Characterization of Methionine Transport in Bovine Intestinal Brush Border Membrane Vesicles

by

Geoffrey Eliot Dahl

thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Masters

in

Dairy Science

APPROVED:

Carl E. Polan, Chairman	Kenneth E. Webb, Jr.
Thomas W. Keenan	William E. Vinson

26 August 1987

Blacksburg, Virginia

Characterization of Methionine Transport in Bovine Intestinal Brush Border Membrane Vesicles

by

Geoffrey E. Dahl

Committee Chairman: C. E. Polan
Dairy Science

Abstract

Characteristics of methionine uptake by brush border membrane vesicles of bovine small intestine were investigated. Alkaline phosphatase marked the brush border membrane fraction obtained through differential centrifugation followed by a sucrose gradient. This preparation yielded a 10-fold enrichment of activity over homogenate. Methionine uptake was found to be into an osmotically active space. A binding constant of 75.4 pmol/mg membrane protein was determined. A significant ($p < .05$) sodium stimulation of methionine uptake was observed. This indicated active (energy dependent) transport in addition to the diffusive component of intravesicular methionine accumulation. Decreasing the pH of buffer medium significantly ($p < .05$) depresses methionine transport. A K_m = .114 mM and V_{max} = 56.5 pmol/s/mg membrane protein were ascertained for methionine.

Acknowledgements

I would like to thank my committee members, Dr. Carl Polan, Dr. Kenneth Webb, Jr., and Dr. Thomas Keenan for their guidance during this project.

Dr. Paul Pocius and Dr. Joseph Herbein deserve thanks for their intellectual stimulation over coffee on many mornings.

I also thank Wendy Wark and Trish Trimble for their assistance in the lab.

To my fellow graduate students in the Animal and Dairy Science Departments, I extend thanks for their friendship and commiserational skills.

I thank Linda Dellers for her assistance with my thesis preparation and her tolerance of me at other times.

Finally, I thank my parents, Carl and Joan Dahl, as well as the rest of my family, for their support and inspiration throughout my education.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

When considering the protein nutrition of a ruminant, it is important to remember the differences compared to a monogastric. Protein from three sources is being presented to the abomasum and intestine for degradation and eventual absorption by the small intestine. The three sources include dietary (bypass) which has survived ruminal degradation, endogenous (sloughed ruminal epithelial cells), and microbial. Another consideration is that of the rumen itself. The very nature of the rumen as a continuous fermentor allows for a rather constant, uniform contribution of digesta to the small intestine, with a 40-100 % microbial protein composition of the dietary fraction (11). Subsequent absorption of amino acids therefore is in turn more consistent and less variable than monogastrics.

Recent research in ruminant protein nutrition has been concerned with learning how the content of digesta available at the small intestine might be controlled by dietary means (3,44,45). However it is not enough to know what is available at the small intestine, one must also understand nutrient absorption. For proteins, this entails understanding amino acid uptake at the cellular level. For this research, it was decided to examine what factors affect uptake of amino acids (specifically

methionine) from the lumen by enterocytes. By combining inferences and results of in vitro and in vivo experiments, it will be possible to set forth the strongest outline of methionine transport in the ruminant.

Brush border membrane (BBM) is luminally oriented in the small intestine. This constitutes the threshold of an animals nutrient uptake. Separation of BBM from the complete enterocyte in form of a vesicle provides a useful tool for the study of nutrient transport. Meanwhile, negative influences of in vitro and in vivo studies with intact cells are eliminated. Examples of these disadvantages would be cellular metabolism or compartmentilization within subcellular organelles of the nutrient in question. Since only the membrane is used to evaluate nutrient uptake, these problems are removed.

Specific objectives of this research were to isolate and purify BBM vesicles (BBMV) from the bovine; then using BBMV to evaluate Met uptake, determine K_m (substrate affinity), a binding constant, presence or absence of sodium stimulation, and effect of pH.

Literature Review

Amino Acid Transport

Membranes consist of a bilayer of polar lipids, acting to selectively inhibit flow of certain compounds, while optimizing the transport of others. Amino acid uptake by the cell occurs at certain protein "transporters" which are interspersed in the bilayer. Stevens et al (50) reported three sodium-dependent (NBB, IMINO, and PHE), two sodium-independent (y^+ and L), and diffusion as amino acid transport pathways in BBM of rabbit jejunum. Typical substrates of the PHE pathway include phenylalanine (Phe) and methionine (Met) (51). Basolateral membranes (BLM) share diffusion and L system pathways, as well as having two sodium-dependent systems of their own (A, ASC). This report is quite an expansion on early work by Christensen (5) presenting the L, LY(y^+), A and PHE systems in Ehrlich cells.

Elucidation of these transport pathways came slowly, as a result of many researchers using a wide variety of methods. Delhumeau et al (13) studied a mixture of 18 L-amino acids uptake from intestinal loops in rats. Met was reported in the group of amino acids which were highly (75-100%) absorbed. A modification of this methodology was used in sheep (53). Again Met was in the group of amino acids which were quickly absorbed. Orten

(39) used a blind loop (Thiry) of ileum to study the disappearance of an equimolar solution of 18 amino acids in a human subject. As reported in rats and sheep previously, Met was determined to be absorbed at the fastest rate. Uptake of Met was found to decrease in Na^+-free medium (4).

Later studies using different methods allowed more control over experimental variables, and as such provided more information on amino acid absorption. Jejunal strips from sheep (27) were used to demonstrate uptake of Met, glycine (Gly), and lysine (Lys) against a concentration gradient. The order of uptake was Met > Lys > Gly. Everted sacs of sheep small intestine (in vitro) were used by Phillips et al (41) to demonstrate an uptake pattern in which Met exceeded valine (Val), which exceeded threonine (Thr). The ileum was reported (40) to be the most active site of amino acid transport using the everted sac method. Bergen (2) later supported this hypothesis using jejunal strips. Working with intestinal strips isolated from cattle (20), methionine uptake was shown to be sodium-stimulated, with maximal uptake occurring in the mid-ileum. Even though there are some species differences, it was reported that overall order of uptake of amino acids in sheep small intestine was similar to that in rat and man (2).

These studies, while providing a great deal of information, do have some disadvantages. Factors such as cellular metabolism and compartmentalization increase the variability of these studies. Phillips et al (40) estimated that only 50% of the amino acids disappearing from the lumen were found in circulating blood. So even though it is possible to estimate available anabolic substrates, the fate of half of the transported nutrients is in question. A system is needed that makes it possible to study transport, while eliminating other cellular functions. Intestinal membrane vesicles provide just such a system.

Studies with Brush Border Membrane Vesicles

In a review of subcellular fractionation, DeDuve (12) profiled isolation schemes emphasizing the use of differential centrifugation. Mircheff (32) employed these theories in outlining a strategy for the analytical fractionation of epithelial cells. Cells were fractured, and then separated physically by exploiting the bouyant densities of their constituents. Certain enzymes, termed marker enyzmes, are specific to certain fractions of cellular components, and are used to distinguish and ascertain the purity of said components.

These methods are used to separate the BBM from BLM and other cellular debris. BBM will then reseal into

vesicles. Orientation of these vesicles has been examined on a morphological and immunological basis in the rat small intestine (22); and substrate accessability in the bovine (9). All studies indicate a predominance of vesicles oriented right-side out (same as in the enterocyte). In a biophysical sense, it would be expected that the folded, rough (increased surface area) brush border would exteriorize, in order to maximize uptake opportunities.

Advantages of membrane vesicles for transport studies include elimination of cellular metabolism, energy, compartmentalization, as well as allowing complete manipulation of intra- and extravesicular fluid (37). These outweigh the disadvantages (i.e. artificial environment or possible changes in transporter systems) to provide the current method of choice for intestinal transport study. Of course by combining both in vitro and in vivo results, we obtain the best view of overall transport and metabolism.

Extensive research has and is continuing to proceed on both brush border and basolateral membrane transport, in both the small intestine and the kidney. Initial transport studies concentrated on sugar uptake characteristics. Hopfer et al (24) found Na^+ stimulation of glucose uptake in rat intestinal brush border

vesicles. No other monovalent cation (i.e. K^+ or Li^+) had any stimulatory effect on glucose uptake. Numerous researchers (9, 34, 50, 55) have reported a similar Na^+-dependent glucose transport in the intestine of ruminants. This transport is thought to be driven by the trans membrane sodium gradient, initially proposed by Crane (7, 8). A natural progression (especially in the small intestine) to amino acids as the next area of research was made. Im and Spector (26) described neutral amino acid transport (Ala and AIB) in Ehrlich cells as requiring a sodium gradient and being stimulated by an electrical potential (intravesicular negative). Leu and AIB were found to be subject to sodium-dependent transport in Balb/c 3T3 mouse cell line culture (19). It is interesting to note that this study reported Li^+ to be intermediate between Na^+ (saturable) and K^+ (diffusive) in stimulatory effect on transport. This work was further supported by Wolffram et al (55) who reported a Na^+ stimulation of Leu uptake in both swine and sheep. Crooker and Clark (9) described a sodium stimulation of Ala uptake in the bovine, supporting work by Sigrist-Nelson et al (48) in the rat with Ala. Sigrist-Nelson et al (48) also noted a Li^+ stimulation of Ala uptake. Working with BBMV from rat hepatocytes, Kilberg et al (28) described Li^+ as stimulatory to amino

acid uptake. The effect was somewhat dependent on both the transporter and amino acid structure. Hopfer (23) reported Na^+ stimulation of L-valine uptake in the rat intestinal brush border membrane. Using rabbit intestinal BBMV, Stevens et al (51) described a Na^+-dependent transporter which favored Phe and Met, later described as the PHE (phenylalanine) pathway (50). They also found no stimulatory effect of Li^+ on transport. Other work with rabbit ileum (42, 43) reported that a 30-40 mV electronegative potential (cell interior) was required for amino acid transport.

Met has been shown to be inhibitory to transport of many neutral amino acids in BBMV (10, 51). Moe (33) reported that no other neutral amino acid was inhibitory toward Met itself. To summarize, there are some generalizations that can be made about amino acid transport in the mammalian small intestine. Accumulation against a concentration gradient requires metabolic energy, which is supplied by a Na^+H^+ exchanger in the brush border and Na^+K^+ATPase in the baso-lateral membrane (17). Lithium may be stimulatory to this transport, by partial replacement of Na^+, depending on the pathway (28) This may be due to Li^+'s smaller crystal-ionic radii when compared to Na^+, as opposed to the larger K^+ which has no stimulatory effect. Electronegative potential on the

vesicular interior seems to provide further impetus for the Na^+-amino acid cotransport by attracting the cation. Methionine itself is inhibitory to many amino acids, although its uptake was not found to be inhibited by any neutral amino acids. Sodium dependent uptake of Met in the brush border of the small intestine is thought to be along the PHE pathway described by Wright et al (56).

Future Studies with BBMV

Characterization of the transport properties of essential amino acids is a primary interest to nutritionists at this time, as well as definition of the transporters themselves. Recent thought (1, 52) has held that simple-peptides (di-, tri-peptides) could be contributing more to amino acid uptake then previously expected. Gardner (18) suggested that intact, biologically active hormone releasing factors (of TH, LH) may be transported across the intestinal brush border. Ganapathy and Leibach (17) proposed that a proton gradient was energizing intestinal peptide transport, since the Na^+ gradient hypothesis is questioned for peptide transport. Manipulation of pH could prove a major consideration in this type of study. This theory, along with Matthews (29) observation of little shared inhibitory properties between peptides and amino acids, connotes different, separate modes of transport.

Of course the use of BBMV's is not limited to studies in protein nutrition. Metabolite (30) and drug (25) transport have been examined and should be intensive areas of future research.

Methionine in Animal Production

An obvious question at this point might be why examine characteristics of Met transport in ruminant small intestine? Most of the previous research with BBMV has concentrated on Ala, which is a major metabolite in gluconeogenesis, an area of interest to ruminant nutritionists. Since research has been completed in this area, it is imperative to now examine other aspects of protein nutrition with influence on production (i.e. milk in dairy cattle). The importance of Met in milk protein production is well documented. Shimbayashi et al (47) implicated Met as the most limiting amino acid to milk production. This was further supported by Fisher (15) who found a significant increase in milk protein production with intravenous infusion of Met. It is also interesting to note that Met is one of the group of amino acids outlined by Mepham (31) as being almost exclusively transferred into milk by the lactating mammary gland. Met, along with Lys, has also been found to be limiting in ruminant growth (38). Thus, manipulation of

post-ruminal availability of Met has shown to be beneficial to production in the ruminant.

Materials and Methods

Tissue Collection

Methods used were similar to those of Moe et al (34) and Wilson (54). Holstein steers of approximately 450 kg liveweight were systematically stunned, exsanguinated and eviscerated. Meter long segments of intestine were collected within 10 min of stunning beginning 2M distal to the pyloric sphincter and proceeding until 2M proximal of the ileo-cecal junction. Segments were rinsed to remove digesta with cold mannitol buffer (300 mM mannitol, 12 mM TRIS, adjusted to pH 7.4 with HCl), everted, and ligated. Intestinal sections were incubated at 37 C in hyaluronidase (EC 3.2.1.35) buffer (1 mg/ml hyaluronidase, 1 mg/ml BSA, 120 mM NaCl, 20mM TRIS, 1 mM $MgCl_2$ and 3 mM K_2HPO_4, adjusted to pH 7.4 with HCl) for 20 min. Tissue was then scraped with a glass slide and washed at 4500 x g in mannitol buffer. Aliquots of mucosal scrapings (3-4 g) were placed in Whirl-pac bags[1] and flash frozen in liquid nitrogen. These were stored at -80 C until used for isolation and purification of membrane fractions.

Vesicle Preparation

During preparation, temperature was between 0-4 C unless otherwise noted. Ten to twelve packets

[1]Nasco, Fort Atkinson, WI

(approximately 40 g total) of tissue were thawed and combined with 25 ml (each packet) of mannitol-succinate buffer (150 mM mannitol, 30 mM succinate, 10 mM TRIS, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 0.1 mM $MnCl_2$; adjusted to pH 7.4 with NaOH). Tissue was then disrupted with a Polytron PT 10/35[2] (probe PTA 205) at setting 6 (15-16,000 rpm) for 15 sec. Resulting suspensions were combined and incubated for 30 min. under gentle agitation. This step allowed for divalent cation aggregation of baso-lateral membrane fragments (24, 46). A differential centrifugation scheme (Figure 1) was employed to isolate brush border membranes. Pellets were resuspended by five strokes of a tissue homogenizer[3] (clearance 0.0889 to 0.1143 mm). The final pellet was resuspended to 10 ml with mannitol-transport buffer (100 mM mannitol, 20 mM HEPES, 2 mM $MgCl_2$; adjusted to pH 7.4 with NH_4OH). This resuspension was placed on a discontinuous (27% and 31%) sucrose gradient (4 mM HEPES, 4 mM $MgCl_2$; pH to 7.4 with TRIS) and centrifuged in a swinging bucket (Beckman,[4] 50.2 Ti) rotor at 105,000 x g for 90 min. Interfacial layers between various sucrose concentrations were washed

[2]Brinkmann Instruments, Westbury, NY

[3]Kontes Scientific Glassware, Vinland, NJ

[4]Beckman Instruments, Palo Alto, CA

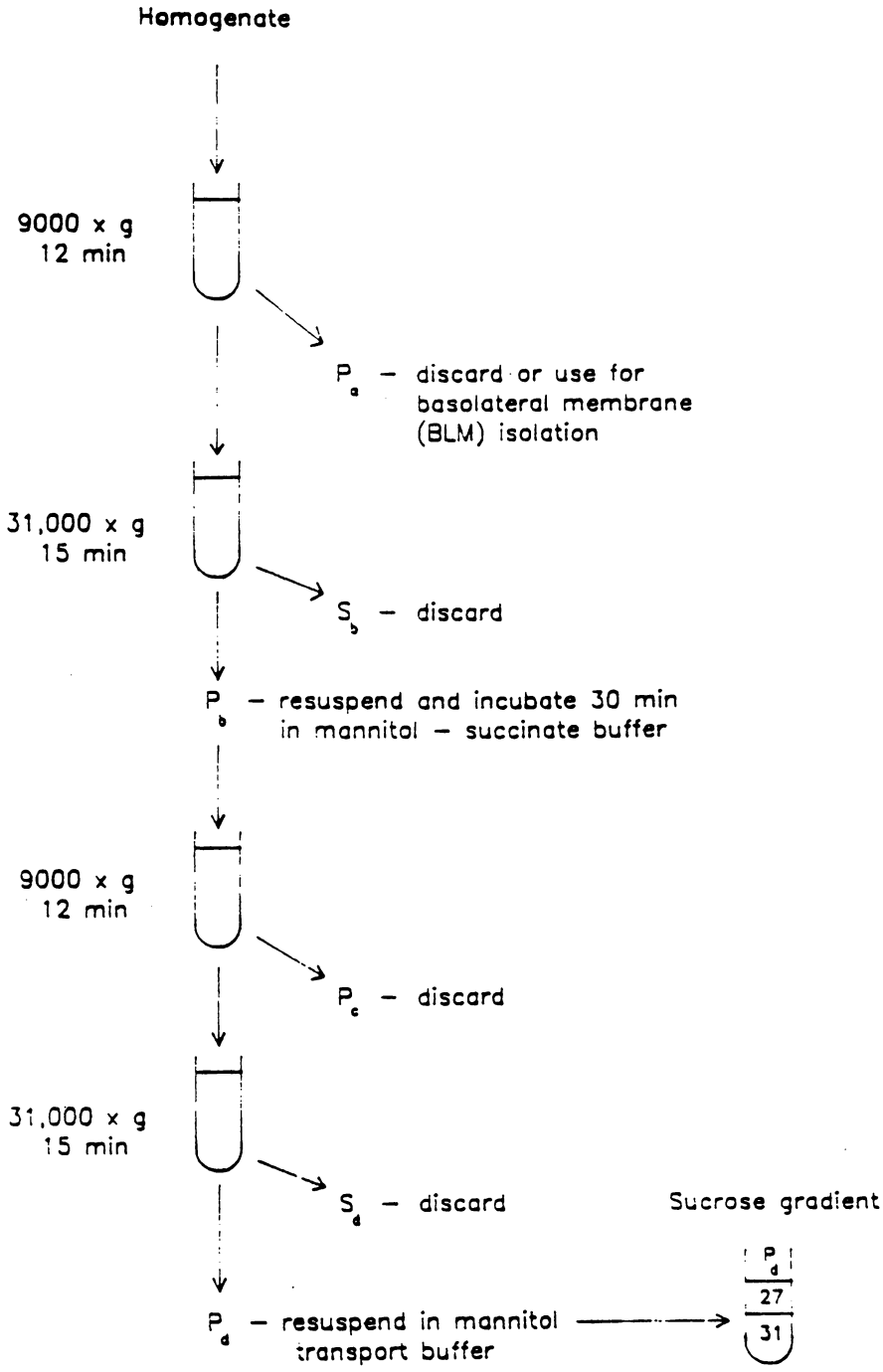


Figure 1 - Differential centrifugation scheme for the separation and isolation of brush border membranes from bovine small intestinal epithelia.

with mannitol-transport buffer at 105,000 x g for 75 min. Resuspended fractions were then suitable for transport experiments or marker enzyme assays. Fractions saved for future transport experiments were aspirated from the gradient, placed in Cryovials[5], flash frozen in liquid nitrogen, and stored at -80 C. Alkaline phosphatase (ALP) was used (EC 3.1.3.1) (Sigma[4] Kit No. 245-10) to detect brush border membranes. It's suitability for this purpose was reported by Eicholz and Crane (14). Ouabain sensitive Na^+K^+ ATPase (E.C. 3.6.1.3) was used as a marker for baso-lateral contamination (36), fractions were analyzed for purity of the isolation and enrichment of activity, relative to the original tissue homogenate.

Transport Assays

A rapid filtration technique was used for methionine transport assays. Sodium- or potassium-thiocyanate transport buffers (100 mM NaSCN or KSCN, 100 mM mannitol, 10 mM HEPES, 2 mM $MgCl_2$; adjusted to pH 7.4 with HCl) were used to elucidate the active (energy dependent) or diffusive (energy independent) transport component respectively. Reaction volumes consisting of 50% 2x

[5]Sarstedt, Princeton, NJ

[4]Sigma, St. Louis, MO

concentration of transport buffer, described above, 30% ^{35}S-Met[7] in distilled water (1 uCi activity per reaction volume), and 20% brush border membrane preparation (previously described) were incubated (after mixing at time zero) at 37 C. Final Met (1120 Ci/mmol specific activity L-methionine). Final Met concentration was 100 uM for all experiments, except when it was varied in the K_m determination. Membrane preparations contained approximately 2.25 mg protein/ml, determined using Pierce[8] protein assay reagent with a BSA standard. All reactions were initiated by adding a membrane aliquot to the buffer/substrate (Met) mixture.

Effect of varying osmolarity on Met transport was determined by examining uptake at equilibrium (45 min.) between active and diffusive components. Inverse osmolarity (osm^{-1}) vs. picomole (pmol) Met transported was plotted. A linear regression was extrapolated from this slope to the origin (infinite osmolarity = zero intracellular volume) to determine Met binding constant. Time course assays were conducted by mixing total volumes of reaction mixture and filtering aliquots at specified timepoints. In addition to NaSCN and KSCN, the effect of LiSCN (100 mM) was examined. Uptake of Met (pmol/mg

[7]N.E.N., DuPont, Boston, MA

[8]Pierce Chemical Co., Rockford, IL

membrane protein) was plotted vs. time. Two different types of buffers were necessary to effectively buffer the pH experimental range. HEPES buffer (100 mM NaSCN or KSCN, 113 mM Mannitol, 10 mM HEPES, 2 mM $MgCl_2$; pH adjusted with HCl) was used at pH 6.0, 6.5, 7.0, and 7.4. Ammonium acetate buffer (100 mM NaSCN or KSCN, 100 mM mannitol, 10 mM ammonium acetate, 2 mM $MgCl_2$; pH adjusted with NH_4OH) was used for pH 5.5 and 6.0. Mannitol concentration between the two is not equivalent. Extra mannitol in the HEPES buffer was required to equilibrate osmolarity with that of ammonium acetate.

A final experiment was undertaken to evaluate Met affinity for the BBM active transporter. Twenty sec time points of Met uptake were determined at varying Met concentrations, with NaSCN buffer. Estimates of K_m and V_{max} were obtained from a plot of inverse velocity (vel^{-1}) vs. inverse substrate (met^{-1}) concentration ($conc.^{-1}$). All filtrations used 0.45 uM Metricel[c] membrane filters[?] on a Millipore filtration manifold. Suction was initiated after the reaction mixture came into contact with the filter. All reactions were terminated with a triplicate 5 ml rinse of stopping solution (150 mM KCl). Filters were placed in scintillation vials along with 10 ml Scintiverse II

[?]Gelman Sciences, Inc., Ann Arbor, MI

cocktail[10] and counted (Beckman[4] LS7500 library program 9, channel 3). Data was analyzed by GLM on SAS (49). Sample calculations and statistical models are presented in Appendices A and B respectively.

[10]Fisher Scientific, Springfield, NJ

Results and Discussion

Marker Enzyme Analysis

Table 1 presents results of marker enzyme analysis to determine purity of the isolated fraction to be used for transport studies. The fraction isolated at the interfacial layer between 27% and 31% sucrose concentration on the gradient was used. This fraction had a ALP:Na^+/K^+ATPase enrichment ratio of 7.67:1.00. These results are in agreement with previous isolation of BBM from the bovine (34).

Osmolarity Study

Results of varying osmolarity on intravesicular substrate accumulation are presented in Figure 2. The sloping line indicates that vesicles were indeed present. This is due to an inverse relationship exhibited between osmolarity and vesicular size. Vesicular size decreases as osmolarity in the buffer medium increases, an adjustment due to the vesicles equilibration with its external environment. Hence, as vesicular size decreases, intravesicular substrate accumulation (in this case, Met) will also decrease. Hopfer (23) outlined this procedure for determining vesicular nature, and it has been used successfully in previous studies with bovine BBMV (33,54).

Table 1 - A presentation of averages of specific activities (S.A.) for alkaline phosphatase (ALP) and ouabain sensitive Na/K ATPase (ATPase) from three isolation preparations. Enrichment (F.E.) of activity over homogenate with standard error (S.E.) is also reported.

	ALP			ATPase		
Fraction	avg. S.A.	F.E.	S.E.	avg. S.A.	F.E.	S.E.
Homogenate	.071	--	--	2.25	--	--
Interface1*	.362	5.44	1.49	6.34	4.00	1.39
Interface2**	.654	9.92	2.63	3.17	1.29	0.65

*Interface between membrane suspension and 27% sucrose layer.
**Interface between 27% and 31% sucrose layer.

Note: Pellet at tube bottom was discarded.
ALP S.A. in Units/mg membrane protein.
ATPase S.A. in μg P liberated/mg membrane protein.

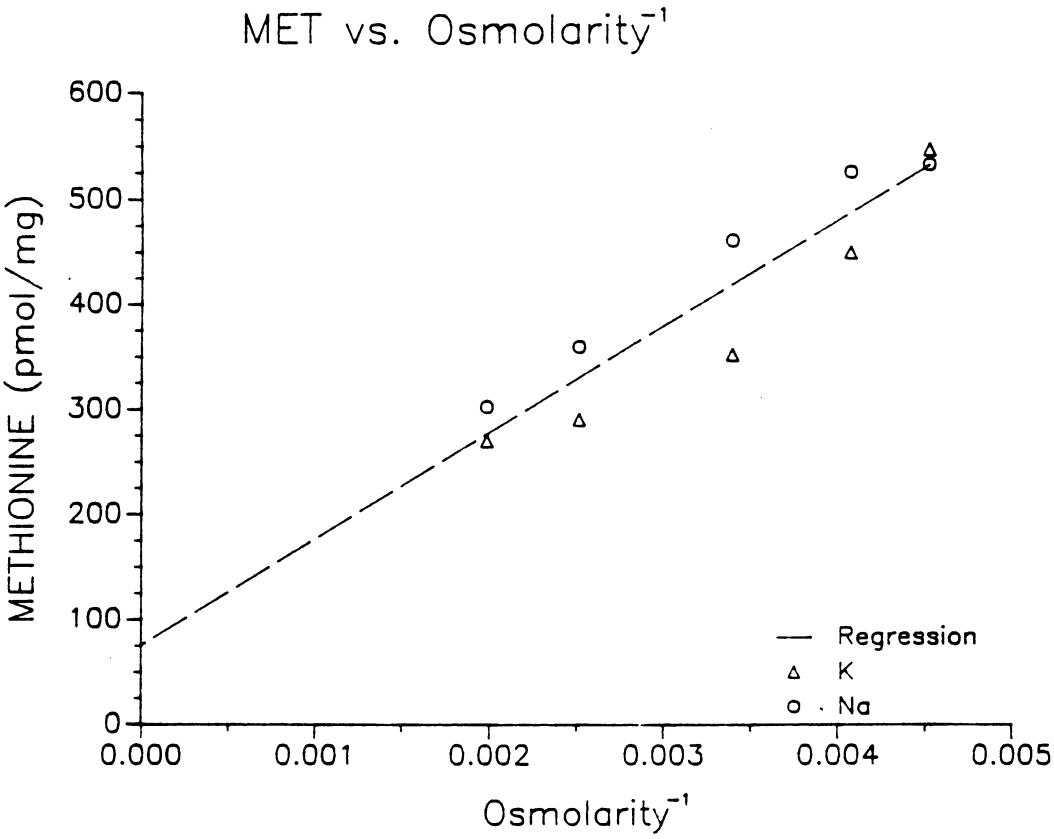


Figure 2 - Effect of osmolarity of incubation buffer on equilibrium (45 min) methionine uptake. The y-intercept represents extravesicular binding (75.4 pmol/mg membrane protein).

A binding estimate was obtained from the plotted line (osm.$^{-1}$ vs. uptake) extrapolated to the y-intercept. This point constitutes infinite osmolarity, which translates to zero intravesicular space. Any transport at this point would be due to binding to the vesicles exterior, rather than actual intravesicular accumulation. Both Na^+ and K^+ based buffers were used in this experiment. Since equilibrium timepoints of 45 min were used with equivalent osmolarity's for both, uptakes and subsequent slopes of plots should be equal. The purpose of this experiment was to check whether other unknowns may have been affecting the results. No significant difference ($p > .05$) was found between the slopes of the two lines. A binding estimate of 75.4 pmol (Fig. 2) of Met per mg of membrane protein was obtained. Careful interpretation of this value is necessary since it may not be applicable to all situations such as lesser time points, different substrate concentrations, other substrates, and particularly other species.

Time Course Study

Figure 3 represents a graph of Met uptake vs. time in BBMV. The Na^+ (saturable) component was significantly different ($p < .05$) than the K^+ (diffusive) component of transport. The overshoot was not as prominent as that of glucose (34), but it was present. Met uptake by BBMV was

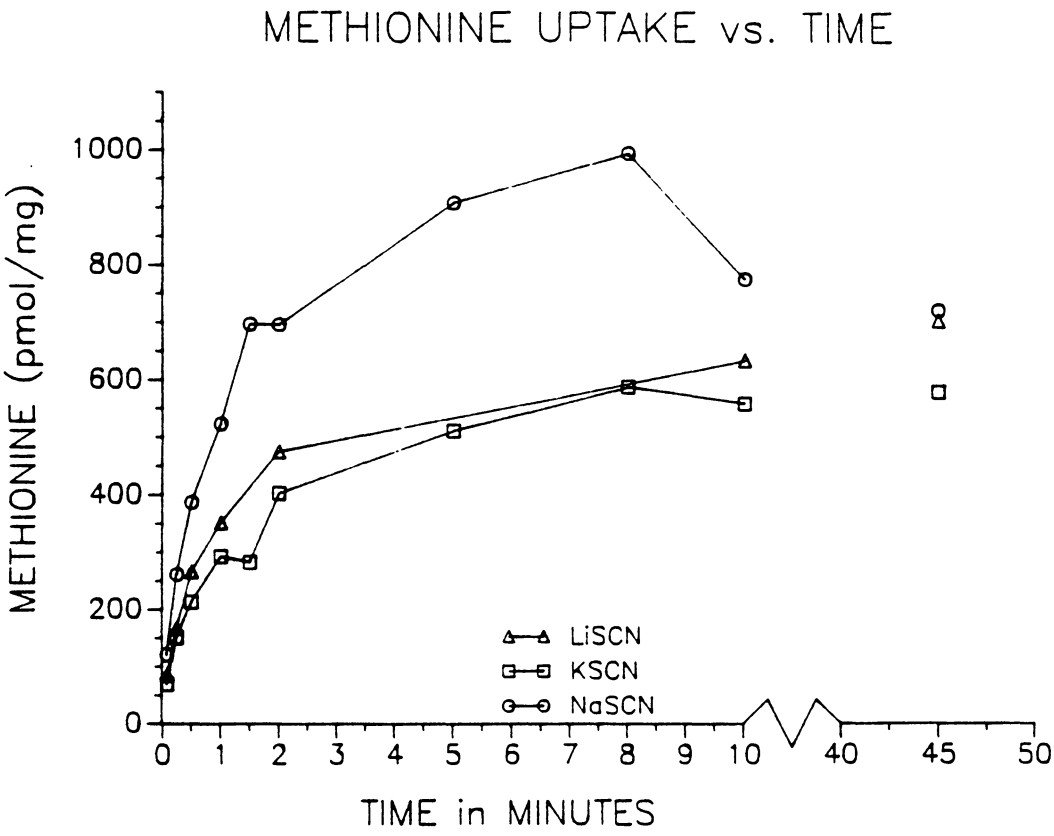


Figure 3 - Time course of methionine uptake in the presence of NaSCN (100 mM), KSCN (100 mM), and LiSCN (100 mM).

stimulated by the presence of Na^+. The sodium gradient was produced in vitro by obtaining vesicles in Na^+ free buffer, and then adding Na^+ extracellularly. To maintain this condition in vivo, energy in the form of ATP must be expended to pump Na^+ out of the cell. Current theory (30) holds that substrate (i.e. Met) is then co-transported into the cell with Na^+.

The data presented agrees with previous findings (21) that Met is transported by both energy dependent (Na^+) and independent (K^+) routes in the bovine small intestine, although (21) used intestinal strips rather than BBMV. Methionine sulfoxide (a derivative of Met oxidation) was shown to be accumulated inside BBMV against a concentration gradient via Na^+-dependent mechanisms (16). In addition, accumulation of Ala (10, 26, 35, 48), Val (23), Leu (19, 55), AIB (19, 26), and Phe (51) were found to be Na^+ dependent in BBMV. From the study of Stevens et al (51), Na^+ dependent transport of Met appears to occur through the PHE or NBB systems in intestinal brush border. Na^+-independent transport would follow the L or diffusion pathways.

Lithium appeared to have the ability to replace Na^+ to some extent in stimulatory effect on Met uptake, but this trend was not statistically significant. Reports on this subject are mixed. Garvey and Babcock (19)

suggested Li^+ to be intermediate to Na^+ and K^+ in stimulatory effect on Leu and AIB uptake in mouse intestinal membrane vesicles. However, Stevens et al (51) found no stimulatory effect of Li^+ on amino acid transport in rabbit BBMV. Li^+ may be able to replace Na^+ in the transporter to some extent due to its smaller crystal ionic radius. The radius of K^+ (larger than Na^+) excludes it from this opportunity. Dependence on amino acid structure and transport pathway has also been noted (28). It is obvious that more research is necessary in this area to clearly define the role of Li^+.

pH Manipulation Study

A significant ($p < .05$) effect of pH on linear rate of Met uptake by BBMV was noted (Figure 4 A-F). At pH 5.5, uptake was depressed and it gradually rose to an expected level as pH increased in the buffer medium. There was no significant ($p > .05$) difference between the two types (ammonium acetate and HEPES) of buffer used, so comparisons over the full range of pH values were considered valid. Conformational changes in the transporter induced by a lower pH may have been responsible for depressing transport. Lower pH may have adversely affected the permeability of SCN^- anion, which is permeant at pH 7.5 (48). Transport of Met may have been depressed by a decrease in intravesicular

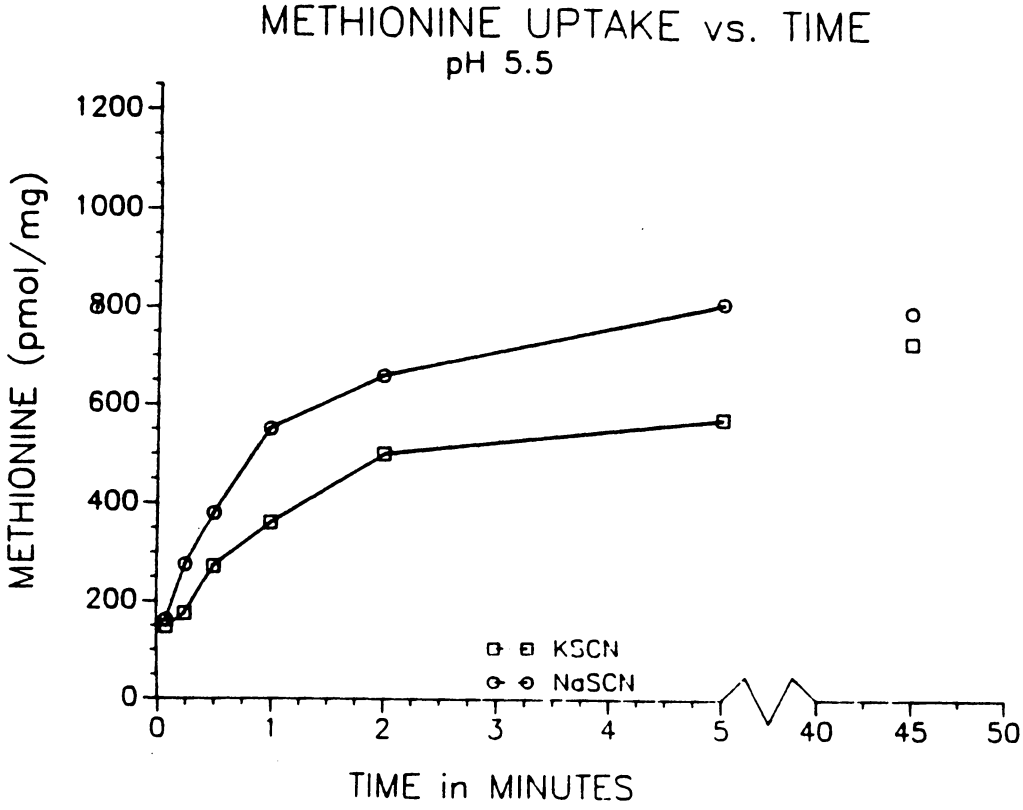


Figure 4 A-F - Effect of pH of incubation buffer (100 mM NaSCN or KSCN) on time course of methionine uptake at pH 5.5 (A), 6.0 Ac (B), 6.0 HE (C), 6.5(D), 7.0 (E), and 7.4 (F). Two medias were employed to effectively buffer the entire pH range; ammonium acetate for pH 5.5 and 6.0 Ac, and HEPES for pH 6.0 HE, 6.5, 7.0, and 7.4. No significant difference was noted between buffer types.

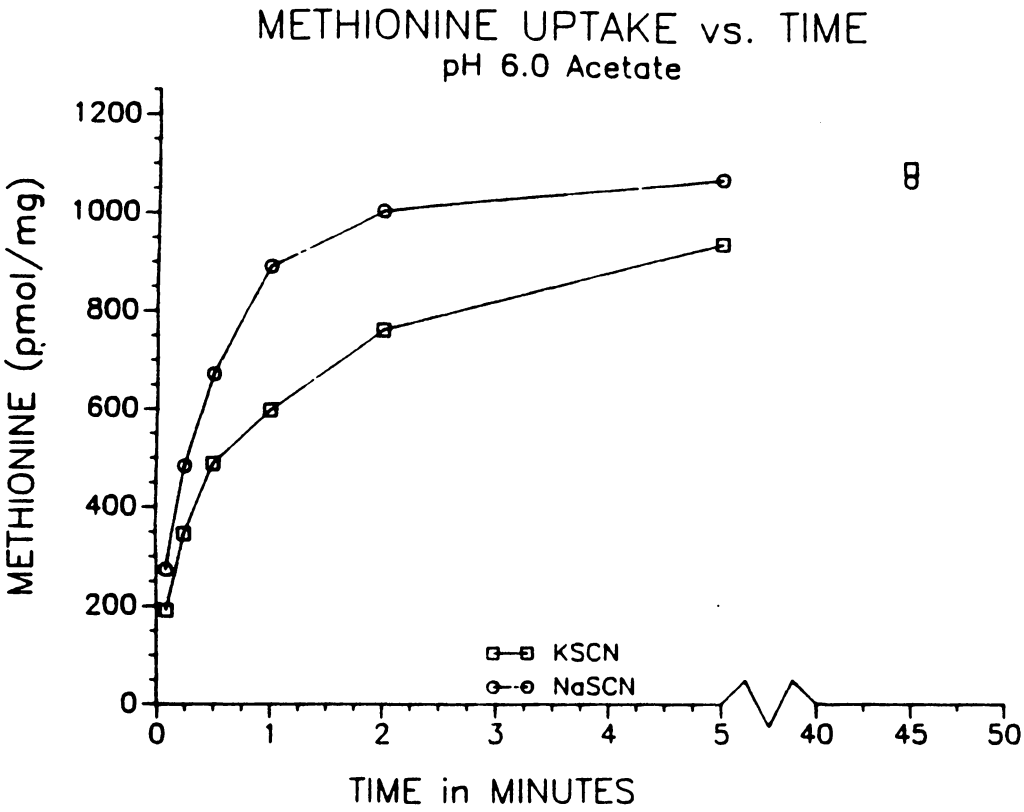


Figure 4B - Effect of pH 6.0 Ac of incubation buffer (100 mM NaSCN or KSCN) on time course of methionine uptake.

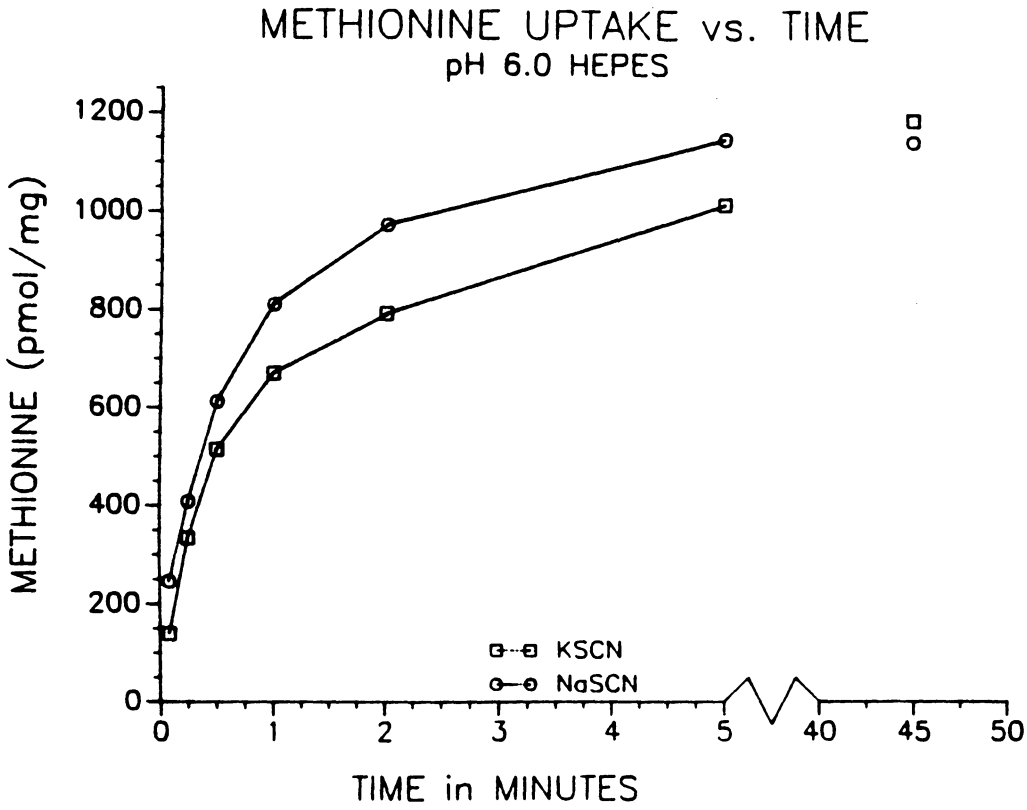


Figure 4C - Effect of pH 6.0 HE of incubation buffer (100 mM NaSCN or KSCN) on time course of methionine uptake.

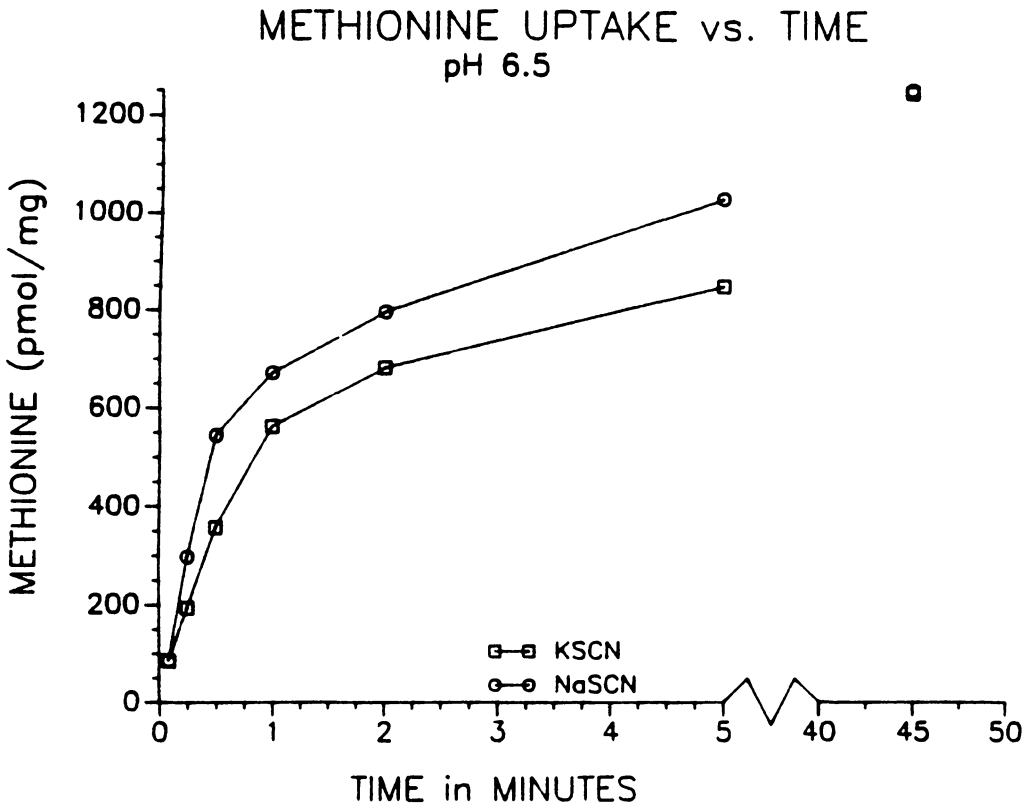


Figure 4D - Effect of pH 6.5 of incubation buffer (100 mM NaSCN or KSCN) on time course of methionine uptake.

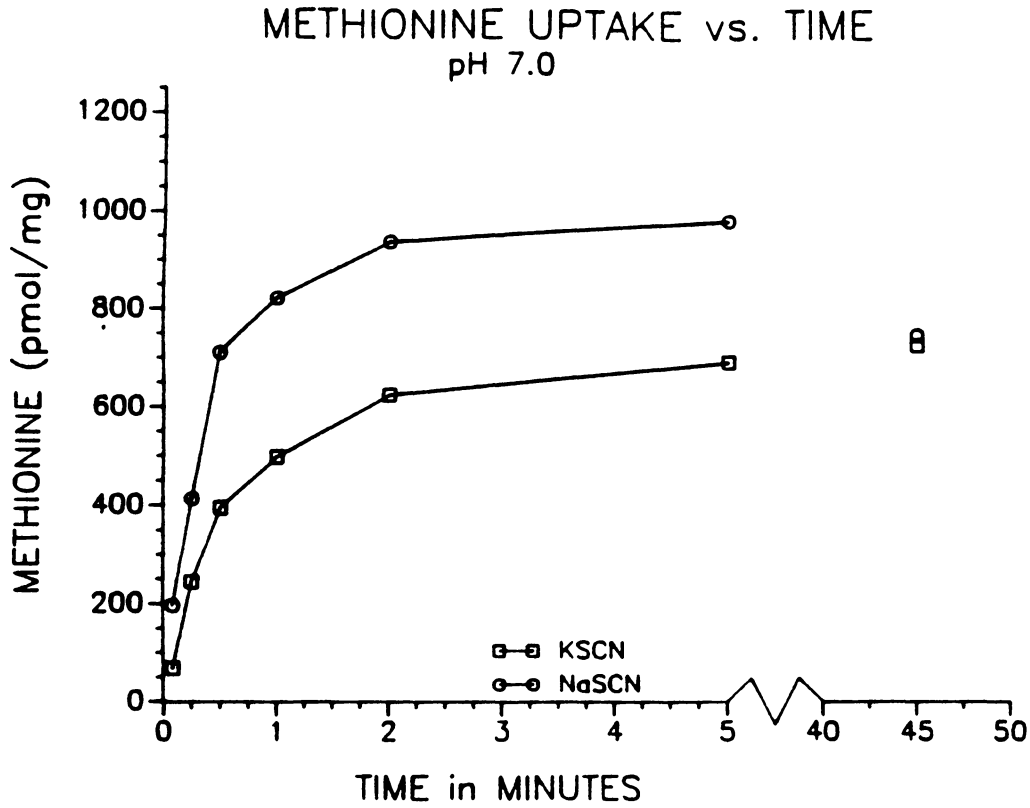


Figure 4E - Effect of pH 7.0 of incubation buffer (100 mM NaSCN or KSCN) on time course of methionine uptake.

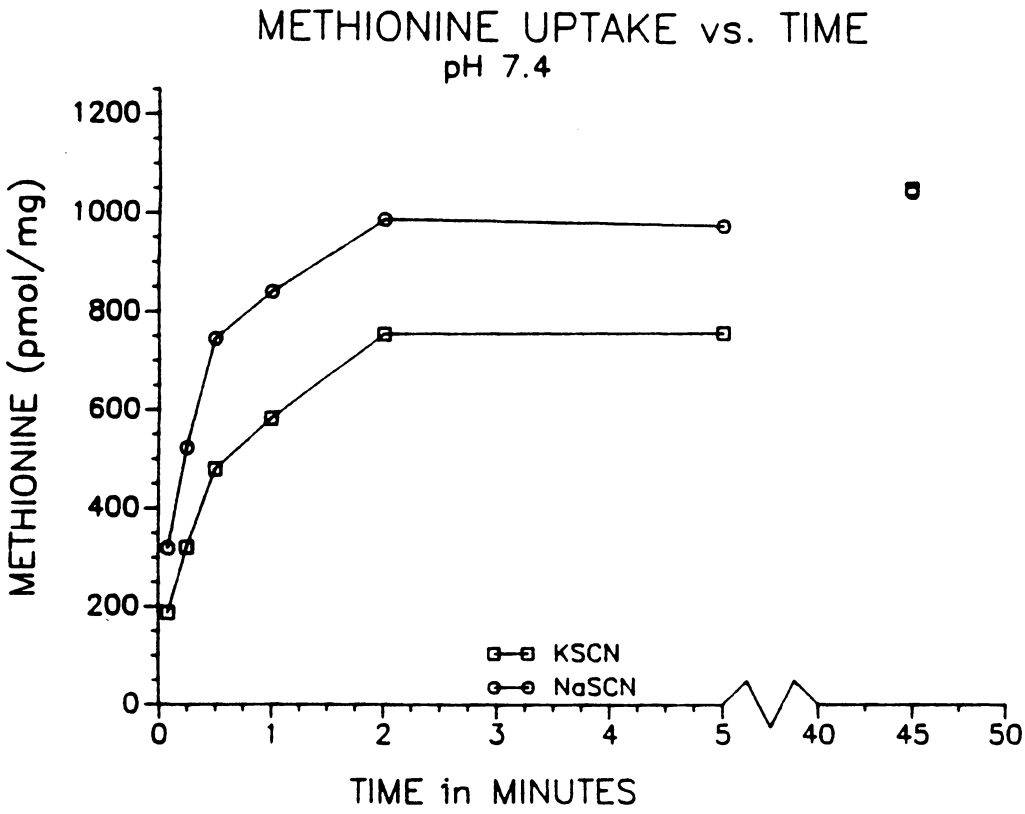


Figure 4F - Effect of pH 7.4 of incubation buffer (100 mM NaSCN or KSCN) on time course of methionine uptake.

electronegativity, thought to be a requirement for active transport (42, 43). It is also possible that the higher H^+ concentration at lower pH may have caused faster dissipation of the trans-membrane Na^+ gradient, with a concurrent lessening of Met transported.

Another trend was observed in this experiment. The disparity between the active (NaSCN) and diffusive (KSCN) components increased as pH increased. Since the NaSCN buffer allows both active and diffusive uptake, it is actually the total transport value. Diffusion accounted for a greater percentage of total uptake with lower pH buffers at the 30 s timepoint (Table 2). This may have been an artifact of the procedure, or it may be explained physiologically. Neutralization in the small intestine of ruminants is slower than that of monogastrics (2). Lower pH values will be found in the duodenum, as well as higher concentrations of transportable substrate. High flux of substrate will occur via diffusion. As the pH increases with movement of digesta down the tract, active transport becomes an aid in scavenging for all substrate available. The pH may have an activational/inhibitional role toward the transporter.

This type of experiment may prove to be of most importance in the study of peptide transport in the small intestine, since it has been proposed (17) that uptake of

Table 2 - Comparison of percentage of total transport (NaSCN) of Met supplied by the diffusive component (KSCN).

pH value	Uptake @ 30 s in pmol/mg memb. prot. NaSCN	KSCN	Diffusion % Total Transport (KSCN/NaSCN)
5.5	379.3	271.5	71.6
6.0 Ac	670.4	487.5	72.7
6.0 HE	611.2	513.7	84.0
6.5	543.6	356.4	65.6
7.0	709.8	395.0	55.6
7.4	743.1	478.6	65.6

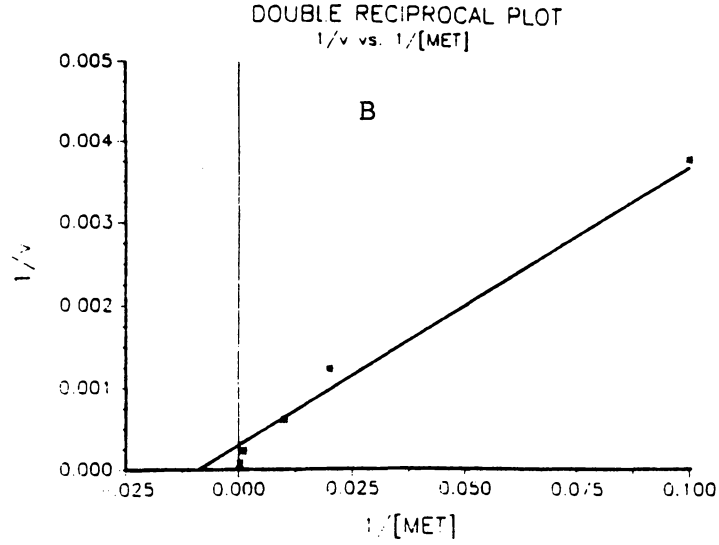
Note: Two types of media were necessary to effectively buffer the entire pH range. Ammonium acetate or Ac (5.5, 6.0) and HEPES or HE (6.0, 6.5, 7.0, 7.4) were used. No significant ($p > .05$) effect of buffer type was found.

peptides is dependent upon a proton/pH gradient rather than a sodium gradient.

Determination of Michaelis Constant

Figures 5-A and 5-B represent double-reciprocal plots of velocity of Met uptake at varying Met concentrations. This type of plot allows the investigator to estimate a Michaelis constant (K_m). Figure 5-B is an explosion of the lower points of Fig. 5-A, it is a clearer presentation of how the K_m was determined. The K_m is an indicator of substrate (in this case, Met) affinity for the transporter. An apparent K_m of 0.114 mM for Met in BBMV, with a V_{max} of 56.5 pmol/s/mg membrane protein was determined. The low K_m suggests Met has a strong affinity for the transporter, in this instance assumed to be the Na^+-dependent pathway.

These data lend support to previous reports of inhibition by Met to transport of other amino acids in BBMV (10, 51) and those which found no amino acid inhibitory to Met (33). The high affinity of Met suggests that it is being bound by transporters preferentially, excluding other substrates (i.e. amino acids). Due to the needs of a dairy cow for large quantities of Met to support milk protein production (15, 31, 47), it is logical that it would be a preferred substrate.



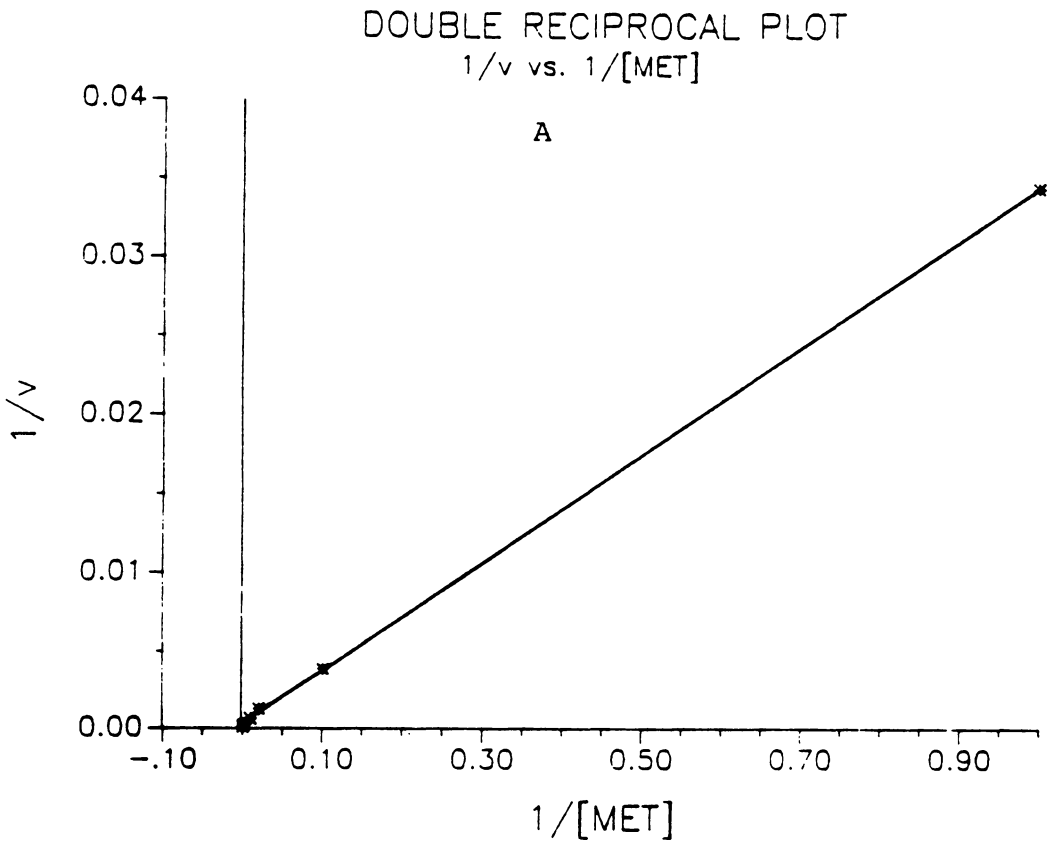


Figure 5(A) - Double-reciprocal plot of uptake velocity (pmol/mg membrane protein) vs. substrate concentration for methionine, used for determination of Michaelis constant (K_m) and maximum velocity (V_{max}) of methionine influx.

(B) - Magnification of data points about the origin of Fig. 5A.

The inhibitory characteristics of Met should be considered with any form of post-ruminal amino acid supplementation. By overemphasizing one or two amino acids, aggravation of other aspects of amino acid nutrition could occur (6). Use of BBMV will prove to be advantageous to further research in this area of amino acid interaction.

Summary

Original research using BBMV to study amino acid transport employed intestinal samples from rats, rabbits, and even human biopsies. Glucose and alanine took precedence as substrates to be examined, possibly due to their simplicity of modeling, availability, and importance to monogastric nutrition. Ruminant nutrition has an emphasis on production of food and fiber. While methionine is not always the limitng amino acid, its availability and metabolism are integral components of protein production.

The K_m value of Met is an indicator of its affinity for the membrane bound transporter. The low value obtained in this research suggests that Met will be transported at a very low concentration from digesta. In other words, Met is a preferred substrate of active uptake in bovine intestinal BBMV. However, accurate inter-substrate comparisons can only be made after values are determined for all substrates. It would seem justified that future research in this area should concentrate on characterizing transport properties of all essential amino acids.

Literature Cited

1. Alpers, D.H. 1986. Uptake and fate of absorbed amino acids and peptides in the mammalian intestine. Fed. Proc. 45:2261.

2. Bergen, W.G. 1978. Postruminal digestion and absorption of nitrogenous components. Fed. Proc. 37:1223.

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

4. Christensen, H.N. 1963. Amino acid transport and nutrition. Fed. Proc. 22:1110.

5. Christensen, H.N. 1965. Relevance of transport across the plasma membrane to the interpretation of the plasma amino acid pattern. Pages 40-52 in Protein Nutrition and Free Amino Acid Patterns. J.H. Leathem, ed. 1968. Rutgers Univ. Press, New Brunswick, NJ.

6. Christensen, H.N. 1966. Methods for distinguishing amino acid transport systems of a given cell or tissue. Fed. Proc. 25:850.

7. Crane, R.K. 1960. Intestinal absorption of sugars. Physiol. Rev. 40:789.

8. Crane, R.K. 1962. Hypothesis of mechanisms of intestinal active transport of sugars. Fed. Proc. 21:891.

9. Crooker, B.A., and J.H. Clark. 1986. Preparation of brush border membrane vesicles from fresh and frozen bovine intestine for nutrient uptake studes. J. Dairy Sci. 69:58.

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

11. Cummins, K.A., J.E. Nocek, C.E. Polan, and J.H. Herbein. 1983. Nitrogen degradability and microbial

protein synthesis in calves fed diet of varying degradability defined by the bag technique. J. Dairy Sci. 66:2356.

12. DeDuve, C. 1975. Exploring cells with a centrifuge. Science. 189:186.

13. Delhumeau, G., G.V. Pratt, and C. Gitler. 1962. The absorption of amino acid mixtures from the small intestine of the rat. J. Nutr. 77:52.

14. Eicholz, A., and R.K. Crane. 1974. Isolation of plasma membranes from intestinal brush borders. Methods in Enzymology, Biomembranes. vol. 31. Academic Press, New York, NY. p. 123.

15. Fisher, L.J. 1972. Response of lactating cows to the intravenous infusion of amino acids. Can. J. Anim. Sci. 52:377.

16. Ganapathy, V., and F.H. Leibach. 1982. Transport and utilization of methionine sulfoxide in the rabbit. Biochem. Biophys. Acta. 693:305.

17. Ganapathy, V., and F.H. Leibach. 1985. Is intestinal peptide transport energized by a proton gradient? Am. J. Physiol. 249 (Gastrointest. Liver Physiol. 12):G153.

18. Gardner, M.L.G. 1983. Evidence for, and implications of, passage of intact peptides across the intestinal mucosa. Biochem. Soc. Trans. 11:810.

19. Garvey, T.Q. III, and A. Babcock. 1979. Amino acid and $^{22}Na^{+}$ uptake in membrane vesicles from confluent simian virus 40 transformed Balb/c3T3 and Balb/c3T3. J. Memb. Biol. 49:139.

20. Guerino, F. and C.R. Baumrucker. 1985. Lysine and Methionine transport systems in the bovine small intestine. J. Anim. Sci. 61(Suppl. 1):457. (Abstr.).

21. Guerino, F., and C.R. Baumrucker. 1987. Identification of methionine and lysine transport systems in cattle small intestine. J. Anim. Sci. 65:630.

22. Haase, W., A. Schafer, H. Murer, and R. Kinne.

1978. Studies on the orientation of brush border membrane vesicles. Biochem. J. 172:57.

23. Hopfer, U. 1977. Isolated membrane vesicles as tools for analysis of epithelial transport. Am. J. Physiol. 233:E445.

24. Hopfer, U., K. Nelson, J. Perrotto, and K.J. Isselbacher. 1973. Glucose transport in isolated brush border membrane from small intestine. J. Biol. Chem. 248:25.

25. Im, W.B., D.W. Misch, D.W. Powell and R.G. Faust. 1980. Phenolphthalein- and Harmaline-induced disturbances in the transport functions of isolated brush border and basolateral membrane vesicles from rat jejunum and kidney cortex. Biochem. Pharmacol. 29:2307.

26. Im, W.B., and A.A. Spector. 1980. Sodium-dependent neutral amino acid transport in native and reconstituted membrane vesicles from Ehrlich cells. J. Biol. Chem. 255:764.

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

28. Kilberg, M.S., H.N. Christensen, and M.E. Handlogten. 1979. Cystiene as a system-specific substrate for transport system ASC in rat hepatocytes. Biochem. Biophys. Res. Commun. 88:744.

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

30. Mengual, R., G. Leblanc, and P. Sudaka. 1983. The mechanism of Na^+-L-lactate cotransport by brush-border membrane vesicles from horse kidney. J. Biol. Chem. 258:15071.

31. Mepham, T.B. 1977. Comparative aspects of lactation. Page 60 in Symp. Zool. Soc. London, No. 41. M. Peaker, ed. Academic Press, New York, NY.

32. Mircheff, A.K. 1983. Empirical strategy for analytical fractionation of epithelial cells. Am. J. Physiol. 244 (Gastrointest. Liver Physiol. 7):6347.

33. Moe, A.J. 1984. Non-electrolyte transport in brush border membrane vesicles from the bovine small intestine. Ph.D. Dissertation. Virginia Polytechnic Institute and State Univ., Blacksburg.

34. Moe, A.J., P.A. Pocius, and C.E. Polan. 1985. Isolation and characterization of brush border membrane vesicles from bovine small intestine. J. Nutr. 115:1173.

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

36. Murer, H., E. Amman, J. Biber, and U. Hopfer. 1976. The surface membrane of the smallintestinal epithelial cell. I. Localization of adenyl cyclase. Biochem. Bipphys. Acta. 433:509.

37. Murer, H., and R. Kinne. 1980. The use of isolated membrane vesicles to study epithelial transport processes. J. Memb. Biol. 55:81.

38. Nelson, K.S., and J.M. Asplund. 1986. Biological value and limiting amino acids, for sheep, in corn protein. Fed. Proc. 45:239. (Abstr.).

39. Orten, A.H. 1963. Intestinal phase of amino acid nutrition. Fed. Proc. 22:1103.

40. Phillips, W.A., K.E. Webb, Jr., and J.P. Fontenot. 1976. In vitro absorption of amino acids by the small intestine of sheep. J. Anim. Sci. 42:201.

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

42. Rose, R.C., and S.G.Schultz. 1970. Alanine and glucose effects on the intracellular electrical potential of rabbit ileum. Biochem. Biophys. Acta. 211:376.

43. Rose, R.C., and S.G. Schultz. 1971. Studies on the electrical potential profile across rabbit ileum. J. Gen. Physiol. 57:639.

44. Rogers, J.A. U. Krishnamoorthy, and C.J. Sniffen. 1987. Plasma amino acids and milk protein production by cows fed rumen-protected methionine and lysine. J. Dairy Sci. 70:789.

45. Satter, L.D. 1986. Protein supply from undegraded dietary protein. J. Dairy Sci. 69:2734.

46. Schmitz, J., H. Preiser, D. Maestracci, B.K. Ghosh, J.J. Cerda, and R.K. Crane. 1973. Purification of human intestinal brush border membrane. Biochem. Biophys. Acta. 323:98.

47. Shimbayashi, K., Y. Ide, and Y. Sano. 1967. Arteriovenous differences in concentrations of free amino acids and some other components across lactating cows udder and the presence of ethanolamine in milk whey. J. Agr. Chem. Soc. Japan. 41:375.

48. Sigrist-Nelson, K., H. Murer, and U. Hopfer. 1975. Active alanine transport in isolated brush border membranes. J. Biol. Chem. 250:5674.

49. Statistical Analysis System. SAS users guide. 1982. SAS Inst. Cary, NC.

50. Stevens, B.R., J.D. Kaunitz, and E.M. Wright. 1984. Intestinal transport of amino acids and sugars: Advances using membrane vesicles. Ann. Rev. Physiol. 46:417.

51. Stevens, B.R., H.J. Ross, and E.M. Wright. 1982. Multiple transport pathways for neutral amino acids in rabbit jejunal brush border vesicles. J. Memb. Biol. 66:213.

52. Webb, K.E., Jr. 1986. Amino acid and peptide absorption from the gastrointestinal tract. Fed. Proc. 45:2268.

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

54. Wilson, J.W. 1986. Preparation of brush border and basolateral membrane vesicles from bovine intestine for nutrient uptake studies. M.S. Thesis. Virginia Polytechnic Institute and State Univ., Blacksburg.

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

56. Wright, E.M., B.R. Stevens, and B.E. Peerce. 1986. Neutral amino acid transport in rabbit intestinal brush-border membranes. Fed. Proc. 45:2450.

APPENDIX

Appendix A

Formula for calculation of correction factor (C.F.) converting c.p.m. to pmol ^{35}S-Met uptake per mg membrane protein.

$(A/B)(C/D)(E/F)(G/H) = X$

$(1/X)(1/Y) = Z$

Where:

A/B = c.p.m. per 25 ul ^{35}S-Met

C/D = 1 liter containing 100 umoles ^{35}S-Met

E/F = 10^6 ul per liter

G/H = 1 umole containing 10^6 pmols

X = c.p.m. per pmol ^{35}S-Met

Y = mg membrane protein per assay point

Z = C.F. for conversion of c.p.m. to uptake in pmol Met/mg membrane protein, following subtraction of background counts

Appendix B

Sample of models used in statistical analysis of data. This particular model was used to analyze the effect of omolarity on Met uptake.

$$Y_{ijk} = \alpha + M_i + R_j + MR_{ij} + \beta_1(X_{ijk} - \bar{X}_{\cdot}) + \beta_{i2}(M_i\ (X_{ijk} - \bar{X}_{\cdot})) + e_{ijk}$$

M_i = effect of i^{th} mineral (Na or K)

R_j = effect of j^{th} Rep

X_{ijk} = osmolarity of k^{th} sample, j^{th} Rep, i^{th} mineral

β_1 = overall regression coefficient of Met uptake on osmolarity

β_2 = regression coefficient within each level of mineral

The vita has been removed from the scanned document