DEMONSTRATION OF PEPTIDE AND FREE AMINO ACID ABSORPTION BY SHEEP FORESTOMACH EPITHELIUM USING PARABIOTIC CHAMBERS AND IDENTIFICATION OF H^+/PEPTIDE AND FREE AMINO ACID TRANSPORT PROTEINS IN SHEEP OMASAL EPITHELIUM AND $b^{O,+}$ AMINO ACID TRANSPORT PROTEINS IN PIG JEJUNAL EPITHELIUM BY EXPRESSION OF mRNA IN *XENOPUS laevis* OOCYTES

by

James C. Matthews

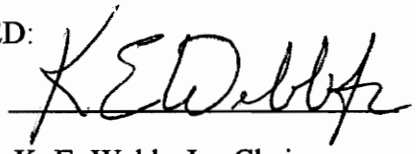
Dissertation submitted to the Graduate Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

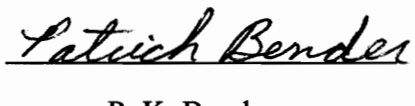
in

Animal Science

APPROVED:

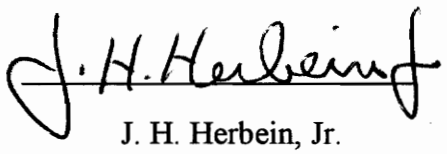


K. E. Webb, Jr., Chairman



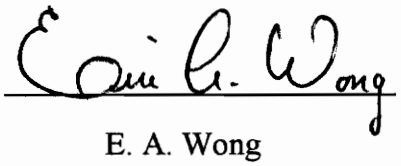
P. K. Bender

F. C. Gwazdauskas



J. H. Herbein, Jr.

E. T. Kornegay



E. A. Wong

April 1995

Blacksburg, Virginia

Key Words: Sheep, Pig, Forestomach, Peptide, Amino Acid, Biological Transport

DEMONSTRATION OF PEPTIDE AND FREE AMINO ACID ABSORPTION BY SHEEP FORESTOMACH EPITHELIUM USING PARABIOTIC CHAMBERS AND IDENTIFICATION OF H^+/PEPTIDE AND FREE AMINO ACID TRANSPORT PROTEINS IN SHEEP OMASAL EPITHELIUM AND $b^{o,+}$ AMINO ACID TRANSPORT PROTEINS IN PIG JEJUNAL EPITHELIUM BY EXPRESSION OF mRNA IN *XENOPUS IAEVIS* OOCYTES

by

James C. Matthews

Committee Chairman: K. E. Webb, Jr.

Animal and Poultry Science

(ABSTRACT)

The absorption of methionine and methionylglycine (Met-Gly) across sheep (average BW = 38 kg) ruminal and omasal epithelia was studied using parabiotic chambers. Ruminal tissue demonstrated a greater ability to accumulate both substrates. Omasal tissue demonstrated a greater ability to translocate methionine and Met-Gly and a greater total absorption of both. Intact Met-Gly was transferred across both tissues. More was hydrolyzed by omasal epithelia. Within tissues, the total absorption of substrates did not differ. Evidence for carrier-mediated absorption was not observed. The ability to express exogenous mRNA in defolliculated *Xenopus laevis* oocytes was developed using sucrose gradient size-fractionated poly(A)$^+$ RNA (RNA) isolated from the jejunal epithelial tissue of pig (average BW = 33.8 kg). Compared to water-injected oocytes, RNA injected oocytes displayed greater rates of Na^+-independent lysine and leucine absorption. RNA-induced uptake of lysine (K_t = 52 *uM*) and leucine (K_t = 97 *uM*) was inhibited by 5 m*M* leucine and lysine, respectively, by .2 m*M* cysteine, but not by 5 m*M*

glutamate. RNA-induced lysine and leucine absorption also was inhibited when oocytes were injected with RNA plus DNA oligomers that were complementary to the cloned human kidney $b^{o,+}$ transporter. Oocytes were injected with RNA isolated from omasal epithelial tissue of sheep (average BW = 67.5 kg) to identify potential peptide and amino acid transport proteins. Injection of specific RNA fractions induced greater rates of glycylsarcosine (Gly-Sar) uptake, as compared to water injection of oocytes. Media pH of less than 6.5 was required for induced Gly-Sar uptake. Induced Gly-Sar uptake required a pH of less than 6.5, was saturable (K_t = .40 m*M*), and was inhibited by 5 m*M* carnosine, Met-Gly, glycylleucine, but not by glycine. The RNA-induced Gly-Sar absorption was completely inhibited when oocytes were co-injected with RNA and DNA oligomers that were complementary to the cloned rabbit H^+/peptide cotransporter. When oocytes were assayed for their ability to absorb lysine, RNA-induced lysine absorption was determined to be Na^+-independent and to display $b^{o,+}$-like transport activity. Collectively, these results indicate that sheep omasal epithelia possess the potential to absorb free and peptide-bound amino acids by non-mediated processes and possess mRNA that encode for H^+-dependent dipeptide and $b^{o,+}$ transport protein activity. mRNA that encodes for $b^{o,+}$-like transport activity was identified in the jejunal epithelium of growing pigs.

ACKNOWLEDGEMENTS

To my advisory committee: It is with great respect and gratitude that I thank you all for your years of service on my behalf. I came to Tech to pursue my professional development in a challenging, yet open and cooperative environment. Your efforts have provided me with many opportunities to achieve this goal. I am very thankful.

I would like to especially thank Dr. K. E. Webb, Jr. for his unfailing support of my professional development through these many years. Because of his uncanny ability to foster a balance of professional guidance and personal friendship, my training under him has been both rewarding and enjoyable. I can only hope to repay my debt to him by emulating his example.

I am deeply indebted to Dr. E. A. Wong for inviting me into his lab, at both a physical and intellectual level. The exceptional training that I received from him allowed me to attempt and to accomplish my research.

I am also indebted to Dr. P. K. Bender for his many keen reviews and engaging discussions of my laboratory protocols and data. His interest in my research has been fundamental to its success.

I am grateful to Dr. J. H. Herbein, Jr. for his years of service on both of my advisory committees. His thoughtful and steady support of my professional and personal lives has been deeply appreciated.

I am grateful to Dr. F. C. Gwasdauskas for his encouragement to continue my training beyond a Master's degree and for his willingness to serve as a committee member.

I am grateful to Dr. E. T. Kornegay for his interest, encouragement, and long standing support of my professional development.

Most dissertational research is demanding, both professionally and personally. Mine was no exception. I am indebted to many faculty and staff members, and graduate students, for their professional conduct and friendliness through several trying periods.

I especially thank Kristin Lee and Donald Shaw, and Drs. Yuanlong Pan, In-Surk Jang, and Shiping Wang for their support and good humor.

I am indebted to Dr. Ludmila Sharova and Kiyoto Kurima for their friendship, many discussions, and help that they have provided, throughout my Ph.D. research.

I am very grateful to Dr. J. R. Bloomquist for teaching me how to measure the membrane potential of oocytes.

I would like to publicly thank Dr. Rashida Karmali (Rutgers University) for sharing with me her fascination of protein structure and function. Without her enthusiastic lectures, I would not have been exposed to the world of transport proteins, which so intrigues me.

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

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

LIST OF APPENDIX FIGURES

Figure

Chapter I

INTRODUCTION

How organisms obtain nourishment from their environments is a fundamental question of biology. Most eucaryotic cells meet their organic N requirements by absorbing amino acids. Intestinal absorption of small, dietary amino acids is now recognized as a major source of amino acid N in non-ruminants (Matthews, 1991a). That peptides may constitute a high proportion of the total amino acids absorbed by ruminants was suggested after very high concentrations of peptides were observed in the plasma of calves (McCormick and Webb, 1982) and after 79% of the total amino acids appearing in the portal circulation were observed to be in the form of peptides (Koeln et al., 1993). The hypothesis that forestomach epithelia absorption of peptides was responsible for contributing the majority of these peptides to the portal circulation was developed after a large net flux of peptide-bound amino acids was measured in the plasma of the non-mesenteric drained viscera, but not in the plasma of mesenteric-drained viscera (DiRienzo and Webb, 1995).

It is accepted that forestomach epithelia have the ability to absorb VFA, electrolytes, ammonia, and water (Van Soest, 1987). The ruminal epithelium of goats and sheep appears to possess the ability to absorb free amino acids (Cook et al., 1965; Bird and Moir, 1972; Leibholz, 1971b; Fejes et al., 1991). The ruminal and omasal epithelia of sheep also appear to possess the ability to absorb dipeptides, by apparently non-mediated processes (Matthews, 1991b). Historically, however, the significance of amino acid N absorption by forestomach epithelia has been regarded to be minor because of the calculation that the rumen was responsible for only 6% of total N absorbed by sheep (Leibholz, 1971a) and because of the known rapid metabolism of

amino acids by rumen microbes. As a consequence, ruminant nutritionists usually discuss the fate of dietary amino acid N in terms of its conversion to ammonia N, incorporation into microbial protein, and subsequent absorption by the small intestine after acid and enzymatic hydrolysis of microbial and dietary "by-pass" proteins (NRC, 1985b).

The quantitative importance of peptide absorption to the nutrition of monogastrics was recognized when mediated absorption of dipeptides was demonstrated using small intestinal loops (Matthews, 1983) and brush border membrane vesicles prepared from enterocytes (Ganapathy et al., 1984). The multi-layered morphological structure of forestomach epithelia, however, prevents the investigation of mediated transport activity with such techniques. The purpose of the present research was to investigate the potential mediated absorption of free and dipeptide amino acids across the epithelium of the sheep forestomach by the use of parabiotic chambers and by the functional expression of isolated forestomach epithelial mRNA by *Xenopus laevis* oocytes.

Chapter II

REVIEW OF LITERATURE

Mechanisms responsible for the selective absorption of free and peptide-bound amino acids have been identified and their function characterized in many species. With the advent of expression cloning techniques, specific proteins responsible for the absorption of amino acids are now being characterized. In ruminants, few amino acid transport mechanisms have been identified or characterized. Even less is known of the potential for free and peptide-bound amino acid absorption across forestomach epithelium. The purpose of this review is to describe mechanisms known to be involved in the absorption of amino acids, so that the potential for the function of similar mechanisms to exist in the forestomach epithelium of ruminants can be evaluated.

Energetics of Carrier-Mediated Transport.

Early biochemical characterization of amino acid absorption processes suggested that transport "systems" were involved in the selective movement of amino acids across gastrointestinal tract tissues. Subsequently, specific proteins have been identified that are thought to require the presence of an amino or imino group and a carboxyl group on the substrate for recognition for free amino acid transport (Spencer et al., 1962; Schultz et al., 1972). The recognition of substrates by peptide transport systems is thought to require the presence of a peptide bond (Sokol, 1990). For both, the size, charge, and(or) configuration of amino acid side chains act as important determinants for substrate transport (Christensen, 1984; Daniel, 1991). For

organisms to have evolved and maintained the biological mechanisms required for the selective (mediated) absorption of amino acids, the relatively high energetic cost of maintaining these mechanisms must have been a more efficient expenditure of energy than that required to maintain mechanisms necessary for de novo synthesis.

The energy for all carrier-mediated transport depends on cellular respiration. The coupling of respiration energy is typically divided into direct (primary) and indirect (secondary) processes. Primary transporters are energized by the direct transfer of chemical energy stored in high-energy phosphate bonds as with ATP hydrolysis to ADP and inorganic phosphate. In contrast, secondary cotransport proteins derive the energy to translocate substrates across membranes by absorbing the electrochemical potential generated (primarily) by transmembrane differences in the electrical and chemical gradients of the cotransported driving Na^+, K^+, and H^+ (Scarborough, 1985). The cell pays for the "free" ride of the solute by expending ATP to fuel the function of primary transporters (Na^+/K^+ ATPase; H^+ ATPase) that reestablish the extrcellular-to-intracellular ion gradients. Although not usually discussed in these terms, facilitative transport (driven by the osmotic gradient of the substrate) ultimately depends on the generation of membrane potential energy in order for transport protein domain movement within the membrane (see below).

The process of mediated transport of substrate molecule(s) through membranes is thought to occur in four distinct steps. After binding to the recognition domain of the transporter, substrates are translocated through the membrane interior and into the cell cytoplasm. Substrates and(or) driving ion then dissociate into the cytosol. Finally, the binding domain of the transport protein is reoriented into the extracellular fluid for future substrate binding. The amount of electrochemical energy available to energize transporter function is thought to be the sum of the steady-state

nonequilibrium ion gradients that are generated by primary transporters (Na^+, K^+ ATPase; H^+ ATPase) and the antagonistic functioning of transmembrane leak channels (Na^+, K^+, H^+, Ca^{2+}, HCO_3^-, Cl^-; Dawson, 1991). How energy is coupled from the resulting transmembrane potential to transporter function is not understood. Currently, two hypotheses are regarded to be plausible. The chemiosmotic theory states that energy derived from the gradient of an ion energizes a conformational change in the transporter, but the biochemistry and mechanism of energy transfer have not been investigated (Tsong, 1992). In contrast, an electroconformational coupling theory proposes that a transporter protein utilizes electric energy from medium or high periodic fields, that were generated by transmembrane electrical potential, to "drive a reaction away from chemical equilibrium" (to change transporter conformation because of an altered internal membrane energy state; Tsong and Astumian, 1988; Tsong, 1992).

As a model for mammalian secondary active transporter function, the electrogenic properties of the cloned Na^+/glucose cotransporter have been studied (Parent et al., 1992). The results showed that glucose (alpha-MDG) binding was dependent upon the magnitude of the transmembrane potential and the extracellular Na^+ concentration. This result, coupled with earlier research (Peerce and Wright, 1984), which showed that the binding of Na^+ to the cotransporter produced a conformational change, suggests that a combination of the above theories is required to account for the manner by which Na^+ and glucose bind to their cotransporter.

Parent and co-workers (1992) additionally demonstrated that reorientation of the empty cotransporter was also dependent upon transmembrane potential, thus indicating that reorientation may be an endothermic process. Based upon dipole strengths of membrane-spanning alpha-helices (strong) and beta-, and random coiled-

helices (weak), it appears that energy for domain translocation must overcome potential alpha-alpha helix interactions (Tsong and Austumian, 1988). As a generic model for membrane permease function, the pair-bonding properties of bacteriorhodopsin alpha-helices (twenty to twenty-one amino acid residues) have been studied and determined to be energetically favorable for the formation of alpha-helices bundles within membranes that tilt and(or) slide (Pullman, 1991). The relative exothermic energies of the helices arranged from an initially closed (tilted) arrangement to a relatively more open configuration decreased as the channel opened. As a consequence, the prediction was made that either the initial translocation event or the resetting of transporters would require energy, but not both. The observation that only the reorientation of the empty Na^+/glucose cotransporter required transmembrane potential, and not the translocation event itself (Parent et al., 1992), indicates that the mammalian Na^+/glucose cotransporter binding domain(s) may share functional homology to the above described bacterial transporter, and that the energy to drive this energetically unfavorable reaction is drawn from the transmembrane potential. If facilitative transport proteins are shown to function in the same manner, then this "energy-independent" carrier-mediated transport mechanism is actually dependent upon cellular respiration energy.

The hypothesis that ATP formation and its conversion into transmembrane potential is coupled through the functioning Na^+,K^+ ATPase (Blond and Whittam, 1964) has been supported by experimental evidence (Nielson, 1979: Harris et al., 1980; Mandel et al., 1981). Subsequently, Na^+/K^+ ATPase activity has been shown to be at least partially regulated by the ADP:ATP ratio (Jones et al., 1992). One result of research that collates O_2 consumption with Na^+/K^+ ATPase function, and, therefore, with energy for secondary transport processes, has been to allow that proportion of

whole-body energy expended to maintain electrochemical gradients to be estimated by measuring oxygen consumption by tissues. In the ruminant, for example, the extent to which whole-body, oubain-sensitive (Na^+,K^+ ATPase-dependent) respiration occurs has been shown to range from 28.5 to 61.6% and to depend on the feeding pattern, nutritional status, tissue type, and physiological status (McBride and Milligan, 1985; Rompala and Hoagland, 1987; McBride and Kelly, 1990).

Mechanisms Involved in the Absorption of Peptide Amino Acids.

Historically, amino acid nourishment of organisms was discussed in terms of how the multitude of biochemically characterized free amino acid transporters collectively functioned to absorb amino acids. It is now well understood that it is not necessary to hydrolyze proteins to free amino acids in order for amino acids to be absorbed by many organisms. Because of the important nutritional and metabolic implications of the observation that di- and tri-peptides are absorbed by many of the epithelia of the small intestine and kidney of animals, and because of the observation that lactamantibiotics successfully compete with small peptides for transport, the study of where and how peptides are absorbed is now a very active field of research by academic and industrial nutritionists and pharmacologists. Accordingly, the following summarizes the diversity of biological mechanisms known to be employed in the absorption of intact peptides by a wide spectrum of organisms existing in a variety of ecoclimes.

Mediated Absorption of Small Peptides by Microbes. For the ruminant nutritionist, an understanding of common and species-specific modes of peptide absorption is particularly germane given the interdependence of microbial forestomach protein metabolism and the potential for gastrointestinal tract peptide

absorption. Primary active peptide transport in microbes has been most thoroughly characterized in the enteric, gram-negative bacteria. *Escherichia coli* and *Salmonella typhimurium* have three genetically distinct peptide transport systems, which have been mapped (Olson et al., 1991). The dipeptide and tripeptide systems are responsible for transporting peptides of two and three amino acid residues, respectively. The oligopeptide system transports peptides of two to five amino acid residues and, during growth, is the transport system responsible for recycling the cell wall (Hiles et al., 1987).

These transport complexes are thought to share a common structure, as indicated by the homology of their gene transcripts. The similarity of the nutritionally important oligopeptide transporter complex for *E. coli* and *S. typhimurium* is highlighted by their functional interchangeability of components (Hiles et al., 1987). These primary transporters belong to a family of osmotic shock-sensitive transporters that share the common features of multiple, soluble, periplasmic substrate-binding receptor proteins (often in the m*M* concentration range), and a single, multi-unit membrane-bound protein complex (composed of three to four proteins) that is capable of binding to different species of substrate-receptor ligands (Hiles et al., 1987; Ames et al., 1990). One functional model for periplasmic transporters postulates that initial binding of a liganded substrate-periplasmic receptor to the transmembrane complex initiates a conformational change in the hydrophobic, membrane-spanning proteins, causing ATP-hydrolysis and subsequent substrate translocation (Pertonilli and Ames, 1991).

A definitive feature of this class of transporters is their cytosolic binding and hydrolyzing of ATP (Bishop et al., 1989), by one or two of the hydrophilic transmembrane complex proteins, to energize the translocation of substrate across the

cytoplasmic membrane. Calculations from an in vivo study indicate that two ATP molecules are hydrolyzed for every substrate molecule transported (Mimmack et al., 1989).

Ames and co-workers (1990) have suggested that these primary ATP-coupling transporters be named "traffic ATPases" in order to acknowledge the close structural and functional homology that these prokaryotic transporters share with eukaryote primary transporters (yeast, insect, and human). Hiles et al. (1987), however, argue that only the ATP-binding subunits of the transmembrane complexes share appreciable homology. Therefore, they suggest that these primary transport systems evolved separately, with each transporter complex recruiting their own ATPase proteins as energy-coupling components. Subsequently, this research group (Higgins, 1990), has proposed that the multiple transmembrane domains of prokaryotic primary transporters (as exemplified by gram-negative oligopeptide transporters) have "fused" into the polypeptide monomer of analogous eukaryotic primary transporters. In this model, the periplasmic binding protein of bacteria is considered to be an adaptive feature not necessary in eukaryotes. More specifically, it has been suggested that these high affinity receptors evolved in prokaryotes to promote efficient scavenging of sparse nutrients, an adaptation not necessary for eukaryotes, which inhabit environments typically rich in nutrients (Petronilli and Ames, 1991).

Overall, little is known about the structure of peptide transport systems of rumen-inhabiting microbial species (Russell et al., 1990). *Bacteroides ruminicola* is thought to be a primary proteolytic bacterium of the rumen (Wallace and Cotta, 1988; Mackie and White, 1990). This gram-negative species preferentially utilizes hydrophilic (Chen et al., 1987) oligopeptides of at least four amino acid residues (Pittman and Bryant, 1964), but less than 2,000 Da (Pittman et al., 1967), and

ammonia as nitrogen sources. A recent advance in the genetic analysis of this organism has identified an indigenous plasmid (Flint, 1988) that has the ability to transfer genes from *B. ruminicola* to *E. coli* and, consequently, to facilitate the "first real system for gene manipulation of a ruminal bacterium" (Mackie and White, 1990). The generation and sequencing of the molecular clones of peptide transporting proteins in *B. ruminicola* (and other proteolytic rumen organisms) should enhance their structural and functional characterization. With this knowledge, the manipulation of peptide concentrations in the ruminant forestomach may become possible.

Secondary, H^+-dependent symport of intact alanylglutamate has been demonstrated in gram-positive bacteria. Cytochrome C oxidase-containing liposomes were fused with *Lactococcous lactis* membrane vesicles to generate the requisite protonmotive force (Smid et al., 1989). These researchers reasoned that H^+-ATPase was responsible for maintaining the membrane potential by pumping the symported H^+ out of the cell. They calculated that the stoichiometry of H^+/peptide symport was charge-dependent with two H^+/alanylglutamate cotransported. Accordingly, they reasoned that one H^+ would be transported for every neutral peptide. In contrast, based on moles of peptide transported/mole of glucose in *Streptococcus faecalis*, Payne (1983) concluded that the amount of energy expended to transport a variety of neutral di- and oligopeptides was equivalent to two H^+ per peptide.

The characterization of peptide transport in fungi has lagged behind that in bacteria. Inherent difficulties caused by variability of subspecies, lack of mutants, N source-induced modulation of transport, and rapid cleavage of transported peptides have been overcome (Island, 1987; Shallow et al., 1991). Consequently, *Candida albicans* has been shown to posses separate dipeptide and oligopeptide systems that

are dependent on a functioning protonmotive force and plasma membrane H^+-ATPase (Shallow et al., 1991). Active (concentrative) transport and carboxyl group-dependent binding of peptides was demonstrated by only the dipeptide permease system. Stimulation of alanylalanine transport was more rapid by cells grown overnight in media containing peptide nitrogen sources as compared to those grown in amino acid- and ammonia-containing media (Payne et al., 1991). These researchers observed that modulation of dipeptide transport rate was reversible and did not require de novo protein synthesis. Accordingly, they postulated that the mechanism by which the extracellular concentration of peptide N affected the rate of dipeptide transport was "a process of exocytotic insertion and endocytic removal of permease components." This substrate concentration-dependent, carrier-mediated regulation of dipeptide uptake is analogous to the model proposed for recruitment of GLUT4 facilitative diffusion glucose transporters in other eukaryote cell types (mammalian adipocytes and skeletal myocytes), when serosal glucose levels are high (Kasanicki, 1990).

Active Absorption of Small Peptides by Animals. In common with the characterized uptake of intact small peptides in gram-positive bacteria and fungi, the brush border membrane of mammalian intestinal absorptive epithelia is known to possess peptide transporters that are electrogenic and that require a protonmotive force to drive translocation (Boyd and Ward, 1982; Ganapathy and Leibach, 1983). The use of hydrolysis-resistant peptides in whole tissue and brush border membrane vesicle (BBMV) experiments has led to the general conclusion that this active transport of intact peptides is mediated by secondary transport proteins that are unique from free amino acid transport proteins (Ganapathy and Leibach, 1982; Hoshi, 1985; Burston et al., 1987). Concentrative H^+/peptide symport has been demonstrated in

rabbit, rat, and guinea pig intestinal BBMV (Ganapathy and Leibach, 1983; Himukai et al., 1983; Said et al., 1988; Iseki et al., 1989).

The active transport of small peptides in the intestinal epithelium has been postulated to be dependent on the existence of a luminal pH gradient (extracellular-to-intracellular) and the coordinated activity of three separate protein complexes (Ganapathy and Leibach, 1985; Hoshi, 1985). Ion transport proteins of this functionally integrated system include the extracellular proton-driven H^+/dipeptide symporter, the extracellular Na^+-driven Na^+/H^+ exchanger, and the ATP-hydrolyzing Na^+/K^+ ATPase. In this model, two protons (Hoshi, 1986) flowing down their electrochemical gradient would bind to the peptide transporter, driving the symport of one peptide across the brush border. Concurrent reduction of the intracellular pH would induce the exchange of intracellular H^+ for extracellular Na^+ by the brush border Na^+/K^+ exchanger, thereby resulting in the restoration of basal intracellular pH and the transmembrane proton gradient. The Na^+/K^+ ATPase would then reestablish the high extracellular Na^+ gradient by antiporting Na^+ out of the cell in exchange for K^+. Additionally, the export of symported H^+ by a primary H^+-ATPase transporter (Hoshi, 1986) would constitute another and(or) alternative, energy-dependent route by which the protonmotive force is reestablished across the brush border, at the cost of ATP hydrolysis.

The requisite components for this proposed model have been identified. The presence of the requisite pH gradient has been measured in the microenvironment of rat jejunal tissue (Lucas, 1983) and brush border Na^+/H^+ exchangers (amiloride-resistant) have been localized in the rat (Murer et al., 1976), rabbit (Knickelbein, 1983), and human (Kikuchi, 1988). Evidence that H^+/peptide symport stimulates H^+/Na^+ antiport is provided by separate reports that peptide uptake stimulated Na^+

uptake, but Na^+ uptake failed to stimulate peptide uptake (Himukai et al., 1983; Cheeseman and Devlin, 1985).

Table 2.1 lists affinity constants of the putative mammalian peptide transporter that have been reported for a variety of substrates and tissue, cells, and membranes. In total, the evidence for the brush border absorption of intact di- and tripeptides by mammalian intestinal ion-gradient transporters is strong. More thorough reviews support this conclusion (Matthews, 1991a; Webb et al., 1992). Until recently, however, little experimental evidence was available to indicate the existence of analogous basolateral mechanisms capable of exporting absorbed peptides into the bloodstream. Therefore, the demonstration (Dyer et al., 1990) of saturable, proton gradient-stimulated glycylproline transport in the basolateral membranes of rabbit proximal intestine was remarkable. Transport of glycylproline across membrane vesicles was by a single transport system (K_t = 2 m*M*) characterized as being competitively inhibited by glycyl-dipeptides and carnosine, free of hydrolysis, and distinct from free amino acid transport. Subsequently, using monolayers of differentiated cultured human colon enterocytes (Caco-2 cells) four other research groups (Burton et al., 1993; Saito and Inui, 1993; Thwaites, et al., 1993; Dantzig et al., 1994) have confirmed the mediated flux of dipeptides and beta-lactam antibiotics across both apical and basolateral membranes. One group has suggested that biochemical characterization of the apical and basolateral transport processes reveals that the two transporters had different functional characteristics (Saito and Inui, 1993). The apical transport system demonstrated maximal Bestatin uptake at pH 6.0 (pH 5.0 to 7.5), whereas the activity of the basolateral system was not affected by pH. A similar differential dependence on a pH gradient for mediated peptide absorption has been identified for two apical (brush border) transport systems in rat kidney

Table 2.1. Affinity Constants for Mediated Peptide Transport in Animals

Animal	Tissue	Experimental Model	Cotransport Substrate	Substrate	K_t (m*M*)	Source[a]
Hamster	Jejunum	Everted rings	H^+	Val-Val	9.6	1
				Gly-Sar	6.1	
				Leu-Leu	5.6	
				Gly-Gly	5.2	
				Ala-Ala	3.2	
Rabbit	Small Intestine	BBMV	None	Gly-Sar	17.3 +/- 1.4	2
			H^+	Gly-Sar	19.5 +/- 2.0	
Rabbit	Jejunum	BLMV	H^+	Gly-Pro	2.0 +/- .20	3
Tilapia (fish)	Intestine	BBMV	None	Gly-Phe	9.8 +/- 3.5	4
Rat	Kidney: Outer medulla and cortical	BBMV	H^+	Gly-Leu	.101 +/- .0209	5
			H^+	Gly-Leu	.003 +/- .0001	
			H^+	Gly-Gln	.003 +/- .0001	
Human	Colon: Caco-2 cells	Apical	H^+	Bestatin	.34	6
		Basolateral	H^+	Bestatin	.71	
Human	Colon: Caco-2 cells	Apical	H^+	Gly-Sar	1.1 +/- .1	7
Rabbit	Small Intestine	Cloned, then expressed in *Xenopus laevis* oocytes	H^+	Gly-Gly-Gly	5.1	8
				Cephalexin	4.2	
				Gly-Gly	2.5	
				Gly-Sar	1.9	
				Ala-Asp	.143	
				Cyclocillin	.137	
				Gly-Leu	.081	
Rabbit	Small Intestine	Cloned, then expressed in *Xenopus laevis* oocytes	H^+	Cefadroxil	1.1 +/- .2	9

[a]1 = Matthews, 1983; 2 = Ganapathy et al., 1984; 3 = Dyer et al., 1990; 4 = Reshkin and Ahern, 1991; 5 = Daniel et al., 1991; 6 = Inui and Saito, 1993; 7 = Brandsch et al., 1994; 8 = Fei et al., 1994; 9 = Boll et al., 1995.

(Daniel et al., 1991). Whereas the pattern of Bestatin competitive inhibition uptake was similar for both systems, co-incubation with several different dipeptides and beta-lactam antibiotics resulted in greater apical than basolateral Bestatin uptake. Additionally, basolateral transport of Bestatin was reduced more by the presence of the sulfhydryl-group inhibitors than was apical transport.

The significance of this in vitro research is that evidence for carrier-mediated translocation of intact peptides from the cytosol of intestinal enterocytes into the bloodstream of mammals has been provided. Therefore, this evidence supports the hypothesis that peptides of dietary origin can be absorbed and presented to all body tissues for potential absorption. That these reports indicate that peptide transport systems may possess functional differences, depending on in which membrane they are localized (apical versus basolateral), and that species differences may exist (rabbit versus human), are analogous to differences in the location and function of many mammalian amino acid transport systems (Kilberg and Haussinger, 1992).

The reported ability of most mammalian peptide transporters to competitively transport multiple peptide and beta-lactamin antibiotic species could be functionally explained by multi-protein transporter complexes that bind multiple substrate binding receptors, analogous to the structure of primary peptide transporters in gram-negative bacteria. Secondary (ion-gradient) transporters, however, are thought to be monomers (Hiles et al., 1987). Alternatively, numerous reports of common beta-lactam antibiotic and di- and tripeptide transport by the putative mammalian H^+/di- and tripeptide intestinal transporter (Burston et al., 1987; Inui et al., 1988; Kramer et al., 1988) suggest that this ion gradient-driven transporter has a promiscuous substrate-binding domain(s). Binding of substrates by this rabbit intestinal peptide transporter, under acidic conditions, is reported to be dependent upon histidyl groups (Kato et al.,

1989; Kramer et al., 1990a), without requiring thiol or sulfhydryl groups. Redox-sensitive dithiol and sulfhydryl groups, however, have been described as being essential for peptide binding by rabbit renal cortex (Miyamoto et al., 1986, 1989) and intestinal epithelial cell (Inui et al., 1992) peptide transporters.

Molecular Structure of Mammalian H^+/Peptide Cotransport Proteins. Whereas the functional structure of mammalian ion gradient-energized peptide transporters have not been elucidated, recent research (Kramer et al., 1990a, 1990b) has resulted in the isolation and purification of a 127 kDa protein from rabbit small intestine that is involved in glycylproline binding and translocation. This protein was determined not to have been involved in the functioning of aminopeptidase N or dipeptidylpeptidase IV. Importantly, this result suggests that the phenomenon of mediated dipeptide uptake and peptide hydrolysis by these common intestinal peptidases are separate events. Similar distinction of function between dipeptide transport and hydrolysis has been reported for the gram-negative bacteria dipeptide transporter (Hiles et al., 1987).

Although the functional structure of peptide transport systems is not known, the primary structure of two proteins reputed to have functional H^+/peptide cotransport has recently been determined. The rabbit intestine H^+/peptide cotransport protein (PepT1) has recently been expression cloned by two different research groups (Fei et al., 1994; Boll et al., 1994). Functional expression of the cRNA for PepT1 in *Xenopus laevis* oocytes results in H^+-dependent, saturable, and competitively inhibitable transport of dipeptides, tripeptides, and beta-lactam antibiotics (Fei et al., 1994; Boll et al., 1994). Electrophysiological measurement of the inward current induced by H^+/glycylsarcosine symport indicated a coupling ratio of 1:1.17 (Fei et al., 1994). This observation, when combined with the proposed role of the Na^+/H^+ and

Na^+/K^+ ATPase in energizing the absorption of peptides (as discussed above) suggests that the indirect cellular energy cost of transporting two moles of amino acid residues by PepT1 is approximately .33 ATP. Other current measurements resulted in the determination of PepT1 substrate affinity values (K_t) for dipeptides (Fei et al., 1994) and beta-lactam antibiotics (Fei et al., 1994; Boll et al., 1994), that are compatible to those obtained using a variety of methodologies for mammalian intestinal and renal tissue, cells, and membranes (Table 2.1).

PepT1 (707 amino acid polypeptide) is predicted to contain one relatively large cytosolic domain and twelve alpha-helical membrane spanning domains. Therefore, the structure of this transporter apparently fits the consensus "rhythm" of two membrane-spanning units, each containing "an array of (about) six transmembrane alpha-helices" that has been hypothesized to exist in all membrane permeases (Maloney, 1990).

In contrast, the HPT-1 H^+/peptide cotransport protein, cloned from Caco-2 cells, is predicted to span the membrane only once (Dantzig et al., 1994). Immunoblot analysis for HPT-1 of SDS-PAGE separated Caco-2 cell membranes identified a single protein band of approximately 120 kDa. This mass is consistent with the apparent identification of a H^+/glycylproline cotransport system, biochemically purified from rabbit enterocytes as a monomer (Kramer et al., 1990a). Other, non-mammalian, H^+-energized transporters also are thought to be functional monomers (Hiles et al., 1987).

Besides their proposed structural differences, tissue-specificity for different isoforms of peptide transport proteins exists. Rabbit PepT1 mRNA (2.9 kb) was identified in the greatest quantity in epithelial cells of the small intestine, lesser amounts in the liver and kidney tissue, and trace amounts in several brain tissues (Fei

et al., 1994). This high-stringency northern analysis, however, failed to reveal the presence of PepT1 mRNA transcripts in the colon, skeletal muscle, or heart tissue. In contrast, the HPT-1 protein was identified by immunohistochemical staining in immortalized human colonic cells (Caco-2, HT-29, COLO 320), pancreatic and small intestinal epithelia, but not in human liver or brain tissue (Dantzig et al., 1994). Also, immunoblot analysis of human adrenal and skin tissues for HPT-1 was negative.

Overall, the use of molecular techniques to screen tissues and cells for the message and(or) proteins responsible for peptide transport activity in tissues confirms the presence of mediated peptide absorption systems previously identified using biochemical techniques. Because the two peptide transporters share little sequence homology (Daniel et al., 1994), it appears that the kidney may possess two different peptide transport proteins. If so, then these results suggest that the biochemically characterized different high and low affinity peptide transport systems in the kidney (Daniel et al., 1991) may be the result of different transport proteins.

The ability of the liver to absorb peptides by carrier-mediated mechanisms is controversial. Hamster liver slices were shown to demonstrate saturable, mediated uptake of carnosine and glycylsarcosine (Matthews, 1991a). In contrast, rat hepatocytes are thought to absorb dipeptides that are less resistant to hydrolysis than glycylsarcosine and carnosine only after hydrolysis, by active uptake of their constituent amino acids (Lochs et al., 1990; Lombardo et al., 1988). Therefore, that liver tissue of rabbits contained the mRNA for PepT1 (Fei et al., 1994) suggests that a species difference exists for peptide transporter expression, or that the presence of PepT1 mRNA does not necessarily mean that it is expressed in rabbit tissue.

Facilitated Absorption of Small Peptides by Animals. Facilitative diffusion transport proteins utilize existing high exterior-to-low interior cell peptide gradients to

drive the downhill transport of peptide substrates into the cell. Accordingly, they are not dependent on the hydrolysis of high energy bonds to energize transport and, consequently, are incapable of transporting peptides against a concentration gradient. This mode of transport is dependent upon the substrate concentration, but unlike random, transmembrane diffusion, the facilitated transport of peptides allows selective absorption when peptides are in surfeit. The characterization of a facilitated diffusion peptide transport mechanism (K_t = 9.8 m*M*) capable of transporting di- and tripeptides has been reported in fish intestinal BBMV (Reshkin and Ahern, 1991). These researchers suggest that another report of peptide transport by a "nonenergized, Na^+-independent, carrier-mediated process" into mouse, rabbit, and human intestinal BBMV also indicates dipeptide absorption by a facilitative transport mechanism. A decrease in dipeptide uptake in the presence of a proton gradient was observed.

Glutathione (GSH) transport has been reported to be Na^+-independent, saturable, GSH-specific in rabbit intestinal BBMV (Vincenzini et al., 1988). Further research revealed that GSH transport was optimal at pH 7.5 in the absence of either inward- or outward-directed proton gradients (Vincenzini et al., 1989). Ca^{2+} and Mn^{2+} divalent cations (5 m*M*) stimulated anionic GSH uptake greater than did the monovalent cations (100 m*M*) Li^+, K^+, and Na^+, with transport of GSH being maximally stimulated by Ca^{2+}. These researchers concluded that the GSH transporter (K_t = .017 m*M*) across the intestinal brush border membrane is pH-dependent, H^+ gradient-independent, and activated by several mono- and divalent cations. The additional observation that presence of these cations resulted in the accelerated "equilibration of GSH without enabling accumulation" suggests that GSH transport was by carrier-dependent, energy-independent facilitative transport.

Similar facilitated diffusion transport of dipeptides (and their methyl derivatives) by cultured human peripheral blood lymphocytes (K_t = .1 to .2 m*M*) was also decreased in the presence of a proton gradient (Thiele and Lipsky, 1990). The difference in functional magnitude between the in vitro K_t values of these concentration-dependent transporters appears to be consistent with predictable in vivo concentrations of peptides. That is, the higher K_t of the intestinal fish transporter is consistent with the higher substrate environment (probably) in which it has evolved as compared to the lower peptide concentration of blood in which the human lymphocyte facilitated diffusion peptide transporter evolved. The low K_t of the intestinal GSH transporter also seems to be consistent with its apparently unique affinity for GSH.

Carrier-mediated peptide transport has been documented in many organisms. Evidently, the energy expense of maintaining substrate-specific uptake mechanisms is warranted to ensure the ability to selectively, and competitively, absorb amino acids as peptides. Alternatively, carrier-independent mechanisms are known to allow comparatively inexpensive absorption of intact peptides.

Carrier-Independent Absorption of Small Peptides by Animals. Because cell membranes are relatively impermeable to hydrophilic molecules, the carrier-independent absorption of small peptides practically depends on envelopment by membrane vesicles (endocytosis), or non-mediated diffusion through existing membrane-spanning channel proteins or paracellular pathways. Consequently, the relative contribution that carrier-independent uptake makes to total peptide absorption is effected by extra- and intracellular peptide concentrations, the magnitude of signals affecting membrane endocytosis and structural protein function, peptide size and charge relative to resident channel protein size and charge, and intracellular energy levels.

Membrane endocytosis is an energy-dependent and carrier-independent process shown to result in absorption of intact peptides and proteins into the bloodstream of mammals (Gardner, 1994). This vesicular transport phenomenon is generally divided into receptor-mediated and non-specific substrate absorption. Non-specific endocytosis of food-type proteins by Caco-2 cells has been shown to occur by a degradative lysosomal pathway and a direct apical-to-basolateral pathway, resulting in less than 10% of absorbed protein being transferred in the intact form (Heyman et al., 1990). The reported ability of this major lysosomal degradative pathway to equally degrade endocytosis vesicles originating from the apical or basolateral membranes (Heyman et al., 1990) is consistent with the reported ability of rat intestinal epithelium to utilize intact plasma peptides (Krzysik and Adibi, 1977). For the neonatal animal, yet to develop other digestive mechanisms, non-specific mucosal "cell drinking" may be an important means of protein absorption and subsequent degradation (Teichberg, 1985).

A general model for paracellular solute flow across tissues whose constituent cells are connected by zonulae occludenes has been postulated (Pappenheimer, 1989). Because zonulae occludenes can "leak" when subjected to high osmotic gradients, absorptive epithelial tissues may display differential rates of intercellular nutrient passage if the integrity of tight junctions is compromised. Therefore, fluctuations in the integrity of tight junctions may provide an alternative mode of absorption for polar compounds whose lipophobic nature reduces their potential for transmembrane diffusion. Dilation of hamster enterocytic tight junctions has been induced by functioning Na^+/glucose cotransporters, resulting in increased absorption of oligopeptides (less than 1,900 Da; Atisook and Madar, 1991). Similar events that dilate tight junctions, including the "stirring" of the unstirred mucosal layer by

cytoskeletal proteins (Mooseker et al., 1984, as cited by Teichberg, 1985), could potentially increase the absorption of peptides into the epithelium, thereby making intact peptides available for absorption into epithelial cells or across the lamina propria into the bloodstream.

Intact peptide absorption by red blood cells has been characterized to occur by non-saturable and non-specific binding (Lochs et al., 1990), suggesting that diffusion was the mechanism responsible for absorption. Transmembrane diffusion of dipeptides ceased after 1 min leading these researchers to conclude that this carrier-independent route of dipeptide absorption, in human red blood cells, is not nutritionally significant. Similarly, the proportion of small peptides (hydrophilic compounds) absorbed across the intestinal epithelial brush border membranes by diffusion is considered to be less than that by active transport (Matthews, 1991a).

For the ruminant, however, non-mediated absorption of peptides across the forestomach epithelium may be important. Recent in vitro experiments have demonstrated transepithelial, non-saturable absorption of intact carnosine and methionylglycine by sheep ruminal and omasal tissue, with no observable equilibration after 4 h (Matthews, 1991b). Accordingly, dipeptide absorption appeared to be linearly dependent on concentration and time. It was not determined whether this carrier-independent uptake process was mediated by paracellular and(or) transmembrane diffusion.

Structural and Functional Aspects of Amino Acid Transport Proteins.

Based upon substrate specificity, kinetics of absorption, and requisite energy sources, many different free and peptide-bound amino acid transport systems have been characterized in both epithelial and non epithelial cells and tissues, using a

variety of experimental methodologies. The specific biochemical criteria and functional classifications of these reputed amino acid transport systems can be found in other reviews (Hoshi, 1985; Christensen, 1990; Cheeseman, 1991; Matthews, 1991a; Webb et al., 1992; Stevens, 1992a; Kilberg et al., 1993; Van Winkle, 1993; Daniel et al., 1994; Ganapathy et al., 1994; Gardner, 1994).

The recent cloning of several proteins thought to be responsible for amino acid absorption is beginning to reveal the molecular structure and function of several biochemically characterized transport "systems". Important features of cloned proteins reported to be responsible for the secondary active transport activity of Na^+/amino acid (A, ASC, X^-_{AG}) are listed in Table 2.2. Features of cloned facilitative amino acid

transport proteins (y^+, $b^{0,+}$ activity) are listed in Table 2.3. One important feature elucidated from the cloning of these transport proteins is that transport systems may be composed of multiple proteins (homomers and(or) heteromers), and, therefore, be encoded for by multiple genes, perhaps contained in gene "cassettes," as has been described for primary transport systems (Higgins, 1990). Subsequently, variable assembly of the subunits may result in different forms and functions of transport systems (isoforms). Alternatively, as noted by Reithmeier (1994), the production of several transcripts from the same gene, by alternative gene promoters or by alternative splicing of transcripts, can result in a diversity of transporter isoforms and their functional characteristics (e.g., K_t values), as observed with the genes for the rat AE1 and AE3 anion exchangers.

Knowledge of the molecular structure of proteins capable of amino acid transport has allowed evolutionary and taxonomic relationships to be established based upon their conserved primary amino acid sequence homology and length, upon

Table 2.2 Cloned Na^+/Amino Acid Cotransporters

Protein Name	Genebank Accession #	Encoded AA (#)	Leucine Zipper?	Predicted MSD	Tissues[a] Screened	Transcript Size (kb)	Transport Activity	Substrate	K_t (μM)	Source
SAAT1	L02900	660	Yes	11	Porcine:		System A	MeAIB	800 +/- 200	Kong et al., 1993
					+LLC-PK1	2.4				
					+ki, li, si, sp.sm	2.4				
					+ki, li, si, sp.sm	3.7				
					+ki, li, si, sp.sm	4.9				
ASCT-1	L14595	532	---	<10	Human:					
					+br, he, ki, li, lu,	2.0	System ASC	Cysteine	29 +/- 6	Arriza et al., 1993
					+pa, pl, sm			Alanine	71 +/- 14	
					+br, he, ki, li, lu,	4.0		Serine	88 +/- 11	
					+pa, pl, sm			Threonine	137 +/- 19	
					+br, he, ki, li, lu,	5.1		Valine	390 +/- 8	
					+pa, pl, sm					
ASCT-1	L14595	532	---	<10	Human:					
					+br, he, ki, li,	2.2, 2.8,	"ASC-like"	Serine	56	Shafqat et al., 1993
					+lu, pa, pl, sm	3.5, 4.8		Alanine, Threonine, but not Cysteine		
EAAC1	(GLTE_Rabbit, SwissProt)	524		10	Rabbit:					
					+du, je, il, ki, li	2.5	System X^-_{AG}	Glutamate	12 +/- 1	Kanai and Hediger, 1992
					- co, sp, lu			Aspartate	63 +/- 7	
					+br, he, ki, li,	3.5				
					+du, je, il					
					-co, lu, sp					
GLAST	X63744	543		>6	Rat:	4.5	System X^-_{AG}	Glutamate	77 +/- 27	Storck et al., 1992
					+br			Aspartate	65 +/- 30	

[a]br = brain; co = colon; du = duondenum; he = heart; il = ileum; je = jejunum;ki = kidney; li = liver; lu = lung; pa = pancreas; pl = placenta; sp = spleen; sm = skeletal muscle; si = small intestine.

Table 2.3. Cloned Na^+-Independent/Amino Acid Cotransporters

Protein Name	Genebank Accession #	Encoded AA (#)	Leucine Zipper?	Predicted MSD	Tissues[a] Screened	Transcript Size (kb)	Transport Activity	Substrate	K_t (μM)	Source
MCAT-1		622	ND	12 to 14	Mouse: +bm, br, he, ki +lu, sp, st, te -li	7.0, 7.9	System y^+	Lysine Arginine Ornithine Histidine	73 +/ 8 77 +/-2 105 +/- 2 1,830 +/- 73	Albriton et al., 1989 Kim et al., 1992 Wang et al., 1992
MCAT-2	L03290	658	ND	14	Mouse: +li	NR	System y^+	Arginine	2,150	Closs et al., 1993
NBAT	M77345	683	Yes	4	Rat: +ki +si enterocyte, enteroendocrine, enteroneuron cells	2.3	System $b^{0,+}$	Leucine Phenylalanine Alanine Methionine Histidine	22 29 50 71 167	Tate et al., 1992, 1994 Pickel et al., 1993
D2	M80804	651	Yes	1	Rat: +ki, du, je, il, -br, he, li, lu, pa -sp, sm	2.3, 4.4	System $b^{0,+}$	Cysteine	67	Wells and Hediger, 1992
D2H	M95548	663	Yes	1	Human: +ki, si	2.3, 4.3	System $b^{0,+}$	Cysteine	30	Lee et al., 1993
rBAT-1	M90096	677	Yes	1	Rabbit: +ki, do, je, il -co	2.2, 3.7	System $b^{0,+}$	Cysteine Arginine Leucine	60 105 128	Bertran et al., 1992 Magagnin et al., 1993
hrBAT	L11696	685	Yes	1	Human: +ki, li (weak) +je, pa +br, he, li (high) +lu, pa, pl, sm	2.5 5.5	System $b^{0,+}$	Cysteine	43	Bertran et al., 1993

[a]bm = bone marrow; br = brain; co = colon; du = duodenum; he = heart; il = ileum; je = jejunum; ki = kidney; li = liver; lu = lung; pa = pancreas; pl = placenta; sp = spleen; si = small intestine;sm skeltal muscle

the topology of their predicted three dimensional structures, and upon their known functional characteristics (Reizer et al., 1993; Reithmeier, 1994; McGiven and Pastor-Anglada, 1994; Reizer et al., 1994). SAAT1 (reported to possess Na^+-dependent neutral amino acid transport capabilities) has been assigned to one of the eleven Na^+/solute symporter superfamilies, which includes the mammalian Na^+/glucose cotransporter (SGLT1) and other transporters predicted to possess at least 660 amino acid residues, 11 to 15 membrane-spanning domains (MSD), and the sodium binding motif of $GX_{35-37}AX_3(EQ)LX_3GR$. Likewise, EACCC1 and GLAST (Na^+-dependent glutamate and aspartate transport proteins) are grouped with other sodium/dicarboxlate symporters, which are predicted to possess 8 to 12 MSD, approximately 547 amino acid residues, and a sodium binding motif of $GX_{47-48}(AS)X_2D(AS)X_3G(KR)$. Because the sodium binding motif has been shown not to be necessary nor sufficient for other known Na^+-dependent symporters (Reizer et al., 1994), other, as yet unidentified, sodium binding motifs must exist in Na^+-dependent transport systems.

Whereas proteins involved in Na^+-coupled transport are generally regarded to share (>25%) homology of sequences (Kong et al., 1993; McGiven and Pastor-Angloda, 1994), little homology appears to be shared between members of the sodium solute family and members of facilitative glucose and amino acid transport proteins. A structural feature shared by all cloned facilitative amino acid transporters is the presence of a carboxy-terminal leucine zipper motif, which provides a potential site for subunit oligomerization (Wells and Hediger, 1992; Kong et al., 1993).

Based upon an analysis of a variety of cloned membrane permeases, it has been suggested that the eventual X-ray crystallographic analyses of transport proteins will reveal them to possess multiple alpha-helix MSD (Maloney, 1990). Accordingly,

it has been argued that amino acid transport proteins predicted to possess a single MSD are not "transporters" per se, but, instead, are type II membrane glycoproteins that either function as regulatory proteins by binding to multiple MSD transport proteins or that are monomer subunits that bind with other subunits to form a transport system (Wells and Hediger, 1992; Shafqat et al., 1993; Kong et al., 1993). Based upon hydrophobicity profile analysis, and assuming a beta-helix structural conformation (21 to 23 amino acid residues), the NBAT, D2, and rBAT family of proteins encoding for $b^{0,+}$ system activity are predicted to possess a single, NH_2-terminus binding, MSD (Wells and Hediger, 1992; Bertran et al., 1993). As a consequence, these researchers have proposed that the proteins are either monomer subunits that self-aggregate into a larger multi-subunit protein or that they are regulatory proteins that bind to another protein(s) that actually comprise the $b^{0,+}$ transport system. Tate and co-workers (1992), however, suggest that if a less stringent hydrophobic profile analysis is employed, then these proteins will possess four MSD. Subsequently, the use of antibodies specific for the putative intracellular and extracellular domains of the NBAT protein appears to have corroborated this topological model (Mosckovitz, 1994).

As with the facilitative $b^{o,+}$ transport proteins, it has been suggested that the Na^+-dependent SAAT1 protein (claimed to be responsible for the ubiquitous System A activity) may not be a transporter per se, but, instead, a protein capable of stimulating system A activity (McGiven and Pastor-Angloda, 1994; M. S. Kilberg, personal communication). The identification of leucine zipper motifs in SAAT1 suggests at least one structural motif in these proteins that can result in subunit dimerization into homo- or hetero-oligomer formations. SAAT1, cloned from immortalized porcine kidney epithelial cells, LLC-PK_1 (Kong et al., 1993), shares

76% sequence homology with the ubiquitous Na^+/glucose cotransporter, which is known to be a functional homotetramer (Stevens et al., 1990). The proteins cloned from rat and rabbit tissues that encode for $b^{0,+}$-like activity (NBAT, D2, D2H, rBAT-1, rBAT-2, hrBAT) share significant homology with regions of the human 4F2C antigen molecule. The 4F2C antigen protein is a heterodimer molecule composed of a 85 kDa heavy and 40 kDa light chain (Wells et al., 1992; Bertran et al., 1993). The implication of the shared homology of primary sequences is that the $b^{0,+}$ transport system may be composed of multiple proteins.

In an attempt to evaluate the evidence for potential monomeric and(or) oligomeric amino acid transport protein function, the isolated and predicted sizes of proteins known to mediate the absorption of amino acids, peptides, and glucose have been compared and summarized in Table 2.4. The obvious discrepancy between the predicted size of the transport protein based upon its amino acid sequence and the reported size of the isolated

protein appears to be attributable to the apparent glycosylation mass of the protein. For example, the HPT-1 (H^+/peptide cotransport) protein has a predicted primary structure MW of 92 kDa and a reported SDS-PAGE value of approximately 120 kDa, as isolated from Caco-2 membranes (Dantzig et al., 1994). Deglycoslylation on SDS-PAGE resulted in a band shift of approximately 20 kDa, thereby indicating a MW of approximately 100 kDa for the primary structure and approximately 20 kDa in glycosylation. Because of this accounting, it appears that the HPT-1 exists as a monomer, and not as an oligomerized protein consisting of multiple subunits. This result is in agreement with the characterization of a 127 kDa protein (identified by photo-affinity labelling) that was isolated from rabbit small intestine and reconstituted in liposomes and that displayed typical H^+/peptide activity (Kramer et al., 1992).

Table 2.4. Comparison of the Predicted to Isolated Mass of Cloned Transport Proteins in Animals

Protein	Transport Activity	Predicted # AA	Predicted (kDa)	Isolated (kDa)	Glycosylation Sites/(kDa)	Source[a]
PepT1	H^+/Peptide	707	71	60	4/11,000	1
HPT-1	H^+/Peptide	832	92	120	7/25,000	2
GLAST	System X^-_{AG}	543	60	66	3/9,000	3
MCAT1	System y^+	622	67	90	4/ ND	4
rBAT-1	System $b^{o,+}$	677	78	85 to 99	7/ upto 22,000	5
SGLT1	Na^+/Glucose	644	70	290	4/15,000	6
GLUT1	Glucose	492	46	55	1/9,000	7

[a]1 = Fei et al., 1994; 2 = Dantzig et al., 1994; 3 = Storck et al., 1992; 4 = Kim et al., 1991; 5 = Bertran et al., 1992; 6 = Stevens, 1992; 7 = Thorens et al., 1993.

Similarly, in vitro translation of PepT1 (H^+/peptide cotransporter) cRNA in the presence of microsomes resulted in the isolation of a 71 kDa protein (Fei et al., 1994). In contrast, translation in the absence of microsomes yielded a protein of 60 kDa, thus indicating that several of the four potential N-glycosylation sites are glycosylated under native conditions.

Similar structural characterizations were reported for the active (GLAST; Storck et al., 1992) and facilitative (rBAT1; Bertran et al., 1992) amino acid transport proteins. Collectively, unless isolation and subsequent SDS-PAGE separation resulted in potential oligomeric protein breakdown, the data in Table 2.4 suggest that proteins capable of amino acid transport do not function as oligomeric proteins. Likewise, the GLUT1 facilitated glucose transporter has been characterized as a functional monomeric protein (Thorens, 1993). In contrast, the 290 kDa Na^+/glucose cotransporter (SGLT1) has been characterized to be a functional homotetramer of four 71 kDa monomeric subunits (Stevens et al., 1990; Wright, 1993).

It is not known whether glycosylation is essential to the function of all solute membrane permeases. However, the function of the active Na^+/glutamate, aspartate (GLAST) amino acid cotransport protein and the Na^+/glucose (SGLT1) cotransporter is not dependent on the presence of their glycosylate moieties (Storck et al.,1992; Wright, 1993). Likewise, the Na^+-dependent exchange of Ca^{2+} and H^+ is reported to be independent of transporter (Na^+/Ca^{2+}, Na^+/H^+) glycosylation (Hryshko et al., 1993; Haworth et al., 1993).

Structural Aspects of mRNA that Encode Amino Acid Transport Proteins. Tissues transcribe mRNA of different sizes that encode for the same transport protein (Tables 2.2 and 2.3). Among proteins, the length of the mRNA transcript does not appear to be associated with transcript abundance. For example, longer messages for

the human (Bertran et al., 1993) hrBAT (5.5 kb) and rabbit (Kanai and Hediger, 1992) EAAC1 (3.5 kb) proteins are found in more tissues and in greater abundance than are their corresponding shorter (2.5 kb) mRNA transcripts, whereas shorter mRNA transcripts for rat (Wells and Hediger, 1992) D2 (2.2 versus 4.3 kb) and rabbit (Magagnin et al., 1992) rBAT (2.2 versus 3.7 kb) proteins were present in the greatest abundance (Table 2.3).

Within proteins, mRNA transcript length also does not appear to affect the abundance of different sized transcripts. For example, human ASCT1 protein transcripts of 2.0, 4.0, and 5.1 kb were identified in all tissues tested (Table 2.2; Arriza et al., 1993). In heart, lung, liver, and kidney tissues, the 4.0 kb transcript was the predominant message. In contrast, placental and pancreas tissues appeared to transcribe equal amounts of the 4.0 and 5.1 kb transcripts and a lesser amount of the 2.0 kb message, whereas equal amounts of all three transcripts were present in the brain and skeletal muscle. Similarly, different-sized mRNA transcripts of varying relative abundance have been observed within tissues that encode the human SATT (Shafqat et al., 1993) and pig SAAT1 (Kong et al., 1993) transport proteins. A comparison of the mRNA transcripts capable of inducing Na^+-dependent (Table 2.2) and Na^+-independent (Table 2.3) transport activities reveals that there appears to be less variety in the size of transcripts encoding Na^+-independent transport activity than observed for Na^+-dependent transporter activity. Specifically, only two mRNA species are reported for the cloned proteins that exhibit $b^{0,+}$ and y^+ amino acid transport activity.

The essential difference between transporter mRNA species encoding the same protein is thought to result from alternative sites of polyadenylation. Markovich and co-workers (1993) have suggested that mRNA species that contain a large non-coding

region rich in AU motifs, proximal to the poly A tail, may be more susceptible to degradation, thus explaining the greater recovery of rBAT-1 (few AU motifs) versus rBAT-2 (many AU motifs) mRNA species from rabbit kidney and intestinal tissues. Recently, researchers have begun the practice of attaching poly A motifs to cRNA, regardless of the presence of AU motifs, which results in the increased expression of exogenous proteins in *Xenopus laevis* oocytes (V. Ganapathy, personal communication). The success of this manipulation suggests that it may be the ratio of AU:poly A motifs that dictates the rate of transcript degradation, and not the presence of the AU region.

Functional Aspects of Amino Acid Transport Protein Distribution.

Preferential distribution of tissue-specific transport protein isoforms appears to be a universal phenomenon of transport protein families. For example, within mammalian solute exchange families (NHE_{1-4}), NHE_1 is the ubiquitously expressed Na^+/H^+ antiporter isoform, NHE_2 is expressed in kidney and intestine epithelial cells, but NHE_3 is expressed only in the apical membrane of enterocytes (Reithmeier, 1994). Likewise, the AE_2 anion exchanger (Cl^-/HCO_3^-) isoform is thought to be expressed by most cells to regulate pH homeostasis, whereas erythrocytes (AE_1) and renal intercalated cells (AE_{1b}) express isoforms with relatively high K_t to facilitate the efficient exchange of HCO_3^- across capillary beds and bicarbonate resorption from the blood, respectively (Reithmeier, 1994). Within the mammalian Na^+-dependent solute transport families, Na^+/glucose symport is currently thought to occur across the apical membranes of intestinal epithelial cells by a single isoform (SGLT1). In contrast, in the kidney, most of the glucose resorption occurs by the functioning of the SGLT2 isoform (K_t 1.6 m*M*) in the early proximal tubule, with SGLT1 isoform (K_t .35 m*M*)

functioning to scavenge any remaining glucose in the more distal regions of the proximal tubule (Kanai et al., 1994).

Other biological models exist where multiple transport protein isoforms result in the specialized function of certain cells and tissues. Within facilitative transport protein families, glucose transport is known to occur by four different isoforms (GLUT1-4; Bell et al., 1990). GLUT1 and GLUT3 (relatively high affinity for glucose) are thought to be constitutively expressed by most cells to allow a basal level of glucose nourishment from the blood (Thorens, 1993). In contrast, the low affinity, basolateral membrane-bound GLUT2 is expressed by hepatocytes and renal and intestinal absorptive epithelial cells, and in enterocytes, is only expressed by the mature cells of the villus tip (Thorens et al., 1990). GLUT4 is the insulin-inducible isoform found in brown and white adipose and cardiac and skeletal muscle tissues (Bourney et al., 1990; Calderhead et al., 1990). Together this variety of isoforms provides the animal with a range of transport affinities and capacity for glucose transport, thus allowing whole-animal homeostasis.

The distribution of mRNA transcripts that encode for specific transport proteins and activities differs between mammalian species, and within tissues of the same species (Tables 2.2, 2.3). For example, the rabbit Na^+/glutamate cotransporter (EAAC1) is expressed in many tissues in the rabbit (Kanai and Hediger, 1992), but not in the colon, spleen, or lung, whereas the Na^+/glutamate cotransporter (GLAST) of the rat and human is found only in the brain (Storck et al., 1992). The cloned protein (MCAT-1) responsible for y^+ activity in most mouse tissues tested is expressed in minimal amounts in the liver, which expresses a different protein (MCAT-2; Closs et al., 1993; Woodard et al., 1994).

Likewise, within facilitative amino acid transport proteins, cloning of the protein(s) responsible for Na^+-independent y^+ activity has identified two isoforms that are preferentially expressed in different tissues that function to ensure a constant supply of cationic amino acids to all tissues. The preferential expression of the low affinity MCAT-2 transporter in liver allows excess cationic amino acids to be absorbed from portal circulation after a high protein meal, but ensures that minimal systemic blood concentrations of arginine, lysine, and histidine are always available for absorption by peripheral tissue, which express the high affinity MCAT-1 transport proteins (Closs et al., 1993). Between tissues that express MCAT-1, the quantity of mRNA that is expressed differs and often correlates with known relative requirements of tissues for cationic amino acids. For example, the relatively high copy numbers of MCAT-1 in testicular tissues are thought to facilitate the relatively greater absorption of lysine and arginine required for the production of large amounts of the arginine- and lysine-rich histone and protamine proteins (Kim et al., 1991).

Potential for Amino Acid Transport Protein Regulation

A primary concern of animal nutritionists is the need to understand what the capacity for absorption of a given nutrient is so that diets can be formulated to provide adequate, but not excessive, nutrients for a given production state. Accordingly, it is imperative to understand the physiological mechanisms that regulate the capacity for nutrient uptake, and through this knowledge, their potential for manipulation.

Compared to microbial species, the ability of animal cells to adjust their absorptive capacity for a given nutrient in response to substrate availability and metabolic demand is regarded to be minor (Diamond, 1991). However, in response to fluctuating extracellular concentrations, mammalian cells are known to alter their

ability to absorb sugars, vitamins, minerals, and amino acids (Boerner et al., 1985; Stevens, 1992b). For amino acids, charged and relatively hydrophilic molecules, the amount of carrier-mediated absorption can vary depending on animal age (Buddington, 1992), extracellular and intracellular amino acid concentrations, cell membrane potential (Cheeseman, 1991) and second messengers (Woodlock et al., 1989; Kulanthaivel et al., 1991), rates of transporter synthesis and degradation, and exposure to hormonal agents (Lerner, 1985).

Hormonal Regulation of Amino Acid Transport Protein Activity. Regulation of amino acid transporter activity by insulin and glucagon appears to be affected by stimulating membrane hyperpolarization (reducing membrane potential) and(or) changing the current number of transport proteins in cell membranes (Shotwell et al., 1983; Lerner, 1985). Glucagon has been shown to promote a biphasic induction of increased alanine transport uptake in hepatocytes (Edmondson and Lumeng, 1980). An immediate and transient valinomycin-sensitive increase was followed by a delayed and prolonged cycloheximide-sensitive increase in alanine absorption. These results indicate that glucagon has the ability to increase the activity of at least one transport system by promoting membrane hyperpolarization and by stimulating the synthesis of cellular proteins responsible for alanine absorption.

Theoretically, the number of resident membrane transport proteins can be increased by the recruitment from, or the removal into, the cytosol of pre-existing transporters, as is the case for insulin and glucocorticoid stimulation of facilitated diffusion glucose transporter movement (Matthaei et al., 1987; Horner et al. 1987). At present, however, there is little evidence to indicate that hormones affect the number of amino acid transporters in the plasma membrane by reversible recruitment of cytosolic vesicle-bound transporters. Instead, research employing inhibitors of

gene transcription (actinomycin D) and protein translation (cycloheximide) indicate that insulin and(or) glucagon act to stimulate de novo synthesis of proteins capable of increasing the rate of system A amino acid transport protein(s) in lamb and pig kidney cortex tissue (Scharrer and Landes, 1978), rat hepatocytes (Fehlmann et al., 1979), human fibroblasts (Longo et al., 1985), and Chinese hamster ovarian cells (Englesberg and Moffett, 1986). That inhibition (tunicamycin) of aspartate-linked glycosylation negated the ability of insulin, glucagon, and glucocorticoids (dexamethasone) to increase amino acid absorption (Barber et al., 1983) provides additional evidence that de novo protein synthesis is the mechanism by which these hormones exert their affect.

Hormones that are capable of stimulating protein kinase C activity (e. g., epinephrine, glucagon, ACTH) may well be able to regulate the rate of transporter function. System A activity was quickly (within 1 min) increased in cells after exposure to 1,2-diacylglycerides (DAG; Woodlock et al., 1989). This stimulatory response was maximal after 30 min and was not inhibitable by cyclohexamide, and appeared to be preferential, as System L (Na^+-independent transport of neutral amino acids) activity was not stimulated until after a 3 h of incubation with DAG. In contrast, using Caco-2 cells, protein kinase C mimentics (phorbol esters) have been shown to inhibit the function of the H^+/peptide cotransporter. Inhibition occurred in a manner that did not disrupt the pH gradient (driving force), did not effect the binding affinity of the transporter, and that was independent of de novo protein synthesis (Brandsch, 1994).

Substrate Regulation of Amino Acid Transport. The presence of high extracellular substrate concentrations increases (up-regulation) the capacity of brush border membranes of intestinal and renal epithelial cells to absorb specific amino

acids (Diamond and Karasov, 1987). This process occurs over a period of 12 h to several d (Diamond, 1991; Kilberg et al., 1993). Similarly, a decrease in the capacity for amino acid transport (down-regulation) gradually occurs over several days and is a function of enterocyte replacement of up-regulated enterocytes with those that have basal levels of transport activity (Stevens, 1992a).

In contrast, not all plasma amino acid transport systems are susceptible to substrate regulation, the type of response may differ, and appear to respond more quickly to substrate abundance. For example, in fibroblasts, the activities of amino acid transport Systems ASC and L were not responsive to amino acid deprivation (Bracy et al., 1986; Stein et al., 1987). In rat hepatocytes, amino acid starvation resulted in the reduction of System ASC activity and an increase in System A (Fong et al., 1989). Low extracellular amino acid concentrations are known to stimulate a rapid (1 to 18 h) increase in the rate of system A amino acid absorption in hepatocytes (Shotwell et al., 1983) and fibroblasts (Gazzola et al., 1981). In cultured hepatocytes, amino acid starvation has resulted in the selective induction of mRNA species (Shay et al., 1990) that encoded for constituent proteins of the ribosomal 60S subunit (Laine et al., 1991). Refeeding of specific amino acids down-regulated these induced mRNA species (Kilberg et al., 1994). As with up-regulation events, down-regulation events in hepatocytes are typically thought to be stimulated within minutes after development of high intracellular amino acid concentrations (Guidotti and Gazzola, 1992) and(or) within 1 h by synthesis of inhibitory proteins (Englesberg and Moffett, 1986).

At the transport protein level, uptake of one amino acid species may be influenced by amino acids of other species. The rate of substrate translocation can be moderated by amino acids acting in a cis- (same side) and(or) trans- (opposite side) regulatory manner (Guidotti et al., 1978; Christensen, 1990). For example, the basal

rate of lysine transport across the basolateral membrane vesicles of rat jejunal epithelia has been shown to be increased by allosteric stimulation by leucine and to be inhibited by the competitive binding of arginine (Lawless et al., 1987). Similar stimulatory effects of methionine and leucine on lysine absorption have been observed in rat, rabbit, and chicken intestinal epithelia (Cheeseman, 1991).

For Na^+-dependent cotransport or facilitated transport of dibasic amino acids (lysine, arginine, histidine), the electrochemical gradient of Na^+ or the charged amino acid is thought to drive the carrier protein towards the cytoplasmic side of the membrane (Dawson, 1991). After movement of the charged transporter through the cell membrane, the transporter is preferentially retained facing the cytoplasm, hence retarding both the return of the transporter to the extracellular face of the membrane and subsequent substrate transport. In a manner not fully understood, high intracellular amino acid substrate levels promote this transinhibitory process (Kilberg et al., 1994). In contrast, low intracellular concentrations act to promote carrier oscillation (Fong et al., 1989).

Models for substrate regulation of System A (Na^+-dependent neutral amino acids) transporter activity by intracellular amino acids have been proposed for fibroblasts (Gazzola et al., 1981), Chinese hamster ovarian cells (Englesberg and Moffett, 1986), and hepatocytes (Kilberg, 1986; Kilberg et al., 1994). Transport activity is hypothesized to be controlled by intracellular binding of the transporter protein, and(or) its gene, by a regulatory protein. The relative equilibrium between active and non-active regulatory proteins would be determined by the binding of intracellular amino acids to the regulatory protein and(or) to its gene. Accordingly, this model allows for system A transport activity to be regulated by its specific substrates or by amino acids transported by other transport systems, thus accounting

for the observed ability of amino acids absorbed by one transport system to moderate the absorption of their own or other transport proteins (as discussed above).

Recent research has provided experimental evidence to suggest that amino acid starvation induces increased system A activity by stimulating the transcription of genes encoding for ribosomal 60S subunit proteins (Shay et al., 1990; Laine et al., 1991). More thorough investigations (Kilberg et al., 1994) revealed that down-regulation of increased levels of these ribosomal protein mRNA was achieved by amino acids that are substrates of System A, and by amino acids thought to be specific substrates of other amino acid transport systems. The pattern of mRNA inhibition that was observed paralleled the pattern of System A transporter inhibition demonstrated in earlier amino acid transport trials (Bracy et al., 1986). Collectively, these experimental results suggest that cellular sensing of amino acid concentrations may occur by a common mechanism.

The purpose of this monitoring does not appear to be to provide the cell with a method of coordinating substrates of protein synthesis with increased amounts of rRNA available for general protein translation. If this were so, then a reduction in ribosomal protein mRNA would be expected to parallel lower levels of amino acids in the cytosol. Instead, reduced levels of cytosolic amino acids results in an increase of mRNA, an increase that appears to be associated with the up-regulation of System A activity (Kilberg et al., 1994). These observations suggest that these specific ribosomal proteins may be involved in the preferential translation of proteins responsible for the increase of System A transporter activity. Obvious candidates for preferential translation would be the mRNA species of regulatory proteins that have been hypothesized (see above) to bind to system A transporters or proteins that bind to the regulatory proteins.

Absorption of Free and Peptide Amino Acids by the Ruminant Gastrointestinal Tract.

Compared to monogastrics, little is known about specific mechanisms responsible for the absorption of proteins in ruminants, especially by the forestomach epithelium. Less is known about their potential for regulation. Research that has identified some of the biochemically characterized amino acid transport systems that probably function in ruminants has been summarized below.

Forestomach Morphology. The potential for the absorption of peptide and free amino acids across the three forestomach organs (rumen, reticulum, omasum) is not well characterized. The keratinized squamous epithelium that lines these organs is thought to have evolved from the squamous gastric mucosa (Stevens, 1973; Martens and Gabel, 1988). Electron microscopy studies have revealed that the forestomach epithelium is composed of four strata of epithelial cells that are organized in the same spatial order in all three forestomach organs (Figure 2.1.B) (Hyden and Sperber, 1965; Henrikson, 1970). Starting proximal to the basement membrane (adjacent to the connective tissue of the forestomach musculature), the four strata of epithelial cells have been classified as the basale, spinosum, granulosum, and corneum (Steven and Marshall, 1970).

Forestomach epithelial tissues structurally differ from skin epithelia by lacking the extremely thick keratinized layer and glandular ability, and differ functionally by their ability to absorb large volumes of water. Epithelial cells of the forestomach differ most dramatically from intestinal epithelia in their morphological arrangement on papillae (Figure 2.1). Functionally, whereas enterocytes rely on the mucous secretion from globlet cells to provide a protective barrier from digesta, the outer strata of dead corneal cells provides protection for the inner strata from the

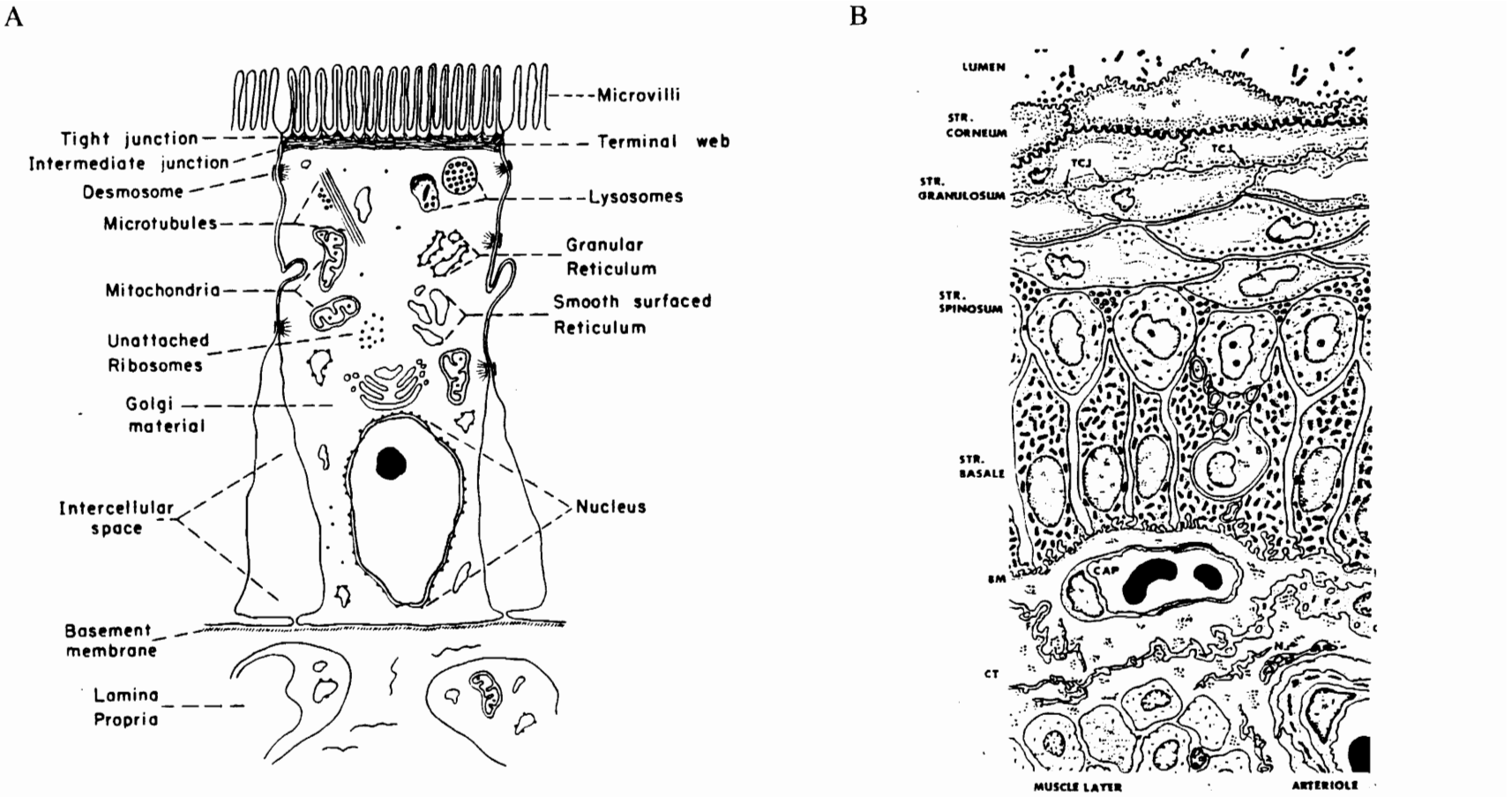


Figure 2.1. Diagramatic representations illustrating mammalian epithelial cells. A; small intestine enterocyte (Madara and Trier, 1987). B; ruminant forestomach epithelia (Steven and Marshall, 1970). BM, Basement membrane; CAP, capillary; CT, connective tissue; F, fibroblast; N, nerve trunk; TCJ, tight cell junction.

mechanically abrasive digesta of the forestomach. Both enterocytes and forestomach epithelia rely on the presence of tight junctions to form solute barriers and allow selective absorption of nutrients. In enterocytes, nutritionally significant amounts of glucose (Pappenheimer, 1993) and oligopeptides (Atisook and Madara, 1991) have been observed to be absorbed by the non-selective, paracellular pathway. Non-mediated absorption of nutrients through the forestomach epithelium is thought to be dependent on an osmotic gradient established by the active transport of Na^+ across the granulosa strata (Keynes, 1969; Gabel et al., 1993), thereby potentially generating the "solvent drag" driving force required for paracellular absorption. Because of the multi-layered cell structure of the forestomach epithelium, and because of the relatively "leaky" tight junctions of the granulosa strata (Fell and Weekes, 1975), paracellular absorption may also be an important mechanism for the absorption of nutrients from the forestomach lumen into the blood.

Omasal epithelial tissues may possess a greater potential for nutrient absorption from the digesta than does ruminal epithelial tissue. Compared to the rumen, it has been observed that the omasal epithelium possesses greater metabolic activity (Engelhardt and Hauffe, 1975), greater numbers of branching cells in the stratum basale (Steven and Marshall, 1970), greater potential electrolyte flux (Martens and Gabel, 1988), and a greater blood supply (blood flow/wet weight of organ, Engelhardt and Hales, 1977). A more detailed review of forestomach epithelium structure and function by this author is available (Matthews, 1991b).

Forestomach Absorption of Free Amino Acids. The ability of the ruminant forestomach to absorb amino acids has not been well characterized. In parabiotic chambers, the absorption of histidine across rumen epithelial sheets was not saturable from .66 to 20 m*M* (Leibholz, 1971a). However, histidine transfer across this tissue

may have been at least partially mediated because methionine (50%), arginine (50%), and glycine (40%) inhibited histidine passage, when coincubated at equal concentrations (.66 m*M*). In hindsight, because a lumen-to-blood Na^+ gradient does not typically exist, that arginine inhibited the absorption of histidine indicates that these substrates may have been competing for recognition by the Na^+-independent y^+ transporter. Alternatively, and(or) concomitantly, the demonstration that arginine, methionine, and glycine inhibited histidine absorption parallels the competitive profile displayed by the Na^+-independent $b^{o,+}$ transporter system (Van Winkle et al., 1988). Additional evidence that ruminal epithelia of sheep may possess proteins capable of facilitative transport of cationic amino acids is supported by the observation that the flux of lysine and arginine across ruminal tissue sheets was saturable from .3 to 30 m*M* (Fejes et al., 1991).

Forestomach Absorption of Peptide Amino Acids. In a study designed to evaluate the potential for forestomach epithelia to absorb dipeptides, ruminal and omasal epithelial tissues mounted in parabiotic chambers were observed to possess the ability to absorb intact L-[^{35}S]-methionylglycine and beta-alanylhistidine (Matthews, 1991b). The omasum demonstrated a markedly (approximately six-fold) greater ability to absorb these dipeptides than did the rumen. For both tissues the total flux of carnosine (6 to 96 m*M*) and methionylglycine (.375 to 12.0 m*M*) across the tissues was not saturable. How the absorption of these dipeptides compared to that of free amino acids was not evaluated.

From a teleological perspective, the lack of demonstrated mediated absorption was surprising. Ions reported to be involved in active, secondary transport of small peptides (H^+, Na^+) in eukaryotes (Webb and Matthews, 1994) are constituents of forestomach liquor. Omasal liquor is essentially acidic (Prins et al., 1972) and

ruminal liquor can develop pH levels of 5.5 or less (Whitelaw, 1970). Proton gradients of this magnitude have been used to demonstrate the presence of carrier-mediated dipeptide transport by intestinal and renal epithelial BBMV (Ganapathy et al., 1983; Daniel, et al., 1991) and in cultured colon cells (Saito and Inui, 1993). Additionally, the Na^+/H^+ exchanger and Na^+/K^+ ATPase proteins, considered to be essential in reestablishing proton gradients in other epithelial cells, are reported to exist and function in both ruminal and omasal epithelia (Martens and Gabel, 1988).

Expected Amino Acid Substrate Concentrations in Forestomach Liquor. A review of the literature indicates that pre-feeding levels of free amino acid concentrations in strained ruminal fluid of .12 to 1.5 mg/dL and post-feeding concentrations of .72 to 6 mg/dL would be available to drive non-mediated absorption of free amino acids in sheep (Table 2.5). Likewise, it is reasonable to expect pre-feeding concentrations of 1.5 to 5 mg/dL of peptide N and post-feeding concentrations of 10 to 27 mg/dL of peptide N in ruminal fluid (Table 2.6). From this information, it appears that a greater osmotic driving force for peptide-bound than free amino acid absorption exists in ruminal liquor, before and after the feeding of common diets. If the data in Tables 2.5 and 2.6 are representative of in vivo substrate concentrations, then the in vivo driving force for peptide amino acid absorption would exceed that for free amino acids. Likewise, if the in vivo absorption capacities of forestomach tissues is primarily dependent upon forestomach liquor concentrations (as characterized by Matthews, 1991b), then proportionately more amino acids would be expected to be absorbed across the forestomach in the form of peptides than as free amino acids. Because omasal liquor amino acid and peptide N concentration values are unknown, it is not possible to predict what the potential relative driving forces for amino acid absorption across the omasal epithelium would be. However, the absorption of water

Table 2.5. Amino acid-N concentrations in strained ruminal fluid

Nitrogen supplement	Species	CP, %	Amino acid-N, mg/dL Pre-Feeding	Post-Feeding	Source
None	Sheep	8.3[b]	.12	.72 (3)[a]	Lewis, 1955
None	Sheep	13.8[c]	.28	.93 (3)	"
Casein	Sheep	21.3[d]	.34	1.4 (3)	"
Casein	Sheep	15.0[e]	1.5	6.0 (2)	Annison, 1956
None	Sheep	4.4[f]	.22	1.0 (1)	Leibholz, 1969
Meat meal	Sheep	16.1[g]	.28	1.8 (1)	"
Skim milk	Sheep	16.1[h]	.32	1.7 (1)	"
None	Sheep	16.1[i]	.50	2.5 (1)	"
None	Sheep	18.2[j]	.48	5.1 (1)	"
Urea	Sheep	15.6[k]	.23	.73 (1)	Broderick and
Casein	Sheep	15.6[l]	.25	.98 (1)	Wallace, 1988

[a]The time (hours) of ruminal fluid collection after feeding. The time listed corresponds to the only (Lewis, 1955; Leibholz, 1969) or maximal (Annison, 1956; Broderick and Wallace, 1988) amino acid-N concnetrations reported by the researchers.

[b]Diet consisted of .9 kg of hay. Animal had access to feed for 2 h.

[c]Diet consisted of .4 kg of hay and .5 kg dried grass. Animal had access to feed for 2 h.

[d]Diet consisted of .7 kg of hay, and .15 kg casein. Animal had access to feed for 2 h.

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

[f]Diet consisted of 1 kg wheat chaff, consumed within 1 h.

[g]Diet consisted of .72 kg wheat chaff and .28 kg meat meal, consumed within 1 h.

[h]Diet consisted of .64 kg wheat chaff and .36 kg dried skim milk, consumed within 1 h.

[i]Diet consisted of .6 kg alfalfa hay and .4 kg barley, consumed within 1 h.

[j]Diet consisted of 1 kg of alfalfa hay, consumed within 1 h.

[k]Diet (as fed) consisted of .67 kg ryegrass and .33 kg of concentrate (32% urea, DM basis), divided over two feedings.

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

Table 2.6. Peptide-bound amino acid-N concentrations in strained ruminal fluid

Nitrogen supplement	Species	CP, %	Peptide-N, mg/dL Pre-Feeding	Post-Feeding	Source
Casein	Sheep	15.0[b]	1.5	10 (2)[a]	Annison, 1956
Casein	Sheep	15.6[c]	1.5	27 (1)	Broderick and Wallace, 1988
SBM[d]-FM[e]	Lactating	17.8[f]	5.4[g]	15 (2)	Chen et al.,
SBM-ESB[h]	cows	17.8[f]	6.0[g]	16 (2)	1987
SBM	"	17.8[f]	5.4[g]	22 (2)	"
None	Dry cows	17.0[i]	—	18[j](0-6)[k]	Broderick
DDGS[l]-CGM[m]	"	18.1[i]	—	22[j](0-6)[k]	et al., 1990
ESBM[n]	"	18.1[i]	—	23[j](0-6)[k]	"
SBM	"	18.9[i]	—	24[j](0-6)[k]	"

[a]The time (hours) of ruminal fluid collection after feeding. The time listed corresponds to the only (Chen et al., 1987; Broderick et al., 1990) or maximal (Annison, 1956; Broderick and Wallace, 1988) peptide-bound amino acid-N concentrations reported by the researchers.

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

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

[d]Soybean meal.

[e]Fish meal.

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

[g]Sampled taken 14 h after the daily feeding.

[h]Extruded soybean meal.

[i]The TMR (DM) contained 58% alfalfa silage, 34% high moisture corn, and 1% mineral mix. The portion of corn grain added depended on the CP percentage supplied by the protein supplements: 7% (no supplement), 1.6% (4.2% DDGS plus 1.5% CGM), 2.6% (4.6% ESBM), and 0% (7.2% SBM).

[j]Values were calculated from millimoles reported per liter peptide-bound amino acid concentrations, amino acid residues:peptide ratios, and assuming 1 g of N per 54.2 mmol of amino acids, as presented.

[k]Values were reported as the mean of five sampling periods (0, 1, 2, 4, and 6 h postfeeding.

[l]Distillers dried grains with solubles.

[m]Corn gluten meal.

[n]Expeller soybean meal.

by omasal tissues would presumably result in the concentration of rumen liquor solutes, thereby potentially reestablishing (or generating greater) omasal liquor-to-blood concentration gradients and solvent-drag forces that were present across ruminal epithelia, depending on the relative water and substrate absorption rates (Smith, 1984).

Intestinal Absorption of Free Amino Acids. The literature abounds with publications enumerating the characterization of free amino acid transport proteins in monogastrics. Although to a lesser extent, similar transport systems have been identified in ruminants. Like monogastrics, the duodenal, jejunal, and ileal regions of the ruminant small intestine appear to have different abilities to absorb amino acids. In the rat (Matthews and Laster, 1965) and pig (Leibholz, 1989) the jejunum has demonstrated greatest potential for the absorption of amino acids. In contrast, in sheep, the ileal region of the intestine has been identified as possessing the greatest potential for free amino acid absorption (Williams, 1969; Johns and Bergen, 1973; Phillips et al., 1979).

The uptake of methionine and lysine by ileal and jejunal BBMV of steers was characterized as having active, facilitative, and diffusional components (Wilson and Webb, 1989). For both ileal and jejunal brush borders, at both 1.25 and 7.5 m*M* concentrations, a greater contribution to total uptake occurred by diffusion than by Na^+-dependent or Na^+-independent mediated transport. It was also observed that transporters had lower affinities and higher capacities for methionine than did transporters for lysine, in both ileal and jejunal tissue.

The ability of 50 m*M* concentrations of alanine, glycine, leucine, lysine, phenylalanine, leucine, and methionine to mutually inhibit the uptake (.1 m*M*) of each other across bovine ileum BBMV has been studied (Moe et al., 1987). The pattern of

mutual inhibition is characteristic of absorption by the neutral and cationic transport family (Van Winkle, 1988). Because uptake was compared in the presence of Na^+, further delineations between Na+-dependent ($B^{o,+}$) and Na^+-independent ($b^{o,+}$) transport can not be determined. Overall, active transport, facilitated diffusion, and simple diffusion accounted for 14, 37, and 49% and 9, 53, and 38% of methionine and lysine transport, respectively.

The observation (Crooker and Clark, 1987) that the uptake of methionine (.1 m*M*) by ileal mucosa was not influenced by the presence of up to 1 m*M* lysine and that the amount of lysine uptake at .1 m*M* was inhibited when coincubated with equal concentrations of arginine and ornithine, but not by 1 m*M* methionine, suggests that bovine intestine expresses a y^+ (Na^+-independent cationic) amino acid transport activity. Together, these observations indicate that the genomes of ruminants are capable of encoding various proteins required for the transport of free amino acids. These studies also indicate that the relative contribution of carrier-mediated amino acid absorption will vary, depending on the concentration of luminal substrates. For peripheral tissues, which are exposed to much lower amino acid concentrations, the contribution of mediated transport typically exceeds that of non-mediated diffusion (Kilberg and Haussinger, 1992).

Summary

The ability to absorb dietary peptides is reputed to be a strong selective advantage to organisms (Olson et al., 1991). The discovery of peptide transport proteins, because of their nutritionally significant capacity for amino acid absorption, demands that we re-evaluate our understanding of how tissue amino acid needs are met. One novel concept that has been proposed (Fisher, 1954) suggests that "peptides,

rather amino acids, may be the protein currency of the body." As discussed, the ruminant small intestine is capable of mediating the absorption of free amino acids. It is not known whether these tissues possess mechanisms involved in the selective absorption of peptides. Also, the relative importance of free versus peptide amino acids and forestomach versus intestinal absorption of total amino acids has not been thoroughly evaluated. However, one study has quantified the relative flux of both free and peptide amino acids across the mesenteric and non-mesenteric viscera of calves and sheep (DiRienzo and Webb, 1995). For both species, the flux of free and peptide amino acids across the jejunum, ileum, and large intestine (mesenteric viscera) was approximately equal and the flux of free amino acids across the forestomach, stomach, duodenum, and spleen (non-mesenteric viscera) was nominal. In contrast, the flux of peptide amino acids across the non-mesenteric viscera accounted for approximately 77% of total portal-drained (mesenteric and non-mesenteric) visceral amino acid absorption. From the results of this study, supported by the in vitro determination that ruminal and omasal epithelia are capable of intact dipeptide absorption (Matthews, 1991b), a hypothesis was developed that the forestomach epithelium may be capable of absorbing nutritionally significant quantities of amino acids, in the form of small peptides (Webb et al., 1992).

This review has focussed on several of the physiological mechanisms thought to influence the ability of cells to absorb amino acids. The physiological mechanisms responsible for the absorption of free and peptide-bound amino acids can be relatively simple (non-mediated diffusion) or complex (mediated transmembrane passage). The relative contribution of each of these mechanisms to amino acid nourishment of animals varies throughout the gastrointestinal and peripheral tissues, depending on substrate supply, cell type, tissue, and(or) whole-animal metabolic status. In the final

analysis, however, it is the aggregate contribution of all these absorption phenomena that supplies the animal with its requisite amino acids. Once the genes, molecular structures, and sites of function of proteins involved in the absorption of free and peptide-bound amino acids are identified, the potential for understanding and manipulating their function will be realized.

CHAPTER III

OBJECTIVES

Little is known about specific mechanisms responsible for the absorption of free and peptide-bound amino acids across the intestinal epithelia of livestock species. Even less is known about the potential function of these mechanisms in ruminant forestomach epithelia, which have recently been hypothesized to possess the ability to absorb nutritionally significant amounts of amino acid N. Therefore, the overall objective of the research presented in this dissertation was to characterize the potential for free and peptide-bound amino acid absorption by the forestomach epithelia of sheep. Specific objectives included:

1) To characterize the absorption of free methionine across ruminal and omasal epithelial tissues of sheep, using parabiotic chambers.

2) To compare this absorption of free methionine to that of peptide-bound methionine (methionylglycine).

3) To develop an assay that could be used to detect the presence of potential free and peptide-bound amino acid transport proteins, based upon functional expression of their mRNA, from any tissue.

4) To employ this assay to identify and characterize the function of Na+-independent amino acid transport proteins in the epithelium of the pig jejunum.

5) To employ this assay to confirm or refute the lack of mediated free and peptide-bound amino acid transport proteins in forestomach epithelial tissues, as characterized by parabiotic chamber studies.

CHAPTER IV

ABSORPTION OF L-METHIONINE AND L-METHIONYLGLYCINE BY ISOLATED SHEEP RUMINAL AND OMASAL EPITHELIAL TISSUE

ABSTRACT

The absorption of methionine and methionylglycine (using [^{35}S]-methionine and [^{35}S]-methionylglycine as representative markers) across ruminal and omasal epithelia collected from seven sheep were studied using parabiotic chambers that were repeatedly sampled over a 240-min incubation. The quantity of all substrates transferred was linearly ($P < .01$) dependent on initial substrate concentration and time. More ($P < .01$) methionine and methionylglycine were transferred across omasal than ruminal epithelia. Methionylglycine was transferred intact across both tissues. A greater ($P < .01$) quantity was hydrolyzed by omasal than ruminal epithelia, after 240 min incubation. Greater ($P < .06$) quantities of methionine and methionylglycine accumulated in ruminal than in omasal tissue after 240 min. Total absorption of methionine and methionylglycine did not differ within tissues, but total absorption of both substrates was greater ($P < .01$) by omasal than by ruminal tissues. There was little evidence for carrier-mediated transport. Therefore, these results indicate that omasal epithelial tissue possesses a greater ability than does ruminal epithelial tissue to absorb both free and bound methionine .

(**Key Words:** Sheep, Rumen, Omasum, Amino Acid, Peptide, Absorption)

Introduction

Historically, the potential for absorption of significant quantities of amino acids from the ruminant forestomach has been regarded as minor. It is generally thought that there is rapid metabolism of amino acids by rumen microbes and that forestomach epithelia have little capacity for amino acid absorption. Consequently, the fate of dietary amino acids has been discussed in terms of their conversions to ammonia N, incorporation into microbial protein, absorption by the intestine, and excretion.

Research from this laboratory (Webb et al., 1993) has caused us to question this understanding. Measurement of the arterial-venous flux of amino acids across the gastrointestinal tract of wethers and steers has identified non-mesenteric drainage as being the primary contributor of total plasma amino acids to the liver in the form of peptide-bound amino acids (DiRienzo and Webb, 1995). In vitro, it has been demonstrated that isolated sheep ruminal and omasal epithelia have the ability to absorb carnosine (Matthews, 1991b). These observations, along with the reported existence of relatively high peptide N concentrations in strained ruminal fluid (Annison, 1956; Broderick and Wallace, 1988), suggested that the forestomach tissue may have evolved the ability to absorb both amino acids and dipeptides.

For non-ruminants, carrier-mediated amino acid and dipeptide transport across the brush border and basalateral membranes of the single cell-layered intestinal epithelium have been characterized (Matthews, 1991a; Gardner, 1994). The present study was designed to investigate and compare the potential for free (methionine) and peptide-bound (methionylglycine) amino acid absorption across the multiple cell-layered ruminal and omasal epithelia of sheep.

Materials and Methods

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

Animals, Feeding and Tissue Collection. Tissues from seven Dorset X Finn X Rambouillet wethers (average BW 38.8 kg) and seven Hampshire ewe lambs (average BW 38.3 kg) were used to determine L-methionine (methionine) and L-methionylglycine (Met-Gly) uptake, respectively. All lambs were fed a mixed diet containing 50% ground, shelled corn, 30% orchardgrass hay, 13.3% soybean meal, 5% molasses, .5% trace mineral salt, .42% defluorinated rock phosphate, and .5% limestone (as-fed basis). Additionally, the diet contained enough decoquinate (Cocci-Control [ix] Crumbles Medicated, Southern States, Inc., Richmond, VA) to supply .5 $mg/day^{-1}sheep^{-1}$. Lambs were housed and fed as a group with continuous access to water. The quantity of diet fed was sufficient to produce .23 kg body weight $gain{\cdot}head^{-1}{\cdot}d^{-1}$ (NRC, 1985a). The lambs were injected with vitamin A (500,000 IU), vitamin D (75,000 IU), vitamin E (3.7 $IU{\cdot}kg^{-1}$), and Se (55 $\mu g{\cdot}kg^{-1}$) approximately 2 wk before the first lamb was removed for tissue collection. The lambs were weighed at 2-wk intervals, and the amount of diet fed was adjusted to maintain rate of growth.

On any given day, tissues were collected from only one lamb. At the time of tissue collection, the heaviest lamb in the group was selected, weighed, stunned with a captive-bolt pistol (Super Cash Mark 2, Accles and Shelvoke LTD., Birmingham, England), and exsanguinated. The abdominal cavity was opened quickly and the

stomach was removed. The rumen was opened along the dorsal surface and the digesta were removed from the rumen and reticulum by rinsing with tap water. The stomach was placed in .85% NaCl and transported to the laboratory. All rinsing solutions and buffers used in the preparation and incubation of tissues were maintained at 39°C.

In the laboratory, the ventral sac of the rumen was removed and serially washed with gentle agitation in five baths containing .85% NaCl to remove adhering digesta particles. The tissue then was placed in oxygenated (O_2) Krebs Ringer Phosphate (KRP) buffer (pH 7.4). Omasal plies were removed and treated in a like manner. Ruminal epithelium was stripped from the underlying muscle layer by careful dissection. Omasal epithelium was prepared by peeling apart the opposing surfaces of individual plies. Epithelial tissues then were cut into sections measuring approximately 4 cm x 4 cm.

Buffer Preparation. Krebs Ringer Phosphate buffer (Umbreit et al., 1964), pH 7.4, was used for tissue preparation and as the initial buffer in both chambers of the parabiotic units. After preparation, the KRP buffer (pH 7.4) was held overnight at 39°C in a waterbath and gassed with O_2 for 1 h before use. For uptake measurements, the initial buffer in the mucosal chamber was replaced with 15 mL KRP buffer (pH 6.0) containing an appropriate concentration of Met or Met-Gly. The initial buffer in the serosal chamber was replaced with 15 mL KRP buffer (pH 7.4) containing 10 m*M* D-glucose and enough D-mannitol to equalize osmolarity among the chambers. Buffers used for uptake measurements were refrigerated overnight in individual 40-mL sealed tubes. Two hours prior to beginning the experiment, these buffers were aspirated into 20-mL syringes (Sherwood Medical, St. Louis, Mo) that

were capped with a 25-gauge needle (Becton Dickinson and Company, Rutherford, NJ), inserted into a neoprene stopper, and placed into a 39°C waterbath.

Uptake Measurement. Parabiotic units consisted of two L-shaped glass chambers of equal volume separated by a neoprene O-ring and held together by a clamp. Epithelial tissues were mounted between the two chambers. The chambers were designated as mucosal or serosal with reference to tissue orientation. The area of exposed tissue was 1.77 cm^2. After tissues were mounted, the mucosal and serosal chambers were loaded with 10 mL of KRP buffer (pH 7.4) and the parabiotic units were placed into a waterbath at 39°C until uptake measurements began. The total elapsed time between stunning of the sheep until beginning uptake measurements was approximately 25 to 30 min.

Uptake measurements were initiated by replacing the initial buffer with appropriate uptake buffers. This point was considered to be time zero. A gassing/sampling apparatus, consisting of two lengths of polypropylene tubing (Atlantic Tubing Co., Patterson, NJ) inserted through a neoprene stopper, was inserted into each chamber. Sampling was performed by attaching a 1-mL syringe to the luer stub adapter of the sampling line of each chamber and withdrawing .6 mL of buffer. All chambers were gassed at a similar rate by passing O_2 through the other polypropylene tubing. After the last sample was taken, tissues were removed and the area exposed to buffers was excised, blotted with paper, and weighed. In a preliminary experiment, the ability of experimental buffers to maintain tissue metabolism was confirmed by the linear increase of beta-hydroxybutyrate in serosal chamber buffers throughout 240 min of incubation.

For each of the seven animals used for methionine or Met-Gly uptake studies, six parabiotic units were prepared for ruminal and omasal epithelial tissues,

simultaneously. The mucosal chamber of each parabiotic unit was loaded with KRP buffer (pH 6.0) containing L-[^{35}S]-methionine (Product No. SJ.235; Amersham Corp., Arlington Heights, IL) or L-[^{35}S]-methionylglycine and enough methionine or Met-Gly to make the final concentration .375, .75, 1.5, 3, 6, or 12 m*M*. The total radioactivity in each mucosal chamber buffer (MCB) was the same within an animal but the amount of radioactivity varied between animals (6.84 to 8.36 *u*Ci, methionine study; 4.13 to 8.02 *u*Ci Met-Gly study). The specific activity of the mucosal buffer of each animal was calculated. Both MCB and serosal chamber buffer samples were collected after 5, 10, 15, 30, 45, 60, 120, 180, and 240 min of incubation. Preliminary experiments demonstrated that the mucosal (pH 6.0) to serosal (pH 7.4) pH gradient across epithelial tissues was maintained throughout the 240-min incubation period.

Sample aliquots of .5 mL were mixed with 5 mL of scintillation fluid (Scintiverse BD; Fisher Scientific Products Corp., Pittsburgh, PA) and ^{35}S content quantified by liquid scintillation counting (LS 5000TA Scintillation Counter; Beckman Instruments, Fullerton, CA) after a minimum of 4 h equilibration time. Recorded cpm were converted to dpm using a standard quench curve. The amount of Met-Gly or methionine appearing in serosal chamber buffers was calculated to be the product of dpm quantified, buffer volume, substrate concentration in the time-zero mucosal buffer, and the specific activity of the time-zero mucosal buffer.

After the last sample was taken (240 min), the remaining buffers were collected by aspiration and stored at -20^{o}C. Cold (2^{o}C) KCl (150 m*M*) was added to each chamber (10 mL) to stop tissue metabolism before removal. The area of tissue that was exposed to the buffers (1.77 cm^2) was excised, weighed, and digested in 1.2 mL distilled water and 2 mL Scintigest (Fisher Scientific Products Corp., Pittsburgh, PA) for 24 h at 50^{o}C in a waterbath. After digestion, 15 mL scintillation fluid

(Scintiverse BOA; Fisher Scientific Products Corp., Pittsburgh, PA) were added. The amount of ^{35}S contained in the tissue was quantified after 20 h of equilibration. The concentration of substrate in the digested tissue samples was calculated by dividing the tissue dpm by the specific activity of the MCB. Because tissues from the parabiotic chambers were digested for the quantification of ^{35}S, separate samples were used to estimate the dry matter content of epithelial tissue. Replicate samples of both the ruminal and omasal tissues were incubated in KRP (pH 7.4) at 39°C for 240 min, simultaneously with the uptake measurement. These tissue were blotted, weighed, and dried at 100°C for 24 h.

Uptake was expressed as nmols/mg dry tissue. Because the amount and rate of nutrient absorption is very likely a function of tissue surface area, it would have been desirable to express absorption on a surface area basis, especially when surface areas vary between tissues. But because no meaningful measure of surface area is available and because it is thought that surface area is quite different between equal cross-sections of ruminal and omasal epithelia, it was decided to express all substrate measurements on a dry tissue basis. Accordingly, it is assumed that mass of dry tissue is more related to surface area than is cross-sectional area (Stevens and Stettler, 1966) and, therefore, is a more accurate basis for comparing ruminal and omasal epithelia uptake of free and peptide amino acid substrates.

For the Met-Gly uptake study, potential hydrolysis of the dipeptide was assayed by characterizing the distribution of ^{35}S among different compounds of the serosal buffer using TLC. Samples from the 12 m*M* parabiotic units obtained at 240 min were removed from the freezer and vacuum evaporated in a cold finger immersed in liquid nitrogen. The concentrated samples were spotted on TLC plates (Silica F_{254}, 150 microns, 60 angstroms; Universal Scientific, Inc., Atlanta, GA) and

developed in methanol:chloroform (3:1, v/v). Reference standards of methionine and Met-Gly were chromatographed simultaneously. Following chromatography, the TLC plates were dried, sprayed with a solution containing .5% ninhydrin (w/v) in ethanol, and placed in an oven at 100°C for 7 min. Areas within each lane on the TLC plate that corresponded to the migration of the reference standards (methionine and Met-Gly), as well as an area encompassing the lane from approximately the origin up to the methionine area were identified. The TLC coating from each of these three areas was scraped from the plate and placed into scintillation vials containing .5 mL of .1 N HCl. Scintillation fluid (Scintiverse BD; Fisher Scientific Products Corp., Pittsburgh, PA) (5 mL) was added and ^{35}S content was quantified. The sum of ^{35}S activity detected in the three areas was calculated and the relative proportions of ^{35}S activity present in each area was calculated by dividing the ^{35}S activity found in an area by the total ^{35}S activity. The 12 m*M* serosal buffer components of the methionine study were not analyzed for fractional ^{35}S representation. For both methionine and Met-Gly studies, the total ^{35}S activity loaded initially into the MCB of the parabiotic units was recovered.

Synthesis of L-[^{35}S]-Methionylglycine. L-[^{35}S]-methionylglycine was synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis techniques (Fields and Noble, 1990). N-Fmoc-L-[^{35}S]-methionine was synthesized using a modified procedure of Atherton and Sheppard (1989). L-^{35}S-methionine (Product No. SJ.235; Amersham Corp., Arlington Heights, IL) (5 mCi) was added with brisk stirring to 1 mL of 8.15% Na_2CO_3 containing 4.43 mg of methionine. One milliliter of 9-fluorenylmethyl succinimidyl carbonate (Calbiochem Corp., LaJolla, CA) (.033 mol/L in 1,2-dimethoxyethane) was added over the course of 1 h. This acylation reaction was allowed to proceed for 19 h. The reaction mixture was filtered

(Teflon syringe-tip filter, .5 micron; Millipore, Bedford, MA), neutralized with HCl to pH 7.0 and vacuum evaporated. Ethyl acetate was added (1.5 mL) and the organic layer was washed with .1 N HCl (four times), then with distilled water saturated with NaCl (three times), and dried by adding $MgSO_4$. The organic layer was then filtered (Teflon syringe-tip filter, .5 micron; Millipore, Bedford, MA) and vacuum evaporated. N-Fmoc-L-[^{35}S]-methionine/N-Fmoc-L-methionine was crystallized out of solution with cold (2^{o}C), re-distilled petroleum ether, recovered as a dry solid after vacuum evaporation of the ether, and stored at -20^{o}C.

N-Fmoc-Gly-Resin (Bachem, Inc., Torrance, CA) (16.6 mg) was added to a silanized reaction vessel equipped with a Teflon-lined screw cap, sintered glass frit, and stopcock for washing according to the procedure of Atherton and Sheppard (1989). Deprotection of the resin compound was performed by adding 200 *u*L of 20% piperidine (Fisher Scientific Products Corp., Pittsburgh, PA) in N,N-dimethylformamide (Fisher Scientific Products Corp., Pittsburgh, PA), shaking (Model 4651 Aliqout Mixer; Miles inc., Diagnostics Division, Elkhart, IN) for 20 min, and then washing with .7 mL of N,N-dimethylformaide (ten times). This deprotection procedure was repeated once.

N,N'-diisopropylcarbodiimide in dimethylformamide (1 mol/L, .5 mL) was added to synthesized N-Fmoc-L-[^{35}S]-methionine/N-Fmoc-L-methionine (2.9 mg), N-Fmoc-L-methionine (Bachem, Torrance, CA) (3.0 mg), and 1-hydroxybenzotriazole (67.6 mg). Ester activation was allowed to proceed for 15 min. This reaction mixture was added to the deprotected resin in the reaction vessel and peptide bond formation (acylation) was allowed to proceed for 1.75 h during constant shaking (Model 4651 Aliqout Mixer; Miles inc., Diagnostics Division, Elkhart, IN). The resulting N-Fmoc-L-[^{35}S]-methionylglycine-resin compound was deprotected as

described above, washed with dichloromethane (three times) and methanol (three times), and vacuum evaporated in the reaction vessel for 4 h. The dipeptide was cleaved from the resin using Reagent K, as described by King et al. (1989). The filtrate was vacuum evaporated (3 h) and stored at -20°C.

L-[^{35}S]-methionylglycine was purified from the filtrate by HPLC using a reverse phase C_{18} column (.9 X 30 cm; Waters Div. of Millipore, Milliford Corp., Milford, MA) with a linear gradient (100:0 to 0:100) formed from .1% trifluoroacetic acid (Fisher Scientific Products Corp., Pittsburgh, PA) and .1% trifluoroacetic acid: acetronitrile (Fisher Scientific Products Corp., Pittsburgh, PA) (50:50), pumped at a rate of 1 mL/min for 75 min. During elution of radioactively labeled peptides, 1-min fractions were collected (Model 328 Fraction Collector; Instrument Specialties Co., Lincoln, NB) from the column and the radioactivity in each was measured, as described above for serosal buffer samples. Four fractions that bracketed the 8.7 min elution time of the peptide (as determined by a reference standard) were dried by vacuum evaporation and stored at -20°C until use.

Statistical Analyses. A split-plot design was used. The effect of substrate was the main plot effect with animal effects nested within substrate effects. Subplot effects of tissue and substrate concentration was imposed using a 2 x 6 factorial design within each animal. Data were evaluated by analysis of variance using the GLM procedure of SAS (1988) after natural log transformation of the means. Transformation of the means was required to stabilize the variance. For the serosal appearance data, the effects of substrate, tissue, substrate concentration, substrate x tissue, substrate x substrate concentration, and tissue x substrate concentration were tested with animal, animal x tissue, animal x substrate concentration, animal x tissue, and animal x substrate concentration error terms, respectively. Because of the

repeated sampling of each parabiotic unit, the effect of time on the above factors was evaluated by the repeated measures option of SAS (1988). For the tissue accumulation and total absorption data, the effect of substrate, tissue, substrate concentration, and tissue x substrate concentration were tested with animal, animal x tissue, animal x substrate concentration, and animal x tissue x substrate concentration error terms, respectively. Orthogonal polynomial contrasts were used to partition the effects of concentration and time. Because of missing observations (i.e., missing sample, lost sample), contrast coefficients were generated by the Matrix function of SAS (1985).

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

Results and Discussion

The abilities of both omasal and ruminal epithelia to absorb methionine in the form of a free or peptide-bound amino acid were compared in separate uptake studies with L-[^{35}S]-methionine and L-[^{35}S]-methionylglycine as representative markers for the absorption and transfer of methionine and Met-Gly, respectively. The data from these separate studies were analyzed, and are presented, together.

Appearance of Substrates in Serosal Buffers. The rate at which ruminal and omasal tissues absorbed methionine and Met-Gly was examined by measuring the

mean appearance of methionine and Met-Gly in serosal chamber buffers as a function of time (Figure 4.1). For both tissues, the amount of methionine and Met-Gly that appeared increased ($P < .01$) with time. The rate of methionine and Met-Gly passage into serosal buffers was greater ($P < .01$, time X tissue interaction) for omasal than that for ruminal tissues. From 5 to 240 min, approximately 7.6 times more methionine and 12.2 times more Met-Gly appeared in omasal than ruminal serosal buffers. Within tissues, from 5 to 240 min, about .9 times more ($P < .01$, time X substrate interaction) methionine than Met-Gly passed into ruminal serosal buffers. In omasal tissues, the magnitude of this preferential methionine appearance was less (.19 times that for Met-Gly). These results indicate that the rate of absorption and transepithelial passage through ruminal and omasal tissue was linearly dependent on time, greater across omasal than ruminal tissues, and greater for methionine than for Met-Gly, at all sampling times.

The potential for mediated substrate passage was evaluated by measuring the ability of the tissues to absorb and translocate methionine and Met-Gly in response to increasing initial mucosal buffer substrate concentrations (Figure 4.2). For both tissues, the mean increase in appearance of methionine and Met-Gly in the serosal buffers had both linear ($P < .01$) and quadratic ($P < .01$) components. Overall, a greater ($P < .01$) quantity of methionine (1.8 to 9.1 times) and Met-Gly (3.0 to 15.2 times) was measured in the serosal buffers of omasal tissue.

The response to increased mucosal methionine and Met-Gly concentrations differed ($P < .01$; substrate X concentration interaction). In all ruminal serosal buffers, more methionine appeared than Met-Gly, ranging from approximately .80 (.375 m*M*) to 1.3 times (12.0 m*M*). In contrast, greater quantities of Met-Gly appeared in the serosal buffers of omasal tissues at the .375, 1.5, and 3.0 m*M* levels

(.47, .18, and .35, respectively) than did methionine. These results suggest that more methionine and Met-Gly appeared in omasal serosal chamber buffers at these concentrations because omasal tissue presented less of a permeability barrier to substrate diffusion.

Within ruminal epithelial tissue, the greater passage of methionine than Met-Gly (at all levels) suggests that more methionine is translocated across its epithelium because of a lesser barrier to methionine diffusion than to Met-Gly. If absorption had been mediated by transporter proteins, then a disproportionately greater amount of these substrates should have been transferred at lower concentrations, compared to quantities transferred at higher concentrations. In contrast, that more Met-Gly than methionine appeared in the omasal serosal chambers at lower substrate concentrations (.375, 1.5, and 3.0 m*M*), but not at the high concentrations (6.0 and 12.0 m*M*), allows for the possibility that Met-Gly appearance in serosal buffers may have been mediated by transport proteins that were saturated at mucosal buffer concentrations greater than 3.0 m*M*.

Tissue Accumulation of Substrates. The amount of Met and Met-Gly that had accumulated in tissues after 240 min of incubation was determined by quantifying the representative ^{35}S after digestion of the tissues (Figure 4.3). The effect of increasing initial mucosal chamber concentrations on tissue accumulation of substrates was both linear ($P < .01$) and quadratic ($P < .01$). Ruminal tissue accumulation of methionine increased in proportion to increased mucosal substrate concentration (from .375 m*M* through 12.0 m*M*). In omasal tissue, increases in accumulation of methionine in tissue linearly increased with mucosal buffer concentrations, whereas the ability to accumulate Met-Gly appears to have saturated at the 6 m*M* level.

Between tissues, approximately 35% more ($P < .01$) methionine and 60% more ($P < .01$) Met-Gly were accumulated by ruminal than by omasal tissue. Except at the .75 m*M* level, this proportionally greater accumulation of methionine was fairly constant across all chambers. In contrast, with the exception at the .75 m*M* level, the ratio of ruminal to omasal tissue accumulation of Met-Gly increased linearly from 67% (.375 m*M*) to 219% (12.0 m*M*), compared with omasal tissue accumulation. The cause of this difference ($P < .01$, tissue X concentration interaction) appears to have been a decreased ability of omasal tissue to accumulate Met-Gly as mucosal chamber buffer concentrations were increased, rather than an increased ability of ruminal tissue to accumulate Met-Gly.

Within ruminal or omasal tissues, the accumulation of methionine and Met-Gly did not differ ($P < .45$). Quantitatively, however, at the 12.0 m*M* level, omasal tissue accumulated 75% more methionine than Met-Gly. An inspection of omasal tissue accumulation of methionine across all concentrations, suggests that the increase in omasal methionine to Met-Gly accumulation is due to decreased omasal tissue capacity for Met-Gly accumulation, rather than an increased capacity for methionine accumulation. This observation, when considered with the greater accumulation of both substrates by ruminal epithelium, indicates that the process of ^{35}S (representing methionine and Met-Gly) passage through these two forestomach tissues may differ. When coupled with the greater appearance of both substrates in omasal serosal buffers (Figures 4.1 and 4.2), these results indicate that the omasal epithelium either possesses a lesser capacity to store substrate or a greater ability to translocate methionine and Met-Gly.

Total Absorption of Substrates. In order to determine and compare the total accumulation and translocation capacities of ruminal and omasal epithelia, the mean

quantities of Met and Met-Gly that appeared in serosal buffers after 4 h (data not shown; equal to the difference between Figure 4.4 and Figure 4.3 values) were summed with the mean quantities that had accumulated in tissues (Figure 4.3). The resulting data (Figure 4.4) represent the total absorption of methionine and Met-Gly. For both tissues, the total absorption of both methionine and Met-Gly increased in a linear ($P < .01$) and quadratic ($P < .01$) manner as their initial concentrations in mucosal chambers were increased. Between tissues, the total absorption of both methionine and Met-Gly by omasal tissues was approximately 3.9 times more ($P < .01$) than by ruminal tissues. For omasal tissue, the vast majority of absorbed methionine (91.4%) and Met-Gly (91.3%) passed through the epithelia into the serosal buffers. In contrast, approximately half of the methionine (54.6%) and Met-Gly (46.1%) absorbed was measured in the serosal buffers and half in the epithelia of ruminal tissues. Quantitatively, both ruminal and omasal epithelia tended to absorb more (23%; $P < .12$) methionine than Met-Gly.

The data in Figure 4.4 indicate that omasal tissue has a greater overall capacity for methionine and Met-Gly absorption than does ruminal tissue, that both substrates pass through omasal tissue in greater quantities than through ruminal, and that both tissues displayed a slightly greater tendency to absorb methionine than Met-Gly.

Potential for Tissue Hydrolysis of Met-Gly. The relative ability of ruminal and omasal tissues to translocate Met-Gly without hydrolysis was compared after 240 min incubation (Figure 4.5). Separation of the 12 m*M* serosal buffer components by TLC showed that approximately 36% of the ^{35}S in ruminal serosal buffers was associated with Met-Gly. A much smaller ($P < .01$) proportion of the radiolabel, 4.6%, was associated with the dipeptide in omasal tissue serosal buffers. The proportion of ^{35}S associated with methionine was nearly twice as great in serosal buffer from omasal

(47.9%) as compared to ruminal (28.6%) epithelium, but the difference was not statistically significant. A relatively large amount of ^{35}S activity was recovered in an unidentified fraction on the TLC plates. This fraction was identified on the plates between the origin and methionine, and accounted for 35.3% and 47.5% of the ^{35}S activity recovered in SCB of ruminal and omasal epithelia, respectively. No ^{35}S was detected on the plates beyond the point of Met-Gly migration.

These results suggest that intact Met-Gly transepithelial passage occurred across both tissues. In addition, the greater proportion of the ^{35}S isotope identified with Met-Gly in ruminal tissue serosal buffers suggests that omasal tissue may possess a greater ability to hydrolyze Met-Gly. The different fate of Met-Gly in serosal buffers suggests that the epithelial cells of ruminal and omasal tissues may possess different biological mechanisms responsible for the appearance and metabolism of [^{35}S]-methionylglycine.

The large proportion of ^{35}S label in the methionine and pre-methionine fractions of both tissues suggests that significant amounts of Met-Gly were hydrolyzed and further metabolized either during, before, or after transepithelial passage. Accordingly, Met-Gly serosal buffer appearance (Figures 4.1 and 4.2), tissue accumulation (Figure 4.3), and total absorption (Figure 4.4) should be evaluated with the knowledge that proportionately more of the ^{35}S label quantified in ruminal parabiotic units was representative of Met-Gly than that of the ^{35}S quantified in the omasal parabiotic units. Whereas the potential metabolism of methionine was not determined in the methionine uptake study, the identification of a ^{35}S fraction that did not correspond to methionine or Met-Gly in both tissues of the Met-Gly study suggests that the absorbed methionine in the methionine study probably was metabolized to some extent after entry into both epithelia.

Our interest in the sites and mechanisms of methionine absorption in sheep exists because of the importance of methionine supplementation to the nutrition of ruminants. Nutrient absorption by forestomach tissue is typically thought to include water, VFA, NH_4^+, and electrolytes (Stevens, 1973; Englehardt and Hauffe, 1975; McSweenney, 1988). The results from the present studies indicate that both ruminal and omasal epithelia are capable of absorbing and translocating methionine, either as a free or peptide-bound amino acid. Therefore, this research identifies forestomach tissues as being potential contributors to the positive fluxes of free (relatively small) and peptide-bound (relatively large) amino acids measured across the non-mesenteric drained viscera in wethers and steers (DiRienzo and Webb, 1995).

Transepithelial passage of methionine across ruminal and omasal epithelia appeared to be non-mediated. Using similar experimental protocols, Leibholz (1971b) failed to demonstrate saturable absorption of histidine from .66 to 20 m*M* concentrations. However, histidine transfer across isolated ruminal epithelial tissues may have been at least partially mediated because methionine (50%), arginine (50%), and glycine 40%) inhibited histidine passage, when coincubated at equal concentrations (.66 m*M*).

In hindsight, because a Na^+ gradient does not typically exist across forestomach tissue, this specificity of substrate passage would presumably be affected by interactions with Na^+-independent transport proteins. That arginine inhibited histidine absorption suggests that these substrates may have been competing for recognition by the "y^+" transporter (Stevens, 1992a). The demonstration that arginine, methionine, and glycine inhibited histidine absorption parallels the competitive inhibition profile displayed by the renal and intestinal $b^{O,+}$ transporter system (Pickel et al., 1993), which is capable of transporting both cationic and neutral amino acids.

The independent observation (Fejes et al., 1991) that the appearance of lysine and arginine in serosal chambers was a saturable process, as mucosal chamber concentrations were increased from .3, to 30 m*M*, supports the concept that the ruminal epithelium of sheep may possess proteins capable of mediating amino acid transport.

From a teleological perspective, it seems reasonable to suggest that forestomach epithelial tissues may have evolved the ability to activate genes capable of encoding for peptide transport proteins (Fei et al., 1994; Dantzig et al., 1994). Ions reported to be involved in active, secondary transport of small peptides (H^+, Na^+) in eukaryotes (Webb and Matthews, 1994) are constituents of forestomach liquor. Omasal liquor is essentially acidic (Prins et al., 1972) and ruminal liquor can develop pH levels of 5.5 or less (Whitelaw, 1970). Proton gradients of this magnitude have been used to demonstrate the presence of carrier-mediated dipeptide transport by intestinal and renal epithelial brush border membranes (Ganapathy et al., 1983) and in cultured intestinal cells (Saito and Inui, 1993). Additionally, the Na^+/H^+ exchanger and Na^+/K^+ ATPase proteins, considered to be essential in reestablishing proton gradients in other epithelial cells, are reported to exist and function in both ruminal and omasal epithelia (Martens and Gabel, 1988).

In this experiment, a pH gradient was established by loading the mucosal and serosal chambers with pH 6.0 and pH 7.4 buffers, respectively. Mediated transport, however, does not appear to have been the primary mechanism responsible for the absorption of Met-Gly. If absorption ocurred because of mediated transport, then potential transport proteins were either saturated by .375 m*M*, or were not saturable at .375 to 12.0 m*M*. With the exception of omasal tissue accumulation of Met-Gly, there was little evidence to indicate that mediated methionine or Met-Gly absorption had

occurred. Another study in this lab (Matthews, 1991b), drew similar conclusions about carnosine translocation across ruminal and omasal epithelia using 6 to 96 m*M* concentrations of carnosine.

Non-mediated absorption of nutrients through the forestomach epithelium has been suggested to be dependent on an osmotic gradient generated by the active transport of Na^+ (Keynes, 1969; Henrikson, 1970; Gabel et al, 1993). In addition, paracellular absorption may contribute to non-mediated absorption. In enterocytes, whose tight junctions are considered to be relatively tighter than forestomach epithelia (Fell and Weekes, 1975) absorption of oligopeptides by this pathway has been demonstrated (Atisook and Madara, 1991). Accordingly, this diffusional pathway may contribute substantially to the absorption of nutrients by forestomach epithelia.

Regardless of the mechanism, omasal epithelium displayed a greater ability to translocate methionine and Met-Gly (Figures 4.1, 4.2, and 4.4), to metabolize Met-Gly (Figure 4.5), and a lesser ability to accumulate Met and Met-Gly (Figure 4.3), as compared to ruminal tissues. These observations suggest that omasal and ruminal tissues may possess different in vivo nutrient absorption abilities. This hypothesis is supported by the observations that the omasal epithelium possesses greater metabolic activity (Engelhardt and Hauffe, 1975), greater numbers of branching cells in the stratum basale (Steven and Marshall, 1970), greater potential electrolyte flux (Martens and Gabel, 1988), and a greater blood supply (blood flow/wet weight of organ, Engelhardt and Hales, 1977) than does ruminal epithelium.

One possible explanation for the greater rate (Figure 4.1) and quantity (Figure 4.2) of Met-Gly translocation across omasal epithelium would be the creation of a larger driving force for non-mediated translocation in omasal than ruminal tissues. A greater removal of Met-Gly by omasal than ruminal epithelium during or after Met-

Gly translocation would reduce the amount of intra-epithelial concentrations of Met-Gly, resulting in a steeper mucosal-to-serosal buffer Met-Gly concentration gradient and a larger driving force for absorption. The greater hydrolysis of Met-Gly by omasal tissue (Figure 4.5) supports this hypothesis. That carnosine is not hydrolyzed during translocation, and that more carnosine appeared in the serosal chambers of omasal tissue than ruminal (Matthews, 1991), suggests that factors other than increased substrate metabolism may participate in the greater ability of omasal tissues to absorb dipeptides. Because the potential metabolism of methionine was not determined in the methionine studies, this explanation for the apparently greater ability of omasal epithelia to translocate free methionine than ruminal tissue can not be offered.

The .375 to 12 m*M* range of substrate concentrations used in this experiment bracket the post-feeding concentrations of free and peptide-bound amino acid N reported to exist in the ruminal liquor of sheep and cows (methionine, .525 to 16.8 mg N/dL, Table 4.1; Met-Gly, 1.05 to 33.6 mg N/dL, Table 4.2). The determination that ruminal and omasal epitheliae have the ability to absorb methionine and Met-Gly by non-mediated processes at these concentrations may have important nutritional implications for the ruminant. If the in vitro characterization of these forestomach tissues accurately represents their functional characteristics in vivo, then the relative absorption of free and peptide amino acids will depend on their relative concentrations in forestomach liquor, and, therefore may be manipulatable.

In conclusion, the results from this parabiotic chamber study indicate that both ruminal and omasal epithelia have the ability to absorb and transfer the amino acid methionine as either a free or dipeptide-bound form (Met-Gly). At the concentrations tested, absorption primarily appears to be a non-mediated event. Within tissues, the

absorption of free and peptide-bound amino acid substrates did not differ. Between tissues, the omasal epithelium apparently has a greater capacity than the ruminal epithelium to absorb and translocate both substrates. The observation that omasal epithelia apparently possesses a greater Met-Gly hydrolytic ability suggests that the amount of peptidase activity and rate of dipeptide metabolism between epithelia of the forestomach may differ.

Implications

If future research confirms the potential of forestomach tissue to absorb peptide-bound amino acids, then the practice of supplementing the amino acid requirements of ruminants with proteins designed to be absorbed by the forestomach epithelia may become an integral component of ruminant diet formulation.

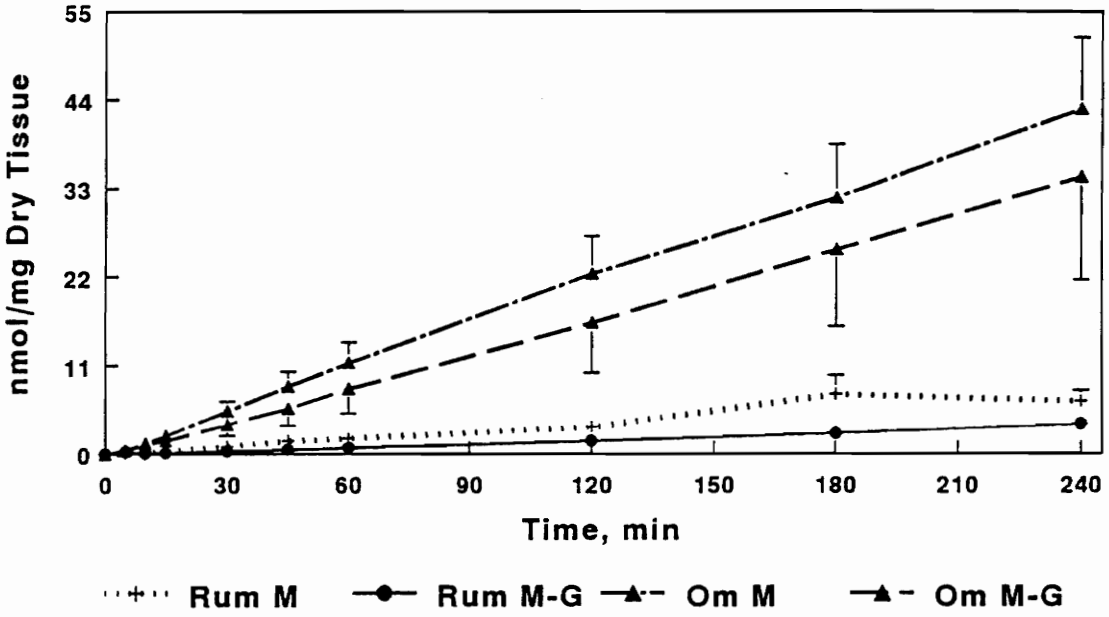


Figure 4.1. Effect of time on methionine and methionylglycine appearance in serosal buffers.

Data were transformed using the natural log function before analysis. Non-transformed means and SE are presented in the figure and are the means of seven animals across concentrations for each methionine (Met) and methionylglycine (Met-Gly) and represent the quantity of Met and Met-Gly that appeared in the serosal chamber of the parabiotic units, using [^{35}S]-methionine and [^{35}S]-methionylglycine as representative markers, respectively.

Rum = ruminal, Om = omasal.
M = methionine, M-G = methionylglycine.
Linear ($P < .01$) and quadratic ($P < .01$) time effect.
Substrates differed ($P < .01$).
Tissues differed ($P < .01$).
Time x substrate interaction ($P < .01$)
Time x tissue interaction ($P < .01$).

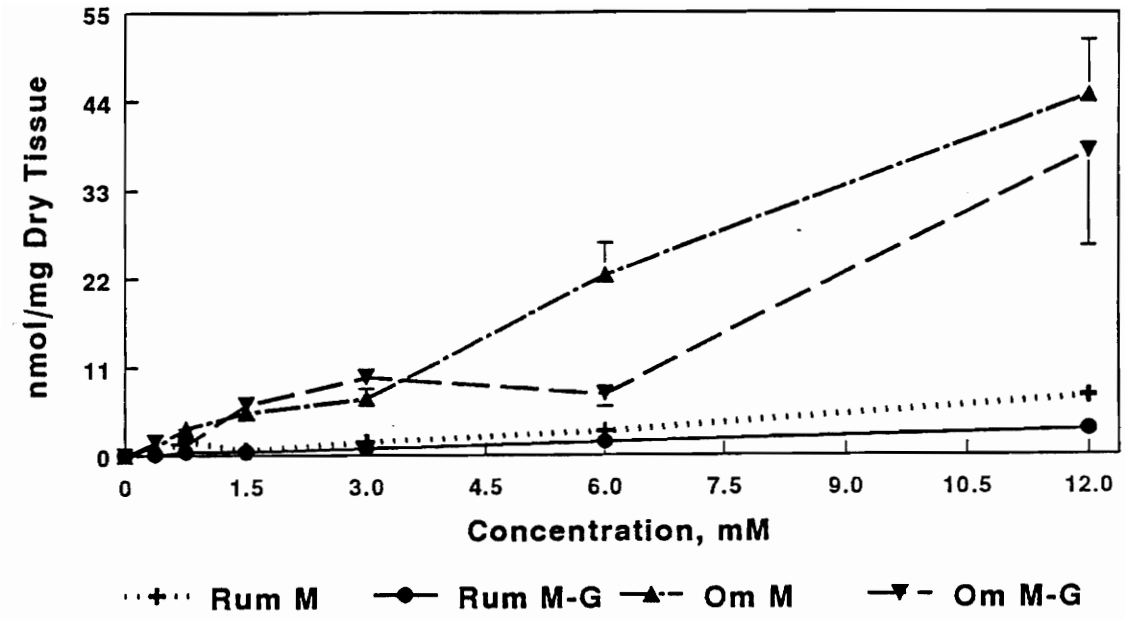


Figure 4.2. Effect of increasing mucosal buffer substrate concentration on methionine and methionylglycine appearance in serosal buffers.

Data were transformed using the natural log function before analysis. Non-transformed means and SE are presented in the figure and are the means of seven animals across time for each methionine (Met) and methionylglycine (Met-Gly) and represent the quantity of Met and Met-Gly that appeared in the serosal chamber of the parabiotic units, using [^{35}S]-methionine and [^{35}S]-methionylglycine as representative markers, respectively.

Rum = ruminal, Om = omasal.
M = methionine, M-G = methionylglycine.
Linear ($P < .01$) and quadratic ($P < .01$) concentration effect.
Tissues differed ($P < .01$).
Substrates differed ($P < .01$).
Substrate x tissue interaction ($P < .06$).
Substrate x concentration interaction ($P < .01$)

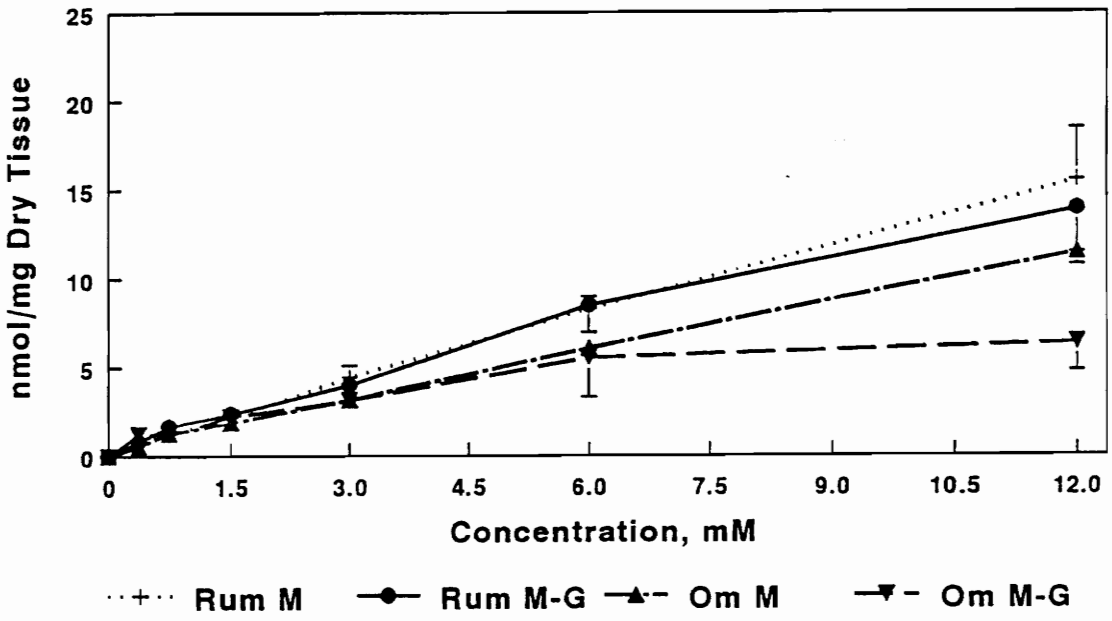


Figure 4.3. Effect of increasing mucosal buffer substrate concentration on the accumulation of methionine and methionylglycine in tissue.

Data were transformed using the natural log function before analysis. Non-transformed means and SE are presented in the figure and are the means of seven animals for each methionine (Met) and methionylglycine (Met-Gly) and represent the quantity of Met and Met-Gly that accumulated in tissues of the parabiotic units after 240 min incubation, assuming all ^{35}S was in the form of [^{35}S]-methionine and [^{35}S]-methionylglycine acting as representative markers for Met and Met-Gly, respectively.

Rum = ruminal, Om = omasal.

M = methionine, M-G methionylglycine.

Linear ($P < .01$) and quadratic ($P < .01$) concentration effect.

Tissues differed ($P < .01$).

Tissue x concentration interaction ($P < .01$).

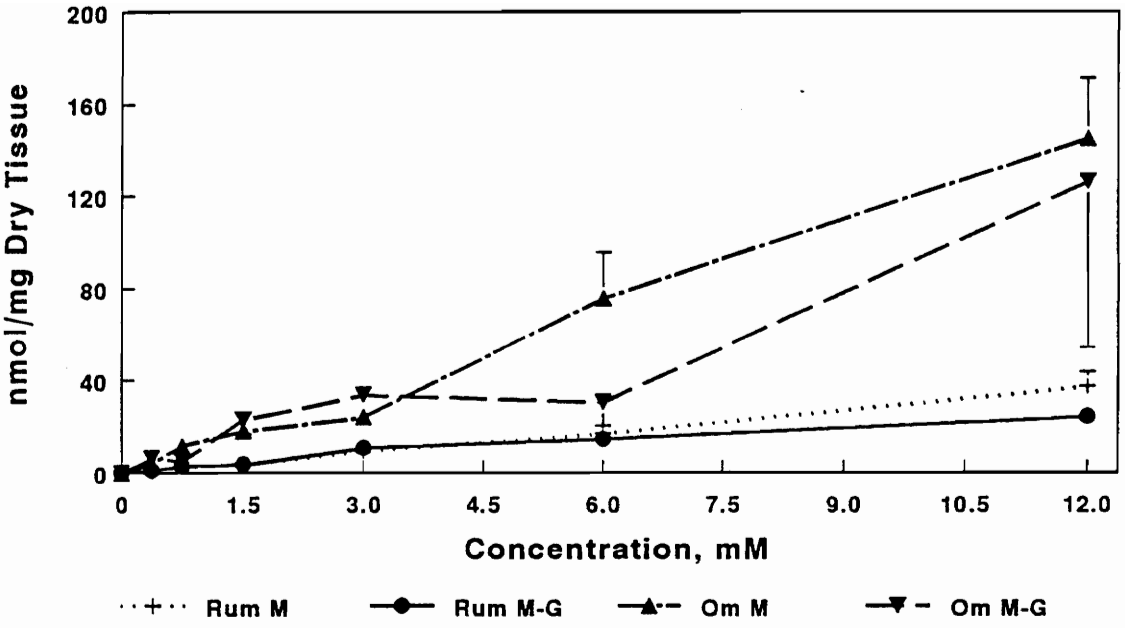


Figure 4.4 Total absorption of methionine and methionylglycine.

Data were transformed using the natural log function before analysis. Non-transformed means and SE are presented in the figure and are the means of seven animals for each methionine (Met) and methionylglycine (Met-Gly) and represent the quantity of Met and Met-Gly that accumulated in tissues and that appeared in the serosal buffers of the parabiotic units after 240 min incubation, assuming all ^{35}S was in the form of [^{35}S]-methionine and [^{35}S]-methionylglycine acting as representative markers for Met and Met-Gly, respectively.

Rum = ruminal, Om = omasal.

M = methionine, M-G = methionylglycine.

Linear ($P < .01$) and quadratic ($P < .01$) concentration effect.

Tissues differed ($P < .01$).

Tissue x concentration interaction ($P < .01$).

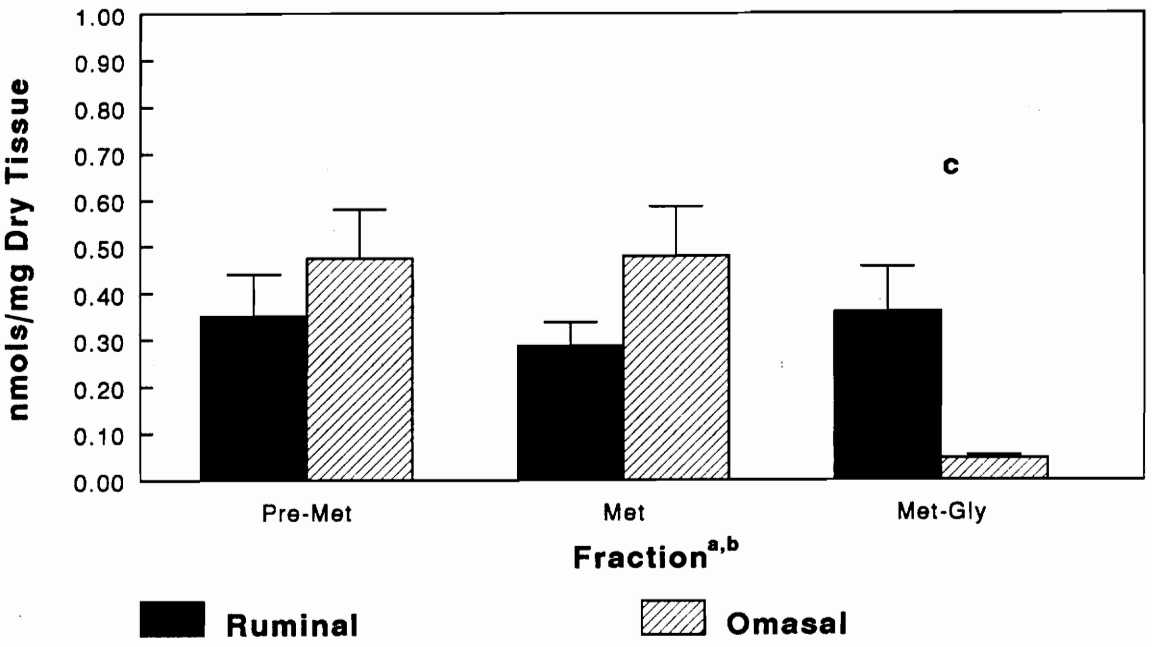


Figure 4.5. Distribution of ^{35}S amoung differing components of 12 m*M* serosal chamber buffer after 240 min incubation.

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

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

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

CHAPTER V

DEMONSTRATION AND CHARACTERIZATION OF A TRANSPORT SYSTEM CAPABLE OF LYSINE AND LEUCINE UPTAKE IN PORCINE JEJUNUM BY EXPRESSION OF mRNA IN *XENOPUS LAEVIS* OOCYTES

ABSTRACT

Defolliculated *Xenopus laevis* oocytes were injected with size-fractionated poly(A)$^+$ RNA (RNA) isolated from the jejunal epithelium of growing pigs (average BW = 33.8 kg) to identify proteins capable of Na^+-independent amino acid transport. The ability of oocytes to absorb L-lysine (lysine) or L-leucine (leucine) from Na^+-free media was quantified in oocyte after injection of RNA fractions or water. Specific RNA fractions were identified that induced saturable uptake of lysine (K_t = 52 *uM*) and leucine (K_t = 97 *uM*), whereas endogenous oocyte uptake was not saturable. Uptake of .05 m*M* lysine and leucine was inhibited ($P < .05$) by 5 m*M* leucine and lysine (68.1 and 83.1%, respectively) and .2 m*M* cystine (38.9 and 23.2%, respectively) and quantitatively stimulated by L-glutamate (18.8 and 60%). To identify mRNA species responsible for this $b^{o,+}$ transporter-like activity, oocytes were co-injected with the RNA fractions and degenerate DNA oligomers complementary (antisense) to the cloned human kidney $b^{o,+}$ amino acid transporter or, as a negative control, with a DNA oligomer complementary to the rabbit intestinal Na^+/glucose cotransporter, or with water. Only those oocytes injected with two specific RNA fractions and the antisense DNA oligomer complementary to the $b^{o,+}$ transporter displayed reduced ($P < .05$) uptake of lysine (45.7, 55.4%) and leucine (44.1, 65.9%). These results indicate that mRNA encoding for a protein capable of stimulating the competitive absorption of lysine and leucine is expressed by the jejunal epithelia of growing pigs.

(**Key Words:** Pig, Jejunum, Lysine, Leucine, Transport, Absorption)

Introduction

The literature abounds with research that documents the need for and proposed manner of dietary supplementation of amino acids to optimize the growth of pigs (Leibholz, 1989; D'Mello, 1993). It is believed that dietary supplementation of some indispensable amino acids may cause the deficiency of others, resulting in reduced feed intake, growth rates, and economic gains (NRC, 1988). Little research, however, has been conducted that attempts to identify specific mechanisms responsible for the absorption of amino acids across the pig small intestine. mRNA extracted from rat, rabbit, and human renal epithelial cells has been expressed in *Xenopus laevis* oocytes, resulting in the identification, functional characterization, and cloning of proteins capable of Na^+-independent recognition and(or) transport of arginine, lysine, leucine, and methionine (Magagnin et al., 1992; Mosekovitz et al., 1994).

Knowledge of the function, capacity, and potential for regulation of proteins capable of indispensable amino acid transport should enhance our ability to supplement diets, without inducing dietary imbalances. The purpose of this study was to identify and characterize the function of proteins in pig jejunum epithelia capable of stimulating the mediated Na^+-independent absorption of lysine and leucine, by expressing their mRNA in *Xenopus laevis* oocytes.

Materials and Methods

Unless noted otherwise, all chemicals, substrates and reagents were of either molecular biology or cell culture tested chemical grades. For all RNA extraction, isolation, fractionation, and suspension procedures, the water used was deionized (Model Unipure II, Solution Consultants Inc., Jasper, GA) and treated with .1% diethyl pyrocarbonate (DEPC) and autoclaved. Additionally, all stock solutions were DEPC-treated after their preparation, except for detergent stock solutions. All media were prepared using deionized water (Milli-Q Reagent Grade, Model No. ZD5211584, Millipore, Bedford, MA). Solution transfers were accomplished using sterile, disposable, glass and plastic graduated pipets (Fisher Scientific Products Company, Pittsburg, PA), or aerosol-resistant tips (Molecular Bio Products, San Diego, CA). All re-usable centrifuge tubes and bottles were rinsed with chloroform after cleaning and then DEPC-treated before reuse. Unless noted otherwise, all RNA was precipitated with isopropanol in the presence of .3 M sodium acetate (pH 5.2).

RNA Extraction. For the initial studies, two crossbred barrows (average BW 38.3 kg) raised at the Virginia Experimental Agricultural Research Center Swine Herd were used. For all subsequent uptake experiments, total RNA was extracted from five Chester White X Yorkshire gilts (average BW 33.7 kg) that had been housed in pens with elevated, expanded metal floors in a temperature- and ventilation-controlled room. All pigs had ad libitum access to a 16% CP corn-soybean diet (Table 5.1) and water for at least 3 wk prior to tissue collection. Pigs were anesthetized by intravenous injection of sodium pentobarbitol (approximately 28.9 mg/kg BW). The

abdominal cavity was opened quickly, the entire intestine was removed and placed on an iced tray, and the animal killed by exsanguination. The duodenum (approximately 1 m distal to the stomach) was discarded. The proximal jejunum (approximately 4 m) was sectioned into twelve to fourteen 30-cm lengths, cut open, and rinsed in 4°C .85% saline. Total RNA was extracted essentially as described by Puissant and Houdebine (1990). The mucosa was scraped from the underlying musculature using a glass slide, divided (approximately 5 g) into a series of 50 mL polypropylene tubes containing 25 mL of 4°C RNA extraction buffer (4 M guanidine thiocyanate, 25 m*M* sodium citrate, .5% sodium lauroylsarcosine, .1 M beta-mercaptoethanol), homogenized (10 cm diameter probe; PT 10/35 polytron, Brinkman Instrument, Wesburg, NY) for 6 to 8 s at setting "7", and held in an ice bath for approximately 15 min.

The homogenized tissue was pooled into four 250 mL centrifuge bottles (approximately 15 g tissue per bottle). To each bottle, 20 mL 2 M acetic acid (pH 4.1), 75 mL water-saturated phenol (pH 4.3; No. #0981, Amresco, Solon, Ohio), and 15 mL water-saturated chloroform were added, vortexing after each addition. After a 40-min ice bath precipitation, the solutions were centrifuged (Model No. RC2-B, Sorval Instrument Company, Newton, CT) for 20 min at 12,000 *x* g and 4°C, and an equal volume of isopropanol was added to the pooled aqueous supernatants in 250 mL bottles. After a precipitation period of at least 6 h at -20°C, the solutions were centrifuged for 20 min at 4,000 *x* g and 4°C. The resulting RNA pellets were pooled, washed by resuspending in 4 M LiCl, and centrifuged for 20 min at 3,600 *x* g and 20°C. The washed pellets were suspended in 25 mL 10 m*M* Tris·HCl, 1 m*M* EDTA, .5 % SDS (pH 7.6), and 25 mL water-saturated chloroform, and then centrifuged in two 30 mL Corex tubes for 20 min at 12,000 *x* g and 20°C. The total RNA-containing

aqueous supernatant phases were pooled and stored at -80°C in 1.5 mL tubes as .3 M sodium acetate (pH 5.2) and ethanol (2.5 volumes) precipitates.

Isolation of Size-Fractionated Poly(A)$^+$ RNA. Poly(A)$^+$ RNA was isolated from total RNA by chromatography on oligo(dT) cellulose (Type 7, Pharmacia Biotech, Piscataway, NJ). Typically, 20 mg of total RNA were suspended in 4 mL of water after recovery from the precipitation solution by centrifugation (Model No. SC100, Savant Instrument Incorporated, Farmingdale, NY) for 20 min at 12,000 *x* g and 10°C, 80% ethanol washing of the pellet, centrifugation for 20 min at 12,000 X g and 10°C, and 5 min vacuum-drying (Model No. RT100, Savant Instrument Inc., Farmingdale, NY) of pellet. The RNA pellet was suspended in 4 mL (typically) of water and quantified at 260 nm (Model U-2000, Spectophotometer, Hitachi Instrument Inc., Japan). No more than 10 mg of total RNA in solution were loaded onto columns (Cat. No. QS-GS, Isolab, Inc., Akron, OH) containing 300 mg of oligo(dT) cellulose. Poly(A)$^+$ RNA was isolated from total RNA as described by Sambrook et al. (1989), except that denaturization of RNA was achieved by 10 min incubations at 65°C and that the elution buffer (1 m*M* EDTA, 10 m*M* Tris·HCl, pH 7.6) lacked SDS. For the two preliminary uptake experiments, the poly(A)$^+$ RNA isolation was performed once, whereas for all subsequent experiments a second enrichment step was performed on a single column containing 60 mg of oligo(dT) cellulose For both, the final eluate was collected in 1.5 mL ultracentrifuge tubes (No. 357448, Beckman Instruments, Inc., Palo Alto, CA) and precipitated with isopropanol for at least 18 h and -20°C. The poly(A)$^+$ RNA precipitate was recovered after centrifugation (Model Optima TL, Beckman Instruments, Inc., Palo Alto, CA) for 30 min at 80,000 *x* g and 2°C.

Poly(A)$^+$ RNA was size-fractionated essentially as described by Sambrook et al. (1989). The pellet was resuspended (> 1 mg/mL; 10 m*M* Tris·HCl, pH 7.6, 1 m*M* EDTA) and loaded onto a 13 mL, 8 to 20% (w,v) linear sucrose gradient formed by loading 6.5 mL of a solution containing 10 m*M* Tris·HCl, 1 m*M* EDTA, 10 m*M* methyl mercuric hydroxide (No. 13395, Johnson Matthey Catalog Company, Ward Hill, MA), plus 8% or 20% sucrose to each chamber of the gradient former (Cat. No. G-030, Ann Arbor Plastics, Ann Arbor, MI) and pumped (Model No. 77, Harvard Apparatus, South Natick, MA) at a rate of .75 mL/min into a 13 mL ultracentrifuge tube (No. 344060, Beckman Instruments, Inc., Palo Alto, CA). The poly(A)$^+$ RNA was centrifuged (Model L5-75 B, Beckman Instrument, Palo Alto, CA) through the gradient for 15.5 h at 80,000 *x* g and 4^{o}C. Typically, forty-eight .27 mL fractions were collected. An equal volume of 5.0 m*M* beta-mecaptoethanol was added to each fraction to reduce the methyl mercuric hydroxide in the gradient buffer, and the size-fractionated poly(A)$^+$ RNA was stored as an ethanol precipitate at -20^{o}C. When appropriate, the integrity and viability of size-fractionated RNA was evaluated by its ability to induce the translation of L-[^{3}H]-leucine-containing proteins in rabbit reticulocyte lysates, per instructions of the manufacturer (Product No. L4960, Promega Corporation, Madison, WI).

Preparation of Oocytes for Injection. Purchased mature female *Xenopus laevis* frogs (Xenopus One, Ann Arbor, MI) were housed in mesh-covered cooler chests containing 15^{o}C de-chlorinated water in a refrigerated (10^{o}C) room, exposed to a 12 h light cycle, and fed a complete diet (Frog Brittle, Nasco, Fort Atkinson, WI) once weekly. Frogs were anesthetized by immersion in an ice bath containing ethyl-3-aminobenzoate (1.5 g/L). Ovaries were exposed by making a small abdominal incision through the skin and muscle (Coleman, 1984). Several ovarian lobes were

removed and placed in petri dishes containing sterile-filtered (.2 micron) Ca^{2+}-free media (96 m*M* NaCl, 2 m*M* KCl, 1 m*M* $MgCl_2$, 5 m*M* HEPES/NaOH, pH 7.6; Miyamoto et al., 1991). The muscle and skin tissues were sutured separately and the frog was placed on an incline in .01% penicillin-G-containing water (room temperature) for several hours before returning to normal housing. The ovarian lobes were pulled apart into pieces containing approximately 30 oocytes each and incubated at room temperature in 1.4 mg Collagenase A/mL Ca^{2+}-free media (Lot No. FFA158, Boerhringer Mannheim Corporation, Indianapolis, IN) for approximately 30 min. Stage IV and V oocytes (Dumont, 1972) were then manually defolliculated with forceps (size 5, George Tiemann and Company, Plainfield, NY). The defolliculated oocytes were rinsed five times and allowed to heal for at least 18 h in sterile-filtered Ca^{2+}-containing media (96 m*M* NaCl, 2.5 m*M* sodium pyruvate, 2 m*M* KCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* HEPES/NaOH, pH 7.6, 1.0 X 10^5 units/L penicillin-G, and 1.67 X 10^5 units/L streptomycin) and cultured in petri dishes at 18°C (Model BK6160 refrigerator, Heraeus Instruments, Inc., South Plainfied, NJ). Preliminary studies showed that oocytes defolliculated and cultured in this manner retained their endogenous Na^+-dependent and Na^+-independent amino acid uptake capabilities (data not shown).

Microinjection of mRNA and DNA Oligomers. The microinjection system consisted of a positive-pressure pump (Trent Walls, Inc., South Gate, CA), micromanipulator (M/W Company, Germany), plunger (10 uL, Model No. 53506-450, VWR Scientific Company, West Chester, PA), and glass micropipets (No. 1B120-6, World Precision Instrument Company, Sarasota, FL), which had been prepared using a micropipet puller (Model No. 720, David Kopf Instruments, Tujunga, CA) and manual snapping of the tips with forceps. DEPC-treated water or

mRNA solutions were pipetted into sterile-filtered (.45 micron) mineral oil (Cat. No. 0121, Fisher Scientific) that had been saturated with media (124.9 m*M* KCl, 10.0 m*M* NaCl, 2.0 m*M* $NaHCO_3$, and 1.0 m*M* KH_2PO_4, pH 7.6; personal communication, D. S. Miller, Laboratory of Cellular and Molecular Pharmacology, NIH, Research Triangle Park, NC) and then aspirated into the micropipet. Oocytes were placed in a cross-scored petri dish containing injection buffer (96 m*M* NaCl, 2 m*M* KCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* HEPES/NaOH, pH 7.6) and injected in the animal pole, near the polar interface, with the aid of a dissection scope (7 to 30X, Bausch and Lomb, Inc., Rochester, NY) and lamp (Model No. 31-33-53, Bausch and Lomb, Inc., Rochester, NY).

Unless noted otherwise, Fractions 36, 37, or 38 of the stored, sucrose-gradient fractionated poly(A)$^+$ RNA (corresponding to the 11 +/- .54% sucrose fractions), were recovered, suspended in DEPC-treated water, and either injected immediately or stored at -20^{o}C for no more than 1 wk before injection. The amount of poly(A)$^+$ RNA injected into oocytes (see Results) varied among fractions and animals because of inherent differences in the amount and molecular size of epithelial mRNA in jejunal mucosa and the subsequent practice of suspending all fraction precipitates in the same volume (usually 6 uL) and the injection of the same volume (usually 100 nL) into oocytes . Within an injection treatment, radiotracer experiments demonstrated that the volume of solution injected per oocyte was approximately the same (+/- 10%).

For the oligomer depletion of uptake experiments, 80 nL of poly(A)$^+$ RNA Fractions 35, 36 ,38, 39, or water were first injected into the oocytes. For lysine uptake, 32 nL of water, the negative control deoxnucleotide oligomer (32 ng; 5'-CACAAAGGCTTCTCTGAGG-3'), or a degenerate deoxynucleotide antisense oligomers to the cloned human renal $b^{o,+}$ transporter (32 ng; 5'-

YTTRTCYTCNGCCATCAGAGTGGC-3'), was then injected into oocytes using the same membrane orifice generated by the injection of poly(A)$^+$ RNA (F35, .32, 1.7; F36, .42, 1.5; F38, .32, 1.8; F39, .6, 1.7 ng/nL). For oligomer depletion of leucine uptake experiments, 65 nL of water, negative control DNA (65 ng), or antisense DNA (65 ng) were injected into oocytes after initial injection of water or poly(A)$^+$ RNA (F35, 1.1, 1.1; F36 1.1, 1.1; F38, 1.2, 1.4; F39, 1.1, 1.1 ng/nL).

After receiving their injection treatments, oocytes were cultured for 5 d at 18°C in Ca^{2+}-containing media. Oocytes of the same injection treatment were cultured in a common 20 mL borosilicate vial. Daily, the media was changed and damaged oocytes were discarded.

Oocyte Uptake Experiments. The standard Na^+-free uptake buffer contained 96 m*M* choline chloride, 2 m*M* KCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* HEPES, pH 7.6, and appropriate quantities of amino acids. Only those oocytes that were healthy and of the same size and stage of maturation were selected for uptake experiments, on the day of assay. This resulted in the use of varying numbers of oocytes in experiments (see figure legends). Prior to assay, oocytes were washed four times in the standard uptake buffer to remove any residual Na^+ and antibiotics. Uptake experiments were initiated by placing all oocytes of a given injection treatment into 7 mL plastic vials that contained .5 mL of experimental uptake buffer (standard uptake buffer plus 6 to 9 *u*Ci/mL of L-[4,5-^{3}H]-lysine (No. TRK.752, 100 Ci/mmol, Amersham Life Science, Arlington Heights, IL) or L-[4,5-^{3}H]-leucine (No. TRK.683, 164 Ci/mmol, Amersham Life Science) and appropriate amounts of non-radiolabeled amino acids (see figure legends). When experimental uptake buffers contained cystine, 10 m*M* dithiothreitol was included to prevent reduction of cystine's disulfide bond (Magagnin et al., 1992).

Experiments were terminated by the addition of 4 mL 4°C stop solution (uptake buffer that included 5 m*M* lysine or leucine) after room temperature incubation for 45 min. For the time course experiments, the periods of incubation varied as indicated. Treatment groups of oocytes were serially washed six times in 1.5 mL of the appropriate stop solutions. Oocytes were individually transferred in 30 *u*L of stop solution into 7 mL plastic scintillation vials containing .2 mL of 10% SDS. Aliquots (30 uL) of the last wash of each treatment group were collected for determination of background radioactivity. After room temperature digestion of the oocytes (minimum of 4 h), 5 mL of scintillation fluid (Scintiverse BD; Fisher Scientific) were added to each vial and allowed to equilibrate (minimum of 4 h) before quantifying the [^{3}H] content by liquid scintillation counting (LS 5000TA Scintillation Counter; Beckman Instruments, Fullerton, CA). Recorded cpm were converted to dpm using a standard quench curve.

Non-injected, water-injected, and RNA-injected oocyte uptake values for lysine and leucine were determined as the product of background-corrected dpm x the specific activity of the buffer. Induced oocyte uptake values were calculated by subtracting the water-injected uptake values from the RNA-injected uptake values. Induced K_t values were generated by fitting the data to the Michaelis-Menton equation $y = V_{max} * X/(K_t + X)$ using the P.Fit computer program (Version 5.1, Fig.P Software Corporation, Durham, NC), where X = concentration of substrate (uM) and y = observed substrate absorption (pmols/oocyte·min^{-1}).

Statistical Analyses. For the time-course experiments, the effects of time, injection treatment, and their interactions on the ability of oocytes to absorb lysine or leucine were evaluated using the general linear model (GLM) procedure of SAS (1988). Orthogonal contrasts were used to test for differences in rates of absorption.

For the determination of K_t experiments, the effects of animal and substrate concentration and their interactions on mean lysine and leucine uptake were evaluated by GLM procedures (SAS, 1988). Orthogonal polynomial contrasts were used to partition the effect of substrate concentration on oocyte uptake of lysine and leucine into linear, quadratic, and cubic components. Because of missing observations, contrast coefficients were generated by the Matrix function of SAS (1985).

For the competitive uptake and oligomer depletion of uptake experiments, the effects of animal, injection treatment, and their interactions on the ability of oocytes to absorb lysine or leucine were evaluated using the general linear model (GLM) procedure of SAS (1988). The effects of competing amino acids on mean lysine or leucine uptake within an mRNA treatment were compared using Tukey's multiple comparison test at the alpha = .05 level. These calculations were manually performed to generate the appropriate standard deviations and critical values for each combination of unequal treatment observations (Lentner and Bishop, 1993). The effects of water, DNA, or antisense co-injection treatments on induced lysine and leucine uptake were evaluated in the same manner.

Results

Initial Experiments. As the initial step to identifying pig jejunal epithelium tissue mRNA capable of encoding for Na^+-independent amino acid transport proteins, the ability of oocytes injected with 100 nL of sucrose gradient-fractionated poly(A)$^+$ RNA (RNA; .6 to 1 ng/nL) to absorb leucine from Na^+-free uptake buffer (50 *uM* L) was compared to each other and that of water-injected oocytes (Figure 5.1). A RNA

injection treatment effect was observed ($P < .001$). Fractions 38, 39, and 40 absorbed 51, 64, and 37%, respectively, more ($P < .05$) leucine than did oocytes injected with water (4.3 pmol/oocyte·30 min-1) or other RNA fractions.

The potential for this increased leucine uptake by oocytes injected with pig jejunal epithelium mRNA to have occurred by leucine-specific transport proteins was investigated by observing the effect of arginine (5 m*M*) or cystine (.2 m*M*) co-incubation on .05 m*M* leucine uptake by oocytes injected with 100 nL of poly(A)$^+$ RNA Fraction 39 (1.05 ng/nL; RNA) or water (Figure 5.2). At .05 m*M* leucine, Na^+-independent leucine absorption by oocytes injected with RNA was greater ($P < .03$) than for water-injected and non-injected oocytes (Figure 5.2). Co-incubation of arginine or cystine reduced ($P < .05$) .05 m*M* leucine uptake by 33.3 and 30%, respectively, for oocytes injected with RNA. In contrast, co-incubation of arginine or cystine did not reduce leucine uptake by water-injected oocytes. A similar response was observed for non-injected oocytes and indicates that the injection of water had no effect on endogenous uptake activity. Thus, it appeared that the enhanced ability of poly(A)$^+$ RNA-injected oocytes to absorb leucine was due to the functioning of a protein capable of stimulating Na^+-independent transport of leucine (neutral) and regulatable (cis-inhibition) by arginine (cationic) and cystine, which was not active in water-injected nor non-injected oocytes. These are characteristics of the "$b^{o,+}$" transporter system (Van Winkle et al., 1988).

In order to test this hypothesis, we conducted experiments designed to characterize the functional uptake of both lysine and leucine by a protein(s) encoded for by mRNA extracted from the jejunal epithelum of growing gilts. The poly(A)$^+$ RNA used in these experiments ostensibly was of greater purity than that used in the preliminary experiments because of their being twice enriched from total RNA. This

extra purification step was performed in order that the injected poly(A)$^+$ RNA would contain less RNA and result in greater enhancement of amino acid uptake by the oocytes and, therefore, increased experimental sensitivity.

Demonstration of mRNA-Inducible Uptake. Time-course trials were conducted to determine the optimal length of time to conduct uptake experiments. The absorption of lysine and leucine from Na^+-free media by oocytes injected with water or poly(A)$^+$ RNA (lysine, 2.6 ng/nL; leucine, .68 to 1.09 ng/nL) increased linearly ($P < .001$) over the course of 1 h (Figures 5.3 and 5.4). A treatment x time interaction ($P < .001$) was observed for both experiments reflecting that oocytes injected with RNA displayed a greater ($P < .001$) ability to absorb lysine and leucine than did water-injected oocytes. RNA-injected oocytes absorbed approximately 3.6, 2.5, 2.6, and 2.4 times more lysine than control oocytes at 15, 30, 45, and 60 min samplings. Leucine uptake averaged 2.3, 2.0, and 2.0 times more than that by water-injected oocytes at 15, 30, and 60 min samplings. Based on the overall linearity of these results, subsequent characterization of endogenous and RNA-inducible uptake of lysine and leucine by oocytes were evaluated using a 45-min uptake period.

Determination of mRNA-Induced K_t. In order to determine whether the enhanced ability of RNA-injected oocytes to absorb lysine and leucine was the result of expressing proteins that mediated absorption, the induced uptake of lysine (Figure 5.5) and leucine (Figure 5.6) by water- and RNA-injected oocytes was studied in Na^+-free media containing 10, 30, 90, 270, or 810 *uM* concentrations of amino acid. Uptake of lysine and leucine was greatest ($P < .001$) by RNA-injected oocytes (1.1 ng/nL). For both substrates, uptake occurred in a quadratic manner, with the relative proportion of substrate taken up decreasing as media concentrations increased. In

contrast, the uptake of both substrates by water-injected oocytes increased linearly as substrate media concentrations were increased.

Induced absorption (the difference between absorption of poly(A)$^+$ RNA-injected oocytes and water-injected oocyte uptake) of both lysine and leucine was greater ($P < .001$) than for water-injected oocytes and displayed Michaelis-Menton (saturable) kinetics. The affinity of induced lysine absorption (K_t = 52 +/- 3.5 *uM*) was almost twice that for leucine (K_t = 97 +/- 30.2 *uM*). At their respective K_t values, induced uptake of lysine and leucine were approximately 7.8 and 3.8 times that of water-injected oocyte uptake.

Characterization of mRNA-Induced Uptake. To further characterize the inducible mediated lysine and leucine transport processes, the effect of co-incubating 5 m*M* leucine or lysine, 5 m*M* glutamate, or .2 m*M* cystine with .05 m*M* levels of lysine or leucine on their uptake in RNA- and water-injected oocytes was evaluated. Lysine uptake (Figure 5.7) at .05 m*M* in water-injected oocytes (endogenous uptake) was reduced ($P < .05$) by the presence of 5 m*M* leucine (56.7%). In contrast, .2 m*M* cystine and 5 m*M* glutamate concentrations had no effect on endogenous lysine uptake. The absorption of lysine (33.31 +/- 2.87 pmol/oocyte·45 min^{-1}) by oocytes injected with RNA (1.1 to 1.3 ng/nL) at .05 m*M* lysine (Figure 5.7) was 3.9 times greater ($P < .001$) than by water-injected oocytes (8.57 +/- 1.04). Induced lysine absorption was reduced ($P < .05$) by leucine (68.1%) and, to a lesser extent, by cystine (38.9%). In contrast, the presence of glutamate quantitatively stimulated lysine uptake by 18.8%.

As shown in Figure 5.8, similar effects were observed for the induced absorption of leucine. Endogenous (water-injected) leucine absorption was not affected by the presence of 5 m*M* lysine or glutamate, but was slightly stimulated ($P <$

.05) by .2 m*M* cystine. At .05 m*M* leucine, oocytes injected with RNA (.7 to .8 ng/nL) absorbed 1.5 times more ($P < .001$) leucine (6.62 +/- .81) compared with oocytes injected with water (4.30 +/- .63). Induced leucine (.05 m*M*) absorption was reduced ($P < .05$) by the presence of 5 m*M* lysine (83.1%). Quantitatively, cystine also reduced leucine uptake by 23.2%, whereas glutamate increased leucine uptake by 60%.

mRNA-Oligomer Depletion of Induced Uptake. To test whether pig jejunal mRNA species share homology with mRNA species from human renal cortex epithelium capable of enhancing $b^{o,+}$ transporter-like activity, oocytes were sequentially injected with RNA fractions F35, F36, F38, or F39, plus a degenerate DNA oligomer (Antisense) that was complementary to the sequence surrounding the initiation site of the cloned human kidney $b^{o,+}$ amino acid transporter (Bertran et al., 1993). A DNA oligomer (Control DNA) complementary to nucleotides 1727 to 1745 of the cloned pig kidney Na^+/glucose cotransporter (Ohta et al., 1990) or water (water) were injected as DNA and volume injection controls, respectively.

The induced absorption of lysine and leucine was compared within injection treatment groups. For oocytes injected with RNA plus water, the quantity of induced uptake of lysine (Figure 5.9) was at least 2.2 times (13.85 +/- 2.19 pmols/oocyte·45 min^{-1}; F38) greater ($P < .001$) than that observed by oocytes injected with only water (6.26 +/- .32; No RNA). Similarly, induced leucine uptake (Figure 5.9) by oocytes injected with RNA plus water was at least 1.0 times (10.93 +/- .80 to 21.0 +/- 5.29 pmols/oocyte·45 min^{-1}; F38) greater ($P < .001$) than for water-injected controls (10.89 +/- .56; No RNA).

For oocytes not injected with RNA, the co-injection of additional water, negative control DNA, or antisense DNA had no effect ($P < .05$) on lysine (Figure

5.9) or leucine (Figure 5.10) uptake. Likewise, induced uptake of lysine or leucine by oocytes co-injected with mRNA fractions F35 and F39 and water or DNA oligomers did not differ. In contrast, induced lysine uptake (Figure 9) by oocytes co-injected with antisense and mRNA fractions F36 or F38 was 45.7 and 44.5% less ($P < .05$) than that observed for water and negative control co-injected oocytes, respectively. The co-injection of antisense DNA also reduced ($P < .05$) induced leucine uptake by 44.1 and 65.9% when co-injected into oocytes with mRNA fractions F36 or F38, respectively.

Discussion

This research was conducted to identify mRNAs that encode for proteins responsible for the absorption of arginine, lysine, and leucine because of 1) the importance of these indispensable amino acids to the nutrition of growing swine, 2) the fact that pigs raised in the USA are typically fed a corn/soybean meal diet that contains relatively high concentrations of these amino acids, and 3) the potential negative interactions among these indispensable amino acids when supplemented in diets (NRC, 1988). Accordingly, the mRNA used in this study was isolated from the jejunal epithelium of growing pigs fed a typical corn/soybean meal "grower" ration. Our interest in identifying lysine and leucine transport proteins whose function is independent of Na^+ was generated by their reputed rate-limiting role in the flux of lysine across intestinal epithelial tissue (Munck, 1989; Lawless et al., 1987; Cheeseman, 1991). The jejunal epithelium was selected as the host tissue because of its important role in the "ileal digestibility" of lysine in pigs (Leibholtz, 1989).

Xenopus laevis oocytes were selected as the expression system and the experimental model for characterizing mediated absorption because of their reliable translation of mammalian mRNA transcripts, processing and targeting of proteins, and proven flexibility of characterizing cell membrane permeases (Hediger et al., 1987; Tarnuzzer et al., 1990; Miyamoto et al., 1991).

The above experiments clearly indicate that the greater uptake of both lysine and leucine by oocytes injected with mRNA isolated from the scraped jejunal epithelium of growing pigs (both barrows and gilts) is because these oocytes have a mediated absorption ability that water-injected oocytes lack. Thus, pig jejunal epithelial cells appear to express the gene for a protein(s) capable of dual cationic and neutral amino acid transport. Cultured pig endothelial cells also are reported to share this ability (Kilberg et al., 1993). Together, these studies indicate that the pig genome possesses a gene(s) that encodes for a protein that either stimulates existing, quiescent lysine and(or) leucine transport processes in oocytes, or that encodes for a discrete, exogenous transport protein(s).

The ability of lysine and leucine to inhibit the induced uptake of each other, both within and between experiments (Figures 5.7 and 5.8) suggests that they are recognized and transported by the same transport protein(s). Because leucine is thought to stimulate the transport of lysine by allosteric binding to the y^+ transporter (Lawless et al., 1987), dipolar amino acids are thought to interact with the y^+ transport protein only in the presence of Na^+ (Ganapathy et al., 1994), and because the characteristic feature of the Na^+-independent $b^{o,+}$ transporter activity is the ability to bind and transport both neutral and cationic amino acids (Kilberg et al., 1993), the observed mutual cis-inhibition of lysine and leucine uptake in Na^+-free media indicates that a protein capable of inducing $b^{o,+}$-like transporter activity was

expressed. Similar magnitudes and patterns of mutual cis-inhibition of cationic and neutral amino acids have been observed in oocytes injected with poly(A)$^+$ RNA isolated from rabbit intestinal mucosa (Magagnin et al., 1992) and human kidney tissue (Bertran et al., 1993).

To date, cloned mammalian proteins reputed to induce $b^{o,+}$-like transporter activity in oocytes (Magagnin et al., 1992; Wells and Hediger, 1992; Bertran et al., 1993) have displayed the functional feature of being susceptible to inhibition by cystine. In the present study (Figures 5.7 and 5.8), the presence of cystine likewise inhibited ($P < .05$) the inducible amount of lysine absorbed by 38.9% and, quantitatively, that of leucine by 23.2% in RNA-injected oocytes. This inhibitory effect was not observed with water-injected oocytes and suggests that the injection of mRNA from pig jejunal epithelium confers to oocytes an enhanced and characteristically different ability to absorb lysine and leucine. In the present study, oocytes that were injected with water appeared to both lack and display endogenous $b^{o,+}$-like transport activity, depending on the amino acid being tested. The existence of endogenous $b^{o,+}$-like transport ability of defolliculated *Xenopus laevis* oocytes is disputed by other researchers. Observed differences most likely occur because of inherent differences between individual and batches of oocytes isolated within and between frogs (Van Winkle, 1993), and because of differences in the manner of oocyte defolliculation (Campa and Kilberg, 1989).

In the present study, the ovarian follicular tissue surrounding the oocytes was manually removed after partial collagenase digestion. Endogenous uptake of leucine was not inhibited by arginine or lysine (Figures 5.2 and 5.8). This failure of cationic amino acids to inhibit uptake of a neutral amino acid in a Na^+-free environment (pH 7.6) would invalidate the possibility of $b^{o,+}$ transporter involvement and, instead,

suggests that leucine uptake was by the L system (Van Winkle, 1988). Analogously, the inability of 10-fold leucine concentrations to inhibit arginine uptake by water-injected oocytes has been interpreted to indicate a lack of endogenous $b^{o,+}$ transport activity in Xenopus oocytes (Bertran et al., 1992).

However, because endogenous lysine uptake was inhibited by leucine (Figure 5.7) in media free of Na^+, an endogenous oocyte $b^{o,+}$-like transport system appears to have been involved in lysine absorption. Similar conclusions have been drawn by other researchers based upon the ability of leucine to inhibit arginine uptake (Campa and Kilberg, 1989) and that the absorption of neutral and cationic amino acids was mutually inhibitive (Wells and Hediger, 1992). Because endogenous lysine uptake was only 43.3% (3.71 +/-.34 vs. 8.57 +/- 1.04 pmol/oocyte·30 min^{-1}) inhibited in the presence of 100 times greater leucine concentrations, these results suggest that oocytes also possess the ability to absorb lysine by the Na^+-independent, cationic transport system (y^+), as has been commonly observed (Campa and Kilberg, 1989; Bertran et al., 1992; Van Winkle, 1993).

Defolliculated oocytes have been shown to lack endogenous, Na^+-independent, mediated cystine uptake abilities (McNarama et al., 1991; Wells and Hediger, 1992). Consequently, the inability of cystine to inhibit the endogenous uptake of leucine or lysine by water-injected oocytes in Na^+-free media has been used to indicate the absence of endogenous $b^{o,+}$-like transporter activity (Wells and Hediger, 1992). By this criteria, the present observations that the presence of four times greater concentrations of cystine failed to affect the uptake of leucine or lysine by oocytes injected with water (Figures 5.2, 5.7, and 5.8) indicates that endogenous $b^{o,+}$ uptake was not observed.

Because mediated glutamate absorption primarily is considered to be dependent on Na^+ cotransport (Lerner, 1987; Kanai and Hediger, 1992), the presence of glutamate in the Na^+-free media was not expected to affect the absorption of lysine or leucine. However, although the response was not significant at the alpha = .05 level, glutamate appeared to quantitatively stimulate the induced uptake of lysine by 18.8% and that of leucine by 60% (Figures 5.7 and 5.8). The magnitude of these responses was similar for all experiments (individual experimental observation values not shown). This result, plus reports that glutamate has no effect on Na^+-independent leucine or arginine uptake by oocytes injected with cRNA encoding for the $b^{o,+}$ transport protein (Wells and Hediger, 1992; Bertran et al., 1993), suggests that the pig jejunal epithelium may express a functionally unique isoform with the characteristic of stimulatory, allosteric binding of glutamate.

These competitive inhibition studies (Figures 5.7 and 5.8) demonstrate that neither lysine nor leucine uptake required Na^+, that lysine and leucine were capable of mutual cis-inhibition, and that cystine was capable of cis-inhibition of lysine and tended to inhibit the absorption of leucine. Therefore, the observed induced lysine and leucine uptake appeared to be the result of oocytes expressing an exogenous mRNA species from pig jejunal epithelium. The experimental results presented in Figures 5.9 and 5.10 demonstrate that increased lysine and leucine uptake induced by the presence of pig jejunal epithelium mRNA for fractions F36 and F38 in oocytes was inhibited when exposed to DNA oligomers that were complementary to a sequence flanking the initiation of translation site of the cloned human renal $b^{o,+}$ transporter. That induced uptake was inhibited for RNA fractions F36 and F38 (which bracket the spectrum of RNA fractions F36, F37, and F38 that have been shown to induce $b^{o,+}$ transporter-like activity in this study), but not for fractions F35

and F39, indicates that mRNA encoding for the $b^{o,+}$ transporter-like activity were enriched in fractions F36, F37, and F38.

Accordingly, it appears that the $b^{o,+}$-like transporter activity characterized in this study is primarily the result of the isolation and expression of mRNA species from pig jejunal epithelium that shares homology to at least the initiation site of the cloned human renal $b^{o,+}$ transporter. A similar pattern of fraction-specific inhibition of rabbit jejunal poly(A)$^+$ RNA-induced arginine (76%) and leucine (52%) uptake has been observed in oocytes also injected with antisense DNA corresponding to the cloned rabbit renal $b^{o,+}$ transporter (Magagnin et al., 1992). The incomplete inhibition of substrate uptake by antisense oligomers could have occurred as the result of incomplete hybridization of the synthetic DNA antisense molecules to the pig jejunum epithelial mRNA species in the oocyte cytoplasm. Alternatively, the incomplete antisense inhibition of induced lysine and leucine uptake (Figures 5.9 and 5.10), and the inability of 100-fold excesses of lysine and leucine to mutually inhibit induced uptake (Figures 5.7 and 5.) suggest that mRNA species that encode for proteins capable of stimulating separate lysine and leucine transport were also isolated in fractions F36 and F38. Likewise, the inducible, but antisense-insensitive uptake of lysine and leucine by oocytes injected with fractions F35 and F39 indicate that these mRNA may have encoded for Na^+-independent cationic, y^+-like and neutral, L-like, amino acid transport systems. That porcine pulmonary endothelial cells have been shown to possess the y^+ transport protein (Woodard et al., 1994) verifies the existence of a gene that encodes for a protein(s) capable of specific Na^+-independent lysine transport in the porcine genome.

The size of the poly(A)+ RNA that induced $b^{o,+}$ transporter-like activity was not determined. The existence of mRNA transcripts of multiple lengths (2.2, 2.3, 2.5,

and 3.9 kb) extracted from rabbit duodenum, jejunum, ileum (Magagnin et al., 1992) and rabbit, rat, and human renal tissue (Bertran et al., 1992; Wells and Hediger, 1992; Markovich et al., 1993) have been reported to be capable of inducing $b^{o,+}$ transporter-like activity when expressed in Xenopus oocytes. The essential difference between these transcripts is thought to be the extent of 3'-polyadenylation, and not that of translatable function (Bertran et al., 1993).

Hydropathy profiles of the homologous cloned renal $b^{o,+}$ transport proteins of the rat (Wells and Hediger, 1992), rabbit (Bertran et al., 1992), and human (Bertran et al., 1993; Lee et al., 1993) have predicted that these proteins will possess one membrane spanning domain (region requiring 21 to 23 predominantly hydrophobic amino acid residues). As a consequence, it was proposed that the protein is not a transporter per se, but, instead, may function as an activating protein that associates with and stimulates existing endogenous oocyte $b^{o,+}$ transporters to increase their permease activity (Wells and Hediger, 1992; Bertran et al., 1992). Because a leucine zipper motif exists at the C-terminus, homologous "single membrane-spanning "$b^{o,+}$" proteins may possess this capacity by forming transporter consortiums of monomeric subunits (Van Winkle, 1993), which mediate the absorption of cationic and neutral amino acids. In the present study, because cystine failed to inhibit endogenous uptake of lysine or leucine (Figures 5.7 and 5.8), it seems unlikely that the pig epithelial mRNA that we have expressed functions as an activator protein, unless the "activation" involves the bestowment of cystine recognition to existing oocyte transport systems.

An alternative interpretation of these hydropathy profiles would be that the transport protein has four membrane-spanning domains of approxiamately nine amino acid residues (Tate et al., 1992). Subsequent testing of this hypothesis with antibodies

directed towards specific peptide sequences of the cloned protein was supportive of a structural model consisting of four intracellular, two extracellular, and four membrane-spanning domains (Mosckovitz, et al., 1994). The functional significance of multiple membrane spanning domains is the capacity to "permeate" the membrane by the orienting of constituent membrane domains to form a channel for directed substrate passage (Stevens, 1992b).

The apparently greater affinity for induced lysine absorption (K_t = 52 *uM*) than for leucine (K_t = 97 *uM*) (Figures 5.5 and 5.6) suggests that lysine would out-compete leucine for transport at equimolar concentrations. Other studies also have demonstrated the greater affinity of induced $b^{o,+}$ transporter activity for cationic than for neutral amino acids (Wells and Hediger, 1992; Bertran et al., 1992). The observed 97 +/- 30 *uM* K_t value for induced leucine uptake by oocytes injected with pig jejunal epithelia RNA in this study (Figure 5.6) is similar to that (K_t = 128 *uM*) demonstrated for a transport protein capable of $b^{o,+}$-like transport activity cloned from rabbit kidney (Bertran et al., 1992) and appears to be less specific than a $b^{o,+}$ transporter cloned from the renal tissue of rats (K_t = 22 *uM*; Tate et al., 1992).

From the present study the cell membrane location of the $b^{o,+}$ transport activity can not be determined. mRNA reported to encode for $b^{o,+}$ transport activity have been located in the intestinal epithelium of human jejunum (Bertran et al., 1993) and ileum (Lee et al., 1993); rabbit duodenum, jejunum, and ileum, but not the colon (Magagnin et al., 1992); and rat duodenum, jejunum, and ileum (Wells and Hediger, 1992). Subsequent protein localization analysis has identified the presence of $b^{o,+}$ transport proteins in the microvilli of the jejunum of rats, in the brush border membranes of both enterocytes and enteroendocrine cells, but not resident in the basolateral membranes (Pickel et al., 1993). These conclusions, drawn from the

expression of identified mRNA, are in agreement with intestinal tissue kinetic characterization that has identified $b^{o,+}$-like activity only in the brush border membrane (as reviewed by Stevens, 1992a; Ganapathy et al., 1994).

In pigs, measurements of the amino acid flux across the small intestine suggest that proteins (and, therefore, their encoding mRNA) capable of Na^+-independent lysine and leucine absorption are expressed in jejunal enterocytes. In growing pigs, using distal duodenal and proximal ileal cannulae, measurement of the apparent absorption of lysine and arginine indicates that the uptake of these cationic amino acids is mutually inhibited (Buraczewski, et al., 1970). Additionally, the flux of lysine across isolated neonatal pig jejunal tissue sheets has been described as having saturable (K_t = 200 *uM*) and non-saturable components (Burton et al., 1980). In both of these studies, uptake was not measured in the absence of Na^+. Therefore, mediated lysine uptake could have occurred by either Na^+-dependent (Y^+, cationic) or Na^+-independent (y^+ cationic; $b^{o,+}$ cationic or neutral) systems. Lysine absorption in the presence of Na^+ by villi-tip enterocytes in the jejunal tissue of pigs (at approximately < 1 through 28 d of age) has been shown to be non-concentrative (James et al., 1987). In the absence of Na^+, an almost identical response was seen for the uptake of alanine. Conversely, enterocytic capacity for alanine uptake in the presence of Na^+ was concentrative. Because concentrative amino acid uptake is thought to be driven by the electrochemical gradient of its cotransporter (typically, Na^+), lysine uptake seems to have occurred by the y^+ (Na^+-independent cationic) and(or) $b^{o,+}$ transport systems. Likewise, non-concentrative alanine uptake was probably by the L and(or) $b^{o,+}$ transport systems and concentrative alanine by the A (Na^+-independent, neutral amino acid) system. Although its dependence on Na^+ gradient was not determined,

the presence of a protein(s) capable of mediated leucine transport (K_t = 490 +/- 60 *uM*) across the jejunal brush border has been demonstrated (Wolffram et al., 1986).

Combined, the above observations describe the potential for mediated flux of lysine and leucine across pig jejunum in a manner that is consistent with the characterized function of proteins encoded by the mRNA expressed in this study. As such, these results provide evidence to indicate that the mRNA isolated from pig jejunal epithelia in the present study are actually expressed in jejunal enterocytes. Because the presence of leucine has been shown to stimulate both the influx and efflux of lysine across the basolateral membranes of enterocytes (Lawless et al., 1987; Cheeseman, 1991), ostensibly by the allosteric binding to the y^+ transport protein, and because the $b^{o,+}$ transport uptake of lysine is inhibitable by leucine (present study), the flux of lysine across pig jejunal enterocytes may be dependent on the luminal and serosal concentrations of leucine. If future research supports this hypothesis, then the determination of an optimal leucine:lysine dietary ratio may promote the more efficient feeding of lysine to pigs.

In summary, we have demonstrated the ability to extract, enrich, and express viable mRNA species from pig jejunal epithelium in *Xenopus laevis* oocytes. Characterization of the function of expressed proteins indicates that mRNA encoding for a protein capable of mediating the transport of lysine and leucine is expressed by the jejunal epithelium of growing pigs fed a corn/soybean diet. In addition, the epithelial cells appear to express mRNA that encode for proteins capable of separate Na^+-independent uptake of lysine and leucine.

Implications

mRNA encoding functionally distinct transport proteins that display the ability to transport lysine and other commonly supplemented indispensable amino acids have been identified in the small intestinal tissue of growing pigs. An increased understanding of the structure, function, and regulatory events of transport proteins that they encode for will affect the feeding of swine by enhancing the establishment of specific dietary amino acid ratios that will allow adequate levels of an indispensable amino acid to be fed without causing deleterious effects on the absorption of others.

Table 5.1. Ingredient composition of diets fed to growing pigs (as fed basis)[a]

Ingredient	Percent
Ground corn (8.7% CP)	76.41
Soybean meal (44% CP)	21.25
Defluorinated phosphate	.93
Ground limestone	.63
Vitamin-Se premix[b]	.25
Salt (plain)	.25
Tylosin (22 g/kg)	.15
Copper sulfate (25%)	.08
Trace mineral premix[c]	.05

[a]Calculated to contain 16.0% CP, .6% Ca, .5% P, 200 ppm Cu, 33 mg/kg tylosin, and .1 ppm Se.

[b]Supplied per kg of diet: 4,400 IU vitamin A, 440 IU vitamin D_3, 11 IU vitamin E, 1.1 mg vitamin K_3 (MPB), 22 ug vitamin B_{12}, 440 mg choline, 22 mg pantothenic acid, 22 mg niacin, 4.4 mg riboflavin, 440 ug biotin, and 1.0 mg selenium.

[c]Supplied per kg of diet: 45.4 mg Zn, 22.7 mg Fe, 12.5 mg Mn, 2.5 mg Cu, and .34 mg I.

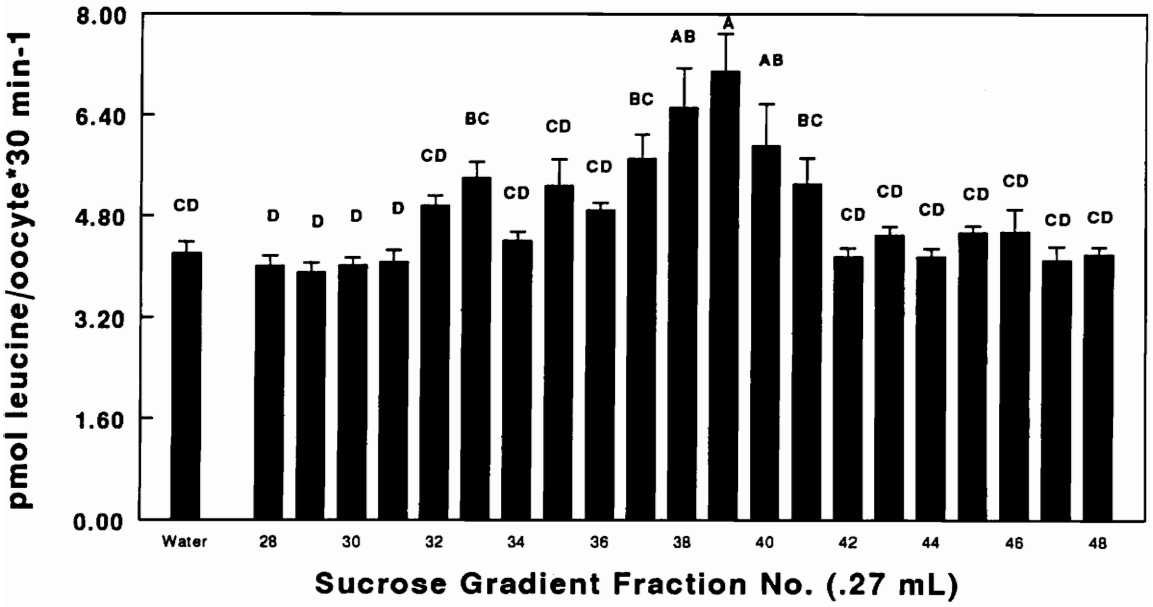


Figure 5.1. Absorption of leucine by defolliculated Xenopus oocytes injected with water or sequential sucrose gradient-fractionated pig jejunal epithelium poly(A)$^+$ RNA.

Oocytes were assayed for leucine absorption in Na^+-free media (50 *uM* leucine). Individual histogram bar values represent animal (n = 1) absorption means +/- SE of oocytes injected with water (n = 11) or RNA (n = 6 to 12).

Injection treatments differed ($P < .001$).

Bars lacking a common letter differ ($P < .05$).

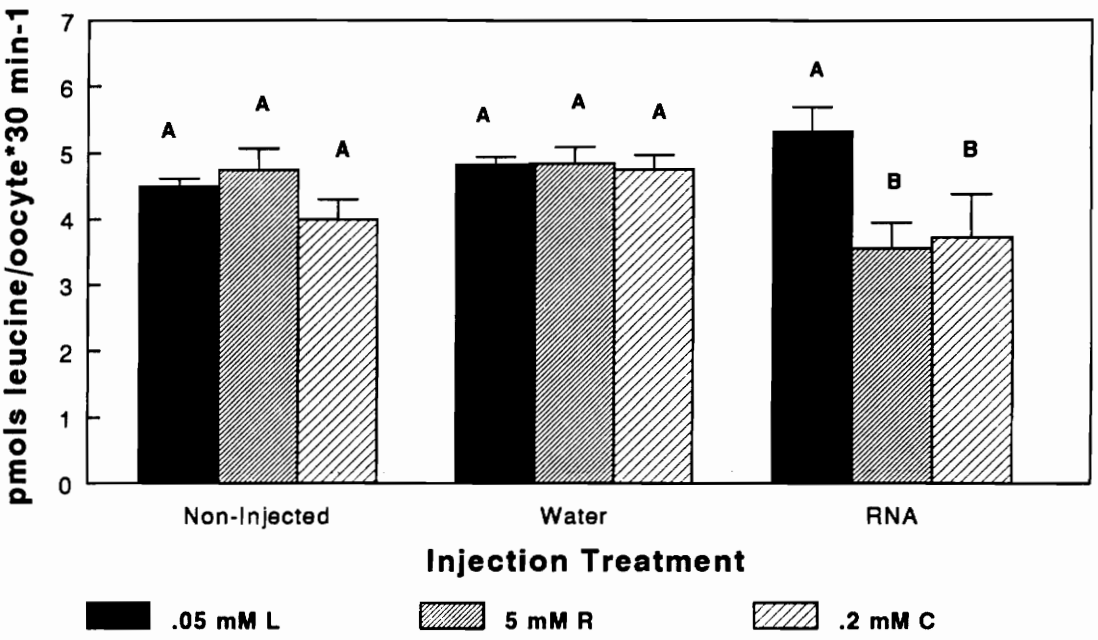


Figure 5.2. Competitive absorption of leucine by defolliculated Xenopus oocytes not injected, or injected with water or size-fractionated pig jejunal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for leucine absorption in Na^+-free media containing .05 m*M* leucine (L) or .05 m*M* L plus L-arginine (R) or L-cystine (C), at indicated concentrations. Individual histogram bar values represent animal (n = 1) absorption means +/- SE of oocytes injected with water (n = 6 to 8) or RNA (n = 8 to 10).

At .05 m*M*, uptake of leucine by oocytes injected with RNA (n = 8) was greater ($P < .03$) than for non-injected (n = 7) or water-injected oocytes (n = 7).

Within treatments, bars lacking a common letter differ ($P < .05$).

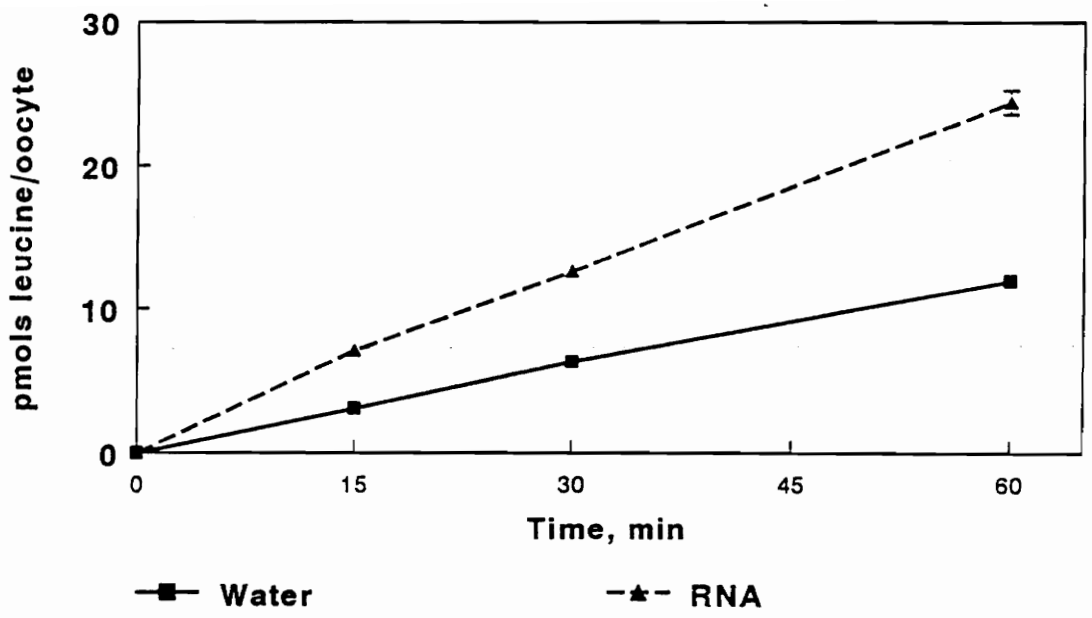


Figure 5.3. Time course for lysine absorption by defolliculated Xenopus oocytes injected with water or pig jejunal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for lysine absorption in Na^+-free media (50 *uM* lysine). Each time period measurement represents animal (n = 1) absorption means +/- SE of oocytes injected with water (n = 4 to 5) or RNA (n = 4 to 8).

Linear time effect ($P < .001$).

Time x treatment effect ($P < .001$).

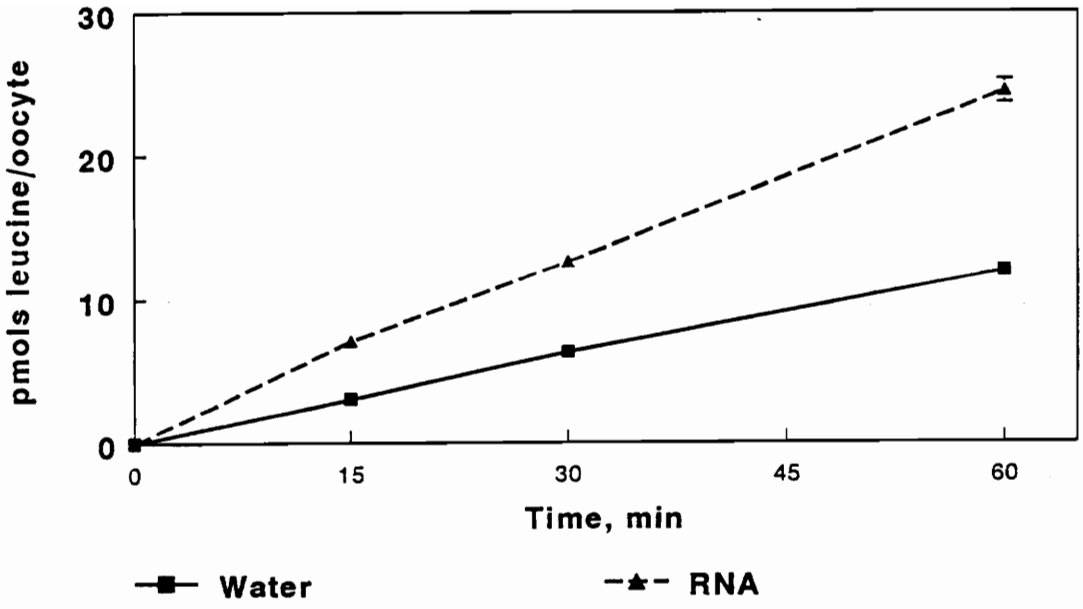


Figure 5.4. Time course for leucine absorption by defolliculated Xenopus oocytes injected with water or pig jejunal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for leucine absorption in Na^+-free media (50 *uM* leucine). Each time period measurement represents across animal (n = 2) absorption means +/- SE of oocytes injected with water (n = 7 to 11) or RNA (n = 16).

Linear time effect ($P < .001$).

Time x treatment effect ($P < .001$).

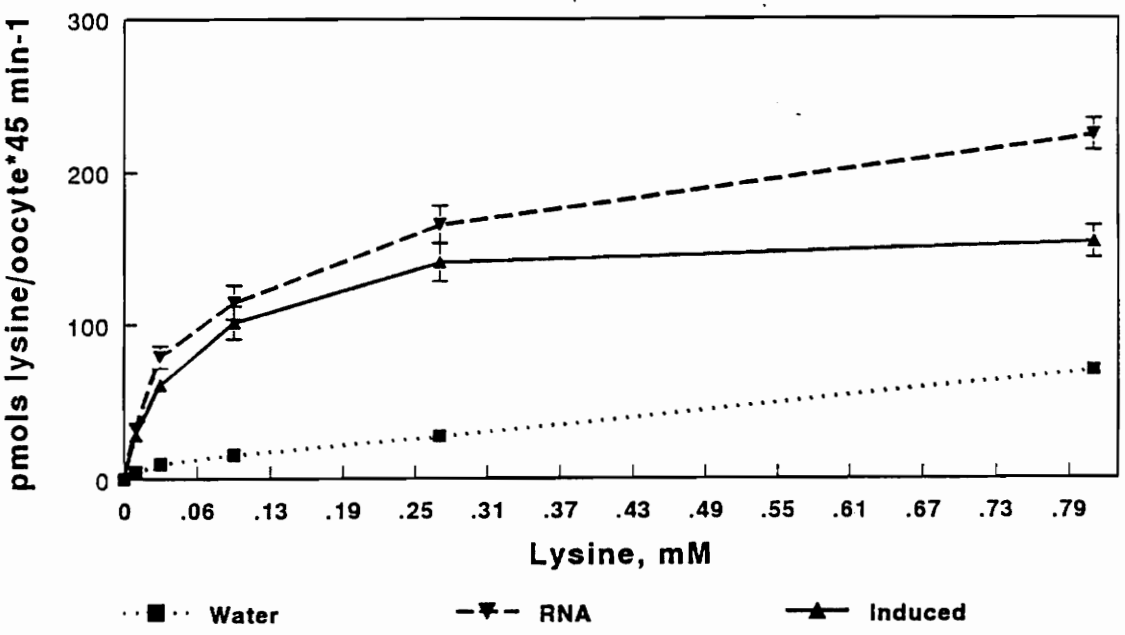


Figure 5.5. K_t characterization (52 +/- 3.5 uM) for induced lysine absorption by defolliculated Xenopus oocytes injected with water or pig jejunal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for lysine absorption in Na^+-free media (50 u*M* lysine). Each concentration measurement represents across animal (n = 3) absorption means +/- SE of oocytes injected with water (n = 7 to 11) or RNA (n = 14 to 18).

Linear ($P < .001$) concentration effect on water-injected oocyte uptake.

Quadratic ($P < .002$) concentration effect on induced uptake.

Concentration x treatment interaction ($P < .001$) on induced uptake.

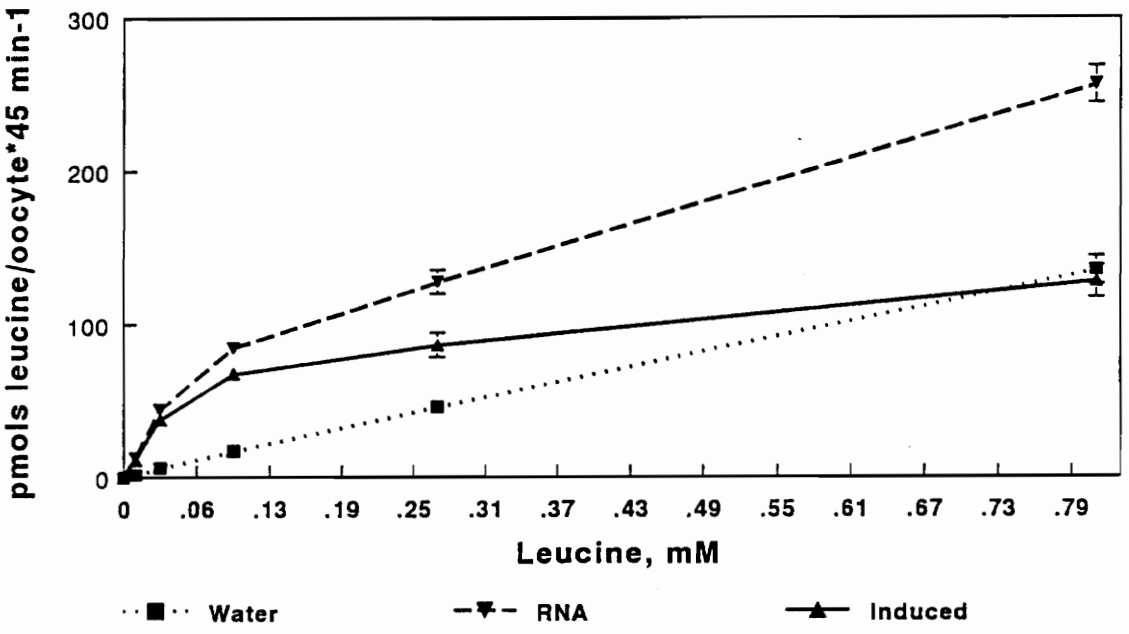


Figure 5.6. K_t characterization (97 +/- 30 uM) for induced leucine absorption by defolliculated Xenopus oocytes injected with water or pig jejunal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for leucine absorption in Na^+-free media (50 u*M* leucine). Each concentration measurement represents across animal (n = 2) absorption means +/- SE of oocytes injected with water (n = 7 to 10) or RNA (n = 14 to 20).

Linear ($P < .001$) concentration effect on water-injected oocyte uptake.
Quadratic ($P < .006$) concentration effect on induced uptake.
Concentration x treatment interaction ($P < .001$) on induced uptake.

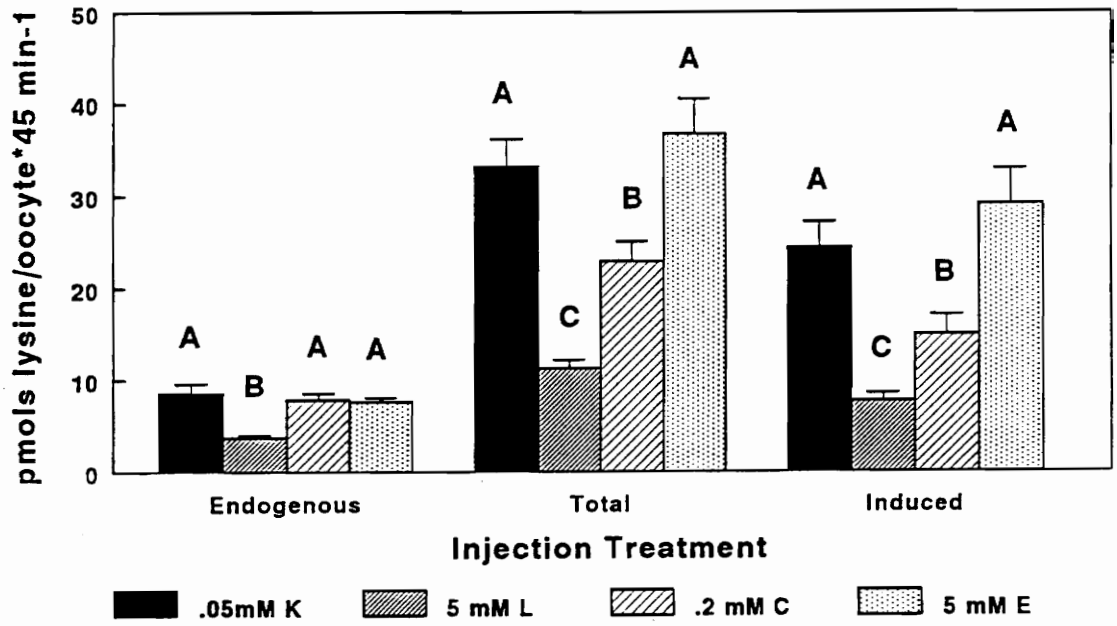


Figure 5.7. Competitive absorption of lysine by defolliculated Xenopus oocytes injected with water (endogenous) or size-fractionated pig jejunal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for lysine absorption in Na^+-free media containing .05 m*M* L-lysine (K) or .05 m*M* K plus L-leucine (L), L-cystine (C), or L-glutamate (E), at indicated concentrations. Individual histogram bar values represent across animal (n = 3) absorption means +/- SE of oocytes injected with water (n = 10 to 19) or RNA (n = 10 to 22).

At .05 m*M*, uptake of lysine by oocytes injected with RNA (n = 20) was greater ($P < .001$) than for water-injected oocytes (n = 19).

Within treatments, bars lacking a common letter differ ($P < .05$).

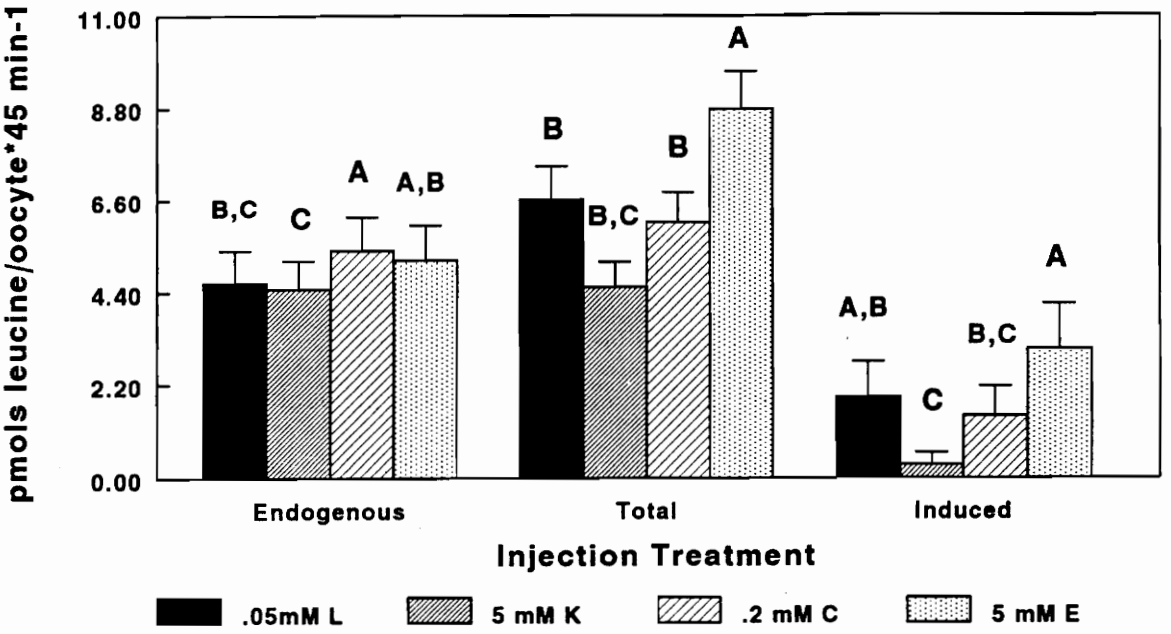


Figure 5.8. Competitive absorption of leucine by defolliculated Xenopus oocytes injected with water (endogenous) or size-fractionated pig jejunal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for leucine absorption in Na^+-free media containing .05 m*M* L-leucine (L) or .05 m*M* L plus L-lysine (K), L-cystine (C), or L-glutamate (E) at indicated concentrations. Individual histogram bar values represent across animal (n = 2) absorption means +/- SE of oocytes injected with water (n = 12 to 15) or RNA (n = 11 to 16).

At .05 m*M*, uptake of leucine by oocytes injected with RNA (n = 11) was greater ($P < .001$) than for water-injected oocytes (n = 12).

Within treatments, bars lacking a common letter differ ($P < .05$).

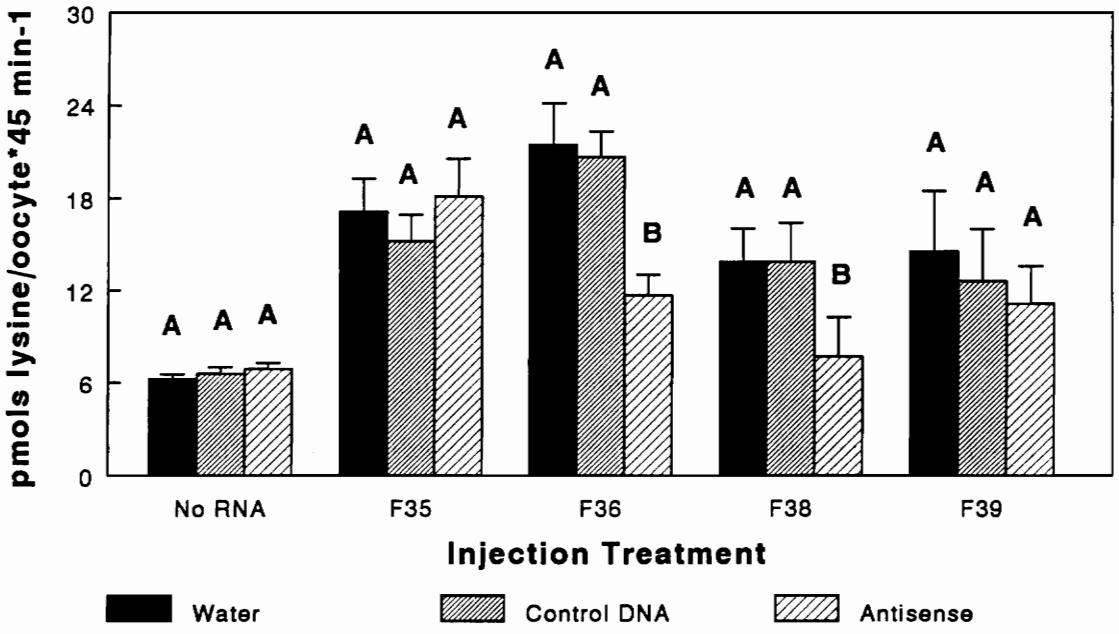


Figure 5.9. Oligomer depletion of induced lysine absorption by defolliculated Xenopus oocytes injected with water or pig jejunal epithelium poly(A)$^+$ RNA fractions 35, 36, 38, or 39, plus water, control DNA, or Antisense DNA.

Oocytes were assayed for lysine absorption in Na^+-free media (50 *uM* lysine). Individual histogram bars represent across animal (n = 2) absorption means +/- SE of oocytes injected with water (No RNA; n = 10 to 12), or injected with RNA (n = 7 to 12).

Among water injection treatments, uptake of lysine by oocytes injected with RNA (n = 8 to 12) was greater ($P < .001$) than by oocytes not injected with RNA (n = 8).

Within treatments, bars lacking a common letter differ ($P < .05$).

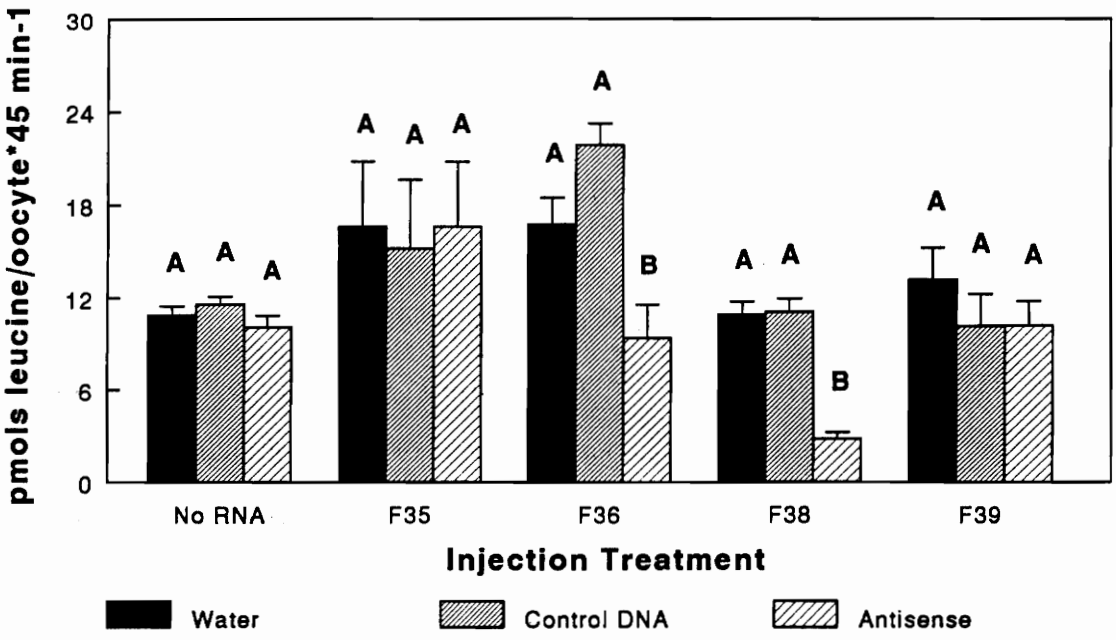


Figure 5.10. Oligomer depletion of induced leucine absorption by defolliculated Xenopus oocytes injected with water or pig jejunal epithelium poly(A)$^+$ RNA fractions 35, 36, 38, or 39, plus water, control DNA, or antisense DNA.

Oocytes were assayed for leucine absorption in Na^+-free media (50 *uM* leucine). Individual histogram bars represent across animal (n = 2) absorption means +/- SE of oocytes injected with water (No RNA; n = 8 to 13), or injected with RNA (n = 8 to 15).

Among water injection treatments, uptake of leucine by oocytes injected with RNA (n = 10 to 15) was greater ($P < .001$) than by oocytes not injected with RNA (n = 11).

Within treatments, bars lacking a common letter differ ($P < .05$).

CHAPTER VI

DEMONSTRATION AND CHARACTERIZATION OF DIPEPTIDE TRANSPORT SYSTEM ACTIVITY IN THE OVINE OMASUM EPITHELIUM BY EXPRESSION OF mRNA IN *XENOPUS LAEVIS* OOCYTES

ABSTRACT

Research from this laboratory has recently demonstrated that the omasal epithelium of sheep is capable of absorbing dipeptides. In order to express proteins potentially responsible for the mediated absorption of small peptides, size-fractionated poly(A)$^+$ RNA (RNA) isolated from omasal epithelial tissue of sheep (average BW = 67.5 kg) were injected into defolliculated *Xenopus laevis* oocytes. The ability of oocytes injected with RNA or water to absorb [^{14}C]glycyl-L-sarcosine (Gly-Sar) from media (usually pH 5.5) was compared. After 4 d ($P < .02$) of culture, specific RNA fractions induced an increased ($P < .02$) rate of Gly-Sar absorption, as compared to water-injected oocytes. The dependency of Gly-Sar uptake on the presence of a pH gradient was evaluated at pH 5.0, 5.5, 6.0, 6.5, and 7.5. Inducible uptake increased linearly ($P < .001$) in the presence of an increasing proton concentrations, whereas endogenous uptake of Gly-Sar decreased ($P < .001$). At pH 5.5, induced Gly-Sar uptake was saturable (K_t = .4 m*M*), but endogenous uptake was not. The specificity of Gly-Sar absorption was studied by the co-incubation of .1 m*M* Gly-Sar with 5 m*M* levels of competing substrates (pH 5.5). Induced uptake was inhibited ($P < .05$) 44% by carnosine, 94% by methionylglycine, 91% by glycylleucine, but not by glycine. Incubation of RNA with DNA oligomers that were complementary to the cloned rabbit intestinal transporter completely inhibited ($P < .05$) induced Gly-Sar uptake.

These results indicate that sheep omasal epithelial cells express mRNA that encode for proteins that are capable of H^+-dependent dipeptide transport activity.

(**Key Words:** Sheep, Omasum, Peptide, Biological Transport, Absorption)

Introduction

Intestinal absorption of small, dietary peptides is now recognized as a major source of amino acid N in non-ruminants (Matthews, 1991a). Paramount to the acceptance of this understanding has been the identification of peptide transport proteins in the small intestine. Recent research has indicated that the forestomach tissues may be the primary site of dietary peptide absorption in ruminants (DiRienzo and Webb, 1995). Little research, however, has been conducted that attempts to characterize the potential for forestomach epithelial absorption of peptide-bound amino acids, which are present in the forestomach liquor in nutritionally significant quantities (Webb and Matthews, 1994).

The use of parabiotic chambers has demonstrated that isolated ruminal and omasal epithelia have the ability to absorb dipeptides, by apparently non-mediated processes (Matthews and Webb, 1995). However, the multi-layered morphological structure of forestomach epithelia presents unique challenges to identifying transport proteins by traditional techniques. The expression of exogenous mRNA by *Xenopus laevis* oocytes has resulted in the identification, by functional characterization, of dipeptide transport activity in mammalian epithelia (Miyamoto et al., 1991; Saito et

al., 1993). The purpose of this study was to identify and to characterize the function of proteins in sheep omasal epithelium that may be capable of mediating the absorption of dipeptides, by expression of their mRNA in *Xenopus laevis* oocytes.

Materials and Methods

Unless noted otherwise, all chemicals, substrates and reagents were of either molecular biology or cell culture tested chemical grades. All reagents, equipment, and standard protocols used in the collection, defollicularization, culture, and microinjection of size-fractionated poly(A)$^+$ RNA into *Xenopus laevis* oocytes have been described thoroughly (Chapter V).

RNA Extraction. RNA was extracted from the omasal epithelium of cross-bred sheep (average BW 67.5 kg) raised at the Virginia Experimental Agricultural Research Center. All sheep had ad libitum access to a mixed pasture, mineral blocks, and water for at least 2 wk prior to tissue collection. In groups of three or four, sheep were individually anesthetized by intravenous injection of sodium pentobarbitol (approximately 28.9 mg/kg BW). The abdominal cavity was opened quickly, the omasum was removed and the animal killed by exsanguination. The omasum was opened through the omasal orifice and the digesta was removed by rinsing in 4°C .85% saline. Order I and II omasal laminae (McSweeney, 1988) were removed from the omasal body and serially washed with gentle agitation in five baths containing cold saline solution to remove adhering particles of digesta.

Omasal mucosa was scraped from the underlying lamina musculature using a glass slide, divided (approximately 6 g) into a series of 50 mL polypropylene tubes

containing 25 mL of 4°C RNA extraction buffer (4 M guanidine thiocyanate, 25 m*M* sodium citrate, .5% sodium lauroylsarcosine, .1 M beta-mercaptoethanol), homogenized, and held in an ice bath for average of 30 min. The total time from anesthezia of the animal to completion of tissue homogenization was less that 15 min. The homogenized tissue was pooled into three 250 mL centrifuge bottles (approximately 12 g tissue per bottle) and the total RNA was extracted essentially as described (Puissant and Houdebine, 1990; Chapter V).

Isolation of Size-Fractionated Poly(A)$^+$ RNA. Poly(A)$^+$ RNA was isolated from total RNA by chromatography on oligo(dT) cellulose (Sambrook et al., 1989; Chapter V). The final eluate was collected in 1.5 mL ultracentrifuge tubes and precipitated with isopropanol for at least 18 h and -20°C. The poly(A)$^+$ RNA precipitate was recovered after centrifugation for 30 min at 80,000 *x* g and 2°C and size-fractionated through an 8 to 20% linear sucrose gradient (Sambrook et al., 1989; Chapter V) by centrifugation for 15 h at 80,000 *x* g and 4°C. Typically, forty-eight .27 mL fractions were collected. An equal volume of 5.0 m*M* beta-mecaptoethanol was added to each fraction to reduce the methyl mercuric hydroxide in the gradient buffer, and the size-fractionated poly(A)$^+$ RNA was stored as an ethanol precipitate at -20°C. When appropriate, the integrity and viability of size-fractionated omasal RNA was evaluated by its ability to induce Na^+-independent uptake of L-[^{3}H]-lysine by defolliculated *Xenopus laevis* oocytes (Appendix A).

Preparation of Oocytes for Injection. Stage IV and V oocytes (Dumont, 1972) were defolliculated after collection from mature female *Xenopus laevis* frogs (Coleman, 1984; Chapter V). The defolliculated oocytes were rinsed five times and allowed to heal for at least 18 h in sterile-filtered Ca^{2+}-containing media (96 m*M* NaCl, 2.5 m*M* sodium pyruvate, 2 m*M* KCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M*

HEPES/NaOH, pH 7.6, 1.0 X 10^5 units/L penicillin-G, and 1.67 X 10^5 units/L streptomycin) and cultured in petri dishes at 18°C. Previous studies showed that oocytes defolliculated and cultured in this manner retained their endogenous Na^+-dependent and Na^+-independent amino acid uptake capabilities (Chapter V; Appendix A).

Microinjection of mRNA and DNA Oligomers. Using a positive pressure microinjection system (Chapter V), defolliculated oocytes were injected in the animal pole, near the polar interface, with fractions F34 through F40 of sucrose-gradient fractionated omasal poly(A)$^+$ RNA (corresponding to the 11 +/- .81% sucrose) that had been stored at -20°C for no more than 3 wk after suspension in DEPC-treated water. The amount of poly(A)$^+$ RNA injected into oocytes (see Results) varied among fractions and groups of animals because of inherent differences in the amount and molecular size of epithelial mRNA in omasal mucosa and the subsequent practice of suspending all fraction precipitates in the same volume (usually 5 uL) and the injection of the same volume (usually 110 nL) into oocytes. Within an injection treatment, radiotracer experiments demonstrated that the volume of solution injected per oocyte was approximately the same (+/- 10%).

Oligomer depletion of uptake experiments were performed essentially as described (Fei et al., 1994). Two *u*L of blended poly(A)$^+$ RNA (fractions F34, F35, and F36; 2.0 ng/nL) or water were incubated at 42°C for 1 h with 2 *u*L of 50 m*M* NaCl, or with 2 *u*L of 50 m*M* NaCl that contained either .5 ng negative control deoxynucleotide sense oligomer (5'-GCCACCATGGGNATGTC-3') or .5 ng degenerate deoxynucleotide antisense oligomer (5'-GACATNCCCATGGTGGC-3'). These solutions were stored in an ice-bath until injection (120 *u*L) into oocytes.

After receiving their injection treatments, oocytes were cultured for 4 d at 18°C in Ca^{2+}-containing media. Oocytes of the same injection treatment were cultured in a common 20 mL borosilicate vial. Daily, the media was changed and damaged oocytes (typically, one-half of those injected) were discarded.

Oocyte Uptake Experiments. The standard uptake buffer contained 96 m*M* NaCl, 2 m*M* KCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 3 m*M* MES, 3 m*M* HEPES, pH 5.5, and appropriate quantities of peptide and free amino acid substrates. Only those oocytes that were healthy and of the same size and stage of maturation were selected for uptake experiments, on the day of assay. This resulted in the use of varying numbers of oocytes in experiments (see figure legends). Prior to assay, oocytes were washed six times in pH 7.6 standard uptake buffer to remove any residual antibiotics. Uptake experiments were initiated by placing all oocytes of a given injection treatment into 7 mL plastic vials that contained .5 mL of experimental uptake buffer (standard uptake buffer plus 5.4 to 11 *u*Ci/mL of [1,2-^{14}C]-glycyl-L-sarcosine, 110 mCi/mmol, custom synthesized, Moravek Biochemicals, Brea, CA) and appropriate amounts of non-radiolabeled amino acid substrates (see figure legends).

Experiments were terminated by the addition of 4 mL 4°C stop solution (pH 7.6 standard uptake buffer that included 5 m*M* nonlabeled glycyl-L-sarcosine) after room temperature incubation for appropriate time periods (see figure legends). Treatment groups of oocytes were serially washed six times in 1.5 mL of the appropriate stop solutions. Oocytes were individually transferred in 30 *u*L of stop solution into 7 mL plastic scintillation vials containing .2 mL of 10% SDS. Aliquots (30 uL) of the last wash of each treatment group were collected for determination of background radioactivity. After room temperature digestion of the oocytes (minimum of 4 h), 5 mL of scintillation fluid (Scintiverse BD; Fisher Scientific) were added to

each vial and allowed to equilibriate (minimum of 4 h) before quantifying the ^{14}C content by liquid scintillation counting (LS 5000TA Scintillation Counter; Beckman Instruments, Fullerton, CA). Recorded cpm were converted to dpm using a standard quench curve.

Non-injected, water-injected, and RNA-injected oocyte uptake values for [1,2-^{14}C]-glycyl-L-sarcosine (Gly-Sar) were determined as the product of background-corrected dpm x the specific activity of the buffer. Induced oocyte uptake values were calculated by subtracting the water-injected uptake values from the RNA-injected uptake values. Induced K_t values were generated by fitting the data to the Michaelis-Menton equation $y = V_{max} * X/(K_t + X)$ using the P.Fit computer program (Version 5.1, Fig.P Software Corporation, Durham, NC), where X = concentration of substrate (m*M*) and y = observed substrate absorption (fmols/oocyte·min^{-1}).

Statistical Analyses. For the serial, sucrose gradient fraction experiment, the effect of injection treatment and specific RNA fractions on the quantity of Gly-Sar absorbed by oocytes was evaluated by the GLM procedure of SAS (1988). For the time-course experiments, the effects of time, injection treatment, and their interactions on the ability of oocytes to absorb Gly-Sar were evaluated by GLM. Orthogonal contrasts were used to test for differences in rates of absorption. For the characterization of K_t and pH-dependence of Gly-Sar uptake experiments, the effects of injection treatment, group and substrate concentration and their interactions on mean Gly-Sar uptake were evaluated using GLM procedures (SAS, 1988). Orthogonal contrasts were used to test for injection treatment differences in the quantity of Gly-Sar absorbed. Orthogonal polynomial contrasts were used to partition the effect of substrate or proton concentration on oocyte uptake of Gly-Sar into linear,

quadratic, and cubic components. Because of missing observations, contrast coefficients were generated by the Matrix function of SAS (1985).

For the competitive inhibition of Gly-Sar absorption experiments, the effects of group, injection treatment, and their interactions on the ability of oocytes to absorb Gly-Sar were evaluated using the GLM procedure of SAS (1988). For the oligomer depletion experiment, the effect of injection treatment was evaluated. Respectively, within an injection treatment, the effects of competitor or co-injected substrate were evaluated using Tukey's multiple comparison test at the alpha = .05 level. These calculations were performed manually to generate the appropriate standard deviations and critical values for each combination of unequal treatment observations (Lentner and Bishop, 1993). Similarly, the effect of injection treatment on the ability of oocytes to absorb Gly-Sar in the oligomer depletion experiment was evaluated using the GLM procedure of SAS, whereas the effect of water, antisense DNA, or sense DNA on endogenous and induced absorption of Gly-Sar were evaluated using Tukey's multiple range test.

Results

Preliminary Experiments. Electrophysiological measurements of reduced membrane of oocytes that were injected with sheep omasal epithelium poly(A)$^+$ RNA indicated that the omasal epithelium of sheep may possess mRNA that encoded for proteins capable of stimulating carnosine absorption (Appendix B). As the initial step to identifying sucrose gradient-fractionated poly(A)$^+$ RNA (RNA) capable of encoding for proteins responsible for mediated Gly-Sar transport, the ability of

oocytes injected with 75 ng of RNA (50 to 200 nL) to absorb Gly-Sar from pH 6.0 uptake buffer (.1 m*M* Gly-Sar) was compared to that of oocytes injected with water (150 nL; Figure 6.1). A RNA injection treatment effect was observed ($P < .03$). Fractions F34 and F40 absorbed 33 and 41%, respectively, more ($P < .03$) Gly-Sar than did oocytes injected with water (508 fmol/oocyte·40 min-1).

Together, these preliminary experiments suggested that the enhanced ability of RNA-injected oocytes to absorb carnosine and Gly-Sar may have been due to the translation and expression, and subsequent functioning of a protein(s) capable of stimulating dipeptide absorption, which had been encoded for by mRNA isolated from sheep omasal epithelium.

Demonstration of mRNA-Inducible, Time-Dependent Uptake. A time-course trial was conducted to determine the optimal length of time to measure the uptake of Gly-Sar. The absorption of Gly-Sar from pH 5.5 media by oocytes injected with water or poly(A)$^+$ RNA (2.9 ng/nL) increased linearly ($P < .001$) over the course of 1 h (Figure 6.2). A treatment x time interaction ($P < .02$) was observed reflecting that oocytes injected with RNA displayed a greater ($P < .001$) rate of Gly-Sar absorption than did water-injected oocytes. RNA-injected oocytes absorbed approximately 2.0, 2.1, 1.4, and 3.1 times more Gly-Sar than control oocytes at 15, 30, 45, and 60 min samplings. Based on the overall linearity of these results, subsequent characterization of endogenous and RNA-inducible uptake of Gly-Sar by oocytes was evaluated using a 60-min uptake period.

To determine the optimal post-injection day of Gly-Sar uptake, and to demonstrate that induced Gly-Sar absorption was dependent on enough time for expression of sheep omasal mRNA, oocytes were assayed for their ability to absorb Gly-Sar (.4 m*M*) on days 1, 2, 3, and 4 after injection of 100 nL water or RNA (2.4

ng/nL; Figure 6.3). Gly-Sar absorption by water-injected oocytes did not increase by day. In contrast, a day x treatment interaction ($P < .02$) was observed, reflecting the 1.5 to 3.0 times greater ($P < .001$) uptake (fmols/oocyte·60 min^{-1}) of Gly-Sar by oocytes injected with RNA on d 3 (1,097 +/- 30) and 4 (2,270 +/- 398), as compared to water-injected oocytes (751 +/- 44 and 752 +/- 49, respectively). These results indicated that a period of 3 to 4 d was necessary for sheep omasal mRNA injections to induce increased Gly-Sar absorption capabilities in oocytes.

Determination of pH-dependent, mRNA-Induced Gly-Sar Uptake. The potential for Gly-Sar uptake by oocytes injected with sheep omasal epithelium mRNA to be dependent on an extracellular-to-intracellular proton gradient was investigated by observing the effect of increasing levels of pH on .4 m*M* Gly-Sar uptake by oocytes injected with 100 nL of poly$(A)^+$ RNA (.97 to 1.2 ng/nL) or water (Figure 6.4). More ($P < .001$) Gly-Sar was absorbed (fmols/oocyte·40 min^{-1}) by RNA-injected (RNA) oocytes than by oocytes injected with water. A pH x treatment interaction ($P < .04$) was observed and reflected the reduced ability of media pH concentrations greater than 6.0 to stimulate the uptake of Gly-Sar by RNA-injected oocytes. In contrast, increasing pH concentrations linearly ($P < .001$) stimulated Gly-Sar uptake by oocytes injected with water.

The effect of pH on endogenous (water) and induced (the difference between individual RNA and averaged Water values) uptake of Gly-Sar also differed ($P < .001$). The quantity of induced Gly-Sar absorption at pH 5.0 (120 +/- 25.4), 5.5 (91.9 +/- 32.2), 6.0 (127 +/- 51.0), and 6.5 (64.8 +/- 25.9), was greater ($P < .05$) than at pH 7.5 (-1.30 +/- 22.2). Thus, Gly-Sar absorption induced by the presence of omasal mRNA increased when a pH gradient of at least 1 log existed between uptake media and oocyte cytosol. In contrast, endogenous absorption of Gly-Sar increased ($P < .05$)

as the extracellular-to-intracellular proton gradient was removed in half-log steps from pH 5.5 to 7.5 (pH 5.0, 102 +/- 7.4; pH 5.5, 144 +/- 11.4; pH 6.0, 297 +/- 36.6; pH 6.5, 389 +/- 31.7; pH 7.5, 485 +/- 27.3). Because maximal induced Gly-Sar absorption occurred at pH levels 5.0 to 6.0, and in order to minimize the contribution of endogenous absorption, all other uptake studies were conducted at pH 5.5.

Determination of mRNA-Induced K_t. In order to determine whether the enhanced ability of RNA-injected oocytes to absorb Gly-Sar was the result of expressing proteins that mediated absorption, the uptake of Gly-Sar (Figure 6.5) from pH 5.5 media containing .1, .4, .8, 1.2, or 6.4 m*M* concentrations of Gly-Sar by water- and RNA-injected oocytes was studied. Uptake of Gly-Sar was greatest ($P < .001$) by RNA-injected oocytes (1.0 to 1.1 ng/nL). Gly-Sar uptake by RNA-injected oocytes increased in a curvelinear ($P < .001$) manner, whereas the uptake of Gly-Sar by water-injected oocytes increased linearly ($P < .001$) as substrate media concentrations were increased. Induced absorption of Gly-Sar displayed Michaelis-Menton (saturable) kinetics, allowing an affinity constant (K_t) of .40 +/- .23 m*M* to be calculated.

Characterization of mRNA-Induced Uptake. To further characterize the inducible mediated Gly-Sar transport processes, the effect of co-incubating 5 m*M* levels of carnosine, methionylglycine, glycylleucine, or glycine with .1 m*M* Gly-Sar levels on Gly-Sar uptake (fmols/oocyte·60 min^{-1}) in RNA- and water-injected oocytes was evaluated (Figure 6.6). Gly-Sar uptake at .1 m*M* in water-injected oocytes (300 +/- 30.9; Endogenous uptake) was reduced ($P < .05$) 62.3% by the presence of methionylglycine and 59.0% by the presence of glycylleucine. In contrast, 5 m*M* concentrations of carnosine and glycine had no effect on endogenous Gly-Sar uptake.

The absorption of Gly-Sar (496 +/- 73.3) by oocytes injected with RNA (.9 to 2.0 ng/nL) at .1 m*M* Gly-Sar (Figure 6.6) was 1.7 times greater ($P < .007$) than by water-injected oocytes. Induced Gly-Sar absorption was reduced ($P < .05$) 44% by carnosine, 94% by methionylglycine, 91% by glycylleucine, but not by glycine, thus demonstrating an apparently greater inhibition than endogenous absorption mechanisms by the competitor dipeptide substrates.

These co-incubation experiments demonstrate that both endogenous and induced Gly-Sar uptake were cis-inhibited by methionylglycine and glycylleucine, but not by the free amino acid glycine. In contrast, only induced Gly-Sar uptake was inhibited by co-incubation with carnosine.

mRNA-Oligomer Depletion of Induced Uptake. To test whether sheep omasal mRNA species share homology with mRNA species from rabbit small intestinal epithelium that encode for H^+/peptide cotransport activity, oocytes were injected with 110 *u*L of a mixture of RNA (fractions F34, F35, and F36; 2.5 ng/nL), or RNA plus degenerate DNA oligomers (Antisense; .25 ng/nL) that are complementary to the sequence flanking the initiation site of the cloned rabbit intestinal H^+/peptide cotransport protein (PepT1; Fei et al., 1994). DNA oligomers (Sense; .25 ng/nL), identical to the sense strand of PepT1 mRNA, or water (water) were injected (110 *u*L) as DNA and volume injection controls, respectively.

The absorption of Gly-Sar (fmols/oocyte·60 min^{-1}) was compared between and within injection treatments (Figure 6.7). For oocytes injected with RNA plus water, the quantity of induced uptake of Gly-Sar was 2.0 times (1,240 +/- 150) greater ($P < .001$) than that observed by oocytes injected with only water (610 +/- 33.4; No RNA). Within injection treatments, for oocytes not injected with RNA, the Gly-Sar uptake did not differ ($P < .05$) in oocytes injected with additional water or antisense

control DNA). Likewise, induced uptake of Gly-Sar by oocytes co-injected with mRNA and water or sense DNA oligomers did not differ ($P < .05$). In contrast, uptake of Gly-Sar by oocytes injected with antisense DNA and RNA (462 +/- 26.2) did not display any induced Gly-Sar absorption ability.

Discussion

This research was conducted to determine whether mRNAs that encode for proteins capable of small peptide transport could be identified in the forestomach epithelia of sheep. The potential for peptide-bound amino acid absorption by these tissues has been identified (DiRienzo and Webb, 1995). mRNA was isolated from omasal, rather than ruminal epithelium, because of the relatively greater in vitro ability of omasal epithelial tissue to absorb dipeptides (Matthews and Webb, 1995). The results show that mRNA that encode for proteins capable of inducing mediated Gly-Sar uptake have been isolated from the scraped omasal epithelium of mature sheep. Therefore, the results from this study indicate that sheep possess and express the gene(s) for a protein(s) capable of mediating dipeptide transport.

[^{14}C]Glycyl-L-sarcosine (Gly-Sar) was used as a representative dipeptide substrate because of its proven role as a model substrate in the identification of peptide transport activity in epithelial tissue (Matthews, 1983), brush border membrane vesicles (Ganapathy et al., 1984), and cells (Brandsch et al., 1994), and because of its well characterized absorption by the cloned mammalian H^+/peptide cotransport protein (Fei et al., 1994). To demonstrate that the representitive ^{14}C radiolabel for [^{14}C]-Gly-Sar absorption was not being absorbed in the form of

unbound [^{14}C]-glycine, the ability of oocytes to absorb Gly-Sar in the presence of 5 m*M* glycine was evaluated (Figure 6.6). That a 50-fold greater concentration of glycine failed to affect the quantity of Gly-Sar absorbed by oocytes injected with water or RNA indicates that the representative ^{14}C radiolabel was absorbed as [^{14}C]Gly-Sar.

Xenopus laevis oocytes were selected as the expression system and the experimental model for characterizing mediated absorption because of their reliable translation of mammalian mRNA transcripts, processing and targeting of proteins, and proven flexibility of identifying and characterizing cell membrane permeases (Hediger et al., 1987; Tarnuzzer et al., 1990; Miyamoto et al., 1991). The endogenous ability of defolliculated *Xenopus laevis* oocytes to absorb Gly-Sar has been reported (Miyamoto et al., 1991), but was not characterized. Oocytes that were injected with water appeared to both lack and possess endogenous mediated peptide transport activity. The data displayed in Figure 6.5 suggest that endogenous Gly-Sar absorption was not saturable from .1 to 6.4 m*M* concentrations, and indicates that Gly-Sar uptake was not mediated. In contrast, that methionylglycine and glycylleucine (Figure 6.6), but not carnosine, competitively inhibited Gly-Sar uptake suggests that these peptide substrates were recognized by a common carrier protein.

The time requirement of 3 to 4 d for the expression of mRNA-induced Gly-Sar transport activity (Figure 6.3) is consistent with that observed to be required for oocyte translation, processing, and insertion into plasma membranes of mammalian peptide permeases by defolliculated *Xenopus laevis* oocytes after injection with rabbit (Miyamoto et al., 1991) and human (Saito et al., 1993) intestinal mRNA species. In these studies, induced peptide transporter activity was increased in the presence of an extracellular-to-intracellular proton gradient. Therefore, these studies appeared to

have identified mRNA species that may encode for the proteins responsible for the reputed concentrative H^+/peptide symport of small peptides across mammalian epithelial membranes (Ganapathy and Leibach, 1983; Said et al., 1988; Daniel et al., 1991).

Cloned mammalian proteins reputed to induce H^+/peptide cotransporter activity in *Xenopus laevis* oocytes have also displayed the functional feature of mediating Gly-Sar (Fei et al., 1994) and antibiotic (Boll et al., 1994) absorption in a H^+-dependent manner. In the present study a similar result was observed (Figure 6.4), with the presence of a proton gradient of at least one-half-log in magnitude being necessary to stimulate induced Gly-Sar uptake. This dependence on H^+ for Gly-Sar uptake was not observed for endogenous oocyte peptide absorption activity. Because Gly-Sar is zwitterionic at pH 6.5 (Daniel et al., 1994), increasing the H^+ concentration of the media should result in a relatively more positively charged molecule. Thus, in media of pH 6.0, 5.5, and 5.0, Gly-Sar would have been increasingly more positively charged, and at pH 7.5, more negatively charged, relative to pH 6.5. Hence, it appears that induced transport activity was enhanced by a charged substrate and(or) is able to utilize free H^+ as an ionic driving force for Gly-Sar uptake. In contrast, endogenous uptake is clearly inhibited by these factors.

Induced uptake of Gly-Sar was saturable, but endogenous absorption was not (Figure 6.5). The affinity .40 +/- .23 m*M* constant for induced Gly-Sar absorption at pH 5.5 is lower than the value of 1.9 m*M* reported for Gly-Sar by oocytes injected with the cRNA for PepT1, as determined by electrophysiological measurement of induced transmembrane currents at pH 5.5 (Fei et al., 1994). If this difference is not due to experimental technique error, then the rabbit and sheep may possess functional isoforms of the same protein.

The ability of carnosine, methionylglycine, and glycylleucine to inhibit induced Gly-Sar uptake (Figures 6.6) indicates that these dipeptides competitively bound and may have been transported by the same transport protein(s). Carnosine and glycylleucine also have been identified as substrates of the cloned H^+/peptide cotransport protein (PepT1; Fei et al., 1994). For methionylglycine and glycylleucine, the magnitude of this inhibitory effect was less for water-injected oocytes and may indicate a difference between the function and structure of proteins capable of stimulating peptide absorption in Xenopus germ cells and ovine epithelial cells.

Combined, the data in Figures 6.4, 6.5, and 6.6 clearly show that the absorption of Gly-Sar by oocytes injected with sheep omasal epithelium mRNA confers to Xenopus oocytes an enhanced and characteristically different ability to absorb Gly-Sar. Induced Gly-Sar uptake was maximal in the presence of a 1 log magnitude proton gradient (Figure 6.4), was mediated by a saturable process (Figure 6.5), and was cis-inhibited by the presence of several dipeptides (Figure 6.6). In contrast, endogenous oocyte absorption of Gly-Sar was progressively inhibited as a proton gradient was established, was not saturable from .1 to 6.4 m*M* Gly-Sar concentrations, and was not inhibited by the presence of carnosine. Therefore, the observed induced Gly-Sar uptake activity appeared to be the result of oocytes expressing proteins encoded by mRNA species that were isolated from sheep omasal epithelium.

Figure 6.7 shows that the effect of incubating omasal poly(A)$^+$ RNA prior to injection results in the complete inhibition of induced Gly-Sar capacity. These observations demonstrate that the increased Gly-Sar uptake activity, induced by the injection of sheep omasal epithelium mRNA, was inhibited when exposed to DNA oligomers that were complementary to regions flanking the initiation of translation

site of PepT1. As such, this experiment indicates that the mRNA isolated from sheep omasal epithelial cells shares at least the initiation of translation site homolous to the PepT1 protein, which has been identified as a H^+/peptide symporter in rabbit and human intestinal tissue, and which shares approximately 70% primary structure homology to the recently cloned human renal H^+/peptide cotransport protein, PepT2 (Liu et al., 1995).

That induced Gly-Sar uptake was inhibited indicates that mRNA encoding for H^+/peptide cotransporter-like activity were enriched in fractions F34, F35, and F36. Fractions F37, F38, F39, and F40, which also demonstrated the ability to induce Gly-Sar uptake in these studies, were not tested for mRNA sequence homology to the cloned rabbit H^+/peptide cotransport protein. The poly(A)$^+$ RNA were isolated from epithelial tissue that had been scraped from omasal laminae. Because the omasal forestomach epithelium is composed of four strata of different cell types (Steven and Marshall, 1970; Figure 2.1), neither the cell nor cell membrane location of potential peptide transport activity can be determined from this study. We are aware of only one study that has investigated and that has identified forestomach epithelial tissue as being capable of small peptide absorption (Matthews and Webb, 1995). Based upon the observation that the absorption of carnosine (6.0 to 96.0 m*M*) and methionylglycine (.375 to 22.0 m*M*) was not saturable, this study concluded that absorption of dipeptides across ruminal and omasal tissue sheets was by non-mediated absorption processes. In hindsight, based on the determination of a K_t constant of .4 m*M* for Gly-Sar by the current study, attempts to identify mediated uptake of carnosine and methionylglycine by demonstration of saturable absorption may have been confounded by the use of substrate concentration levels that probably were in excess of mediated transport capacity.

In summary, we have demonstrated the ability to extract, enrich, and express viable mRNA species from sheep omasal epithelium in *Xenopus laevis* oocytes. Characterization of the function of induced transporter activity indicates that mRNA encoding for a protein capable of mediating the transport of Gly-Sar, and of recognizing other dipeptides, is expressed by the omasal epithelium of mature, grass-fed sheep.

Implications

The observations of this study indicate that the omasal epithelium possesses mRNA that encode for mediated dipeptide transport. Therefore, these results describe the identification and functional characterization of a biological mechanism that may act to absorb small peptides from the forestomach liquor of ruminants. If so, then an important, alternative route for the supplementation of amino acids may have been identified.

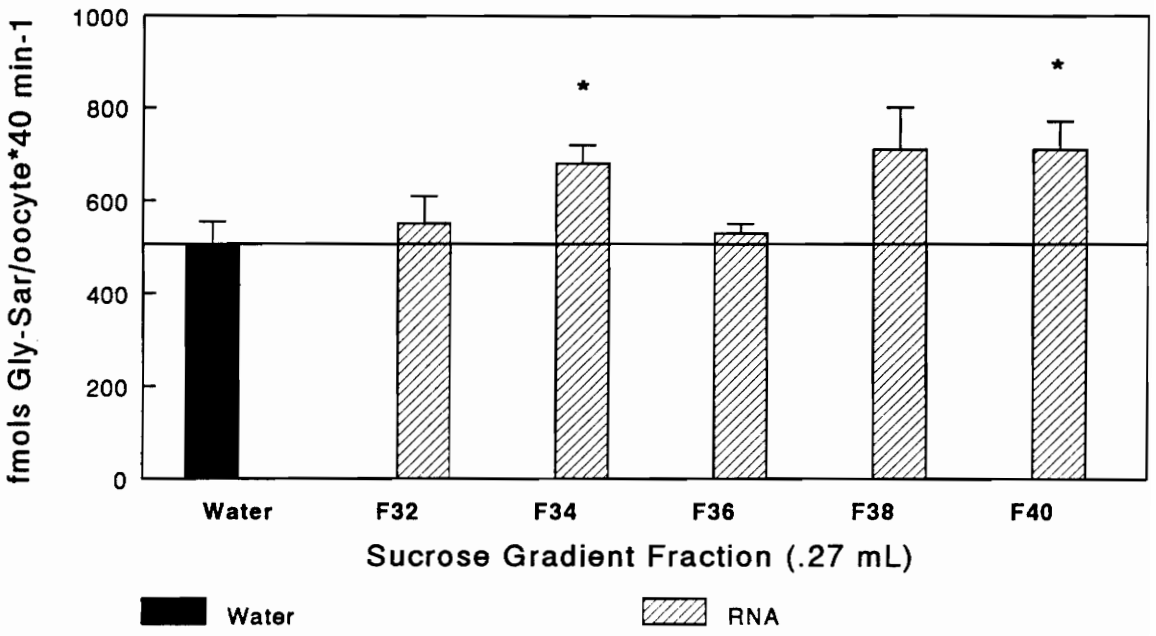


Figure 6.1. Absorption of glycyl-L-sarcosine (Gly-Sar) by defolliculated Xenopus oocytes injected with water or sequential sucrose gradient-fractionated sheep omasal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for Gly-Sar absorption in pH 6.0 media (.1 m*M* Gly-Sar). Individual histogram bar values represent group (n = 1) absorption means +/- SE of oocytes injected with water (n = 5) or RNA (n = 5 to 8).

Injection treatments differed ($P < .03$).

*Different from water treatment ($P < .03$).

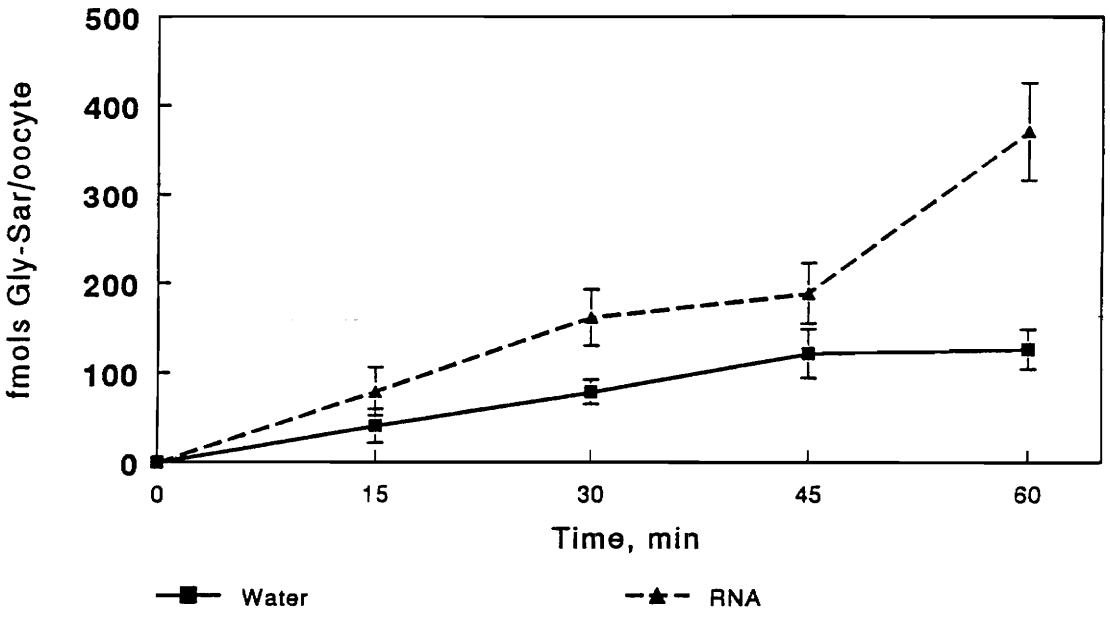


Figure 6.2. Time course for glycyl-L-sarcosine (Gly-Sar) absorption by defolliculated Xenopus oocytes injected with water or size-fractionated sheep omasal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for Gly-Sar absorption in pH 5.5 media (.05 m*M* Gly-Sar). Each time period measurement represents group (n = 1) absorption means +/- SE of oocytes injected with water (n = 5) or RNA (n = 4 to 7).

Linear time effect ($P < .001$).

Time x treatment effect ($P < .016$).

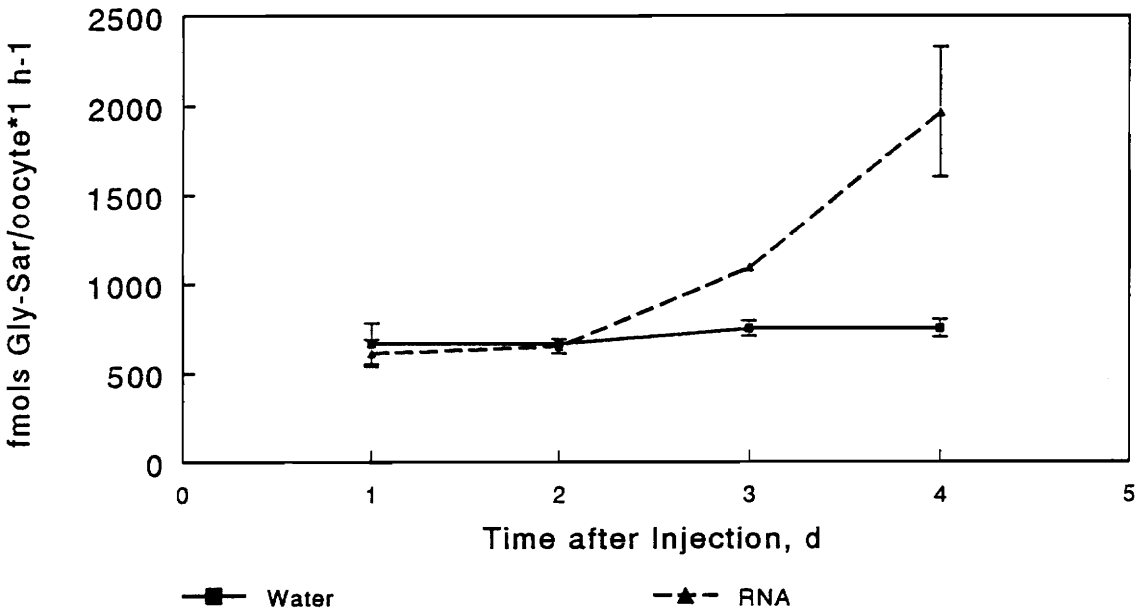


Figure 6.3. Time-dependent expression of glycyl-L-sarcosine (Gly-Sar) absorption activity by defolliculated Xenopus oocytes injected with water or size-fractionated sheep omasal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for Gly-Sar absorption in pH 5.5 media (.4 m*M* Gly-Sar) 1, 2, 3, or 4 d after injection. Each time period measurement represents group (n = 1) absorption means +/- SE of oocytes injected with water (n = 3 to 5) or RNA (n = 3 to 7).

Day x Treatment effect ($P < .001$) on oocytes injected with RNA.

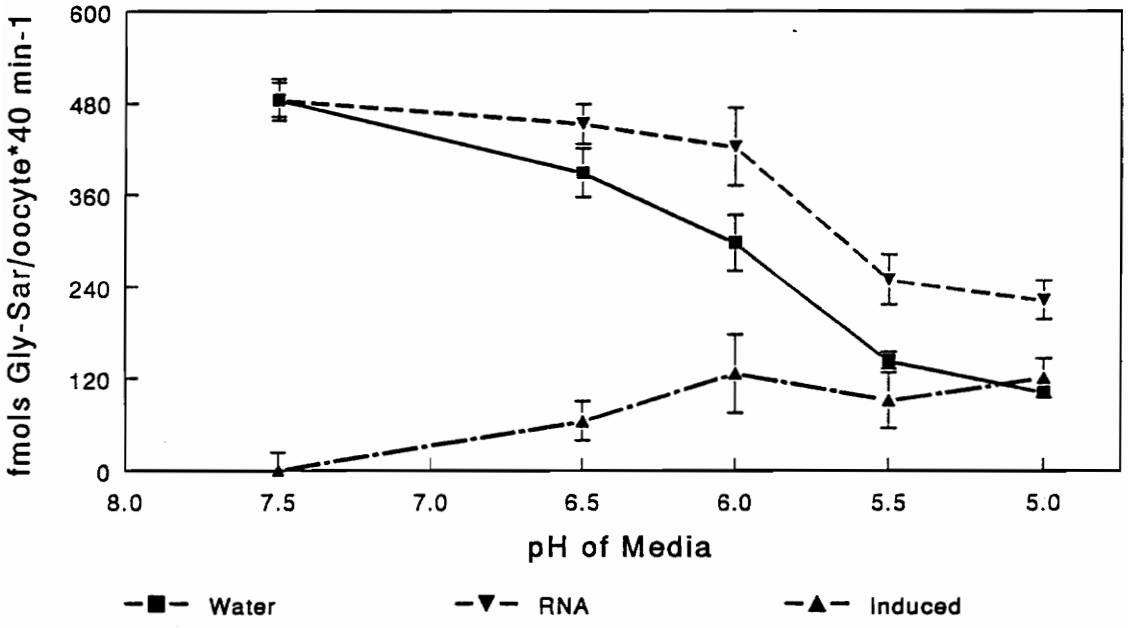


Figure 6.4. pH-dependent glycyl-L-sarcosine (Gly-Sar) absorption by defolliculated Xenopus oocytes injected with water or size-fractionated sheep omasal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for Gly-Sar absorption in media (.1 m*M*) of varying pH. Individual histogram values represent across group (n = 3) absorption means +/- SE of oocytes injected with water (n = 9 to 14) or RNA (n = 9 to 13).

Uptake of Gly-Sar was greater ($P < .001$) by oocytes injected with RNA than for oocytes injected with water.

pH x treatment interaction ($P < .04$).

Linear ($P < .001$) pH effect on water-injected and induced oocyte uptake.

Quadratic ($P < .01$) pH effect on RNA-injected oocyte uptake.

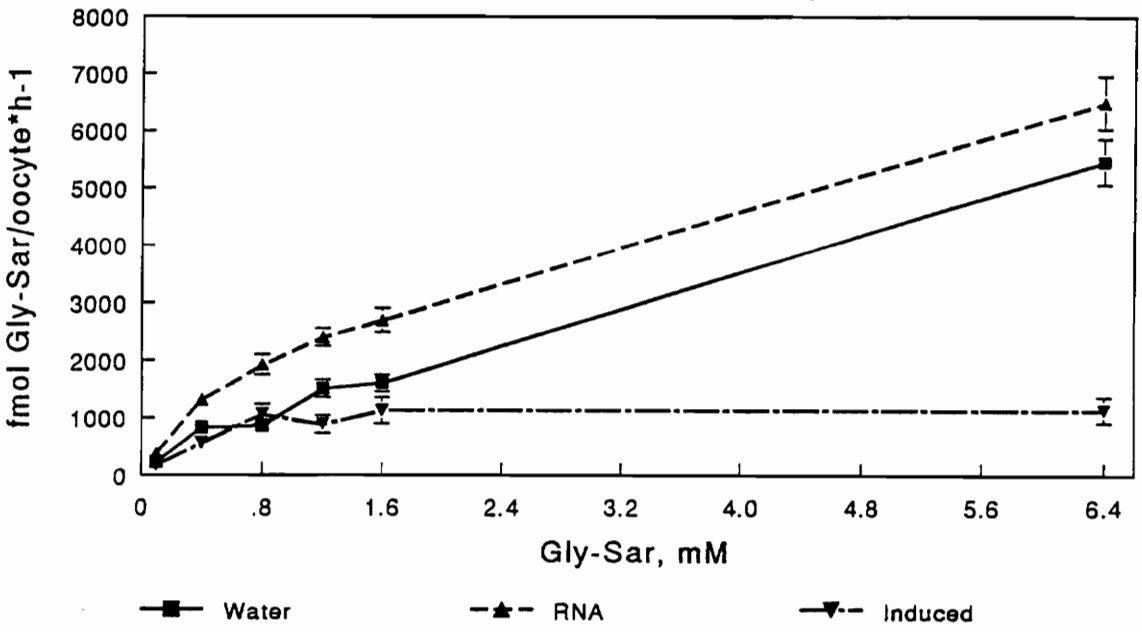


Figure 6.5. K_t characterization (.40 +/- .23 m*M*) for induced glycyl-L-sarcosine (Gly-Sar) absorption by defolliculated Xenopus oocytes injected with water or size-fractionated sheep omasal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for Gly-Sar absorption in pH 5.5 media. Each concentration measurement represents across group (n = 2) absorption means +/- SE of oocytes injected with water (n = 9 to 13) or RNA (n = 10 to 13).

Uptake of Gly-Sar was greater ($P < .001$) by oocytes injected with RNA than for oocytes injected with water.

Linear ($P < .001$) concentration effect on water-injected oocyte uptake.

Quadratic ($P < .001$) concentration effect on induced uptake.

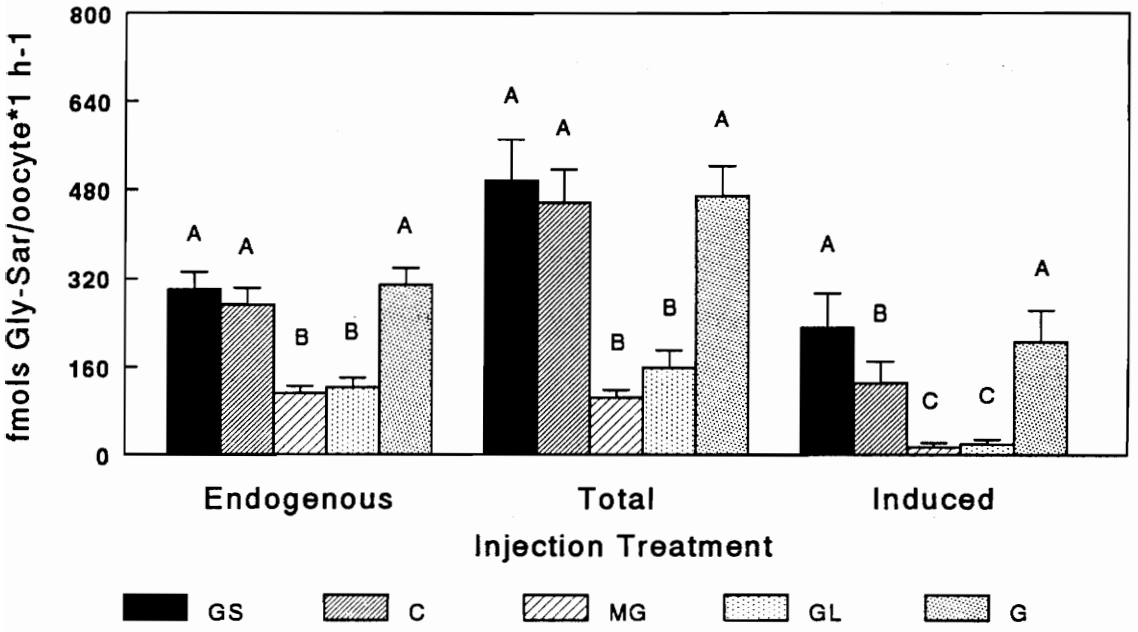


Figure 6.6. Competitive absorption of glycyl-L-sarcosine (Gly-Sar) by defolliculated Xenopus oocytes injected with water (endogenous) or size-fractionated sheep omasal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed for Gly-Sar absorption in pH 5.5 media containing .1 m*M* Gly-Sar (GS) or .1 m*M* GS plus 5 m*M* L-carnosine (C), L-methionylglycine (MG), glycyl-L-leucine (GL), or L-glycine (G). Individual histogram bar values represent across group (n = 3) absorption means +/- SE of oocytes injected with water (n = 7 to 12) or RNA (n = 7 to 10).

At .1 m*M*, uptake of Gly-Sar by oocytes injected with RNA (n = 10) was greater ($P < .007$) than for water-injected oocytes (n = 12).

Within treatments, bars lacking a common letter differ ($P < .05$).

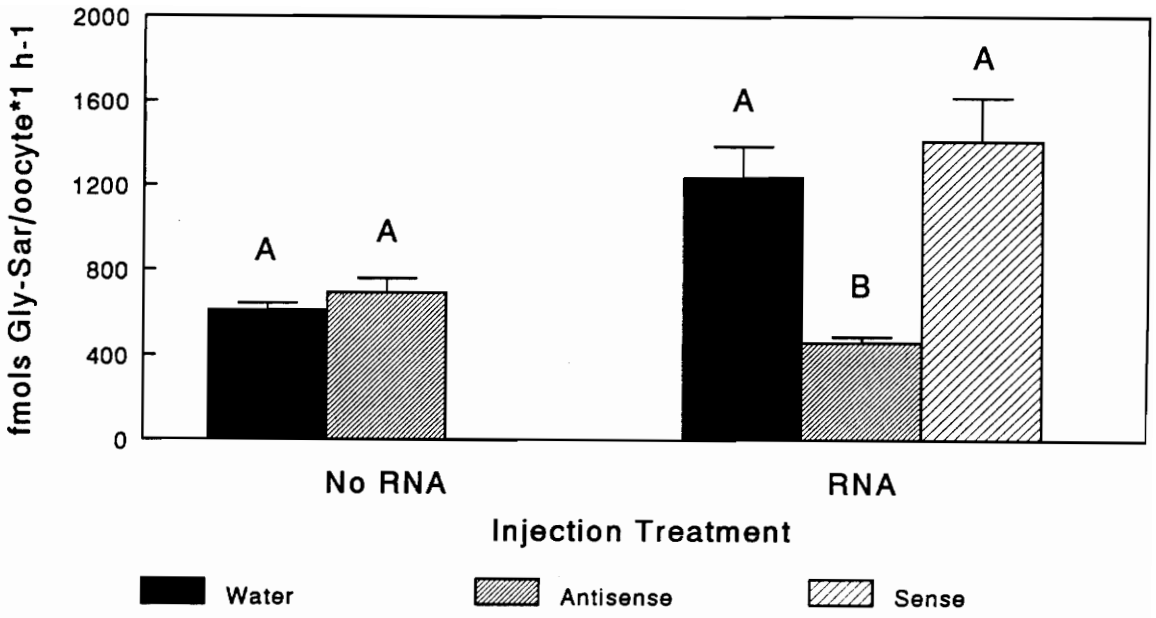


Figure 6.7. Oligomer depletion of induced glycyl-L-sarcosine (Gly-Sar) absorption by defolliculated Xenopus oocytes injected with water or Antisense DNA, or with size-fractionated sheep omasal epithelium poly(A)$^+$ RNA plus water, Antisense DNA, or Sense DNA.

Oocytes were assayed for Gly-Sar absorption in pH 5.5 media (.4 m*M* Gly-Sar). Individual histogram bars represent group (n = 1) absorption means +/- SE of oocytes injected with water (No RNA; n = 5 to 10), or injected with RNA (n = 6 to 9).

Between water injection treatments, uptake of Gly-Sar by oocytes injected with RNA (n = 9) was greater ($P < .001$) than by oocytes not injected with RNA (n = 10).

Within treatments, bars lacking a common letter differ ($P < .05$).

CHAPTER VII

EPILOGUE

The identification of omasal peptide (Chapter VI, Appendix B) and amino acid (Appendix A) transporters, by expression of their mRNA in *Xenopus laevis* oocytes, is the primary accomplishment of this research. Combined with the data in Chapter IV, these results indicate that forestomach epithelia should possess the potential to absorb free and peptide-bound amino acids, by both mediated and non-mediated processes. Accordingly, forestomach epithelia (at least the omasum) should possess the ability to absorb both peptide-bound and free amino acid N when forestomach liquor concentrations are high and low.

A comparison of Tables 2.5 and 2.6 reveals that a greater driving force for peptide-bound than free amino acid absorption commonly exists in ruminal liquor, before and after feeding. In order to evaluate the potential effect of this differential driving force for free and peptide-bound amino acid absorption across epithelial tissues, these literature values were used as the independent concentration variable (c) in the equation $Y = a + b * c$ to generate a range of values (mg N absorbed/mg dry tissue) that represent the potential absorption of amino acid and peptide N from forestomach liquors (Table 7.1) The Y intercept (a) and slope (b) coefficients were determined by regression analysis of the effect of mucosal chamber substrate concentration on total absorption of substrate, as determined in the methionine and Met-Gly studies (Chapter IV). A linear equation was used because it essentially describes the dependence of the total substrate absorption on initial substrate concentration.

Table 7.1 Relative potential for the absorption of peptide-bound and free amino acid across forestomach epithelia

Form[a]	Tissue	Reported[b]	Y intercept[c]	Slope[c]	Absorption[d]
			Pre-feeding[e]		
Peptide		mg N/dl			mg N/mg dry tissue
	Rumen	1.5 to 6.0	.86	.219	3.4 to 10.7
	Omasum	1.5 to 6.0	1.94	.201	11.1 to 38.2
Free					
	Rumen	.12 to 1.5	.85	.254	2.4 to 3.4
	Omasum	.12 to 1.5	2.03	.269	7.8 to 11.1
					Ratio
Peptide:Free					
	Rumen				.4 to 3.1
	Omasum				1.4 to 3.4
			Post-Feeding[f]		
Peptide		mg N/dl			mg N/mg dry tissue
	Rumen	10 to 27	.86	.219	21.1 to 869
	Omasum	10 to 27	1.94	.201	52.0 to 1595
Free					
	Rumen	.72 to 6.0	.85	.254	2.8 to 8.8
	Omasum	.72 to 6.0	2.03	.269	8.0 to 23.2
					Ratio
Peptide:Free					
	Rumen				7.5 to 98.8
	Omasum				6.5 to 68.8

[a]Peptide-bound (peptide) or free (free) amino acid-N.

[b]Reported strained ruminal fluid concentrations of free and peptide-bound amino acid-N (see Tables 4.1 and 4.2). The same concentrations were used for ruminal and omasal tissues because of the lack of reported values for strained omasal fluid.

[c]Coefficients were generated from the fitted linear regression equation, $Y = a + (b \cdot c)$, where Y equals the natural log values (nanomol/mg dry tissue$\cdot h^{-1}$) observed for the mean (n = 7) sum of methionine or Met-Gly appearance in serosal chamber buffers of parabiotic chambers, and where the slope (c) equals the initial mucosal chamber substrate concentrations (.375, .75, 1.5, 3.0, 6.0, or 12.0 mM).

[d]Potential uptake values (nanomol/mg dry tissue$\cdot h^{-1}$) were calculated from the linear equation $Y = a + (b \cdot c)$. Values (e^Y) were generated using reported concentrations of free and peptide-bound amino acid-N in strained ruminal fluid as the slope coefficient (c), and where a and b are the experimentally generated Y intercept and slope constants, respectively.

[e]Values are the reported concentrations of free and peptide-bound amino acid-N in strained ruminal fluid prior to feeding (see Tables 1 and 2).

[f]Values are the reported concentrations of free and peptide-bound amino acid-N in strained ruminal fluid 1 to 3 h after feeding (see Tables 1 and 2).

The results of these calculations (Table 7.1) indicate that, at pre-feeding forestomach liquor concentrations, the relative absorption of N in the form of peptide-bound to free amino acids would be similar for both tissues, with .4 to 3.4 times more peptide-bound amino acid N being absorbed. However, post-feeding, the relatively greater peptide AA N absorption would increase, ranging from 6.5 to 98 times the absorption of free AA.

The use of ruminal liquor amino acid N concentration values for omasal liquor concentrations was necessary because of the lack of reported amino acid and peptide N values for omasal liquor. As a consequence, the ruminal to omasal substrate absorption ratios in Table 7.1 may overestimate the proportional in vivo contribution of omasal tissue to total sheep forestomach absorption of these substrates because the omasum will receive most of the ruminal liquor after potential ruminoreticular absorption of substrates has occurred. However, the absorption of water by omasal tissues would presumably result in the concentration of rumen liquor solutes, thereby potentially reestablishing (or generating greater) omasal liquor-to-blood concentration gradients and solvent-drag forces that were present across ruminal epithelia, depending on the relative water and substrate absorption rates (Smith, 1984).

The identification of omasal peptide and amino acid transporters by expression of their mRNA in Xenopus laevis oocytes does not allow the site of their function in the multi-layered forestomach cell strata to be determined. If these proteins are functionally expressed in granulosa cells (where the barrier to solute passage is thought to be established), then the relative proportion of transport at the lower concentrations may be greater than that predicted in Table 7.1. Also, it is not known whether similar transport proteins exist in rumen epithelial tissue. In future studies, the site of mRNA expression and functional translation can, theoretically, be

demonstrated within and between forestomach epithelia by the use of in situ hybridization and antibody-binding techniques (respectively).

Because the present research has demonstrated the specific isolation of mRNA that are responsible for inducing mediated transport in certain sucrose-gradient fractions, the future cloning of the proteins responsible for the uptake of peptide-bound and free amino acids has been facilitated. This is especially true for the sheep forestomach peptide and the pig jejunal $b^{o,+}$ transport proteins because of the demonstration that they share some homology to the cloned rabbit intestinal H+/peptide cotransport protein (PepT1) and human renal $b^{o,+}$ transporter (hrBAT).

The identification that mRNA exist in the pig jejunal epithelium that encode for "$b^{o,+}$-like" cationic and neutral amino acid transport activity should facilitate the eventual cloning of this gene in pigs. As a consequence, studies designed to elucidate how this gene is regulated may lead to the ability to control the functional activity of this transporter, and allow the functional activity of the transporter to be coordinated with that of available substrate levels. The application of such research would be especially appropriate for breeds of pigs known to possess different maintenance energy costs and to require different amino acid profiles for optimal muscle accretion.

In summary, this research has identified and characterized the potential for non-mediated methionine and methionylglycine, and mediated glycylsarcosine transport in the sheep omasal epithelium, and the potential for mediated cationic and neutral amino acid transport in the sheep omasal and pig jejunal epithelia.

Literature Cited

Albritton, L. M., L. Tseng, D. Scadden, and J. M. Cunningham. 1989. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell 57:659-666.

Ames, G. F.-L., C. S. Mimura, and V. Shyamala. 1990. Bacterial periplasmic permeases belong to a family of transport proteins operating from Escherichia to human: traffic ATPases. FEMS Microbiol. Rev. 75:429-446.

Annison, E. F. 1956. Nitrogen metabolism in the sheep. Protein digestion in the rumen. Biochemistry 64:705-714.

Arriza, J. L., M. P. Kavanaugh, W. A. Fairman, Y.-N. Wu, G. H. Murdoch, R. A. North, and S. G. Amara. 1993. Cloning and expression of a human neutral amino acid transporter with a structural similarity to the glutamate transporter gene family. J. Biol. Chem. 268:15329-15332.

Atherton, E., and R. C. Sheppard. 1989. Solid Peptide Synthesis. A Practical Approach. IRL Press, Oxford.

Atisook, K., and J. L. Madara. 1991. An oligopeptide permeates intestinal tight junctions at glucose-elicited dilations. Implications for oligopeptide absorption. Gastroenterology 100:719-724.

Barber, E. F., M. E. Handlogten, and M. S. Kilberg. 1983. Induction of amino acid transport system A in rat hepatocytes is blocked by tunicamycin. J. Biol. Chem. 19:11851-11855.

Bell, G. I., T. Kayano, J. B. Buse, C. F. Burant, J. Takeda, D. Lin, H. Fukumoto, and S. Seino. 1990. Molecular biology of mammalian glucose transporters. Diabetes Care 13:198-208.

Bertran, J., A. Werner, M. L. Moore, G. Stange, D. Markovich, J. Biber, X. Testar, A. Zorzano, M. Palacin, and H. Murer. 1992. Expression cloning of a cDNA from rabbit kidney cortex that induces a single transport system for cystine and dibasic and neutral amino acids. Proc. Natl. Acad. Sci. USA 89:5601-5605.

Bertran, J., A. Werner, J. Chillaron, V. Nunes, J. Biber, X. Testar, A. Zorzano, X. Estivill, H. Murer, and M. Palacin. 1993. Expression cloning of a human renal cDNA that induces high affinity transport of L-cystine shared with dibasic amino acids in Xenopus oocytes. J. Biol. Chem. 268:14842-14849.

Bird, P. R., and R. J. Moir. 1972. Sulpher metabolism and excretion studies in ruminants. VIII. Methionine degradation and utilization in sheep when infused into the rumen or abomasum. Aust. J. Sci. 25:835-848.

Bishop, L., R. Agbayani, Jr., S.V. Ambudkar, P. C. Maloney, and G. F.-L. Ames. 1989. Reconstruction of a bacterial periplasmic permease in proteoliposomes and demonstration of ATP hydrolysis concomitant with transport. Proc. Natl. Acad. Sci. USA 86:6953-6957.

Blond, D. M., and R. Whittam. 1964. The regulation of kidney respiration by sodium and potassium ions. Biochem. J. 92:158-167.

Boll, M., D. Markovich, W. M. Weber, H. Daniel, H. Muer. 1994. Cloning of a cDNA from rabbit small intestine related to the transporter for peptides and beta-lactam antibiotics. Pflugers Arch.-Eur. J. Physiol. 429:146-149.

Boerner, P., and M. H. Saier, Jr. 1985. Adaptive regulatory control of system A transport activity in a kidney epithelial cell line (MDCK) and in a transformed variant (MDCK-T). J. Cell Physiol. 122:308-315.

Bourney, R. E., L. Koranyi, D. E. James, M. Mueckler, and M. A. Permutt. 1990. Effects of altered glucose homeostasis on glucose expression in skeletal muscle of the rat. J. Clin. Invest. 86:542-547.

Boyd, A. R., and M. R. Ward. 1982. A micro-electrode study of oligopeptide absorption by the small intestinal epithelium of *Necturus maculosus*. J. Physiol. 324:411-428.

Bracy, D. S., M. E. Handlogten, E. F. Barber, H.-P. Han, and M. S. Kilberg. 1986. Cis-inhibition, trans-inhibition, and repression of hepatic amino acid transport mediated by system A. Substrate specificity and other properties. J. Biol. Chem. 261:1514-1520.

Brandsch, M., Y. Miyamoto, V. Ganapathy, and F. H. Leibach. 1994. Expression and protein kinase C-dependent regulation of peptide/H^+ co-transport system in the caco-2 human colon carcinoma cell line. Biochem. J. 299:253-260.

Broderick, G., D. B. Ricker, and W. M. Craig. 1990. Expeller soybean meal and corn by-products versus solvent soybean meal for lactating dairy cows fed alfalfa silage as a sole forage. J. Dairy Sci. 73:453-462.

Broderick, G. A., and R. J. Wallace. 1988. Effects of dietary nitrogen source on concentrations of ammonia, free amino acids and fluorescamine-reactive peptides in the sheep rumen. J. Anim. Sci. 66:2233-2238.

Buddington, R. K. 1992. Intestinal nutrient transport during ontogeny of vertebrates. Am. J. Physiol. 263:R503-R509.

Buddington, R. K., and J. M. Diamond. 1989. Ontogenic development of intestinal nutrient transporters. Annu. Rev. Physiol. 51:601-619.

Buraczewski, S., A. G. Chamberlain, F. Horszczaruk, and T. Zebrowska. 1970. Lysine and arginine interactions affecting the absorption from the duodenum of the pig. Nutrition Society Proc. 29:51A-52A.

Burton, K. A., P. S. James, M. W. Smith, and J. D. Young. 1980. Different pathways for lysine transport across neonatal pig intestine. J. Physiol. 308:451-464.

Burton, P. S., R. A. Conradi, A. R. Hilgers, and N. F. H. Ho. 1993. Evidence for a polarized efflux system for peptides in the apical membrane of caco-2 cells. Biochem. Biophys. Res. Commun. 190:760-766.

Burston, D., Addison, J. M. and Matthews, D. M.. 1987. Effects of sodium replacement on uptake of the dipeptide glycylsarcosine by hamster jejunum in vitro. Clin. Sci. 73: 61-68.

Calderhead, D. M., K. Kitagawa, L. I. Tanner, G. D. Holman, and G. E. Lienhard. 1990. Insulin regulation of the two glucose transporters in 3T3-L1 adipocytes. J. Biol. Chem. 265:13800-13808.

Campa, M. J., and M. S. Kilberg. 1989. Characterization of neutral and cationic amino acid transport in *Xenopus* oocytes. J. Cell. Physiol. 141:645-652.

Cheeseman, C. I. 1991. Molecular Mechanisms involved in the regulation of amino acid transport. Prog. Biophysiol. Molec. Biol. 55:71-84.

Cheeseman, C. I., and D. Devlin. 1985. The effects of amino acids and dipeptides on sodium-ion transport in rat enterocytes. Biochim. Biophys. Acta 812:767-773.

Chen, G. H., C. J. Sniffen, and J. B. Russell. 1987. Concentration and estimated flow of peptides from the rumen of dairy cattle: effects of protein quantity, protein solubility, and feeding frequency. J. Dairy Sci. 70:983-992.

Christensen, H. N. 1984. Organic ion transport during seven decades: the amino acids. Biochim. Biophys. Acta 779:255-269.

Christensen, H. N. 1990. Role of amino acid transport and countertransport in nutrition and metabolism. Physiol. Rev. 70:43-77.

Closs, E. I., L. M. Albritton, J. W. Kim, and J. M. Cunningham. 1993. Identification of a low affinity, high capacity transporter of cationic amino acids in mouse liver. J. Biol. Chem. 268:7538-7544.

Coleman, A. 1984. Translation of eukaryotic messenger RNA in Xenopus oocytes. In B. D. Hames and S. J. Higgins (Ed.) Transcription and Translation. A Practical Approach. p 271-302. IRL Press, Oxford, England.

Cook, R. M., R. E. Brown, and C. L. Davis. 1965. Protein metabolism in the rumen. I. absorption of glycine and other amino acids. J. Dairy Sci. 48:475-483.

Crooker, B. A., and J. H. Clark. 1987. Inhibition of L-alanine uptake into bovine jejunal brush border membrane vesicles by L-amino acids. J. Dairy Sci. 70:963-969.

Daniel, H., M. Boll, and U. Wenzel. 1994. Physiological importance and characteristics of peptide transport in intestinal epithelial cells. VIth International Symp. on Digestive Physiol. in Pigs, Vol. I. No. 80:1-7.

Daniel, H., E. L. Morse, and S. A. Adibi. 1991. The high and low affinity transport systems for dipeptides in kidney brush border membrane respond differently to alterations in pH gradient and membrane potential. J. Biol. Chem. 266:19917-19924.

Daniel, H., E. L. Morse, and S. A. Adibi. 1992. Determinants of substrate affinity for the oligopeptide/H+ symporter in the renal brush border membrane. J. Biol. Chem. 267:9565-9573.

Dantzig, A. H., J. Hoskins, L. B. Tobas, S. Bright, R. L. Shepard, I. L. Jenkins, D. C. Duckworth, J. R. Sportsman, D. Mackensen, P. R. Rosteck, Jr., and P. L. Skatrud. 1994. Association of intestinal peptide transport with a protein related to the cadherin superfamily. Science 264:430-433.

Dawson, D. C. 1991. Principles of membrane transport. In : B. B. Rauner (Ed.) The Gastrointestinal System. Intestinal Absorption and Secretion. Vol IV. p 1-44.

Diamond, J. M., and W. H. Karasov. 1987. Adaptive regulation of intestinal nutrient transporters. Proc. Natl. Acad. Sci. USA 84:2242-2245.

Diamond, J. 1991. Evolutionary design of intestinal nutrient absorption: Enough but not too much. News Physiol. Sci. 6:92-96.

DiRienzo, D. B., and K. E. Webb, Jr. 1995. Free and peptide amino acid fluxes across the mesenteric-drained and non-mesenteric-drained viscera of sheep and calves. J. Dairy Sci. (submitted).

D'Mello, J. P. F., 1993. Amino acid supplementation of cereal-based diets for non-ruminants. Anim. Feed Sci. Technol. 45:1-18.

Dumont, J. N. 1972. Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. J. Morph. 136:153-180.

Dyer, R. B. Beechey, J.-P. Gorvel, R. T. Smith, R. Wootton, and S. P. Shirazi-Beechey. 1990. Glcyl-L-proline transport in rabbit enterocyte basolateral-membrane vesicles. Biochem J. 269:565-571.

Edmondson, J. W., and L. Lumeng. 1980. Biphasic stimulation of amino acid uptake by glucagon in hepatocytes. Biochem. Biophys. Res. Comm. 96:61-68.

Engelhardt, W. V., and J. R. S. Hales. 1977. Partition of capillary blood flow in rumen, reticulum, and omasum of sheep. Am. J. Physiol. 232(1):E53-E56.

Engelhardt, W. V., and R. Hauffe. 1975. Role of the omasum in absorption and secretion of water and electrolytes in sheep and goats. In: I. W. McDonald and A. C. I. Warner (Ed.) Digestion and Metabolism in the Ruminant. p 216-230. Proceedings of the 5th International Symposium on Ruminant Physiology, August 1974, Sidney, Australia, University of New England Publishing Unit, Armidale.

Englesberg, E., and J. Moffett. 1986. A genetic approach to the study of neutral amino acid transport in mammalian cells in culture. J. Membr. Biol. 91:199-212.

Fehlmann, M., A. Le Cam, and P. Freychet. 1979. Insulin and glucagon stimulation of amino acid transport in isolated rat hepatocytes. J. Biol. Chem. 254:10431-10437.

Fei, Y.-Y., Y. Kanai, S. Nussenberger, V. Ganapathy, F. H. Leibach, M. F. Romero, S. Singh, W. F. Boron, and M. A. Hediger. 1994. Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature 368:563-566.

Fejes, J., Z. Faixova, J. Varady, and M. Cibula. 1991. In vitro transport of amino acids across the rumen mucosa in sheep. Vet. Med. Praha. 36(9):551-558.

Fell, B. F., and T. E. C. Weekes. 1975. Food intake as a mediator of adaptation in the ruminal epithelium. In: I. W. McDonald and A. C. I. Warner (Ed.) Digestion and Metabolism in the Ruminant. p 101-118. Proceedings of the IV International Symposium on Ruminant Physiology, August 1974, Sidney, Australia, University of New England Publishing Unit, Armidale.

Fields, G. B., and R. L. Noble. 1990. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int. J. Protein Res. 35:161-214.

Fisher, R. B. 1954. Protein Metabolism. Methuen, London.

Flint, H. J., A. M. Thomson, and J. Bisset. 1988. Plasmid-associated tranfer of tetracycline resistance in *Bacteriodes ruminacola*. Appl. Environ. Microbiol. 54:855-860.

Fong, A. D., M. E. Handlogten, and M. S. Kilberg. 1989. Substrate-dependent adaptive regulation and trans-inhibition of system A-mediated amino acid transport. Studies using rat hepatoma plasma membrane vesicles. Biochim. Biophys. Acta 1022:325-332.

Gabel, G., S. Volger, and H. Martens. 1993. Mechanisms of sodium and chloride transport across isolated sheep reticulum. Comp. Biochem. Physiol. 105A:1-10.

Ganapathy, V, and Leibach, F. H. 1982. Peptide transport in rabbit kidney. Studies with L-carnosine. Biochim Biophys. Acta 691:362-366.

Ganapathy, V., and F. H. Leibach. 1983. Role of pH gradient and membrane potential in dipeptide transport in intestinal and renal brush-border membrane vesicles from the rabbit. J. Biol. Chem. 258:14189-14192.

Ganapathy, V., and Leibach, F. H. 1985. Is intestinal peptide transport energized by a proton gradient? Am. J. Physiol. 249:G153-G160.

Ganapathy, V., G. Burckhardt, and F. H. Leibach. 1984. Characteristics of glycylsarcosine transport in rabbit intestinal brush-border membrane vesicles. J. Biol. Chem. 259:8954-8959.

Ganapathy, V., M. Brandsch, and F. H. Leibach. 1994. Intestinal transport of amino acids and peptides. In L. R. Johnson (Ed.) Physiology of the Gastrointestinal Tract (3rd edition). p 1773-94. Raven Press, Inc., NY.

Gardner, M. L. G. 1994. Absorption of intact proteins and peptides. In L. R. Johnson (Ed.) Physiology of the Gastrointestinal Tract (3rd edition). p 1795-1820. Raven Press, Inc., NY.

Gazzola, G. C., V. Dall'asta, and G. G. Guidotti. 1981. Adaptive regulation of amino acid transport in cultured human fibroblasts. Sites and mechanism of action. J. Biol. Chem. 256:3191-3198.

Guidotti, G. G., A. F. Borghetti, and G. C. Gazzola. 1978. The regulation of amino acid transport in animal cells. Biochim. Biophys. Acta 515:329-366.

Guidotti, G. G., and G. C. Gazzola. 1992. Amino acid transporters: Systematic approach and principles of control. In: M. S. Kilberg, D. Haussinger (Ed.) Mammalian Amino Acid Transport: Mechanisms and Control. p 3. Plenum Press, New York.

Harris, S. I., R.S. Balaban, and L. J. Mandel. 1980. Oxygen consumption and cellular ion transport: Evidence for adenosine triphosphate to O_2 ratio near 6 in intact cell. Science 208:1148-1150.

Haworth, R. S., O. Frohlich, and L. Fliegel. 1993. Multiple carbohydrate moieties on the Na^+/H^+ exchanger. J. Biochem. 289:637-640.

Hediger, M. A., T. Ikeda, M. Coady, C. B. Gundersen, and E. M. Wright. 1987. Expression of size-selected mRNA encoding the intestinal Na/glucose cotransporter in *Xenopus laevis* oocytes. Proc. Natl. Acad. Sci. USA 84:2634-2637.

Henrikson, R. C. 1970. Ultrastructure of ovine ruminal epithelium and localization of sodium in the tissue. J. Ultrastr. Res. 30:385-401.

Heyman, M., A.-M. Crain-Denoyelle, S. K. Nath, and J.-F. Desjeux. 1990. Quantification of protein transcytosis in the human colon carcinoma cell line CaCo-2. J. Cell. Physiol. 143:391-395.

Higgins, C. F. 1990. The role of ATP in binding-protein-dependent transport systems. Res. Microbiol. 141:353-360.

Hiles, I. D., M. P. Gallagher, D. J. Jamieson, and C. H. Higgins. 1987. Molecular characteriazatioin of the oligo peptide permease of Salmonella typimurium. J. Mol. Biol. 195:125-142.

Himukai, M., Kameyama, A., and Hoshi, T. 1983. Interaction of glycylglycine and Na^+ at the mucosal border of guinea pig small intestine. Biochim. Biophys. Acts 732:659-667.

Horner, H. C., A. Munck, and G. E. Lienhard. 1987. Dexamethasone causes translocation of glucose transporters from the plasma membrane to an intracellular site in human fibroblasts. J. Biol. Chem. 262:17696-17702.

Hoshi, T. 1985. Proton-coupled transport of organic solutes in animal cell membranes and its relation to N^+ transport. Jap. J. Physiol. 35:179-191.

Hoshi, T. 1986. Proton-coupled transport of dipeptides across renal and intestinal brush border membranes. In: F. Alvarado and C. H. van Os (Ed.) Ion Gradient-Coupled Transport. p 183-190. Elsevier Science Publishers, Amsterdam.

Hryshko, L. V., D. A. Nicoll, J. N. Weiss, and K. D. Philipson. 1993. Biosynthesis and initial processing of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. Biochim. Biophys. Acta 1151:35-42.

Hyden, S., and I. Sperber. 1965. Electron microscopy of the ruminant fore-stomach. In: R. W. Dougherty (Ed.) Physiology of Digestion in the Ruminant. p 51. Butterworths, Washington.

Inui, K. I., T. Okano, H. Maegawa, M. Kato, M. Takano, and R. Hori. 1988. H+ coupled transport of p.o. cephalosporins via dipeptide carriers in rabbit intestinal brush-border membranes:difference of transport characteristics between cefixime and cephradine. J. Pharm. Exp. Ther. 247:235.

Inui, K. I., M. Yamamoto, and H. Saito. 1992. Transepithelial transport of oral cephlosporins by monolayers of intestinal epithelial cell line Caco-2: specific transport systems in apical and basolateral membranes. J. Pharm. Exper. Ther. 261:195-201.

Iseki, K., Sugawara, H. Saitoh, K. Miyazaki, and T. Arita. 1989. Comparison of transport characteristics of amino beta-lactam antibiotics and dipeptides across rat intestinal brush border membrane. J. Pharmacol. 41:628-632..

Island, M. D., F. Naider, and J. M. Becker. 1987. Regulation of dipeptide transport in *Saccharomyces cerevisiae* by micromolar amino acid concentrations. J. Bacteriol. 169:2132-2136.

James, P. S., M. W. Smith, D. R. Tivey, and T. J. G. Wilson. 1987. Dexamethasone selectively increases sodium-dependent alanine transport across neonatal piglet intestine. J. Physiol. 393:569-582.

Johns, J. T., and W. G. Bergen. 1973. Studies on amino acid uptake by ovine small intestine. J. Nutr. 103:1581-1586.

Jones, D. P., X. Shan, and Y. PArk. 1992. Coordinated multisite regulation of cellular energy metabolism. Annu. Rev. Nutr. 12:327-343.

Kanai, Y., and M. A. Hediger. 1992. Primary structure and functional characterization of a high-affinity glutamate transporter. Nature 360:467-471.

Kanai, Y., W.-S. Lee, G. You, D. Brown, and M. A. Hediger. 1994. The human kidney low affinity Na+/glucose cotransporter SGLT2. Delineation of the major renal resorptive mechanism for D-glucose. J. Clin. Invest. 93:397-404.

Kasanicki, M. A., and P. F. Pilch. 1990. Regulation of glucose transporter function. Diabet. Care 13:219-227.

Kato, M., H. Maegawa, T. Okano., K. Inui, and R. Hori. 1989. Effect of variouos chemical modifiers on H+ coupled transport of cephradine via dipeptide carriers in rabbit intestinal brush-border membranes: Role of histidine residues. J. Pharm. Exp. Ther. 251:745.

Keynes, R. D. 1969. From frog skin to sheep rumen: a survey of transport of salts and water across multicellular structures. Quart. Rev. Biophys. 2:177-281.

Kikuchi, K., N. N. Abumrand, and F. K. Ghishan. 1988. Na+/H+ exchange by brush border membrane vesicles of human ileal small intestine. Gastroenterology. 95:388-393.

Kilberg, M. S. 1986. Amino acid transport in eukaryotic cells and tissues. Fed. Proc. 45:2438-2440.

Kilberg, M. S., and D. Haussinger. 1992. Mammalian Amino Acid Transport. Plenum Press, New York.

Kilberg, M. S., R. G. Hutson, and R. O. Laine. 1994. Amino acid-regulated gene expression in eukaryotic cells. FASEB J. 8:13-19.

Kilberg, M. S., B. R. Stevens, and D. A. Novak. 1993. Recent advances in mammalian amino acid transport. Annu. Rev. Nutr. 13:137-165.

Kim, J. W., E. I. Cross, L. M. Albritton, and J. M. Cunningham. 1991. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. Nature 352:725-728.

King, D. S., C. G. Fields, and G. B. Fields. 1989. A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. Int. J. Peptide Protein Res. 36:255-266.

Knickelbein, R., P. S. Aronson, W. Atherton, and J. W. Dobbins. 1983. Sodium and chloride transport across rabbit ileal brush border. I. Evidence for Na+-H+ exchange. Am. J. Physiol. 245:G504-G510.

Koeln, L. L., T. S. Schlagheck, and K. E. Webb, Jr. 1993. Amino acid flux across the gastrointestinal tract and liver of calves. J. Dairy Sci. 76:2275.

Kong, C.-T., S.-H. Yet, and J. E. Lever. 1993. Cloning and expression of a mammalian Na^+/amino acid cotransporter with sequence homology to Na^+/glucose cotransporters. J. Biol. Chem. 268:1509-1512.

Kramer, W., F. Girbig, U. Gutjahr, S. Kowalewski, F. Adam, and W. Schiebler. 1992. Intestinal absorption of beta-lactam antibiotics and oligopeptides. Functional and stereospecific reconstruction of the oligopeptide transport system from rabbit small intestine. Eur. J. Biochem. 204:923-930.

Kramer, W., F. Girbig, I,. Leipe, and E. Petzoldt. 1988. Direct photoaffinity labelling of binding proteins for beta-lactam antibiotics in rabbit intestinal brush border membranes with ^{3}H-benzylpenicillin. Biochim. Biophys. Acta 37:2427-2435.

Kramer, W., F. Gutjahr, F. Girbig, and I,. Leipe. 1990a. Intestinal absorption of dipeptides and beta-lactam antibiotics. II. Purification of the binding protein for dipeptides and betalactam antibiotics from rabbit small intestinal brush border membrane. Biochim. Biophys. Acta 1030:50-59.

Kramer, W., F. Gutjahr, F. Girbig, and I. Leipe. 1990b. Intesinal absorption of dipeptides and beta-lactam antibiotics. I. Intestinal uptake system for dipeptides and betalactam antibiotics is not part of a brush border peptidase. Biochim. Biophys. Acta 1030:41-49.

Kryzsik, B. A., and S.A. Adibi. 1977. Comparison of metabolism of glycine injected intravenously in free and dipeptide form. Metabolism 28:1211.

Kulanthaivel, P., D. R. Cool, S. Ramamoorthy, V. B. Mahesh, F. H. Leibach, and V. Ganapathy. 1991. Transport of taurine and its regulation by protein kinase C in the JAR human placental choriocarcinoma cell line. Biochem. J. 277:53-58.

Laine, R. O., P. J. Laipis, N. F. Shay, and M. S. Kilberg. 1991. Identification of an amino acid-regulated mRNA from rat liver as the mammalian equivalent of bacterial ribosomal protein L22. J. Biol. Chem. 266:16969-16972.

Lawless, K., D. Maenz, and C. Cheeseman. 1987. Is leucine an allosteric modulator of the lysine transporter in the intestinal basolateral membrane? Am. J. Physiol. 253:G637-G642.

Lee, W.-S., R. G. Wells, R. V. Sabbag, T. K. Mohandas, and M. A. Hediger. 1993. Cloning and chromosomal localization of a human kidney cDNA involved in cystine, dibasic, and neutral amino acid transport. J. Clin. Invest. 91:1959-1963.

Leibholz, J. 1969. Effect of diet on the concentration of free amino acids, ammonia and urea in the rumen liquor and blood plasma of sheep. J. Anim. Sci. 29:628-633.

Leibholz, J. 1971a. The absorption of amino acids from the rumen of the sheep. I. The loss of amino acids from solutions placed in the washed rumen in vivo. Aust. J. Agric. Res. 22:639-645.

Leibholz, J. 1971b. The absorption of amino acids from the rumen of the sheep. II. The transfer of histidine, glycine, and ammonia across the rumen epithelium in vitro. Aust. J. Agric. Res. 22:647-653.

Leibholz, J. 1989. The utilization of free and protein-bound lysine. In M. Friedman (Ed.) The Utilization of Amino Acids. p 175-186. CRC Press, Inc., Boca Raton, FL.

Lentner, M., and T. Bishop. 1993. Experimental Design and Analysis. p. 90. Valley Book Company, Blacksburg, VA.

Lerner, J. 1985. Effectors of amino acid transport processes in animal cell membranes. Comp. Biochem Physiol. 81A:713-739.

Lerner, J. 1987. Acidic amino acid transport in animal cells and tissues. Comp. Biochem. Physiol. 87B:443-457.

Lewis, D. 1955. Amino-acid metabolism in the rumen of the sheep. Br. J. Nutr. 9:215-230.

Lochs, H., E. L. Morse, and S. A. Adibi. 1990. Uptake and metabolism of dipeptides by human red blood cells. Biochem. J. 271:133-137.

Lombardo, Y. B., E.L. Morse, and S. A. Adibi. 1988. Specificity and mechanism of influence of amino acid residues on hepatic clearance of oligopeptides. J. Biol. Chem. 263:12920-12926.

Longo, N., R. Franchi-Gazzola, O. Bussolati, V. Dall'Asta, P. P. Foa, G. G. Guidotti, and G. C. Gazzola. 1985. Effect of insulin on the activity of amino acid

transport systems in cultured human fibroblasts. Biochim. Biophys. Acta. 844:216-223.

Liu, W., R. Liang, S. Ramamoorthy, Y. J. Fei., M. E. Ganapathy, M A. Hediger, and F.H. Leibach. 1995. Cloning of PepT 2, a new member of the H+/peptide cotranporter family, from human kidney. FASEB J. 9(3):A310.

Lucas, M. 1983. Determination of acid surface pH in vivo in rat proximal jejunum. Gut 24:734-739.

Mackie, R. I., and B. A. White. 1990. Recent advances in rumen ecology and metabolism: Potential impact on nutrient outlook. J. Dairy Sci. 73:2971-2995.

Madara, J. L., and J. R. Pappenheimer. 1987. Structural basis for physiological regulation of paracellular pathways in intestinal epithelia. J. Membrane Biol. 100:149-164.

Madara, J. L., and J. S. Trier. 1987. Functional morphology of the small intestine. In: L. R. Johnson (Ed.) Physiology of the Gastrointestinal Tract. p 1209-1249. Raven Press, New York.

Magagnin, S., J. Bertran, A. Werner, D. MArkovich, J. Biber, M. Palacin, and H. Murer. 1992. Poly(A)+ RNA from rabbit intestinal mucosa induces $b^{0,+}$ and y^+ amino acid transport activities in *Xenopus laevis* oocytes. J. Biol. Chem. 267:15384-15390.

Maloney, P. C. 1990. A consensus structure for membrane transport. Res. Microbiol. 141:374-383.

Mandel, L. J., and R. S. Balaban. 1981. Stoichiometry and coupling of active transport to oxidative metabolism in epithelial tissue. Am. J. Physiol. 240:F357-F371.

Matthaei, S., T. Garvey, R. Horuk, T. P. Hueckstaedt, and J. M. Olefsky. 1987. Human adipocyte glucose transport system. Biochemical and functional heterogeneity of glucose carriers. J. Clin. Invest. 79:703-709.

Markovich, D., G. Stange, Bertran, J., M. Palacin, A. Werner, J. Biber, and H. Murer. 1992. Two mRNA transcripts (rBAT-1 and rBAT-2) are involved in system $b^{o,+}$-related amino acid transport. J. Biol. Chem. 268:1362-1367.

Martens, H., and G. Gabel. 1988. Transport of Na and Cl across the epithelium of ruminant forestomachs: rumen and omasum. A review. Comp. Biochem. Physiol. 90A:569-575.

Matthews, D. M. 1983. Intestinal absorption of peptides. Biochem Soc. Trans. 11:808-810.

Matthews, D. M. 1991a. Protein Absorption. Development and Present State of the Subject. Wiley-Liss, Inc., New York.

Matthews, D. M., and L. Laster. 1965. Competition for intestinal transport among five neutral amino acids. Am. J. Physiol. 208:601-606.

Matthews, J. C., and K. E. Webb, Jr. 1995. The absorption of L-carnosine, L-methionine, and L-methionylglycine by isolated sheep ruminal and omasal epithelial tissue. J. Anim. Sci. (Accepted).

Matthews, J. C. 1991b. Absorption of carnosine and methionylglycine by sheep ruminal and omasal epithelia. M. S. Thesis. Virginia Polytechnic Inst. and State Univ., Blacksburg.

McBride, B. W., and L. P. Milligan. 1985. Influence of feed intake and starvation on the magnitude of Na+, K+ ATPase (EC 3.6.1.3)-dependent respiration in duodenal mucosa of sheep. Br. J. Nutr. 53:605-614.

McBride, B. W., and J. M .Kelly. 1990. Energy cost of absorption and metabolism in the ruminant gastrointestinal tract and liver: A review. J. Anim. Sci. 68:2997-3010.

McCormick, M. E., and K. E. Webb, Jr. 1982. Plasma free erythrocyte free and plasma peptide amino acid exchange of calves in steady state and fasting metabolism. J. Nutr. 112:276-282.

McGiven, J. D., and M. Pastor-Anglada. 1994. Regulatory and molecular aspects of mammalian amino acid transport. Biochem. J. 299:321-334.

McNamara, P. D., C. T. Rea, and S. Segal. 1991. Expression of rat jejunal cystine carrier in *Xenopus* oocytes. J. Biol. Chem. 266:986-989.

McSweeney, C. S. 1988. A comparative study of the anatomy of the omasum in domesticated ruminants. Aust. Vet. J. 65:205-207.

Mimmack, M. L., M. P. Gallagher, S. C. Hyde, S. R. Hyde, S. R. Pierce, I. R. Bo, and C. F. Higgins. 1989. Energy-coupling to periplasmic binding protein-dependent transport systems: Stoichiometry of ATP hydrolysis during transport. Proc. Natl. Acad. Sci. USA 86:8257-8261.

Miyamoto, Y., V. Ganapathy, and F. H. Leibach. 1986. Identification of histidyl and thiol groups at the active site of rabbit renal dipeptide transporter. J. Biol. Chem. 261:16133-16140.

Miyamoto, Y., C. Tiruppathi, V. Ganapathy, and F. H. Leibach. 1989. Involvement of thiol groups in the function of the dipeptide/proton cotransport system in rabbit renal brush-border membrane vesicles. Biochim. Biophys. Acta 978:25-31.

Miyamoto, Y., Y. G. Thompson, E. F. Howard, V. Ganapathy, and F. H. Leibach. 1991. Functional expression of the intestinal peptide-proton co-transporter in *Xenopus laevis* oocytes. J. Biol. Chem. 266:4742-4745.

Moe, A. J., P. A. Pocius, and C. E. Polan. 1987. Transport of L-amino acids by brush border membrane vesicles from bovine small intestine. J. Dairy Sci. 70:290-297.

Mooseker, M. S., E. M. Bonder, K. A. Conzelman, D. J. Fishkind, C. L. Howe, and T. C. S. Keller, III. 1984. Brush border cytoskeleton and integration of cellular functions. J. Cell Biol. 99:104s-112s.

Mosckovitz, R., S. Udenfriend, A. Felix, E. Heimer, and S. S. Tate. 1994. Membrane topology of the rat neutral and basic amino acid transporter. FASEB 8:1069-1074.

Munck, B. G. 1989. Amino acid transport across the hen colon: interactions between leucine and lysine. Am. J. Physiol. 256:G532-G539.

Murer, H., U. Hopfer, and R. Kinne. 1976. Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. Biochem. J. 154:597-604.

Nielson, R. 1979. A 3 to 2 coupling ratio of the Na^+, K pump responsible for the transepithelial Na transport in frog skin disclosed by the effect of Ba. Acta Physiol. Scand. 107:189-191.

NRC. 1985a. Nutrient Requirements of Sheep (6th ed.). National Academy Press, Washington, DC.

NRC. 1985b. Ruminant Nitrogen Usage. National Academy Press, Washington, DC.

NRC. 1988. Nutrient Requirements of Swine (9th ed.). National Academy Press, Washington, DC.

Ohta, T., K. J. Isselbacher, and D. B. Rhoads. 1990. Regulation of glucose transporters in LLC-PK cells: Effects of D-glucose and monosaccharides. Mol. Cell Biol. 10:6491-6499.

Olson, E. R., D. S. Dunyak, L. M. Jurss, and R. A. Poorman. 1991. Identification and characterization of dppA, an *Escherichia coli* gene encoding a periplasmic dipeptide transport protein. J. Bacteriol. 173:234-244.

Pappenheimer, J. R. 1989. Flow and diffusion through biological membranes. In: D. C. Toteson (Ed.) Membrane Transport. People and Ideas. p 363. Waverly Press Inc, Baltimore.

Pappenheimer, J. R. 1993. On the coupling of membrane digestion with intestinal absorption of sugars and amino acids. Am. J. Physiol. 265:G409-G417.

Parent, L., S. Supplisson, D. D. F. Loo, and E. M. Wright. 1992. Electrogenic properties of the cloned Na^+/glucose transporter: I. Voltage clamp studies. J. Membr. Biol. 125:49-62.

Payne, J. W. 1983. Peptide transport in bacteria: Methods, mutants, and coupling energy. Biochem. Soc. Trans. 11:794-798.

Payne, J. W., K. J. Barrett-Bee, and D. A. Shallow. 1991. Peptide substrates rapidly modulate expression of dipeptide and oligopeptide permeases in *Candida albicans*. FEMS Microbiol. Lett. 79:15-20.

Peerce, B. E., and E. M. Wright. 1984. Sodium-induced conformational changes in the glucose transporter of intestinal brush borders. J. Biol. Chem. 259:14105-14112.

Petronilli, V. and G. F-L. Ames. 1991. Binding protein-independent histidine permease mutants. Uncoupling of ATP from transmembrane signaling. J. Biol. Chem. 266:16293-16296.

Phillips, W. A., Webb, Jr., K. E. and Fontenot, J. P. 1979. Characteristics of threonine, valine and methionine absorption in the jejunum and ileum of sheep. J. Anim. Sci. 48:926-933.

Pickel, V. M., M. J. Nirenberg, J. Chan, R. Mosckovitz, S. Udenfriend, and S. S. Tate. 1993. Ultrastructural localization of a neutral and basic amino acid transporter in rat kidney and intestine. Proc. Natl. Acad. Sci. USA 90:7779-7783.

Pittman, K. A., and M. P. Bryant. 1964. Peptides and other nitrogen sources for growth of *Bacteriodes ruminicola*. J. Bacter. 88:401-410.

Pittman, K. A., A. S., Lakshmanan, and M. P. Bryant. 1967. Oligopeptide uptake by *Bacteriodes ruminicola*. J. Bacteriol. 93:1499-1508.

Prins, R. A., R. E. Hungate, and E. R. Prast. 1972. Function of the omasum in several ruminant species. Comp. Biochem. Physiol. 43A:155-163.

Puissant, C., and L.-M., Houdebine. 1990. An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. BioTechniques 8:148-149.

Pullman, A. 1991. Contribution of theoretical chemistry to the study of ion transport through membranes. Chem. Rev. 91:793-812.

Reithmeier, R. A. F. 1994. Mammalian exchangers and co-transporters. Curr Opin. Cell Biol. 6:583-594.

Reizer, J., K. Finley, D. Kakuda, C. L. MacLeod, A. Reizer, and M. H. Saier, Jr. 1993. Mammalian integral membrane receptors are homologous to facilitators and antiporters of yeast, fungi, and eubacteria. Protein Science 2:20-30.

Reizer, J., A. Reizer, and M. H. Saier, Jr. 1994. A functional superfamily of sodium/solute symporters. Biochim. Biophys. Acta 1197:133-166.

Reshkin, S. J., and G. A. Ahearn. 1991. Intestinal glycyl-L-phenylalanine and L-phenylalanine transport in euyhaline teleost. Am .J. Physiol. 260:R563-R569.

Rompala, R. E., and T. A. Hoagland. 1987. Effect of level of alimentation on visceral organ mass and morphology and Na+, K+ adenosinetriphosphate activity in the intestinal mucosa in lambs. J. Anim. Sci. 65:1058-1065.

Russell, J. B., H. J. Strobel, and S. A. Martin. 1990. Strategies of nutrient transport by ruminal bacteria. J. Dairy Sci. 73:2996-3012.

Said, H. M, F. K. Ghishan, and R. Redha. 1988. Transport of glycyl-L-proline in intestinal brush border membrane vesicles of the suckling rat: Characteristics and maturation. Biochim. Biophys. Acta 94:232-240.

Saito, H., and K.-I. Inui. 1993. Dipeptide transporters in apical and basolateral membranes of the human intestinal cell line Caco-2. Am. J. Physiol. 265:G289-G294.

Saito, H., T. Ishii, and K.-I. Inui. 1993. Expression of human intestinal dipeptide transporter in *Xenopus laevis* oocytes. Biochem. Pharm. 45:776-779.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual (2nd edition). p 7.26-7.36. Cold Spring Harbor Press, Cold Spring Harbor Press, NY.

SAS User's Guide:Statistics 5th ed. 1985. SAS Inst. Inc., Cary, NC.

SAS User's Guide:Statistics, Release 6.03. 1988. SAS Inst. Inc., Cary, NC.

Scarborough, G. A. 1985. Binding energy, conformational change and the mechanism of transmembrane solute movements. Microbiol. Rev. 49:214-231.

Scharrer, E., and C. Landes. 1978. Amino acid accumulation in kidney cortex of lambs and piglets as affected by insulin. Comp. Biochem Physiol. 61A:407-410.

Schultz, S. G., L. Yu-Tu ,and C. K. Strecker. 1972. Influx of neutral amino acids across the brush border of rabbit ileum. Stereospecificity and the roles of the alpha-amino and alpha-carboxylate groups. Biochim. Biophys. Acta 288:367.

Shafqat, S., B. Tamarapoo, M. S. Kilberg, R. S. Puranam, J. O. McNamara, A. Guadano-Ferraz, and R. T. Fremeau, Jr. 1993. Cloning and expression of a novel Na^+-dependent neutral amino acid transporter structurally related to mammalian Na^+/glutamate cotransporters. J. Biol. Chem. 268:15351-15355.

Shallow, D. A., K. J. Barrett-Bee, and J.W. Payne. 1991. Evaluation of the dipeptide and oligopeptide permeases of *Candida albicans* as uptake routes for synthetic anticandidal agents. FEMS Microbiol. Lett. 79:9-14.

Shay, N. F., H. S. Nick, and M. S. Kilberg. 1990. Molecular cloning of an amino acid-regulated mRNA (amino acid starvation-induced) in rat hepatoma cells. J. Biol. Chem. 265:17844-17848.

Shotwell, M. A., M. S. Kilberg, and D. L. Oxender. 1983. The regulation of neutral amino acid transport in mammalian cells. Biochim. Biophys. Acta. 737:267-284.

Smid, E. J., R. Plapp, and W. N. Konings. 1989. Peptide uptake is essential for growth of lactococcus on the milk protein casein. J. Bacteriol. 171:6135-6140.

Smith, R. H. 1984. Microbial activity in the omasum. Proc. Nutr. Soc. 43:63-68.

Sokol, P. P. 1990. Effect of DQ-2556, a new cephalosporin, on organic ion transport in renal plasma membrane vesicles from the dog, rabbit and rat. J. Pharmacol. Exper. Therap. 255:436-441.

Spencer, R. P., T. M. Bow, and M. A. Markulis. 1962. Amino group requirement for in vitro intestinal transport of amino acids. Am. J. Physiol. 202:271.

Stein, E. D., S. D. Chang, and J. M. Diamond. 1987. Comparison of different dietary amino acids as inducers of intestinal amino acid transport. Am. J. Physiol. 252:G626-G635.

Steven, D. H., and A. B. Marshall. 1970. Organization of the rumen epithelium. In: A. T. Phillipson (Ed.) Physiology of Digestion and Metabolism in the Ruminant. p 80-100. Proceedings of the 3rd International Symposium, Cambridge, England. August 1969. Oriel Press, New Castle Upon Tyne, England.

Stevens, B. R. 1992a. Amino acid transport in intestine. In: M. S. Kilberg and D. Haussinger (Ed.) Mammalian Amino Acid Transport. p 149-163. Plenum Press, New York.

Stevens, B. R. 1992b. Vertebrate intestine apical membrane mechanisms of organic nutrient transport. Am. J. Physiol. 263:R458-R463.

Stevens, B. R., A. Fernandez, B. Hirayama, and E. M. Wright. 1990. Intestinal brush border membrane Na^+/glucose cotransporter functions as a homoheteramer. Proc. Natl. Acad. Sci. USA 87:1456-1460.

Stevens, C. E. 1973. Transport across rumen epithelium. In: H. H. Ussing and N. A. Thorn (Ed.) Transport Mechanisms in Epithelia. p 405-426. Academic Press, New York.

Stevens, C. E., and B. K. Stettler. 1966. Transport of fatty acid mixtures across rumen epithelium. Am. J. Physiol. 211:264-271.

Storck, T., S. Schulte, K. Hoffmann, and W. Stoffel. 1992. Proc. Natl. Acad. Sci. USA 89:10955-10959.

Tarnuzzer, R. W., M. J. Campa, N.-X. Qian, E. Englesberg, and M. S. Kilberg. 1990. Expression of the mammalian system A neutral amino acid transporter in Xenopus oocytes. J. Biol. Chem. 265:13914-13917.

Tate, S. S., N. Yang, and S. Udenfriend. 1992. Expression cloning of a Na^+-independent neutral amino acid transporter from rat kidney. Proc. Natl. Acad. Sci. USA 89:1-5.

Teichberg., S. 1985. Morphological alterations in small intestinal eptihelial surfaces during transport. Nutrition for Special Needs in Infancy. pp 59-75.

Thiele, D. L., and P. E. Lipsky. 1990. The action of leucyl-leucine methyl ester on cytotoxic lymphocytes requires uptake by a novel dipeptide-specific facilitated transport system and dipeptidyl peptidase I-mediated conversion to membranolytic products. J. Exp. Med. 172:183.

Thorens, B. 1993. Facilitated glucose transporters in epithelial cells. Annu. Rev. Physiol. 55:591-608.

Thorens, B., Z.-Q. Cheng, D. Brown, H. F. Lodish. 1990. Liver glucose transporter: a basolateral protein in hepatocytes and intestine and kidney cells. Am. J. Physiol. 259:C279-C285.

Thwaites, D. T., C. D. A. Brown, B.H. Hirst, and N. L. Simmons. 1993. H+-coupled dipeptide (glycylsarcosine) transport across apical and basal borders of human intestinal Caco-2 cell monolayers display distinctive characteristics. Biochim. Biophys. Acta 1151:237-245.

Tsong, T. Y. 1992. Molecular recognition and processing of periodic signals in cells: Study of activation of membrane ATPases by alternating electric fields. Biochem. Biophys. Acta. 1113:53-70.

Tsong, T. Y., and R. D. Astumian. 1988. Electroconformational coupling: How membrane-bound ATPase transduces energy from dynamic electric fields. Annu. Rev. Physiol. 50:273-290.

Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. Manometric Techniques. A Manual Describing Methods Applicable to the Study of Tissue Metabolism. p 132. Burgess Publishing Company, Minneapolis.

Van Soest, P. J. 1987. Nutritional Ecology of the Ruminant: Ruminant Metabolism, Nutritional Strategies, The Cellulytic Fermentation and the Chemistry of Forages and Plant Fiber. Comstock Publishing Associates, Ithaca, NY.

Van Winkle, L. J. 1988. Amino acid transport in developing animal oocytes and early conceptuses. Biochim. Biophys. Acta 947:173-208.

Van Winkle, L. J. 1993. Endogenous amino acid transport systems and expression of mammalian amino acid transport proteins in *Xenopus* oocytes. Biochim. Biophys. Acta 1154:157-172.

Van Winkle, L. J., A. L. Campione, and J. M. Gorman. 1988. Na^+-independent transport of basic and zwitterionic amino acids in mouse blastocysts by a shared system and by processes which distinguish between those substrates. J. Biol. Chem. 263:3150-3163.

Vincenzini, M. T., F. Favilli, and T. Iantomasi. 1988. Glutathione-mediated transport across intestinal brush border membranes. Biochim. Biophys. Acta 942:107-114.

Vincenzini, M. T., T. Iantomasi, and F. Favilli. 1989. Glutathione transport across intestinal brush-border membranes: Effects of ions, pH, membrane potential, and inhibitors. Biochim. Biophys. Acta 987:29-37.

Wallace, R. J., and M. A. Cotta. 1988. Metabolism of nitrogen-containing compounds. In: P. N. Hobson (Ed.) The Rumen Microbial Ecosystem. p 217. Elsevier Publishing Co., Inc., New York.

Wang, H., M. P. Kavanaugh, R. A. North, and D. Kabat. 1991. Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. Nature 352:729-731.

Webb, K. E., Jr., D. B. DiRienzo, and J. C. Matthews. 1993. Recent developments in gastrointestinal absorption and tissue utilization of peptides: A review. J. Dairy Sci. 76:351-361.

Webb, K. E., Jr., and J. C. Matthews. 1994. The absorption of amino acids and peptides. In J. M. Asplund (Ed.) Principles of Protein Nutrition of Ruminants. p 127-146. CRC Press, Inc., Boca Raton, FL.

Webb, K. E., Jr., J. C. Matthews, and D. B. DiRienzo. 1992. Peptide Absorption: A review of current concepts and future perspectives. J. Anim. Sci. 70:3248-3257.

Wells, R. G., and M. A. Hediger. 1992. Cloning of a rat kidney cDNA that stimulates dibasic and neutral amino acid transport and has sequence similarity to glucosidases. Proc. Natl. Acad. Sci. USA 89:5596-5600.

Whitelaw, F. G., J. Hyldgaard-Jensen, R. S. Reid, and M. G. Kay. 1970. Volatile fatty acid production in the rumen of cattle given an all-concentrate diet. Br. J. Nutr. 24:179-195.

Williams, V. J. 1969. The relative rates of absorption of amino acids from the small intestine of the sheep. Comp. Biochem. Physiol. 29:865-870.

Wilson, J. W., and K. E. Webb, Jr. 1990. Lysine and methionine transport by bovine jejunal and ileal brush border membrane vesicles. J. Anim. Sci. 68:504-514.

Wolffram, S., E. Eggenberger, and E. Schaarrer. 1986. Kinetics of D-glucose and L-leucine transport into sheep and pig intestinal brush border membrane vesicles. Comp. Biochem. Physiol. 84A:589-593.

Woodard, M. H., W. A. Dunn, R. O. Laine, M. Malandro, R. McMahon, O. Simell, E. Block, and M. S. Kilberg. 1994. Plasma membrane clustering of system y^+ (CAT-1) amino acid transporter as detected by immunohistochemistry. Am. J. Physiol. 266:E817-E824.

Woodlock, T. J., G. B. Segel, and M. A. Lichtman. 1989. Diacylglycerol and calcium induce rapid enhancement of A-system amino acid transport by independent mechanisms in human T-lymphocytes. J. Cell Physiol. 141:33-39.

Wright, D. E., and R. E. Moir. 1967. Amino acid concentrations in rumen fluid. Appl. Microbiol. 15:148-151.

Wright, E. M. 1993. The intestinal Na^+/glucose cotransporter. Annu. Rev. Physiol. 55:575-589.

APPENDIX A

Na^+-Independent Lysine Absorption by Defolliculated *Xenopus laevis* Oocytes Injected with Water or Sheep Omasum Epithelial Poly(A)$^+$ RNA

The purpose of these experiments was to demonstrate or refute the potential for the mediated absorption of lysine in omasal forestomach epithelial tissue, as characterized by the expression of their mRNA in *Xenopus laevis* oocytes. All experimental materials, methods and procedures used in these experiments have been described (Chapter VI), except that the uptake media contained 5 *u*Ci/mL L-[^{3}H]-lysine (5 Ci/mmol) and 50 u*M* total L-lysine, and that 96 m*M* of choline chloride replaced NaCl, when appropriate.

The potential for mediated lysine absorption was tested because of its importance to the nutrition of ruminants and because it is reported to be present in relatively high concentrations in rumen liquor (Wright and Moir, 1967). Compared to water-injected oocytes, oocytes injected with omasum epithelial poly(A)$^+$ RNA displayed a greater ($P < .001$) ability to absorb (pmols/oocyte·35 min^{-1}) lysine from both Na^+-free or Na^+-containing buffers (Figure A-1). Within RNA fractions, the amount of induced lysine absorbed did not differ ($P > .05$) between buffers. Therefore, it was concluded that induced uptake was by Na^+-independent processes. The linear ($P < .001$) rate of lysine uptake from Na^+-containing media (.05 m*M* lysine) by RNA-injected oocytes was approximately 2 times greater ($P < .001$) than that demonstrated by oocytes injected with water (Figure A-2). In order to determine whether this RNA-induced uptake was the result of increased mediated absorption, the uptake of .05 m*M* lysine by water- and RNA -injected oocytes was evaluated in the presence of Na^+-containing media that also contained 5 mM leucine, 5 mM glutamate

and .2 mM cysteine (Figure A-3). The absorption of lysine (pmols/oocyte·35 min^{-1}) by oocytes injected with water or RNA was, essentially, completely inhibited ($P < .05$) by leucine, and not affected by glutamate, thus displaying characteristic $b^{o,+}$-like transport activity. However, that cystine stimulated ($P < .05$) the uptake of lysine in oocytes injected with omasal RNA suggests that the omasal epithelium may possess an unusual isoform of this transport protein.

From these results, it is preliminarily concluded that the omasum epithelial tissue possesses mRNA that are capable of mediating the Na^+-independent absorption of lysine.

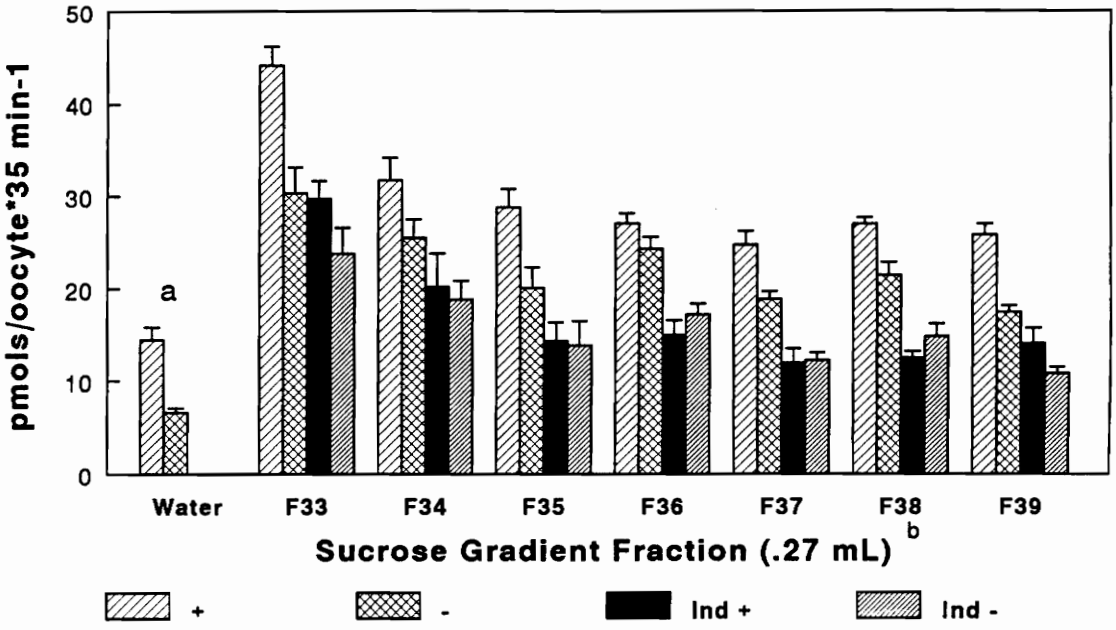


Figure A.1. Absorption of lysine by defolliculated Xenopus oocytes injected with water or sequential sucrose gradient size-fractionated sheep omasal epithelium poly(A)$^+$ RNA (RNA).

Oocytes were assayed in a media lacking (-) or containing (+) Na^+ (.05 m*M* L-lysine). Individual histogram bar values represent group (n = 1) absorption means ± SE of oocytes injected with water (n = 10) or RNA (RNA; n = 6 to 13).

[a]Between water-injected oocytes, buffer treatments differed ($P < .001$).

[b]Within buffers, injection treatments differed ($P < .001$).

Within RNA fractions, and within induced uptake, lysine uptake in the presence of Na^+ (+) did not differ from uptake in Na^+-free (-) media.

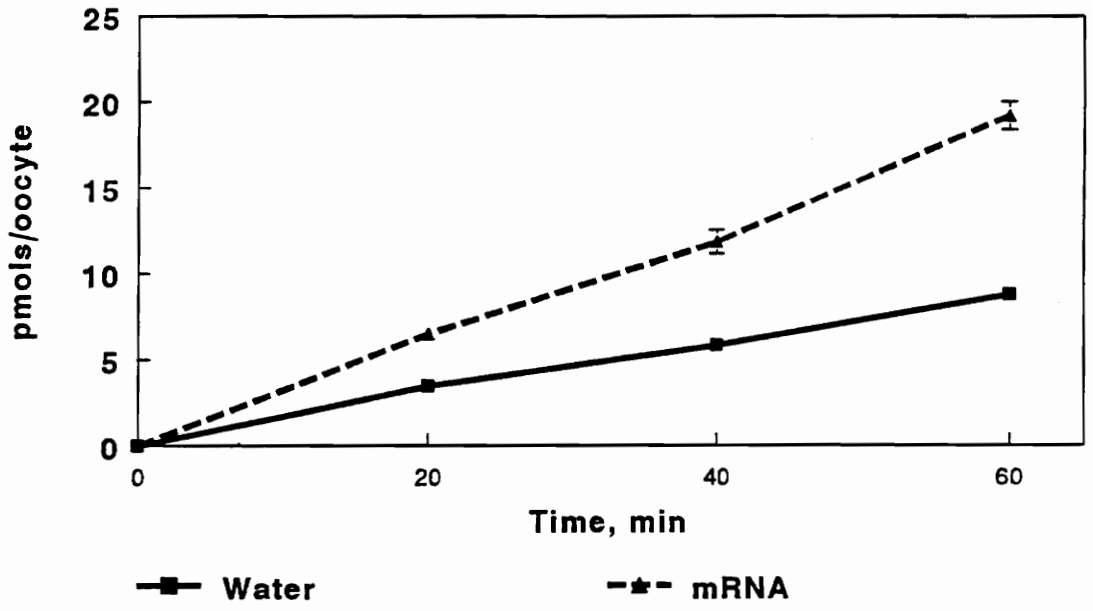


Figure A.2. Time course for lysine absorption by defolliculated Xenopus oocytes injected with water or size-fractionated sheep omasal epithelium RNA (RNA).

Oocytes were assayed in a media containing .05 m*M* L-lysine. Each time period measurement represents group (n = 1) absorption means ± SE of oocytes injected with water (n = 4 to 5) or RNA (n = 4 to 5).

Linear time effect ($P < .001$).

Time x treatment effect ($P < .001$).

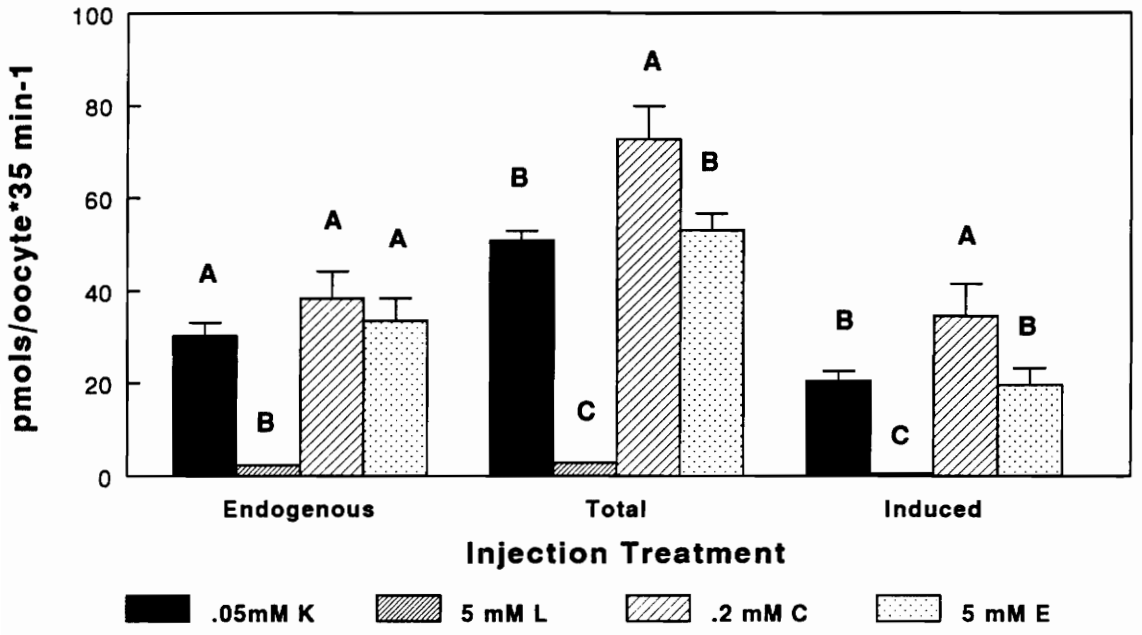


Figure A.3. Competitive lysine absorption by Xenopus oocytes injected with water (endogenous) or size-fractionated sheep omasal epithelium RNA (Total).

Oocytes were assayed in a media containing .05 m*M* L-lysine (K), L-leucine (L), L-Cysteine, (C), or L-glutamate (E), at indicated conentrations. Individual histogram values represent across group (n = 2) absorption means ± SE of oocytes injected with water (n = 5 to 7) or RNA (n = 11 to 19).

At .05 m*M*, uptake of lysine by oocytes injected with RNA (n = 15) was greater ($P < .001$) than for water-injected oocytes (n = 5).

Within treatments, bars lacking a common letter differ ($P < .05$).

APPENDIX B

Preliminary Electophysiological Measurement of Reduced Membrane Potential in Defolliculated *Xenopus laevis* Oocytes Injected with Sheep Omasal mRNA Upon Incubation with Carnosine

As the initial step in identifying sheep omasal epithelium tissue mRNA capable of encoding for peptide transport proteins, 4 d post injection with sucrose-gradient size-fractionated (fraction F34) omasal poly(A)$^+$ RNA (RNA; Chapter VI), fluctuation in the membrane potential (mV) of oocytes was measured in the presence of .5 mL pH 5.5 uptake buffer (Chapter VI) prior to impalement with voltage recording electrode, after impalement, and throughout a 4-min incubation in pH 5.5, 1 m*M* carnosine uptake buffer (Figure B-1). The initial membrane potential of -35 mV was reduced 10 to 30 mV, in two of three RNA-injected oocytes evaluated (Figure B-1A, B-1B), but not in two water-injected control oocytes (Figure B-1C). Because these RNA-injected oocytes had been randomly selected from a group of oocytes that had demonstrated an RNA-induced ability to absorb [^{3}H]-L-lysine, thereby establishing the translatability of the isolated RNA, it was concluded that the reduction of membrane potential was probably because of an enhanced ability of oocytes to absorb carnosine (positively charged at pH 5.5) and(or) H^+.

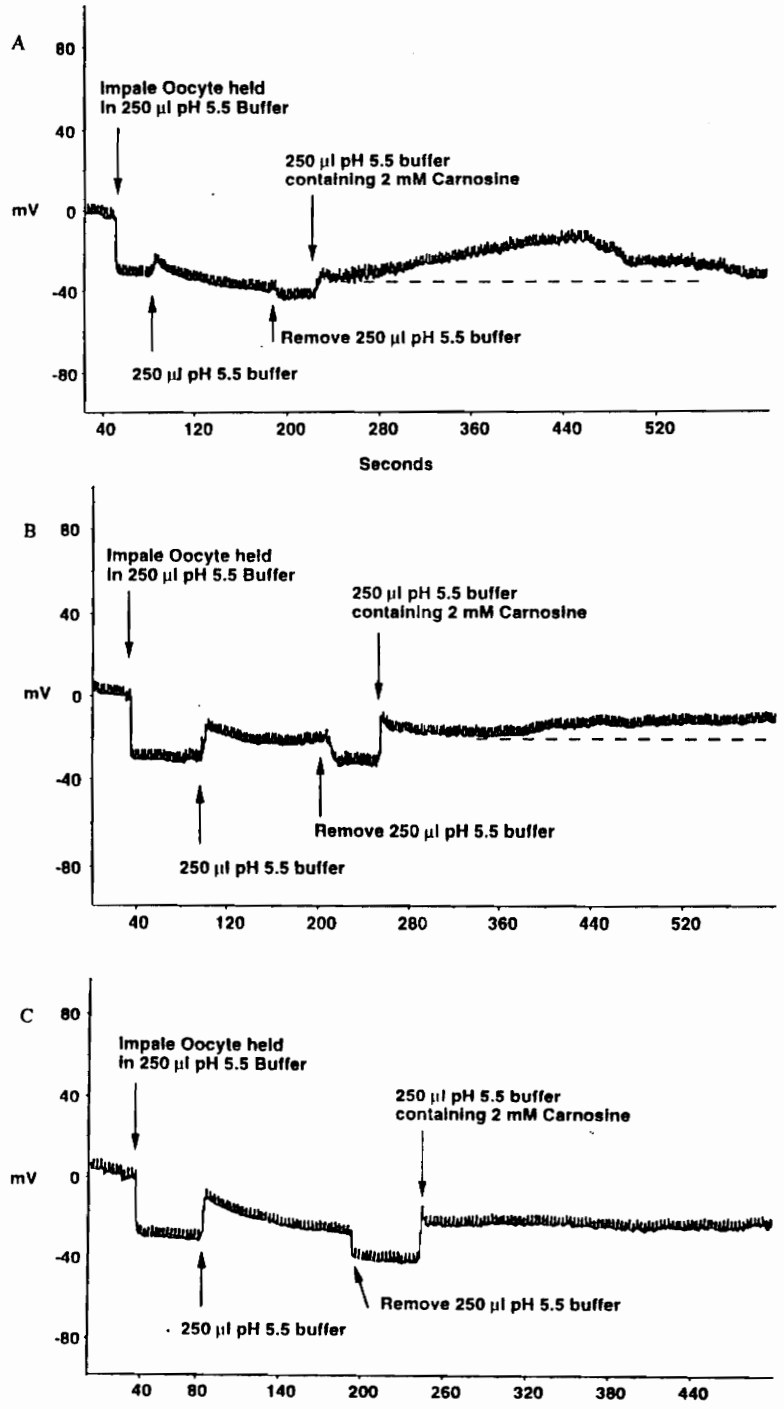


Figure B.1. Absorption of L-carnosine by defolliculated *Xenopus laevis* oocytes injected with sucrose gradient size-fractionated sheep poly(A)+ RNA (A, B) or with water (C).

Oocytes were assayed in pH 5.5 media (1 m*M* carnosine). Tracings are the recordings of individual oocytes.

APPENDIX C
Statistical Examples

Example C.1. ANALYSIS OF VARIANCE FOR COMPARISON OF METHIONINE AND METHIONYLGLYCINE (SOURCE) APPEARANCE IN RUMEN AND OMASUM SEROSAL CHAMBERS AFTER NATURAL LOG TRANSFORMATION

General Linear Model Procedure

Dependent variable: Glycylsarcosine (pmols/oocyte·1h)

Source	DF	Sum of squares	Mean squares	F value	PR > F
Model	327	6362.6	19.46	70.00	.0001
Error	1184	329.1	.2780		
Corrected total	1511	6691.7			

r^2	CV	SD	Transformed Mean
.95	-1186.4	.53	-.0444

Source	DF	Sum of squares	Mean squares	F value	PR > F
Source	1	96.15	96.15	345.89	.0001
Animal(Source)	12	56.15	56.15	16.89	.0001
Tissue	1	1148.03	1148.03	4129.90	.0001
Conc	5	1656.85	331.36	1192.07	.0001
Tissue*Source	1	.01	.01	.01	.9028
Source*Conc	5	39.71	7.94	28.57	.0001
Animal*Tissue(Source)	12	107.59	8.97	32.25	.0001
Animal*Conc(Source)	60	164.85	2.75	9.88	.0001
Tissue*Conc	5	31.82	6.36	22.90	.0001
Time	8	2686.60	335.83	1208.09	.0001
Source*Time	8	33.23	4.15	14.95	.0001
Animal*Time(Source)	96	80.12	.83	3.00	.0001
Tissue*Time	8	16.41	2.05	7.38	.0001
Time*Conc	40	60.85	1.52	5.47	.0001
Animal*Tissue*Conc(Source)	65	183.98	2.83	10.18	.0001
Tests of hypothesis using the Type III MS for Animal(Source) as an error term					
Source	1	96.15	96.15	20.48	.0007
Tests of hypothesis using the Type III MS for Animal*Tissue(Source) as an error term					
Tissue	1	1148.03	1148.03	128.04	.0001
Conc	5	1656.48	331.37	36.96	.0001
Tissue*Source	1	.01	.01	0	.9832
Tests of hypothesis using the Type III MS for Animal*Conc(Source) as an error term					
Conc	5	1656.48	331.37	120.61	.0001
Source*Conc	5	39.72	7.94	2.89	.0210
Contrast					
Linear Conc	1	932.60	932.60	339.44	.0001
Quadratic Conc	1	385.52	385.52	140.32	.0001
Tests of hypothesis using the Type III MS for Animal*Tissue*Conc(Source) as an error term					
Source	1	96.14	96.14	33.97	.0001
Tissue*Conc	5	31.82	6.36	2.25	.0598

Example C.2. REPEATED MEASURES ANALYSIS OF VARIANCE FOR EFFECT OF TIME ON COMPARISON OF METHIONINE AND METHIONYLGLYCINE (SOURCE) APPEARANCE IN RUMEN AND OMASUM SEROSAL CHAMBERS AFTER NATURAL LOG TRANSFORMATION

Repeated Measures Procedure

Univariate tests for hypothesis for within subjects effects.

Source	DF	Sum of squares	Mean squares	F value	Adjusted P> F G-G[1]	H-F[2]
Time	8	2686.60	335.83	1539.11	.0001	.0001
Time*Source	8	33.23	4.15	19.04	.0001	.0001
Time*Animal(Source)	96	80.12	0.83	3.82	.0001	.0001
Time*Tissue	8	16.41	2.05	9.41	.0003	.0001
Time*Conc	40	60.85	1.52	6.97	.0001	.0001
Time*Tissue*Source	8	6.15	.77	3.53	.0384	.0052
Time*Source*Conc	40	7.12	.18	.82	.5994	.7111
Time*Animal*Tissue(Source)	96	44.85	.45	2.14	.0057	.0001
Time*Animal*Conc(Source)	480	132.82	.28	1.27	.1070	.0218
Time*Tissue*Conc	40	24.71	.62	2.83	.0052	.0001
Error	520	113.46	.22			

[1]Greenhouse-Geisser Epsilon = .2191
[2]Huynh-Feldt Epsilon = .5780

Example C.3. REPEATED MEASURES ANALYSIS OF VARIANCE OF CONTRAST VARIABLES FOR THE EFFECT OF TIME ON METHIONINE AND METHIONYLGLYCINE (SOURCE) APPEARANCE IN RUMEN AND OMASUM SEROSAL CHAMBERS AFTER NATURAL LOG TRANSFORMATION

Repeated Measures Procedure

Analysis of variance contrast variables.

Source	DF	Sum of squares	Mean squares	F value	PR > F
Linear contrast					
Mean	1	2677.05	2677.05	5675.29	.0001
Source	1	10.54	10.54	22.35	.0001
Animal(Source)	12	45.75	3.81	8.08	.0001
Tissue	1	9.22	9.22	19.54	.0001
Conc	5	20.81	4.16	8.82	.0001
Tissue*Source	1	3.43	3.43	7.27	.0089
Source*Conc	5	1.11	.22	.47	.7965
Animal*Tissue(Source)	12	17.83	1.49	3.15	.0014
Animal*Conc(Source)	60	27.73	.46	.98	.5305
Tissue*Conc	5	11.69	2.34	4.96	.0007
Error	65	30.66	.47		
Quadratic contrast					
Mean	1	.01	.01	0	.9610
Source	1	18.71	18.71	31.67	.0001
Animal(Source)	12	15.92	1.33	2.25	.0191
Tissue	1	4.21	4.21	7.12	.0096
Conc	5	22.64	4.53	7.66	.0001
Tissue*Source	1	1.32	1.32	2.24	.1392
Source*Conc	5	1.79	.36	.61	.6956
Animal*Tissue(Source)	12	11.46	.95	1.62	.1089
Animal*Conc(Source)	60	43.56	.73	1.23	.2076
Tissue*Conc	5	8.06	1.61	2.73	.0268
Error	65	38.40	.59		

Example C.4. ANALYSIS OF VARIANCE FOR COMPARISON OF GLYCYLSARCOSINE UPTAKE BY OOCYTES INJECTED WITH WATER OR SIZE-FRACTIONATED SHEEP OMASAL POLY(A)$^+$ RNA

General Linear Model Procedure

Dependent variable: Glycylsarcosine (pmols/oocyte·1h)

Source	DF	Sum of squares	Mean squares	F value	PR > F
Model	18	507716074.0	28206448.6	67.15	.0001
Error	117	49143433.4	420029.3		
Corrected total	135	556859507.4			

r^2	CV	SD	Glycylsarcosine Mean
.91	29.4	648.1	2201.3

Source	DF	Sum of squares	Mean squares	F value	PR > F
Trt	1	20868166.1	20868166.1	49.68	.0001
Conc	5	454375854.3	90875170.9	216.35	.0001
Group	1	14454578.3	14454578.3	34.41	.0001
Trt*Conc	5	3725813.3	745162.7	1.77	.1235
Trt*Group	1	8507.7	850707	.02	.8871
Conc*Group	5	606182.7	1212365.4	2.89	.0171

Tests of hypothesis using the Type III MS for Conc*Group

Conc	5	454375854.3	90875170.9	74.96	.0001

Example C.5. ANALYSIS OF VARIANCE FOR EFFECT OF CONCENTRATION ON GLYCYLSARCOSINE UPTAKE BY OOCYTES INJECTED WITH WATER

General Linear Model Procedure

Dependent variable: Glycylsarcosine (pmols/oocyte·1h)

Source	DF	Sum of squares	Mean squares	F value	PR > F
Model	11	187444563.1	17040414.8	71.13	.0001
Error	52	12456855.4	239554.9		
Corrected total	63	199901418.5			

r^2	CV	SD	Glycylsarcosine Mean
.94	27.9	489.4	1752.3

Source	DF	Sum of squares	Mean squares	F value	PR > F
Conc	5	82394898.9	36478979.8	152.3	.0001
Group	1	6844658.1	6844658.1	28.57	.0001
Conc*Group	5	2707302.1	541460.4	2.26	.0619

Tests of hypothesis using the Type III MS for Trt*Conc*Group

Contrast					
Linear Conc	1	176894556.9	176894556.9	453.32	.0001
Quadratic Conc	1	168391.1	168391.1	.43	.5138
Cubic Conc	1	165492.5	165492.5	.42	.5175

Example C.6. COMPARISON OF DNA OLIGOMER INHIBITION OF SIZE-FRACTIONATED SHEEP OMASAL POLY(A)$^+$ RNA-INDUCED GLYCYLSARCOSINE UPTAKE[1] USING TUKEY'S MULTIPLE RANGE TEST

Treatment	n	mean	Treatment comparison	SD[2]	TK[3]	Difference in means	Difference significant?
1 = Water	9	1239	1 to 2	186	470	777	yes
2 = Antisense	8	462	1 to 3	201	510	175	no
3 = Sense	6	1414	2 to 3	206	522	952	yes

MSE[4] = 146049
r = 3.58; (.05, 3, 22)

[1]pmols/oocyte·1h
[2]SD = square root [MSE $1/k_i + 1/j_i$)]
[3]TK value = (SD * r)/ square root [2]
[4]From General Linear Model procedure (F value: = 13.21; Pr > F = .0002; r^2 = .60)

VITA

James Clyde Matthews, son of Ann Orr and Griffith C. Matthews, was born May 26, 1958, in Princeton, New Jersey. He married Elizabeth A. Lehman on September 7, 1981, in East Waterboro, Maine. He was graduated from Rutgers, The State University of New Jersey, with a Bachelor of Science degree in Animal Science in December, 1988. He was graduated from Virginia Polytechnic Institute and State University with a Master of Science degree in Ruminant Nutrition, in November, 1991, where he initiated his Ph.D. program of study in Ruminant Nutrition in December, 1991. He received financial support from the John Lee Pratt Animal Nutrition Program for three years and was the recipient of a 1993 Carl S. Akey Nutrition Scholarship.

He is a member of The American Society Of Animal Science, American Institute of Nutrition, Phi Sigma Biological Honor Society, and Gamma Sigma Delta, The Honor Society of Agriculture.