. About 60% of the proteolytic activity is cytosolic, but the peptide was observed penetrating all intestinal segments with more than 80% of it remaining intact, thus absorption must have occurred paracellularly (Yen and Lee, 1994). The Pz-peptide also induced penetration of mannitol and atenolol (paracellular markers) and decreased TER in Caco-2 monolayers by 20% (Yen and Lee, 1994). Yen and Lee (1995) demonstrated that Na^+ is involved in the paracellular transport of the Pz-peptide. They proposed that the peptide facilitates its own paracellular transport by stimulating Na^+ entry into epithelial cells which increases water flux, which is Na^+-dependent, and creates solvent drag.

Regulators of Paracellular Absorption

ATP. The presence or depletion of ATP has been implicated in the control of tight junction expansion and therefore the absorption of nutrients via the paracellular pathway. As energy was depleted for 10 to 30 min from cultured Madin-Darby canine kidney (MDCK) cells, a decrease in TER was observed suggesting an increased permeability of the tight junctions (Mandel et al., 1993). With the removal of ATP from

the medium of cultured MDCK cells for 60 min, disruption of the tight junction strands was observed along with the decrease in TER (Bacallao et al., 1994). After the depletion of ATP for 60 min, the strands of proteins in the tight junction were visible only as short rows formed from five to six particles that were folded together (Bacallao et al., 1994). This disruption may be related to the actin cytoskeleton network which comprises the actin ring around the cell, a portion of the junctional complex. In culture, actin fibers form a ring that circumscribes the plasma membrane and this ring is in the same level as intercellular junctions (Bacallao et al., 1994). Disruption of the perijunctional actin will disrupt the paracellular barrier created by tight junctions (Anderson and Van Itallie, 1995). Although ATP is not directly required in the expansion of tight junctions or for solvent drag to occur, ATP is necessary for some of the Na^+-coupled transporters, during Na^+ movement out of the cell with the Na^+/K^+ ATPase, and to maintain the integrity of the actin cytoskeleton network.

Cytoskeleton. As described above, disruption of the cytoskeleton leads to disruption of the functions of the tight junction. Changes in the cytoskeleton alter the tension of the junctional complex therefore increasing permeability (Ballard et al., 1995). Cytochalasins disrupt actin microfilaments and inhibit actin filaments from interacting with each other. In the presence of 10 μg/mL of cytochalasin D, the TER of intestinal absorptive cells decreased and freeze fracture findings showed decreased strand number (Madara et al., 1986). Cytochalasin D reduced strand to strand cross-linking and

subsequently the strands failed to impede movement (Madara et al., 1986). Because cytochalasin D increased tight junction permeability to a level comparable to that observed in the presence of glucose, it is suggested that the cytoskeleton influences tight junctional integrity (Ballard et al., 1995).

Phosphorylation of Proteins. Changes in the phosphorylation state of the junctional proteins also affect the interactions of these proteins resulting in increased permeability (Ballard et al., 1995; Staddon et al., 1995). Pervanadate and phenylarsine oxide both stimulated tyrosine phosphorylation of ZO-1 and ZO-2 (protein components of the junctional complex) and led to an decrease in TER, or an increase in tight junction permeability (Staddon et al., 1995). The proposed mechanism involves the cadherin/catenin complex. These transmembrane proteins mediate intercellular adhesiveness. Tyrosine phosphorylation of the components of the cadherin/catenin complex appears to decrease cadherin adhesiveness which leads to the opening of the tight junctions (Staddon et al., 1995). Or, because the phosphorylation occurs on the ZO-1 protein, maybe the phosphorylation of the protein components directly associated with the tight junction result in the expansion of the tight junctions. Anderson and Van Itallie (1995) suggest that the increase in tyrosine phosphorylated proteins causes contraction of the actin and results in the degradation of the paracellular barrier.

Calcium. Intracellular Ca^{2+} is needed for tight junction assembly and to maintain tight junction resistance (Ballard et al., 1995; Contreras et al., 1995). Madin-Darby

canine kidney cells incubated in Ca^{2+}-free medium had a markedly decreased TER and resistance was restored when Ca^{2+} was added (Gumbiner and Simons, 1986). In another study, removal of extracellular Ca^{2+} led to the rapid opening of the tight junctions (Staddon et al., 1995). It has been suggested that Ca^{2+} is indirectly related to the phosphorylation of the myosin regulatory light chain, a portion of the cytoskeleton (Anderson and Van Itallie, 1995), or to the perijunctional actin ring (Turner and Madara, 1995). Removal of intracellular Ca^{2+} affects phosphorylation of the myosin regulatory light chain through the actions of Ca^{2+}-calmodulin-activated myosin light chain kinase (Anderson and Van Itallie, 1995). After intracellular Ca^{2+} is removed, contraction of the perijunctional actin is observed and an increased paracellular permeability is the result. Myosin light chain kinase is activated by intracellular Ca^{2+} spikes. The activated kinase phosphorylates myosin regulatory light chain, increasing cytoskeletal tension and producing increased tight junction permeability (Turner and Madara, 1995).

Other Regulators. Assembly and barrier properties of epithelial cells are influenced by second messengers and signaling pathways such as tyrosine kinases, Ca^{2+}, protein kinase C, calmodulin, adenosine 3',5'-cyclic monophosphate (cAMP), and phopholipase (Anderson and Van Itallie, 1995). Some of these activators have been discussed. Their influences on paracellular permeability tend to act upon the proteins of the tight junction and they interact with each other. Protein kinase C inhibitors decrease paracellular permeability (Anderson and Van Itallie, 1995). The suggested mode of

function is related to Ca^{2+}, actin, and the cadherin-mediated cell to cell contacts. Changes in the number of and depth of junctional strands can be associated with increases in intracellular cAMP (Ballard et al., 1995). These changes increase TER which increases tight junction resistance. However, cAMP may also increase tight junction resistance by collapsing lateral spaces (Ballard et al., 1995).

The point of this discussion has been to present information so far accepted in the control and regulation of the junctional complex, and therefore paracellular absorption. It must be understood that many interactions occur between components of the junctional complex and commonly found intracellular metabolites that can affect permeability of the tight junction in several ways, often related to one another. Much is yet to be determined which may result in additional regulators of permeability being elucidated and lead to specific alterations that may influence the uptake of nutrients.

If the paracellular pathway is truly involved in the absorption of various solutes and large quantities of these solutes, this pathway must be considered as another mode of nutrient absorption. Understanding this mechanism and how it could interact with other transport mechanisms is necessary to thoroughly understand and investigate nutrient absorption.

While many different components and mechanisms may control and regulate the junctional complex and tight junction permeability, there are two necessary conditions currently accepted as being necessary for paracellular absorption to occur. Those

conditions include a Na^+-coupled, or Na^+ related, carrier-mediated transport system for the nutrient that is being absorbed that can initiate the expansion of the tight junctions; and the high luminal concentration of the nutrient that is being absorbed, so that solvent drag can pull the nutrients through the expanded tight junctions. Since we know that the necessary components for paracellular absorption are present in the forestomach of ruminants (Matthews et al., 1996a), paracellular absorption may be occurring in this portion of the GIT, although no current research is available that supports this idea.

Chapter III

ABSORPTION OF LYSINE BY SHEEP RUMINAL AND OMASAL EPITHELIA

ABSTRACT

In order to study the absorption of lysine across ruminal and omasal epithelia, tissue was collected from six wethers (avg. BW = 58.5 kg) and mounted in parabiotic chambers that were repeatedly sampled throughout a 60-min incubation. The appearance of lysine (using ^{3}H-*L*-Lysine as a representative marker) in serosal buffers increased linearly ($P < .001$) with time for both tissues. More ($P < .001$) lysine appeared in omasal serosal buffers than in ruminal. The tissues responded differently ($P < .001$) to increases in the mucosal buffer concentrations of lysine. The appearance of lysine in the serosal buffers of ruminal tissue increased proportionally as the concentration of lysine increased in the mucosal buffers. However, the appearance of lysine in the serosal buffers of omasal tissue increased proportionally to a substrate concentration of 1.5 m*M*, then plateaued. After 60-min of incubation, the accumulation of lysine in the ruminal epithelium was greater ($P < .001$) than the accumulation in the omasal epithelium. For both tissues, the accumulation of lysine increased linearly ($P < .011$) as mucosal substrate concentrations were increased. Total absorption of lysine (tissue accumulation plus serosal appearance) was greater ($P < .001$) for omasal than for ruminal epithelium. For ruminal tissue, total absorption increased linearly; however, for omasal tissue, absorption increased linearly up to 1.5 m*M* ($P < .001$) then plateaued. The linear increase in total

absorption of lysine by the ruminal tissue suggests lysine transport is likely not mediated. In contrast, that lysine absorption by the omasal tissue was saturable suggests carrier-mediated transport of lysine.

Key Words: Absorption, Lysine, Sheep, Rumen, Omasum

Introduction

Currently it is accepted that the forestomach has the ability to absorb VFA, electrolytes, ammonia, and water; however, amino acid absorption from the forestomach has been speculatively minimal. Still, amino acid absorption from the rumen was observed by Leibholz (1971), and she described some of this absorption as being selective. Lysine absorption by ovine abomasal epithelium has been observed, but the concentrations examined were well above what would be expected physiologically (Fejes et al., 1991). Methionine absorption has been observed across the ovine rumen and omasum, but no evidence of a saturable mechanism was found in this study (Matthews and Webb, 1995). The significance of amino acid absorption is still considered as minimal, largely due to the low concentrations of amino acids in the forestomach digesta due to the rapid metabolism of amino acids by the ruminal microbes (Matthews et al., 1996); therefore, suggesting no need for amino acid absorption in the forestomach. However, this does not mean that the capability of amino acid absorption does not exist, nor that such a capability has no apparent purpose. Recent research is challenging the assumption that the forestomach does not have the ability to absorb amino acids and small peptides and the present study was conducted to evaluate the absorption and transport of lysine by ruminal and omasal epithelia.

Materials and Methods

Unless otherwise noted, all chemicals, substrates, and reagents used in the preparation of buffers were purchased from Sigma Chemical (St. Louis, MO). Standard protocols were used in the care of animals. Collection of tissues and measurement of lysine uptake were previously described (Matthews and Webb, 1995).

Uptake Measurements

Ruminal and omasal tissues were collected on separate days from six Dorset x Suffolk x Finn wethers (avg. BW = 58.5 kg) and mounted in parabiotic chambers to determine the uptake of lysine by ovine ruminal and omasal epithelia. For uptake measurements, the mucosal chamber was loaded with Krebs Ringer Phosphate (KRP) buffer (pH 6.0) containing appropriate concentrations of ^{3}H-*L*-Lysine. Serosal chambers were loaded with KRP buffer (pH 7.4) containing 10 m*M* glucose and enough mannitol to equalize osmolarity across chambers. Twelve parabiotic units, six for ruminal and six for omasal epithelium, were prepared simultaneously for each lamb. The mucosal chamber of each parabiotic unit was loaded with KRP buffer (pH 6.0) containing ^{3}H-*L*-Lysine (New England Nuclear, Wilmington, DE) and enough *L*-lysine to make final concentrations of 0, .09375, .1875, .375, .75, 1.5, and 3.0 m*M*, with an average specific activity of 0, 24, 48.4, 96.9, 193.7, 387.4, and 774.8 dpm/nmol, respectively. Samples were collected from both chambers of the parabiotic units after 5, 10, 15, 30, 45, and 60 min of incubation. Sample aliquots were prepared and the ^{3}H content quantified. The ^{3}H activity associated with

the epithelial tissue at the end of the incubation was determined following digestion. Uptake was expressed as nmol/mg dry tissue.

Statistical Analysis

The effects of animal, concentration, tissue, and their interactions on the appearance of lysine in the serosal buffer and in the tissue were analyzed using the general linear model of SAS (1988). The repeated measures option of SAS was used to determine the effect of time, because of the repeated sampling of each parabiotic unit. Orthogonal polynomial contrasts were used to partition the effects of concentration and time.

Results and Discussion

During the 60-min incubation period, the appearance of lysine in the serosal buffer increased linearly ($P < .001$), although more ($P < .001$) lysine appeared in omasal than in ruminal serosal buffers (Figure 3.1). The tissues responded differently ($P < .001$) to increases in the mucosal buffer concentrations of lysine (Figure 3.2). As the mucosal buffer concentration of lysine increased from 0 to 3.0 m*M*, the appearance of lysine in the serosal buffer with ruminal tissue increased proportionally. However, with omasal tissue, the appearance of lysine in the serosal buffer increased proportionally to a substrate concentration of 1.5 m*M* then plateaued. The effect of time and mucosal substrate concentrations on serosal appearance of lysine resulted in different responses from the ruminal and omasal epithelia, establishing the two tissues as different from one another.

Both tissues appear to have the ability to transfer and absorb lysine; the ability to do so being greater in omasal epithelium. Methionine appearance in serosal buffers was also greater with omasal tissue than ruminal tissue in a similar study (Matthews and Webb, 1995), suggesting that the omasum has a greater capacity for amino acid absorption than the rumen.

After 60-min of incubation, the accumulation of lysine in the ruminal epithelium was greater ($P < 001$) than the accumulation in the omasal epithelium (Figure 3.3). Similarly, when methionine accumulation in ruminal and omasal tissue was quantified, methionine was accumulated more by ruminal than by omasal tissue (Matthews and Webb, 1995). For both tissues, the accumulation of lysine increased linearly ($P < .01$) as mucosal buffer substrate concentration increased. This suggests that both tissues have the ability to transport lysine.

To compare the total accumulation and translocation capacities of ruminal and omasal tissues, the mean quantity of lysine appearance in serosal buffers after 60 min was added to the mean quantity of lysine accumulation in tissues. The resulting values represent total absorption of lysine for each tissue. Total absorption of lysine after 60 min of incubation increased in both tissues as the lysine concentration in the mucosal buffer increased. Total absorption was greater ($P < .001$) for omasal than ruminal epithelium (Figure 3.4). For ruminal tissue, total absorption increased linearly; however, for omasal tissue, absorption increased linearly ($P < .001$) up to 1.5 m*M* of mucosal

lysine then plateaued. In total, the omasum seems to have a greater ability to transfer and absorb lysine. The linear increase in the serosal appearance of lysine by the ruminal tissue suggests that the pathway of lysine transport was likely not mediated. That saturation of the transport capability of lysine occurred in the omasal epithelium suggests the presence of a carrier-mediated mechanism capable of lysine transport in the omasal epithelium. This is further supported by a study in which oocytes injected with poly $(A)^+$ RNA from ovine omasal epithelium were observed to display a greater ability to absorb lysine than did water-injected oocytes. The induced uptake of lysine was approximately 7.8 times that of water-injected oocytes (Matthews, unpublished data). This observation provides evidence that the ovine omasal epithelia has the genetic coding for a transport mechanism specific for lysine. Additional evidence from the current study supports this idea and suggests that this transport mechanism can be saturated.

The results obtained in the present study and from earlier work in this laboratory (Matthews, unpublished data), provide the first available evidence for a mediated transport mechanism for an amino acid in the forestomach, although several amino acid transporters have been reported to exist in intestinal enterocytes (Atisook and Madara, 1991). The significance of a mediated lysine transport system in the omasum has not yet been determined. Generally, it is assumed that lysine concentrations in the forestomach digesta are low and, therefore, the potential for free lysine to be absorbed is minimal (Matthews et al., 1996a). The principal function of the mediated transport system may

be to insure the functionality of the epithelium or it may provide an initializing mechanism for other nutrient absorptive pathways. This idea is based on research that may suggest that a mediated transport mechanism is necessary to expand the tight junctions of epithelial cells so that solutes from the digesta can be absorbed paracellularly (McCollum and Webb, 1997).

In conclusion, the present study confirms the ability of the ovine rumen and omasum to transport and absorb lysine. These results combined with the observation of methionine absorption by the same epithelia, provides evidence for amino acid absorption in the ovine forestomach. Because lysine absorption can be saturated at a concentration of 1.5 mM suggests that a mechanism for mediated transport exists in the omasum capable of transporting lysine. The present study, along with the study by Matthews (unpublished data), provide *in vitro* and molecular evidence for saturable lysine absorption in the omasum. While the significance for such a mechanism is yet to be determined, the present study provides the first evidence for a saturable amino acid transport system.

Implications

A saturable, mediated transport mechanism for amino acids in the ruminant forestomach has yet to be characterized. To define a system of this type would present the first available evidence for amino acid absorption by the forestomach epithelia and the

beginning of the characterization of amino acid transport systems in the ruminant animal. While the significance of such a discovery has yet to be determined, the existence of the system alone is of major importance to the study of protein transport and absorption.

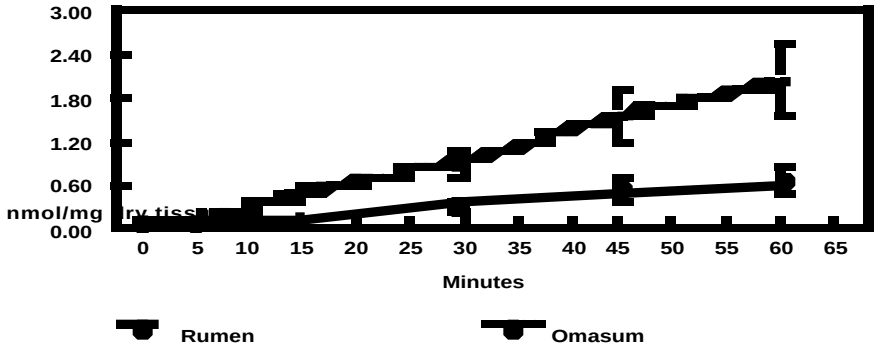

Figure 3.1. Effect of length of incubation on appearance of lysine in serosal buffer. Samples were collected at 0, 5, 10, 15, 30, 45, and 60 min of incubation. The appearance of lysine in serosal buffers increased linearly ($P < .001$) with time for both tissues. Each data point represents the mean of 36 observations (six animals times six substrate concentrations).

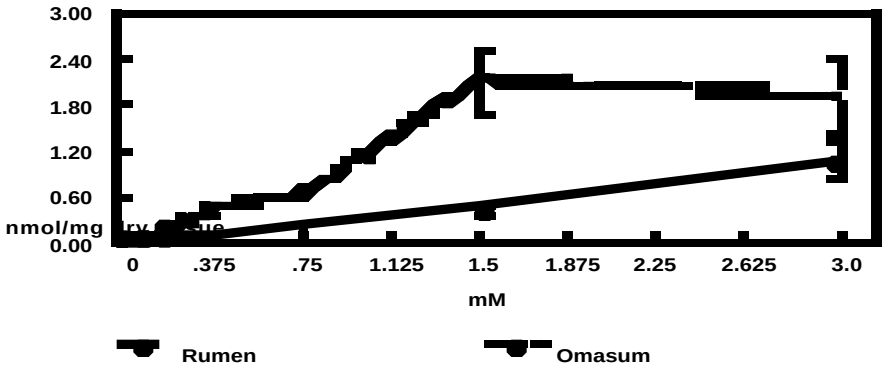

Figure 3.2. Effect of substrate concentration on appearance of lysine in serosal buffers. The tissues responded differently ($P < .001$) to increases in the mucosal buffer concentrations (0, .09375, .1875, .375, .75, 1.5, and 3.0 m*M*) of lysine. Each data point represents the mean of 36 observations (six animals times six time increments).

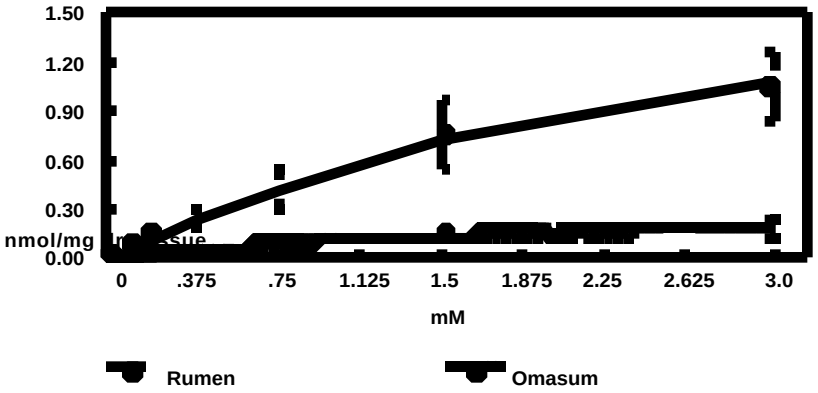

Figure 3.3. ^{3}H associated with tissues after 60 min of incubation. After 60-min of incubation, the accumulation of lysine in the ruminal epithelium was greater ($P < .001$) than the accumulation in the omasal epithelium. For both tissues, the accumulation of lysine increased linearly ($P < .011$) as mucosal substrate concentrations were increased. Each data point represents the mean of 6 observations (six animals).

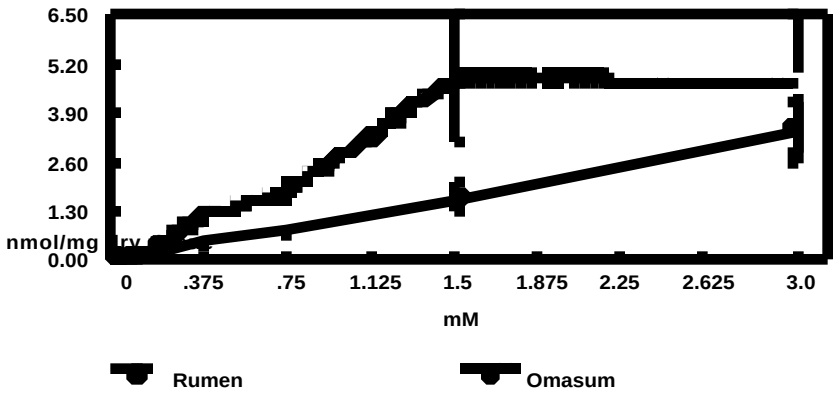

Figure 3.4. Total absorption of lysine after 60 min of incubation. Total absorption is the appearance of lysine in serosal buffers plus lysine accumulation in the tissue after 60-min of incubation. For ruminal tissue, total absorption increased linearly; however, for omasal tissue, absorption increased linearly up to 1.5 m*M* ($P < .001$) then plateaued. Each data point represents the mean of 6 observations (six animals).

Chapter IV

Glycyl-*L*-Sarcosine Absorption Across Ovine Omasal Epithelium During Co-Incubation with Other Peptide Substrates and Volatile Fatty Acids

ABSTRACT

To define the interactions between the absorption of glycyl-*L*-sarcosine (Gly-Sar; .1m*M*) and glycine, *L*-methionylglycine, glycyl-*L*-leucine, *L*-carnosine, or *L*-methionylglycyl-*L*-methionyl-*L*-methionine (each at 5 m*M*), ovine omasal epithelium was collected from eight wethers (avg. BW = 69 kg) and mounted in parabiotic chambers. [1,2]-[^{14}C]Glycyl-*L*-sarcosine was used as a marker to monitor the presence of Gly-Sar. Accumulation of Gly-Sar in the omasal epithelium was greatest ($P < .05$) when only Gly-Sar was present. Glycine inhibited ($P < .05$) Gly-Sar accumulation by 20%, while peptide substrates inhibited ($P < .05$) Gly-Sar accumulation by 60 to 85%. The appearance of Gly-Sar in serosal buffers increased linearly ($P < .001$) during the 60-min incubation. There was a tendency for Gly-Sar appearance in serosal buffers to be stimulated by the presence of other substrates . In a second experiment, ovine omasal epithelium was collected from four lambs (avg. BW = 47 kg) to determine the interactions between the absorption of Gly-Sar (.1m*M*) alone or co-incubated with either 10 m*M* butyric acid, or a mixture of VFA (50 m*M* acetic acid, 40 m*M* propionic acid, and 10 m*M* butyric acid). Accumulation of Gly-Sar in the omasal epithelia was greatest ($P < .05$) when only Gly-Sar was present; butyric acid inhibited ($P < .05$) Gly-Sar accumulation by

50%, while the VFA mixture inhibited ($P < .05$) Gly-Sar accumulation by 84%. These results support the idea that peptides can be absorbed across omasal epithelium and the mechanism involves mediated as well as possibly paracellular transport.

Key Words: Omasum, Paracellular, Peptide, VFA, Nutrient Transport, Absorption

Introduction

The investigation of transport mechanisms in the ruminant forestomach has begun to uncover clues suggesting that small peptides may be absorbed from this portion of the gastrointestinal tract. It is accepted that the microbial population of the rumen metabolizes protein into smaller peptides (Annison, 1956; Broderick and Wallace, 1988), and the concentration of these peptides in ruminal digesta is high enough to support them being absorbed (Matthews et al., 1996a). While VFA, electrolytes, and water are absorbed by the forestomach epithelia (Van Soest, 1987), it is still unclear to what extent protein components may be absorbed by these epithelia. A large net flux of peptide-bound amino acids was observed across the nonmesenteric-drained viscera of calves (DiRienzo, 1990). This net flux of peptide-bound amino acids potentially originated from the forestomach and abomasum. Ruminal and omasal epithelia have the ability to absorb dipeptides and methionine (Matthews and Webb, 1995). The omasal epithelium has also been shown to possess mRNA that encodes for a protein(s) capable of mediating the transport of glycyl-*L*-sarcosine (Gly-Sar; Matthews et al., 1996b). Because a protein capable of transporting Gly-Sar may also transport other peptides, the present study was conducted to assess the possible interaction between Gly-Sar transport by omasal epithelium and other substrates.

Materials and Methods

Unless otherwise noted, all chemicals, substrates, and reagents used in the preparation of buffers were purchased from Sigma Chemical Company (St. Louis, MO). Standard protocols were used in the care of animals. Collection of tissues and measurement of dipeptide uptake were described previously (Matthews and Webb, 1995).

Uptake Measurements

Glycyl-L-Sarcosine Co-incubation with Peptide Substrates. Omasal tissue was collected on separate days from eight Dorset x Suffolk x Finn lambs (avg. BW = 68.9 kg) and mounted in parabiotic chambers to determine the uptake of Gly-Sar, a dipeptide highly resistant to hydrolysis, by ovine omasal epithelium in the presence of other peptides. For uptake measurements, the mucosal chamber was loaded with Krebs Ringer Phosphate (KRP) buffer (pH 6.0), Gly-Sar, and one of the following substrates: *L*-glycine (Gly), *L*-methionylglycine (Met-Gly), glycyl-*L*-leucine (Gly-Leu), *L*-carnosine (Car), or *L*-methionylglycyl-*L*-methionyl-*L*-methionine (Met-Gly-Met-Met). Serosal chambers were loaded with KRP buffer (pH 7.4) containing 10 m*M* glucose and enough mannitol to equalize osmolarity across chambers. Twelve parabiotic units loaded with omasal epithelium were prepared simultaneously for each lamb. The mucosal side of duplicate chambers (one in each of two water baths) was loaded with KRP buffer (pH

6.0) containing [1,2]-[^{14}C]glycyl-*L*-sarcosine (custom synthesized, Moravek Biochemicals, Brea, CA) and enough Gly-Sar to make a final concentration of .1 m*M*, plus one of the other substrates at a concentration of 5 m*M*. The specific activity (avg. 9816 dpm/nmol) in the mucosal chambers was the same within an experiment, but differed among experiments. Samples were collected from both chambers of the parabiotic units after 5, 10, 15, 30, 45, and 60 min of incubation. Sample aliquots were prepared and the ^{14}C content quantified. The ^{14}C activity associated with the epithelial tissue at the end of the incubation was determined following digestion. Uptake of Gly-Sar was expressed as nmol/mg dry tissue.

Glycyl-L-Sarcosine Co-incubation with VFA. Omasal tissue was collected from three Dorset ewe lambs and one Dorset wether (avg. BW = 47 kg) to determine the uptake of Gly-Sar by ovine omasal epithelium in the presence of VFA. Tissue from two lambs were prepared on a given day with six parabiotic units loaded with omasal epithelium from a single animal. For uptake measurements, the mucosal chambers were loaded with KRP buffer (pH 6.0) containing [1,2]-[^{14}C]glycyl-*L*-sarcosine and enough Gly-Sar to make a final concentration of .1 m*M* alone or co-incubated with either 10 m*M* butyric acid, or a mixture of VFA containing 50 m*M* acetic acid, 40 m*M* propionic acid, and 10 m*M* butyric acid. Serosal chambers were loaded with KRP buffer (pH 7.4) containing 10 m*M* glucose and enough mannitol to equalize osmolarity across chambers.

Sampling and quantification of ^{14}C content were as described for Gly-Sar co-incubated with peptide substrates.

Statistical Analysis

The main effects and their interactions on the appearance of Gly-Sar in the serosal buffer and the accumulation of Gly-Sar in the omasal epithelium were analyzed using the general linear model of SAS (1988) for both experimental models. The main effects for Gly-Sar co-incubated with peptides were animal, water bath, substrate, and time. The main effects for Gly-Sar co-incubated with VFA were animal, substrate, and time. The models also included all two-way interactions. Because the data for the appearance of Gly-Sar in the serosal buffer did not pass the test for sphericity (SAS, 1988), the data were analyzed using the mutlivariate analysis procedure of SAS (1988). The distribution of the data was determined to be normal, therefore transformation of the data was not necessary. Differences between the various substrates on the accumulation of Gly-Sar in the tissue were determined using Tukey's multiple comparison test at $\alpha = .05$. The repeated measures option of SAS (1988) was used to determine the effect of time on the above. Orthogonal polynomial contrasts were used to partition the effects of time into linear and quadratic components.

Results and Discussion

The accumulation of Gly-Sar in the omasal epithelium was quantified after 60-min of incubation. Accumulation of Gly-Sar in the tissue was greatest ($P < .05$) when only Gly-Sar was present (Figure 4.1). Glycine inhibited ($P < .05$) Gly-Sar accumulation in the omasal epithelium by about 20%. Peptide substrates, Car, Met-Gly, Met-Gly-Met-Met, and Gly-Leu inhibited ($P < .05$) Gly-Sar accumulation in the tissue by 60, 78, 81, and 85%, respectively. Because the substrates competed with Gly-Sar, as indicated by inhibition, suggests that a mediated transport system is likely present for Gly-Sar and that this mediated system(s) is capable of transporting the other substrates as well. Competition between peptide substrates is expected if they share a common transport system. Competition from free amino acids is not expected because, to date, no transport protein is recognized to exist that is capable of transporting both peptides and amino acids (Webb and Bergman, 1991). In previous work from this laboratory it was shown that absorption of Gly-Sar by *Xenopus laevis* oocytes injected with RNA isolated from ovine omasal epithelium was reduced 44% by Car, 94% by Met-Gly, 91% by Gly-Leu, but not by Gly (Matthews et al., 1996b). It is unclear why Gly inhibited Gly-Sar absorption in the present study. An animal x treatment interaction was observed during analysis of the accumulation of Gly-Sar in the tissue. For six of the eight animals there was little accumulation of Gly-Sar in the omasal epithelium in the presence of the various substrates, with little variation among the observations. For the other two animals, the

presence of Car and Gly in the mucosal buffers resulted in a substantial enhancement of the accumulation of Gly-Sar in the omasal epithelium.

Appearance of Gly-Sar in the serosal buffer was also quantified. The appearance of Gly-Sar in the serosal buffer increased linearly ($P < .001$) during the 60-min incubation (Figure 4.2) indicating that omasal epithelium has the ability for transepithelial passage of Gly-Sar. The appearance of Gly-Sar in the serosal buffer was not affected by the presence of Gly, Met-Gly, Gly-Leu, Car, nor Met-Gly-Met-Met, but there was a tendency for the presence of these substrates to increase absorption of Gly-Sar into the serosal buffer. Numerically, the appearance of Gly-Sar in the serosal buffer was lowest when Gly-Sar was present alone. Also, the variability of Gly-Sar appearance in the serosal buffer was the least when Gly-Sar was present alone throughout the 60-min incubation for all eight animals. In the presence of Gly and the peptide substrates, larger standard errors were associated with the measurement of Gly-Sar appearance in the serosal buffer. This variation certainly contributed to our inability to detect differences among substrates with regard to their influence on Gly-Sar appearance in the serosal buffer.

The ability of Car, Met-Gly, Gly-Leu, and Met-Gly-Met-Met to inhibit the accumulation of Gly-Sar in the epithelial tissue suggests that these peptides are transported by the same mediated transport mechanism(s), however, the inability of these same substrates to decrease (when they apparently increased) the appearance of Gly-Sar

in the serosal buffer conflicts with this hypothesis. Historically held beliefs of protein transport mechanisms suggest that free amino acids and individual, small peptides are transported by different transport proteins that recognize the substrates based on size and charge. There is minimal reason to expect much positive influence of one substrate on the absorption of the other (Webb and Bergman, 1991).

In the present and in a previous study (Matthews and Webb, 1995), a substantial amount of peptide absorption by omasal epithelium appeared to be occurring other than directly by a transport protein. How, then, can we explain the possible positive effect Gly and the peptide substrates had on Gly-Sar appearance in the serosal buffer? One possible explanation might be offered if the potential for paracellular transport is considered (Madara and Pappenheimer, 1987). They observed that, in the presence of glucose, dilatations between absorptive cells of hamster small intestinal epithelia occurred. These dilatations were observed between the cells at the site of the tight junctions, a portion of the cytoskeleton of epithelial cells. After perfusion with 25 m*M* glucose, dilatations were measurable from < .1 to .5 μm in width. Because the transport of glucose is known to be a Na^+-coupled process, they suggested that the function of Na^+-coupled solute transport is to increase permeability of tight junctions with a concomitant expansion of lateral spaces providing optimal conditions for transport of luminal nutrients in bulk by solvent drag through these paracellular channels. They arrived at their hypothesis because Na^+-dependent transport of glucose is saturated at 10 to 20 m*M*, but

after a meal the concentration of glucose in the duodenum is closer to 100 to 300 m*M* (Madara and Pappenheimer, 1987). At these high concentrations, the greater proportion of absorption of hydrophilic nutrients is by solvent drag and may include some peptides. This mechanism of nutrient absorption is called paracellular absorption. Glucose elicited dilatations of the tight junctions provided for the permeation of the intercellular space by an eleven amino acid hemepeptide tracer (1,900 Da), but not a heme-conjugated macromolecule (40,000 Da; Atisook and Madara, 1991). They postulate that turnover of the Na^+-coupled glucose cotransporter initiated the dilatations of the tight junctions and increased their permeability, therefore promoting paracellular absorption of nutrients. When glucose was not present, the smaller peptide was not observed penetrating the tight junctions (Atisook and Madara, 1991). When a Pz-peptide (4-phenylazobenzyloxcarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg) was absorbed through the paracellular pathway, Yen and Lee (1995) demonstrated that Na^+ was involved in the paracellular transport. They proposed that the peptide facilitated its own paracellular transport by stimulating Na^+ entry into the cells which increased water flux into the intercellular space and created solvent drag. Peptides are transported on H^+-cotransporters, however, a H^+/Na^+ exchanger is responsible for removing the H^+ from the cell, so that transcellular peptide absorption ultimately results in Na^+ entry into the cell. Based on these studies, it has been suggested that the mediated transport of a nutrient is

necessary to initiate the expansion of the tight junctions which makes the paracellular pathway available for absorption of nutrients.

It can be argued that the components, mediated transport of the stimulating substrate and a high substrate gradient, necessary for paracellular transport (as proposed by Madara and Pappenheimer, 1987, and Atisook and Madara, 1991) are present in our examination of peptide transport in the omasal epithelium. First, research from this laboratory has confirmed that mediated absorption of Gly-Sar occurs in the omasal epithelium. *Xenopus laevis* oocytes injected with poly(A)$^+$RNA isolated from the omasal epithelium of sheep had a greater ability to absorb Gly-Sar than did water-injected oocytes (Matthews et al., 1996b). In the present study, the effects of competing substrates on Gly-Sar accumulation in the omasal tissue also support the idea that mediated transport of peptides occurs in the omasum. Because there is mediated transport of peptides in the omasal epithelium, this may provide the initializing mechanism for the paracellular movement of Gly-Sar and other substrates.

This same rationale may explain why, in the present study, Gly appeared to stimulate the transport of Gly-Sar into the serosal buffer. Data have been accumulated from mRNA expression studies and a study using parabiotic chambers indicating that mediated transport of lysine occurs in ovine omasal epithelium (Matthews and McCollum, unpublished data). Amino acids, when present in high concentrations, may stimulate the opening of tight junctions in omasal epithelium, thus permitting paracellular

absorption of substrates including peptides. Practically, however, it is not likely that free amino acids play a large role in this regard because their concentrations in forestomach contents are quite low (Matthews et al., 1996a).

In the present study the appearance of Gly-Sar in the serosal buffer, when incubated alone, was associated with low variability (S.E. = .006 nmol/mg dry tissue) when compared with the variability of the values for Gly-Sar incubated with the other substrates (S.E. = .02 to .05 nmol/mg dry tissue). If paracellular absorption is occurring, the expansion of the tight junctions may not be as uniform a process as is transport via a protein. It is also critical to note that studies of the Na^+ flux rates from mucosal to serosal sides of ruminal and omasal epithelium suggest that the omasum has more leaky tight junctions compared to the tight junctions of the rumen and reticulum, which themselves are moderately "tight" when compared to other epithelial tight junctions (Gabel et al., 1993).

Because VFA absorption is so extensive from the forestomach, it was decided to examine the effect of these substrates on Gly-Sar transport. For many years, absorption of VFA was assumed to be by diffusion (Dougherty et al., 1965), but more current research has suggested that H^+-dependent, carrier-mediated processes may result in the absorption of VFA and other monocarboxylic acids (Takanaga et al., 1994; Tsuji et al., 1994). Knowing how these substrates are absorbed may lend support to the proposal of paracellular absorption. Butyric acid was examined alone because of its known extensive

metabolism by the epithelial tissue (Fell and Weekes, 1975). Accumulation of Gly-Sar in the omasal epithelial tissue was greatest ($P < .05$) when Gly-Sar was present without any of the VFA (Figure 4.3). Butyric acid inhibited ($P < .05$) Gly-Sar accumulation in the omasal epithelium by 50%, while VFA inhibited ($P < .05$) Gly-Sar accumulation by 84%. The appearance of Gly-Sar in the serosal buffer increased linearly ($P < .001$) during the 60-min incubation (Figure 4.4). The appearance of Gly-Sar in the serosal buffer was not affected by butyric acid alone nor by the presence of the VFA mixture. Using a similar parabiotic system as was used in the present study, Tempelmann (1993) was able to demonstrate that the addition of short-chain fatty acids decreased the paracellular permeability of mannitol across the abomasal epithelium. Therefore, butyric acid and VFA may be serving to attenuate paracellular transport, while amino acids and peptides are stimulating this transport process.

While the quantitative significance of peptide absorption from the ruminant stomach remains to be clearly established, it now appears certain that mechanisms for peptide absorption are present in the ruminant omasal epithelium. Evidence to support the presence of mediated transport as well as the non-mediated processes of simple diffusion and paracellular transport is accumulating. The relative importance of each of these mechanisms for peptide absorption is yet to be determined.

Implications

The suggestion that paracellular absorption of peptides is responsible for a portion of the absorption of the nitrogenous end products of protein digestion in the forestomach of the ruminant is new. If paracellular absorption is proven to be a contributing factor to the absorption of peptides by the ruminant forestomach, it will be necessary to learn what impact this process has on the overall absorption and utilization of protein digestion products.

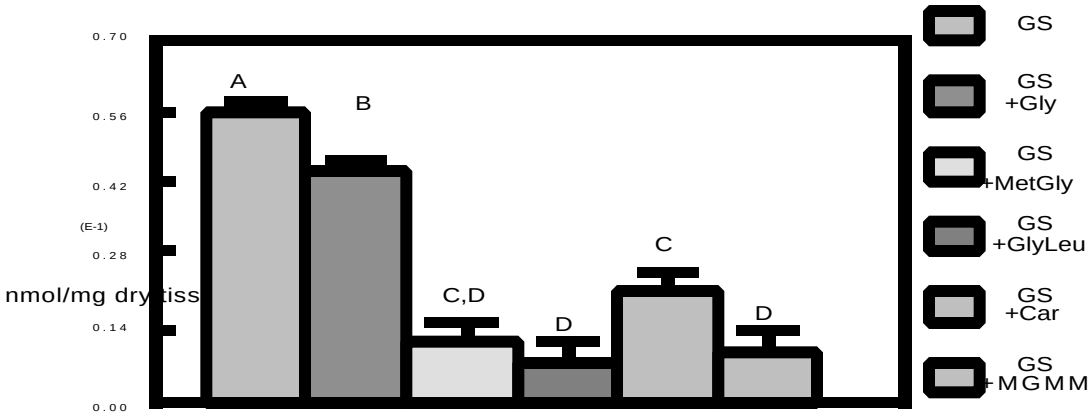

Figure 4.1. Accumulation of Gly-Sar in ovine omasal epithelium during co-incubation with Gly, Met-Gly, Gly-Leu, Car, and Met-Gly-Met-Met. Gly-Sar was present at a concentration of .1 m*M* during co-incubation with one of the other substrates (5 m*M*). Bars lacking a common letter differ ($P < .05$). GS = Gly-Sar, MGMM = Met-Gly-Met-Met. Each bar represents 16 observations (eight animals times duplicates).

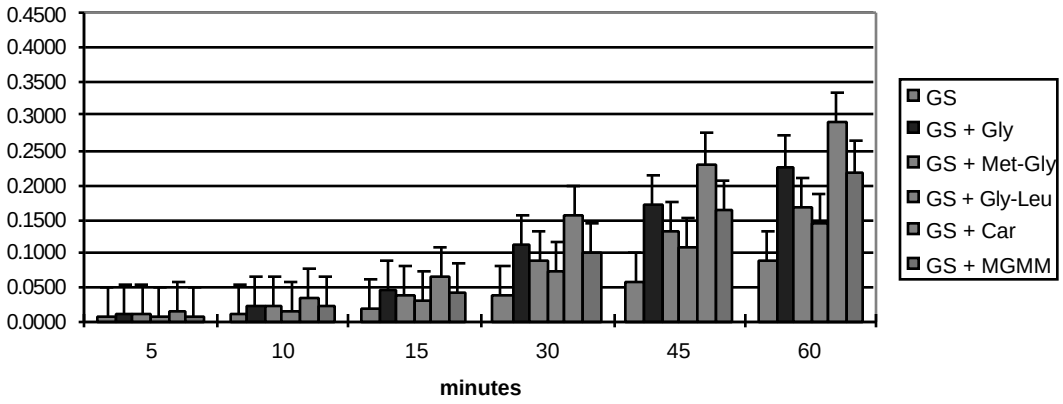

Figure 4.2. Appearance of Gly-Sar in serosal buffers during co-incubation with Gly, Met-Gly, Gly-Leu, Car, and Met-Gly-Met-Met. Gly-Sar was present at a concentration of .1 m*M* during co-incubation with one of the other substrates (5 m*M*). GS = Gly-Sar, MGMM = Met-Gly-Met-Met. Each bar represents 16 observations (eight animals times duplicates).

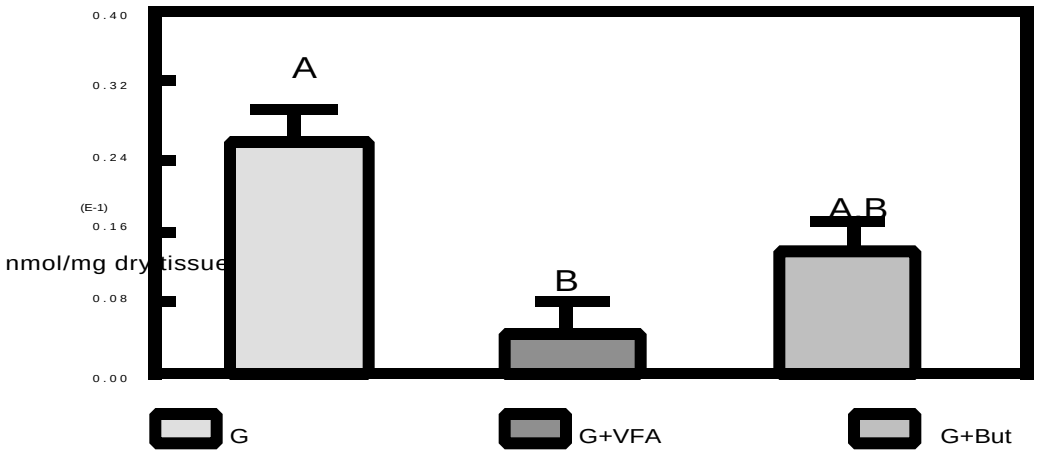

Figure 4.3. Accumulation of Gly-Sar in ovine omasal epithelium either alone (.1 m*M*), or co-incubated with either butyric acid (10 m*M*) or a mixture of VFA (50 m*M* acetic acid, 40 m*M* propionic acid, and 10 m*M* butyric acid). Bars lacking a common letter differ ($P < .05$). G = Gly-Sar, But = Butyric acid. Each bar represents 8 observations (four animals times duplicates).

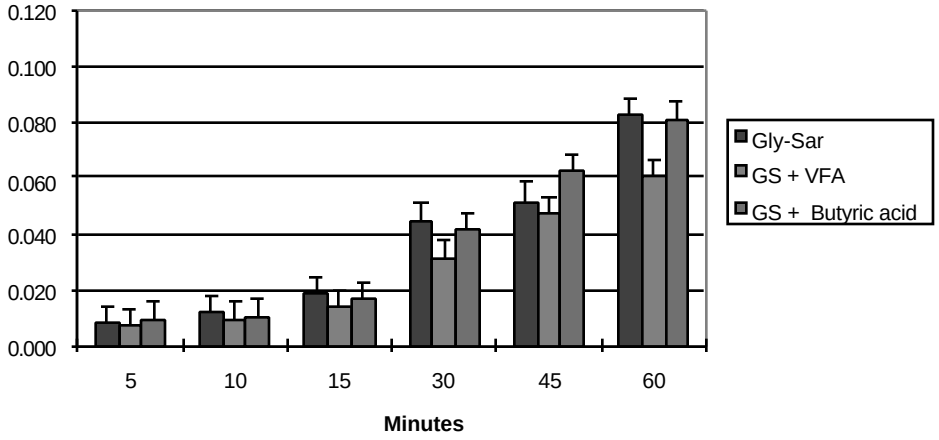

Figure 4.4. Appearance of Gly-Sar in serosal buffers either alone (.1 m*M*), or co-incubated with either butyric acid (10 m*M*) or a mixture of VFA (50 m*M* acetic acid, 40 m*M* propionic acid, and 10 m*M* butyric acid). G = Gly-Sar, But = Butyric acid. Each bar represents 8 observations (four animals times duplicates).

Chapter V

EPILOGUE

The two major contributions associated with the research reported in this thesis are the discovery of the presence of a saturable transport system in omasal epithelium specific for lysine absorption (Chapter III) and the accumulation of evidence suggesting that paracellular absorption may be an important mechanism for peptide absorption across omasal epithelium (Chapter VI).

One way to investigate further whether paracellular absorption is indeed occurring in the omasum would be to perform serosa to mucosa absorption studies. If paracellular absorption occurs simply as the result of an opening between cells that allows for nutrient movement, either radiolabled or fluorescent markers could be used to measure the bidirectionality of this process. Serosal to mucosal fluxes of mannitol, an extracellular marker, were measured in the presence of cytochalasin D, a cytoskeleton disruptor (Madara, 1996). Introduction of a substrate, known to be transported through a mediated process, on the mucosal side, but measuring the movement of a marker in the serosa to mucosa direction could provide further evidence that the substrate results in the expansion of the tight junction. This measurement could also indicate whether the movement can occur in the opposite direction.

While many questions remain as to the exact mechanisms involved in the absorption of amino acids and peptides from the ruminant forestomach, one thing remains clear - the existence of multiple mechanisms, which offer a vast array of absorptive opportunities within the forestomach. These opportunities may discriminate on the basis of molecular size or charge, or on the basis of energetics, but they allow for the needs of the animal to be met and it is up to science to more clearly understand how that occurs on a daily basis.

A greater understanding of paracellular absorption needs to be available in order for manipulation of this absorptive process to occur and in order to more efficiently provide nutrients to the animal.

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Appendix A

Statistical Examples

Example A.1. ANALYSIS OF VARIANCE OF LYSINE APPERANCE IN SEROSAL BUFFERS AFTER NATURAL LOG TRANSFORMATION

General Linear Model Procedure

Dependent Variable: Transformed data of lysine (nmol/mg dry tissue)

Source	DF	Sum of Squares	Mean Squares	F value	PR > F
Model	131	1931.606	14.745	29.27	0.0001
Error	300	151.152	0.504		
Corrected Total	431	2082.758			

r^2	C.V.	Root MSE	Transformed Mean
0.927	-31.179	0.710	-2.277

Source	DF	Type III SS	Mean Square	F value	PR > F
Animal	5	221.920	44.384	88.09	0.0001
Tissue	1	242.565	242.565	481.43	0.0001
Concentration	5	708.256	141.651	281.14	0.0001
Animal*Tissue	5	40.650	8.130	16.14	0.0001
Animal*Conc	25	77.839	3.114	6.18	0.0001
Tissue*Conc	5	16.158	3.231	6.41	0.0001
Animal*Tissue*Conc	25	55.263	2.211	4.39	0.0001
Time	5	541.267	108.253	214.86	0.0001
Animal*Time	25	11.474	0.459	0.91	0.5904
Tissue*Time	5	4.615	0.923	1.83	0.1064
Time*Conc	25	11.599	0.464	0.92	0.5764

Contrast	DF	Contrast SS	Mean Square	F value	PR > F
Linear	1	0.358	0.358	0.04	0.8420
Quadratic	1	180.908	180.908	22.25	0.0053

Example A.2. REPEATED MEASURES ANALYSIS OF VARIANCE OF TIME ON LYSINE APPEARANCE IN SEROSAL BUFFERS AFTER NATURAL LOG TRANSFORMATION

Univariate tests of hypotheses for within subject effects.

Source	DF	Type III SS	Mean Square	F Value	PR > F	Adj G - G	PR > F H - F
Time	5	540.976	108.195	244.94	0.0001	0.0001	0.0001
Error (Time)	125	55.215	0.442				

Greenhouse - Geisser Epsilon = 0.4578
Huynh - Feldt Epsilon = 1.4339

Example A.3. ANALYSIS OF VARIANCE OF LYSINE ACCUMULATED IN RUMINAL OR OMASAL TISSUE AFTER NATURAL LOG TRANSFORMATION

General Linear Model Procedure

Dependent Variable: Transformed data of Lysine (nmol/mg dry tissue)

Source	DF	Sum of Squares	Mean Squares	F value	PR > F
Model	46	170.244	3.701	15.26	0.0001
Error	25	6.061	0.242		
Corrected Total	71	176.305			

r^2	C.V.	Root MSE	Transformed Mean
0.966	-21.265	0.492	-2.316

Source	DF	Type III SS	Mean Square	F value	PR > F
Animal	5	19.806	3.961	16.34	0.0001
Tissue	1	55.311	55.311	228.13	0.0001
Concentration	5	77.251	15.450	63.72	0.0001
Animal*Tissue	5	5.903	1.181	4.87	0.0030
Animal*Conc	25	10.816	0.433	1.78	0.0773
Tissue*Conc	5	1.157	0.231	0.95	0.4641

Contrast	DF	Contrast SS	Mean Square	F value	PR > F
Linear Conc	1	0.028	0.028	0.02	0.8843
Quadratic	1	18.382	18.382	15.57	0.0109

Example A.4. REPEATED MEASURES ANALYSIS OF VARIANCE OF GLYCYLSARCOSINE APPEARANCE IN SEROSAL BUFFERS DURING CO-INCUBATION WITH GLYCINE AND PEPTIDE SUBSTRATES

General Linear Model Procedure

Test for Sphericity: Mauchly's Criterion = 8.847E-10
Chisquare Approximation = 1523.8296 with 14 df
Prob > Chisquare 0.0000

Manova Test Criteria and Exact F Statistics for the Hypothesis of no TIME Effect
H = Type III SS&CP Matrix for TIME E = Error SS&CP Matrix
S=1 M=1.5 N=34.5

Statistic	Value	F	Num DF	Den DF	PR>F
Wilks' Lambda	0.60525302	9.2613	5	71	0.0001
Pillai's Trace	0.39474698	9.2613	5	71	0.0001
Hotelling-Lawley Trace	0.65220158	9.2613	5	71	0.0001
Roy's Greatest Root	0.65220158	9.2613	5	71	0.0001

Manova Test Criteria and F Approximations for the Hypothesis of no TIME*TRT Effect
H = Type III SS&CP Matrix for TIME*TRT E = Error SS&CP Matrix
S=5 M=0.5 N=34.5

Statistic	Value	F	Num DF	Den DF	PR>F
Wilks' Lambda	0.81769293	0.5907	25	265.2552	0.9417
Pillai's Trace	0.19256410	0.6008	25	375	0.93721
Hotelling-Lawley Trace	0.21061865	0.5847	25	347	0.9462
Roy's Greatest Root	0.12859560	1.9289	5	75	0.0995

Note: F Statistic for Roy's Greatest Root is an upper bound.

Tests of Hypotheses for Between Subjects Effects

Source	DF	Type III SS	Mean Square	F value	PR > F
Block	15	2.27257718	0.15150515	1.66	0.0780
Treatment	5	0.49806872	0.09961374	1.09	0.3719
Error	75	6.84087964	0.09121173		

Example A.5. REPEATED MEASURES ANALYSIS OF VARIANCE FOR EFFECT OF TIME ON GLYCYLSARCOSINE APPEARANCE IN SEROSAL BUFFERS DURING CO-INCUBATION WITH GLYCINE AND PEPTIDE SUBSTRATES

Repeated Measures Procedure

Univariate tests of hypotheses for within subject effects.

Source	DF	Sum of Squares	Mean Square	F Value	PR > F	Adjusted PR > F G-G[1]	Adjusted PR > F H-F[2]
Time	5	2.547	0.509	40.46	0.0001	0.0001	0.0001
Time*Animal	35	0.918	0.026	2.08	0.005	0.0525	0.0362
Time*Water bath(Animal)	40	0.691	0.017	1.37	0.0714	0.2191	0.1999
Time*Treatment	25	0.323	0.013	1.03	0.4313	0.4100	0.4163
Error	375	4.722	0.013				

[1] Greenhouse-Geisser Epsilon = 0.2090
[2] Huynh-Feldt Epsilon = 0.2658

Contrast	DF	Sum of Squares	Mean Square	F Value	PR > F
Linear	1	2.543	2.543	123.13	0.0001
Quadratic	1	0.002	0.002	0.09	0.7594

Example A.6. ANALYSIS OF VARIANCE OF GLYCYLSARCOSINE ACCUMLATED IN OMASAL TISSUE DURING CO-INCUBATION WITH GLYCINE AND PEPTIDE SUBSTRATES

General Linear Model Procedure

Dependent Variable: Glycylsarcosine (nmol/mg dry tissue)

Source	DF	Sum of Squares	Mean Squares	F value	PR > F
Model	47	0.074	0.002	16.44	0.0001
Error	48	0.005	0.0001		
Corrected Total	95	0.079			

r^2	C.V.	Root MSE	Y Mean
0.941	38.641	0.010	0.025

Source	DF	Type I SS	Mean Square	F value	PR > F
Animal	7	0.020	0.003	29.54	0.0001
Treatment	5	0.031	0.006	64.56	0.0001
Animal*Treatment	35	0.023	0.0007	6.94	0.0001

Example A.7. ANALYSIS OF VARIANCE OF GLYCYLSARCOSINE ACCUMULATION IN OMASAL TISSUE DURING CO-INCUBATION WITH GLYCINE AND PEPTIDE SUBSTRATES

General Linear Model Procedure

Tukey's studentized test for variable: Glycylsarcosine (nmol/mg dry tissue)

Alpha = 0.05, DF = 48, Mean Square Error = 0.000096
Critical value of studentized range = 4.197
Minimum significant difference = 0.0103

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	0.0550	16	1
B	0.0445	16	2
C	0.0218	16	5
C,D	0.0123	16	3
D	0.0104	16	6
D	0.0084	16	4

Example A.8. REPEATED MEASURES ANALYSIS OF VARIANCE OF GLYCYLSARCOSINE APPEARANCE IN SEROSAL BUFFERS DURING CO-INCUBATION EITHER ALONE OR DURING CO-INCUBATION WITH BUTYRIC ACID OR A MIXTURE OF VFA

General Linear Model Procedure

Test for Sphericity: Mauchly's Criterion = 2.9705E-7
Chisquare Approximation = 106.7085 with 14 df
Prob > Chisquare 0.0000

Manova Test Criteria and Exact F Statistics for the Hypothesis of no TIME Effect
H = Type III SS&CP Matrix for TIME E = Error SS&CP Matrix
S=1 M=1.5 N=1.5

Statistic	Value	F	Num DF	Den DF	PR>F
Wilks' Lambda	0.06112260	15.3606	5	5	0.0047
Pillai's Trace	0.93887740	15.3606	5	5	0.0047
Hotelling-Lawley Trace	15.36055928	15.3606	5	5	0.0047
Roy's Greatest Root	15.36055928	15.3606	5	5	0.0047

Manova Test Criteria and F Approximations for the Hypothesis of no TIME*TRT Effect
H = Type III SS&CP Matrix for TIME*TRT E = Error SS&CP Matrix
S=2 M=1 N=1.5

Statistic	Value	F	Num DF	Den DF	PR>F
Wilks' Lambda	0.18794378	1.3067	10	10	0.3402
Pillai's Trace	1.07361793	1.3907	10	12	0.2903
Hotelling-Lawley Trace	2.92903832	1.1716	10	8	0.4193
Roy's Greatest Root	2.33234086	2.7988	5	6	0.1212

Note: F Statistic for Roy's Greatest Root is an upper bound.
Note: F Statistic for Wilks' Lambda is exact.

Tests of Hypotheses for Between Subjects Effects

Source	DF	Type III SS	Mean Square	F value	PR > F
Treatment	2	0.00112272	0.00056136	0.32	0. 7332
Error	9	0.01572424	0.00174714		

Example A.9. REPEATED MEASURES ANALYSIS OF VARIANCE FOR EFFECT OF TIME ON GLYCYLSARCOSINE APPEARANCE IN SEROSAL BUFFERS EITHER ALONE OR DURING CO-INCUBATION WITH BUTYRIC ACID OR A MIXTURE OF VFA

Repeated Measures Procedure

Univariate tests of hypotheses for within subject effects.

Source	DF	Sum of Squares	Mean Square	F Value	PR > F	Adjusted PR > F G-G[1]	Adjusted PR > F H-F[2]
Time	5	0.043	0.009	44.31	0.0001	0.0001	0.0001
Time*Treatment	10	0.001	0.0001	0.52	0.8678	0.6329	0.6753
Error	45	0.009	0.0002				

[1] Greenhouse-Geisser Epsilon = 0.2285
[2] Huynh-Feldt Epsilon = 0.2980

Contrast	DF	Sum of Squares	Mean Square	F Value	PR > F
Linear	1	0.043	0.043	221.79	0.0001
Quadratic	1	0.000002	0.000002	0.01	0.9218

Example A.10. ANALYSIS OF VARIANCE OF GLYCYLSARCOSINE ACCUMLATED IN OMASAL TISSUE EITHER ALONE OR DURING CO-INCUBATION WITH BUTYRIC ACID OR A MIXTURE OF VFA

General Linear Model Procedure

Dependent Variable: Glycylsarcosine (nmol/mg dry tissue)

Source	DF	Sum of Squares	Mean Squares	F value	PR > F
Model	5	0.001	0.0002	5.28	0.0334
Error	6	0.0002	0.00004		
Corrected Total	11	0.001			

r^2	C.V.	Root MSE	Y Mean
0.815	43.740	0.006	0.014

Source	DF	Type I SS	Mean Square	F value	PR > F
Animal	3	0.00009	0.00003	0.76	0.5538
Treatment	2	0.001	0.0005	12.04	0.0079

Example A.7. ANALYSIS OF VARIANCE OF GLYCYLSARCOSINE ACCUMULATION IN OMASAL TISSUE EITHER ALONE OR DURING CO-INCUBATION WITH BUTYRIC ACID OR A MIXTURE OF VFA

General Linear Model Procedure

Tukey's studentized test for variable: Glycylsarcosine (nmol/mg dry tissue)

Alpha = 0.05, DF = 6, Mean Square Error = 0.00004
Critical value of studentized range = 4.339
Minimum significant difference = 0.0136

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Treatment
A	0.0258	4	1
A,B	0.0134	4	3
B	0.004	4	2

VITA

Martha Quinn McCollum, daughter of J. T. and Carolyn A. Quinn, was born September 25, 1971, in Roanoke, Virginia. She married William Christopher McCollum on May 23, 1992, in Christiansburg, Virginia. She was graduated from Virginia Polytechnic Institute and State University with a Bachelor of Science degree in Animal Science in May 1994. She received financial support from the John Lee Pratt Animal Nutrition Program during her undergraduate studies to perform research and was the recipient of the Cyrus McCormick Merit Scholarship, Lorenz Neuhoff, Jr., Pork Industry Student Leader Scholarship, Animal Science Scholarship, and the Philanthropic Educational Organization's Scholarship. She is a member of The American Society of Animal Science, American Institute of Nutrition, and Gamma Sigma Delta, The Honor Society of Agriculture.