Characterization of Glycyl-Sarcosine Uptake by Ovine Intestinal Brush Border Membrane Vesicles

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

Master of Science
in
Animal and Poultry Sciences

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August 19, 1997
Blacksburg, Virginia

Keywords: Sheep, Jejunum, Ileum, Peptide, Absorption, Transport

CHARACTERIZATION OF GLYCYL-SARCOSINE UPTAKE BY OVINE INTESTINAL BRUSH BORDER MEMBRANE VESICLES

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(ABSTRACT)

In order to characterize peptide transport in the ovine small intestine, [^{14}C]-glycyl-sarcosine uptake by tissue collected from five sheep was studied through the use of brush border membrane vesicles (BBMV). Preliminary experiments determined that incubation in hyaluronidase is not necessary in order to separate mucosal tissue from the basement membrane and that the stop solution used in the uptake study needed to be buffered. Uptake was examined in proximal (denoted jejunal) and distal (denoted ileal) halves of the intestine at four times (15, 30, 45, and 60 s) and at three extravesicular pH levels (6.4, 7.0, and 7.5). An intravesicular pH of 7.5 was used throughout the study. The two tissue sites differed ($P < .02$), with BBMV from jejunal tissue showing greater uptake than ileal. Uptake plateaued after 45 s, resulting in a quadratic ($P < .005$) effect of time. The effect of changes in extravesicular pH was also quadratic ($P < .04$), with uptake being greatest at pH 6.4, lowest at pH 7.0, and intermediate between the two at pH 7.5. Peptide uptake by sheep jejunal and ileal BBMV was demonstrated, but there was no clear evidence for increased uptake with decreasing extravesicular pH

ACKNOWLEDGEMENTS

I would especially like to thank Dr. K. E. Webb, Jr. for providing a learning experience of the highest caliber in a manner that was professional yet understanding and supportive.

I would also like to thank Drs. J. H. Herbein, Jr. and E. T. Kornegay for serving on my committee and for the use of their laboratory facilities.

I am indebted to Dr. J. C. Matthews for patiently answering my questions and for helping me learn my way around the laboratory.

I am indebted also to Kristin Lee and Donald Shaw for their unfailing support and assistance with my research.

I am grateful to Dr. N. E. Jack for helping me keep things in perspective and for allowing me to serve as her teaching assistant.

I am, in addition, grateful to Dr. T. A. Gorell (Colorado State University) for years of friendship and advice, and for encouraging me to pursue graduate studies.

I would also like to express my sincere appreciation to my husband, Michael R. Bowers, for his love and for his flexibility and patience throughout my graduate program.

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Chapter I

Introduction

Economic factors and waste disposal problems are driving a need for increased efficiency in the livestock feeding industry (Axe et al., 1987; MacRae, 1996). Many ingested nutrients are either not absorbed or, if absorbed, are not completely utilized within the animal's body. This inefficiency results in reduced profitability for the producer, as well as the problem of safe and economical disposal of waste. In addition, nutrients which are absorbed into and metabolized by the organism are not always utilized for the production of marketable products, but instead are laid down as fat. Again, this results in an economic loss for the producer as well as a waste product for disposal.

When a predominantly forage-based diet provides a substantial portion of nitrogen intake, ruminant protein utilization is inefficient. It has been estimated that even high yielding dairy cows on this type of diet ultimately convert less than 20% of ingested protein into a saleable product (MacRae, 1996). This inefficiency results in nitrogenous waste for disposal. There is, therefore, a need to design feedstuffs in such a manner that the animal's needs are well met, but the production of waste is minimized. This, however, first requires a thorough understanding of the processes of digestion, absorption, and metabolism.

It is now known that glucose, amino acids, and long chain fatty acids are all absorbed from the small intestine by means of transporters in the brush border membrane. The use of Na^+-dependent active transport mechanisms for the transport of these nutrients allows for metabolic and hormonal control of uptake. Recently, it has also been discovered that small peptides are absorbed across the brush border by means of carrier-mediated transport. Unlike the nutrients mentioned above, however, peptides are thought to be co-transported with protons. This H^+-peptide co-transport represents an evolutionary link between solute transporters in prokaryotes, which couple transport to the influx of protons, and the large number of Na^+-coupled transporters in vertebrates (Miyamoto et al., 1985; Ganapathy and Liebach, 1991; Ganapathy et al., 1994). The use, in mammals, of a Na^+-dependent system for amino acid absorption and H^+ co-transport for peptide absorption removes competition for energy, allowing the two systems to operate in parallel (Ganapathy et al., 1994).

Current and future research will continue to broaden our understanding of protein digestion, absorption, and metabolism and will, thereby, assist in the endeavor to make livestock feeding more efficient. If nutrient intake, absorption, and metabolism can be matched to animal nutrient needs, environmental contaminants in animal waste can be minimized while profitability and percentage of marketable product can be maximized. The purpose of the present research is to examine characteristics of dipeptide transport across the ovine brush border membrane.

Chapter II

Review of Literature

Transcellular Absorption from the Small Intestine

Protein Digestion and Absorption in the Small Intestine. The absorptive epithelia of the small intestine is specialized in the transcellular transfer of dietary nutrients (Murer et al., 1974). This process includes the uptake of nutrients across the brush border membrane, movement of these substrates through the cytoplasm, and release of the nutritional components through the basolateral membrane into the portal blood. Transfer across the two membranes is accomplished by diffusion and(or) mediated transport (Argiles and Lopez-Soriano, 1990; Wilson and Webb, 1990a). Substrate-dependent regulation and regulation under physiological conditions associated with changing energy requirements (e.g., pregnancy, lactation, decreased environmental temperature) avoid costs of extra transporters (Diamond, 1991; Olson et al., 1991; Ganapathy et al., 1994). With a combination of transcellular and paracellular mechanisms, the small intestine is able to almost perfectly match absorptive to digestive load (Diamond, 1991; Pappenheimer, 1993).

Because of ruminal fermentation, protein reaching the small intestine of the ruminant, the majority of which is of microbial origin, arrives with a more constant flow and is of a more uniform composition than in the monogastric animal (Ben-Ghedalia et al., 1974; Harmon, 1992). After reaching the small intestine, feed particles are broken down in size and are absorbed into the animal in much the same manner as they are in the monogastric. However, copious acid secretion from the abomasum coupled with less pancreatic juice in proportion to body weight causes the contents of the ruminant intestine to reach neutrality slower than in simple stomached animals (Moe et al., 1987; Church, 1988). While in humans the pH of digesta reaches neutrality in the upper jejunum (Kay, 1969), in the ruminant it may not reach pH 7 until it is in the ileum (Lennox and Garton, 1968; Hecker, 1983).

Brush border-bound proteases and peptidases are part of a hydrolytic barrier which complements the physical barrier of the brush border membrane itself (Wilson and Webb, 1990a; Gardner, 1994). The pH of the lumen of the small intestine affects protease and peptidase activity. Pepsin is most active between pH 1 and 3 which, in sheep, corresponds to the region from the pylorus to the entry of the common bile and pancreatic duct (Kay, 1969). The production of trypsin and chymotrypsin from their precursors only occurs above pH 5 (Kay, 1969), causing the concentrations of these enzymes to increase gradually from the duodenum through the jejunum. A pH of 7, usually reached between the mid-jejunum and the proximal ileum, is optimal for trypsin, chymotrypsin and carboxypeptidase A (Ben-Ghedalia et al., 1974; Church, 1988). These enzymes decrease in activity gradually throughout the remainder of the intestine as they are inactivated by the alkaline medium of the lower regions (Ben-Ghedalia et al., 1974; Church, 1988).

Luminal digestion by proteases reduces protein to peptides, which in turn can be hydrolyzed by peptidases of the brush border membrane in preparation for absorption by enterocytes (Ganapathy et al., 1994). In the monogastric animal, peptides predominate

over free amino acids as the major products of post-prandial luminal and membrane-associated protein digestion (Said et al., 1988; Daniel et al., 1994; Gardner, 1994), with most peptides containing two to six amino acids (Ganapathy et al., 1994). The absorptive capacity for small peptides in monogastric animals is greater in the proximal intestine than in the distal (Ganapathy et al., 1994). Brush border peptidase activities are higher and the duration of contact between peptide substrates and peptidases increases as the intestinal contents move from the jejunum to the ileum. The rate of appearance of free amino acids in the lumen, therefore, gradually increases while the concentration of small peptides decreases (Ganapathy et al., 1994). In the human and the rat, maximum amino acid absorption occurs in the jejunum (Moe et al., 1987). Less work has been done to investigate the appearance and disappearance of peptides and amino acids in ruminant intestine but it has been shown that, in sheep, peak activity of dipeptidases occurs in the mid-ileum (Ben-Ghedalia et al., 1974) and that maximum amino acid absorption also occurs in the ileum (Phillips et al., 1979; Moe et al., 1987). This difference between monogastric and ruminant amino acid absorption is probably due to the effects of lower luminal pH in the ruminant (Moe et al., 1987).

The insoluble mucous gel that adheres to the mucosal surface of intestinal enterocytes is a component of the unstirred water layer. The mucous coating appears to help maintain the acid microclimate adjacent to the brush border (Thomson et al., 1989). The highest H^+ concentration occurs below the tip of the villus in the zone of digestive and absorptive epithelial cells (Thomson et al., 1989). The mucous layer may also act as a mixing barrier and(or) may retard backdiffusion into the lumen (Argiles and Lopez-Soriano, 1990; Pappenheimer, 1993). In addition, it is possible that the mucous layer acts as a zone of accumulation, favoring the transport of certain substances across the brush border (Argiles and Lopez-Soriano, 1990).

Transport of Glucose, Amino Acids, and Long Chain Fatty Acids. The transport of major energy-yielding substrates such as glucose, amino acids, and long chain fatty acids provides a site for metabolic and hormonal control of metabolism (Abumrad et al., 1984; Nunn et al., 1986; Stremmel, 1989). Glucose resulting from small intestinal starch hydrolysis is normally transported across the brush border by either a Na^+-dependent process or by diffusion (Moe et al., 1985). The Na^+-dependent process is a secondary active transport system in which energy is not consumed for the uptake of glucose itself, but is consumed for an outward flux of Na^+ from the enterocyte. This Na^+ then re-enters the cell down a concentration gradient while coupled to a carrier molecule which transports the glucose (Church, 1988). There is some evidence to suggest that when Na^+ is absent, its role may be filled by H^+ (Abe et al., 1987). When the young ruminant is weaned to dry feed, the rumen assumes the task of carbohydrate fermentation. The small amount of starch presented to the intestine of the adult ruminant is composed of microbial polysaccharides (Owens et al., 1986) and some molecules that escape the rumen when high-grain diets are fed (White et al., 1971; Chittenden et al., 1984). Due to the smaller quantity of glucose traditionally available for absorption, the transport capacity for glucose in the ruminant decreases after weaning causing the adult animal to have less ability to absorb glucose than does the non-ruminant (White et al., 1971; Nocek and Tamminga, 1991; Reynolds et al., 1992).

Amino acid uptake from the small intestine most often involves a Na^+-dependent secondary active transport system (Argiles and Lopez-Soriano, 1990). As with glucose, ATP is involved in maintaining the driving forces for uphill transfer rather than directly energizing transport (Church, 1988; Ganapathy et al., 1994). Because of their widely differing physiochemical properties, multiple carriers are needed to move the different free amino acids across the brush border (Ganapathy et al., 1994; Webb and Matthews, 1994). All of the amino acid carriers, however, require a substrate with an amino or imino group (Webb, 1990). Individual amino acids may inhibit or stimulate the absorption of other amino acids by interacting with each other during the process of absorption (Phillips et al., 1979; Moe et al., 1987; Webb and Bergman, 1991). Competition is greater among amino acids for which a particular carrier has greater affinity (Webb, 1990). It is also possible that the uptake of essential amino acids may be favored over the uptake of non-essential (Webb and Matthews, 1994). Intestinal amino acid transporters are able to adapt to dietary and metabolic changes (Argiles and Lopez-Soriano, 1990) in order to provide needed nutrients in an efficient manner.

Recently, it has been determined that at least a portion of long chain fatty acid (LCFA) absorption in the gut of some monogastric animals is mediated by a plasma membrane fatty acid binding protein (FABP-PM; Stremmel, 1988a). In 1979, Chow and Hollander found that LCFA absorption by rat jejunal enterocytes is a concentration-dependent process, exhibiting saturable uptake at physiologic luminal concentrations. Since that time, the FABP-PM has been located in the apical and lateral portions of rat jejunal enterocytes (Stremmel et al., 1985; Stremmel, 1988a). This transporter shows competence for oleic, arachidonic, linoleic, palmitic, and myristic acids, but not for caproic, caprylic, capric, and lauric acids. The transport determinant, therefore, may be the structure of the monomeric hydrocarbon chain, through steric and(or) hydrophobic interactions, rather than the carboxylic group (Stremmel, 1988a). Because the free fatty acid chemical gradient across the membrane is assumed to be small due to extensive binding inside and outside the cell and because LCFA uptake is electrically unfavorable, LCFA transport is coupled to the influx of Na^+, which is favored both electrically and chemically (Weisiger et al., 1989), in a secondary active transport system. Regulation of cellular expression of the carrier under conditions such as fasting, high calorie diet, and metabolic disorders still needs to be examined, as does the potential existence of such a carrier in the ruminant.

Characteristics of Peptide Absorption from the Small Intestine

Peptide Transport Across the Brush Border. Dipeptides appear to be absorbed from the small intestine more rapidly than are free amino acids (Webb, 1990; Reshkin and Ahearn, 1991; Webb et al., 1993). Peptide uptake is a carrier-mediated process which exhibits saturation kinetics and competitive inhibition (Ganapathy et al., 1984). A variety of di- and tripeptides, but no free amino acids, are transferred across the brush border membrane on this transporter (Hoshi, 1985; Burston and Matthews, 1987; Webb, 1990). It is not yet known the extent to which peptide transport contributes to overall absorption of amino acids in comparison to free (Daniel et al., 1994), but it has been suggested that peptides may be the primary amino acid source for ruminants (Webb et al., 1993). It is known, however, that certain genetic defects which result in the

malabsorption of amino acids do not result in symptoms of protein malabsorption or malnutrition because peptide absorption is adequate to meet the patient's needs (Daniel et al., 1994; Ganapathy et al., 1994). No genetic disorder has been reported in which the primary defect involves peptide transport (Ganapathy et al., 1994).

Peptide uptake is thought to be accomplished when protons, flowing down an electrochemical gradient, bind the H^+/peptide co-transporter, causing the symport of one peptide molecule (Ganapathy and Leibach, 1985; Hoshi, 1986; Webb and Matthews, 1994). The acid microclimate at the absorptive zone in the upper villus causes an inward H^+-gradient which can be used to drive uphill transport into the enterocyte, the intracellular pH of which is approximately 7.2 (Tiruppathi et al., 1988a; Daniel et al., 1994; Ganapathy et al., 1994). Glycyl-sarcosine, which is resistant to hydrolysis (Ganapathy et al., 1984), and glycyl-proline, which is relatively poorly hydrolyzed (Cheeseman and Devlin, 1985), are the peptides most often used in uptake studies. These peptides have two ionizable groups but exist mostly as dipolar ions with zero net charge between pH 5.5 and 7.5 (Ganapathy and Leibach, 1983; Ganapathy et al., 1984; Daniel et al., 1994). Peptide uptake appears to be optimum at an extracellular pH of 5.5 to 6.0, where the concentration of transportable zwitterionic species is maximum. Further acidification would decrease the zwitterion concentration and, as a result, also probably decrease transport despite the enhanced inward H^+-gradient (Ganapathy and Leibach, 1985; Saito and Inui, 1993).

Because of the co-transport of H^+, peptide transport induces a net transfer of positive charge across the membrane, making the process electrogenic (Ganapathy and Leibach, 1983; Ganapathy et al., 1984, 1994). This increase in intracellular pH activates the electroneutral Na^+/H^+ antiport system in the brush border (Cassano et al., 1984; Daniel et al., 1994). The Na^+/H^+ antiporter is driven by the *in vivo* Na^+-gradient (Tiruppathi et al., 1988b) and results in the exchange of intracellular H^+ for extracellular Na^+ in order to restore pH and the proton gradient (Cheeseman and Devlin, 1985; Webb and Matthews, 1994). The Na^+-gradient is restored through the action of ATP-hydrolyzing Na^+/K^+-ATPase found on the basolateral membrane (Ganapathy et al., 1994). Because ATP is required to maintain the Na^+-gradient, which in turn maintains the H^+-gradient, which energizes peptide transport, this system is considered an example of "tertiary active" transport (Ganapathy and Leibach, 1986; Tiruppathi et al., 1987; Ganapathy et al., 1994).

Some early work with intact intestinal tissue seemed to suggest a dependency of the peptide transporter on Na^+, while other work did not find this dependency (Boyd and Ward, 1982; Himukai et al., 1983; Cheeseman and Devlin, 1985). Presently, the inhibition of peptide transport caused by the removal of Na^+ from intact tissue preparations is considered to be due to interference with the operation of the Na^+/H^+-antiport (Ganapathy and Leibach, 1983; Burston and Matthews, 1987; Daniel et al., 1994). In the absence of Na^+, intracellular pH recovery can occur, although more slowly, through the action of H^+/K^+-ATPase (Thwaites et al., 1993a).

Another question concerns the actual number of H^+ transported along with the peptide. Results generally fall into the range of 1.17 to 2 protons transported for each peptide molecule absorbed (Webb et al., 1992; Fei et al., 1994). However, work with the

Caco 2 human colon carcinoma cell line seems to suggest that H^+/peptide symport causes a flux of three protons for every transported peptide (Thwaites et al., 1993c). There is also evidence that carnosine may be transported in a 1:1 ratio with H^+ (Abe et al., 1987). The conflicting results may be due to the presence of more than one peptide transporter, or they may be due to differences in experimental design. The Caco 2 cell line, for instance, is selected for the ability to grow continuously in culture. The mechanism for this adaptation is not clear, nor is the potential effect on the validity of results from these cells (Dayton and Allen, 1987).

The H^+/peptide co-transporter appears to have a somewhat greater affinity for dipeptides than for tripeptides (Ganapathy et al., 1985; Brandsch et al., 1994). At this point, it seems that the 400 possible dipeptides and 8000 possible tripeptides available from the 20 dietary amino acids may all be transported using the same uptake system. The transporter must have very broad specificity to be able to accept this huge number of substrates with their great variation in molecular size and hydrophobic/hydrophilic properties (Daniel et al., 1994). There are also some studies which suggest tetrapeptides may be transported to a small extent (Ganapathy et al., 1994), however, there are other results which suggest that they are not (Fei et al., 1994).

While most studies show that a proton gradient stimulates peptide transfer across the brush border, other studies fail to show this stimulation (Rajendran et al., 1987; Reshkin and Ahearn, 1991). If dipeptides are actively transported with H^+, overshoot uptake should be observed in brush border membrane vesicles when a sufficiently large proton gradient is imposed across the membrane, demonstrating accumulation against a concentration gradient (Hoshi, 1985). The ability to demonstrate an overshoot has, through the years, eluded researchers working with intestinal tissue (Ganapathy et al., 1984; Calonge et al., 1990). It is possible, therefore, that peptide transport may be able to operate both downhill, in a facilitated diffusion process driven by the transmembrane substrate gradient, or uphill using the electrochemical H^+ gradient (Reshkin and Ahearn, 1991; Daniel et al., 1994).

Comparisons to Peptide Transport in the Kidney. A peptide transport system has been identified in tubular epithelial cells of the kidney. Luminal hydrolysis of oligopeptides produces smaller peptides and amino acids which are absorbed across the brush border, allowing for reclamation of peptide nitrogen and regulation of plasma levels of small peptides (Ganapathy and Leibach, 1986; Miyamoto et al., 1988). Many of the characteristics of this transporter are similar to the intestinal peptide transporter (Miyamoto et al., 1986; Tiruppathi et al., 1990; Daniel and Adibi, 1994). Of notable exception, however, is the fact that the overshoot phenomenon has been demonstrated in renal brush border membrane vesicle studies (Hoshi, 1985; Miyamoto et al., 1985; Daniel et al., 1991). Another unique aspect of renal peptide uptake is the discovery that there seem to be two different types of transporters, a high affinity/low capacity type and a low affinity/high capacity type (Daniel et al., 1991). The concentration of dipeptide in the medium determines the system on which it will primarily be carried (Daniel et al., 1991). The low affinity system, which is operational in the absence of a pH gradient, is considered to be the type most similar to that in the intestine (Daniel et al., 1991, 1992).

Fate of Peptides Entering Enterocytes. Between 65% and 78% of the amino acids appearing in the portal blood of steers and sheep is in peptide form (Seal and Parker,

1991; Webb and Bergman, 1991; Koeln et al., 1993). The origin of these peptides has not been determined. They may be degradation products from protein turnover in the intestine and(or) other tissues; they may result from synthetic activity in tissues drained by the portal vein; or, they may be absorbed from the intestinal lumen (Webb, 1990; Koeln et al., 1993). The observation that ruminants seem to have a higher proportion of peptide-bound amino acids in the portal blood than do non-ruminants (Seal and Parker, 1991) may be due in part to the more acidic nature of the ruminant small intestine and its relationship to protein digestion and absorption (Webb and Bergman, 1991).

Despite the large proportion of peptides in the portal blood, it is currently believed that the majority of peptides absorbed by the enterocyte are released into the blood as free amino acids (Ganapathy et al., 1994). Once inside the enterocyte, small peptides are thought to be rapidly hydrolyzed by cytoplasmic peptidases (Webb, 1990; Ganapathy et al., 1994). This cytosolic peptidase activity maintains a downhill peptide gradient across the brush border membrane (Webb et al., 1992). In monogastrics, cytoplasmic peptidase activity is highest in enterocytes of the proximal to middle intestine (Ganapathy et al., 1994), corresponding to the area of greatest peptide absorption. The amino acid products of peptide hydrolysis may be utilized by the cell, or transported across the basolateral membrane into the blood by a number of group specific amino acid transport systems (Daniel et al., 1994; Ganapathy et al., 1994).

The enterocyte does, however, also appear to have a mechanism by which a small but significant quantity of peptides may cross the basolateral membrane intact (Dyer et al., 1990; Ganapathy and Leibach, 1991; Koeln et al., 1993). Proton-coupled transporters on the basolateral membrane appear to be able to mediate small peptide transfer from the cytoplasm into the portal circulation (Thwaites et al., 1993b,d).

Other Transport Possibilities. In Caco 2 cells, an electrogenic, H^+-coupled, pH independent peptide uptake system allows intracellular accumulation of peptides across the basolateral membrane (Thwaites et al., 1993b). This is thought to occur by facilitated diffusion because no overshoot has been demonstrated (Saito and Inui, 1993). Affinity and capacity of the brush border uptake system are higher than affinity and capacity of the basolateral system, as evidenced by the fact that competitive inhibition by dipeptides is greater across the brush border and that there is low intracellular accumulation of dipeptide across the basolateral membrane (Saito and Inui, 1993; Thwaites et al., 1993b).

The brush border of Caco 2 cells is also able to transfer peptides from the enterocyte into the intestinal lumen when low luminal peptide concentrations are present. This is a substrate specific, carrier-mediated system (Burton et al., 1993). Secretion is enhanced by raising the apical pH and lowering the basolateral pH. Under conditions found in the intestine, however, where apical pH would be approximately 6.0 and basolateral pH approximately 7.4, net absorption should occur (Thwaites et al., 1993d).

Peptide Transporters in the Intestine. Polar compounds that are able to penetrate the lecithin- and cholesterol-containing lipid barrier that is the plasma membrane often do so by means of transporters (Scarborough, 1985). These transporters, which are solute-specific protein components of the membrane are subject to biological regulation and show saturation kinetics (Argiles and Lopez-Soriano, 1990). Transport molecules provide the means by which binding sites for ligands alternately become exposed to the aqueous media on either side of the membrane (Scarborough, 1985). In general, all

transporters are thought to have a pair of membrane-embedded domains, each of which contains an array of approximately six transmembrane helical elements. Substrates interact with a pathway bordered by the opposing surfaces of paired subunits or domains (Maloney, 1990).

A 127 kDa brush border membrane glycoprotein (PepT1) has been isolated from rabbit small intestine. The PepT1 molecule is a binding protein for dipeptides and is directly involved in the uptake process, but does not have peptidase activity (Kramer et al., 1990a,b). Recently, PepT1 has been "expression cloned" using *Xenopus laevis* oocytes (Boll et al., 1994; Fei et al., 1994). *Xenopus laevis* oocytes are able to translate injected mRNA from eukaryotic sources and carry out post-translational modifications and targeting for the foreign proteins. In this way, the mammalian intestinal H^+/peptide co-transporter has been expressed in a functionally competent form (Miyamoto et al., 1991). This transporter shows broad substrate specificity for dipeptides, tripeptides and possibly some tetrapeptides, but not for free amino acids or for peptides of more than four amino acids. Optimal peptide length is two amino acids (Fei et al., 1994). It can transport acidic, basic or hydrophobic substrates, but prefers bulky aliphatic side chains (Fei et al., 1994). Uptake is Na^+-independent and saturable, with maximum transport occurring when external pH is 5.5 (Boll et al., 1994; Fei et al., 1994). This implicates a proton motive force as part of the driving force. It is interesting to note, however, that the endogenous *X. laevis* transporter fails to show H^+-dependence (Miyamoto et al., 1991). PepT1 appears to be the only glycyl-sarcosine transporter in enterocytes of the rabbit and is located primarily in the duodenum and jejunum. In *X. laevis* oocytes, glycyl-sarcosine is co-transported with protons in a ratio of 1: 1.17 (Fei et al., 1994). The PepT1 primary structure from rabbit intestine predicts 12 membrane-spanning domains and an unusually large hydrophilic loop which is probably external (Fei et al., 1994). The PepT1 sequence shows weak similarity to the peptide permease from *Saccharomyces cerevisiae* (Fei et al., 1994).

A 92 kDa membrane protein (HPT 1) associated with acquisition of peptide transport activity by transport deficient Caco 2 cells has also been isolated (Dantzig et al., 1994). PepT1 and HPT 1 are very different. While PepT1 seems to fit the general model for transporters with its 12 membrane-spanning domains, HPT 1 has only one membrane-spanning region. HPT 1 is similar in this regard, however, to two recently described amino acid transporters (Dantzig et al., 1994). It has been suggested that HPT 1 may use cysteine residues in its extracellular region to associate with other membrane proteins and form a multimeric transporter (Dantzig et al., 1994).

Mechanisms of Transport. The mechanism of the transport process has not yet been elucidated. It may involve the binding of the ligand to a preformed site, followed by a conformation change in the transporter molecule which causes translocation of the binding site and ligand from one side of the membrane to the other. Or, the mechanism may involve a ligand binding site that is not preformed in which the conformation transition that brings about the access change is driven and stabilized by the ligand-binding reaction itself (Scarborough, 1985).

In rabbit intestine, histidine residues are essential components at or near the substrate binding site, while sulfhydryl groups and tyrosine residues contribute little to H^+-coupled transport (Kato et al., 1989). In Caco 2 cells, however, sulfhydryl groups

which are localized at the external surface of the cell appear to be essential (Inui et al., 1992; Saito and Inui, 1993). In addition, protein kinase C decreases transport in Caco 2 cells and may serve as a post-translational regulatory control through phosphorylation/ dephosphorylation reactions (Brandsch et al., 1994). Transporters in rabbit renal cortex have histidyl and thiol groups present at or near the substrate binding site (Miyamoto et al., 1986; Daniel et al., 1991). The thiol groups are thought to exist in the reduced form to maintain maximum activity. When they are in the oxidized form, the transport system is inactive (Miyamoto et al., 1989). Thiol groups have not at this time been identified in the intestinal transporter.

The structural features of di- and tripeptides which appear to be necessary for uptake by the intestinal transporter, as well as the kidney and microbial transporters include a protonated primary amino group, a free carboxyl terminal, L-stereospecificity, and an alpha-peptide bond (Olson et al., 1991; Daniel et al., 1992; Daniel et al., 1994). Side chain properties are not critical, although it does appear that the transporter prefers hydrophobic substrates with bulky side chains (Matthews, 1991; Olson et al., 1991; Daniel et al., 1994). There is, however, no correlation between affinity for the carrier and molecular size (Abe et al., 1987).

Interaction of a dipeptide with the binding site on the transporter may be facilitated by the electronegative carbonyl function and the electropositive amide function of the rigid peptide bond acting as an anchor. With the substrate anchored into the correct position, hydrogen bonding with functional groups at the carrier site can occur (Daniel et al., 1992). Amino acid side chains would be of less importance in the overall interaction because of their free rotation along the alpha-carbons (Daniel et al., 1992).

Energy for transport processes can be temporarily stored in the form of ionic gradients (Tsong, 1992). Free energy release occurs when the ions move down their chemical and(or) electric gradient (Ganapathy and Leibach, 1991). In bacterial systems the principal coupling ion is H^+, but there are also some Na^+-coupled systems. In animal cells, Na^+ is the principal, but not exclusive, coupling ion (Ganapathy and Leibach, 1991). Various amino acid transport systems in renal brush border membrane and the glucose transporter in intestinal brush border membrane appear to accept either Na^+ or H^+ (Ganapathy and Leibach, 1991). The exact mechanism of the transport process has not yet been elucidated, nor has the site/method of action for energy generated by the ion gradient (Scarborough, 1985; Tsong, 1992).

Further Considerations

Age-Related Changes in Transport Capacities. Some intestinal nutrient transporters are present in their highest levels at birth, or shortly thereafter, when nutrient and energy requirements of the animal are highest (Buddington, 1992). Postnatal changes in the activities of transporters often correspond to the species' evolutionary diet and the age-related shift in nutritional needs for growth and metabolism. Glucose transport in lambs, for instance, normally decreases to negligible levels at the time of weaning, corresponding to the onset of fermentation in the rumen and the associated decrease in the amount of glucose entering the intestine (Buddington, 1992). Age-related changes in transport rates may be caused by changes in the types of transporters, density of

transporters, activity of transporters and(or) changes in the physiochemical characteristics of the membrane (Buddington, 1992).

The intestinal transport of many dipeptides exhibits a peak at the time of birth and then decreases slowly with increasing age (Himukai et al., 1980; Guandalini and Rubino, 1982; Ganapathy et al., 1985). It has been shown in rabbits that the fetal period of fast-paced increase, while coincident with rapid development of villus and microvillus structure, is not merely the result of an increasing amount of brush border membrane per unit area (Guandalini and Rubino, 1982). While newborn and adult animals appear to have analogous K_t's for dipeptide uptake, V_{max} is much higher in the newborn than the adult, providing very efficient dipeptide influx in the newborn (Guandalini and Rubino, 1982). This increase in V_{max} could be the result of a larger number of carrier sites per unit area or increased translocation velocity (Guandalini and Rubino, 1982). As the animal ages, V_{max} and the capacity to absorb peptides decrease (Ganapathy et al., 1994).

High capacity for peptide absorption in the young animal may be required because of incomplete protein digestion and(or) increased need for amino acids during the postnatal growth period of the animal. Peptidase activity is less developed and, therefore, protein digestion is incomplete during the suckling period (Tiruppathi et al., 1987; Said et al., 1988), causing a need for increased peptide transport in the immature animal. Peptide influx of amino acids is remarkably higher than free amino acid influx in the newborn, allowing peptides to play a predominant role in the protein digestion-absorption processes of the youngster (Guandalini and Rubino, 1982).

Medical Applications. The H^+/dipeptide transporter is also able to transport peptide mimetics, such as orally active beta-lactam antibiotics (Iseki et al., 1989; Daniel et al., 1994). Aminopenicillins and aminocephalosporins are completely ionized in the gastrointestinal tract pH range and, therefore, have low lipid solubility (Kramer et al., 1988). These compounds have alpha-amino and carboxyl groups and are highly resistant to enzyme hydrolysis by intestinal mucosa (Inui et al., 1992). The PepT1 membrane polypeptide is thought to be a constituent of the transport system for orally effective beta-lactam antibiotics in rabbit intestinal brush border membrane vesicles (Kramer et al., 1988). Beta-lactam antibiotics lacking alpha-amino groups also seem to be transported by the H^+/dipeptide system, although at a much lower pH optimum (Inui et al., 1988; Kramer et al., 1988), suggesting that conformation of the transport protein may change with pH (Inui et al., 1988).

In addition, the effect of an inward H^+ gradient appears to vary by substrate and by experimental animal. For instance, the effect of a H^+ gradient on cephalosporin uptake is greater in rabbit brush border than in rat (Iseki et al., 1989). In the rat, a H^+ gradient stimulates cephalosporin uptake to a lesser extent than it does dipeptide uptake, but does not alter ampicillin uptake. In the absence of a H^+ gradient, cephalosporin uptake in the rat is greater than dipeptide uptake (Iseki et al., 1989).

Another medicinal application of the peptide transporter involves the delivery of needed amino acids to seriously ill patients who are being “tube fed”. Certain necessary free amino acids must be left out of enteral diet formulations because of insolubility (tyrosine) and instability (glutamine and cysteine; Adibi, 1989; Ganapathy et al., 1994). These insolubility and instability problems are alleviated when amino acids are in peptide

form. Other benefits of enteral diet formulations which provide amino acids in peptide form include greater absorption speed, more even appearance of amino acids in the blood, less competition during transport, conservation of metabolic energy, and prevention of diarrhea due to lower osmotic load (Ohkohchi et al., 1990; Daniel et al., 1994; Ganapathy et al., 1994).

A similar system could enhance absorption of drugs by presenting them to the small intestine as pro-drugs which would subsequently be hydrolyzed intracellularly (Ganapathy et al., 1994). Pharmaceutical delivery of peptides and drugs via enteral routes would, however, be more efficient if proteases and peptidases could be inhibited or if formulations that protect against intestinal degradation could be devised (Gardner, 1994).

Paracellular Absorption from the Small Intestine. In addition to the transcellular route, there is also a possibility that small- to medium-sized peptides and beta-lactam antibiotics can travel from the intestinal lumen to the blood via paracellular routes (Atisook and Madara, 1991; Inui et al., 1992; Pappenheimer, 1993). Absorption of dietary nutrients causes an osmotic force and triggers the reversible opening, through cytoskeletal contractions, of tight junctions which allow transjunctional water flow (Atisook and Madara, 1991; Pappenheimer, 1993; Gardner, 1994). As water passes through the permeable tight junctions, small molecules are carried along by solvent drag (Gardner, 1994). The paracellular route would, therefore, be more important at higher substrate concentrations (mM; Thwaites et al., 1993d).

Paracellular transport itself is a passive process, requiring no oxidative energy (Thwaites et al., 1993b). Energy is required, however, to create the transjunctional concentration gradient and to energize the contraction of cytoskeletal elements (Pappenheimer, 1993). Saturable, instead of linear, kinetics can be observed for a solute being carried paracellularly by solvent drag when increasing mucosal solute concentration causes decreasing solvent drag (Gardner, 1994).

Brush Border Membrane Vesicles as a Method of Studying Transport

Brush border membrane vesicles (BBMV) allow the formation of intravesicular aqueous spaces separated from the external medium by a membrane (Hopfer et al., 1973). This allows transport properties of the brush border to be evaluated independent of the unstirred water layer, cellular metabolism and energy, intracellular compartmentalization, the basolateral membrane, and paracellular transport routes (Hopfer et al., 1973; Argiles and Lopez-Soriano, 1990; Keelan et al., 1992). The composition of fluids on both sides of the membrane can be controlled (Webb, 1990), as can the direction and magnitude of ion gradients (Moe et al., 1987). In addition, BBMV increase efficiency by allowing many transport experiments to be performed using tissue from a single animal (Wilson and Webb, 1990b).

On the other hand, however, the percentage of brush border membrane actually incorporated into vesicles is low because most of the microvilli remain part of large pieces of tissue which are lost in the sediment of low-speed centrifugation steps (Murer et al., 1974). Possible alterations in the biochemical composition of membranes may accompany the process of cell lysis, cation precipitation, and sealing of vesicles (Ganapathy et al., 1981; Cassano et al., 1984; Calonge et al., 1990). In addition, vesicle preparation procedures may enhance membrane permeability, resulting in dissipation of chemical and ionic gradients (Cassano et al., 1984; Calonge et al., 1990). Finally, due to

the removal of environmental effects of the intestinal lumen, the loss of endocrine effects of the live animal and the lack of associative effects of other nutrients, uptake parameters determined through the use of BBMV cannot be directly extrapolated to *in vivo* conditions (Wilson and Webb, 1990a).

Chapter III

CHARACTERIZATION OF GLYCYL-SARCOSINE UPTAKE BY OVINE INTESTINAL BRUSH BORDER MEMBRANE VESICLES

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ABSTRACT

In order to characterize peptide transport in the ovine small intestine, [^{14}C]-glycyl-sarcosine uptake by tissue collected from five sheep was studied through the use of brush border membrane vesicles (BBMV). Preliminary experiments determined that incubation in hyaluronidase is not necessary in order to separate mucosal tissue from the basement membrane and that the stop solution used in the uptake study needed to be buffered. Uptake was examined in proximal (denoted jejunal) and distal (denoted ileal) halves of the intestine at four times (15, 30, 45, and 60 s) and at three extravesicular pH levels (6.4, 7.0, and 7.5). An intravesicular pH of 7.5 was used throughout the study. The two tissue sites differed ($P < .02$), with BBMV from jejunal tissue showing greater uptake than ileal. Uptake plateaued after 45 s, resulting in a quadratic ($P < .005$) effect of time. The effect of changes in extravesicular pH was also quadratic ($P < .04$), with uptake being greatest at pH 6.4, lowest at pH 7.0 and intermediate between the two at pH 7.5. Peptide uptake by sheep jejunal and ileal BBMV was demonstrated, but there was no clear evidence for increased uptake with decreasing extravesicular pH.

Key Words: Sheep, Jejunum, Ileum, Peptide, Absorption, Transport

Introduction

The processes of protein digestion, absorption, and metabolism provide an essential source of nitrogen as well as important energy-producing substrates for members of the animal kingdom. Historically, it has been thought that proteins and peptides reaching the intestine had to be broken down to free amino acids before absorption into enterocytes could occur. Recent work, however, has shown that small peptides, especially dipeptides and some tripeptides, can be transported intact across the brush border membrane of the small intestine (Ganapathy et al., 1994).

Peptides appear to predominate over free amino acids as the major products of post-prandial luminal and membrane-associated protein digestion (Said et al., 1988; Daniel et al., 1994; Gardner, 1994). Peptide uptake has been found to be a carrier-mediated process exhibiting saturation kinetics and competitive inhibition (Ganapathy et al., 1984), and the Pep T1 peptide transporter has been isolated and sequenced (Fei et al., 1994). Peptide transport is enhanced in the presence of a H^+-gradient across the brush border (Ganapathy and Leibach, 1983) and is thought to be accomplished when a proton flowing down its electrochemical gradient binds the H^+/peptide co-transporter and causes the symport of a peptide molecule (Ganapathy and Leibach, 1985; Hoshi, 1986; Fei et al., 1994). The acid microclimate at the absorptive zone in the upper villus causes the inward H^+-gradient which is used to drive transport into the enterocyte, the intracellular pH of which is 7.2 (Tiruppathi et al., 1988a; Daniel et al., 1994; Ganapathy et al., 1994). While most peptide transport experiments have been performed using tissue from monogastric organisms, ruminants also have been shown to have the capability to absorb peptides (Koeln et al., 1993; Backwell et al., 1995; Matthews and Webb, 1995; Matthews et al., 1996; Pan et al., 1997). The present study considers the ruminant small intestine in its entirety in an attempt to characterize peptide uptake along its length.

Materials and Methods

Animals and Feeding. Intestinal tissue was collected from five cross-bred sheep (avg wt 50 kg). Sheep were fed a diet consisting of 50% ground, shelled corn, 30% orchardgrass hay, 13.3% soybean meal, 5% molasses, .5% trace mineral salt, .42% defluorinated rock phosphate, and .5% limestone on an as-fed basis. Sheep were weighed every 2 wk and the quantity of feed was adjusted to maintain .23 kg body weight gain·head^{-1}·d^{-1}. Sheep were housed and fed as a group with free access to water. Every 2 mo, sheep were injected with vitamin A (500,000 IU), vitamin D (75,000 IU), vitamin E (3.7 IU/kg), and selenium (55 μg/kg).

The following procedures are modifications of those reported by Wilson and Webb (1990b). Detailed protocols for these procedures can be found in the appendices.

Tissue Harvest. Sheep were killed by anesthetization with sodium pentobarbital followed by exsanguination. The small intestine was dissected from the animal, placed on an ice-cold tray, and divided into proximal and distal halves. In all subsequent procedures, the proximal half is referred to as the jejunum and the distal half is referred to as the ileum. Each half was segmented into lengths of approximately 30 cm and then

opened longitudinally. Lengths were washed in four successive baths of cold buffer consisting of 300 m*M* mannitol and 12 m*M* Tris at pH 7.4. Mucosal tissue was scraped from the underlying layers with a glass slide, frozen in liquid nitrogen, and stored at -80^{o}C.

In a pre-trial experiment, an incubation with hyaluronidase used in previous studies (Wilson and Webb, 1990b; Jang, 1993) in order to make the mucosa easier to scrape from the basement membrane was tested for effectiveness by harvesting half the tissue from a single animal with the hyaluronidase incubation and half without. Brush border membrane vesicles prepared from tissue harvested in each manner were compared on the basis of protein recovery, alkaline phosphatase (ALP) activity, and Na^{+}/K^{+}-ATPase activity. Based on the results from this experiment, the hyaluronidase incubation was removed from the tissue harvest procedure.

Preparation of Brush Border Membrane Vesicles (BBMV). All steps in this procedure were performed in a manner which maintained a tissue temperature of approximately 4^{o}C. Weighed mucosa was homogenized in a buffer containing 150 m*M* mannitol, 30 m*M* succinate, 10 m*M* Tris, 5 m*M* $MgCl_2$, 5 m*M* K_2HPO_4, and .1 m*M* $MnCl_2$ at pH 7.4. Sequential homogenization, stirring, and centrifugation at 8,700 *x* g (12 min) and 31,000 *x* g (15 min) were used to separate brush border membrane from basolateral membrane and membranes from cellular organelles. After the final 31,000 *x* g centrifugation step, brush border membrane pellets were homogenized, suspended in buffer consisting of 305 m*M* mannitol, 20 m*M* Hepes, and 2 m*M* $MgCl_2$ at pH 7.4 and 331 mOsm, and centrifuged at 105,000 *x* g on a solution containing 31% sucrose in 2 m*M* $MgCl_2$ and 4 m*M* Hepes at pH 7.4. The resulting band and associated sucrose layer were harvested and stored at -80^{o}C until used for transport studies, while the aqueous layer and the pellet were discarded.

Characterization of Brush Border Membrane Vesicles. Samples for characterization were taken at the beginning and the end of the BBMV preparation procedure. Samples for the protein assay were stored at -20^{o}C while samples for the alkaline phosphatase and Na^{+}/K^{+}-ATPase assays were stored at -80^{o}C.

Protein concentration was determined using a BCA (bicinchoninic acid) Protein Assay Kit (Pierce Chemical, Rockford, IL). Purity of BBMV preparations was determined by assays of the brush-border enzyme ALP and the basolateral enzyme Na^{+}/K^{+}-ATPase. Alkaline phosphatase was analyzed using a diagnostic kit (Sigma Chemical, St. Louis, MO), while Na^{+}/K^{+}-ATPase activity was determined based upon the concentration of inorganic phosphate liberated from ATP (Eibl and Lands, 1969). Vesicle preparations were not analyzed for contamination by membranes from cellular organelles because these assays had been performed previously in this laboratory for this BBMV preparation procedure (Jang, 1993).

Uptake Study. Prior to the uptake study, pre-trial experiments were conducted to determine the effect of manifold position on uptake, the necessary amount of stabilization time before counting radioactivity, and the effect of filter orientation within scintillation vials on counting efficiency. The appropriateness of unbuffered stop solution as compared to buffered stop solution was also evaluated. Subsequently, two buffered stop solutions were compared to each other and one selected for use in the uptake study.

Uptake experiments were performed using a rapid filtration technique. Uptake was initiated by mixing 50 μL of vesicle suspension with 250 μL of a medium containing [^{14}C]-glycyl-sarcosine (Moravek Biochemicals, Brea, CA). The 50 μL of vesicle suspension contained of .31 mg vesicular protein in a solution consisting of 2 m*M* $MgCl_2$, 20 m*M* Hepes, and 305 m*M* mannitol at pH 7.5 and 331 mOsm. The glycyl-sarcosine medium contained 2 m*M* $MgCl_2$, 10 m*M* Hepes, 10 m*M* Mes, 304.7 m*M* mannitol, and .3m*M* glycyl-sarcosine at 331 mOsm and at a pH which, when combined with the 50 μL of vesicle solution, yielded the desired experimental pH. Uptake was stopped by addition of ice-cold stop solution containing 210 m*M* KC1, .5 m*M* Hepes, and .5 m*M* Tris at pH 7.5, as described by Miyamoto et al. (1989). An aliquot from each reaction vessel was filtered. Filters were then washed three times with ice-cold stop solution and subsequently counted for associated radioactivity.

Statistical Analyses. The uptake study employed a split plot design. Treatments were applied using a randomized complete block design where each individual animal served as a block. Within each animal, treatments were blocked again by tissue site. Because animal was the highest-ranking block, animal itself and the animal by treatment interactions could not be tested. Thus, the effects of pH, time, tissue site, and their two- and three-way interactions on [^{14}C]-glycyl-sarcosine uptake by BBMV were evaluated by analysis of variance using the General Linear Models procedure of SAS (1988). Orthogonal polynomial contrasts were used to partition the effects of pH and time. Contrast coefficients were generated by the Matrix function of SAS (1985).

In evaluating the differences between the tissue harvest procedure with hyaluronidase incubation or without hyaluronidase incubation, the T Test procedure of SAS (1988) was used. Differences between stop solutions, differences between uptake at pH 5.5 vs. 7.5, and background binding experiments were evaluated by analysis of variance using the General Linear Models procedure of SAS (1988).

Results and Discussion

Changes to Tissue Harvest Procedure. Previous work in this laboratory utilized an incubation at 37^{o}C for 20 min in hyaluronidase buffer (Wilson and Webb, 1990a; Jang, 1993) as part of the tissue harvest procedure. This incubation was meant to make it easier to separate intestinal mucosal cells from the basement membrane. After cells were harvested, the hyaluronidase buffer had to be removed by centrifugation before the mucosal tissue could be frozen. In order to reduce the time spent harvesting mucosal cells, the incubation in hyaluronidase buffer and the related centrifugation step were removed from the procedure. In the absence of hyaluronidase, mucosal cells were scraped from the basement membrane with no noticeable difference in difficulty as compared to cells harvested with the hyaluronidase incubation. Protein recovery and Na^+/K^+-ATPase enrichment values of BBMV prepared from tissue harvested without hyaluronidase did not differ from BBMV prepared from tissue harvested with hyaluronidase (Table 1). Values for ALP enrichment, however, were higher ($P < .05$) in BBMV prepared from tissue harvested without hyaluronidase. Because ALP is an enzyme of the brush border membrane and an indicator of vesicle purity, this higher

enrichment value is preferred. The increased enrichment is possibly due to better preservation when tissue is kept consistently cool.

Table 1. Characterization of BBMV Prepared from Jejunal Tissue Harvested Either With or Without Hyaluronidase

	Protein recovery[c]		Alkaline phosphatase[d]		Na^+/K^+-ATPase[e]	
Procedure	%	S E	Enrichment	S E	Enrichment	S E
With hyaluronidase incubation[a]	.51	.08	1.58	.14	.82	.21
Without hyaluronidase incubation[b]	.48	.09	2.49	.36	.79	.28

[a] Average of four isolations from jejunal tissue.
[b] Average of three isolations from jejunal tissue.
[c] Recovery did not differ ($P < .84$)
[d] Enrichment differed ($P < .05$).
[e] Enrichment did not differ ($P < .93$).

Characterization of Isolation Procedure. In an effort to observe the fate of enterocyte membrane components, a pre-trial study characterized each step of the isolation procedure in terms of protein recovery, ALP enrichment, and Na^+/K^+-ATPase enrichment. As expected, ALP, a brush border enzyme, was ultimately enriched while Na^+/K^+-ATPase, an enzyme of the basolateral membrane, was ultimately reduced (Table 2).

Characterization of Brush Border Membrane Vesicles. The relative activity of ALP, an indicator of vesicle purity, increased in vesicles prepared from both jejunal and ileal mucosal tissue, while the relative activity of Na^+/K^+-ATPase, an indicator of vesicle contamination, was reduced (Table 3). The Na^+/K^+-ATPase enrichment value for BBMV prepared from jejunal mucosal tissue, however, was influenced by difficulty in accurately measuring Na^+/K^+-ATPase activity. This difficulty resulted from incomplete homogenization in samples taken before the first low-speed centrifugation step. Nevertheless, the enrichment ratio shows the vesicles to be composed primarily of brush border membrane with little contamination from basolateral membrane.

Table 2. Characterization of Each Step of Isolation Procedure[a,b]

Fraction	Fate	Protein recovery (%)	ALP specific activity (units/mg prot)	ALP enrichment	Na^+/K^+-ATPase specific activity (units/mg prot)	Na^+/K^+-ATPase enrichment
Homogenate	Begin	100	.72	-	.15	-
Cent. 1 supernatant	Keep	58.4	.31	.44	.02	.14
Cent. 1 pellet	Discard	not solubilized	-	-	-	-
Cent. 2 supernatant	Discard	50.35	.19	.26	.008	.06
Cent. 2 pellet	Keep	5.82	1.33	1.87	.08	.55
Cent. 3 supernatant	Discard	1.55	.87	1.21	.08	.49
Cent. 3 pellet	Keep	3.76	1.53	2.15	.12	.89
Cent. 4 supernatant	Keep	1.31	1.82	2.59	.11	.67
Cent. 4 pellet	Discard	2.61	1.37	1.90	.06	.79
Cent. 5 supernatant	Discard	.22	.93	1.34	.05	.35
Cent. 5 pellet	Keep	.80	2.00	2.80	.23	1.45
Cent. 6 above band[c]	Discard	.01	.85	1.35	0	0
Cent. 6 pellet[c]	Discard	.46	2.26	3.61	.28	1.4
Brush border band	Keep	.36	1.20	1.70	.07	.45

[a] Average of two isolations from jejunal tissue, unless otherwise noted.
[b] Performed using tissue harvested with hyaluronidase incubation. See section pertaining to "Changes to Tissue Harvest Procedure." [c] Values from only one isolation.

Table 3. Characterization of Brush Border Membrane Vesicles

	ALP		ALP		Na^+/K^+-ATPase		Na^+/K^+-ATPase		ALP:Na^+/K^+-ATPase
Tissue	SP ACT (units/mg prot)	S E	Enrich.	S E	SP ACT (units/mg prot)	S E	Enrich.	S E	Enrichment ratio
Jejunal[a]	2.20	.10	3.04	.11	.08	.006	.99[b,c]	.14[b,c]	3.1:1[b]
Ileal[d]	.28	.02	2.39	.12	.10	.03	.43[e]	.04[e]	5.56:1

[a] Average of 23 isolations, unless otherwise noted.
[b] Homogenate samples used in calculation of enrichment contained chunks of tissue and, therefore, Na^+/K^+-ATPase assay was not accurate.
[c] Average of 19 isolations, due to two missing values and two outliers which were removed before calculating the mean.
[d] Average of 30 isolations, unless otherwise noted.
[e] Average of 27 isolations, due to one missing value and two outliers which were removed before calculating the mean.

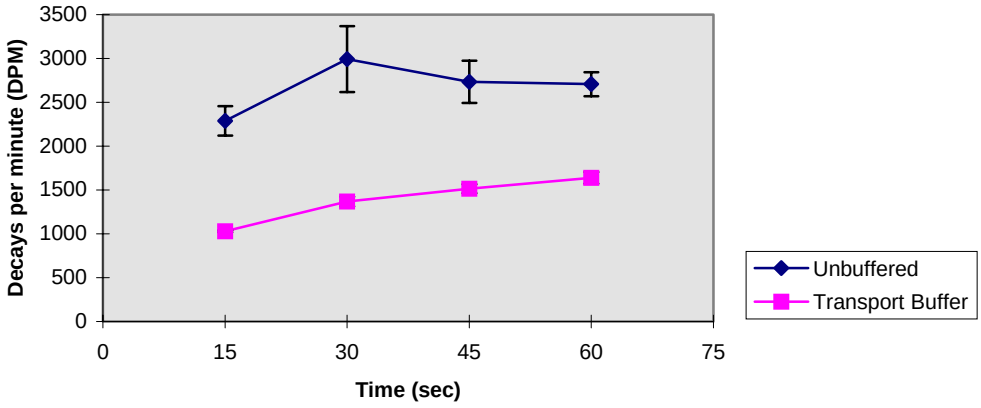
Experiments Prior to Uptake Study. Elevated uptake values were noted at manifold positions further from the vacuum source and, therefore, only the first three positions on the manifold were used for the uptake study. Scintillation vials with filters were fully stabilized and ready for counting after 4 h. Filters leaning at an angle within scintillation vials, as opposed to flat on the bottom, did not interfere with counting.

Previous work with this uptake procedure in our laboratory was done using individual amino acids as substrates (Jang, 1993). Because amino acid transport across the brush border is not a H^+-dependent process (Webb and Matthews, 1994), the stop solution used for the amino acid studies was not buffered (Wilson and Webb, 1990a; Jang, 1993). Peptide uptake, however, is considered to be a H^+-dependent process and, therefore, a buffered stop solution is generally used (Ganapathy et al., 1981), even though it is the temperature of the solution, rather than its components, that inhibits uptake. The

ice-cold unbuffered stop solution used in the previous amino acid work (150m*M* KCl, .5m*M* Phloridzin) was tested against ice-cold mannitol-transport buffer (305m*M* mannitol, 20m*M* Hepes, 2m*M* $MgCl_2$ at pH 7.5) used as a stop solution (Figure 1). The two stop solutions differed ($P < .0001$), with the buffered solution showing more of the desired straight-line response and smaller standard errors. The reason for the high values seen with the unbuffered stop solution is unclear at this time.

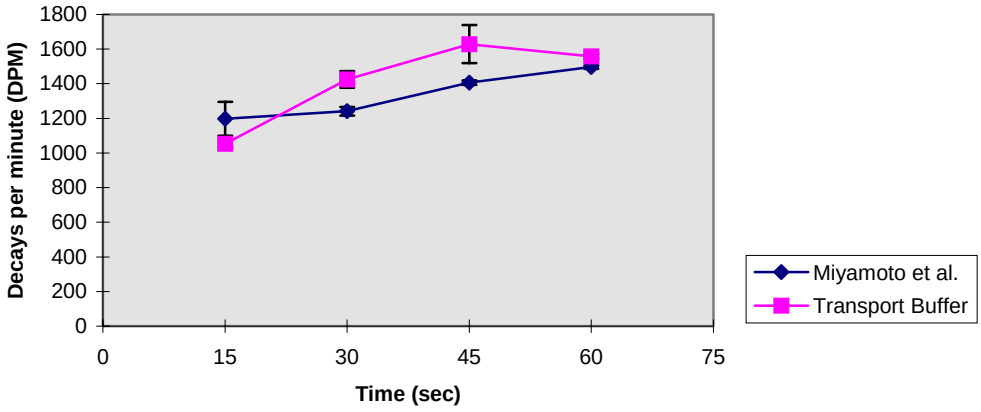
Ice-cold mannitol-transport buffer was further tested against the ice-cold stop solution (210 m*M* KCl, .5m*M* Hepes, .5m*M* Tris at pH 7.5) used by Miyamoto et al. (1989; Figure 2). The two stop solutions were not different ($P < .08$) and, therefore, based on current literature, the Miyamoto et al. (1989) stop solution was chosen to be used in the uptake study.

Figure 1. Unbuffered Stop Solution vs. Transport Buffer as Stop Solution[a,b,c,d]



[a] Composite of ileal tissue from four animals.
[b] Extravesicular pH 6.4.
[c] Each point is mean of three observations.
[d] Stop solutions differed ($P < .0001$).

Figure 2. Miyamoto et al. (1989) Stop Solution vs. Transport Buffer as Stop Solution[a,b,c,d]



[a] Composite of jejunal tissue from two animals.
[b] Extravesicular pH 6.4.
[c] Each point is mean of three observations.
[d] Stop solutions not different ($P < .08$).

Uptake Study. The majority of the variation in this experiment was due to differences among animals (Appendix A), however, there were also differences due to tissue site, time, and pH (Table 4). Significant effects are dealt with individually in Figures 3, 4 and 5.

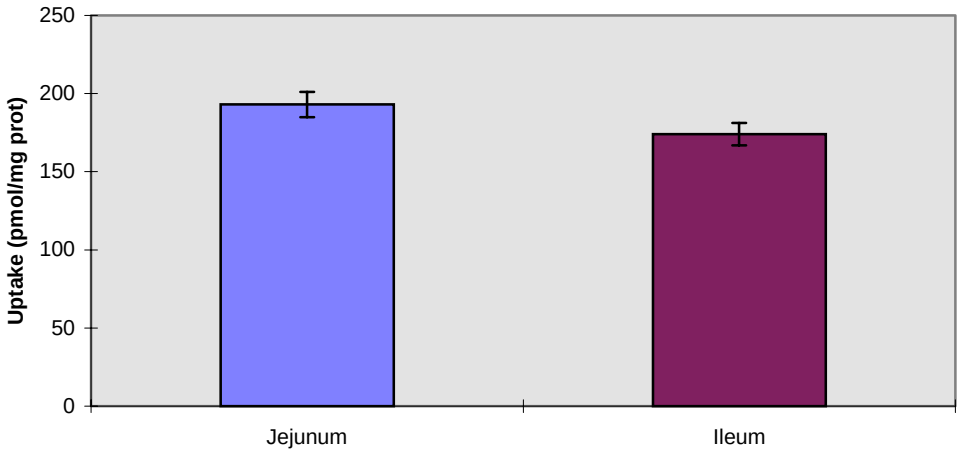
Table 4. Uptake of Glycyl-Sarcosine by Brush Border Membrane Vesicles[a,b]

pH[c]	Time[d] (s)	Site[e] Jejunum (pmol/mg prot)		Ileum (pmol/mg prot)		Mean across sites (pmol /mg prot)	
6.4	15	171.78		148.93		160.36	
	30	202.93		180.66		191.80	
	45	212.47		209.57		211.02	
	60	215.75		189.12		202.44	
			X=200.73		X=182.07		X=191.41
7.0	15	152.91		138.38		145.65	
	30	185.86		170.02		177.94	
	45	209.15		173.66		191.41	
	60	205.23		171.86		188.55	
			X=188.29		X=163.48		X=175.89
7.5	15	166.00		164.34		165.17	
	30	183.09		177.93		180.51	
	45	204.88		180.76		192.82	
	60	209.14		181.89		195.52	
			X=190.78		X=176.23		X=183.51
		X=193.27		X=173.93			

[a] 120 observations from five animals. Each observation is mean of three replicates.
[b] Values include background binding to membranes and filters.
[c] Quadratic effect of extravesicular pH ($P < .04$).
[d] Quadratic effect of time ($P < .005$).
[e] Sites differed ($P < .02$).

Greater uptake ($P < .02$) was seen in BBMV from jejunal intestinal tissue than from ileal (Figure 3). This is not surprising since peptides are broken down to free amino acids as they travel the length of the intestine. A greater concentration of peptides would be presented to absorptive cells in the proximal portion of the intestine and, therefore, this region appears to have more peptide transport capability. The distal portion of the intestine is the site of the majority of free amino acid transport (Phillips et al., 1976, 1979; Moe et al., 1987) and, therefore, less peptide transport capability would be expected in this region because of the decreased concentration of peptides presented to absorptive cells.

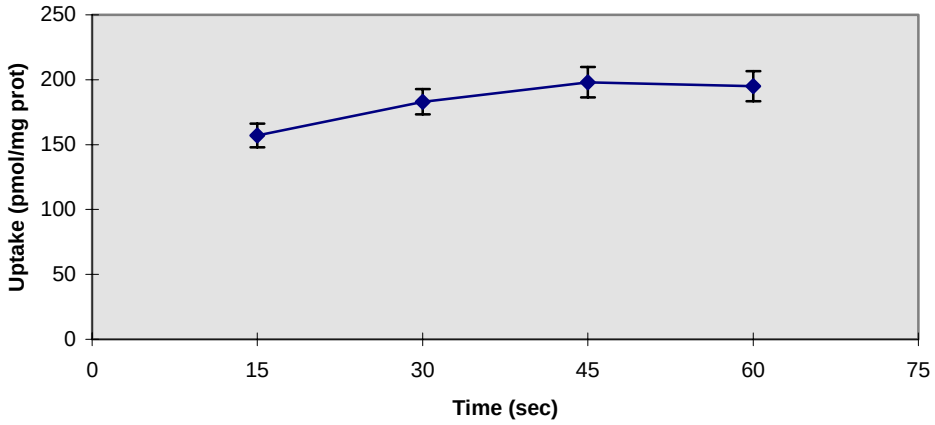
Figure 3. Effect of Tissue Site on Glycyl-Sarcosine Uptake[a,b,c,d]



[a] 120 observations from five animals. Each observation is mean of three replicates.
[b] Values include background binding to membranes and filters.
[c] Consolidation of data for pH 6.4, 7.0, and 7.5 at 15, 30, 45, and 60 s.
[d] Sites differed ($P < .02$).

Uptake plateaued after 45 s, resulting in a quadratic ($P < .005$) effect of time (Figure 4). The time course study does not establish whether observed uptake is a result of a membrane-associated carrier mechanism or of diffusion (Stremmel and Berk, 1986). Kinetic and competitive inhibition studies must be conducted in order to elucidate the method of uptake. Because these studies need to be limited to the linear portion of the uptake curve, to be sure that a unidirectional translocation process is being measured (Stremmel 1988a, b), future work should be conducted at incubation periods of 15 s or less. Incubation periods of less than 15 s, however, are extremely difficult with the techniques used in the present study.

Figure 4. Time Course of Glycyl-Sarcosine Uptake[a,b,c,d]

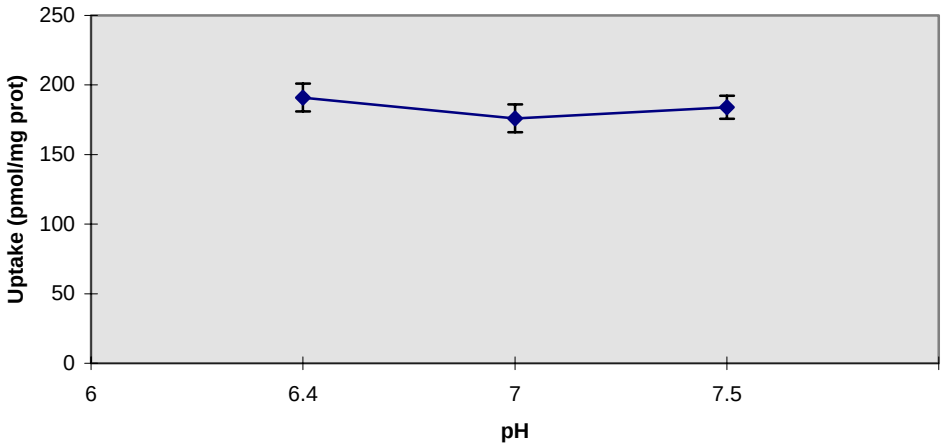


[a] 120 observations from five animals. Each observation is mean of three replicates.
[b] Values include background binding to membranes and filters.
[c] Consolidation of data for jejunal and ileal tissue at pH 6.4, 7.0, and 7.5.
[d] Quadratic effect of time ($P < .005$).

There was no evidence of the overshoot phenomenon characteristic of active transport into brush border membrane vesicles. The overshoot response typically consists of a rapid influx phase representing accumulation against a concentration gradient followed by a slow decline until equilibrium is reached (Stremmel et al., 1992). If dipeptides are actively transported with H^+, an overshoot is expected when a large proton gradient is imposed across the membrane (Hoshi, 1985). The present study, however, is not unique in its lack of demonstration of the overshoot phenomenon. Unlike renal brush border membrane studies, in which overshoot values for H^+-dependent peptide uptake exceed equilibrium values by severalfold (Daniel et al., 1991), no transient accumulation is generally observed in intestinal brush border membrane studies even though many of these studies indicate that peptide transport is stimulated by the presence of H^+ (Ganapathy et al., 1984; Calonge et al., 1990).

A quadratic effect ($P < .04$) of extravesicular pH was seen, with pH 6.4 showing the greatest uptake, pH 7.0 showing the least, and pH 7.5 intermediate between the two (Figure 5). If peptide transport is a H^+-dependent process, uptake would be expected to decrease as the pH of the external buffer increases.

Figure 5. Effect of Extravesicular pH on Glycyl-Sarcosine Uptake[a,b,c,d]



[a] 120 observations from five animals. Each observation is mean of three replicates.
[b] Values include background binding to membranes and filters.
[c] Consolidation of data for jejunal and ileal tissue at 15, 30, 45, and 60 s.
[d] Quadratic effect of extravesicular pH ($P < .04$).

A subsequent study evaluating uptake at extravesicular pH 5.5 compared to pH 7.5 was also conducted (Table 5). There was no effect of pH ($P < .81$). The lack of evidence for H^+-dependence in the present study may have been due to any of a number of things. The vesicle preparation procedure used in the present study had not previously been employed to study peptide uptake and, while glucose and amino acid transporters appear to survive this procedure well enough, the same may not hold true for the H^+-dependent peptide transporter. Also, the vesicles in the present study may have been leaky for H^+, causing the gradient to dissipate (Ganapathy et al., 1984). Additionally, low activity of the transporter may have hidden the H^+ effect (Miyamoto et al., 1991).

Table 5. Uptake at Extravesicular pH 5.5 Compared to pH 7.5[a,b,c]

pH	Uptake (pmol/mg prot)	S E (pmol/mg prot)
5.5	151.39	5.45
7.5	152.28	6.8

[a] Mean of two replicates at each of three time periods (15, 30, and 45 s) using jejunal tissue from a single animal.
[b] Values include background binding to membranes and filters.
[c] No effect of pH ($P < .81$).

The possibility that peptide transport may not actually depend on a H^+ gradient must also be considered. Some research fails to show H^+ gradient stimulation of peptide uptake (Rajendran et al., 1987; Reshkin and Ahearn, 1991), while other research has found that the H^+ gradient effect varies with substrate being transported and with experimental animal being studied (Iseki et al., 1989). Cytosolic peptidase activity

maintains a downhill peptide gradient across the brush border of enterocytes (Webb et al., 1992) and, therefore, the use of the H^+ gradient to drive transport may not be necessary. It is interesting to note that in *Xenopus laevis* oocyte studies, the incorporated foreign transporter shows H^+-dependence while the endogenous peptide transporter fails to show this dependence (Miyamoto et al., 1991; Fei et al, 1994).

These conflicting results are reminiscent of early work examining intestinal peptide uptake, some of which seemed to indicate a Na^+-dependency while some did not (Boyd and Ward, 1982; Himukai et al., 1983; Cheeseman and Devlin, 1985). Later, it was found that Na^+ was necessary for operation of the Na^+/H^+-antiport, which assists with intracellular pH recovery, rather than for peptide transport itself (Ganapathy and Leibach, 1983; Burston and Matthews, 1987; Daniel et al., 1994).

It is possible that pH could play an indirect role, through maintenance of substrates in zwitterionic form, rather than a direct role in peptide transport. It is also possible that intestinal peptide transport is able to operate downhill, driven by the transmembrane substrate gradient as well as uphill using an electrochemical H^+ gradient (Reshkin and Ahearn, 1991; Daniel et al., 1994). The kidney, in fact, appears to have two types of transporters, one of which operates in the presence of and the other of which operates in the absence of a pH gradient (Daniel et al., 1991, 1992).

At first glance, the results of the present study concerning effect of extravesicular pH conflict with the results reported by Backwell et al. (1995) from a study conducted on ovine duodenal tissue, which showed that transport is stimulated by an extravesicular pH of 6.0 compared to an extravesicular pH of 8.4 when vesicles are loaded with a pH 8.4 internal buffer. It must also be noted that the study reported by Backwell et al. (1995) was very limited and that the extravesicular pH of 6.0 in that study may not actually have been 6.0 as claimed. When 20 μL of the pH 8.4 vesicle solution was added to 100 μL of the pH 6.0 uptake buffer, the resulting pH would likely have been closer to 7.0 than to 6.0. Furthermore, an extravesicular pH of 8.4 would not be encountered physiologically and would, in fact, cause many of the peptide molecules to be negatively charged. Because peptide molecules need to be in zwitterionic form in order to be transported (Ganapathy and Leibach, 1985), the results of the Backwell et al. (1995) study may show a reduction in uptake due to the charged nature of the peptide substrate at pH 8.4, rather than an enhancement of uptake in the presence of a pH gradient.

Both the Backwell et al. (1995) study and the present study, however, show that a portion of the products of protein digestion absorbed from the ovine small intestine is in the form of small peptides. Because the capability for peptide absorption appears to be greater in the proximal portion, proteins which are more easily hydrolyzed to small peptides by intestinal peptidases may be more likely to be presented to peptide transporters. Proteins which are not as easily homogenized may not be able to be reduced to peptide form until they have already moved past the region of greatest peptide transport, making it more likely that they would need to be broken down to individual amino acids in order to be absorbed. If both amino acid and peptide uptake are active transport processes, the cost of restoring the Na^+ gradient, per amino acid, is greater for free amino acid uptake than for peptide uptake. Differences in the ease with which proteins are hydrolyzed to small peptides may help explain why two proteins composed of the same amino acids, but in different sequences, can vary in their biological value.

Implications

Based upon the results of this study, it appears that the ruminant small intestine has the ability to absorb intact dipeptides along its entire length, although the proximal half has somewhat greater capability than the distal. The lack of evidence for increasing uptake with decreasing extravesicular pH, however, is disturbing in light of the current literature. It is possible that the procedure used in the present study damaged the peptide transporter and(or) resulted in vesicles that were leaky for H^+ and, therefore, is not appropriate for further peptide transport studies.

Chapter IV

Epilogue

Observed uptake into BBMV can be due to transport into the intravesicular lumen and(or) to binding on either membranes or filters (Hopfer et al., 1973). In an effort to determine the amount of binding to membranes and filters, stop solution was added to reaction vessels prior to the addition of the BBMV solution. An effect of time ($P < .009$) was seen (Table 6) but no effect of extravesicular pH was observed ($P < .15$). It must be noted that this was a small data set and that the model accounted for only 46% of the variation in the data.

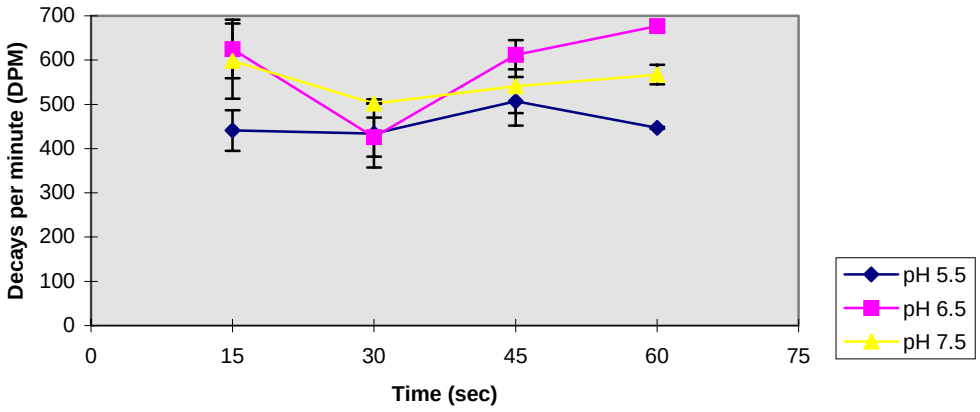
Table 6. Background Binding to Membranes and Filters - Effect of Time[a,b]

Time (s)[c]	Decays per minute (DPM)[d]	S E
15	610[e]	32
30	580	44
45	750	42
60	725[e]	32

[a] Composite of jejunal tissue from five animals.
[b] Consolidation of data for pH 5.5, 6.5, and 7.5.
[c] Linear effect of time ($P < .009$).
[d] Mean of nine observations at each time period, unless otherwise noted.
[e] Mean of eight observations due to missing values.

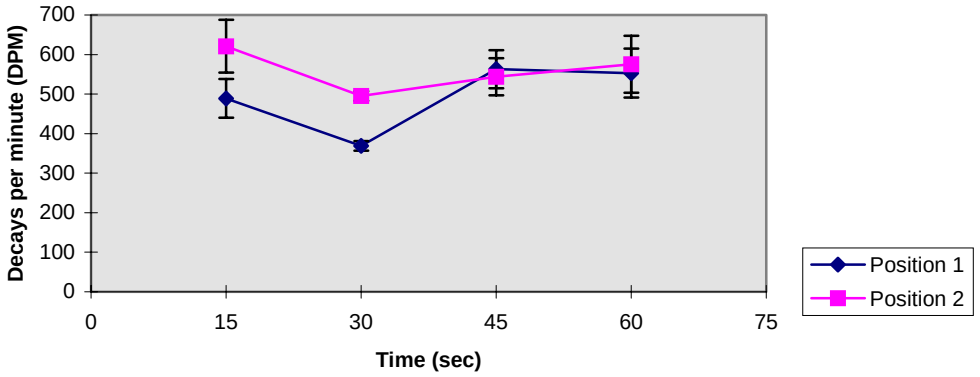
In a further effort to characterize background binding, binding to filters was determined in the absence of BBMV. A pH by time interaction ($P < .08$; Figure 6) and a manifold position by time interaction ($P < .05$; Figure 7) were noted. While 96% of the variation in the data was accounted for by the model, this was a very small data set and more extensive work needs to be conducted in the future. It is worth noting, however, that background binding to filters, and hence to membranes and filters combined, was generally lowest at 30 s. The reason for this is unclear, but merits future attention.

Figure 6. Background Binding to Filters - pH by Time[a,b,c]



[a] No vesicles in reaction vessels.
[b] Each point is mean of two observations, except pH 7.5 at 30 s which is a single observation.
[c] pH*Time interaction ($P < .08$).

Figure 7. Background Binding to Filters - Manifold Position by Time[a,b,c,d]



[a] No vesicles in reaction vessels.
[b] Each point is mean of three observations, except manifold position 1 at 30 s which is mean of two observations.
[c] Consolidation of data for pH 5.5, 6.5, and 7.5.
[d] Manifold Position*Time interaction ($P < .05$).

A corrected value for glycyl-sarcosine uptake can be obtained by subtracting background binding from values determined in the uptake study (Table 7). While the background binding experiment itself did not show a significant effect of pH ($P < .15$), overall background binding to membranes and filters was lower at pH 6.4 than at pH 7.5. This, combined with somewhat greater uptake at pH 6.4 compared to pH 7.5, causes a higher corrected uptake for pH 6.4 relative to pH 7.5. The corrected uptake

values could be interpreted as a trend toward increased uptake with decreasing pH, or it could be a result of the percentage of peptide in zwitterionic form. Because the isoelectric pH of glycyl-sarcosine is 5.68 (Ganapathy et al., 1984), a greater percentage of peptide will be in zwitterionic form at pH 6.4 than at pH 7.5. Because the peptide needs to be in zwitterionic form in order to be transported (Ganapathy and Leibach, 1985), more of it will be available to be transported at pH 6.4 than at pH 7.5. While the majority of the peptide molecules will still be zwitterions at pH 7.5, a greater percentage will carry a negative charge than at pH 6.4. The charged molecules would be more likely to bind to the membranes and filters.

Table 7. Subtraction of Background Binding from Glycyl-Sarcosine Uptake[ab]

pH	Time (s)	Uptake[d] (pmol/mg protein)	Background binding[e] (pmol/mg protein)	Corrected uptake (pmol/mg protein)
6.4[c]	15	171.8	80.2	91.6
	30	202.9	74.4	128.6
	45	212.5	88.1	124.4
	60	215.8	99.7[f]	116.1
7.5	15	166.0	84.2	81.8
	30	183.1	89.1	94.0
	45	204.9	103.6	101.3
	60	209.1	111.7	97.4

[a] Background binding experiment performed on a different day than any of the uptake studies.
[b] Uptake data from jejunal tissue used to calculate corrected uptake because background binding values solely for jejunal tissue.
[c] Used values for background binding to membranes and filters at pH 6.5.
[d] Each value is mean of three observations on jejunal tissue from each of five animals.
[e] Each value is mean of three observations on jejunal tissue combined from five animals, unless otherwise noted.
[f] Mean of two observations.

Further work using the procedures in the present study should separate duodenal tissue from jejunal tissue in an effort to more accurately characterize intestinal peptide uptake. The question of the role of protons in the peptide transport process, however, may not be able to be answered using the experimental approach of the present study. The process of BBMV preparation alters the membrane physically and, possibly, chemically. The lack of internal components and reactions of the cell may also affect the transport process. Other approaches, such as the *X. laevis* oocyte work, in which intact cellular machinery is present may be more beneficial for elucidating the finer points of the peptide transport system.

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APPENDIX A

General Linear Models Procedure for Uptake Study

Dependent Variable: UPTAKE

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	31	357727.83	11539.61	15.29	.0001
Error	88	66424.99	754.83		
Corrected Total	119	424152.83			

R-Square	C.V.	Root MSE	UPTAKE Mean
0.84	14.96	27.47	183.60

Source	DF	Type I/III SS	Mean Square	F-Value	Pr>F
Anim[a]	4	301937.10	75484.28	100.00	0.0001
Site[a]	1	11219.71	11219.71	14.86	0.0002
Anim*Site[a]	4	2494.92	623.73	0.83	0.5119
pH	2	4818.39	2409.19	3.19	0.0459
Time	3	31969.02	10656.34	14.12	0.0001
pH*Time	6	1697.79	282.96	0.37	0.8932
Site*pH	2	533.61	266.80	0.35	0.7032
Site*Time	3	1210.23	403.41	0.53	0.6599
Site*pH*Time	6	1847.07	307.84	0.41	0.8720

[a] Because of Split Plot (RCBD,RCBD) Design with Anim as highest level block, Anim and any Anim interactions cannot be tested. Site, being the block beneath Anim block, can be tested as seen below.

Tests of Hypotheses using the Type III MS for Anim*Site as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr>F
Site	1	11219.71	11219.71	17.99	0.0133

Orthogonal Contrasts

Contrast	DF	Contrast SS	Mean Square	F Value	Pr>F
Linear Time	1	25477.95	25477.95	33.75	0.0001
Quadratic Time	1	6426.52	6426.52	8.51	0.0045
Linear pH	1	1384.36	1384.36	1.83	0.1791
Quadratic pH	1	3434.03	3434.03	4.55	0.0357

APPENDIX B

HARVEST OF BRUSH BORDER MEMBRANE (1-12-95)

Procedure

Several days in advance, order animal by # and set delivery time.

Check space in basement.
- Room 58: clean, work table present, scales to weigh animal available.
- Room 48: clean, lots of counter space.

On morning of harvest, unlock room and make sure pen available for delivery.
- Make sure feeder is in pen so animal cannot jump out.

Weigh and mark beakers for mucosal tissue and leftovers.

Get ice trays set up before beginning procedure.
- Two surgical trays with ice between; one set in Room 58 and two sets in Room 48.
- Replenish ice as needed.

Measure and record animal weight.

Shave hair on animal's neck.

Anesthetize animal with sodium pentobarbital (approx. 30 mL).

Exanguinate animal.

Open abdominal cavity carefully and quickly.
- Hole with knife, then scissors. Careful not to cut intestine.

Tie beginning of duodenum and end of ileum with labeled strings.
- Can tie ileum after remove mesentery and find it.

Remove entire intestine by carefully tearing the mesenteric membranes.
- In surgical tray with ice in tray below.
- Tear starting at duodenum.

Divide intestine into two halves. The proximal half will be referred to as the jejunum and the distal half will be referred to as the ileum.

Keep jejunum separate from ileum for rest of procedure.

Segment each half into lengths of approx. 30 cm.

Open each piece of intestine by cutting lengthwise with scissors.

Wash intestine using four successive baths of cold Mannitol Buffer.
- Be very gentle so not lose mature cells.
- Let drip after each wash. Do not squeeze.

Scrape segments with a glass slide to harvest mucosal cells.

In surgical trays with ice in tray below.
Two people work on jejunum while two people work on ileum.

Place harvested cells in glass 600 mL beakers.
KEEP BEAKERS IN ICE SLURRY.

Save leftovers in 1000 mL plastic beakers.

Measure and record mucosal cell weights (jejunum and ileum).

Mix by stirring beakers of mucosal cells with glass rods.

Divide mucosal tissue into 35 to 40 g aliquots, place in labeled bags and seal.
Keep as much air out as possible.
Not need to mark weight on bag unless it is outside range.

Freeze bags of tissue in liquid nitrogen.
If leave in liquid nitrogen too long, bag shatters.

Store in dry ice until reach ultralow (-80^{o}C) freezer.
Thaws easily.

Measure and record leftover weight (jejunum and ileum).

Solutions Used in Harvest Procedure

Mannitol Buffer

Compound	MW	m*M*	g/L
Mannitol	182.2	300	54.65
Trizma Base	121.1	12	1.45

Adjust pH to 7.4 with HCl. (Approx. 2 mL 4 N HCl for 1 L.)
Store in carboy in walk-in cooler.

Saline Solution
Dissolve .9 g NaCl in 100 mL distilled water.

Sodium Pentobarbital Solution
Dissolve 3.25 g pentobarbital (sodium salt) in 50 mL saline solution. Store at Room T in serum bottle in locked cabinet.

Chemicals Used in Harvest Procedure

Company	Catalog #	Compound	MW
Sigma	M4125	Mannitol	182.2
Sigma	T1503	Trizma Base	121.1
Fisher	S271	Sodium Chloride (NaCl)	58.44
Sigma	P3761	Pentobarbital (sodium salt)	248.3

Equipment for Harvest Procedure (for one animal)

Assistants (4-6)
Paper towels (1 package)
Latex gloves, assorted sizes for self and assistants
Beakers (weigh and label as jejunum or ileum): 2 glass 600 mL for tissue, 2 plastic 1000 mL for leftovers
Surgical trays (6)
Ice (full cooler)
Paper to record animal #, animal weight, jejunal mucosal cell weight, ileal mucosal cell weight, jejunal leftovers weight, ileal leftovers weight, and other observations of note.
Clippers
Extension cord for clippers
Sodium pentobarbital solution
Needles (2)
Syringe
Knives (box from Don)
Labeled strings to tie beginning and end of small intestine
Scissors (2+)
Mannitol Buffer (8 L/animal)
Carboy (for Mannitol Buffer)
Eight polypropylene containers for Mannitol Buffer washes (label four for jejunum and four for ileum)
Glass slides for scraping (4)
More Beakers: 2 glass 250 mL to hold bags on scales, 2 plastic 2000 mL for ice slurry, 2 extra plastic 1000 mL
Glass rods to stir tissue
Balances (2)
Small plastic bags labeled with animal #, name, date, jejunal or ileal tissue (approx. 15 of each)
Tweezers (2) to load bags
Sealing device (4 pieces) for bags
Liquid nitrogen (approx. 5 L)
Dewar, gloves and set of grabs for freezing with liquid nitrogen
Dry ice in appropriate container
Large labeled plastic bag to hold small bags of tissue in ultralow (-80^{o}C) freezer
Pyrex dish for carrying hot sealing device
Bone saw
Bags for carcass (6+)

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APPENDIX C

ISOLATION OF BRUSH BORDER MEMBRANE VESICLES (5-11-95)

Can do two isolations simultaneously.

Preparation

On day before procedure, check liquid nitrogen supply.

Refrigerate both rotors for Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge overnight.

Procedure (Keep <u>everything</u> in ice slurry at <u>all</u> times.)

Adjust centrifuge temperature (red knob at -2^{o}C and blue knob at +2^{o}C).

Put rotor for large tubes in centrifuge.

Posts on bottom of rotor should **not** line up with holes on spindle.
Spin by hand clockwise to make sure goes easily.

Put bottle of Mannitol-Succinate Buffer in ice slurry.

Label assay tubes and add detergent solutions.

Label with animal number, assay type, date, jejunal or ileal, H or BB (homogenate or brush border).
(See below for size of tube and amount and type of detergent for each particular assay.)
Keep in ice slurry until needed.

Thaw tissue in warm water until still cool, but not frozen.

Tissue bag is inside zip-lock bag.
Mix frequently to make sure it thaws evenly.

Put on ice.

Weigh tissue in 600 mL beaker.

Suspend 35 to 40 g of tissue in 245 to 280 mL of Mannitol-Succinate Buffer (7 mL buffer/g tissue).

Stir by hand with glass stir bar and then homogenize with model PT 10/35 Brinkman Polytron Homogenizer (Westburg, NY) equipped with a 20 mm diameter generator (PTA20S), for 20 sec at setting six. Rinse with Mannitol-Succinate Buffer.

Gets foamy. Pre-homogenize at low speed and then use Level 6 for just barely 20 sec.
Not work well if tissue wraps around generator.
When finished, clean homogenizer with warm water and with ethanol (3 times for 30 sec with each).

Stir gently for 30 min with stir bar at level 3.5 (on Multi Stir 9 magnetic stirrer, Bellco Biotechnology, Vineland, NJ).

Beaker with sample is in 2000 mL beaker of ice water.

[While stirring, remove 2 mL sample from beaker and homogenize with 12 strokes of a glass-glass homogenizer. Use this homogenized sample to make assay tubes.

Enzyme assays (esp. Na^+/K^+-ATPase) do not work well on samples with large pieces of tissue. We have not yet tried this. It may or may not solve the problem of samples with large pieces of tissue.]

Make tubes for assays (duplicates).

Make sure pipetter really getting correct amount. Clumps clog tip.

ALP (in 1.5 mL Fisherbrand microcentrifuge tube, 05-407-10): 200 µL Triton-X-100 in Mannitol-Succinate Buffer + 200 µL sample. Freeze in liquid nitrogen and take to ultralow (-80°C) freezer.

Na^+/K^+-ATPase (in 1.5 mL Fisherbrand microcentrifuge tube): 900 µL Imidazole Incubation Buffer + 100 µL sample. Freeze in liquid nitrogen and take to ultralow (-80°C) freezer.

Protein (in 0.6 mL Fisherbrand microcentrifuge tube, 05-407-16): 100 µL homogenate. Goes in conventional freezer. No liquid nitrogen.

After stirring, measure and record volume. (Add volume used to make assay tubes.)

Some of the foam goes away while stirring, therefore, can measure volume more accurately after stirring.

Pour so not make foam.

Centrifuge the homogenate at 2°C and 8,700 *x* g (7,300 rpm) for 12 min.

Use 2 large tubes.

Balance tubes by using Mannitol-Succinate Buffer.

Put tubes in, set time and rpm, start.

Let get up to speed (adjust if necessary) and set time exactly.

Turn brake to "on".

When take out samples, turn brake to "off".

Collect the supernatant.

Use 8 smaller tubes.

Keep cold. Be quick.

All not have to weigh same, but ones across from each other do.

Weigh all with lids on.

Change to small rotor. Posts on bottom of rotor should **not** line up with holes on spindle.

Centrifuge at 2°C and 31,000 *x* g (16,000 rpm) for 15 min.

If doing two isolations simultaneously, need to stagger this step.

Discard the supernatant carefully.

Resuspend the pellet in Mannitol-Succinate Buffer.

Wash 3 times with Mannitol-Succinate Buffer. Use disposable pipets. Keep pipet in Mannitol-Succinate Buffer separate from pipet used for washing. Do not let pieces of pellet get in bulb of pipet.

Do not use too much buffer.

Transfer to tube for teflon-glass homogenizer. Tube is in drawer below homogenizer.

Homogenize with 12 strokes (up and down) of a teflon-glass homogenizer (Size 24 Tissue Grinding Tube and Pestle, Kontes Scientific Glassware/Instruments, Vineland, NJ).

Wipe grease off pestle before using.

Go slow and keep tube in plastic bag of ice.

(InSurk Jang did not do this step.)

Centrifuge at 2°C and 31,000 *x* g for 15 min.

If only doing one isolation, use tube of water for balance.
(Supernatant cloudy; leaving stuff in it?)

Discard the supernatant with pipet.

Resuspend the pellet in Mannitol-Succinate Buffer

Use disposable pipets. Keep pipet in Mannitol-Succinate Buffer separate from pipet used for washing. Do not let pieces of pellet get in bulb of pipet.
Wash three times. Use plenty of buffer.
Transfer to tube for teflon-glass homogenizer.

Homogenize with 12 strokes (up and down) of teflon-glass homogenizer. Go slow and keep in bag of ice.

Stir the resuspended sample gently for 30 min.

30 mL beaker with sample is in 1000 mL beaker of ice water, with stir bar at level 2.5 (on Multi Stir 9 magnetic stirrer, Bellco Biotechnology, Vineland, NJ).
30 mL beaker should be a little over half full; add more Mannitol-Succinate Buffer if needed.

While stirring, put rotor (SW40Ti) and buckets for ultracentrifuge in Dr. Herbein's walk-in refrigerator, make sure spacer disk is on bottom of rotor, check oil level in ultracentrifuge and make sure overflow pan not full.

After stirring, centrifuge at 2°C and 8,700 *x* g (8,500 rpm) for 12 min.

Have at least 43 g in tubes.
Leave brake off.

Collect the supernatant.

Supernatant = milky white.
Be careful. Use disposable pipet.
Very difficult.

Centrifuge at 2°C and 31,000 *x* g for 15 min.

While centrifuging, turn on ultracentrifuge (two switches together).

Low needle always approximately 2°C; high needle approx. 10°C.
Isolation speed = 24,300 rpm.
Take balance and set up on counter near ultracentrifuge.
Open back door to lab.

Discard the supernatant very carefully.

Use pipet.

Resuspend the pellet in **Mannitol-Transport Buffer** (approximately 4 mL divided among three washes).

Transfer to tube for glass-glass homogenizer.

Homogenize with 12 strokes of a glass-glass homogenizer (7 mL Pyrex Tissue Grinder, Corning Inc., Corning, NY).

In ice slurry.
Flutter plunger a few times first to draw large pieces off bottom.
Keep plunger from touching anything until after rinsing homogenizer with additional 1 mL of buffer.

Put sample in graduated cylinder to determine how much sucrose needed.

Put approx. 7.0 mL of 31% Sucrose Solution into an ultracentrifuge tube (13 mL, Beckman, 344060) with care. Add to extra tube also when doing one isolation.

Take everything to ultracentrifuge (tubes with sucrose and sample covered with parafilm in a container of ice, disposable pipets, racks for holding and weighing tubes, Mannitol-Transport Buffer, marking pen.

Place the final homogenized solution onto the surface of the 31% Sucrose Solution. Care should be taken to avoid mixing the two layers at the interface.

Avoid making bubbles.
Weigh tubes and balance them.
If only doing one isolation, add water to extra tube to balance.
All buckets go on rotor, but not all have to have something in them.

Centrifuge for 90 min at 105,000 *x* g (24,300 rpm) in Beckman L5-75B ultracentrifuge using SW40Ti rotor.

Give small clockwise turn when put rotor on spindle.
Set time.
Push start button.
Will not start until temperature in range.
Speed goes up after vacuum kicks in.
Buttons = auto, slow brake, auto.
Balance light = on; accel. = fast.

Check on ultracentrifuge after 30 min to make sure everything working properly.

Label assay tubes and add detergent solutions (as above). Label 3 cryovials (2 mL, Nalge Company, 5000-0020) for storage of BBMV.

Mark cryovials with animal #, tissue type, I. D. #, and date.
Keep assay tubes in ice slurry until needed.

Harvest the membrane band and sucrose layer under the band.

Take to Dr. Herbein's lab: forceps, beaker with ice slurry, small beaker for waste, several pipets, graduated cylinder, parafilm, swabs
Get rid of as much of aqueous layer above membrane band as possible.
Harvest membrane band and as much of sucrose layer below the band as possible into graduated cylinder. Keep in ice slurry.
Discard pellet.
Wash and dry (Kimwipes around swabs) buckets that held samples; dry centrifuge and un-used buckets.

Check and record volume.

Solution should be clear-ish?

Invert 10 times to homogenize. [Better method might be to homogenize using glass-glass homogenizer?]

Make tubes for assays (as above). Store rest of BBMV in cryovials.

Record volume stored in each cryovial.

For everything except protein tubes, freeze in liquid nitrogen and store in ultralow (-80^{o}C) freezer; protein tubes go directly into conventional freezer.

The degree of purification of BBMV can be determined by assaying for the marker enzymes alkaline phosphatase and sucrase (non-ruminants). The degree of contamination of isolated BBMV can be determined by assaying for the marker enzymes Na^{+}/K^{+}-ATPase (basolateral membrane), acid phosphatase

(lysosomes), NADP-cytochrome c reductase (microsomes), cytochrome c oxidase (mitochondrial membrane), and lactate dehydrogenase (cytosol).

Solutions Used in Isolation Procedure

Mannitol-Succinate Buffer

Compound	MW	m*M*	g/L
Mannitol	182.2	150	27.35
Succinate	118.1	30	3.55
Trizma Base	121.1	10	1.2
$MgCl_2$	203.31	5	1.0
K_2HPO_4	174.2	5	0.87
$MnCl_2$	197.9	0.1	0.02

Adjust pH to 7.4 using NaOH.
1 L enough for 1 isolation.
Good approx. 3 weeks.
Store pH probes in electrode solution because need ions; dH_20 water not have ions.

2X Buffer (4 m*M* Hepes and 2 m*M* $MgCl_2$ when make 31% Sucrose Solution because diluted by 1/2.)

Compound	MW	m*M*	g/L
Hepes	238.3	8	1.91
$MgCl_2$	203.31	4	0.81

Adjust pH to 7.4 with NH_3OH.
250 mL will do a lot of isolations.
Remake when make mannitol buffers.

31% Sucrose Solution

% Sucrose	Sucrose (g)	2X Buffer (mL)	Total Volume (mL)
31	31	50	100

Sucrose solutions become contaminated easily. **Make new solution on weekly basis.**
Use dried sucrose (50^{o}C for 5 h).
Start with approx. 35 g in a beaker. Cover with aluminum foil with holes. Keep in desiccator after dry.
Dissolve sucrose in 2X Buffer in a beaker.
Bring up to 100 mL with ultra-pure water.

Mannitol-Transport Buffer

Compound	MW	m*M*	g/L
Mannitol	182.2	305	55.6
Hepes	238.3	20	4.8
$MgCl_2$	203.31	2	0.41

Adjust pH to 7.4 with NH_3OH.

Adjust osmolarity to 331 mOsm with distilled water.

Automatic Osmometer (Osmette A, Precision Systems, Inc.):

Lift probe and remove its tube. Add fluid until overflows into petri dish.
Turn to "operate" and let come to temperature.
Put 2 mL of high standard in a regular tube, push probe and tube down slowly, when "read" light on turn to "special" and adjust with knob I to 500.
Tube should get fluid on bottom when pushed down.
Change back to "operate". Carefully raise probe and remove tube.
Dry two sections of probe with Kimwipe. Let probe come back to room temperature.
Put 2 mL of low standard in a tube, lower probe and tube, when "read" light on turn to "special" and adjust to 100 with knob II.
Change back to operate. Carefully raise probe and remove tube.
Dry two sections of probe with Kimwipe. Let probe come back to room temperature.
Test 2 mL sample, raise probe, remove tube, dry probe and let come to room temperature. Calculate and add amount of water needed to bring solution to 331 mOsm; mix.
Check 2 mL sample of diluted solution (as above).
When finished, turn off machine, wash probe, put probe tube in machine, lower probe.

Good approx. 3 weeks.
200 mL will do a lot of isolations.

2% Triton-X-100 in Mannitol-Succinate Buffer

See Alkaline Phosphatase Assay

Imidazole Incubation Buffer

See Na^+/K^+-ATPase Assay

Chemicals Used in Isolation Procedure

Company	Catalog #	Compound	MW
Sigma	M4125	Mannitol	182.2
Sigma	S7501	Succinate	118.1
Sigma	T1503	Trizma Base	121.1
Fisher	M33	Magnesium Chloride ($MgCl_2$)	203.31
Sigma	P3786	Potassium Phosphate (K_2HPO_4)	174.2
Sigma	M3634	Manganese Chloride ($MnCl_2$), tetrahydrate	197.9
Sigma	H3375	Hepes	238.3
Sigma	S9378	Sucrose	342.3

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APPENDIX D

PROTEIN ASSAY (11-18-94)

Principles

Protein concentration is determined using the BCA (bicinchoninic acid) Protein Assay Reagent Kit (Pierce). In this assay, proteins react with alkaline copper II to produce copper I. BCA then reacts with copper I to form an intense purple color at 562 nm. TCA precipitation precedes the BCA Protein Assay to remove sugars (especially sucrose used in the Isolation Procedure) from the samples. The reducing properties of sugars interfere with the BCA Protein Assay. Bovine serum albumin (BSA) is used to establish a standard curve of protein concentrations.

Experimental Procedure

Protein is in brush border membrane and in contaminants.
Will be expressing specific activity of marker enzymes as U/mg protein.
Not on ice at all.

Heat water bath to 90^{o}C.
Takes a while to reach 90^{o}C.
Verify temperature with thermometer.

Take samples out of freezer and thaw on counter.

<u>TCA Precipitation</u>

Add 20 μL of .125% DOC to 100 μL sample in .5 mL microcentrifuge tube. Vortex.

Sit on counter at Room T for 10 min.

Add 80 μL of 15% TCA. Vortex.

Centrifuge samples for 10 min using microcentrifuge (IEC Centra-M).
Tubes fit very tightly into plate with small holes.
Strong pellet.

Discard supernatant completely with pipets. Almost all can be removed.

Add 400 μL of .1 N NaOH to the precipitated protein.

Using water bath at 90^{o}C, heat sample to solubilize the pellet .
Vortex occasionally (approx. every 20 min).
BBMV done first so take out and sit on counter until H ready.
Takes approximately 45 min for BBMV and 3 h for H.

Let come to room T.

<u>BCA Protein Assay</u>

Make .4 mg/mL BSA Standard.

For the standard curve, make the following dilutions in 1.5 mL microcentrifuge tubes:

	0	80	160	240	320	400 μg/mL
.4 mg/mL sol'n	0	40	80	120	160	200 μL
.1 N NaOH	200	160	120	80	40	0 μL

Make BCA Working Reagent.

Dilute 100 μL of sample using .1 N NaOH as diluent (in 1.5 mL microcentrifuge tube). Put NaOH in first.
H: 10X; BBMV: 4X.
10X = 100 μL sample + 900 μL NaOH; 4X = 100 μL sample + 300 μL NaOH.
Clean pipet tip with solution.

Vortex tubes before transferring to plate.

Transfer 50 μL of each standard (in duplicate), or sample (in triplicate) into appropriate microtiter plate wells (Marsh Biomedical, 2-69620).

Add 200 μL of BCA Working Reagent to each well.
Use multichannel pipetter.
Tap plate gently on counter to get drops off sides of wells. Swirl to mix.

Cover microtiter plate and sit on counter at Room T for 2 h.

Turn on computer and microtiter plate reader (Titertek Multiskan MCC/340) and check paper supply in printer.
Warm up 15 to 20 min and initialize.

Read absorbance at 540 nm (because program not have filter for 562 nm) with microtiter plate reader.
Skansoft: 1 - 8 - 6 - 11 - 3 - put in plate - 1 - 7 - 6 - 1 - 5 - 9.
"Change filter" twice to make sure correct one is in machine.

Calculations

1. Determination of Protein Concentration (μg/mL):
   The linear portion of the standard curve is from 0 to 400 μg/mL. Intercept and slope of the standard curve can be computed using simple regression. The standard curve is used to determine the protein concentration (C) of each sample.

2. Determination of Total Protein:

   Protein (mg) = C * 4 * D * VF

   Where: C = Protein concentration (determined above and converted to mg/mL)
   4 = Initial dilution value (from NaOH addition)
   D = Dilution factor (10 for H; 4 for BBMV)
   VF = Volume of H or BBMV fraction measured during Isolation Procedure (mL)

3. Determination of Recovery Percentage:

$$\% \text{ Recovery} = \frac{\text{Total Protein of BBMV (mg)} * 100}{\text{Total Protein of H (mg)}}$$

4. Determine and record amount of protein stored in individual cryovials.

Solutions Used in Protein Assay

.125% (W/V) Deoxycholic Acid (DOC) Solution

Dissolve .125 g DOC in 100 mL distilled water. Store in refrigerator.

15% (W/V) Trichloroacetic Acid (TCA) Solution

Dissolve 15 g TCA in 100 mL distilled water. Store in refrigerator.

Weigh into beaker because melts weighing dish.

.1 N NaOH Solution

Dissolve 2 g NaOH in 500 mL distilled water. Store in refrigerator.

Make with acid washed glassware so can use for Na^+/K^+-ATPase assay.

.4 mg/mL BSA (Bovine Serum Albumin) Standard

Dilute the stock BSA solution (2 mg/mL) by emptying 1 mL vial into 30 mL beaker and adding 4 mL .1 N NaOH (= 5X dilution). Swirl to mix.

Wrap paper around bottle and break at thin part, below line.
Use pipet to get BSA out of vial.
Clean pipet tip with solution.
Bubbles easily. Bubbles = denature?
Prepare fresh daily?

BCA Working Reagent

Mix 30 mL Reagent A (= 50 parts) with .6 mL Reagent B (= 1 part). Prepare fresh daily.

Can do whole microtiter plate with this amount.
Make in deep half of petri dish.
Clean pipet tip with solution after adding reagent B to reagent A.
Slide tray in circular motion to mix.

Chemicals Used in Protein Assay

Company	Catalog #	Compound	MW
Sigma	D6750	Deoxycholic Acid (DOC), sodium salt	414.5
Fisher	A322	Trichloroacetic Acid (TCA)	163.39
Fisher	S318	Sodium Hydroxide (NaOH)	40.0
Pierce	23225	BCA Protein Assay Reagent Kit (Reagent A, Reagent B, Bovine Serum Albumin (BSA) Standard)	

References

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APPENDIX E

MEMBRANOUS ALKALINE PHOSPHATASE ASSAY (11-18-94)

Principles

Alkaline phosphatase (EC.3.1.3.1), a marker enzyme of brush border membrane, is analyzed using a Sigma Diagnostic Kit. Alkaline phosphatase (ALP) hydrolyzes p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate. P-nitrophenol has a maximum absorbance at 405 nm. The microtiter plate reader (Titertek Multiskan MCC/340) detects continuous increase in absorbance at 405 nm while the computer program "Micro-Kinetics" (version 1.0) verifies linearity of the enzyme reaction and computes the increase in absorbance. Specific activity will be expressed per milligram of protein.

Previous to Assay

During isolation procedure, samples were diluted 2X with Mannitol-Succinate Buffer containing 2% Triton X-100.

100 μL sample + 100 μL detergent = 200 μL total

Samples were stored in ultra-low freezer after freezing with liquid nitrogen.

Experimental Procedure

Keep on ice until otherwise noted.

Assay not designed for use on samples with large pieces of tissue.

Run blank before thawing to make sure correct filter is in plate reader.

Melt samples at Room T. Keep on ice thereafter.

Add 90% of the recommended volume of distilled water to the ALP Reagent. Dissolve by gentle swirling.

Two bottles do almost whole 96-well plate.

Room T 20 to 30 min to completely dissolve.

Temperature of the reaction mixture should be maintained at 24^{o}C.

Turn on computer (at outlet box) and microtiter plate reader (make sure light shows through side) and check paper supply in printer.

Vortex sample.

Dilute sample with distilled water (in 1.5 mL microcentrifuge tube). Put water in first.

Mark tubes before filling.

After sample goes into water, draw up to clean pipet.

For **Jejunum** - H: 100 μL sample + 900 μL water = 10X.

BBMV: 40 μL sample + 960 μL water = 25X.

For **Ileum** - 10X for H and BBMV.

Samples with large pieces of tissue show more variation among replicates.

Pipet 20 μL of diluted samples and of blank (H_2O) into microtiter plate wells (Marsh Biomedical,

2-69620).

Plate not on ice.
Vortex tubes before transferring to plate.
Run assay in triplicate. (If samples have large pieces of tissue, use five wells per sample and throw out highest and lowest values.)
Let come to Room T.

Transfer 180 μL of reaction mixture to each microtiter plate well when instructed to by computer.

Use multichannel pipetter. Test pipetter several times to make sure all pipet tips on properly.
Add by columns.
Reagent in pipetting reservoir.
Load while plate sitting on counter, tap plate on counter to get drops off sides of wells, swirl to mix, and place in machine. Be quick. No bubbles.

Read microtiter plate using "Micro-Kinetics".

After initial delay of 60 sec, read the increase in absorbance at 405 nm for 5 consecutive cycles (4 min).
Go through #'s 3, 4, 5, 6.
Disk = Han: Enzyme Kinetics.

Skansoft - initialize - end program (a) - b: - Han - 3 - info. - 4 - 5 - 60 - 60 - 5 - 5 - 2 - 6 - R - add reagent - reads - 8 - 7 - 9 - a: - turn off and cover.

Can change "time to add reagent" to 7 sec. if have full tray.
"Change filter" twice to make sure correct one is in plate reader.

Calculations

1. Determination of Conversion Factor (C):

Because using vertical spectrophotometer instead of horizontal.

$C = S/TV$ Where: S = Surface area of bottom of well (.342119 cm^2)
TV = Total volume (.2 mL)

2. Determination of ALP Activity:

$$T\ ACT\ (U) = \frac{A/min * C * 2 * D * TV * VF}{18.45 * SV}$$

Where: A/min = Change in absorbance per minute (from computer print-out)
C = Conversion factor (determined above)
2 = Initial dilution factor (Triton-X-100 addition)
D = Dilution factor (10 or 25, see experimental procedure)
TV = Total volume (.2 mL)
VF = Volume of H or BB fraction measured during isolation procedure (mL)
18.45 = Millimolar absorptivity of p-nitrophenol at 405 nm
SV = Sample volume (.02 mL)

One unit of ALP activity is defined as the amount of enzyme which will produce 1.0 μmole of p-nitrophenol per minute at 24°C.

3. Determination of Specific Activity:

$$\text{SP ACT} = \frac{\text{T ACT determined above (U)}}{\text{Total Protein as determined in Protein Assay (mg)}}$$

4. Determination of Enrichment:

$$\text{ENRICH} = \frac{\text{Specific Activity of BBMV (U/mg)}}{\text{Specific Activity of H (U/mg)}}$$

Solutions Used in ALP Assay

Mannitol-Succinate Buffer (see Isolation Procedure)

2% (V/V) Triton-X-100 in Mannitol-Succinate Buffer (for Isolation Procedure)
Dissolve 1 mL Triton-X-100 in 50 mL Mannitol-Succinate Buffer. Store in refrigerator.
Make Triton solution in beaker, not volumetric flask. Stir slowly to avoid bubbles.
Need: 100 mL beaker, storage bottle, stir bar, pipet.

Chemicals Used in ALP Assay

Company	Catalog #	Description
Sigma	245-10	Alkaline Phosphatase Procedure No. 245
Fisher	BP151	Triton-X-100 (liquid)

Reference

Bowers, G. N., Jr., and R. B. McComb. 1966. A continuous spectrophotometric method for measuring the activity of serum alkaline phosphatase. Clin. Chem. 12:70.

APPENDIX F

MEMBRANOUS Na^+/K^+-ATPase ASSAY (11-18-94)

Principles

Ouabain-sensitive Na^+/K^+-ATPase (EC 3.6.1.3) is used as a marker enzyme of the basolateral membrane. This enzyme catalyzes the hydrolysis of ATP to ADP and inorganic phosphate (P_i). Na^+/K^+-ATPase activity is determined using an end-point assay. The end-point product, P_i, is measured colorimetrically using Malachite Green. To determine the P_i released by Na^+/K^+-ATPase, P_i concentration in the presence of ouabain is subtracted from the total P_i concentration in the absence of ouabain. Specific activity is expressed as units of Na^+/K^+-ATPase activity per mg protein at 37°C.

Experimental Procedure

Glassware and plastic (no metal) should be acid washed to avoid phosphorus contamination (or use disposable).

Assay not reliable for samples with large pieces of tissue.

Accurate pipetting very important.

Use microtiter plates sideways so that all wells for each sample are read by the plate reader at approximately the same time.

Can do four sets of samples at once.

During isolation procedure, .1 mL sample was added to .9 mL of Imidazole Incubation Buffer (= 10X dilution).

Remove 10X diluted samples from ultralow freezer, thaw on counter, invert several times, and refrigerate for 24 h at 4°C.

(Han: During incubation, the enzyme becomes accessible for the substrate (ATP); Bovine Albumin minimizes loss in Na^+/K^+-ATPase activity.)

After approximately 22 h, dilute homogenate samples (H) to 50X.

Keep cold.

Add .1 mL of 10X diluted sample to .4 mL Imidazole Incubation Buffer. Use 1.5 mL microcentrifuge tube.

Clean pipet tip with solution.

Mix by inverting.

Take Histidine Buffer and NaOH out of refrigerator to warm up.

Make ATP Solutions With and Without Ouabain.

Make 4-fold diluted P_i Standard Solution.

Four-fold diluted = .3 mL Histidine Buffer + .1 mL P_i Standard (.645 μmole/mL) in 1.5 mL microcentrifuge tube. Clean pipet with solution and vortex.

Filter Malachite Green Working Solution and pour back into its jar.

For standard curve, pipet 0, 4, 8, 12, 16 and 20 μL of 4-fold diluted P_i Standard Solution into wells of a microtiter plate (Marsh Biomedical, 2-69620). Bring up to 20 μL with Histidine Buffer.

Use plate sideways.
Run standard in duplicate - once at top of plate and once at bottom.
After loading standard, put this plate aside until later.

Pipet 20 μL of ATP Solution Without Ouabain into six plate wells **of a second microtiter plate** for blank test.

Use plate sideways.
Is this really the proper solution to use for the blank?
Blanks are tested to calculate the spontaneous hydrolysis of ATP occurring during the reaction.

Put ATP Solutions in 37°C water bath.

PUT PLATE ON ICE SLURRY.

Mix sample tubes by inverting and pipet 20 μL of cold sample into six microtiter plate wells **of the second plate**.

Invert again after filling three of the wells.
Six wells per sample are required, three for triplicate analysis with ouabain and three for triplicate analysis without ouabain.
Alternate rows of H and BBMV.
(J. Wilson: Use supernatant. InSurk Jang: Not disturb pellet?, use refrigerated centrifuge if needed.)
Much of the Na^+/K^+-ATPase activity is in the large pieces of tissue; need to find way to homogenize sample.

Add 200 μL of preincubated (5 min in water bath at 37°C) ATP Solution With Ouabain or Without Ouabain to appropriate wells.

Sit tray on counter. **No ice.**
Three wells of each sample and of blank receive ATP Solution With Ouabain and three wells receive ATP Solution Without Ouabain.
Use multichannel pipetter. Use two small, brand-new petri dishes and put three pipets into each dish.

Tap plate gently on counter to get drops off sides of wells, swirl to mix, cover the microtiter plate with lid, and incubate in a water bath at 37°C for exactly 10 min.

Be careful not to let water into plate.

Immediately following incubation, put microtiter plate on ice slurry to slow down spontaneous hydrolysis of ATP occurring at low pH.

To stop the reaction, add 50 μL of ice-cold 20% TCA. Swirl to mix.

Be quick.
Use multichannel pipetter and pipetting reservoir.

Transfer a 20 μL aliquot of each well to an empty well **in the plate that contains the standard.**

Accurate pipetting is critical. For accuracy, do **not** use multichannel pipetter.
Keep everything on ice slurry.

Add 180 μL of Malachite Green Working Solution to the standards and to the transferred samples and blanks.

Use multichannel pipetter and pipetting reservoir.
The microtiter plate should be kept on ice slurry. Malachite Green Working Solution is very acidic and will induce spontaneous hydrolysis of ATP.

Keep the microtiter plate on ice slurry for 10 min with occasional swirling.

Water just barely reaching top of ice.

Turn on computer and microtiter plate reader (Titertek Multiskan MCC/340) and check paper supply in printer.

Read the absorbance at 620 nm (Han: 645 nm) using the computer program "Skansoft".

#1-8-7-11-3-put in-1-7-6-1-5-9
"Change filter" twice to make sure correct one gets put in machine.
Water/condensation should be wiped off the bottom of the microtiter plate before reading.
Run through twice, wiping off condensation each time.
Use first or second set of optical densities, preferably first.
NOTE: Sometimes plate looks fine but plate reader does not read it correctly. Especially on days when lab is hot/humid (air handling units off?)? Possible condensation inside plate reader when ice-cold plate is put in warm plate reader? By the time standard values come back to normal range, plate is warm enough that spontaneous hydrolysis of samples has begun. Refrigerated plate reader might help?

Calculations

1. Determination of P_i Concentration (μmoles/mL):

   The approximately linear portion of the standard curve is from 0 to .09675 μmoles/mL. Intercept and slope of the standard curve can be computed using simple regression. The standard curve is used to determine the P_i Concentration (C) of each sample and blank.

2. Determination of Na^+/K^+-ATPase Activity of a Sample:

$$\text{T ACT (U)} = \frac{[(C_t - B_t) - (C_O - B_O)] * D * TV * VF}{SV * 10}$$

Where:

C_t = P_i concentration in sample without ouabain (μmoles/mL)
B_t = P_i concentration in blank without ouabain (μmoles/mL)
C_O = P_i concentration in sample with ouabain (μmoles/mL)
B_O = P_i concentration in blank with ouabain (μmoles/ mL)
D = Dilution factor (50 for H; 10 for BBMV)
TV = Total volume (.27 mL)
VF = Volume of H or BBMV fraction measured during Isolation Procedure (mL)
SV = Sample volume (.02 mL)
10 = Reaction time (min)

One unit of Na^+/K^+-ATPase activity is defined as the amount of enzyme which will liberate 1.0 μmole of inorganic phosphate from ATP per minute at pH = 7.5 and T = 37°C.

3. Determination of Specific Activity:

$$\text{SP ACT} = \frac{\text{T ACT determined above (U)}}{}$$

Total Protein as determined in Protein Assay (mg)

4. Determination of Enrichment:

$$\text{ENRICH} = \frac{\text{Specific Activity of BBMV (U/mg)}}{\text{Specific Activity of H (U/mg)}}$$

Solutions Used in Na^+/K^+-ATPase Assay

ACID WASH dH_2O BEAKER FOR MAKING SOLUTIONS.

.025 *M* EDTA Stock Solution

Dissolve .93 g EDTA (InSurk: .221 g) in 100 mL distilled water. Store in refrigerator.

Acid Wash: 100 mL beaker, stir bar, 100 mL vol. flask, storage bottle.

.25 *M* Imidazole Stock Solution

Dissolve 3.404 g imidazole in 200 mL distilled water. Store in refrigerator.

Acid Wash: 250 mL beaker, stir bar, 200 mL vol. flask, storage bottle.

.111 *M* Potassium Chloride Stock Solution

Dissolve 4.142 g potassium chloride in 50 mL distilled water. Store in refrigerator.

Acid Wash: 50 mL vol. flask, 100 mL beaker, stir bar, storage bottle.

.167 *M* L-Histidine Stock Solution

Dissolve 12.933 g L-histidine in 500 mL distilled water. Store in refrigerator.

(2.5866 g L-histidine in 100 mL distilled water.)

Acid Wash: 500 mL beaker, 500 mL vol. flask, stir bar, storage bottle.

.333 *M* Magnesium Chloride Stock Solution

Dissolve 1.694 g magnesium chloride in 25 mL distilled water. Store in refrigerator.

Acid Wash: 50 mL beaker, 25 mL vol. flask, stir bar, storage bottle.

1.445 *M* Sodium Chloride Stock Solution

Dissolve 21.103 g sodium chloride in 250 mL distilled water. Store in refrigerator.

Acid Wash: 250 mL vol. flask, stir bar, storage bottle.

20% (W/V) Trichloroacetic Acid (TCA) Solution

Dissolve 20 g TCA in 100 mL distilled water. Store in refrigerator.

Weigh into beaker because melts plastic weighing dish.

Acid Wash: 100 mL vol. flask, 100 mL beaker, stir bar, storage bottle.

2% (V/V) Triton-X-100 Solution

Dissolve 2 mL Triton-X-100 in 100 mL distilled water. Store in refrigerator.

Stir slowly to avoid bubbles.

Acid Wash: 100 mL beaker, 100 mL vol. flask, stir bar, storage bottle.

Imidazole Incubation Buffer (.125 mg/mL DOC and 1% Bovine Albumin)

(1) Dissolve .0125 g deoxycholic acid (DOC) and 1 g of bovine albumin in distilled water. (2) Add 8 mL of EDTA Stock Solution to 20 mL of Imidazole Stock Solution. Mix (1) and (2) together and bring up to a final volume of 100 mL. Adjust to pH 7.0 with HCl. Store in refrigerator.

Use disposable pipets. Very easy to exceed volume. Go light on water.

Bovine albumin is sticky and denatures easily. Use stir bar gently. Bubbles = denature?

Takes very little 4 N HCl. Finish with .1 N HCl.

Acid Wash: 100 mL beaker, stir bar, 100 mL vol. flask, storage bottle.

L-Histidine Buffer

Add 5 mL potassium chloride stock solution, 2.5 mL magnesium chloride stock solution and 25 mL sodium chloride stock solution to 50 mL L-histidine stock solution. Bring up to a volume of 250 mL using distilled water. Store in refrigerator.

Use disposable pipets.

Acid Wash: 250 mL vol. flask, storage bottle.

4 N HCl Solution

Add 50 mL (one part) concentrated HCl (12 N) to 100 mL (two parts) distilled water. Store at Room T in acid cabinet.

Prepare in hood. Double glove. Pipet out of bottle with disposable pipet.

Acid Wash: 250 mL beaker, 100 mL vol. flask, glass rod, storage bottle.

2.5% (W/V) Ammonium Molybdate in 4 N HCl

Dissolve .625 g ammonium molybdate in 25 mL 4 N HCl.

Use disposable pipet.

Acid Wash: 50 mL beaker, stir bar.

.045% (W/V) Malachite Green Solution

Dissolve .0338 g malachite green in 75 mL distilled water.

Use disposable pipet for water.

Let stir for a few minutes to make sure it dissolves completely.

Acid Wash: 150 mL beaker, stir bar.

Malachite Green Working Solution

Mix three parts (75 mL) malachite green solution with one part (25 mL) ammonium molybdate in 4 N HCl. Stir at least 30 min. until color turns from dark brown to yellow. Filter using #40 Whatman filter paper. After filtration, add .2 mL of 2% Triton-X-100 Solution for every 10 mL of filtrate. Store at Room T in amber bottle wrapped with aluminum foil. Refilter before each use.

Can do six plates with this amount.

Acid Wash: 150 mL beaker (from above), funnel, 100 mL graduated cylinder, stir bar (from above), amber storage bottle and cap.

ATP in L-Histidine Buffer (without 1 m*M* ouabain)

Dissolve .1835 g ATP in 100 mL L-histidine buffer. Adjust pH to 7.5 at 37^{o}C with NaOH. Prepare solution fresh daily.

Be careful of static when weighing.

Use heating stir plate. Also need to heat Standard pH 7.0 Solution for pH meter. Adjust pH meter temperature setting to 37^{o}C. The Standard pH 7.0 Solution should be calibrated to pH 6.99 at 37^{o}C.

The pH starts at approx. 6.58.

Takes about 6 mL of .1 N NaOH (from Protein Assay).

Can run at least two plates per day with this amount.

Acid Wash: 100 mL vol. flask, 100 mL beaker, stir bar.

ATP in L-Histidine Buffer with 1 m*M* Ouabain

Dissolve .0364 g ouabain in 50 mL L-histidine buffer containing ATP (from above). Prepare solution fresh daily.

Double glove.

Can run at least two plates per day with this amount.

Acid Wash: 100 mL beaker (from above), 50 mL vol. flask, stir bar (from above).

Additional Acid Washed Items Needed During Assay:

Funnel and erlenmeyer flask for filtering, 30 mL beaker, 2 pipetting reservoirs.

NOTE: Pipetting reservoirs should only be left in 50% Concentrated Nitric Acid bath overnight because their acid resistance is rated as "fair".

Chemicals Used in Na^+/K^+-ATPase Assay

Company	Catalog #	Compound	MW
Sigma	A-4503	Bovine Albumin, fraction V powder	
Sigma	A-5394	Adenosine 5' Triphosphate, disodium salt	551.1
Fisher	A-674	Ammonium Molybdate	1235.86
Sigma	D-6750	Deoxycholic Acid (DOC), sodium salt	414.5
Sigma	ED2SS	EDTA, disodium salt, dihydrate	372.2
Sigma	H-8000	L-Histidine	155.2
Fisher	A144-212	Hydrochloric Acid, normality approx. 12.1	
Sigma	I-0125	Imidazole	68.08
Fisher	M-33	Magnesium Chloride	203.31
Sigma	M-6880	Malachite Green, oxalate salt	929.0
Sigma	O-3125	Ouabain, octahydrate	728.8
Sigma	661-9	Phosphorus Standard Solution (.645 μmole/mL)	
Sigma	P-4504	Potassium Chloride	74.55
Fisher	S-271	Sodium Chloride	58.44
Fisher	A-322	Trichloroacetic Acid (TCA)	163.39
Fisher	BP151	Triton-X-100	628

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APPENDIX G

INCORPORATION OF [^{14}C]-GLYCYL-SARCOSINE BY ISOLATED BRUSH BORDER MEMBRANE VESICLES (May 19, 1995)

Procedure for Membrane Transport Study and Radioactivity Safety Control
(Adapted from procedure of InSurk Jang, 7/14/92)

Principles

The purpose of this study is to measure uptake of peptides by isolated brush border membrane vesicles (BBMV). While muscle produces sarcosine, a derivative of methionine, Glycyl-Sarcosine is not a naturally occurring peptide and is non-hydrolyzable by peptidases.

Previous to Experiment

Prepare buffers.
Make sure stop watches available and functional. Batteries = #303 Eveready.
Dry down [^{14}C]-Gly-Sar to get rid of ethanol, resuspend to desired concentration, and aliquot in daily amounts (see attached Dry Down Procedure).
Create spread sheet so just have to plug numbers into computer.

Day Before Experiment

Put rotor for Beckman L5-75B ultracentrifuge in Dr. Herbein's walk-in refrigerator at least **24 h in advance**.
- Rotor = TY50.2Ti. Holds 12 (25 mL) tubes.
- Make sure spacer disk on bottom of rotor.

Decide how many cryovials to thaw.
- Only thaw as much as can use within 2 h of resuspension.
- # reaction vessels x .3125 mg prot/reaction vessel = mg protein needed

Label reaction vessels (12 mm x 75 mm disposable polystyrene culture tubes with plug caps, Scientific Products, T1226-52) in triplicate and put in plastic racks.
- When doing kinetics experiment, need six reaction vessels for 100 mM concentration.

Label six reaction vessels (7 mL scintillation vials with caps, Fisher, 03-337-1) for each background binding run. Put in plastic racks.
- Will run background binding for each pH and each different time period of uptake.
- Can also do background binding runs with Internal Buffer instead of BBMV Solution to determine how much is due exclusively to filters.

Put 20 mL scintillation vials (Fisher, 03-341-71A) into marked racks. Do not write on vials.
- One for each reaction vessel plus additional 6 for determination of specific activity.
- Labeling walls of scintillation vials causes aberrant counts.

Pour scintillation fluid (ScintiVerse BD, SX 18-4, Fisher) into dispensing bottle.
- If there is a precipitate present, it should settle to the bottom over time.
- Precipitate = secondary fluor; solution saturated. Should not affect counting.

Transfer aliquoted Radiolabelled Substrate Solution from freezer to refrigerator to allow thawing.

Morning of Experiment

Turn on ultracentrifuge (2 switches together).
Needs at least 1 h to come to temperature.
Low needle approx. 2°C; high needle approx. 10°C.
Check oil level in ultracentrifuge and make sure drip pan not overflowing.
Buttons = auto, slow brake, auto. Balance light on. Acceleration = fast.
Let [^{14}C]-Gly-Sar come to room temperature.
Bring water bath to 39°C.
Put paper towels near vortex for drying reaction vessels, and put extra sheets of absorbent paper on high-use counter work areas.

I Tissue preparation. (Takes approximately 3 h)
KEEP EVERYTHING IN ICE SLURRY unless otherwise noted.
When working with radioactivity, personal protection devices should be used (i.e., double gloves, safety glasses, lab coat with rubber bands around sleeves).
Not need to work in fume hood when using ^{14}C.

1. Remove needed cryovials from ultralow (-80°C) freezer and allow to thaw on counter.
Invert often.
When thawed, put in ice slurry.
(Can thaw in slightly warm water, if needed.)

2. While thawing, put 125 μL External Buffers into reaction vessels.
External Buffers differ from each other by concentration of Gly-Sar and(or) by pH.

3. Suspend BBMV in 10 volumes of Internal Buffer, pH 7.5.
i.e., 2 mL BBMV + 20 mL Buffer
Empty each cryovial into its own ultracentrifuge tube. Rinse cryovial with 1 mL buffer. Add additional 19 mL of buffer to each ultracentrifuge tube.
Dr. Akers has ultracentrifuge tubes and caps.

4. Weigh tubes with their caps; balance using buffer.
Not all have to weigh same, but ones across from each other do.

5. Centrifuge at 105,000 *x* g (29,500 rpm) for 60 min at 4°C to remove sucrose from BBMV.
Keep tubes in ice slurry until load into cold rotor.
Dry outside of tubes and load them into rotor while in walk-in refrigerator.
Give small turn when put rotor on.
Set speed by centering knob on 30K rpm.
Set time. Takes 10 min to come up to speed.
Temperature will rise for awhile. Wait until temperature starts to fall before pushing start button.
Check to make sure temperature stays in range while rotor is accelerating, or centrifuge will shut off.

6. While centrifuging, load 125 μL Radiolabelled Substrate Solution into reaction vessels.
Do not get drops on side of reaction vessel.
Final concentration in transport assay will be 2 μCi/.3 mL = 6.7 μCi/mL.

7. Put racks of reaction vessels into water bath.
Use lead to weight trays if they float.

8. Load 20 μL Radiolabelled Substrate Solution into each of five 20 mL scintillation vials, to verify actual daily activity.

Add 15 mL scintillation fluid using scintillation fluid dispenser. Swirl to mix.
Control = 20 μL ultra-pure water.

9. When centrifuge stops, discard the supernatant from ultracentrifuge tubes.
Done in walk-in refrigerator.
Take container with ice slurry, beaker for waste, several pipets, and swabs to walk-in refrigerator.
Tool for removing tubes from rotor is in cabinet with other ultracentrifuge supplies.
Dry centrifuge and clean and dry (Kimwipes around swabs) buckets of rotor.

10. Resuspend the pellet in Internal Buffer,
Amount of buffer = (.050 mL x # reaction vessels) + .2 mL.
Very little buffer available for resuspending BBMV and then rinsing homogenizer. Be efficient.
Transfer to glass-glass homogenizer tube (Pyrex Tissue Grinder, 7727-7, Corning).
Protein concentration should be approximately 6.25 mg/mL.
Will correct for differences in protein concentration when do calculations.

11. Homogenize using 12 strokes of glass-glass homogenizer.
Flutter plunger to pull pieces off bottom. Avoids getting pieces stuck to bottom of tube.

12. Put BBMV in centrifuge tube with screw-on cap and place in rack in 39^{o}C water bath.
Needs to all be below water level. Use lead to weight rack if necessary.
Takes approx. 15 min to come to temperature, with swirling. Do not vortex; causes bubbles.

13. Soak filters in shallow Rubbermaid container of stop solution at room temperature.
Keep filters right-side-up.
Filters brittle when dry.

II. Determination of [^{14}C]-Glycyl-Sarcosine Uptake
Alternate jejunal and ileal tissue so not start with same type each day.

1. Place stop solution in ice slurry.
1000 mL beaker of stop solution in 2000 mL beaker ice slurry for washes.
100 mL beaker of stop solution in pint container of ice slurry for filling 3 mL syringe.
All of stop solution needs to be below water level in ice slurry.

2. Place three .45 μm pore size nitrocellulose filters (HAWP 025 00, Millipore Corp., Bedford, MA) on manifold towers (Fisher, PVC vacuum manifolds, 87.6 x 12 x 17 cm, 09-753-39B) using forceps.
Filters need to be centered carefully.
Assistant 1 does this.
There is an effect of manifold position on uptake, therefore, in order to compare triplicates, only the first three towers should be used.

3. Add 50 μL of BBMV suspension to reaction vessel.
Desired pH reached when add to 250μL already in reaction vessel.
Swirl BBMV solution before each triplicate.
Blot water off bottom of reaction vessel with paper towels.
Do not start until Assistant 1 is ready.
Assistant 1 runs stop watch. Say "ready", "start" for Assistant 1.
Use fresh tip each time. Do not rinse pipet in BBMV solution.
Release BBMV on wall of container just above solution. Try not to touch side where ^{14}C has touched. Treat tip as contaminated.
Total reaction volume is 300 μL.
4. Vortex for 2 to 3 seconds.
Set vortex at level that will not allow liquid to make it more than halfway up tube.

This 2 to 3 sec is part of uptake period.
Dry vortex as needed.

5. Terminate the reaction after desired time with 3 mL ice-cold stop solution.
   Dispense stop solution by using 3 cc syringe.
   For short uptake periods (i.e., 15 s, 30 s), Assistant 2 is needed to load the syringe.
   3.3 mL now in reaction vessel.

6. Mix solution by drawing up 3X into 1 mL pipet.
   Be quick but avoid causing turbulence in pipet tip.

7. Remove two 1 mL aliquots from reaction vessel and vacuum filter on manifold.
   Manifold becomes contaminated during filtration. It should be cleaned externally and labeled for radioisotope use only. Clean absorbent paper should be kept under manifold.
   Vacuum apparatus consists of vacuum pump, hoses, two arm Erlenmeyer flasks (1^{st} to collect washes and 2^{nd} in case of overflow), and activated charcoal in-line particle removal filter (Gelman Acro 50, 45 μm)

8. Wash filters with three 5 mL aliquots or five 2 1/2 mL aliquots (use automatic pipetter) of ice-cold stopping solution.
   Assistant 1 washes.
   Make sure filter is vacuumed dry between washes.
   Washes being caught in Erlenmeyer. Be careful not to let it get too full.

9. Place the filters in liquid scintillation vials using curved tip, fine point forceps.
   Try not to contaminate forceps.
   Try to get filters in vials right side up?
   (Can dissolve filters with a special scintillation fluid if needed. (i.e., when using ^{3}H?))
   Assistant 1 takes off clamps and investigator puts filters into scintillation vials.
   Assistant 1 puts in new filters and reclamps. Towers have been washed with stop solution, therefore, consider clean and just put in fresh filter.

10. After all reactions finished, add 10 mL scintillation fluid to each vial containing a filter.
    Put vial caps on securely and mark them to correspond to what is in them.
    Allow vials to sit until filters become transparent.
    When transparent, mix well by swirling.
    Fluid should completely cover filter.
    Do not let bubbles get caught under filter.

11. Allow vials with filters and daily activity vials to stabilize overnight and then count for radioactivity using liquid scintillation counter (LS 5000 TA, Beckman Instruments, Fullerton, CA)
    Choose a user program (#4 for ^{14}C) and set time (2 min). Put calibration rack with "auto" card in first. Put card for user program in first rack of vials. Put "halt" rack in last. Make sure cards in rack are all the way down. Push "auto count." Enter the run into the log book.
    Do not throw away vials. ^{14}C stable so store in locked cabinet until whole experiment finished.

12. Put leftover Radiolabelled Substrate Solution from aliquot back into freezer.
    Can combine with other leftover amounts to be used later.

13. Do calculations after each uptake study until sure everything working properly.

14. Record in Radioactive Isotope Notebook.

III. Determination of Background Binding to Filters and BBMV

Subtract from everything.

1. Add 3 mL of stopping solution to six background reaction vessels (7 mL scintillation vials) **before** adding BBMV.
    Do at same time as everything else, but do six replicates instead of three.
    Using larger reaction vessels in order to vortex larger volume safely.

2. Remove 2 mL aliquot from each background reaction vessel. Filter and count as described above.

IV. Determination of Diffusion

The permeability constant is used to estimate the diffusion constant. Diffusion is determined by measuring the initial velocity of accumulation at a substrate concentration of 100 mM. The slope of the line formed by extrapolating uptake back to zero serves as the diffusion constant. Multiplying the diffusion constant by the substrate concentration yields an estimation of diffusion which would occur at that substrate concentration.
    Do at same time as do other concentrations, but do six replicates instead of three.
    Subtract background value before extrapolating uptake back to zero.

V. Clean-up

1. Clean up plastic/glassware with three successive baths of water (1000 mL water in each of 3 buckets).
    Third bath is distilled water.
    Dispose of water from first wash in liquid waste container from Radiation Safety Office (RSO).

2. Check out area with survey meter.

3. Carefully dispose of liquid waste left in reaction vessels and in erlenmeyer that collects washes.
    Very radioactive.
    Put in liquid waste container from RSO.

4. Use leftover water from second wash of plastic/glassware to rinse erlenmeyer.
    Dispose of in liquid waste container from RSO.
    If algae growing in erlenmeyer, kill by rinsing with Lysol. Do not use Clorox. Clorox interferes with scintillation counting.

5. Clean outside of erlenmeyer with decontaminating foam.

6. Count (1 min) liquid waste, and water in third wash and in water bath.
    1 mL liquid in 20 mL scintillation vial with 15 mL scintillation fluid.
    Use 1 mL distilled water for control.
    Mix by swirling.
    Let sit to stabilize before counting.
    If third wash comes out same as control, dishes clean and third wash water can be poured down drain.
    Use liquid waste value to do calculation for Radioactive Isotope Notebook.

7. Swipe area and equipment, count (1 min), record.
    Use piece of Kimwipe to swipe. Put in 7 mL scintillation vial with 5 mL scintillation fluid.
    Control = clean piece of Kimwipe.
    Be careful not to pass contamination from one swipe to the next.
    Use forceps for stuff that is known to be hot. Make sure forceps not contaminate subsequent swipes.
    Swirl to mix and let sit to stabilize before counting.
    Mark run in scintillation counter log book.
    Mark individual values and do calculations in radioactive isotope notebook.

Save printout in envelope in file drawer.

8. Decontaminate and re-swipe if needed.

9. Dispose of "swipe" vials in liquid scintillation vial container from RSO.

10. A 1.5 gal bucket lined with 2 plastic bags is used for bench top collection of solid waste. Put full plastic bags from 1.5 gal bucket into 20 gal barrel (supplied by RSO). Record additions on sheet of paper on lid of barrel. Put new plastic bags in bucket.

11. Call RSO for waste pick-up as needed.

Calculations

1. Determine protein concentration (mg protein/reaction vessel):
   For use in Uptake calculations.

$$\frac{\text{mg prot}}{\text{mL}} * \frac{.05\ \text{mL}}{\text{rx vess}} = \frac{\text{mg prot}}{\text{rx vess}}$$

   Where:
   - mg prot/mL = amount of protein thawed (as determined from Protein Assay)/ volume to which suspended after centrifugation (Should be approximately 6.25 mg/mL.)
   - .05 mL = volume of BBMV in Internal Buffer added to each reaction vessel

2. Determine specific activity (SP ACT) at each Gly-Sar concentration (pmol/dpm):
   For use in Uptake calculations.

   Daily SP ACT of Radiolabelled Substrate Solution

$$\frac{\text{ave dpm - ctr dpm}}{20\ \mu\text{L}} * \frac{125\ \mu\text{L}}{\text{rx vess}} = \frac{\text{dpm}}{\text{rx vess}}$$

   SP ACT at particular concentration of Gly-Sar

$$\frac{\text{mmol}}{\text{L}} * \frac{\text{L}}{1000\ \text{mL}} * \frac{1\text{x}10^{9}\ \text{pmol}}{\text{mmol}} * \frac{.3\ \text{mL}}{\text{rx vess}} * \frac{\text{rx vess}}{\text{dpm}} = \frac{\text{pmol}}{\text{dpm}}$$

   Where:
   - ave dpm = as determined from experiment
   - ctr dpm = control value as determined from experiment
   - 20 μL = volume of solution used for daily specific activity determination
   - 125 μL = volume of Radiolabelled Substrate Solution added to each reaction vessel
   - mmol/L = particular Gly-Sar concentration at which experiment conducted
   - .3 mL = volume in reaction vessel during experiment

3. Determine Uptake (pmol/mg prot) for each time period at each pH of a particular concentration:

$$\frac{\text{ave dpm - bk dpm}}{2\ \text{mL}} * \frac{\text{pmol}}{\text{dpm}} = \frac{\text{pmol}}{2\ \text{mL}}$$

$$\frac{\text{pmol}}{\text{2 mL}} * \frac{\text{3.3 mL}}{\text{rx vess}} * \frac{\text{rx vess}}{\text{mg prot}} = \frac{\text{pmol}}{\text{mg prot}}$$

Where:

ave dpm	= as determined from experiment
bk dpm	= average background binding as determined from experiment
2 mL	= sample volume put on vacuum tower
pmol/dpm	= determined above
3.3 mL	= volume in reaction vessel after addition of stop solution
rx vess/ mg prot	= inverse of value determined above

4. Plot uptake (pmol/ mg prot) vs. time (sec) for each pH of a particular concentration.

Solutions Used in Transport Study

Internal Buffer
Same as Mannitol-Transport Buffer from Isolation Procedure for BBMV.
Adjust pH to 7.5 and osmolarity to 331 mOsm.

Radiolabelled Substrate Solution
Suspend [^{14}C]-Glycyl-Sarcosine in ultra-pure H_2O so that concentration is 2μCi/125μL.

External Buffer

Compound	MW	m*M*	g/L
$MgCl_2$	203.31	4	0.8132
Mes	213.2	20	4.264
Hepes	238.3	20	4.766
Mannitol	182.2	610-x	(610-x) 182.2/1000
Gly-Sar	146.1	x	(x) 146.1/1000

Adjust mannitol and Gly-Sar to obtain solutions which will have final Gly-Sar concentrations of .075, .15, .3, .6, 1.2, 2.4, 4.8, 9.6 and 100 mM in the experiment.

Final concentration of Gly-Sar in the experiment will be .42 of sum of concentrations in External Buffer and Radiolabelled Substrate Solution.

Concentration of [^{14}C]-Gly-Sar in Radiolabelled Substrate Solution may or may not be negligible depending on specific activity.

Concentrations of $MgCl_2$, Mes and Hepes will be halved after combining External Buffer with Radiolabelled Substrate Solution.

Adjust pH with NH_3OH so that when External Buffer, Radiolabelled Substrate Solution and Internal Buffer are added together in experiment final pH will be 5.5 (External Buffer pH = 4.5), 6.5 (External Buffer pH = 6.0), or 7.5 (External Buffer pH = 7.5).

Adjust osmolarity to 662 mOsm.

Osmolarity will be halved after combining External Buffer with Radiolabelled Substrate Solution.

Stop Solution

Compound	MW	m*M*	g/L
Potassium Chloride	74.55	210	15.65

Hepes	238.3	0.5	0.12
Tris	121.1	0.5	0.06

Adjust pH to 7.5 at 4°C.

Chemicals Used in Transport Study

Company	Catalog #	Compound	MW
Sigma	M4125	Mannitol	182.2
Sigma	T1503	Trizma Base	121.1
Fisher	M33	Magnesium Chloride ($MgCl_2$)	203.31
Sigma	H3375	Hepes	238.3
Sigma	P4504	Potassium Chloride	74.55
Sigma	M5287	Mes, monohydrate	213.2
Sigma	G3127	Glycyl-Sarcosine	146.1
Moravek	MC100	[^{14}C]-Glycyl-Sarcosine	146.1

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APPENDIX H

DRY DOWN OF RADIOLABELLED SUBSTRATE (5/19/95)

Procedure

Takes at least 6 hours.
Personal protection devices should be used (i.e., double gloves, safety glasses, lab coat with rubber bands around sleeves).

Swipe and count surfaces of Dewar and styrofoam plug. If clean, take off tape and remove from "radioactive" hood.

Take Dewar, plug, and gloves for liquid nitrogen downstairs to Judy's lab.

Put Dewar on shelf in stainless steel container to cool for 10 min.

While waiting for Dewar to cool, return to "radioactive" hood and check oil level in pump, make sure belt moves, make sure there is a thin layer of grease with no foreign particles on sealing edges of desiccator, make sure cardboard holder and absorbent paper with hole(s) for vials are in desiccator and make sure waste bucket is in hood.

When Dewar is cool, fill with liquid nitrogen while sitting on shelf in stainless steel container.

Put styrofoam plug in Dewar and fill out liquid nitrogen log sheet.

Take Dewar to "radioactive" hood, remove plug, lower cold-finger into Dewar very slowly so it doesn't break when it gets cold.
Lay plug on counter where it will not get contaminated.

Put gauze around cold finger at top of Dewar and hold down with lead weights.

Get vial of [^{14}C]-Gly-Sar out of freezer. Mark starting level and desired finishing level on outside of vial with marker.
Use water and a clean vial to determine desired finishing level.
Throw cap from vial away.
In this particular case starting with 10 mL of 50 μCi/mL and drying to 3 mL.
Can count 1 μL in triplicate if not sure of starting specific activity. Swirl to mix before sampling.

Put vial in cardboard holder in desiccator. Hold absorbent paper down with lead weights.

Put lid on desiccator. Rotate to make sure seal is good (i.e., difficult to rotate)
Do not jostle vial.
Lid has lip to keep it in place.

Plug in pump. Oil level in window should come all the way up.
Pump might leak a little oil at first.

With desiccator valve off, hook vacuum hose to desiccator.
Pump will draw vacuum and be quieter.

Start opening valve on desiccator slowly.
Pump makes gurgling sound when drawing.

Open valve slowly until approximately halfway open (i.e., 45^{o} angle).
Opening valve too fast can cause solution to bubble and escape from vial.

Turn on hood to vent heat made by pump. Lower door of hood.

Check every minute at first and every 10 min later on, to make sure no bubbling.

Check progress of drying frequently. As progress slows, open valve a little more.
After opening more, check often to make sure not bubbling.
After several hours, valve will be all the way open.

Solution may or may not freeze after ethanol gone
For case of 40% ethanol, should be gone by the time there are only 3 mL left in vial.

When dry, turn off valve on desiccator, disconnect hose, unplug pump.

Let air slowly into desiccator through valve.

Open desiccator and put new cap on vial.

Use decontaminating foam on Kimwipes to decontaminate vial.

Remove vial from hood.
Not need to work in fume hood when using ^{14}C.

Remove cold-finger from Dewar, clamp to ring stand and let thaw into beaker.
Absorbent paper on arms of clamp.

When thawed, put 1 mL of liquid from cold finger into 20 mL scintillation vial with 15 mL of scintillation fluid. Swirl to mix. Let sit to stabilize. Count for 1 minute.
Control = 1 mL water + 15 mL scintillation fluid.

Measure volume of liquid remaining in cold finger with pipet and calculate μCi. Dispose in radioactive liquid waste container from RSO.
Should be very few μCi in liquid from cold-finger.

Swipe and count (1 min) everything in hood. Put bags from garbage bucket into solid waste container from RSO and record on sheet of paper on lid of barrel. Replace bags in garbage bucket.

When finished with all dry-down procedures, clean cold-finger with methanol. Count and dispose of liquid as described above.

Resuspension
2 μCi of [^{14}C]-Gly-Sar will be used per evaluation point. Swirl vial to mix before sampling.

Put 7 μL aliquot into each of four 7 mL scintillation vials. Add 5 mL scintillation fluid. Cap well. Swirl to mix. Let sit until it stabilizes. Count (1 min).
Use pipet tips with filters and swipe pipetter when finished.
Do not let scintillation fluid dispenser touch side of vial.
Control = scintillation fluid.

After counting, determine volume of dried down solution and calculate total μCi:

$$\frac{\text{ave dpm}}{7\ \mu L} * \frac{1000\ \mu L}{1\ mL} * \frac{\mu Ci}{2.22 \times 10^6\ dpm} * \text{remaining mL of solution} = \text{Total } \mu Ci$$

Determine and add amount of ultra-pure water needed to bring remaining solution to 16 μCi/mL
Add water with disposable pipet.
Remember to subtract remaining mL of dried down solution from amount determined.

In order to minimize the amount of freezing and thawing, aliquot resuspended solution into amounts needed for daily experiments.

Use disposable pipet.

Aliquot into 20 mL screw capped plastic scintillation vials.

(.125 mL x # reaction vessels) + .5 mL = amount aliquoted.

VITA

Sharon Hayden Bowers was born to Patricia and Thomas Hayden on February 1, 1963 in Chicago, Illinois. She graduated With Highest Distinction from Colorado State University in Fort Collins, Colorado with a Bachelor of Science degree in Biological Science on May16, 1987. She was granted a teaching certificate with a Secondary Science endorsement from the State of Colorado on July 16, 1987. Sharon married Michael R. Bowers on September 9, 1989 in Estes Park, Colorado. She initiated her program of graduate study at Virginia Tech in September, 1992. Sharon is a member of Phi Beta Kappa, Phi Kappa Phi and Sigma Xi.