LYSINE AND METHIONINE TRANSPORT BY BOVINE JEJUNAL AND ILEAL BRUSH BORDER MEMBRANE VESICLES

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(ABSTRACT)

Purified brush border and basolateral membranes were isolated from homogenized intestinal enterocytes of Holstein steers by divalent cation precipitation followed by differential and sucrose density gradient centrifugation. Membrane marker enzymes were used to determine the effectiveness of the fractionation procedure. Alkaline phosphatase and sodium-potassium adenosine triphosphatase served as the marker enzymes for the brush border and basolateral membranes, respectively. The brush border fraction was enriched 5.1-fold over the cellular homogenate. Purification of 10.1-fold over cellular homogenate was obtained for the basolateral membrane fraction. Electronmicrographs and osmotic response data were used to confirm the vesicular nature of the membrane

preparations. Brush border membrane vesicles from bovine jejunal and ileal tissue were used to evaluate lysine (LYS) and methionine (MET) transport. Total transport of LYS and MET was divided into mediated and diffusion components. Mediated uptake was further divided into sodium dependent (Na^+) and sodium independent (Na^-) systems. Total LYS and MET uptake by ileal tissue tended to be higher than jejunal tissue at all concentrations evaluated but differences were significant ($P<.05$) at 2.5 and 7.5 mM for LYS and 5, 12.5 and 15 mM for MET. The greater capacity of ileal tissue appeared to be due to the Na^+ component of LYS uptake and the diffusion component of MET uptake. Methionine transporters had lower affinities and higher capacities than the corresponding LYS transporters in both ileal and jejunal tissue. Methionine transport was greater ($P<.05$) than LYS transport in both ileal and jejunal tissue when initial amino acid concentration was 7.5 mM. When initial amino acid concentration was 1.25 mM, MET uptake was greater ($P<.13$) than LYS uptake in jejunal but not ileal tissue. The relative contribution of mediated and diffusion uptake systems to total MET and LYS uptake was found to be dependent of substrate concentration.

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Chapter I

Introduction

Intensive management programs and genetic progress have resulted in elevated production levels of the ruminant species, including more efficient production of meat and milk. To support the production demands, it has become common practice to increase the amount of concentrate and decrease the amount of roughages utilized in the diets of intensively managed ruminants (Blaxter, 1973). This practice takes the ruminants out of their traditional role as forage utilizers and places them in direct competition with the more efficient nonruminants for feedstuffs. The efficiencies of feed to food conversion for beef steers, finishing lambs, hogs and fish have been reported to be in the order of 10:1, 9:1, 4.9:1 and 1.6:1, respectively (Ensminger, 1969). To justify these feeding practices, improvements in the efficiency of protein utilization by ruminants are essential.

Increased performance in ruminant species has been attained through the feeding of by-pass proteins and rumen protected amino acids. The realization that it is feasible to increase the quantity and quality of proteins reaching the small intestine of ruminants has spurred interest in this area of ruminant protein nutrition. Research that has arisen in this area includes processing of feedstuffs and

rumen manipulation, both of which are designed to increase the quantity and possibly the quality of protein reaching the small intestine. By improving the quality or balance of amino acids reaching the small intestine, the animal can more efficiently utilize the protein component of the diet.

The composition of a given combination of amino acids presented to the small intestine will not necessarily be the same as the combination of amino acids absorbed into the enterocyte, since individual amino acids may differ in their rate of uptake and competition may exist between amino acids for uptake at the brush border surface. The characterization of the transport systems regulating uptake at the brush border surface will allow nutritionists to better assess the protein quality of ruminant diets.

The in depth evaluation of amino acid transport properties requires a suitable investigative technique. In vivo methods such as arterio-venous differences and substrate disappearance from intestinal segments have proven too complex to delineate transport mechanisms. The interpretation of in vitro methods such as mucosal scrapings and everted intestinal sacs are complicated by cellular metabolism, compartmentalization and multiple membrane types. The use of the membrane vesicle technique alleviates these complications and allows for the determination of transport mechanisms.

Methionine and lysine have been implicated as the first limiting amino acids for production in ruminant species on maize based diets. Therefore, this study was designed to provide basic information on the mechanisms and site of methionine and lysine transport by the bovine small intestine.

Chapter II

Review of Literature

General Amino Acid Absorption. Production responses dependent on protein accretion are determined by the availability of amino acids at the cellular level. The cellular supply of amino acids is dictated by the rate of absorption from the lumen of the gastrointestinal tract into the circulation and removal of amino acids from circulation by peripheral tissue. Amino acid nutrition at the cellular level has been reviewed by Christensen (1982) and Baumrucker (1985). The remainder of this literature review concentrates on intestinal amino acid transport.

Essentially all the early work on characterizing intestinal amino acid transport occurred in nonruminant species. One of the earliest theories on protein absorption was the Liehig theory (Van Slyke, 1917). This theory stated that proteins found in food were incorporated directly into tissue. This theory persisted even though evidence that proteins were digested in the gastrointestinal tract was available. As stated in a review by Matthews (1975) the digestion of protein into free amino acids in the intestinal lumen was recognized in 1865 by Kolliker and Muller. The discovery of trypsin and erepsin by the early 1900's made it

clear that proteins were at least partially hydrolyzed to amino acids.

Early laboratory procedures could not detect amino acids and peptides in portal blood and this led to two theories being developed as to the fate of amino acids and peptides. It was first believed that amino acids and peptides were broken down into ammonia and nonnitrogenous residues, while the second theory stated that proteins were synthesized in the intestinal wall (Van Slyke, 1917). These theories latter were proven false when analytical procedures were improved. The classical belief has been that proteins are completely hydrolyzed into amino acids and are then absorbed into the hepatic-portal blood as amino acids (Matthews, 1975). This theory was prominent even though there was no evidence that peptides were not absorbed in addition to the free amino acids. Newey and Smyth (1960) proposed the presently accepted theory of protein absorption. The theory states that amino acids, dipeptides and tripeptides are all absorbed across the brush border membrane. The peptide fractions are then hydrolyzed within the epithelial cell and free amino acids are released into the bloodstream. Although absorption of dipeptides and tripeptides has become an accepted fact, the fate of the peptide once in the enterocyte is still under dispute. For a review of intestinal peptide transport, the reader is referred to Matthews (1975) and Webb (1986).

The mechanism of amino acid absorption was first thought to be simply passive diffusion. Reports that rates of absorption between L-isomers and racemic mixtures of amino acids were not different (Chase and Lewis, 1934) and the finding that absorption of amino acids from the intestinal lumen into lymph vessels and capillaries followed the law of diffusion (Bolton and Wright, 1937), supported the passive diffusion theory.

An early contradiction of the passive diffusion theory was presented by Hober and Hober (1937). They found that the percentage of amino acid absorbed by the small intestine decreased with greater amino acid concentrations in the gut, thus indicating saturation of the system and suggesting the presence of a mediated transporter. Gibson and Wiseman (1951) showed the rates of disappearance of the L-isomers of 13 amino acids to be greater than that of the D-isomers. These data provided evidence for a specific absorption mechanism for the L-amino acids. Studies which demonstrated a transporter preference for L-amino acids and concentrative uptake of L-amino acids which was sensitive to metabolic inhibitors provided further proof of mediated and active transport of amino acids by the small intestine (Agar et al., 1953, 1954, 1956; Wiseman, 1953, 1955, 1956).

The first viable explanation for the mechanisms of Na-dependent active transport was presented by Crane (1961). The Crane hypothesis originally explained the active

transport of glucose but was extended to amino acid active transport by Curran et al. (1967). This theory proposed that the accumulation of amino acids against a concentration gradient was driven by the energy potential of a Na gradient. For this to occur the carrier complex of the active transport system must cotransport a Na ion and an amino acid. The Na gradient, high extracellular and low intracellular Na concentration, is maintained by Na^+/K^+ ATPase located in the basolateral membrane of the enterocyte (Quigley and Gotterer, 1969; Fujita et al., 1972). Unlike free amino acids, the active transport of peptides is thought to be coupled to a proton gradient (Ganapathy and Leibach, 1985). The proton gradient is thought to be maintained by a Na^+/H^+ exchanger in the brush border membrane.

Classification of amino acid transport systems is based on substrate preference. Transport by a particular system seems to be determined by size, charge and(or) configuration of amino acid side chains. However, amino acids with quite diverse structures often share a transport system. Christensen (1984) listed 12 amino acid transport systems which are suggested to occur in animal tissue. The A, ASC, L and Y^+ systems have been identified in both epithelial and nonepithelial tissues. The Gly, N, ß, L_1, T, X^-_{AG}, X^-_A and x^-_G systems have been identified in nonepithelial tissue but not in epithelial tissues to date.

Stevens et al. (1984) has identified three additional transport systems (NBB, PHE, and IMINO) in the intestinal mucosa. For further information on amino acid transport systems, the reader is referred to Christensen (1975, 1982, 1984) and Stevens et al. (1984). Since the present research was concerned with methionine and lysine uptake by bovine small intestine tissue only, intestinal transport systems for these two amino acids will be reviewed.

The two Na-dependent systems found in intestinal tissue are the A and ASC systems (Stevens et al. 1984). These two systems are primarily responsible for neutral amino acid transport (Guidotti et al., 1978). The ASC system prefers neutral amino acids with hydroxyl or sulfur containing side chains. Among those amino acids carried are alanine, serine and cysteine, the amino acids from which the name is derived. The A system prefers amino acids with linear side chains (Stevens et al. 1984). The ASC system is insensitive to pH changes and intolerant of N-methyl groups on substrates while the A system is very sensitive to pH and tolerant of N-methyl groups on substrate (Guidotti et al., 1978; Christensen, 1984).

Two Na-independent systems have been identified, the cationic system (Y^+) and the neutral L system. The Y^+ system is very specific for amino acids with a positively charged side group such as lysine, arginine and histidine (White and Christensen, 1982). The Y^+ system is pH

independent, stereoselective and capable of bidirectional transport. The Y^+ system is capable of concentrative uptake but the energy source has not yet been identified, it is not derived from the sodium gradient (White et al., 1982).

The neutral L system is both pH and Na insensitive and operates by an exchange mechanism (Oxender and Christensen, 1963; Oxender et al., 1977). The system functions by exchanging intracellular amino acids for extracellular amino acids. The neutral L system transports neutral branched chain amino acids and aromatic amino acids such as leucine, valine and tyrosine (Christensen, 1984). This system is not capable of concentrative uptake and its apparent function is to maintain the quality or balance of the intracellular amino acid pool (Oxender et al., 1977). The neutral L system has also been implicated to serve an osmoregulatory function (Tramacere et al., 1984).

The transport of amino acids by intestinal enterocytes occurs by simple diffusion, facilitated diffusion and active transport (Stevens et al., 1984). The quantitative significance of each route is dependent on the concentrations of substrate present. Active transport is generally the most quantitatively important route of amino acid uptake when amino acid concentration is low. Diffusion makes a progressively larger contribution to

total uptake as substrate concentration increases (Stevens et al., 1984).

Two traits common to all known mediated transport systems are that they are saturable and that competition for uptake among amino acids occurs. The competition among amino acids for uptake is utilized to identify individual transport systems. Competition may determine the extent to which amino acids are absorbed by the small intestine and made available to the whole animal. It has been clearly demonstrated that amino acids do compete for intestinal transport (Matthews and Laster, 1965). Competition for transport has been seen between amino acids which share and those which do not share the same transporter (Munck, 1972). The effect of competition on intestinal absorption and amino acid availability to the tissue is unclear at this time. Patterson et al. (1981) speculated that the low basic amino acid transport capacity of the small intestine could be partly due to the interaction between the basic and neutral amino acids at the brush border membrane. Understanding the impacts of these potential interaction may allow producers to adjust the diets of meat animals to result in a more efficient usage of protein resources.

Amino Acid Absorption by Ruminants. Research to determine the mechanism of absorption and amino acid requirements of ruminant species has not keep pace with

similar research in nonruminant species. This may be partially attributed to the landmark work of Loosli et al. (1949) which demonstrated that rumen microbes could synthesize all the essential amino acids from non protein nitrogen (NPN) and an energy source. The conclusion drawn was that ruminants had no dietary requirement for essential amino acids and therefore dietary protein quality was not a nutritional concern.

Subsequent research showed greater growth rates and feed efficiencies were obtained with steers feed a soy-supplemented ration compared to NPN ration containing the same crude protein levels (Oltjen and Putman, 1966). Similar production responses were demonstrated by Virtanen (1966) in dairy cows when an oat and silage diet was compared to a NPN diet. Little and Mitchell (1967) found greater responses to post-ruminal supplementation of quality preformed proteins than to rumen infusions of the same proteins. These findings and those from numerous other studies led researchers to conclude that quantity and quality of dietary protein which escapes the rumen will directly influence production levels of the ruminant species.

In an effort to increase production with protein nutrition, bypass protein became a popular research area. These research efforts were primarily directed toward increasing the protein quantity reaching the small

intestine by decreasing the degradability of the protein supplement in the rumen. The use of naturally occurring bypass proteins such as fish meal combined with chemical and physical treatments (heating, formaldehyde, aldehyde and tannin) of feedstuffs to improve bypass qualities have been investigated (Chalmers et al. 1964; Sherrod and Tillman 1964; Clark et al., 1974; Schmidt et al., 1974; Driedger and Hatfield; 1972). These procedures have been successful in improving N retention and milk protein production in a variety of ruminant species.

The ability to elicit a production response by increasing the protein quality reaching the small intestine depends on the level of metabolic demand for protein synthesis and the ability to determine and supply the limiting amino acid(s). The first limiting amino acid may be determined by infusing graded levels of individual and combinations of amino acids into the abomasum. The first limiting amino acid of sheep fed a typical maize based diet was found to be methionine (Nimirick et al., 1970), while methionine and lysine were found to be co-limiting in growing steers (Fenderson and Bergen, 1975; Richardson and Hatfield, 1978). Methionine has been implicated as the first limiting amino acid for lactation (Hogan, 1975). Supplementation of ruminant diets with rumen protected (RP) methionine, RP lysine and a mixture of RP methionine and RP lysine resulted in mixed success in improving growth and

milk production (Kreuzer and Kirchgessner, 1986; Martin et al., 1986;Oke et al., 1986; Rogers et al., 1987; Casper et al., 1987; Wright and Loerch, 1988). Generally, basal diets were of adequate protein quality for animals in a stage of production requiring low levels of protein accretion including finishing steers or cows in late lactation. Production responses were seen in situations where tissue demand exceeded dietary supply and amino acids became limiting to production. In these cases, supplying the limiting amino acid(s) elicited a production response. This situation is prevalent in growing steers and high-producing dairy cows. The importance of providing appropriate quantity and quality of protein to the ruminant will likely become magnified with the use of beta agonist agents and bovine somatotropin, both of which increase protein accretion (Bauman et al., 1982; Ricks et al., 1984; Hanrahan et al., 1986).

Research efforts in ruminant protein nutrition have classically concentrated on the supply of amino acid to the small intestine, i.e., bypass protein and RP amino acids. There has been relatively little research to determine the site and properties of amino acid absorption or the interaction among the amino acids for absorption.

In ruminants, the small intestine is considered to be the primary site of amino acid absorption. However, research has indicated that the rumen is permeable to amino

acids (Demaux et al., 1961; Cook et al., 1965) and evidence for the presence of amino acid transport systems was obtained by Liebholz (1971) using in vitro preparations of rumen epithelium. The ability to induce increased amino acid transport activity under nutrient stress served as a further indication that amino acid transporters were present. Despite the presence of amino acid transport, it is assumed that ammonia is the major N form absorbed from the rumen and, under normal conditions, most of the amino acid absorption occurs in the small intestine (Liebholz, 1971; Klouster and Bochholt, 1972).

Intestinal amino acid absorption in ruminant species has been studied with both in vivo and in vitro techniques. The in vivo techniques utilized have included intestinal cannulation (Coelho Da Silva et al., 1972; Phillips et al., 1979; Prange et al., 1984; Santos et al., 1984; Stern et al., 1985) and arterio-venous differences (Hume et al., 1972; Sniffen and Jacobson, 1975; Tagari and Bergman, 1978; Koeln, 1982). In vitro techniques include whole tissue strips (Johns and Bergen, 1973), everted gut sacs (Phillips et al., 1976), mucosal scrapings (Guerino and Baumrucker, 1987a) and brush border membrane vesicles (Moe et al., 1985; Crooker and Clark, 1986).

The majority of intestinal cannulation techniques have been utilized to test the effects of diet on amino acid composition reaching the small intestine and subsequent

disappearance of these amino acids. The apparent absorption of amino acids reaching the small intestine under a variety of dietary conditions has ranged from 58 to 77% (Chapula, 1984). The amino acid supply reaching the small intestine is generally higher than the amounts found in the diet. This is especially apparent with lysine and methionine. This is explained by the fact that the profile of amino acids entering the small intestine resembles the profile of amino acid in microbial protein (Chalupa, 1984). In fact, microbial protein represents 60 to 80% of the total amino acid N reaching the small intestine under normal circumstances (Storm et al., 1983). The amino acid composition of rumen microorganisms has been shown to remain constant under a large number of dietary conditions (Harrison et al., 1973; Keyser, 1976; Storm and Orskow, 1983).

Phillips et al. (1979), using double reentry cannulated sheep, investigated intestinal uptake of threonine, methionine and valine. The test solutions infused contained combinations of all three amino acids over a range of concentrations, .56 to 8.44 mM. They found that one amino acid could both inhibit and stimulate the uptake of another amino acid depending on the concentration of the third amino acid. High levels of valine stimulated methionine uptake and high levels of threonine stimulated valine uptake when the third amino acid was at low levels.

Methionine inhibited both valine and threonine uptake. This experiment illustrates the complex nature of amino acid absorption in the ruminant small intestine.

The arterio-venous difference technique has been used to identify amino acid transport systems. Sniffen and Jacobson (1975), using correlations of amino acid absorption, demonstrated the presence of at least one neutral L-type system and a cationic transport system. There were 39 significant correlations between amino acids. They suggested that this indicated a great deal of transport system sharing between amino acids. The presence of competition for absorption between neutral and cationic amino acid was demonstrated by Hume et al. (1972). The abomasal infusion of leucine resulted in elevated portal blood concentrations of leucine and decreased levels of lysine. Johns and Bergen (1973) also demonstrated leucine inhibition of lysine uptake using ovine intestinal strips. The large demand of intestinal tissue for protein synthesis and energy metabolism may be indicated by the findings of Tagari and Bergman (1978) who demonstrated that only 30 to 80% of the amino acids absorbed could be accounted for in the portal blood.

There have been relatively few in vitro experiments on intestinal amino acid uptake with ruminant tissue. Johns and Bergman (1973) and Phillips et al. (1976), using intestinal strips and everted sacs, respectively,

demonstrated that ileal tissue was the most active and efficient site of intestinal amino acid absorption. These findings were confirmed by Guerino and Baumrucker (1987a), using incubations of intestinal mucosal scrapings. In vivo confirmation of the superior transport ability of ileal versus jejunal tissue was obtained by Phillips et al. (1979). These findings demonstrated that physiological differences may occur between ruminant and nonruminant species with regard to amino acid transport since the jejunum appears to be the region of most active amino acid transport in nonruminants (Wiseman, 1974).

Johns and Bergen (1973) investigated the absorption of glycine, lysine and methionine in the ovine small intestine. The affinity and maximal velocity for the transport systems of each of these amino acids was determined using the intestinal strips technique. The ranking of the relative rates of transport indicated that the uptake of methionine > lysine > glycine. Guerino and Baumrucker (1987a) showed bovine intestinal tissue also had a greater capacity for methionine than lysine transport.

Moe et al. (1987) using brush border membrane vesicles of the bovine demonstrated that lysine, alanine, methionine, phenylalanine and proline all had Na-dependent components of their total uptake. The diffusion component was found to account for a large proportion, 33 to 62%, of the total uptake at a substrate concentration of .1 mM. A

proline transport system independent from the neutral amino acids was identified and methionine was found to be a potent inhibitor of the uptake of all the other amino acids. Alanine uptake by a Na-dependent transporter was also demonstrated by Crooker and Clark (1987). In addition, Crooker and Clark (1987) demonstrated the presence of at least two Na-dependent transport systems capable of alanine uptake. All of the alanine Na-dependent systems were shared by methionine, valine and isoleucine, while glycine shared at least one but not all of these transport systems. Stingelin et al. (1986) demonstrated a clear Na-dependence for leucine uptake but only a slight Na-dependence for lysine and glutamate in ovine duodenal strips.

Guerino and Baumrucker (1987b) investigated lysine and methionine uptake by bovine mucosal scrapings. Lysine uptake occurred primarily by a Na-independent system. The relative uptake of Lysine at .1 mM was 63% Na-independent, 17% Na-dependent and 20% by diffusion systems. The relative methionine uptake at .1 mM was found to be 54% Na-independent, 43% Na-dependent and 25% by diffusion systems, and the total of 122% indicated some error involved in the estimation. Physiological concentrations of inhibitors decreased lysine uptake but not methionine uptake, indicating that bovine small intestine had a higher capacity for methionine than lysine.

The effects of pH on lysine, leucine and glutamate transport were investigated in ovine duodenal tissue by Stingelin et al. (1986). Lysine uptake was depressed while glutamate uptake was stimulated at pH 4 compared to pH 5.5 or 7. Leucine uptake was not affected by changes in pH. Both the depression and stimulation of uptake were likely due to changes in diffusion properties of the amino acid. Permeability of lysine in brush border membranes would be depressed since at pH 4, lysine would be positively charged and hydrophilic, while the charge of glutamate would approach neutrality at pH 4 and be more hydrophobic, thus increasing the permeability of glutamate. The primary route of absorption of lysine appeared to be diffusion while leucine and glutamate utilized saturatable uptake mechanisms at pH 4.

Membrane Vesicle Studies. To accurately and efficiently characterize nutrient transport properties, suitable experimental techniques are required. Historically, the in vitro techniques such as intestinal rings, everted sacs, ligated intestinal segments and isolated mucosal cells have allowed for partial characterization of nutrient transport (Fisher and Parsons, 1949; Agar et al., 1954; Wilson and Wiseman, 1959; Kimmich, 1975; Phillips et al., 1976). These techniques have limitations in that cellular metabolism is occurring, and

that the experimenter cannot regulate the intracellular substrate and electrolyte concentration gradients. In order to develop an understanding of the complex multicompartmented and multicomponent transcellular transport systems of the intestinal epithelial tissue, the complex system needs to be dissected into its components. In this way the behavior of the whole system might be explained by studying the properties of the individual components under well defined conditions.

Kaback (1960) pioneered the first attempts in this area by demonstrating the transport of amino acids into ghost bacterial cells. This landmark research was the first to show that fractured membranes would reform in a vesicular nature, free of cytoplasmic components and still retain their transport capabilities. Subsequently, researchers have not only isolated the plasma membranes of enterocytes but they have been able to separate this heterogeneous plasma membrane into its membrane fractions due to the differing compositions of the membranes. The different membrane compositions (different ratios of protein and lipids) resulted in differences in buoyant densities, surface properties such as different surface receptors, surface charge density and surface hydrophobicity (Murer and Kinne, 1980). The brush border and basolateral membranes also resist shearing forces to varying degrees and therefore form different sized

fragments when the enterocytes are homogenized (Murer and Kinne, 1980). Researchers have been able to use these physical differences between brush border and basolateral membranes to isolate each of these membrane fractions independent of the other. The purity of the membrane fractions are monitored using marker enzymes, which are unique to the membrane in question. Disaccharidase and alkaline phosphatase serve as the marker enzymes for the brush border membrane (Eicholz and Crane, 1974) and Na^+/K^+ATPase is the marker for the basolateral membrane (Stirling, 1972; Mircheff and Wright, 1976).

Miller and Crane (1961) were the first to apply these techniques to intestinal epithelial cells. The goal of their study, as with most early studies, was isolation of brush border membrane vesicles. The procedure applied was cell homogenization in the presence of hypotonic EDTA solution followed by differential centrifugation. Modifications of this procedure have been used by Forstner et al. (1968) and Hopfer et al. (1973) to isolate purified brush border membrane vesicles. Eicholz (1969) used a similar procedure which called for cell disruption in tris buffer followed by density gradient centrifugation. All of these techniques relied on the membranes differing response to shearing forces which results in brush border membranes forming larger vesicles than the basolateral membranes. Therefore the two vesicle populations sediment at different

rates. Unfortunately, these vesicle preparations were plagued by contamination with internal membranes.

The method presently used to separate brush border from basolateral and internal membranes is the differential precipitation technique. This technique developed by Schmitz et al. (1975) utilizes the divalent cations of Mg^{++} and Ca^{++} to precipitate internal membranes. The difference in the charge density of the cellular membrane allows for the divalent cation to cross link the membranes of intracellular organelles and basolateral membranes but not brush border plasma membranes. This allows for the isolation of brush border membrane vesicles while minimizing the cross contamination of other cellular membranes (Schmitz et al., 1975; Kessler et al., 1978). Other methods applied to isolate purified brush border membrane vesicles include free-flow electrophoresis (Murer and Kinne, 1980) and thiocynate treatment followed by differential centrifugation (Hopfer et al., 1983).

Early work to isolate and purify basolateral membrane vesicles was carried out in the laboratories of Douglas et al. (1972) and Fujita et al. (1972). These scientists utilized differential precipitation followed by differential centrifugation with a final purification step of density gradient centrifugation to achieve purified basolateral membrane vesicles. Free-flow electrophoresis, which takes advantage of differences in electrophoretic

mobility, is also used to separate basolateral membrane vesicles from brush border and intracellular organelles (Murer et al. 1974)

The use of these isolated basolateral and brush border membrane vesicles in transport studies has great advantages over procedures utilizing intact tissue in that the substrate composition of the external and internal regions of the vesicles can be manipulated. In order to take advantage of this capability the orientation of the vesicles needs to be known. The use of morphological, enzymatic and immunological techniques has revealed that brush border membrane vesicles are generally right side out or, in other words, the same orientation as found in the intact epithelial cells (Haas et al. 1978).

The determination of the orientation of the basolateral membrane has proved difficult. The basolateral membrane, unlike the brush border, lacks easily identifiable morphological characteristics and unique enzymes which would allow for the determination of whether the internal or external surface of the basolateral membrane is oriented outward in the vesicles. Del Castillo and Robinson (1982) found basolateral membranes from guinea-pig to be 60 % inside out vesicles and 40 % membrane sheets. Determination of the orientation of basolateral membranes in other laboratories has not been reported.

The uptake of solutes by membrane vesicles is most often studied by the rapid filtration techniques (Hopfer et al. 1973 and Kaback, 1974). The earliest time point which can be evaluated consistently with this technique is after 5 s of incubation. This technique can be used to measure uptake of a solute or efflux of a solute after preloading of vesicles. Column chromatography has also been used to separate vesicles from extravesicular medium following the incubation period. The major drawback to this procedure is the susceptibility of vesicles to solute leakage during separation from the extra vesicular fluid.

Before the transport systems of the intestinal epithelial tissue could be investigated with isolated membrane vesicles, researchers needed to be certain that the permeability properties of the membrane were maintained in the resealed vesicles. This was accomplished by monitoring changes in fluorescence of dyes as the electrical potential across the membrane changed as transport occurred (Burckhardt et al., 1980). Proton sensitive dyes and arenazo III have been used to monitor H^+ and Ca^{++} fluxes, respectively (Sachs, 1977; Yingst and Hoffman 1978). Transport phenomena can also be monitored by measuring changes in extravesicular ion concentration with ion selective electrodes (Murer et al., 1977). All of these techniques have been used to demonstrate that the

permeability properties of the membrane remain intact when membrane vesicles are formed.

Before using isolated brush border and basolateral membranes in transport studies it is important to demonstrate that these membranes are of vesicular nature. Hopfer et al. (1973) demonstrated vesicular nature by showing the vesicles were osmotically active. The amount of substrate uptake was decreased with increasing osmolality of the incubation buffer; therefore, indicating a decrease in intravesicular volume. If uptake was unaltered by osmolality then it could be concluded that binding was occurring on the surface membranes, instead of transport into the intravesicular compartments.

The membrane vesicle techniques are limited in that quantitative comparisons with intact epithelial preparations are not possible, since the membrane surface areas of both types of preparation are unknown. Research which compared the Na-dependent transport properties of brush border membrane vesicles isolated from different intestinal segments found a correlation to the transport activities of these segments in vivo (Hopfer et al., 1976; Kessler et al., 1978; Lucke et al., 1978; and Murer et al., 1980). The factors which change transepithelial phosphate transport in vivo have been demonstrated to alter the Na-dependent transport of inorganic phosphate into brush border membrane vesicles. Therefore, it appears that

membrane vesicles are a valid model for qualitative comparisons such as Na effect of kinetic parameters, stereospecificity, dependence on membrane potential and segmental differences of epithelial transport.

The use of plasma membrane vesicles isolated from intestinal epithelial tissue in transport studies offers several advantages over the previous methods which utilized intact tissue in vivo or in vitro. Isolation of plasma membrane from both luminal and serosal surfaces of the cell allows for the transport properties of these surfaces to be investigated independent of each other. The removal of cytoplasmic components would allow for the mechanisms of transepithelial transport of metabolizable substrates, such as peptides, to be evaluated. Vesicle preparations allow for manipulation of the composition of intravesicular and extravesicular fluids which is necessary for the complete characterization of the driving forces for the transport systems. The composition of the intravesicular fluid can be adjusted by altering the buffer used in the resuspension of the membrane, through the use of ionophores and by loading vesicles via a preincubation procedure (Schnieder, 1979; Stevens et al., 1982; Ganapathy and Leibach, 1983). By manipulating the driving forces which determine the transport properties of the brush border and basolateral membranes, the site of regulation for transepithelial transport can be defined.

Isolated brush border and basolateral membrane vesicles have been successfully used to evaluate the transport properties of the small intestine and kidney of several nonruminant species (Douglass et al., 1972; Hopfer et al., 1973; Im et al., 1980; Ganapathy et al., 1981; Ling et al., 1981). Only recently has the membrane vesicle technique been applied to the ruminant species. To date three other laboratories have reported successful isolation and characterization of brush border membrane vesicles for use in transport studies (Kaunitz and Wright, 1984; Moe et al., 1985; Crooker and Clark, 1986). Del Castillo and Robinson (1982) reported the simultaneous preparation of basolateral and brush border membrane vesicles in nonruminants, but simultaneous isolation in ruminant species has yet to be reported. Nor has there been a reported successful attempt to isolate and characterize the transport systems of basolateral membrane vesicles from the bovine.

Chapter III

Running head: Brush Border and Basolateral Membrane Vesicles

SIMULTANEOUS ISOLATION AND CHARACTERIZATION OF BRUSH BORDER AND BASOLATERAL MEMBRANE VESICLES FROM BOVINE JEJUNAL AND ILEAL TISSUE

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ABSTRACT

Purified brush border and basolateral membranes were isolated from homogenized intestinal enterocytes of Holstein steers by divalent cation precipitation followed by differential and sucrose density gradient centrifugation. Membrane marker enzymes were used to determine the effectiveness of the fractionation procedure. Alkaline phosphatase and Na/K adenosine triphosphatase served as the marker enzymes for the brush border and basolateral membranes, respectively. The brush border fraction was enriched 5.1-fold over the cellular homogenate. Purification of 10.1-fold over cellular homogenate was obtained for the basolateral membrane fraction. Electron micrographs obtained with transmission electron microscopy confirmed the vesicular nature of the membranes and revealed that basolateral membrane vesicles were generally smaller and more irregular in shape than brush border membrane vesicles. The response of equilibrium methionine accumulation by membrane preparations to increasing osmolality of the incubation buffer confirmed the vesicular configuration of the isolated membranes. The enrichment of brush border and basolateral membrane fractions compared to the initial homogenate and the vesicular configuration of both preparations indicate that the isolated brush border and

basolateral membrane preparations were suitable models for evaluating nutrient transport properties of bovine small intestine. With respect to the number of transport experiments possible per animal, the membrane vesicle technique was found to be many times more efficient than more conventional in vitro techniques, i.e. intestinal rings or everted sacs.
(Key Words: Brush Border, Basolateral, Bovine, Vesicles, Jejunum, Ileum.)

Introduction

The basis for the majority of information on the mechanisms of nutrient transport has arisen through the use of in vitro techniques. These techniques have largely been applied to nonruminant laboratory species. Consequently, the knowledge of the mechanisms of transport in the nonruminant species is substantially more extensive than that of the ruminant species.

In order to accurately and efficiently characterize nutrient transport properties, suitable experimental techniques are required. Historically, the in vitro techniques such as intestinal rings, everted sacs, ligated intestinal segments and isolated mucosal cells have allowed for partial characterization of nutrient transport (Fisher and Parsons, 1949; Agar et al., 1954; Wilson and Wiseman,

1959; Kimmich, 1975; Phillips et al., 1976). These techniques have limitations in that cellular metabolism is occurring, and that the experimenter cannot regulate the intracellular substrate and electrolyte concentration gradients.

The recent use of isolated membrane vesicles has overcome some of these problems and has given researchers the ability to characterize nutrient transport systems at both the luminal and serosal side of the mucosal lining. The transport characteristics of the luminal surface of the enterocyte are measured with brush border (BB) membrane vesicles. Transport characterization of the serosal surface is determined with basolateral (BL) membrane vesicles. Isolation of plasma membrane from both the luminal and serosal surfaces of the cell allows for the transport properties of these surfaces to be investigated independent of one another (Stevens et al., 1984).

The removal of the cytoplasmic components allows the mechanisms of transepithelial transport of metabolizable substrates, such as peptides, to be evaluated. By manipulating the driving forces, such as ion gradients, which determine the transport properties of the BB and BL membranes, the site of regulation for transepithelial transport can be defined (Hopfer, 1977).

Isolated BB and BL vesicles have been successfully used to evaluate the transport properties of the small

intestine and kidney of several nonruminant species (Douglas et al., 1972; Hopfer et al., 1973; Im et al., 1980; Ganapathy et al., 1981; Ling et al., 1981). Only recently has the membrane vesicle technique been applied to the ruminant species. Three other laboratories have reported successful isolation and characterization of BB membrane vesicles for use in transport studies (Kaunitz and Wright, 1984; Moe et al, 1985; Crooker and Clark, 1986). Del Castillo and Robinson (1982) reported the simultaneous preparation of BL and BB membrane vesicles in nonruminants, but simultaneous isolation in ruminant species has yet to be reported. In order to study the mechanisms involved in transport of organic solutes across intestinal epithelia, from the lumen to the circulation, both BB and BL membranes must be isolated. Simultaneous isolation of BB and BL membranes minimizes treatment differences that could occur during processing. Tissue is also conserved with simultaneous isolation since both the BB and BL membranes of the initial enterocyte are isolated.

These experiments were conducted to develop a simple and rapid procedure for the isolation of functional BB and BL membrane vesicles from the same starting material.

Materials and Methods

Animals. Holstein steers weighing an average of 400 kg were used as the tissue donors for the experiments. Steers were fed to gain .9 kg a day. The diet consisted of 50% ground corn (IFN 4-02-931), 30% orchardgrass hay (IFN 1-03-438), 13.3% soybean meal (IFN 5-04-604), 5% molasses (IFN 4-04-696), .42% deflourinated rock phosphate (IFN 6-01-780), .78% limestone (IFN 6-02-632) and .5% trace mineral salt on an as fed basis, until 12 h before slaughter. A total of 14 steers were used to develop various aspects of the vesicle isolation procedures but the data presented here are based on the evaluations of combined tissue from three steers.

Tissue preparation. The procedure for isolation of membrane vesicles is a modification of that from Moe et al. (1985) for bovine intestine. Steers were mechanically stunned then killed by exsanguination. Viscera were removed from steers within 12 min of stunning. Small intestine was removed beginning 2 m distal of the pyloric valve and proceeding distally to 2 m proximal of the ileocecal junction. The isolated intestinal section was divided in half by doubling the section back on itself and cutting the jejunum (proximal half) free from the ileum (distal half). Intestine was cut into 1 m segments and flushed free of digesta with buffer containing 300 mM

mannitol and 12 mM tris base, pH was adjusted to 7.4 with HCl (mannitol buffer).

Tissue was maintained at 4°C throughout the procedure unless otherwise specified. The intestinal segments were then everted and incubated in buffer containing 1 mg/ml hyaluronidase , 1 mg/ml bovine serum albumin (BSA), 120 mM NaCl, 20 mM tris base, 1 mM $MgCl_2$ and 3 mM K_2HPO_4 (pH was adjusted to 7.4 with HCl) for 20 min at 37°C. Following incubation, the mucosal lining was harvested by scrapping the intestinal segments with a glass slide. The use of hyaluronidase incubation of the isolated intestinal segments was implemented to separate mucus from luminal lining and to facilitate the harvesting of enterocytes (A.J. Moe et al, personal communication). Residual hyaluronidase was removed from enterocytes by twice resuspending cells in equal volumes of mannitol buffer and centrifuging at 4500 · g for 12 min. All centrifugations were performed at 4°C in refrigerated centrifuges. Isolated enterocytes were divided into 3-4 g aliquots, placed in whirl-pac bags and flash frozen in liquid N_2. Enterocytes were stored at -80°C in an ultralow freezer[1] for future use. The jejunal and ileal mucosal scrapings were kept separate throughout this procedure.

[1]Biofreezer, Forma Scientific, Marietta, OH.

Marker enzymes. Alkaline phosphatase (EC 3.1.3.1) was the marker for brush border (BB) membranes. Activity of alkaline phosphatase (AP) was determined with an enzyme assay kit[2]. Activity was expressed on a per mg of protein basis.

Sodium-potassium adenosine triphosphatase (EC 3.6.1.3) (Na/K ATPase) was the marker for basolateral (BL) membranes. The assay used to measure Na/K ATPase activity was a modification of the procedure of Fugita et al. (1971). The incubation medium, final volume 1 ml, contained 100 mM tris base, 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 3 mM NaEDTA, 3 mM Na_2ATP, with or without 1 mM ouabain (pH was adjusted to 7.4 with HCl and incubation temperature was 37° C). The reaction was initiated by adding .1 ml of membrane suspension to .9 ml incubation buffer and vortexing. A shaking water bath was used for incubation to assure membranes remained suspended. After 30 min, the reaction was terminated by adding 1 ml of ice cold 10% (w/v) TCA and vortexing. Liberated orthophosphate concentration was determined spectrophotometrically by the method of Eibl and Lands (1969). All samples were analyzed in triplicate. The activity of Na/K ATPase was obtained by subtracting ouabain sensitive ATPase activity from total ATPase activity which was measured in the absence of ouabain.

[2]Kit 246, Sigma Chemical, St. Louis, MO.

Activity of Na/K ATPase was expressed on a microgram of orthophosphate per milligram of protein basis.

Moe et al. (1985) and Crooker and Clark (1986) demonstrated that the majority of internal membranes were removed by low speed centrifugation and, therefore, did not present a cross-contamination problem during isolation of BB and BL membrane fractions. Therefore, the specific activities of these membranes were not measured in the present experiments.

Tissue fractionation. The enterocytes were suspended at a concentration of 3 g of tissue per 24 ml of buffer containing 5 mM $MgCl_2$, 150 mM mannitol, 10 mM tris base, 30 mM succinate, 5 mM potassium phosphate and .1 mM $MnCl_2$, pH was adjusted to 7.4 with NaOH (mannitol-succinate buffer) and homogenized with a polytron[3], equiped with a 20 cm diameter probe, for 15 s at setting six. A total of 36 g of tissue were used per preparation. The 36 g of tissue was a composite of twelve g of tissue from three different steers. The tissues of three steers were combined to compensate for animal variation. The BB and BL membranes were then isolated with the use of differential and density gradient centrifugations (Figure 1). The homogenate was incubated for 30 min with gentle stirring to allow Mg^{2+} aggregation of internal (i.e. endoplasmic reticulum,

[3]PT 10/35 polytron, Brinkman instruments, Westburg, NY.

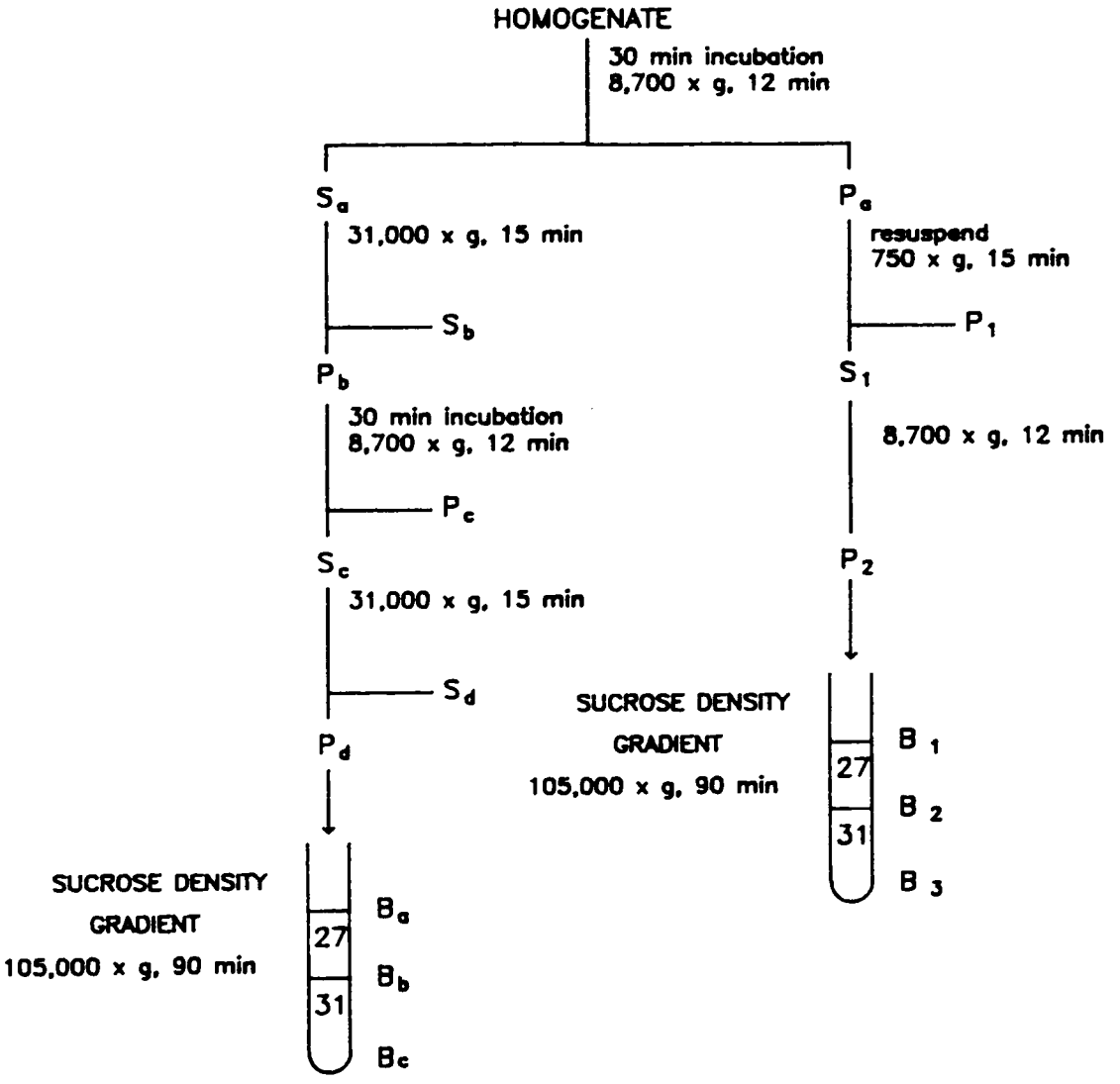

Figure 1. Scheme for the isolation of bovine brush border and basolateral membrane vesicles

lysosomes and mitochondria) and BL membranes (Schmitz et al.,1975; Kessler et al, 1978). After incubation, the homogenate was centrifuged at 8700 · g for 12 min. Through the use of marker enzymes, the resulting pellet (P_a) was found to contain a majority of internal and BL membranes, while BB membranes remained primarily in the supernatant (S_a). Brush border membranes were further enriched by applying the double precipitation technique of Orsenigo et al. (1984). This technique consisted of first harvesting BB membranes from S_a by centrifuging at 31,000 · g for 15 min. The resulting pellet (P_b) was then resuspended in mannitol-succinate buffer with 12 strokes of a teflon-glass homogenizer[4] (.0889 - .1143 mm clearance) and incubating for 30 min with mild agitation. This second Mg^{++} precipitation facilitates the removal of BL membrane cross-contamination. The suspension was centrifuged at 8,700 · g for 12 min to precipitate aggregated membranes into a pellet (P_c). The supernatant (S_c) was then centrifuged at 31,000 · g for 15 min to harvest the BB membranes in pellet (P_d).

The BL membrane fraction, P_a, was resuspended in a buffer containing 272 mM mannitol, 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and 2 mM $MgCl_2$, pH 7.4 (mannitol-transport buffer) with 12 strokes of a teflon-glass homogenizer. The suspension was

[4]Kontes Scientific Glassware, Vineland, NJ.

then centrifuged at 750 · g for 15 min, precipitating the internal membranes into pellet (P_1). The supernatant (S_1) was then centrifuged at 8,700 · g for 12 min yielding the BL membrane fraction in the pellet (P_2).

Pellets P_d and P_2 or BB and BL membrane fractions, respectively, were resuspended in mannitol-transport buffer to a protein concentration of 2-3 mg protein/ml and applied to a sucrose gradient of 27 and 31% sucrose (wt/wt). Sucrose solutions were prepared with buffer containing 4 mM $MgCl_2$ and 4 mM HEPES (pH 7.4, adjusted with NH_3OH). Approximately 5 ml of membrane suspension were applied to density gradients made up of 3.5 ml 27% sucrose solution layered on 3.5 ml 31% sucrose solution and centrifuged at 105,000 · g for 90 min in a IEC model M-60 ultracentrifuge[5]. The resulting bands were then collected by aspirating the membrane band and underlying sucrose layer. Bands were placed either in cryovials[6] and frozen in liquid N_2 or diluted to 40 ml with mannitol-transport buffer and centrifuged at 105,000 · g for 60 min. The resulting washed pellets were resuspended in mannitol transport buffer and used for either marker enzyme analysis or transport experiments. The membranes originally frozen in liquid N_2 were stored at -80 C in an ultralow freezer for future transport experiments. Moe et al. (1985) and

[5]International Equipment Company, Needham Heights, MA.
[6]Nunc cryotubes, Vangard International Inc., Neptune, NJ.

Crooker and Clark (1986) found no difference between the transport properties of fresh and frozen membrane preparations.

Protein. The membrane protein levels were determined using Pierce protein assay reagent[7]. Bovine serum albumen was used to construct the standard curve with a range of 25 to 150 ug of protein. All membrane solutions were diluted prior to assay so that the protein concentration was within the standard range.

Transport assay. Transport experiments were conducted with the membrane filtration technique of Murer et al. (1974) and Kimmich (1975) . Prior to the start of the transport assay, two to three cryovials of sucrose-membrane suspension were removed from -80°C storage and thawed in warm tap water and then suspended in 10 volumes of mannitol-transport buffer and centrifuged for 60 min at 105,000 · g to wash sucrose from the membranes. The number of cryovials used was dependent on the size of the assay; 2 to 3 mg of protein per ml was needed in the final membrane solution. The pellet was resuspended in a volume of mannitol-transport buffer that yielded the desired protein concentration.

[7]Protein Assay Reagent 23200, Pierce Chemical, Rockford, IL.

The composition of the intravesicular space was a function of the buffer used for the resuspension of the membranes. For the purposes of the present experiments, a Na-free buffer was required so that a Na gradient could be formed when the vesicles were added to the Na containing reaction mixture. Mannitol-transport buffer was a suitable buffer for this purpose.

Reaction vessels were prepared by adding 175 ul of transport buffer and 105 ul of methionine solution to the vessel and incubating at 37°C. Transport was initiated by the addition of 70 ul of membrane solution to the reaction vessel. The transport buffer was prepared so that the final incubation medium contained 100 mM NaSCN, 2 mM $MgCl_2$, 10mM HEPES and mannitol at each of the following concentrations: 25, 50, 100, 200 and 300 mM. The pH was adjusted to 7.4 with HCL. The osmolalities of the incubation media were determined with an osmometer[8]. The methionine solution was prepared so that the final methionine concentration would be .1 mM, including 2.5 uCi of ^{35}S-methionine per reaction vessel used as the radiotracer.

Reactions were terminated after 60 min by removing 100 ul aliquots of reaction mixture and placing on a .45 um nitrocellulose filter[9] and applying vacuum. Filters were

[8]Osmette A, Precision Systems Inc., Sudburg, MA.
[9]HAWP .45 uM pore size, Millipore Corporation, Bedford, MA.

then washed with three, 5 ml aliquots of ice cold 150 mM KCl solution. Three, 100 ul aliquots were filtered for each reaction vessel with four reaction vessels per treatment. The filters were air-dried and placed in 20 ml scintillation vials and mixed with 9 ml of Ecoscint scintillation fluid[10]. Radioactivity was determined by liquid scintillation counting[11]. Non-specific radioactivity retention on the membrane filters was determined by applying 30 ul of substrate solution (equivalent to the radioactive methionine present in 100 ul of reaction mixture) to the filter and washing with three, 5 ml aliquots of 150 mM KCl. Filter non-specific binding was subtracted from total activity of all data points prior to calculations of picamoles (pmoles) of substrate retention by vesicles.

L-[^{35}S]-Methionine was obtained from New England Nuclear (Boston, MA). Enzyme substrates and buffers were obtained from Sigma Chemical Co. (St. Louis, MO) and all other chemicals were reagent grade from standard sources.

Microscopy. Both fresh and frozen mucosal scrapings were evaluated with a differential interference contrast microscope[12]. After obtaining a sample of fresh mucosal

[10]LS-271, National Diagnostics, Manville, NJ.
[11]Model SR7500, Beckman Instruments, Palo Alto, CA.
[12]Leitz Ortholux 2, Bunton Instrument company Inc., Rockville, MD

scrapings a subsample was placed in a whirl pac bag and flash frozen in liquid N_2. Frozen tissue was thawed at room temperature. Wet mounts of tissue from fresh and frozen origin were fitted with cover slips and placed under an oil emersion lense for evaluation. Total magnification was 1250.

Electron microscopy. Brush border and BL membrane vesicle preparations were separated from sucrose solution by diluting with ten volumes of mannitol-transport buffer followed by ultracentrifugation at 105,000 · g for 60 min. Supernatant was then decanted and membranes were resuspended in 6 ml of mannitol-transport buffer with ten strokes of a 10 ml teflon-glass homogenizer. The suspension was centrifuged at 31,000 · g for 15 min. The resulting supernatant was decanted and the pellet was fixed for 12 h with 5 ml of buffer containing 5% gluteraldhyde, 3% formaldehyde, 2.75% picric acid and .05 M Na cacodylate (fixation buffer). The pellet was washed twice in .1 M cacodylate in fixation buffer. The pellet was post-fixed in 1 % osmium tetraoxide in fixation buffer. Post-fixation was followed by two washes in fixation buffer, 10 min each. The pellet was then dehydrated through 15, 30, 50, 70, 95 and 100 % ethanol, 15 min each. The pellet was then placed in propylene oxide for 15 min followed by infiltration in

50:50 propylene oxide:polybed 812 resin[13] for 12 h. Infiltration with pure resin was carried out for 8 h and followed by embedding in flat molds. Embedded tissue was allowed to cure for 24 h. Blocks were stained with 2% aqueous urinyl acetate, 12 min, followed by Reynolds lead citrate, 5 min (Reynolds, 1963). Tissue blocks were then sliced with an LKB microtome[14] and evaluated with a JEOL 100 CX stem transmission electron microscope[15]. The procedures used for electron microscopy are that of Dr. Caceci, College of Veterinary Medicine, Virginia Polytechnic Institute and State University.

Results and Discussion

The development of an isolation scheme which results in the simultaneous isolation of BB and BL membranes from the same initial homogenate required modification of the procedure of Moe et al. (1985). This procedure relies on the differences in the surface charge densities which exist between BB and BL membranes (Schmitz et al., 1975 and Kessler et al., 1978). The BB membranes have a surface charge density which allows for the incorporation of both of the positive charges of divalent cations, such as Mg^{++}. This property minimizes the aggregation of BB membrane

[13]Polysciences Inc, Warrington, Pa.
[14]Ultrotome Nova, LKB, Sweden.
[15]100 CX Stem, JEOL Ltd., Tokyo, Japan.

fragments which would result from Mg^{++} cross linking. To the contrary, the BL membrane surface charge density can accommodate only one of the positive charges of a divalent cation, thus facilitating cross linking between two fragments and subsequent precipitation of BL membrane fragments.

This difference in surface membrane property allows for separation of BL from BB membranes with differential centrifugation (Figure 1). The initial 30 min incubation of enterocyte homogenate with a divalent cation-containing buffer (mannitol-succinate buffer) allows for BL membranes to be separated from BB membranes by a 9,000 · g centrifugation. The BL membranes in the resulting pellet can then be separated from internal cellular membranes with a two-step differential centrifugation. The resulting membrane fraction, P_2, was then placed on a sucrose density gradient for further BL membrane purification.

The isolation of a BB membrane fraction suitable for sucrose density gradient centrifugation required an additional divalent cation incubation. The resuspension and incubation of P_b in mannitol-succinate buffer followed by centrifugation at 8700 · g for 15 min served to remove BL membranes which carried over from the initial incubation. The BL membranes in the resulting pellet, P_c, appeared to have characteristics expected of BL and BB membrane hybrids in that Na/K ATPase and AP were enriched

to a similar extent in this fraction. The centrifugation of S_c at 31,000 · g yielded a membrane fraction, P_d, which was placed on a sucrose density gradient to further enhance the enrichment of the BB membrane preparation.

The higher density sucrose gradients recommended by Moe et al. (1985) and Crooker and Clark (1986) proved to be unfit for further enrichment of BB and BL membrane with this preparation. The use of a sucrose gradient consisting of 27 and 31% sucrose bands proved suitable for both BB and BL membrane isolation. The different results may be due to differences in homogenization procedures which could produce vesicles of different sizes and therefore different buoyant densities. The application of the membrane fractions from differential centrifugation to the 27% and 31% sucrose gradient and centrifugation at 105,000 · g for 90 min resulted in the formation of two bands, one between the interface of the buffer and the 27% sucrose layer, and another at the interface of the 27 and 31% sucrose layers. The third fraction formed was a pellet at the bottom of the 31% sucrose layer. When P_d was applied to the sucrose density gradient, the three membrane fractions from top to bottom were referred to as B_a, B_b and B_c, while the fractions resulting from centrifugation of P_2 (BL membrane fraction) were referred to as B_1, B_2 and B_3.

The B_b fraction of the BB sucrose density gradient was found to have the highest BB enrichment, 5.1-fold AP (Table

1). This enrichment is similar to that achieved by Moe et al. (1985) and slightly lower than that of Crooker and Clark (1986). The B_a fraction contained nearly equal proportions of BB and BL membranes as indicated by the 2.6-fold enrichment in AP and 2.2-fold enrichment of Na/K ATPase, respectively. The B_c fraction was enriched 4.0- and 1.4-fold for AP and ATPase, respectively. The B_b fraction contained the highest relative proportions of the BB membranes compared to BL membranes and therefore was chosen for further characterization.

The B_1 fraction of the BL membrane density gradient was the most suitable for use as a model for BL membrane in transport studies having 10.1-fold Na/K ATPase enrichment and only a .5-fold AP cross contamination (Table 2). The high ratio of Na/K ATPase enrichment:AP cross contamination, 20.2 indicates that transport measurements by vesicles of this fraction represent substrate uptake via BL membranes. The Na/K ATPase enrichment for B_2 and B_3 were 5.5- and 2.6-fold, respectively, while AP enrichments were found to be 2.1- and 2.3-fold for B_2 and B_3, respectively. The low enrichments and high cross-contamination made these fractions unsuitable for use in transport studies.

The enrichment of enzyme markers did not guarantee that the membrane fractions were suitable for measuring transport properties of the isolated membranes. The

Table 1. ENRICHMENT OF NA/K ATPASE AND ALKALINE PHOSPHATASE IN BRUSH BORDER MEMBRANE FRACTIONS RELATIVE TO INITIAL HOMOGENATE

	Enzyme enrichment[a]	
Fraction	Na/KATPase	Alkaline phosphatase
B_a	2.2 (.8)	2.6 (.6)
B_b	1.6 (.3)	5.1 (.6)
B_c	1.4 (.7)	4.0 (.3)

[a] Reported enrichments are means of five separate isolation experiments with standard deviations in parentheses. Enrichment is determined by dividing the specific activity of enzyme in membrane fraction by the specific activity of that enzyme in the homogenate.

Table 2. ENRICHMENT OF NA/K ATPASE AND ALKALINE PHOSPHATASE IN BASOLATERAL MEMBRANE FRACTIONS RELATIVE TO INITIAL HOMOGENATE

	Enzyme enrichment[a]	
Fraction	Na/K ATPase	Alkaline phosphatase
B_1	10.1 (3.5)	.5 (.1)
B_2	5.5 (2.9)	2.1 (.6)
B_3	2.6 (1.7)	2.3 (.2)

[a] Reported enrichments are means of five separate isolation experiments with standard deviations in parentheses. Enrichment is determined by dividing the specific activity of enzyme in membrane fraction by the specific activity of that enzyme in the homogenate.

membranes must also be of a vesicular nature as opposed to globular or membrane sheets. The vesicular nature of the membrane preparations were confirmed by both visual and experimental appraisals.

Electron micrographs obtained with transmission electron microscopy show that both BL and BB membrane preparations were of vesicular orientation (Figure 2). The BL membrane vesicles appeared to be consistently smaller and more irregular in shape and size than the BB membrane vesicles. This is similar to results obtained by Fugita et al. (1972) using isolated BB and BL membrane vesicles from rat intestines. The higher magnification of the membrane preparations revealed that the vesicles were formed by a well preserved trilaminar plasma membrane. Although there appears to be a rougher outer texture (microvilli) in the BB membrane preparation, the visual evidence is not conclusive enough to state that this is the brush border surface. Neither BB nor BL membrane preparations had any recognizable organelle contamination, i.e. mitochondria.

The vesicular nature of the membrane preparations were also tested experimentally by monitoring the osmotic responsiveness of the membrane preparations. The assumption made with this technique was that equilibrium uptake by vesicles would be proportional to the intravesicular space and that the intravesicular space will be determined by the osmolality of the incubation buffer

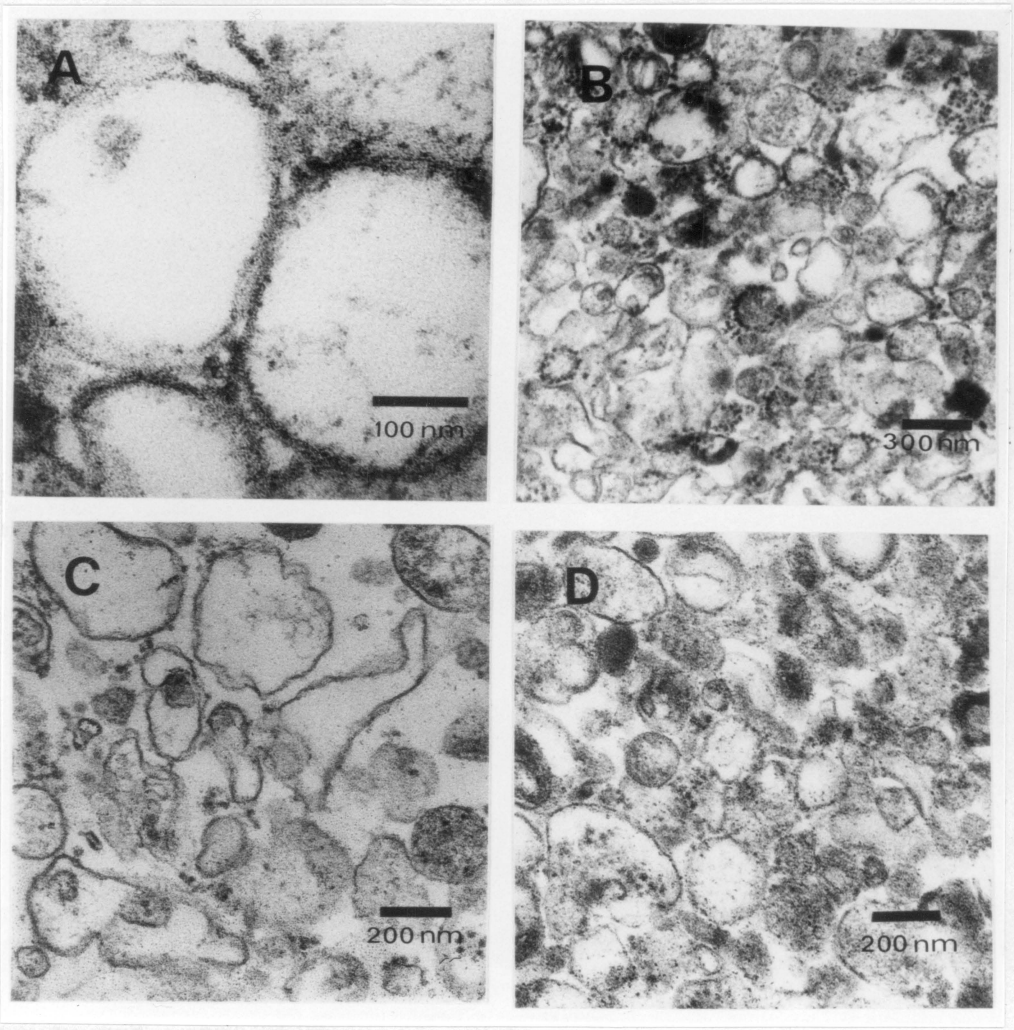

Figure 2. Transmission electron micrographs of brush border and basolateral membrane preparations. A and B represent brush border membranes magnified 100000 X and 19000 X (before enlargement), respectively. C and D represent basolateral membranes magnified 36000 X and 29000 X (before enlargement), respectively.

(Munck, 1966). Therefore, by monitoring the equilibrium accumulation of a substrate at various osmolalities by a membrane preparation, one can determine the configuration of that membrane preparation. If uptake decreases with increasing osmolality of incubation buffer, then the membrane preparation is considered to be at least partially vesicular. If uptake is not affected by osmolality, the preparation is considered to be nonvesicular.

Methionine was the substrate utilized to experimentally test the vesicularity of BB and BL membrane preparations. Plotting equilibrium accumulation by both BB and BL membrane preparation versus inverse osmolality revealed a linear relationship with a positive slope (Figure 3). The regression equations for BB and BL membrane preparations were $Y = 140\ X + 276$, $r^2 = .992$ and $Y = 171\ X + 222$, $r^2 = .994$, respectively. The linearity and positive slope of the equilibrium methionine accumulation versus inverse of increasing osmolality indicate that methionine uptake was occurring into an osmotically active space, vesicles, with both BB and BL membrane preparations

Substrate accumulation was plotted against inverse osmolality to allow for the extrapolation of uptake data to infinite osmolality, Y-intercept, in order to predict the level of substrate which was present due to surface binding but not due to uptake into the vesicle. It is assumed that at infinite osmolality, there would be no intravesicular

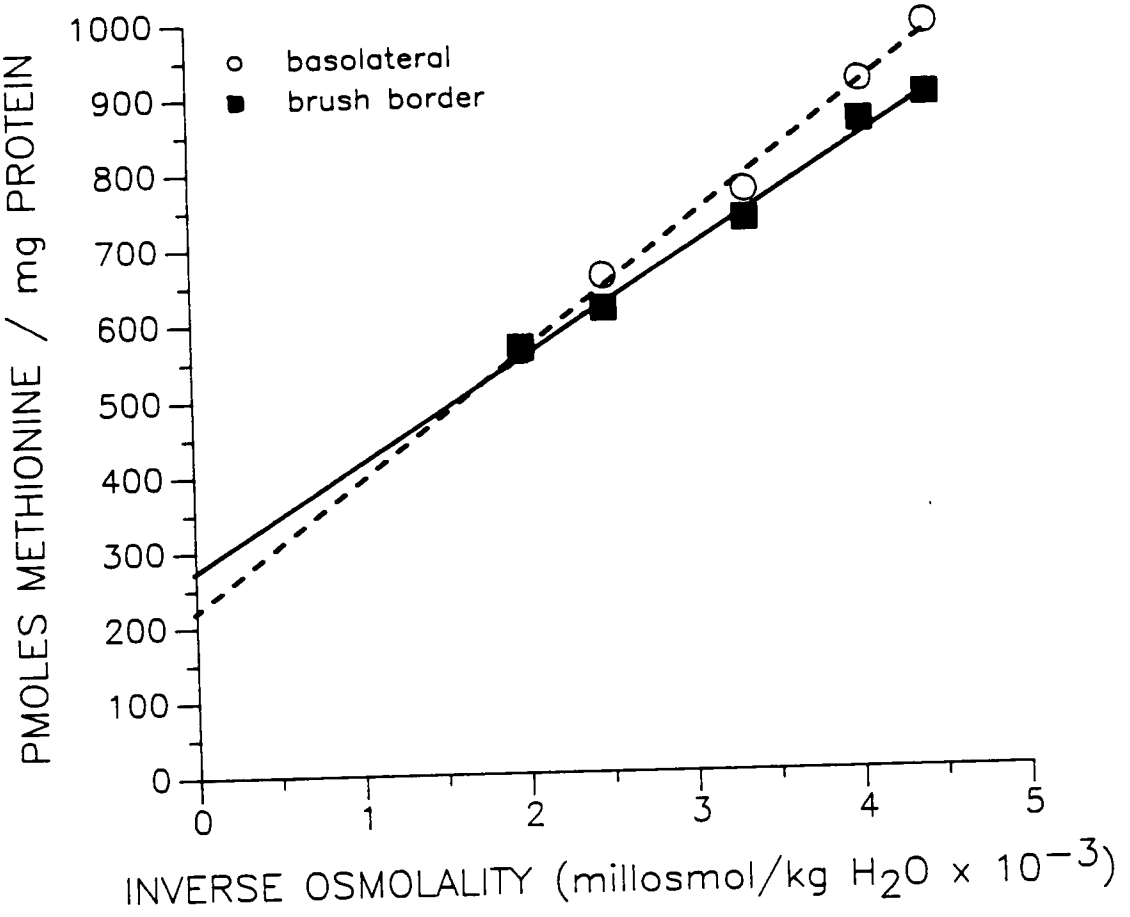

Figure 3. Effect of osmolalities of incubation buffers on equilibrium (60 min) net fluxes of labeled L-methionine (100 uM) by brush border (■) and basolateral (o) membrane preparations. Osmolalities were adjusted by changing the concentrations of D-mannitol. Data are means of twelve observations.

space (Faust et al. 1968; Eicholz et al. 1969). The BB and BL membrane surface binding after 60 min of incubation was determined to be 276 and 222 pMoles of methionine/mg of protein, respectively.

Among the advantages of the membrane vesicle technique are efficient use of animals and the ability to characterize transport properties of intestinal enterocytes in the absence of cellular metabolism.

The scraping of jejunal and ileal intestinal segments resulted in mucosal tissue yields of 958 and 598 g, respectively, per steer. This corresponds to a minimum of 70 transport experiments per steer for each cell surface, BB and BL. When compared to a conventional technique, such as the everted sac procedure of Phillips et al. (1976), where only three transport experiments were performed per animal, the membrane vesicle technique is a far more efficient use of experimental animals. The large number of experiments estimated per steer with the membrane vesicle technique is possible because mucosal scrapings can be frozen prior to BB and BL membrane separation. Freezing of mucosal scrapings was found to have no detrimental effects. In fact, enrichment of BL membranes was enhanced by freezing as indicated by the increased Na/K ATPase: AP ratio of 4.3 and 2.9 for frozen and fresh tissue preparations, respectively. These results are in agreement with the findings of Crooker and Clark (1986). The

advantage of frozen tissue may be due to freeze fracturing of isolated enterocytes. Examination of previously frozen tissue with a differential interference contrast microscope showed uniform cell fragments with no intact cells. Therefore, homogenization of frozen tissue may result in membrane fragments of different size and composition than the same homogenization of fresh tissue. Freeze fracturing makes the use of homogenization techniques, such as N_2-cavitation, impractical when frozen tissue is being used.

The BB and BL membranes were found to be osmotically active and therefore of a vesicular nature. Thus, the BB and BL membrane vesicles are suitable tools for characterizing the transport properties of the bovine intestinal enterocyte. The BB membrane vesicles can be used to evaluate the transport properties regulating entry of nutrients into the enterocyte, while BL membrane vesicles can be used to monitor transport properties leaving the enterocyte and entering the circulation. In both cases the absence of cellular metabolism allows for the characterization of transport properties of metabolizable substrates.

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Chapter IV

Running Heading: Lysine and Methionine Transport

LYSINE AND METHIONINE TRANSPORT BY BOVINE JEJUNAL AND ILEAL BRUSH BORDER MEMBRANE VESICLES

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ABSTRACT

Lysine (LYS) and methionine (MET) transport were studied using brush border membrane vesicles from bovine jejunal and ileal tissue. Total transport of LYS and MET was divided into mediated and diffusion components. Mediated uptake was further divided into sodium dependent (Na^+) and sodium independent (Na^-) systems. Total LYS and MET uptake by ileal brush border (BB) vesicles tended to be higher than jejunal BB vesicles at all concentrations evaluated but differences were significant ($P<.05$) at 2.5 and 7.5 mM for LYS and 5, 12.5 and 15 mM for MET. The greater capacity of ileal BB vesicles appeared to be due to the Na^+ component of LYS uptake and the diffusion component of MET uptake. Methionine transporters had lower affinities and higher capacities than the corresponding LYS transporters in both ileal and jejunal tissue. Methionine transport was greater ($P<.05$) than LYS transport in both ileal and jejunal BB vesicles when the initial amino acid concentration was 7.5 mM. When the initial amino acid concentration was 1.25 mM, MET uptake was greater ($P<.13$) than LYS uptake in jejunal but not ileal BB vesicles. The relative contribution of mediated and diffusion uptake systems to total MET and LYS uptake was found to be dependent of substrate concentration.

(Key Words: Methionine, Lysine, Jejunal, Ileal, Bovine, Brush Border Membrane Vesicles.)

Introduction

It is currently believed that the primary site of amino acid absorption in ruminant species is the small intestine (Bergen, 1978). Relative to the laboratory species, very little information is available on the amino acid transport systems of the ruminant. There is evidence that the properties of amino acid absorption may differ between ruminant and nonruminant species. The ileum has been identified as the intestinal region of maximal amino acid absorption in the ovine (Johns and Bergen, 1973; Phillips et al., 1976), while the jejunum fills this role in nonruminants (Wiseman, 1974). This difference may have evolved due to the nature of intestinal secretions and digesta flow in ruminants compared to nonruminants. The continuous nature of digesta flow in the ruminant results in a more stable amino acid concentration and a lower average pH in the small intestine relative to nonruminants.

The brush border (BB) membrane is the first membrane barrier nutrients must transverse during intestinal absorption. The method of transfer across the BB membrane is either diffusion or mediated transport or a combination of both. The rate of diffusion of a substrate is a

function of the extracellular concentration and solubility of that substrate in the lipid component of the BB membrane (Moe et al, 1987). Mediated transport is regulated by enzyme-like transporters which are protein components of BB membrane. Mediated transporters can be divided into Na-dependent (Na^+) and Na-independent (Na^-) systems. Na-dependent systems are generally considered active, energy requiring, and include the A, ASC, and N systems which have been characterized in nonruminants (Collarini and Oxender, 1987). The Na^- systems which have been characterized in the nonruminants includes the L, Y^+ and X^- systems (Christensen, 1984). The presence of transport systems similar to these in ruminant species has been suggested by Baumrucker (1985) and Guerino and Baumrucker (1987).

The influence of substrate concentration on the relative contribution of Na^+ and Na^- and diffusion transport to total uptake has not been addressed for the bovine small intestine. Stevens et al. (1984) suggested that in rabbits, the relative importance of various routes of uptake depends on substrate concentration. This information could make researchers reconsider the substrate concentrations at which they carry out their transport experiments.

The present research was conducted to determine the transport characteristics of ileal and jejunal BB membrane vesicles for methionine (MET) and lysine (LYS).

Materials and Methods

Holstein steers with an average weight of 400 kg were used as tissue donors. The steers were fed to gain .9 kg a day on an orchardgrass hay, corn, soybean meal diet. The procedure used for isolation of brush border membrane vesicles was previously reported in Chapter IV. Briefly, jejunal and ileal tissue were excised and divided into 1 m segments. The digesta was flushed from the segments with a buffer composed of 300 mM mannitol and 12 mM tris base, pH was adjusted to 7.4 with HCL, (mannitol buffer). The segments were everted with the aid of a glass rod then the residual digesta was rinsed away with mannitol buffer. Segments were then incubated in hyaluronidase buffer (120 mM NaCl, 20 mM tris base, 1 mM Mg_2Cl, 3 mM K_2HPO_4, 1 mg/ml bovine serum albumin and 1 mg/ml hyaluronidase, pH adjusted to 7.4 with HCl) for 15 min at 37°C. The mucosal lining was then harvested by gently scraping the intestinal segments with a glass microscope slide. The hyaluronidase was then washed from the harvested cells by suspending the cells with one volume of mannitol buffer and then collecting the cells with a 3000 · g centrifugation for 15 min, process was repeated twice. Mucosal cells were divided into 3 to 4 gram aliquots and placed in whirl-pac

bags, frozen in liquid N_2 and stored in an ultra-low freezer[1].

Membrane isolation procedures were initiated by thawing 36 g of mucosal tissue (either jejunal or ileal depending upon the experiment), 12 g from each of three steers. Tissue was combined and diluted then homogenized with a polytron[2] homogenizer. A buffer containing 150 mM mannitol, 30 mM succinate, 5 mM $MgCl_2$, 5 mM $KHPO_4$,.1 mM $MnCl_2$ and 10 mM Tris-HCl, pH 7.4,(mannitol-succinate buffer) was used to dilute enterocytes. The homogenized tissue was incubated at 4°C for 30 min while gently stirring. This allowed for Mg^{++} aggregation of internal and basolateral membranes. Incubation was followed by a two-step differential centrifugation 9,000 and 31,000 · g, 12 and 15 min, respectively, and a second incubation in mannitol-succinate buffer of the resulting pellets. Following the second incubation, the suspension was centrifuged at 8,700 and 31,000 · g, 12 and 15 min, respectively. The final pellet was resuspended in buffer containing 272 mM mannitol, 20 mM HEPES and 2 mM $MgCl_2$, pH was adjusted to 7.4 with NH_3OH. The membrane suspension was layered on a density gradient consisting of 27 and 31% sucrose (wt/wt) in a solution of 4 mM $MgCl_2$ and 4 mM HEPES, pH was adjusted to 7.4 with NH_3OH. The density gradient

[1]Biofreezer, Forma Scientific, Marietta, OH.
[2]PT 10/35 polytron, Brinkman instruments, Westburg, NY.

was centrifuged at 4°C for 90 min at 105,000 · g in an ultracentrifuge[3]. Enzyme analyses indicated the highest enrichment (5.1-fold) over homogenate of alkaline phosphatase (brush border membrane marker enzyme) was at the interface of 27 and 31% sucrose layers of the gradient. Membranes from this band were used in all transport assays.

Transport properties of BB membrane vesicles were evaluated using the millipore filtration technique (Murer et al., 1974; Kimmich, 1975). The incubation buffer for transport experiments was formulated to contain final concentrations of 100 mM mannitol, 10 mM HEPES, 2 mM $MgCl_2$ and 100 mM of either NaSCN or KSCN, pH was adjusted to 7.4 with NH_3OH. Substrate concentrations evaluated were .313, .625, 1.25, 2.5 and 7.5 mM for LYS and .125, 1.25, 2.5, 5.0, 7.5, 10, 12.5, and 15 mM for MET. When these substrate concentrations were added to incubation buffers, enough mannitol was removed to maintain equal osmolality among the buffers.

Transport was initiated by adding BB membrane vesicles to a reaction vessel containing incubation buffer and approximately 1 uCi radioisotope, pre-incubated at 37°C. The total reaction volume was 250 ul and contained 150-175 ug of protein. Following 15 sec of incubation, uptake by vesicles was terminated by adding 1 ml of ice cold stopping solution (150 mM KCl). Two, .5 ml aliquots of the

[3]International Equipment Company, Needham Heights, MA.

resulting mixture were then filtered to collect BB membrane vesicles which contained transported substrate. The filters were washed with three, 5 ml aliquots of stopping solution to minimize the background binding to the filters. Transport was quantified by liquid scintillation counting of the transported isotope which remained within the vesicles on the filters. The scintillation fluid used was LS-271 Ecoscint[4]. Radiochemicals were 4, 5-[^{3}H]-L-Lysine and ^{35}S-methionine[5]. Protein was assayed with Pierce protein reagent 23200[6].

The initial influx rates for MET and LYS transport systems were determined at 15 s. Shorter time spans are sometimes recommended for measuring initial influxes but the 15 s incubation chosen for our experiments best fit our facilities and equipment. The 15 s incubation used in our experiments was far below the 30 s limit set by Kessler and Toggenburger (1979) to measure initial influx rates with BB membrane vesicles.

The total uptake of LYS and MET was divided into three components Na^{+}, Na^{-} and diffusion. In order to separate systems, three parallel experiments for each amino acid were necessary. In experiment 1, total uptake at each substrate concentration was determined by incubating vesicles with a Na-containing incubation buffer. In

[4]National Diagnostics, Manville, NJ.
[5]NEN Research Products, Boston, MA.
[6]Pierce Chemical, Rockford, IL.

experiment 2, Na^- plus diffusion uptake at each substrate concentration was measured by incubation of vesicles in Na-free incubation buffer (potassium buffer). In experiment 3, a permeability constant for the amino acid being evaluated was determined as described by Hopfer (1981). The permeability constant was used to estimate diffusion at each substrate concentration evaluated. The permeability constant was determined to be equivalent to the slope of the line formed when the uptake at 100 mM substrate level was extrapolated through the origin. The assumption being that at 100 mM, an overwhelming percentage of the total uptake was due to diffusion. The permeability constant will have the units liters $\cdot$ mg protein^{-1} $\cdot$ sec^{-1}.

Sodium-dependent uptake for each substrate concentration was calculated as the difference between uptake for a substrate concentration in experiment 1 and experiment 2. The difference between experiment 2 and experiment 3 for a substrate concentration was assumed to be equivalent to Na^- uptake. Mediated uptake, which is the combination of Na^+ and Na^-, was determined by difference between experiment 1 and experiment 3 for each substrate concentration.

Statistical analysis was by the general linear model procedure of the statistical analysis system computational package (SAS, 1982). Data were analyzed within amino acid and tissue type. Differences among least square means for

different amino acid concentrations and tissue types were tested using two-tailed paired t-test (Fisher, 1925).

Results and Discussion

The decision to divide total amino acid uptake into Na^+, Na^- and diffusion systems was based on the desire for a rapid and accurate method to characterize the routes of amino acid transport in intestinal tissue. Researchers have subdivided these systems into groups based upon the affinity and capacity of the transporters for the amino acids (Christensen, 1984; Collarini and Oxender, 1987). This type of transporter classification is based on inhibition analysis of transport which involves complex experimental designs that rely on a number of assumptions and interpretations of multiple interactions in order to identify the transport system. The use of three more general categories of amino acid transport systems yields the information necessary to make conclusions as to what type of transporters are responsible for what proportion of uptake in which tissue.

The total uptake (Na^+, Na^- and diffusion) of MET and LYS tended to be higher for ileal than jejunal tissues (Figure 1). Lysine uptake by ileal BB membrane was greater ($P<.05$) than jejunal uptake at the two highest substrate concentrations, 2.5 and 7.5 mM. Uptake of MET by ileal BB

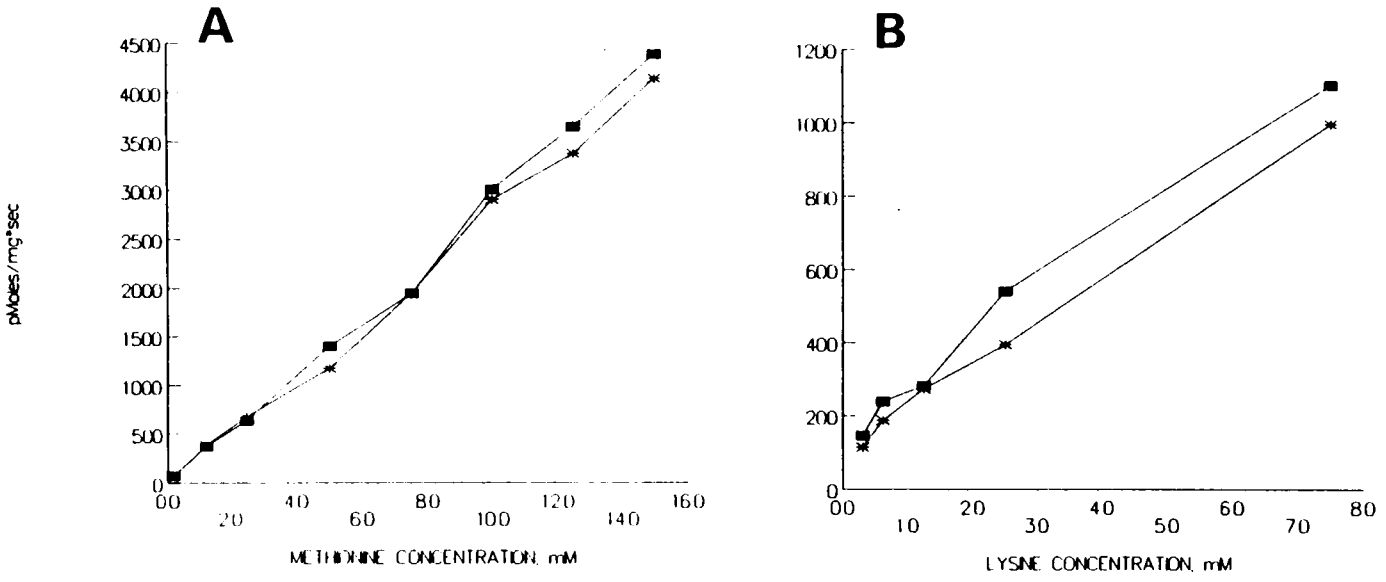

Figure 1. Total uptake of methionine (A) and lysine (B) by jejunal (*) and ileal (■) brush border membrane vesicles as influenced by increasing substrate concentration. Data presented are least square means for a minimium of 10 observations.

was higher (P<.03) than jejunal uptake at 5, 12.5, and 15 mM MET concentrations. Phillips et al. (1976 and 1979) using both in vivo and in vitro techniques found ileal amino acid uptake to exceed jejunal amino acid uptake in the ovine. Guerino and Baumrucker (1987) also made similar observations. They observed ileal uptake of MET and LYS to be greater than jejunal uptake in the bovine.

Although total uptakes of both MET and LYS appear to be higher in ileal versus jejunal tissue, especially at the highest substrate concentrations, the transport systems responsible for greater uptake may differ for each of the amino acids. Lysine uptake by diffusion was found to be virtually identical in ileal and jejunal tissue across all substrate concentrations. This indicates that the cationic amino acid, LYS, had the same solubility properties in ileal as jejunal BB membranes. Methionine diffusion was found to be higher (P<.002) in ileal than jejunal BB tissue at all substrate concentration evaluated (Figure 2). This seems to indicate that the neutral amino acid MET had a greater solubility in ileal than jejunal BB membranes.

Lysine transport by mediated systems, Na^+ and Na^-, was found to be higher (P<.12) in ileal than jejunal BB tissue at 2.5 and 7.5 mM LYS concentration with a tendency for greater ileal uptake at the lower concentrations (Figure 3). Methionine uptake by mediated systems was greater (P<.15) in jejunal than ileal tissue at 2.5, 7.5, 10, 12.5

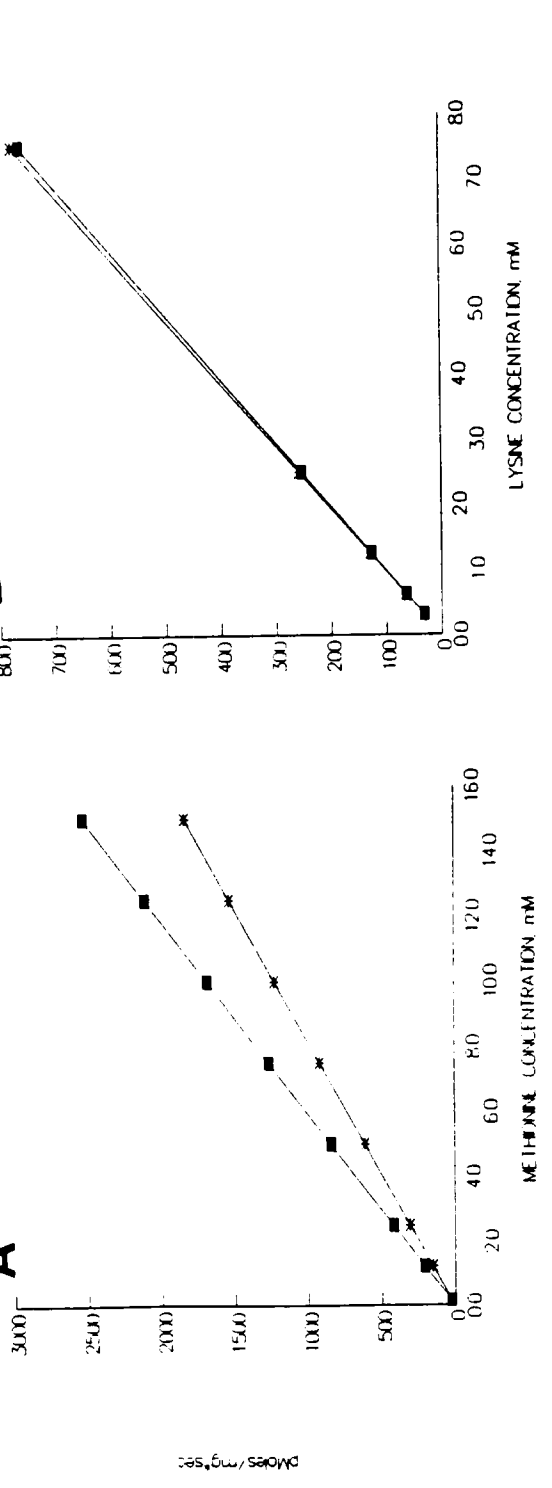

Figure 2. Diffusion uptake of methionine (A) and lysine (B) by jejunal (*) and ileal (■) brush border membrane vesicles as influenced by increasing substrate concentration. Data presented are least square means for a minimium of 16 observations.

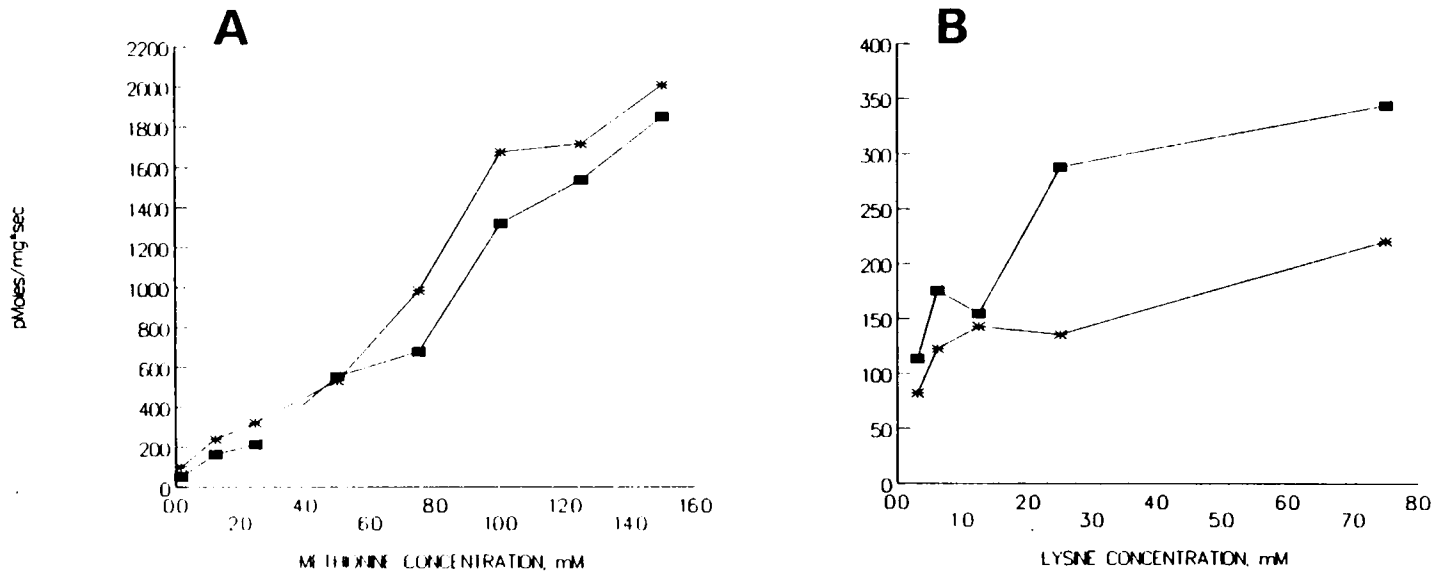

Figure 3. Mediated uptake of methionine (A) and lysine (B) by jejunal (*) and ileal (■) brush border membrane vesicles as influenced by increasing substrate concentration. Data presented are least square means for a minimium of 15 observations.

and 15 mM MET concentrations. The site differences for maximal mediated LYS and MET uptake may indicate that transporters are organized to minimize potential competition between these essential amino acids. Methionine has been shown to be a potent inhibitor of LYS as well as other amino acid uptake (Phillips et al, 1979; Moe et al et al, 1987). Therefore, maximized MET uptake by jejunal tissue would reduce competition at the site where LYS uptake is maximized, the ileal tissue.

Mediated uptake was separated into Na^+ and Na^- systems to determine which system or systems were responsible for the differences in mediated uptake between tissues for MET and LYS. Sodium-independent LYS uptake in ileal tissue was greater ($P<.11$) than jejunal tissue only at 2.5 mM LYS concentration (Figure 4). Sodium-independent methionine uptake was greater ($P<.12$) in jejunal tissue than ileal tissue at 5, 10, 12.5 and 15 mM MET concentrations. The Na^+ uptake of LYS tended to be higher in ileal tissue compared to jejunal tissue, with greater uptake ($P<.12$) occurring at the 7.5 mM LYS concentration (Figure 5). The sodium-dependent MET uptake by ileal BB was greater ($P<.14$) than jejunal uptake at 5 mM MET concentration, but was not different at other concentrations evaluated. These results suggest that Na^- systems are responsible for the greater mediated MET uptake in jejunal tissue while Na^+ systems are responsible for the greater mediated uptake, as well as

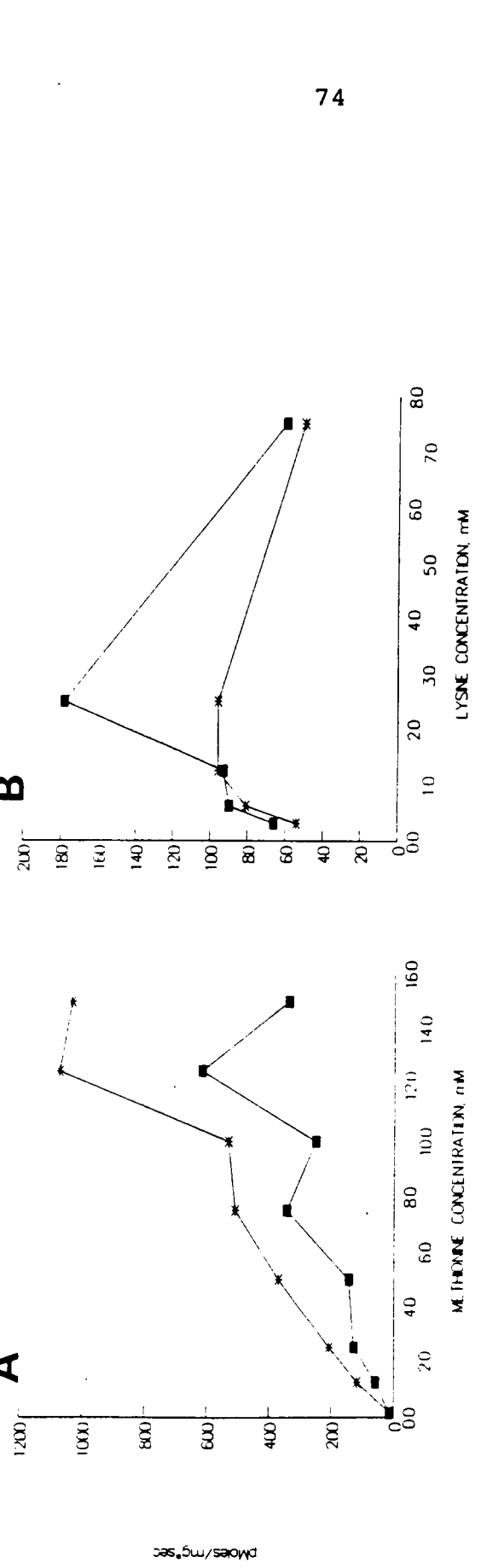

Figure 4. Sodium-independent uptake of methionine (A) and lysine (B) by jejunal (*) and ileal (■) brush border membrane vesicles as influenced by increasing substrate concentration. Data presented are least square means for a minimium of 15 observations.

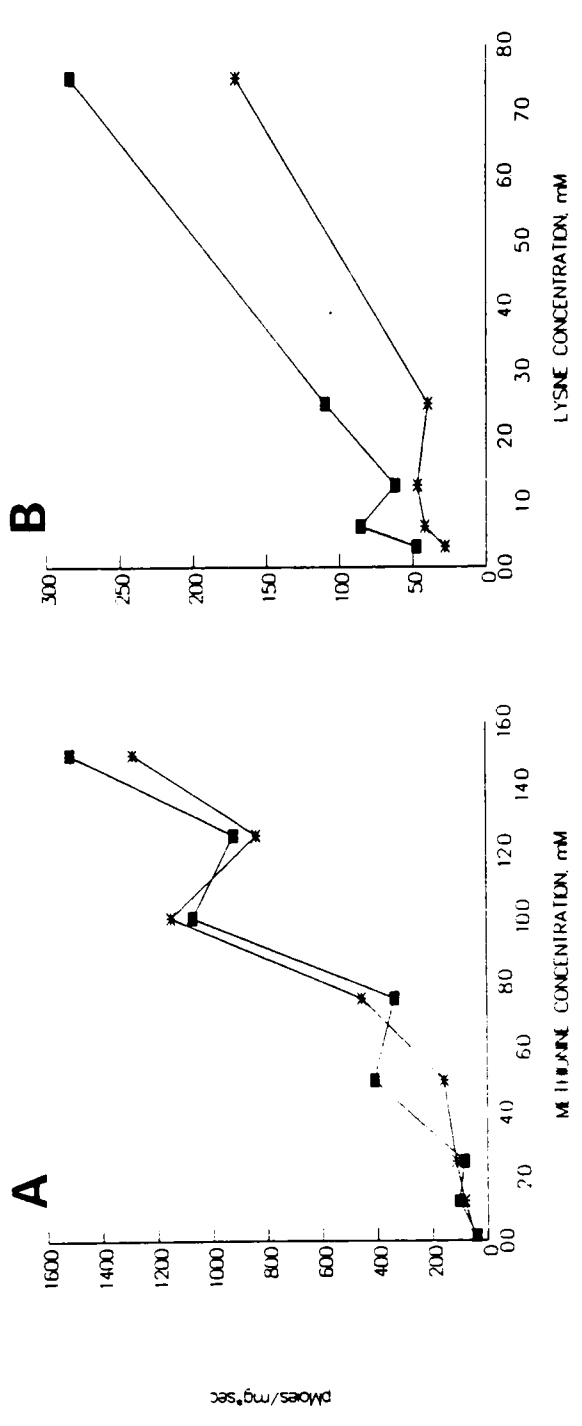

Figure 5. Sodium-dependent uptake of methionine (A) and lysine (B) by jejunal (*) and ileal (■) brush border membrane vesicles as influenced by increasing substrate concentration. Data presented are least square means for a minimium of 12 observations.

total uptake, of LYS in ileal BB tissue. The greater uptake of MET by ileal tissue as compared to jejunal tissue seems to be a function of the diffusion properties of the BB membranes.

The kinetic parameters of Na^+ and Na^- systems for MET and LYS transport were determined by the methods of Dowd and Riggs (1965) in both jejunal and ileal tissue (Table 1). The K_m is a measure of the substrate concentration required for the transporter to achieve half the maximal velocity (V_{max}). The K_m value is a measure of the transporters affinity for the substrate, i.e., a high K_m reflects a low affinity for the substrate. The capacity of a transporter is reflected by the V_{max} for that substrate (Dowd and Riggs, 1965).

The jejunal Na^+ and Na^- transporters for LYS had greater affinities, as reflected by lower K_m's, than ileal tissue but had a lower capacity as indicated by the lower V_{max}'s. The lower affinities and higher capacities of the transporters in ileal tissue may explain the greater LYS uptake by ileal tissue as compared to jejunal tissue.

The higher uptake by the MET Na^- systems in jejunal versus ileal tissue could be predicted from kinetic parameters of this transport system. The Na^- system of jejunal BB tissue had similar affinities, K_m, for MET but had almost twice the capacity, V_{max}, as ileal Na^- systems. The Na^+ system for MET had similar K_m and V_{max} in both

TABLE 1. KINETIC PARAMETERS, K_m AND V_{max}, OF SODIUM-DEPENDENT AND SODIUM-INDEPENDENT METHIONINE AND LYSINE UPTAKE BY JEJUNAL AND ILEAL BRUSH BORDER MEMBRANE VESICLES

		Methionine		Lysine	
System[a]	Parameter[b]	Jejunal	Ileal	Jejunal	Ileal
Na^+	K_m	11.0	9.7	.52	.66
	V_{max}	.76	.75	.07	.15
Na^-	K_m	11.0	11.6	.36	.45
	V_{max}	1.19	.62	.12	.15

[a]System refers to the sodium-dependent system (Na^+) and sodium-independent systems (Na^-)
[b]K_m and Vmax are reported as millimolar and nmoles · mg protein^{-1} · sec units^{-1}, respectively.

ileal and jejunal tissues, which explains the similar uptake patterns seen in both tissues. Both Na^+ and Na^- transporters for MET, have lower affinities and higher capacities than the corresponding LYS mediated transporters.

Methionine was transported at a greater ($P<.05$) rate than LYS in both ileal and jejunal tissue when the initial amino acid concentration was 7.5 mM (Table 2). When the initial amino acid concentration was 1.25 mM, total MET uptake was greater ($P<.13$) than LYS uptake in jejunal but not ileal tissue (Table 3). Methionine uptake by diffusion was greater ($P<.08$) in ileal and jejunal tissue at both 1.25 and 7.5 mM amino acid concentrations. The transport of MET and LYS by mediated systems, Na^+ and Na^-, was not different in jejunal or ileal tissue at 1.25 mM substrate concentration. The 7.5 mM incubations in jejunal tissue resulted in the transport of MET at a greater ($P<.005$) rate than LYS with both the Na^+ and Na^- systems. In the ileal tissue the Na^- system but not the Na^+ system had higher ($P<.03$) MET uptake. The comparisons of uptake rates of mediated transport for LYS and MET does not infer that both amino acids are transported by the same transport system(s), but that both amino acids have mediated components for their uptake. The differences seen in transport systems in relation to total uptake as substrate was increased from 1.25 to 7.5 mM indicate that the

Table 2. Transport by methionine and lysine uptake systems in jejunal and ileal brush border membrane vesicles at a substrate concentration of 7.5 millimolar

	pmoles . mg^{-1} . sec^{-1}			
	Jejunal		Ileal	
System[a]	Methionine	Lysine	Methionine	Lysine
Total	1935±76[b]	998±32[c]	1946±81[b]	1106±40[d]
Na^+	523±100[b]	171±43[c]	336±113[b]	284±53[b]
Na^-	509±94[b]	50±40[c]	343±101[b]	60±59[c]
Diffusion	921±68[e]	777±29[f]	1267±63[g]	762±47[f]

[a]Total = total uptake, Na^+ = sodium-dependent uptake, Na^-= sodium-independent uptake.
[bcd]Means with different superscript within rows are different ($p<.05$).
[egf]Means with different superscript within rows are different ($p<.1$).

Table 3. Transport by methionine and lysine uptake systems in jejunal and ileal brush border membrane vesicles at a substrate concentration of 1.25 millimolar

	pmoles . mg^{-1} . sec^{-1}			
	Jejunal		Ileal	
System[a]	Methionine	Lysine	Methionine	Lysine
Total	392±65[b]	273±37[c]	374±78[b]	282±35[bc]
Na^+	107±93	47±47	104±111	62±51
Na^-	132±68	96±29	59±79	93±38
Diffusion	153±11[e]	130±5[f]	211±11[g]	127±8[f]

[a]Total = total uptake, Na^+ = sodium-dependent uptake, Na^- = sodium-independent uptake.
[bcd]Means with different superscript within rows are different ($p<.2$).
[egf]Means with different superscript within rows are different ($p<.1$).

relationship between transport systems is not static across all substrate concentrations.

The comparison of the relative contribution of Na^+, Na^- and diffusion systems to total uptake as substrate concentration increased illustrates that the relative importance of transport systems to total uptake of amino acids is dependent on amino acid concentration presented to the transporter. Diffusion appears to be a significant pathway for LYS uptake by jejunal BB tissues at all substrate concentrations evaluated, accounting for 28 to 78% of total LYS uptake depending on LYS concentrations (Figure 6). While the Na^- system predominates at LYS concentrations < .75 mM in jejunal BB tissue, the percent contribution of this system to total uptake decreases with increasing LYS concentration. The percent contribution to total LYS uptake by the Na^+ system accounts for the least amount of LYS uptake at substrate concentrations up to 5.5 mM. In ileal BB tissue the percent contribution to total LYS uptake by Na^- systems > Na^+ systems > diffusion at concentrations < .75 mM. When LYS concentration was > .75 mM but < 5.3 mM, diffusion predominated, Na^- systems were intermediate and Na^+ system made the least contribution to total uptake. When LYS concentration was above 5.3 mM, the Na^+ system and the Na^- system reverse roles in regard to the percent contribution to total uptake. The mediated systems, Na^+ and Na^-, for LYS uptake are the predominate

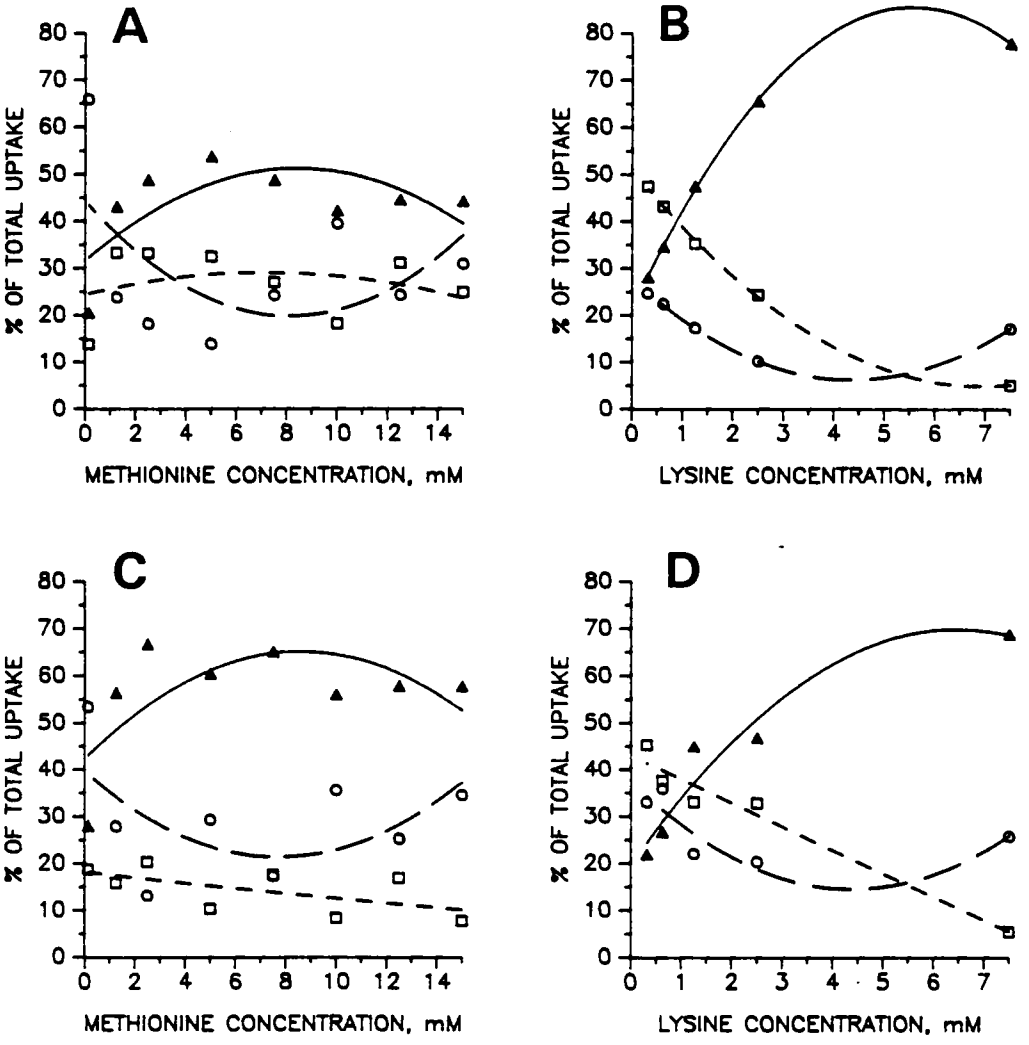

Figure 6. Relative contribution of Na-dependent (O), Na-independent (□) and diffusion (▲) systems to total uptake of methionine (A) and lysine (B) by jejunal tissue and methionine (C) and lysine (D) by ileal tissue.

routes of uptake only when LYS concentrations are at the lower end of the range evaluated, presumably this is the lysine concentration found in the ileum, where the mediated LYS uptake was found to be the most active. The increase in the relative contribution of diffusion to total uptake with increasing substrate concentrations may be explained by the saturation of the mediated transport systems at the higher substrate levels.

Methionine uptake by jejunal and ileal BB tissue was predominated by diffusion at all concentrations evaluated except .125 mM (Figure 6). The Na^+ system was the predominate route of MET uptake at .125 mM in both jejunal and ileal BB tissues. In jejunal BB tissue when the MET concentration was > .125 mM, the Na^- system tended to be intermediate to diffusion and Na^+ systems in relative contribution to total uptake. While in ileal tissue the Na^+ system tended to be intermediate to diffusion and Na^- systems in relative contribution to total uptake when MET concentration was > .125 mM. Although mediated uptake systems dominant only at lower concentrations, the mediated systems make significant contributions to total uptake throughout the physiological concentration range. The difference seen in response of MET and LYS mediated systems to increased concentration of substrate may be explained by the lower affinity and higher capacity of the mediated MET transport systems.

Comparison of uptake data with literature values are difficult due to the practice of monitoring uptake properties at only one substrate concentration. Guerino and Baumrucker (1987) found that of the mediated systems in bovine ileal tissue, the Na^- system made the greatest contribution to total uptake for both LYS and MET, at 1 mM substrate concentration. This was in agreement with our data for ileal LYS uptake but not with MET data, at 1.25 mM substrate concentration. Comparisons as percent of total uptake for Na^+, Na^- and diffusion systems are not possible with this data (Guerino and Baumrucker, 1987) because sodium dependency was determined by the concentrative uptake technique, which does not divide transport into its component systems.

Moe et al. (1987) using bovine ileal BB membrane vesicles found that the uptake by Na^+, Na^-, and diffusion systems to be 14, 37 and 49% of the total MET uptake and 9, 53 and 38% of total LYS uptake. These uptake data were determined at a substrate concentration of .1 mM. These findings are in general agreement with our LYS uptake data for the LYS concentrations of .313 mM, the lowest substrate concentration evaluated in ileal BB tissue. The Na^+, Na^- and diffusion systems contributed 33, 45 and 22% of total LYS uptake. When MET concentration was .125 mM the Na^+, Na^- and diffusion systems accounted for 53, 19 and 28% of total MET uptake. The Na^+ systems of both LYS and MET

contributed more to total uptake than what Moe et al. (1987) had suggested.

The relative contribution of Na^+, Na^- and diffusion systems to total uptake at substrate concentration of 1.25 mM was 28, 16, and 56% for MET and 22, 33 and 45% for LYS. Comparisons of these values to our data obtained at .125 and .313 mM substrate concentration, MET and LYS, respectively and to the data of Moe et al. (1987) obtained at .1 mM substrate concentrations demonstrates how the relative contribution to total uptake can vary even within the physiological concentration range. The conclusion drawn about the relative importance of Na^+, Na^- and diffusion transport to total transport would clearly differ depending on which initial amino acid concentration was used to evaluate transport. Therefore, caution should be exercised when making conclusions about intestinal amino acid transport when uptake at only one substrate concentration has been evaluated.

The transport of LYS and MET by jejunal and ileal tissue was characterized utilizing BB membrane vesicles. Caution should be exercised in the extrapolation of these determinations of the kinetic parameters for LYS and MET to the in vivo situation since the potential interactions and competitive effects of other amino acids were not addressed in this study. Other parameters which may alter in vivo amino acid transport properties include associative effects

of other nutrients, environmental condition of the intestinal lumen and endocrine status of the animal. By coupling the determination of the site of maximum absorption and kinetics of absorption for an amino acid with studies addressing these other parameters, predictions can be made as to where in the small intestine and in what combination amino acids should be made available in order to maximize the efficiency of amino acid absorption.

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Chapter V

Epilogue

Previous chapters have discussed the isolation of BB and BL membrane vesicles and the use of these vesicles in the evaluation of MET and LYS transport by the jejunum and ileum. The discussion in this chapter will focus on some of the limitations of the membrane vesicle technique and suggest modifications which may alleviate these problems. Also, some of the MET and LYS transport properties that have not yet been addressed will be discussed.

Data were presented in Chapter IV, Figure 6, that demonstrated the percentage contribution of the various transport systems (Na^+, Na^- and diffusion) is dependent on substrate concentration. This is an important consideration when trying to characterize the uptake of a particular amino acid, since characteristics of uptake may appear different with changes in substrate concentration. Evaluation of transport systems on a percentage contribution basis is not without problems. One of the problems is the perception that the same absolute amount of uptake is occurring at the various substrate concentrations; this may not be the case. Therefore, even though the percentage contribution of a system decreases with increasing substrate concentration, the absolute

uptake by this system does not necessarily decrease. Presenting the same data as stacked bar graphs allows for the visualization of the percentage contribution to total uptake as well as total uptake.

Lysine (LYS) uptake by jejunal and ileal BB tissue is presented in Figure 1, while methionine (MET) uptake by jejunal and ileal BB tissue is presented in Figure 2. The total uptake of MET and LYS increased with increasing substrate concentration in both jejunal and ileal BB tissue. Since the level of diffusion is a function of substrate concentration, the amount of uptake occurring by diffusion increased proportionally with increasing substrate concentration, irrespective of amino acid or tissue. The absolute amount of uptake by the mediated uptake systems, Na^+ and Na^-, tended to increase or remain the same as substrate concentration was increased.

In an environment free of competitors for mediated transporters, at what concentration of substrate will physiological needs be met? The assumption can be made that amino acid uptake occurring when amino acid concentration is at normal physiological levels, approximately 2.5 mM, is adequate to meet metabolic demand. Obviously, the amino acid requirement will vary with the level of production, but this approximation is likely valid under normal conditions. A starting point for comparisons can be obtained using this assumption, that amino acid

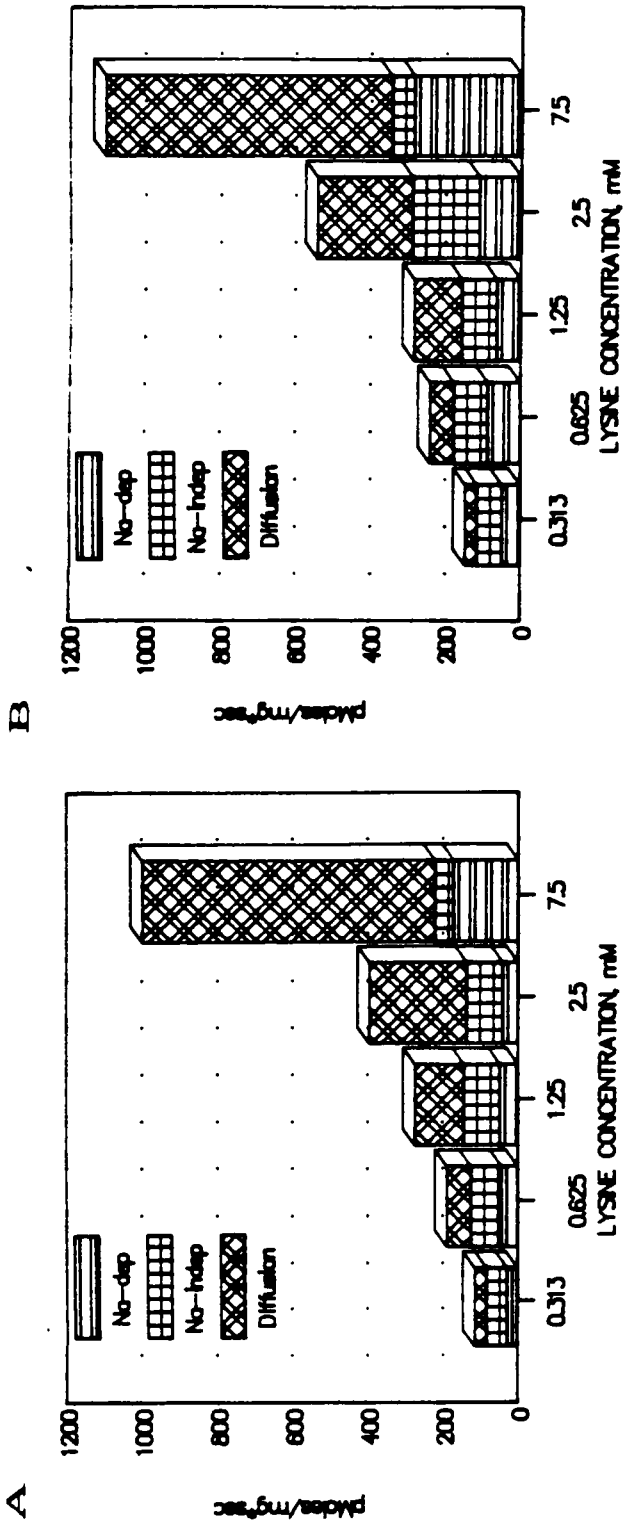

Figure 1. Lysine uptake by jejunal (A) and ileal (B) brush border tissue as influenced by increasing substrate concentration.

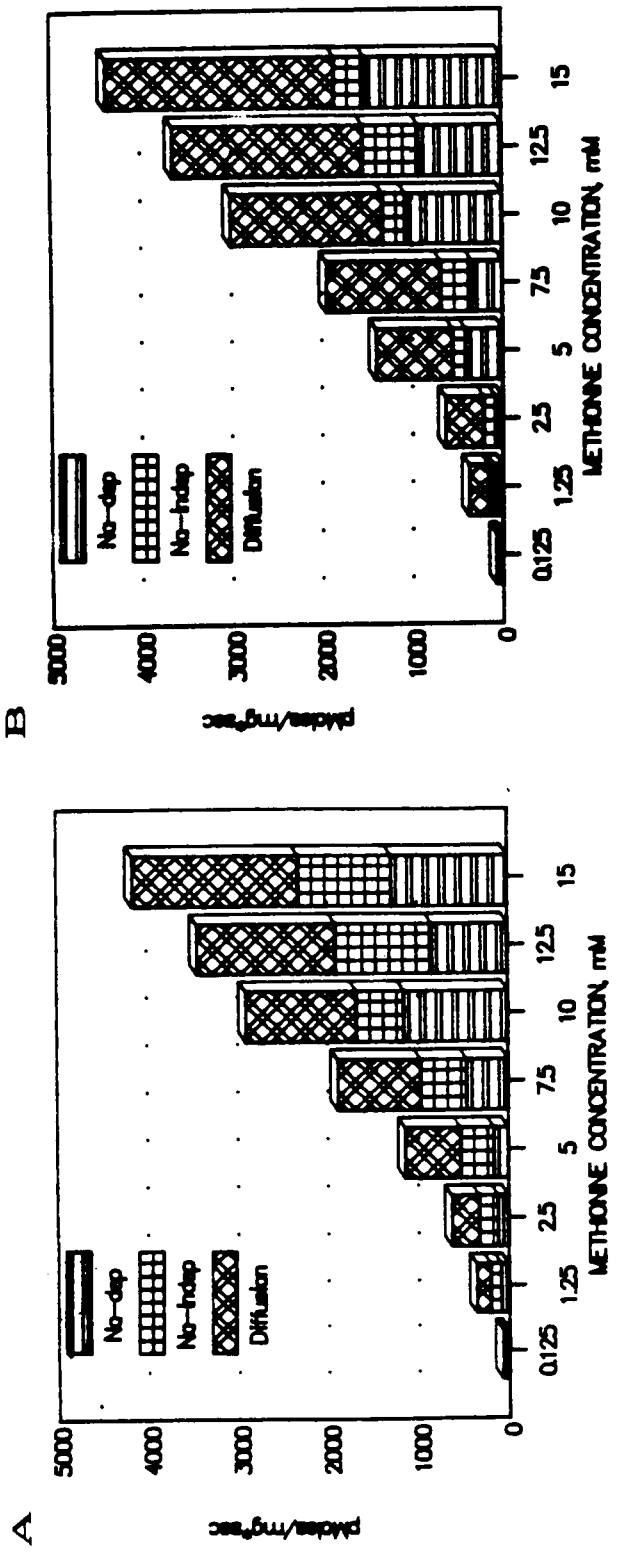

Figure 2. Methionine uptake by jejunal (A) and ileal (B) brush border tissue as influenced by increasing substrate concentration.

uptake will meet physiological demand when amino acid concentration presented to the transporters is 2.5 mM. Total MET uptake by jejunal and ileal tissue was 680 and 634 pmoles/mg·sec, respectively, when substrate concentration was 2.5 mM. The MET uptake due to diffusion for jejunal and ileal BB tissue was 614 and 845 pmoles/mg·sec, respectively, when substrate concentration was 5.0 mM. Thus it appears that physiological needs can be met by diffusion when MET is presented at a concentration of 5 mM or greater.

The total uptake of LYS in jejunal and ileal BB tissue was found to be 395 and 542 pmoles/mg·sec, respectively, when LYS concentration was 2.5 mM. Lysine uptake at a substrate concentration of 5 mM was not evaluated, but data obtained at 7.5 mM indicated that results would be similar to MET data. Diffusion uptake of LYS in jejunal and ileal BB tissue was 777 and 762 pmoles/mg·sec, respectively, when substrate concentration was 7.5 mM. It appears that concerns about amino acid interaction and competition for mediated transporters limiting amino acid availability to the point of reduced productivity are unfounded when amino acid concentration is in excess of 5 mM. Since the amino acid uptake by diffusion appears capable of meeting amino acid requirements and diffusion is not known to be influenced by competition.

Interactions and competition would seem to be of greater concern at and below 2.5 mM substrate concentrations. When evaluating amino acid transport on a physiological requirement basis, the biological importance of the contribution of the mediated systems increased at lower substrate concentrations. The increase significance of the mediated transport systems at lower amino acid concentrations appears to be more a function of the lower diffusion contribution than an elevation of uptake via the mediated systems.

It should be pointed out that the previous speculations on amino acid absorption have been based on the observations of the transport properties of two amino acids, LYS and MET, and the assumption that uptake at 2.5 mM amino acid concentrations meets the physiological demands. Based on these data and assumptions, it can be concluded that the diffusion component of uptake can meet physiological demands when amino acid concentration is above 5 mM. It appears that the investigation of amino acid interaction and competition for mediated uptake should occur at a substrate concentration below 2.5 mM, since this is the concentration range were these systems make significant contribution toward meeting the physiological demands.

Among the problems encountered with the membrane vesicle technique are the physical and time constraints

inherent in the equipment. Even when there are six transport towers available, only one transport reaction can be started at a time. With this some overlap in washing procedure between towers may occur. Therefore the average time for one evaluation or data point is approximately 50 s. Since membranes are incubated at 39 C during the experiment, concern arises as to potential protease damage becoming significant after the first hour of incubation. This time constraint limits the size of experiments to approximately 75 evaluations points, which, depending on the type of experiment, is three to six repetitions of each treatment. This may be adequate but it is not ideal with this type of experimentation.

Another problem is the potential for inconsistency between repetitions and sub-sampling that accompanies experiments requiring multiple pipetting. The multiple hand pipettings for each reaction vessel that occur prior to the evaluation of transport results is a large potential variation between repetitions. The added pressure of time constraints when stopping and sub-sampling reactions increases the potential for rushed and inaccurate pipetting.

The ideal technique for measuring transport properties using membrane vesicles would allow for multiple evaluations during one time period. In addition, the technique should have consistent reaction mixture pipetting

and allow for the stopping of reactions and collection of vesicles with a minimum of time constraint pressure. The criterion of the ideal technique may be met to a large degree by using the wells of microtiter plates as reaction vessels and the automated cell harvester to collect vesicles for quantification of uptake.

The microtiter plates consist of eight rows and twelve columns of wells, each well has a 300 ul capacity. The cell harvester consists of 24 paired wash outlets and aspirator inlets arranged in such a way as to allow for the collection of the contents of two rows of wells (24 wells) on the microtiter plate. The wash outlet flushes contents of the well free for aspiration. The aspirator inlets lead to a liquid waste trap via filter paper. Thus, the potential exists to stop and collect 24 transport experiments simultaneously.

Advantages of this system include: more evaluations per unit time, uniform washing for up to 24 wells, the use of smaller less expensive scintillation vials, the use of less scintillation fluid, allows for larger scale experiments within a shorter time interval, and minimizes experimental variation. With proper planning of experiments this procedure also maximizes the use of vesicles and isotope. Since there will be no need for including the extra half reaction volume in each reaction vessels, as is done in the current procedure.

Even though the time required to perform the experiment will be reduced, the need for preparation and planning the experiment will be increased. Some possible problem areas include: residual radiation in cell harvester tubing, background binding to filter paper, finding filter paper with appropriate pore size for trapping vesicles, assuring uniform mixing of reaction wells, maintaining incubation temperatures and designing pipette troughs for multi-tip pipettors. Also, the larger scale experiment will have a greater financial and material (vesicles) investment, therefore, a mistake in buffers, mix up in scintillation vials or an experiment which does not work will have a larger cost.

The advantages of this potential technique far outweigh the disadvantages. Therefore, the development of this technique should have a high priority in the arena of membrane vesicle research.

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APPENDIXES

APPENDIX A

Slaughter and Tissue Harvesting Technique

The steer is stunned with a single shot between the eyes from a .22 caliber pistol. The animal is exsanguinated by inserting a knife blade just above the brisket and extending the cut dorsally. Once violent kicking has stopped, approximately 3 min, a leg chain is place around the rear leg just above the hock and the steer is hoisted so that the shoulders are just above the ground. The anus is washed and bunged, with care being taken to avoid cutting the rectum, then a ligature is place on the caudal end of the rectum. The anus is pushed into the body cavity approximately 1 m, tearing large intestine and rectum free from suspensory fat and fascia. The peritoneal cavity is opened with a midline ventral incision starting at the caudal most end of the abdominal cavity and extending to the sternum. To avoid cutting viscera during incision, the hand should be placed inside the cavity with the blade of the knife facing away from viscera. Viscera is removed by pulling intestines and rumen free from suspensory tissue, then cutting the esophagus, allowing viscera to fall to the floor.

The abomasum is located, found on right side of rumen, and traced to the jejunum. This junction is the pyloric sphincter. The duodenum is followed distally for 2 m, then

ligated with twine. The duodenum is severed on the caudal side of the ligature and stripped free from mesentery in a caudal direction. An associate should locate a point 2 m rostral to ileocecal junction and begin striping the intestine free of the mesentery in a rostral direction. Care should be taken not to perforate the intestine during the striping procedure. The entire segment is then folded in half and rostral and caudal segments designated jejunum and ileum, respectively. These procedures should be accomplished within 30 min of stunning.

The intestine is divided into two segments at the midpoint. These segments are separated and digesta is poured out of each segment (segment approximately 8 m). Segments are placed in appropriately labeled polypropylene pans for short term storage (5-20 min).

Ileal and jejunal sections are sub-divided into segments 1 m in length. One meter segments are flushed with a bolus of mannitol buffer[1] (room temperature) until the effluent appears colorless. Segments are then everted with aid of a glass rod. Everted segments are then washed in a series of three polypropylene pans containing mannitol buffer. Segments remain in the last wash until space is available in hyaluronidase incubation solution. Three, 1 liter glass beakers containing 330 ml of hyaluronidase incubation buffer in a 37 °C water bath serve as incubation

[1] Composition of all buffers found in appendix B.

baths. Following 20 min incubation, each segment is removed from the bath and placed on surgical trays which are located on a bed of crushed ice. Mucosal lining is then harvested by scraping the tissue with a glass slide. The harvested mucosal cells are placed in beakers and stored on ice.

Harvested cells are suspended in mannitol buffer and then centrifuged for 10 min at 1,000 · g to wash residual hyaluronidase and digesta from the cells. Two washes using 250 ml centrifuge bottles yield satisfactory results. Cell yields (g) are determined for intestinal sites following washings. The washed cells are divided into 3-4 g aliquots and placed into whirl-pac bags. Sealed bags are frozen by submerging in liquid N_2. Frozen mucosal scrapings are then placed in 3.78 liter freezer bags and placed in the ultra-low freezer (-80°C) for long term storage. Freezer bags are labeled with date of slaughter, animal number and tissue origin. An equipment checklist for slaughter and tissue collection is given in Figure 1.

Vesicle Isolation

Brush Border. Isolation begins with the homogenization of mucosal scrapings. Previously isolated and frozen mucosal scrapings of three animals are composited. The composite consists of one packet (3 g) of mucosal tissue from each of the animals placed into a 150

Materials:	chain hoist	water bath	stop watch
	leg chain	rat cages	scissors
	stun gun	glass slides	parafilm
	knives	labeling tape	surgical trays (2)
	glass rod	string	glass beakers (1 L)
	sharpies	carboy	plastic bags (3.8 L)
	centrifuge	ice chest	centrifuge bottles
	steer	liquid N_2	whirl pack bags
	balance	freezer bags	disposable pipet
	ice	assistants (3)	ultra low freezer
	N_2 tank	plastic beakers (1 L)	

Buffers: hyaluronidase buffer (1 L)

mannitol buffer (30 L)

Figure 1. Equipment checklist for tissue harvesting.

ml beaker then 75 ml of mannitol-succinate[1] buffer is added. This process is repeated three additional times, with the result being four 150 ml beakers, each containing membrane composites of three animals and 75 ml of mannitol-succinate buffer. After the mucosal scrapings mixture is allowed to thaw, approximately 7 min, they are homogenized for 15 sec with a polytron at setting six. The homogenates are combined in a 500 ml wide-mouth Erhlenmyer flask and placed on ice. The homogenate is incubated at 4°C for 30 min while being mildly agitated on a stir plate. Note that a total of 12 packets of mucosal scrapings are utilized and the total volume is 300 ml. This volume was chosen due to the volume constraints of the centrifuge utilized for differential centrifugation.

Following the incubation, the homogenate is distributed equally between eight, 50 ml centrifuge tubes. Homogenate is then centrifuged at 8700 · g for 12 min. All centrifugations are performed at 4°C. The resulting pellet contains a majority of basolateral (BL) and internal membranes. The BL membranes may be isolated by a method described in BL membrane isolation section. The supernatant from each tube is poured into a clean 50 ml centrifuge tube, one supernate per tube. The eight tubes containing supernatant are centrifuged at 31,000 · g for 15 min. The resulting supernatant is discarded. The pellets

[1] Composition of all buffers found in appendix B.

containing the brush border (BB) membranes are combined for resuspension with a total of 35 ml mannitol-succinate buffer for all eight pellets. The resuspension is accomplished with six to eight strokes of a 40 ml capacity teflon-glass homogenizer.

This homogenate is incubated at 4°C for 30 min. The second divalent cation incubation is for the removal of BL that escaped the initial divalent cation precipitation. Following incubation the mixture is centrifuged at 8700 · g for 12 min. The resulting supernatant is poured off into clean 50 ml centrifuge tubes and the pellet is discarded. The supernatant is centrifuged at 31,000 · g for 15 min. The resulting supernatant is discarded while the pellet is resuspended with 15 ml mannitol-transport buffer. This 15 ml figure may be changed if multiple isolations are preformed, see flow chart (Figure 2). The pellet is resuspended with six to eight strokes of 40 ml capacity teflon-glass homogenizer. The homogenate is equally distributed on a sucrose density gradient consisting of 27 and 31% sucrose, and if any space remains the void is filled with mannitol-transport buffer. Generally, four gradients are prepared for isolation starting with 300 ml of initial homogenate. If the following procedure is used for multiple isolations, six gradients are required for every two initial homogenizations. The sucrose density gradients are then centrifuged for 90 min at 105,000 · g.

Comments	Time	Batch 1	Batch 2	Batch 3	Batch 4
	:00	homogenize			
	:30	incubation			
batch 1 pellets on ice	1:05	8700 · g 12 min 31000 · g 15 min	homogenize incubation		
	1:40		8700 · g 12 min 31000 · g 15 min	homogenize incubation	
combine pellets of B1 and B2	2:15	resuspend pellets in mannitol-succinate buffer, incubate 30 min		8700 · g 12 min 31000 · g 15 min	
batch 3 pellets on ice	2:50	8700 · g 12 min 31000 · g 15 min			homogenize incubate
		ultra centrifugation sucrose density gradient 105000 · g, 90 min			8700 · g 12 min 31000 · g 15 min
combine pellets of B3 and B4	3:25			resuspend pellets in mannitol-succinate buffer, incubate	
				8700 · g 12 min 31000 · g 15 min	
	4:30			ultra centrifugation sucrose density gradient 105000 · g, 90 min	
	5:15				
	6:30	harvest bands and freeze in liquid N_2			

Figure 2. Centrifuge schedule for the isolation of multiple batches of brush border membrane vesicles

An equipment check list for membrane isolation is given in Figure 3.

Basolateral: The homogenization and initial divalent cation incubation are the same as those followed for brush border isolation. The technique for BL membrane isolation diverges from BB membrane isolation after the initial 8700 · g centrifugation. The supernatants from this centrifugation are utilized for BB isolation while the pellets (eight pellets) are used for BL isolation. These pellets are resuspended in two, 30 ml aliquots of mannitol buffer with 10 strokes of a teflon-glass homogenizer. The resulting homogenate is placed in two 40 ml centrifuge tubes and centrifuged at 750 · g for 12 min. The resulting pellets contain internal membranes and organelles of the cell (nucleus, mitochondria and endoplasmic reticulum) while the BL membranes remain in the supernatant. The supernatants are decanted into clean centrifuge tubes and the BL membranes are collected with a 8700 · g centrifugation for 12 min.

The resulting supernates are discarded and the pellets are resuspended in 20 ml mannitol buffer with six to eight strokes of a teflon-glass homogenizer and then placed on four sucrose density gradients consisting of 27 and 31% sucrose. If multiple preparations are occurring, the BL membrane enriched pellets from the first homogenization are placed on ice until BL membrane enriched pellets from

Materials:	labeling tape	glass beakers (250 ml)
	sharpies	refrigerated centrifuge
	ice	centrifuge tubes (40 ml)
	liquid N_2	mucosal scrapings
	balance	disposable pipet
	N_2 tank	ultra low freezer
	cryovials	plastic beakers (1 l)
	ultracentrifuge	ultracentrifuge tubes (13 ml)
	stir plate	magnetic stirrer

Buffers: mannitol-succinate buffer (2 L)
mannitol-transport buffer (2 L)
sucrose solution 27 and 31% (25 ml)

Figure 3. Materials checklist for membrane isolation.

second homogenizations are ready for the sucrose density gradients. The two sets of pellets are combined and resuspended in 30 ml of mannitol buffer with six to eight strokes of a teflon-glass homogenizer and placed on six sucrose density gradients and centrifuged at 105,000 · g for 90 min. Preparation of sucrose gradients and band collection are addressed in future sections. An equipment check list for membrane isolation is given in Figure 3.

Gradient Preparation and Band Collection

A typical gradient consisted of 27 and 31% sucrose[1] with the highest density sucrose comprising the lower layer. The gradient is prepared by layering 3.5 ml of 31% sucrose into a 13 ml ultra centrifuge tube with a 13.5 cm polyethylene transfer pipet. The volume of sucrose solution to be used is measured with a 5 ml graduated pipet into a 25 ml beaker and then transferred to the gradient tube with the transfer pipet. When applying the 3.5 ml of 27% sucrose solution, care must be taken to avoid mixing the two layers at the interface. This is accomplished by placing the pipet tip against the side of the tube at the level of the previous layer and applying slight pressure. This allows the first drop to gradually leave the pipette and spread across the surface of the previous layer. The

completed gradient should have a sharp interface and if there is turbid appearance then that gradient should be discarded. The sucrose density gradient should not be prepared more than 1 h prior to use since the gradient interface will dissipate with time. The membranes to be purified are divided equally among prepared gradients. Care is taken to avoid mixing when membranes are layered on the gradient. If any space remains in gradient, fill the tube with mannitol-transport[1] buffer.

Following ultracentrifugation of the sucrose gradient, the membrane bands are harvested. The membrane band and sucrose layer under the band is collected with a 13.5 cm polyethylene transfer pipet. For example, the 27 % membrane band consists of the membranes on the surface of the 27% sucrose layer and the sucrose material down to, but not including, the membrane band on top of the 31% sucrose. The aspirated membranes are placed in cyrovials properly labeled with vesicle type, tissue origin, date of isolation and % sucrose of band. Cyrovials are flash frozen in liquid N_2 and stored for future use at -80°C in the ultralow freezer.

Transport Studies with Membrane Vesicles

[1] Composition of all buffers found in appendix B.

Uptake is initiated by adding membrane vesicles to a reaction vessel. The reaction vessel contains substrate, cofactors, buffer and isotope. Following the incubation period, uptake is terminated with the addition of stopping solution. The resulting mixture is sub-sampled and the level of uptake by vesicles is determined by liquid scintillation counting.

Although this overview of transport evaluation seems simplistic, there are several underlying considerations that must be addressed in order to properly prepare for a transport experiment. Some of these considerations include: what volume of membranes, which isotope and reaction buffers are needed and at what concentration? How to maintain osmolality of the reaction buffer across treatments and how to calculate uptake from CPM are also important consideration. These problems and others will be addressed in the following section.

Reaction Volume. The total volume of reaction mixture is determined by the number of points evaluated per vessel. Generally the volume of one evaluation point is set at 100 ul. An additional 50 ul of total volume is included per reaction vessel to minimize the possibility of being short due to adherence of mixture to the reaction vessel. It can be seen that as the number of reaction points per vessel is increased, the percentage of unused

(wasted) isotope decreases. The desire to minimize waste needs to be balanced with the need for repetition. For this reason, reaction vessels generally contain two evaluation points. Therefore the reaction vessel will have a final volume of 2.5 · 100 ul or 250 ul.

This 250 ul will be composed of transport buffer, isotope solution and membrane solution. In preparing the reaction mixture, the transport buffer is added at 50%, isotope 33% and membranes 17% of total volume. Note that these percentages are arbitrarily chosen for convenience.

Transport Buffer. The transport buffer is formulated to have the final concentrations of Mg_2Cl, 2 mM; HEPES, 10 mM; and either NaSCN or KSCN at 100 mM. Transport buffer will also contain substrate and mannitol but their final concentration will be variable. Substrate concentration will vary with design of the experiment while mannitol concentration will vary inversely with that of substrate, since mannitol is used to maintain equal osmolalities across substrate concentration. Equal osmolalities can be maintained if the summation of the concentrations of mannitol and substrate equals 100 mM. Note that the osmolality of all buffers should be measured prior to their use in an experiment. This will avoid bias in uptake data due to differences in vesicle size across treatments and serves as a check to see if buffers were properly prepared.

The stock transport buffer is prepared at twice the desired final concentration. Remember that a stock transport buffer needs to be prepared for each substrate concentration evaluated. If Na-dependent uptake is to be evaluated, two sets of every substrate concentration needs to be prepared, one with NaSCN and one with KSCN.

Isotope Handling. Isotope solution is prepared by adding distilled water to the desired amount of isotope. The amount of isotope used in the experiment is dictated by the number of points being evaluated. The role of thumb followed is 1 uCi of isotope per evaluation point. The amount of distilled water used to dilute the isotope also depends on the size of the assay. Generally, the total volume of isotope required is calculated from assay size and then 200 ul more volume is added to the amount of distilled water used in dilution.

Isotope is stored either frozen or refrigerated. Frozen isotopes are generally aliquoted into 7 ml glass scintillation vials in assay size fractions (50 uCi, 75 uCi and 100 uCi aliquots) at the time of arrival at the laboratory. This practice minimizes the amount of freezing and thawing of the isotope. At the time of the assay the vial containing the appropriate level of isotope is removed from the freezer and the predetermined amount of distilled water is added to isotope. Refrigerated isotope is stored

as one pooled sample at the time of experiment, the desired level of isotope is removed from the pool and placed in a 7 ml glass scintillation vial. Isotope is then diluted to desired volume with distilled water for use in assay.

Membrane Solution. The volume of membrane solution prepared is also dependent on assay size. The goal is to deliver .06 to .08 mg of protein per evaluation point. This can be provided by preparing membrane solution at a protein concentration of 3-4 $g \cdot ml^{-1}$. The total volume of membrane solution needed can be calculated from the number of reaction vessels being prepared for the assay plus 200 ul for protein concentration determination assay. Again, waste should be minimized since membranes are an important commodity.

To prepare the membrane vesicle solution, three to four cyrovials of frozen membrane vesicles are removed from freezer and thawed. Three cryovials are used if the experiment contains less than seventy-five evaluation points, while four vials are used for experiments of 75-125 evaluation points. Thawing is accelerated by placing cyrovials containing vesicles in a beaker of warm water, 45°C. After vesicles are thawed, they are pooled into 35 ml ultracentrifuge tube and diluted to volume with mannitol-transport buffer. A counter-balanced tube is prepared and vesicles are centrifuged for 60 min at 105,000

· g. The resulting supernatant is discarded and the pellet is resuspended in mannitol-transport buffer at the predetermined volume with ten strokes of 10 ml teflon-glass homogenizer. The membrane solution is then placed in a 7 ml glass scintillation vial for storage during the experiment. The experiment should be preformed within 2 h of vesicle resuspension.

Experimental Design. The reaction vessels used in most transport experiments are 12 · 75 mm disposable culture tubes. Before the experiment can be performed, reaction vesicles are labeled and organized in test tube rack. The organization of the reaction vessels is generally such that the Na^+ and K^+ vesicles are paired within concentration. A repetition consists of evaluating all concentrations in presence of Na^+ and K^+. A minimum of three repetitions is recommended. After transport buffer and isotope solution are added to the reaction vessels, they are placed in a water bath at 39°C, bovine body temperature.

The vial containing membranes is also placed in the water bath. Transport is evaluated after membrane solution and reaction vessels have equilibrated to 39°C, approximately 10 min of incubation. To initiate transport, the membrane solution is added to the reaction vessel while contents are being vortexed. A stopwatch is started at

time of addition. Vortexing is continued for 3-5 sec. The reaction mixture is held at rest until 12 sec time when vortexing is repeated. Termination of transport occurs at 15 sec with the addition of 1 ml of stopping solution. The resulting mixture is vortexed and then sub-sampled. The volume of sub-sample depends on the number of evaluation points in the reaction vessel. If there were two evaluation points then one evaluation volume (100 ul) would be 40% of total volume (250 ul). Thus two 500 ul aliquots of final mixture would be evaluated from each reaction vessel.

Uptake Quantification. The amount of uptake by the vesicles is determined by collecting the vesicles present in the 500 ul aliquot on a nitrocellulose membrane filter, .45 um pore size. The vesicles are washed with three, 5 ml aliquots of stopping solution. For convenience, stopping solution is delivered with a repipetor. The assumption is that by washing the vesicles, the isotope not contained within the vesicles is removed from the filter. Thus all isotope on the filter is contained within the vesicles and the result of transport. One known flaw in this assumption is that background binding to the filter does occur. This binding is thought to be due to ionic interaction and therefore will vary with type of substrate and substrate concentration. For this reason, background binding needs

to be evaluated under all experimental conditions. This is done by making background reaction vessels the same as experimental reaction vessels but then adding water instead of membranes prior to the termination step. Note, isotope volume but not membrane volume needs to be accounted for when calculating total volume required for these fractions.

The nitrocellulose filters used to harvest membrane vesicles need to be pre-soaked in stopping solution prior to their use in experiments. To minimize filter variation, the presoak time should be consistent between experiments. This consistency can be achieved by placing filters in presoak while vesicles are thawing. The result will be a 2 to 3 h presoaking. The number of filters presoaked is experiment dependent but as a general rule preparing at least ten more filters than the anticipated need is a good idea.

Miscellaneous. Some details of the transport manifold technique which have been omitted from previous sections but need to be stated are presented next. The transport manifold is a structure composed of a primary tube with six secondary tubes, transport towers. Each tower has an independent valve for controlling the vacuum applied to that tower. House vacuum is applied via a hydroscopic filter and a double trap to the primary tube. It is important to be sure the house vacuum is on before

the experiment is started. The vacuum source to the tower remains closed until vesicles are applied. The vacuum remains open until the last of the third washing is pulled through the filter, at which time the valve is closed. The filter is then removed from filter platform with, radioactive, filter forceps and placed in a 20 ml scintillation vial. Note that scintillation vials are organized and their box labeled so that treatments will not become confused or misplaced. The vacuum is turned on and the filter platform is rinsed with distilled water from a wash bottle. The vacuum is turned off and the nonradioactive filter forceps are used to place a new filter on the platform. The tower is clamped in position and at this time the manifold is ready for the next sample. Remember that the manifold has six towers so for greater time efficiency, all six towers should be used and then filters collected and replaced at same time.

Liquid scintillation counting is used to quantify uptake following the completion of the transport assay. Samples are prepared by adding 9 ml of scintillation fluid to every vial containing isotope. Vials are capped and labeled, label caps only and avoid handling sides of vials. Next, vortex vigorously for a minimum of 20 sec. Vortexing enhances the probability that the isotope is free of the filter and uniformly distributed throughout the flour solution.

Samples are allowed to equilibrate for a minimum of 2 h before they are placed in the scintillation counter for quantification. All isotopes, ^{35}S, ^{3}H and ^{14}C, are evaluated with program 9 for 5 min and 1% error. When ^{35}S is evaluated, cpm from total window are used in calculations. When ^{3}H or ^{14}C are evaluated, only cpm in their respective windows are used in calculations.

Note that these experiments all involve the use of radioisotopes. Therefore, strict adherence to the guidelines outlined in the radiation safety manual is essential. Some of the procedures which should be followed during transport experiments include: placing clean blotter paper under manifold area before each experiment, placing radioactive pipet tips in plastic bag for dry waste disposal, and trapping all liquid waste in first vacuum flask to avoid possibility of contaminating house vacuum. Following the experiment, residual fluids of reaction vessels are placed in the liquid waste and the reaction vessels themselves are placed in the dry waste along with the blotter paper and pipet tips. After transport studies are completed, the area is wiped clean with soapy water and the manifold and forceps are thoroughly rinsed. Background swipes are taken to assure the safety of the operator and others who use the area.

Uptake by vesicles is reported as pmoles $\cdot$ mg protein^{-1} $\cdot$ sec^{-1}. This practice standardizes uptake as a function

of protein, thus allowing for across experiment comparisons. To calculate pmoles · mg protein^{-1} · sec^{-1} from cpm obtained from scintillation counting, the specific activity (cpm/pmole) of the substrate needs to be determined.

Protein concentration is determined by assaying membrane solution with Pierce protein solution[2]. The concentration of protein in membrane solution is then multiplied by 17 ul, the volume of membrane solution used for each evaluation point. This yields mg protein at each evaluation point.

The specific activity of substrate is determined by measuring the CPM of a known volume of substrate at a known concentration. This can be accomplished by pooling the residual reaction solution and sub-sampling a known volume, usually five repetitions of 25 ul, and determining CPM. This allows for the calculation of a constant with the units of CPM · ul^{-1}. This value will be constant across all reaction vessels since the same level of isotope was added to all reaction vessels. By dividing the concentration of substrate in a reaction mixture by this constant, the specific activity, pmoles · CPM^{-1}, of the mixture can be determined. It is very important to calculate the specific activity of all concentrations evaluated in the experiment. The multiplication of CPM

2 Assay procedure outlined in appendix C.

times specific activity yields the apparent substrate uptake in pmoles. The subtraction of background binding for this substrate concentration yields true substrate uptake. The rate of uptake is found by dividing uptake by incubation time, in sec. This value is then standardized by dividing by level of protein per evaluation point. It is my recommendation that a spread sheet program be prepared to perform these tedious calculations. An equipment checklist for transport experiments is given in Figure 4.

The importance of pre-planning experiments should be apparent by this time. To maximize the chance of success and to avoid waste, pre-planning is needed to design the layout of the experiment, to prepare proper amounts and types of buffers, to determine the transport system to be evaluated, to determine volume of membrane and isotope solutions required and number of filters to prepare. The following is an example of the type of experimental outline which should be prepared before any transport experiment is preformed.

Experimental Outline .

Objectives. To determine the relative contribution of Na-dependent, Na-independent and diffusion components of lysine uptake to total lysine accumulation at different substrate levels.

Equipment:	ultracentrifuge	water bath
	transport manifold	ice bath
	wash bottle	ultracentrifuge tubes
	repipetor	vortex
	Pipetteman	test tube rack
	-1000 ml (2)	beaker (2 l)
	-200 ml (1)	pipet tips (in rack)
	-100 ml (1)	teflon-glass homogenizer
Materials:	nitrocellulose filters	membrane vesicles
	scintillation vials	transport buffers
	-20 ml	stopping solution
	-7 ml	isotopes
	Scintillation fluid	culture tubes
	plastic bag (3.8 L)	blotter paper

Figure 4. Equipment checklist for transport assays.

Hypothesis. Total lysine accumulation is assumed to be composed of three components: Na-dependent, Na-independent and diffusion uptake. Thus by determining total uptake (Na-buffer), total uptake minus Na-dependent uptake (K-buffer) and diffusion, all three components of total transport can be determined. Na-dependent uptake is determine by the difference between uptake in presence of Na and K at a given concentration of lysine. Na-independent uptake is equivalent to the difference in uptake in presence of K and diffusion at given concentration of lysine. The diffusion component of uptake for each concentration of lysine evaluated is determined indirectly with the use of a calculated diffusion constant. Diffusion constant is determined by measuring rate of lysine uptake at a lysine concentration which greatly exceeds the capacity of the mediated transport systems. A concentration of 100 mM generally meets this criterion. The slope of the line formed from extrapolating this uptake value back to zero serves as the diffusion constant. Multiplying the diffusion constant times the substrate concentration yields an estimate of the amount of diffusion which would occur at that substrate concentration.

Procedure. Determine total lysine accumulation by measuring the initial velocity , uptake first 15 sec, of

lysine transport in the presence of Na buffer at following concentrations: .313, .625, 1.25, 2.5 and 7.5 mM.

Na-independent component of total uptake is determined by measuring the initial velocity of lysine accumulation in the presence of K buffer at lysine concentrations of .313, .625, 1.25, 2.5 and 7.5 mM.

Diffusion is determined by measuring initial velocity of lysine accumulation with K buffer at a lysine concentration of 100 mM.

Protocol. Three repetitions, two evaluation per reaction vessel, a total of six evaluations at each concentration. Two repetitions for background determination. A diagram of this experiment is presented in Figure 5.

		CONCENTRATION, mM						
REP	BUFFER	.313	.625	1.25	2.5	7.5	100	tmt
1	Na	x	x	x	x	x	x	a,b
	K	x	x	x	x	x	x	a,b
2	Na	x	x	x	x	x	x	a,b
	K	x	x	x	x	x	x	a,b
3	Na	x	x	x	x	x	x	a,b
	K	x	x	x	x	x	x	a,b
BKG	K	x	x	x	x	x	x	b
		x	x	x	x	x	x	b

Figure 5. Diagram of a transport experiment. Note there are 36 reaction vessels which require membrane solution (a) and 48 reaction vessels which require isotope (b).

Example Calculations

1. Reaction volume: 100 ul·pt^{-1} · 2.5 pts·rx^{-1} = 250 ul

Components	% of total vol.	Vol (ul)
transport buffer	50	125
isotope	33	83
membrane	17	42

2. Transport buffer: volume required: 125 · 4 = 600 ul
   generally prepared in excess prior to experiment.

3. Isotope solution: Labeled Lysine = 100 uCi (in .1 ml)
   total volume required: 48 · .083 ml = 3.98 ml
   3.98 + .2 ml (cushion amount)= 4.18 ml
   Note: add only 4.08 ml water to isotope since .1 ml of solution with isotope.

4. Membrane solution, total volume required
   36 · .042 ml = 1.51
   1.51 + .2 ml(cushion) = 1.71 ml
   Note this is volume of mannitol-transport buffer added to membrane pellet for resuspension.

5. Number of filters required:
   48 rx vessels · 2 filters/rx vessel = 96 filters
   96 + 10 (cushion)= 106 filters total

6. Stopping solution: unless experiment is large than 125 evaluation points, 2 liters will be adequate.

7. Specific activity calculations: Need to save final .25 ml of reaction solution for specific activity determination

Flow chart.

1) Add transport buffer and isotope to reaction vessel and bring to 39 C.
2) Kill transport with 1 ml stopping solution, vortex.
3) Filter two .5 ml aliquots
4) Save last .25 ml for specific activity determination
5) Wash with three 5 ml aliquots of stopping solution
6) Repeat for all concentrations
7) Pool remaining reaction solution, remove 25 ul sub -sample and place in scintillation vial for determination of specific activity. Replicate six times.

APPENDIX B

Isolation Buffers.

TABLE 1. MANNITOL BUFFER.

Compound	MW	mM	Grams 2 L	4 L	6 L
Mannitol	182.2	300	109.3	218.6	327.9
Tris	121.1	12	2.9	5.8	8.7

pH adjusted to 7.4 with HCl

TABLE 2. MANNITOL-SUCCINATE BUFFER.

Compound	MW	mM	Grams .5 L	1 L	2 L
Mannitol	182.2	150	13.7	27.3	54.7
Succinate	118.1	30	1.8	3.5	7.1
Tris	121.1	10	.6	1.2	2.4
$MgCl_2$	203.1	5	.5	1.0	2.0
K_2HPO_4	174.2	5	.44	.87	1.74
$MnCl_2$	197.9	.1	.01	.02	.04

pH adjusted to 7.4 with NaOH

TABLE 3. MANNITOL-TRANSPORT BUFFER

Compound	MW	mM	Grams .5 L	Grams 1 L	Grams 2 L
Mannitol	182.2	272	24.8	49.5	99.0
Hepes	238.3	20	2.4	4.8	9.5
$MgCl_2$	203.1	2	.2	.4	.8

pH adjusted to 7.4 with NH_3OH

TABLE 4. GRADIENT BUFFER TWICE CONCENTRATED.

Compound	MW	mM	Grams .5 L	Grams 1 L	Grams 2 L
Hepes	238.3	4	.95	1.91	3.82
$MgCl_2$	203.1	4	.41	.82	1.63

pH adjusted to 7.4 with NH_3OH

TABLE 5. SUCROSE GRADIENT SOLUTIONS.

% sucrose	Sucrose g	ml [2x] Gradient buffer	ml Total volume[1]
27	30.05	50	100
31	35.09	50	100

[1] Slurry of sucrose and gradient buffer brought to 100ml with double distilled water.
Note: Sucrose solutions are prone to mold, so therefore do not make gradient solutions more than a week prior to usage

Transport Assays.

TABLE 6. TRANSPORT ASSAY BUFFER.

Compound	MW	mM	g/l	2*[ ] g/l	4*[ ] g/l
NaSCN	81.1	100	8.11	16.22	32.4
MgCl2	203.1	2	.41	.81	1.62
Hepes	238.3	10	2.38	4.76	9.52
Mannitol	182.2	100	18.2	36.4	72.8
KSCN[1]	97.2	100	9.72	19.44	38.8
Substrate[2]	X	X	X	X	X

[1] Either NaSCN or KSCN are used but not both in same buffer pH adjusted to 7.4 with NH_3OH

[2] Concentration of substrate is experiment dependent, when added to buffer it replaces mannitol isoosmoticly

TABLE 7. OSMOLARITY BUFFERS.

			4*[ ] Stock
mM Mannitol	g/l	2*[ ] g/dl	Transport buffer ml[1]
25	4.6	.91	50
50	9.1	1.82	50
100	18.2	3.64	50
200	36.4	7.28	50
300	54.6	10.97	50

[1] Mannitol is disolved in 50 ml of 4*[ ] stock transport buffer, transport buffer without mannitol, total volume is then adjusted to 100 ml

TABLE 8. STOPPING SOLUTION.

Compound	MW	mM	grams/L
KCl	75.56	150	11.18
Phloridzin	436.4	.5	.22

Table 9. HYALURONIDASE BUFFER

			Grams	
Compound	MW	[ ]	500 ml	1 liter
NaCl	56.4	120 mM	3.38	6.77
tris base	121.0	20 mM	1.21	2.42
Mg_2Cl	203.0	1 mM	.10	.20
K_2HPO_4	174.2	3 mM	.26	.52
BSA	-	1 mg/ml	.50	1.0
Hyaluronidase	-	1 mg/ml	.50	1.0

pH was adjusted to 7.4 with HCl

APPENDIX C

Na/K ATPase Assay.

TABLE 1. ATPASE INCUBATION BUFFER

Compound	MW	mM[1]	Grams		
			.1 L	.5 L	1 L
NaCl	58.4	100	.65	3.24	6.49
KCl	74.6	10	.08	.41	.83
$MgCl_2$	203.1	5	.11	.57	1.13
Tris	121.1	100	1.35	6.73	13.46
NaEDTA	292.3	3	.10	.49	.97
Na_2ATP	551.2	3	.18	.92	1.84
Ouabain	738.6	1	.082	.41	.82

pH adjusted to 7.4 with HCl

[1] concentrations are in reference to the final concentration of the incubation reaction mixture, which contains 90% incubation buffer and 10% membrane fraction.

Procedure:

1. Place .9 ml incubation buffer in a 15 ml test tube, recommend running three with ouabain and without ouabain reaction vessels per tissue fraction.
2. Allow reaction vessels containing incubation buffer to equilibrate to 37 C in a shaking water bath.
3. Start reactions by adding .1 ml of diluted membrane fraction to reaction vessel and vortex.
4. Allow reaction to proceed with water bath shaker mechanism activated. This will assure the membranes do not settle out of solution.

5. Stop reaction after 30 min by adding 1 ml of ice cold 10 % TCA solution followed by vortexing. Be sure to stop reactions in same order that they were started.

6. Centrifuge reaction vessels at 10,000 x g for 10 min to remove membranes from solution.

7. Assay for liberated inorganic phosphorous.

Assay for Inorganic Phosphorous.

TABLE 2. AMMONIUM MOLYBDATE SOLUTION.

			Grams[1]		
Compound	MW	[w/v]	.1 L	.5 L	1 L
Ammonium molybdate	1235.9	2.5	2.5	12.5	25.0

[1] brought to volume with 6N H_2SO_4

TABLE 3. STOCK TRITON-X SOLUTION.

		Grams[1]		
Compound	[w/v]	.1 L	.5 L	1 L
Triton-X	1	1	5	10

[1] brought to volume with double distilled water

TABLE 4. TRITON-X WORKING SOLUTION.

Compound	[w/v]	ml[1] .1 L	.5 L	1 L
1% Triton-X	.012	1.22	6.12	12.2

[1] brought to volume with double distilled water

Procedure:

1. Add 5 ml of .012 % triton-X solution to a 15 ml test tube. Prepare one reaction vessel for each reaction vessel used during Na+/K+ ATPase assay.

2. Add .2 ml of supernatant from Na+/K+ ATPase reaction vessel to the vessel containing the triton-X solution and vortex.

3. Initiate reaction by adding .6 ml of molybdate solution and vortexing. Important to stagger start these reactions to correspond with the time required to determine the absorbance of the samples since the reaction does not reach a stable end point.

4. Absorbance determined after 20 min at a wavelength of 660 nm.

Calculations:

[ATPase] = (ug Pi /volume of supernatant) x dilution factor of membrane fraction

Total ATPase activity = [ATPase] x total volume of membrane fraction

Na+/K+ ATPase activity = ATPase activity without ouabain - ATPase activity with ouabain

Assay for Alkaline Phosphatase.

	Compound	Amount
A.	p-nitrophenol phosphate	316 umoles
B.	2-amino-2-methyl propanol	1.25 M

Procedure:

1. Add 31 ml of reagent A to vial containing reagent B
2. Add 3 ml of the resulting solution to cuvette
3. Add .1 ml of membrane to cuvette and mix
4. Monitor reaction for 3 min at 405 nm

Calculations:

AP activity (u/l) = change in absorbance over 3 min x conversion factor (550)

Protein Assay.

Procedure:

1. Add 5 ml of pierce protein assay reagent to a 15 ml test tube.
2. Initiate the reaction by adding .1 ml of membrane solution to the reaction vessel and vortexing
3. Read absorbance at 595 nm after 5 min

Calculations:

[Protein] = mg protein in aliquot / volume of aliquot

Total protein = [protein] x total volume of membrane fraction

Appendix D

Uptake data were analyzed using the General Linear Models (GLM) option of the Statistical Analysis System (SAS, 1982). The model accounted for day of experiment, substrate concentration, presence or absence of sodium, substrate concentration by presence or absence of sodium interaction and error.

Total uptake (uptake in the presence of sodium) and Na-independent (Na^-) plus diffusion uptake (uptake in the presence of potassium) were experimentally determined while diffusion data for each substrate concentration evaluated were determined using extrapolations of the data obtained by monitoring uptake at a substrate concentration of 100 mM. The Na-dependent (Na^+) uptake was determined by taking the difference of total uptake and uptake in presence of potassium. The Na^- uptake was the difference of uptake in the presence of potassium and diffusion at that substrate concentration. Mediated uptake was the difference of total uptake and diffusion at that substrate concentration. Therefore, total uptake and uptake in the presence of potassium were the only data with least-square (LS) means and standard errors (SE) calculated directly by SAS. The LS means and SE for diffusion, Na^+, Na^- and mediated data were calculated using the SAS determined LS means and SE.

Example calculations.

Diffusion. The diffusion at each concentration was determined by multiplying the diffusion coefficient (ß) times the substrate concentration. The ß was the slope of the line regressed from the total uptake (x) when substrate concentration was 100 mM (c) through the origin.

$$\beta = x/c \qquad SE(\beta) = 1/c\ SE(x)$$

$$\text{Diffusion rate} = D = \beta * (\text{substrate concentration})$$

$$SE(D) = (\text{substrate concentration}) * SE(\beta)$$

note: x and SE(x) are LS means and SE, respectively, of uptake data when substrate concentration was 100 mM.

Mediated systems. Least-square means (Z) and SE(Z) for mediated, Na^+ and Na^-, data were all determined with the same type of calculations (Tables 1-8). In all cases, the calculations involved taking the difference of two uptake LS means U_1 and U_2, and their associated SE, $SE(U_1)$ and $SE(U_2)$.

$$\text{LS mean } Z = U_1 - U_2$$

$$SE(z) = \{[SE(U_1)]^2 + [SE(U_2)]^2\}^{\frac{1}{2}}$$

Note: U_1, U_2, $SE(U_1)$ and $SE(U_2)$ were determined by SAS or calculated using equations outlined in diffusion section of this appendix.

Test Statistic: Pair-wise comparisons of LS means were made using the Students T test.

$$T = (X_1 - X_2) / \{[SE(X_1)]^2 + [SE(X_2)]^2\}^{\frac{1}{2}}$$

Degrees of Freedom: The degrees of freedom (df) for data determined directly were equivalent to (n - 1), while df for all calculated data were determined by the following equation (Tables 9-16).

$$df = 1/[(1/df_1 + 1/df_2) \cdot \tfrac{1}{2}]$$

$$df_1 = n_1 - 1 \qquad df_2 = n_2 - 1$$

Probabilities: The probabilities for the T-statistics were determined in SAS using the PROBT function. These were two tailed tests so the statement was (1-PROBT(ABS(x),DF))*2. The x refers to test statistic and DF refers to df associated with that test statistic.

TABLE 1. LEAST-SQUARE MEANS AND STANDARD ERRORS FOR LYSINE UPTAKE IN JEJUNAL BRUSH BORDER TISSUE[a].

	Sodium[b]		Potassium		Diffusion	
[MET]	Uptake[c]	S.E.	Uptake	S.E.	Uptake	S.E.
.313	114	30.2	86	31.0	32	1.2
.625	188	36.1	146	31.0	65	2.4
1.250	273	37.0	226	29.0	130	4.8
2.500	395	28.3	355	28.3	259	9.6
7.500	998	32.0	827	28.3	277	28.7

[a]Statistics determined by SAS from experimentally measured parameters.
[b]Sodium refers to uptake in the presence of Na, total uptake, K refers to uptake in the absence of Na, diffusion is determined by extrapolation techniques.
[c]Uptake is reported as pmoles·mg of protein^{-1}·sec^{-1}.

TABLE 2. LEAST-SQUARE MEANS AND STANDARD ERRORS FOR LYSINE UPTAKE IN ILEAL BRUSH BORDER TISSUE[a].

	Sodium[b]		Potassium		Diffusion	
[MET]	Uptake[c]	S.E.	Uptake	S.E.	Uptake	S.E.
.313	146	34.7	98	34.7	32	2.0
.625	240	36.8	154	34.7	64	3.9
1.250	282	34.7	220	36.8	127	7.9
2.500	542	34.7	432	35.6	254	15.8
7.500	1106	39.6	822	34.7	762	47.3

[a]Statistics determined by SAS from experimentally measured parameters.
[b]Sodium refers to uptake in the presence of Na, total uptake, K refers to uptake in the absence of Na, diffusion is determined by extrapolation techniques.
[c]Uptake is reported as pmoles·mg of protein^{-1}·sec^{-1}.

TABLE 3. LEAST-SQUARE MEANS AND STANDARD ERRORS FOR MEDIATED LYSINE UPTAKE SYSTEMS IN JEJUNAL BRUSH BORDER TISSUE[a].

	Mediated[b]		Na^+		Na^-	
[MET]	Uptake[c]	S.E.	Uptake	S.E.	Uptake	S.E.
.313	82	30.2	28	43.3	54	31.0
.625	123	36.2	42	47.6	81	31.1
1.250	143	37.3	47	47.0	96	29.4
2.500	136	29.9	40	40.0	96	29.9
7.500	221	43.0	171	42.7	50	40.3

[a]Statistics were calculated using the statistics determined by SAS from experimentally measured parameters.

[b]Mediated uptake = sodium uptake - diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion.

[c]Uptake is reported as pmoles·mg of protein^{-1}·sec^{-1}.

TABLE 4. LEAST-SQUARE MEANS AND STANDARD ERRORS FOR MEDIATED LYSINE UPTAKE SYSTEMS IN ILEAL BRUSH BORDER TISSUE[a].

	Mediated[b]		Na^+		Na^-	
[MET]	Uptake[c]	S.E.	Uptake	S.E.	Uptake	S.E.
.313	114	34.8	48	49.1	66	34.8
.625	176	37.0	86	50.6	90	34.9
1.250	155	35.6	62	50.6	93	37.6
2.500	288	38.1	110	49.1	178	38.9
7.500	344	61.7	284	52.7	60	58.7

[a]Statistics were calculated using the statistics determined by SAS from experimentally measured parameters.

[b]Mediated uptake = sodium uptake - diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion.

[c]Uptake is reported as pmoles·mg of protein^{-1}·sec^{-1}.

TABLE 5. LEAST-SQUARE MEANS AND STANDARD ERRORS FOR METHIONINE UPTAKE IN JEJUNAL BRUSH BORDER TISSUE[a].

	Sodium[b]		Potassium		Diffusion	
[MET]	Uptake[c]	S.E.	Uptake	S.E.	Uptake	S.E.
.125	77	68.2	35	66.6	15	1.1
1.250	392	64.5	285	66.6	153	11.4
2.500	680	68.2	514	64.5	307	22.7
5.000	1173	66.6	983	64.5	614	45.5
7.500	1953	76.3	1430	64.5	921	68.2
10.00	2904	64.5	1756	64.5	1228	90.9
12.50	3367	68.6	2473	81.6	1535	113.1
15.00	4132	72.6	2782	70.6	1842	136.4

[a]Statistics determined by SAS from experimentally measured parameters.
[b]Sodium refers to uptake in the presence of Na, total uptake, K refers to uptake in the absence of Na, diffusion is determined by extrapolation techniques.
[c]Uptake is reported as pmoles·mg of protein^{-1}·sec^{-1}.

TABLE 6. LEAST-SQUARE MEANS AND STANDARD ERRORS FOR METHIONINE UPTAKE IN ILEAL BRUSH BORDER TISSUE[a].

	Sodium[b]		Potassium		Diffusion	
[MET]	Uptake[c]	S.E.	Uptake	S.E.	Uptake	S.E.
.125	75	78.2	35	75.2	21	1.1
1.250	374	78.3	270	78.3	211	10.5
2.500	634	75.2	551	78.3	422	21.1
5.000	1398	78.3	989	75.2	845	42.1
7.500	1946	81.2	1610	78.3	1267	63.2
10.00	3009	75.2	1939	81.2	1689	84.2
12.50	3647	75.2	2728	84.1	2111	105.3
15.00	4384	78.3	2871	81.2	2534	126.2

[a]Statistics determined by SAS from experimentally measured parameters.
[b]Sodium refers to uptake in the presence of Na, total uptake, K refers to uptake in the absence of Na, diffusion is determined by extrapolation techniques.
[c]Uptake is reported as pmoles·mg of protein^{-1}·sec^{-1}.

TABLE 7. LEAST-SQUARE MEANS AND STANDARD ERRORS FOR MEDIATED METHIONINE UPTAKE SYSTEMS IN JEJUNAL BRUSH BORDER TISSUE[a].

	Mediated[b]		Na^+		Na^-	
[MET]	Uptake[c]	S.E.	Uptake	S.E.	Uptake	S.E.
.125	62	68.6	42	95.6	20	66.6
1.250	239	65.5	107	92.7	132	67.6
2.500	373	72.3	166	94.2	207	68.4
5.000	559	80.7	190	92.7	369	78.9
7.500	1032	102.3	523	99.9	509	93.9
10.00	1676	111.5	1148	91.2	528	111.5
12.50	1832	132.7	894	106.6	938	139.9
15.00	2290	154.5	1350	101.3	940	153.6

[a]Statistics were calculated using the statistics determined by SAS from experimentally measured parameters.

[b]Mediated uptake = sodium uptake - diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion.

[c]Uptake is reported as pmoles·mg of protein^{-1}·sec^{-1}.

TABLE 8. LEAST-SQUARE MEANS AND STANDARD ERRORS FOR MEDIATED METHIONINE UPTAKE SYSTEMS IN ILEAL BRUSH BORDER TISSUE[a].

	Mediated[b]		Na^+		Na^-	
[MET]	Uptake[c]	S.E.	Uptake	S.E.	Uptake	S.E.
.125	54	78.2	40	108.5	14	75.2
1.250	163	79.0	104	110.7	59	79.0
2.500	212	78.1	83	108.6	129	81.1
5.000	553	88.9	409	108.6	144	86.2
7.500	679	102.9	336	112.8	343	100.6
10.00	1320	112.9	1070	110.7	250	117.0
12.50	1536	129.4	919	112.8	617	134.8
15.00	1850	148.6	1513	112.8	337	150.2

[a]Statistics were calculated using the statistics determined by SAS from experimentally measured parameters.

[b]Mediated uptake = sodium uptake - diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion.

[c]Uptake is reported as pmoles·mg of protein^{-1}·sec^{-1}.

TABLE 9. DEGREES OF FREEDOM FOR LYSINE UPTAKE DATA IN ILEAL BRUSH BORDER MEMBRANE TISSUE.

[LYS]	Na[a]	K	Na^+	Na^-	Diff.	Mediated
.313	14.0	15.0	14.5	18.5	24.0	17.7
.625	13.0	15.0	13.9	18.5	24.0	16.9
1.25	14.0	13.0	13.5	16.9	24.0	17.7
2.5	14.0	14.0	14.0	17.7	24.0	17.7
7.5	14.0	14.0	14.0	17.7	24.0	17.7

[a]Na = uptake in presence of sodium (experimentally determined), K = uptake in the presence of potassium (experimentally determined), Diff. = estimated diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion, Mediated uptake = sodium uptake - diffusion uptake.

TABLE 10. DEGREES OF FREEDOM FOR LYSINE UPTAKE DATA IN JEJUNAL BRUSH BORDER MEMBRANE TISSUE.

[LYS]	Na[a]	K	Na^+	Na^-	Diff.	Mediated
.313	14.0	14.0	14.0	17.7	24.0	17.7
.625	11.0	13.0	11.9	16.9	24.0	15.1
1.25	10.0	15.0	12.0	18.4	24.0	14.1
2.5	16.0	16.0	16.0	19.2	24.0	19.2
7.5	13.0	16.0	14.3	19.2	24.0	16.9

[a]Na = uptake in presence of sodium (experimentally determined), K = uptake in the presence of potassium (experimentally determined), Diff. = estimated diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion, Mediated uptake = sodium uptake - diffusion uptake.

TABLE 11. DEGREES OF FREEDOM FOR METHIONINE UPTAKE DATA IN ILEAL BRUSH BORDER MEMBRANE TISSUE.

[LYS]	Na[a]	K	Na^+	Na^-	Diff.	Mediated
.125	15.0	16.0	15.5	16.0	16.0	15.5
1.25	15.0	15.0	15.0	15.5	16.0	15.5
2.5	16.0	15.0	15.5	15.5	16.0	16.0
5.0	15.0	16.0	15.5	16.0	16.0	15.5
7.5	14.0	15.0	14.5	15.5	16.0	14.9
10.0	16.0	14.0	14.9	14.9	16.0	16.0
12.5	16.0	14.0	14.9	14.9	16.0	16.0
15.0	15.0	14.0	14.5	14.9	16.0	15.5

[a]Na = uptake in presence of sodium (experimentally determined), K = uptake in the presence of potassium (experimentally determined), Diff. = estimated diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion, Mediated uptake = sodium uptake - diffusion uptake.

TABLE 12. DEGREES OF FREEDOM FOR METHIONINE UPTAKE DATA IN JEJUNAL BRUSH BORDER MEMBRANE TISSUE.

[LYS]	Na^a	K	Na^+	Na^-	Diff.	Mediated
.125	18.0	19.0	18.5	17.4	16.0	16.9
1.25	20.0	19.0	19.5	17.4	16.0	17.8
2.5	18.0	19.0	19.0	17.8	16.0	16.9
5.0	19.0	20.0	19.5	17.8	16.0	17.4
7.5	16.0	20.0	17.8	17.8	16.0	16.0
10.0	20.0	20.0	20.0	17.8	16.0	17.8
12.5	18.0	17.0	17.5	16.5	16.0	16.9
15.0	17.0	18.0	17.5	16.9	16.0	16.5

[a]Na = uptake in presence of sodium (experimentally determined), K = uptake in the presence of potassium (experimentally determined), Diff. = estimated diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion, Mediated uptake = sodium uptake - diffusion uptake.

TABLE 13. DEGREES OF FREEDOM FOR THE COMPARISON OF LYSINE UPTAKE DATA OF ILEAL AND JEJUNAL BRUSH BORDER MEMBRANE TISSUE.

[LYS]	Na[a]	K	Na^+	Na^-	Diff.	Mediated
.313	14.0	14.5	14.2	18.1	24.0	17.7
.625	11.9	13.9	12.8	17.6	24.0	15.9
1.25	11.7	13.9	12.7	17.6	24.0	15.7
2.5	14.9	14.9	14.9	18.4	24.0	18.4
7.5	13.5	14.9	14.2	18.4	24.0	17.3

[a]Na = uptake in presence of sodium (experimentally determined), K = uptake in the presence of potassium (experimentally determined), Diff. = estimated diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion, Mediated uptake = sodium uptake - diffusion uptake.

TABLE 14. DEGREES OF FREEDOM FOR THE COMPARISON OF METHIONINE UPTAKE DATA OF ILEAL AND JEJUNAL BRUSH BORDER MEMBRANE TISSUE.

[LYS]	Na^a	K	Na^+	Na^-	Diff.	Mediated
.125	16.4	17.4	16.9	16.7	16.0	16.2
1.25	17.1	16.8	17.0	16.4	16.0	16.6
2.5	16.9	17.1	17.0	16.6	16.0	16.5
5.0	16.8	17.8	17.3	16.8	16.0	16.4
7.5	14.9	17.1	16.0	16.6	16.0	15.5
10.0	17.8	16.5	17.1	16.2	16.0	16.8
12.5	16.9	15.4	16.1	15.7	16.0	16.5
15.0	15.9	15.8	15.8	15.9	16.0	16.0

[a] Na = uptake in presence of sodium (experimentally determined), K = uptake in the presence of potassium (experimentally determined), Diff. = estimated diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion, Mediated uptake = sodium uptake - diffusion uptake.

TABLE 15. DEGREES OF FREEDOM FOR THE COMPARISON OF LYSINE AND METHIONINE UPTAKE DATA IN ILEAL BRUSH BORDER MEMBRANE TISSUE.

[LYS]	Na^a	K	Na^+	Na^-	Diff.	Mediated
1.25	14.5	13.9	14.2	16.1	19.2	16.5
2.5	14.9	14.5	14.7	16.5	19.2	16.8
7.5	14.0	14.9	14.2	16.5	19.2	16.2

[a]Na = uptake in presence of sodium (experimentally determined), K = uptake in the presence of potassium (experimentally determined), Diff. = estimated diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion, Mediated uptake = sodium uptake - diffusion uptake.

TABLE 16. DEGREES OF FREEDOM FOR THE COMPARISON OF LYSINE AND METHIONINE UPTAKE DATA IN JEJUNAL BRUSH BORDER MEMBRANE TISSUE.

[LYS]	Na^a	K	Na^+	Na^-	Diff.	Mediated
1.25	13.3	16.8	14.9	17.9	19.2	15.7
2.5	16.9	17.8	17.4	18.5	19.2	18.0
7.5	14.3	17.8	15.9	18.5	19.2	16.4

[a]Na = uptake in presence of sodium (experimentally determined), K = uptake in the presence of potassium (experimentally determined), Diff. = estimated diffusion uptake, Na^+ = sodium uptake - potassium uptake, Na^- = potassium uptake - diffusion, Mediated uptake = sodium uptake - diffusion uptake.

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