PREPARATION OF BRUSH BORDER AND BASOLATERAL MEMBRANE ESICLES FROM BOVINE INTESTINE FOR NUTRIENT UPTAKE STUDIES

BY

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INTRODUCTION

Carbohydrates, proteins, minerals, vitamins, fats and water are considered the nutrient components required by animals to sustain life. Before these nutrients can be utilized by the cells of the organism they must first be digested ,then absorbed. Much of this absorption occurs in the intestine. Therefore, an understanding of the interrelationships and characteristics of nutrient absorption across the epithelial lining of the intestine is an important component of understanding nutritional and physiological considerations.

The characterization of sugar and amino acid absorption in ruminants has not kept pace with the work done in monogastrics. Among the reasons for this is that the microbes of the rumen ferment the dietary carbohydrates; therefore, very little available glucose reaches the small intestine. These microbes are also able to utilize carbon chains from carbohydrates and nitrogen from nonprotein origin to synthesize essential amino acids required by the animal. Therefore, scientists tended to overlook the need to understand the mechanism of absorption of these dietary nutrients at the

level of the small intestine in the ruminant. But with the increased interest in the use of by-pass protein sources, protected amino acids and the possibility of synthetic peptides in ruminant diets, it has become evident that a further understanding of the digestion and absorption mechanisms of the ruminant small intestine is necessary.

In order to characterize the transport properties of a nutrient a suitable experimental technique is required. In vivo techniques used in the past have included arterio-venous differences, reentry cannulas and isolated intestinal loops. These techniques allow for crude approximations of transport mechanisms but complete characterization of transport has been impossible due to interferences by endogenous and dietary components. The development of in vitro techniques such as intestinal rings, everted sacs, ligated intestinal segments and isolated mucosal cells have allowed for further characterization of nutrient transport. These techniques are limited not only by the fact that cellular metabolism is occurring but the experimenter cannot regulate intracellular substrate and electrolyte concentration gradients. The relatively recent techniques of isolated membrane vesicles has overcome these problems and has

given researchers the ability to characterize nutrient transport from the luminal to the serosal side of the mucosa lining. The transport characteristics of the luminal surface of the enterocyte is measured with brush border membrane vesicles. Transport characterization of the serosal surface is determined with basolateral membrane vesicles.

The purpose of this study was to develop a procedure for the isolation of brush border and basolateral membrane vesicles from the epithelial cells of the bovine small intestine. The application of this method would allow characterization of nutrient transport into the enterocyte under a wide range of easily manipulated conditions. It would also allow characterization of the transport systems out of the enterocyte without interference from intracellular metabolism; this becomes important when one considers the evaluation of peptide transport. Further understanding of the mechanisms and interrelationships involved in transporting nutrients from the lumen to the circulation should result in applications which will improve animal performance.

LITERATURE REVIEW

Progress in understanding the nature of transport systems characteristic of intestinal tissue has been directly related to the development of methods which allow the various aspects of transport to be evaluated. By reviewing the progression of methodology used to evaluate intestinal transport one realizes that innovation often serves as the driving force for further innovation, thereby increasing our understanding of basic science.

As early as the mid 1800's researchers where studying the disappearance of intestinal nutrients in anesthetized or surgically prepared animals. Thiry (1864) used animals with surgically prepared intestinal blind sacs to follow the uptake of solutes. Nutrients to be studied were placed in the intestine via a fistula and the contents of the sac were sampled at the time of initial introduction and after a predetermined time interval. By difference in solute concentrations the uptake was determined. This technique was modified by Vella (1888) so nutrient disappearance along an intestinal segment could be studied. In this technique

two ends of an intestinal segment were cannulated so that nutrients could be introduced at one end and disappearance determined by sampling digesta at the other end. This type of procedure lead to the development of in vivo perfusion techniques, in which the researcher had greater control of the material being presented into the lumen. In this technique an intestinal segment is isolated by dual cannulation and the digesta is flushed from the segment. The substrate to be studied can either be perfused through the segment once or can be recycled. Absorption rate is then determined from initial and final solute concentrations and times and rates of the perfusion (Sheff and Smyth, 1955; Fullerton and Parson, 1956). A similar technique was demonstrated by Miller and Abbott (1934) and Cummins and Jussila (1955). This method introduces a solution containing the substrate into the isolated intestinal loops and measures the disappearance over time with no attempt made to simulate intestinal flow. These techniques allowed for the characterizations of absorption in different regions of the small intestine but gave no indication of the fate of the absorbed solutes.

To determine the fate of the nutrients after their disappearance from the lumen, investigators cannulated

the portal vein and lymphatic system draining the intestine. Cannulation of the portal vein established that sugars and amino acids are absorbed into the venous blood (London, 1929; Dent and Schilling, 1949 and Shoemaker, 1963). Long chain fatty acids, cholesterol and triglycerides were found to be drained by the lymphatic system (Bloom et al., 1950 and Blomstrand, 1959). Matthews and Smyth (1954) modified these techniques by cannulating the mesenteric vein draining a ligated intestinal loop, thus allowing quantitative collection of material absorbed from the isolated loop. Using this technique researchers determined that most amino acids (Matthews and Smyth 1954) and monosaccharide sugars (Shoemaker et al, 1963) reached the blood stream in unaltered form. Oligosaccharides (Wilson and Vincient, 1955), oligopeptides (Levenson et al., 1959; Wiggans and Johnson, 1959) and some monosaccharides such as fructose, reached the portal blood as metabolites of the nutrients introduced to the ligated loop. This indicates that intestinal disappearance is not a complete definition of the transport system but represents a combination of metabolism by intestinal epithelium and transport capabilities. In these in vivo techniques the investigator is unable to differentiate between transport

and metabolism and therefore is limited in his ability to characterize transport systems of the intestine.

Researchers realized that if tissue could be excised and maintained under laboratory conditions greater manipulation and control over the conditions influencing transport mechanisms could be achieved than with the in vivo techniques. Reid (1901) developed an early predecessor to the Ussing-type flux chamber by partitioning two glass compartments with an intestinal segment. This technique enables researchers to monitor transmural solute fluxes by measuring the changes in solute concentration over time on both sides of the membrane. Fischer and Parson (1949) were able to overcome some of the problems of tissue viability which often occurred in in vitro perfusion techniques by oxygenating the medium which bathes both sides of the isolated intestinal loop. Using this technique they were able to demonstrate transmural glucose transport against a concentration gradient. However, a large portion of the glucose disappearing from the luminal compartment could not be accounted for in the serosal compartment thus indicating tissue accumulation and/or metabolism.

Modifications of the perfusion techniques were used to demonstrate that nutrient uptake required active

metabolism of epithelial tissue and the presence of sodium (Darlington and Quastel, 1953; Wiseman, 1953). Metabolic inhibitors which blocked energy transducing capability of the epithelial cells were used to demonstrate the energy dependence of transmural transport. As data accumulated showing epithelial tissue to have an active role in nutrient absorption it became clear that a method which allowed sampling of tissue accumulation was required to gain further information about the transport systems of the small intestine. The perfusion technique was unsuitable for such experiments since only one time point could be evaluated following initiation of transport. The use of a series of segments was of questionable validity since the transport and metabolic capabilities differ markedly in segments from different regions of the intestine (Fischer and Parsons, 1949; Cummins and Jussilia, 1955)

The everted sac technique is probably the best known and most widely used in vitro technique which allows for tissue samples to be taken so accumulation can be determined (Wilson and Wiseman, 1959). This technique also requires one segment for each time point but the segments are smaller and therefore, several sacs can be prepared from one intestinal region. The first technique

developed exclusively for the measurements of tissue accumulation was the intestinal ring technique of Agar (1954). In this technique everted intestinal tissue is cut transversely into segments 1 to 2 mm in width. These segments are then incubated with radioactively labeled substrate for a series of time intervals after which the segments are rinsed and the absorbed solutes are extracted from the tissue, thus allowing the amount of isotope present to be quantified. The use of these two procedures allowed researchers to demonstrate that intestinal tissue can accumulate solutes against a concentration gradient. Bihler and Crane (1962) and Shultz et al. (1966) demonstrated that only the mucosal lining of the whole tissue preparations were capable of accumulating amino acids and sugars against a concentration gradient. This accumulation was shown to depend on the presence of sodium and could be inhibited by ouabain. The submucosal layers of the intestine showed no tendency to accumulate solutes. This suggested that the solute transport capabilities of the intestinal tissue are localized in the brush border membrane of the mucosal epithelial cell.

These early experimental approaches concentrated on the phenomenon of intestinal absorption and evolved into

an investigation of the molecular mechanisms by which intestinal epithelial cells accomplish active transport. Data from transmural and unidirectional flux measurements have lead to the development of models which suggest energy coupling to the sodium-dependent transport systems of amino acids and sugars (Curran et al., 1967; Goldner et al., 1969). This model is similar to the coupling mechanisms first suggested by Crane et al (1960). The model suggests that solute accumulation is dependent on the presence of a transmembrane sodium gradient. While kinetic and inhibitor data support this concept, (Schultz and Curran, 1970; Kimmich, 1973), the intact tissue techniques do not allow for extensive testing of the hypothesis. The complete evaluation of the hypothesis requires manipulation of the sodium gradient of an epithelial cell to see if the transport system exhibits the predicted changes in rate and direction of solute flux with changes in the sodium gradient. In the intact tissue procedures, precise manipulation of epithelial cell sodium concentration is hampered by the presence of several additional tissue layers and the large extracellular space in the lamina propia region of the villus. For this reason isolation of epithelial tissue

free of submucosal and other tissue is necessary to further study the details of transport mechanisms.

Investigators have been able to separate epithelial cells from underlying layers of the intestine in the form of mucosal sheets and mucosal cells (Schultz et al., 1966; Harrison and Webster, 1969; and Kimmich, 1975). The use of isolated mucosal cells has allowed the effects of sodium and substrate gradients on substrate accumulation to be evaluated extensively. Since the cells are free from other tissue contamination the sodium and substrate gradients can be rigidly controlled. This allows for complete characterization of transport properties of the epithelial cells. This does not necessarily reflect the transport systems regulating uptake from the lumen, since the epithelial cells of the intestinal lining have a heterogeneous plasma membrane. The surface exposed to the lumen is referred to as the brush border membrane, while the serosal surface is known as the basolateral membrane. The brush border membrane regulates the entry of nutrients into the cell from the lumen while the basolateral membrane determines the flux of nutrients into the circulation. These two membranes have different functions and therefore can be expected to have different transport properties. Therefore, in order

to study the mechanisms regulating the transport systems entering and leaving the cells, the brush border and basolateral membranes need to be studied independently of each other.

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 can 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 are reflected in the different buoyant densities and different surface properties such as different surface receptors, different

surface charge density and different surface hydrophobicity. The brush border and basolateral membranes also resist shearing forces to varying degrees and therefore form different sized fragments when the enterocyte is homogenized. 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 specific marker enzymes. Disaccharidase and alkaline phosphatase are 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 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 (1965)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 population sediment at different rates. Unfortunately, these vesicle preparations were plagued by contamination with internal membranes.

The method most used presently to separate brush border from basolateral and internal membranes is the differential precipitation technique. This technique developed by Schmitz et al. (1973) 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 free from 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.

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 cytoplasmic region of the vesicles can be manipulated extensively. In order to take advantage of this capability the orientation of the vesicles needs to be known. The use of morphological 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 orientation of the basolateral membrane has not as yet been established.

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 sec 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 susceptibilty 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 osmolarity of the incubation buffer; therefore, indicating a decrease in intravesicular volume. If uptake was unaltered by osmolarity then it could be concluded 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 if preparation are unknown and the temperature at which the experiments are performed with the different experimental approachs are not identical.

Research which compared the sodium-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 sodium-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 sodium 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 allows the mechanisms of transepithelial transport of metabolizable substrates, such as peptides, to be evaluated. Vesicle

preparations allow for easy manipulation of the composition of intravesicular and extravesicular fluids which is necessary for the complete characterization of the driving forces for the transport systems. 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 monogastric species (Douglass et al., 1972; Hopfer et al., 1973; Im et al., 1980; Ganapathy et al., 1981; Ling et al., 1981). Only recently the membrane vesicle technique has been applied to the ruminant species. To date three laboratories have reported successful isolation and characterization of brush border membrane vesicles for use in transport studies (Kannitz and Wright, 1984; Moe et al., 1984; Crooker and Clark, 1986). It appears that there have been no successful attempts to isolate and characterize the transport systems of basolateral membrane vesicles from the bovine.

Objectives

To develop a methodology which would allow for the evaluation of peptide and amino acid transport capabilities of bovine basolateral and brush border membranes.

The specific objectives were to:

1. Isolate basolateral membrane vesicles
2. Isolate brush border membrane vesicles
3. Study ability of vesicles to accumulate L-alanine

Materials and Methods

Isolation of Intestinal Tissue. The procedure employed for the isolation and partial purification of brush border and basolateral membrane vesicles was a modification of the procedure of Moe. (1984). Segments of small intestine were removed from Holstein calves that weighed 125 to 300 kg. Three to four meters of intestine were isolated beginning 1 m proximal to the ileal-cecal junction and proceeding proximally. This was performed within 12 min of stunning the steers. Isolated intestine was segmented into 1 m lengths and flushed with cold mannitol buffer ,(Appendix A) immediately following isolation (figure 1). Segments were everted with the aid of a glass rod (figure 2) and placed in cold mannitol buffer for transport to the laboratory.

Isolation and Homogenization of Enterocytes. All procedures henceforth mentioned were carried out at 0 to 4 C unless otherwise specified. Mucosal linings of everted intestinal segments were blotted dry with matting

Figure 1. Flushing of digesta from intestinal segment with ice cold mannitol buffer.

Figure 2. Intestinal segments being everted with the aid of a glass rod.

paper and then scraped free from musculature with a glass slide with care being taken to avoid tearing the muscle lining (figure 3). The enterocytes harvested were then divided into 3 to 4 g aliquots which were either placed in Whirl pac bags and flash frozen in liquid nitrogen and stored at -80 C[1] for future experiments or added to 24 ml of mannitol-succinate buffer (Appendix A), 1 g membrane per 8 ml buffer and homogenized for 15 sec with a polytron[2] on setting six (figure 4). A total of 40 g of harvested tissue were used in each preparation. Homogenized fractions were combined in a 500 ml wide mouth Erlenmeyer flask which was then placed on a stir plate for 30 min of mild agitation. This step allowed for the aggregation and precipitation of internal membranes. At the end of this step the total volume of the homogenate was measured and a 2 ml subsample was taken for determining initial enzyme and protein contents. A similar subsampling procedure was followed for each terminal step in the differential centrifugation scheme.

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

Figure 3. Harvesting of mucosal cells from everted intestinal segment.

Figure 4. Polytron homogenization of mucosal cells.

Differential Centrifugation. Several types of differential centrifugation schemes were applied during the evolution of a technique which results in the isolation of membrane fractions enriched in brush border and basolateral membranes. A typical differential scheme is illustrated in figure 5. Following incubation, the homogenate was distributed into 50 ml centrifuge tubes and centrifuged at 6500 x g for 12 min. The resulting supernatant (S_a) was saved and the pellet (P_a) was resuspended in mannitol transport buffer (Appendix A) and homogenized with 15 strokes of a teflon glass homogenizer[3] (clearance .0889 to .1143mm). Resuspended P_a was then centrifuged at 6500 x g for 12 min. The supernatant (S_b) was combined with S_a to be used for brush border membrane vesicles (BBMV) isolation and P_b was resuspended in mannitol transport buffer with 15 strokes of a teflon glass homogenizer and used for basolateral membrane vesicle (BLMV) isolation. The first two steps were washing steps designed to remove a majority of BBMV from the BLMV fraction. The resuspended P_b was then centrifuged at 750 x g for 12 min to remove aggregated intercellular membranes and nuclei. The pellet P_1 was resuspended and subsampled with the

[3] Kontes Scientific Glassware, Vineland, NJ.

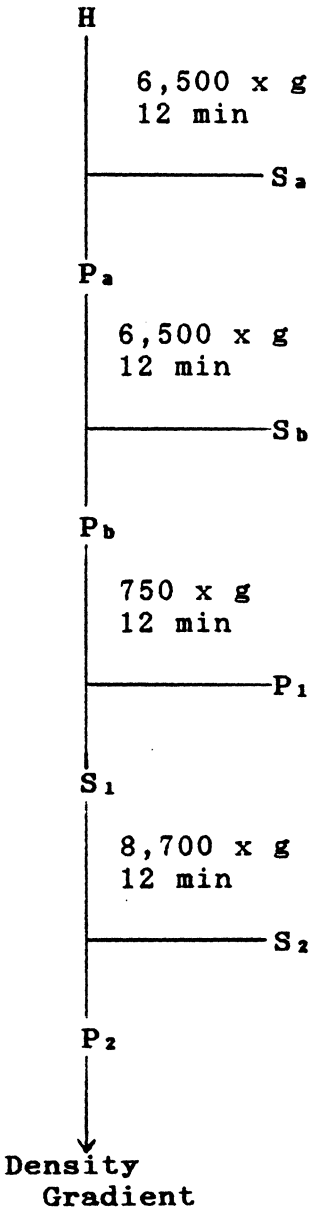

FIGURE 5. Typical differential centrifugation scheme.

supernatant S_1 being centrifuged at 8700 x g for 12 min. The resulting pellet (P_2) was applied to a sucrose density gradient for further purification of BLMV, and the supernatant (S_2) was subsampled and discarded. The S_a and S_b fractions were combined to form S_c and centrifuged at 37,000 x g to form a pellet (P_d). Fraction P_d was then applied to a sucrose density gradient for further purification of BBMV.

Density Gradient. The density gradients used were discontinuous sucrose gradients. A typical gradient of this type consisted of 31, 34, and 38% sucrose, with the highest density of sucrose at the lower layers (Appendix A). The gradient was prepared by layering 2.5 ml of 38% sucrose into a 13 ml ultra centrifuge tube with a 13.5 cm polyethylene transfer pipette. When applying the second layer, 34% sucrose, care was taken to avoid mixing of the two layers at the interface. To accomplish this the pipette tip was placed against the side of the tube at the level of the previous layer of sucrose. Then slight pressure was applied to the bulb to allow the first drop to gradually leave the pipette and spread across the surface of the previous layer. After completing the

second layer, the third layer was applied using the same technique. The completed gradient had sharp interfaces between sucrose layers. If an interface had a turbid appearance then mixing had occurred and the gradient was discarded and remade. After placing the tube containing the density gradient into the centrifuge bucket, the remainder of the tube was filled by layering the membrane to be purified onto the gradient. Before layering the membrane from the pellets onto the gradient the pellets were resuspended in mannitol transport buffer to a total volume of 30 ml and homogenized with 15 strokes of a teflon glass homogenizer. This volume allows 5.5 ml of membrane suspension, containing approximately 25 mg of protein, to be layered onto each gradient. Typically six density gradients were used for each preparation.

The density gradients with membranes applied were centrifuged at 105,000 x g for 90 min in an ultracentrifuge[4] equipped with a swinging bucket rotor[5]. Membrane bands which resulted from density gradient centrifugation were collected by aspirating the individual sucrose layers with a transfer pipette (figure 6). Band 1 refers to membrane band on top of 31% sucrose layer down to but not including the membrane band on top

[4] Model L5-75B, Beckman Instruments. Palo Alto, CA.
[5] SW-40 rotor. Beckman Instruments. Palo Alto, CA.

of the 34% sucrose layer. Band 2 refers to the membrane band on top of 34% sucrose layer down to but not including the membrane band on top of the 34% layer. Band 3 refers to the membrane band on top of 38% sucrose layer down to but not including the pellet on the bottom of the tube. Band 4 refers to the remainder of the fluid in the tube and the pellet at the bottom of the tube. If time did not permit transport studies at the time of isolation, the bands of interest were placed in 1.8 ml cyro vials[6] and frozen in liquid nitrogen and stored at -80 C. For transport studies, collected bands were placed in 40 ml ultracentrifuge tubes along with approximately 25 ml of mannitol transport buffer. These tubes were then centrifuged at 105,000 x g for 90 min. This step washed the sucrose from the membrane fractions. The resulting pellets were then resuspended in mannitol transport buffer to achieve a final membrane protein concentration of 3 to 4 mg/ml.Normally, this required 2 ml of transport buffer. Resuspension was accomplished by repeated aspiration with a pasteur pipette. The resuspended pellets were then used either for transport studies or enzyme assays to determine enrichment values

[6] Nunc cryotubes, Vangard International Inc. Neptune NJ.

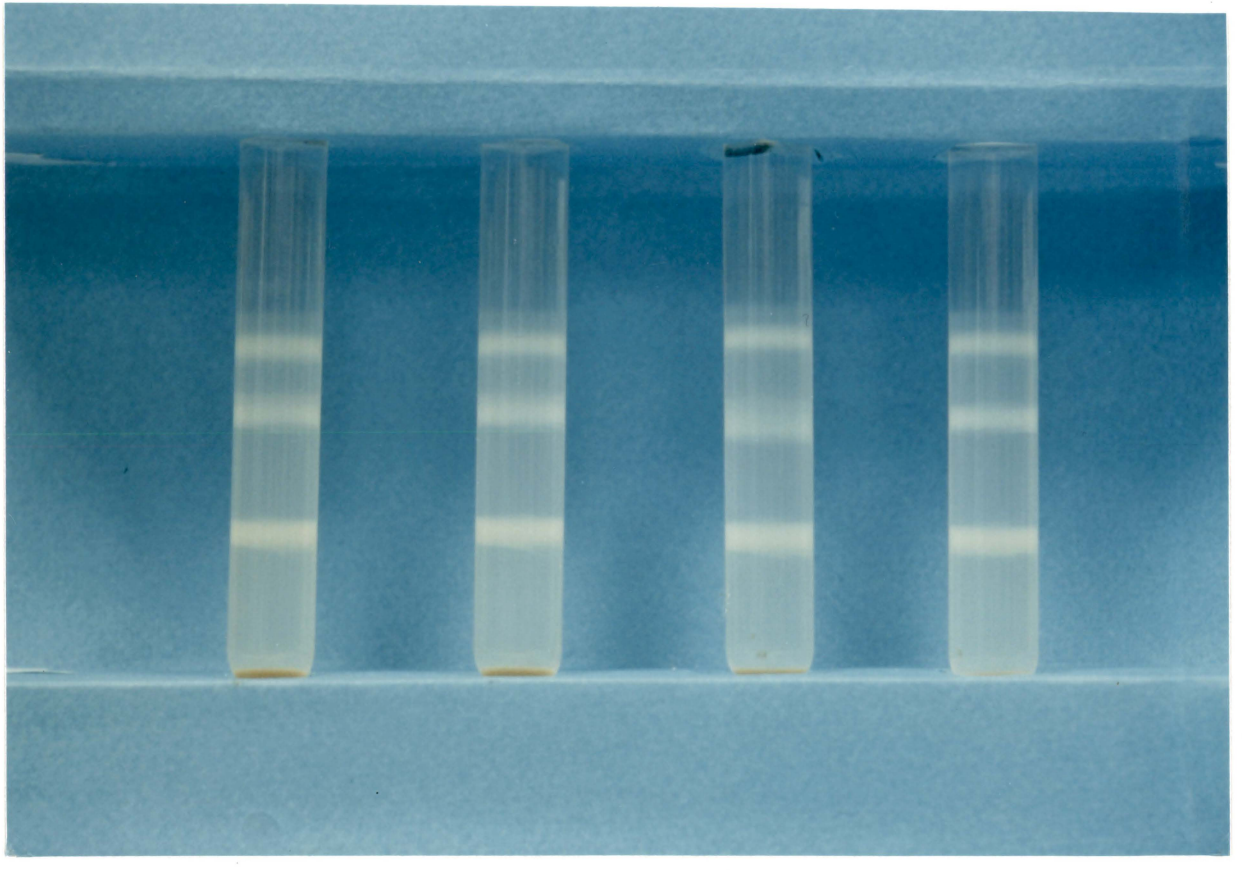

Figure 6. Membrane bands resulting from, centrifugation of membrane fraction on a sucrose step gradient .

Na⁺/K⁺ATPase. The enzyme marker for the basolateral membrane is Na⁺/K⁺ATPase. The assay used to measure the activity of Na⁺/K⁺ATPase was a modification of the procedure of Fugita et al.(1970). This procedure relies on the fact that Na⁺/K⁺ATPase can be selectively inhibited by ouabain. Therefore, by concurrent measurements of total ATPase activity and ATPase activity in the presence of ouabain, the activity of Na⁺/K⁺ATPase of a membrane fraction can be determined by the difference.

To measure the Na⁺/K⁺ATPase activity of a membrane fraction, the fraction was diluted to a protein concentration of approximately 1 mg/ml. Generally, this can be accomplished with a 10-fold dilution for pelleted fractions and a fivefold dilution for supernatant fractions. The reaction was initiated by adding .1 ml of membrane to .9 ml of ATPase incubation buffer both with and without .1 mM ouabain, (Appendix B) equilibrated to 37 C and then vortexing the mixture. The incubation was carried out in a shaking water bath to assure the membranes remained suspended throughout the incubation period. The reaction was stopped after 30 min by the addition of 1 ml of ice cold 10% (w/v) trichloroacetic acid (TCA). Once the reaction was stopped the reaction

vessels (15 ml tube) were centrifuged at 9,000 x g for 10 min to remove precipitated membranes. Assays for each membrane fraction were carried out in triplicate, both with and without ouabain.

Since Na^+/K^+ATPase activity was determined as the difference between total ATPase and inhibited ATPase, minute differences between incubation buffers could result in erroneous results. To minimize errors associated with differences between the with and without ouabain buffers, a stock ATPase buffer was prepared with all components except Na_2ATP and ouabain.

To determine the ATPase activity of a membrane fraction, the level of inorganic phosphorous hydrolyzed from ATP during incubation was measured. Inorganic phosphorous was determined by the method of Eibl and Lands, (1969). This assay measures the turbidity produced by the reaction of inorganic phosphorous, molybdate and triton-X solution. First, .2 ml of supernatant from the incubated reaction vessels were added to 15 ml tubes containing 5 ml of a .012 % triton-X solution. The reaction was initiated by addition of .6 ml of molybdate solution (Appendix C) and vortexing immediately. The starting of the reaction was staggered since the reaction does not stabilize at an endpoint and

must be read after 20 min. The absorbance was measured at a wavelength of 660 nm with a spectrophotometer[7]. The values obtained for the samples were compared to a standard curve consisting of 0, .75, 1.5, 3, 5, and 10 ug phosphorous to determine the level of inorganic phosphorous liberated from ATP during incubation. If phosphorous levels were not within the standard curve values the amount of supernatant added was adjusted accordingly.

After a value was determined for the inorganic phosphorous in the sample it was corrected for dilution factor and divided by volume used in the phosphorous assay to give activity on a concentration basis. The concentration of enzyme activity was multiplied by total volume of that fraction to yield the total activity contained in that fraction. This value was used to calculate percent recoveries and specific activities.

Alkaline Phosphatase. Alkaline phosphatase (E.C. 3.1, 3.1) is the marker enzyme for the brush border membrane. Activity of alkaline phosphatase (AP) was determined with an enzyme assay kit[8]. The assay measures

[7] Spectronic 1001, Bausch and Lomb. Rochester, NY.t
[8] Aklaline Phosphatase, kit 246. Sigma Chemical. St. Louis, MO.

the rate at which AP in the sample converts colorless p-nitrophenyl phosphate to yellow p-nitrophenol, which can be monitored at 405 nm on a spectrophotometer (Appendix D). Generally, dilutions of threefold to fivefold for pelleted fractions and onefold to threefold for supernatant fractions yielded AP concentrations which fell into the linear range of the assay. The reaction was initiated by the addition of .1 ml of membrane to a 5 ml tube containing 3 ml of p-nitrophenyl phosphate solution. Solutions were mixed by inversion and after 1 min, initial absorbance was determined. Three minutes later, final absorbance was determined. Absorbance changes over 3 min were multiplied by a conversion factor (551) provided by Sigma to yield the activity of AP in units per liter. Total activity was then calculated by multiplying activity by total volume of the fraction. The total activity of the fraction was used to calculate specific activities and percent recoveries.

Proteins. The membrane protein levels were determined with coomassie blue G-250[9] which becomes bound to proteins in acidic solution. The binding produced an

[9] Protein assay reagent 23200, Pierce Chemical, Rockford, IL.

absorbance shift from 465 to 595 nm which was monitored on the spectrophotometer (appendix E).

The membrane fractions were prepared for this assay by diluting pelleted fractions 10-fold and supernatant fractions fivefold. The reaction was initiated by the addition of .1 ml of membrane solution to a 15 ml tube containing 5 ml of protein assay reagent followed by vortexing. Absorbance was read at 595 nm on a spectrophotometer. Protein concentration was determined by comparing the absorbance of the sample to the absorbances of a standard curve consisting of 0, 25, 50, 100, 150, and 200 ug of bovine serum albumin per reaction vessel. If absorbance did not fall within the range of the standard curve, the amount of sample added was altered.

Transport Assays. Transport experiments were conducted with the membrane filtration technique of Kimmich (1975) and Murer et al., (1974). The incubation medium contained 100 mM NaSCN, 100 mM mannitol, 2 mM $MgCl_2$ and 10 mM Hepes-Tris, membrane substrate, pH 7.4. When Na-independent transport was measured, KSCN replaced NaSCN equimolarly in the incubation medium. All components of the incubation media except the membranes

and substrates were supplied via transport buffer (Appendix F). Transport buffer was prepared at twice the concentration stated above for the incubation medium. Therefore, one-half of the total volume of the incubation medium could be transport buffer and still result in the final concentration stated above for the incubation medium.

The total reaction volume per time point was 120 ul, (60 ul transport buffer, 20 ul isotope solution (.25 u Ci), 20 ul membrane solution and 20 ul distilled water). The isotope solution was prepared by adding cold substrate at a volume and concentration such that the addition of 20 ul of this isotope solution would result in a final substrate concentration of 100 uM in the incubation medium. Generally, several time points were contained in each reaction vessel. For example, if six time points were to be measured in a time course uptake study, a single vessel would contain enough incubation medium to measure all six time points. The vessel contained 780 ul of incubation medium (6.5 x 120 ul), composed of 390 ul transport buffer (50%), 130 ul isotope solution (16.7%), 130 ul distilled water (16.7%), and 130 ul of membrane solution (16.7%). This allowed for six time point measurements of 120 ul each with 60 ul of

reaction mixture remaining. The membrane vesicle solution contained 2 to 3 mg/ml protein, which resulted in 50 to 70 ug of protein per time point. Typically, this transport reaction was initiated by adding membrane vesicle solution to incubation medium. Transport assays were incubated at 22 C with periodic agitation to assure membranes remained suspended. All transport studies were performed on a three port filtration manifold[10] (figure 7). Transport reactions were stopped at appropriate times by adding 120 ul of incubation media to the stopping tower which contained 3 ml of cold (2 C) 150 mM KCl solution. In glucose studies, 100 mM phloridzin[11] was added to the stopping solution. The tower was immediately opened to allow buffer to filter through a millipore filter[12] under constant vacuum (380 mm Hg). The filter was then washed with 6 ml of stopping solution. The filters trapped the vesicles, containing transported isotope and allowed isotope that had not been transported into the vesicles to be washed free. Care was taken to subject all test points to the same washing procedures and vacuum pressure. Once the last wash

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[10] Millipore Corporation. Bedford, MA.
[11] Sigma Chemical. St. Louis, MO.
[12] HAWP .45 um pore size, Millipore Corporation. Bedford,
MA.

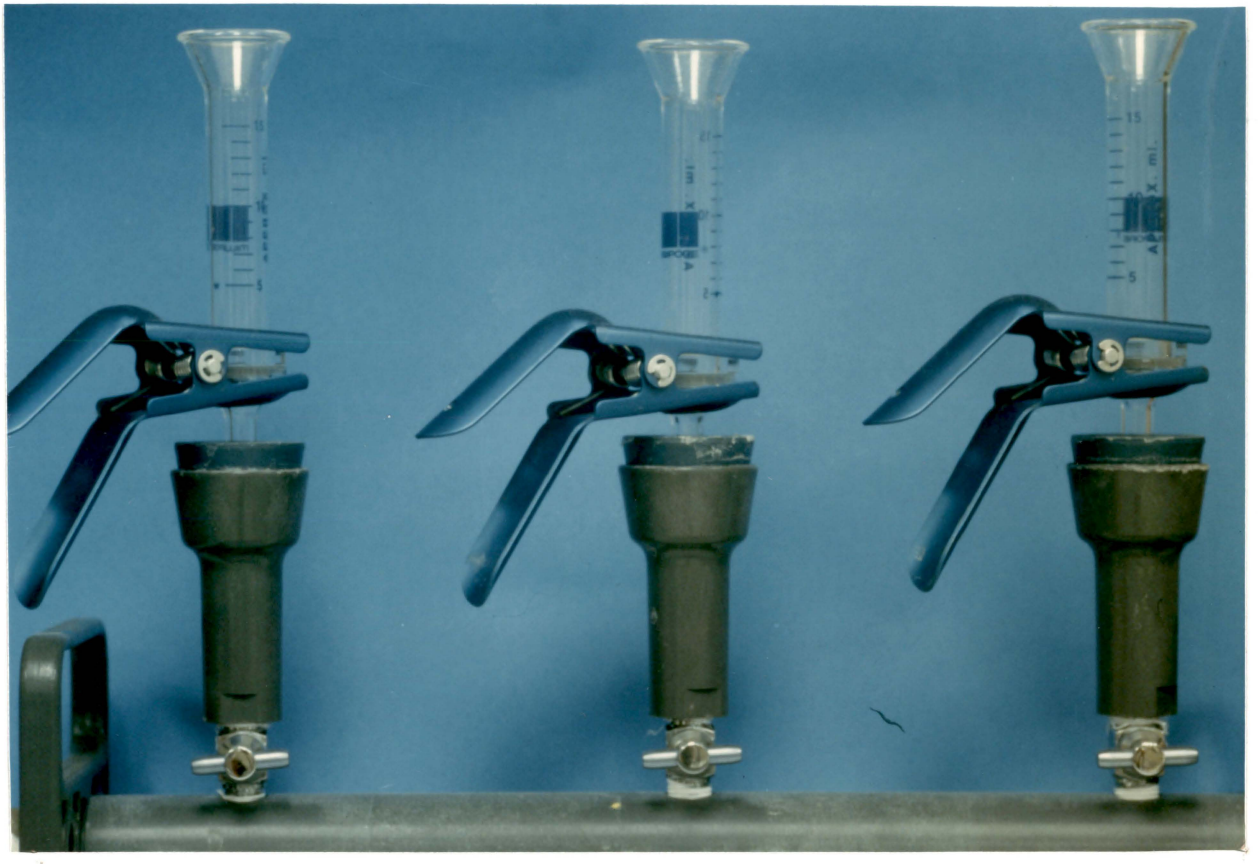

Figure 7. Three tower millipore filtration manifold.

volume was pulled through the filter the valve to that tower was closed.

The filter containing isotope was then placed in a scintillation vial to dry overnight. Then 10 ml of scintillation fluid[13] was added to each vial containing a filter. The vial was capped and then vortexed vigorously to free isotope containing vesicles from the filter, thus assuring uniform distribution of isotope in scintillation fluid. Vials were allowed to stabilize for at least 5 h and were then counted on a beta counter[14].

The substrate uptake by vesicles was then determined by multiplying by a factor which converted counts per minute (CPM) to pmoles of substrate. The conversion factor (pmoles/cpm) was determined by counting a known volume and concentration of substrate.

To demonstrate that membranes were of vesicular nature the glucose concentration at equilibrium was determined in the presence of 25, 50, 100, 200, and 300 mM mannitol (Appendix F). The osmolarities of the incubation media were determined with an osmometer[15]. The percent of substrate binding to membrane was determined by plotting pmoles of glucose uptake per mg of

[13] Scinti-verse II, Fischer Scientific. Orangeburg, NY.
[14] Model SR 7500, Beckman Instruments. Palo Alto, CA.
[15] Osmette A, Precision Systems Inc. Sudburg, MA.

protein (y-axis) versus inverse osmolarity (x-axis) and extrapolating the line to infinite osmolarity (zero).

RESULTS AND DISCUSSION

Whenever investigators attempt to develop a system to isolate and purify a subpopulation from a larger population, be it hydrocarbons from petroleum or constituent membranes from the cell, the investigator must have a technique which allows for monitoring of the components of interest at every step of the isolation procedure. In the field of membrane biology the monitoring system of choice consist of marker enzymes, which are enzymes unique to the membranes of interest. Therefore, the relative amounts of the various membrane components of the cell can be determined in each of the steps of a differential and density gradient centrifugation scheme by determining the specific activity (SA) of the marker enzyme. The specific activity of the enzyme refers to units of enzyme activity per unit of protein present. This standardizes the enzyme activities so that relative enrichments of the various fractions can be determined by comparison to the homogenate. For example, if the specific activity of an isolated membrane fraction was three times the specific activity of the homogenate, then this fraction was

enriched threefold. Since the specific activity of the homogenate represents the specific activity of the intact enterocyte it can be concluded that the isolated fraction contains the membranes of interest in proportions three times as great as those found in the intact enterocyte.

The enrichment of brush border (luminal surface) and basolateral (serosal surface) membranes during the isolation procedures was monitored by following the specific activity of alkaline phosphatase and Na^+/K^+ATPase, respectively. Moe (1984) and Crooker and Clark (1986) demonstrated that the majority of the internal membranes, i.e. endoplasmic reticulum, lysosomes and mitochondria, are removed by the initial low speed centrifugation and therefore do not present a cross contamination problem during isolation of brush border (BBM) and basolateral membrane (BLM) fractions. Therefore, the specific activities of these membranes were not measured in these experiments.

When attempting to compare and contrast transport properties of brush border and basolateral membrane vesicles it is important to isolate the two membrane fractions under identical conditions. Simultaneous separation and isolation of brush border and basolateral membrane minimizes the possible differences which may

result from inactivation of transport properties during the isolation procedure and maximizes utilization of the isolated tissue. The evolution of experimental techniques used to achieve suitable BLM and BBM preparations can be divided into five stages.

Stage 1. First attempts to simultaneously isolate BBM and BLM utilized Mg^{++} precipitation followed by a three step differential centrifugation which separated membrane fractions strictly by decreasing density, i.e., highest density membrane in the first pellet and lowest density membrane in the last pellet (figure 8). This technique was used by Moe (1984) to isolate functional BBM vesicles enriched up to 10-fold. Therefore, the original thrust of this investigation was geared toward the isolation of BLM vesicles using the isolation procedure of Moe (1984).

To determine if this relatively simple differential centrifugation procedure would allow for isolation of purified BLM, the Na^+/K^+ATPase activity sedimentation pattern was determined (table 1). The initial centrifugation step, 750 x g, resulted in a pellet, P1, which contained 59.9% of the total protein and 80.0% of the total Na^+/K^+ATPase (BLM). Although this fraction is

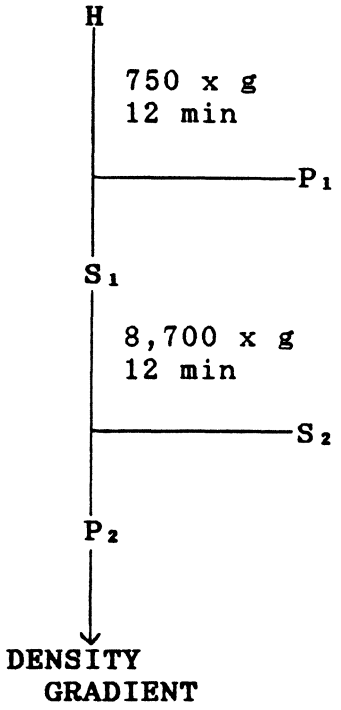

FIGURE 8. Stage 1 differential centrifugation scheme.

TABLE 1. ENRICHMENT AND PERCENT OF INITIAL ACTIVITY OF ALKALINE PHOSPHATASE, NA^+/K^+ ATPASE AND PROTEIN LEVEL OF STAGE 1 DIFFERENTIAL CENTRIFUGATION STEPS, SUBJECTED TO EITHER TEFLON-GLASS OR POLYTRON HOMOGENIZATION.

Homogenization[a] procedure	Differential[b] fraction	Component[c]	Percent of[d] initial	Enrichment[e]
Teflon-glass homogenizer	P_1	Protein	59.9	---
		Na^+/K^+ATPase	80.0	1.3
		AP	----	---
	P_2	Protein	10.0	---
		Na^+/K^+ATPase	6.8	.7
		AP	----	---
	S_2	Protein	29.0	---
		Na^+/K^+ATPase	9.8	.6
		AP	----	---
Polytron	P_1	Protein	32.6	---
		Na^+/K^+ATPase	42.5	1.3
		AP	26.5	.8
	P_2	Protein	6.4	---
		Na^+/K^+ATPase	11.9	1.9
		AP	12.6	2.0
	S_2	Protein	60.6	---
		Na^+/K^+ATPase	18.0	.3
		AP	65.4	1.1

[a] Indicates method used to homogenize isolated enterocytes

[b] Membrane fractions resulting from differential centrifugation: P_1-pellet resulting from 750 x g centrifugation of homogenate, P_2-pellet resulting from 8700 x g centrifugation of the supernatant from P_1, S_2 was supernate from P_2. S_2 values were estimated in some cases by addition of the values of P_2 and S_2 , which resulted from a 31000 x g centrifugation of S_1.

[c] Protein, Na+/K+ATPase, and alkaline phosphatase components of the membrane fractions were analyzed so that enrichment of BLM and BBM could be monitored.

[d] Percent of initial determined by dividing the total amount or activity of each component found in a fraction by the total amount or activity of that component found in the initial homogenate.

[e] Enrichment represents specific activity of component in a membrane fraction divided by specific activity of that component in the homogenate.

enriched 1.3 fold with BLM it also contains the internal membranes of the homogenized enterocytes and therefore the BLM of this fraction were not considered suitable for further purification. The pellet resulting from the 8,700 x g spin, P_2, contained 10.0% of the total protein and 6.8% of the total basolateral membranes, only a .7-fold enrichment. The supernatant, S_2, contained 29.0% of the total protein and only 9.8% of the total BLMV. This was the fraction from which brush border membrane vesicles were isolated by Moe (1984). Thus, none of the isolated fractions were suitable for further purification via density gradient purification.

The homogenization of the enterocytes for the above experiments were accomplished with a teflon-glass, tight fitting homogenizer. This method of homogenization was abandoned because the homogenizer became worn rapidly due to the grit content of the cell suspension. This wearing increased the clearance of the homogenizer, thus introducing variability between preparations due to poor reproducibility of the homogenization step.

The variability between preparations along with the cost of frequent replacement of the homogenizer prompted the use of an alternative homogenization procedure. The alternative method chosen for enterocyte homogenization

was the polytron. The differential sedimentation profile of protein and Na^+/K^+ATPase activity following polytron homogenization differed greatly from the pattern seen following teflon-glass homogenization.

The initial pellet following polytron homogenization contained 32.6% of the total protein and 42.5% of the total Na^+/K^+ATPase compared to 59.9% of the total protein and 80.0% of the total Na^+/K^+ATPase found following teflon-glass homogenization. The result was less of the protein and, more importantly, the BLM were lost in the initial low speed centrifugation step. The Na^+/K^+ATPase level in the 8,700 x g pellet (P_2) increased from 6.8 to 11.9% while the percent protein dropped from 10 to 6.4% when using polytron homogenization. The net result was an increase in specific activity of Na^+/K^+ATPase in P_2 from .7 to 1.9-fold. Unfortunately the P_2 alkaline phosphatase level was enriched 2.0-fold, thus indicating brush border cross contamination.

The shift in membrane distribution with the method of homogenization may be attributed to the degree to which the membranes are fragmented. The polytron technique appears to break the membranes into smaller fragments, thereby releasing more of the BLM from the

internal membranes and thus reducing the amount of BLM lost in the first centrifugation step.

The P_2 fraction was subjected to a discontinuous sucrose density gradient, consisting of 31, 34, 38, 42, and 46% sucrose, in an attempt to further enrich BLM level by separating the BBM from the BLM fractions. The discontinuous or step density gradient separates the membrane components when the fractions migrate to the sucrose interface corresponding to their buoyant density (table 2). The highest specific activity of Na^+/K^+ATPase was found at the 31% sucrose interface, where an 8.9-fold enrichment was achieved but the alkaline phosphatase activity was also enriched 3.9-fold. Therefore, even though BLM were isolated relatively free from cross contamination, the fact that the BBM were also enriched to a high degree made this preparation unsuitable for transport experiments.

The similar sedimentation and density migration for BBM and BLM, even though the physical properties of these membranes vary, can possibly be explained by two theories. The homogenization procedure may fracture the plasma membrane of the enterocyte in such a way that the vesicles formed are hybrids containing the enzyme activity of both the BBM and BLM (Berger and Slackton,

Table 2. ENRICHMENT OF Na^+/K^+ATPASE AND ALKALINE PHOSPHATASE IN THE MEMBRANE FRACTIONS OBTAINED FOLLOWING SUCROSE DENSITY GRADIENT CENTRIFUGATION OF PELLET 2 FROM STAGE 1 PREPARATION.

Percentage[a] sucrose	Enrichment[b]		Ratio[c]
	Na^+/K^+ATPase	AP	Na^+/K^+ATPase:AP
31	8.9	3.9	2.3
34	4.2	4.7	.9
38	4.4	2.5	1.8
42	1.1	1.6	.7
46	1.3	1.5	.9
Pellet	.9	1.0	.9

[a] Percentage of sucrose refers to the concentration of sucrose at each step of the gradient, pellet refers to membranes which migrated to the bottom of the gradient.

[b] Enrichment represents specific activity of an enzyme in a membrane fraction divided by specific activity of that enzyme in the homogenate.

[c] Enrichment of Na^+/K^+ATPase in a membrane fraction divided by enrichment of alkaline phosphatase in the same fraction.

1970). Another possibility is that homogenization produces BBM vesicles of uniform size while BLM vesicles tend to be variable in size (Heidrich et al. 1972; Evers et al. 1984). The vesicles being different sizes would allow membranes of different composition to have the same density, since density is a weight per volume property.

If either or both of these theories are valid it would be futile to attempt to isolate BLM using differential centrifugation procedure of stage 1. Fortunately only 11.9% of the total Na^+/K^+ATPase activity was evolved in this isolated BLM fraction, P_2. Therefore, by developing a procedure which removes the 11.9% of the BLM which have similar density properties as BBM, it should be possible to isolate purified BLM from the remaining 88.1% of the BLM.

Stage 2. The technique developed relies on the Mg^{++} binding properties of the cellular membranes. The BBM have the ability to accommodate the two positive charges of the divalent cation within their surface while the other cellular membranes can only accommodate one of the charges of the Mg^{++}. Thus the Mg^{++} forms a cross link between these membranes (Kessler et al. 1972; Schmitz, 1975). This phenomenon was used in the stage 1 step wise

differential centrifugation scheme to remove internal membranes and a majority of the BLM from the BBM in the initial low speed centrifugation. The BLM which did not precipitate have density properties similar to brush border membranes. Therefore, a series of washing steps were introduced which would remove a majority of the lower density BBM, and theoretically, the BLM which shared BBM density properties, in the first steps of the differential centrifugation procedure (figure 9).

The wash steps consisted of a 6,500 x g centrifugation following the 30 min incubation with Mg^{++} buffer. The resulting pellet was resuspended and centrifuged a second time at 6,500 x g. When the supernatants were combined and analyzed they were found to contain 65.0% of the total protein, 51.0% of the total Na^{+}/K^{+}ATPase activity and 50.0% of the total alkaline phosphatase activity (table 3). The Mg^{++} was washed from the membranes of the resulting pellet by resuspension in transport buffer. Once the Mg^{++} concentration is diluted the BLM can be separated from the internal membranes using differential centrifugation since the density of the BLM is much less than the internal membranes due to a lower protein content.

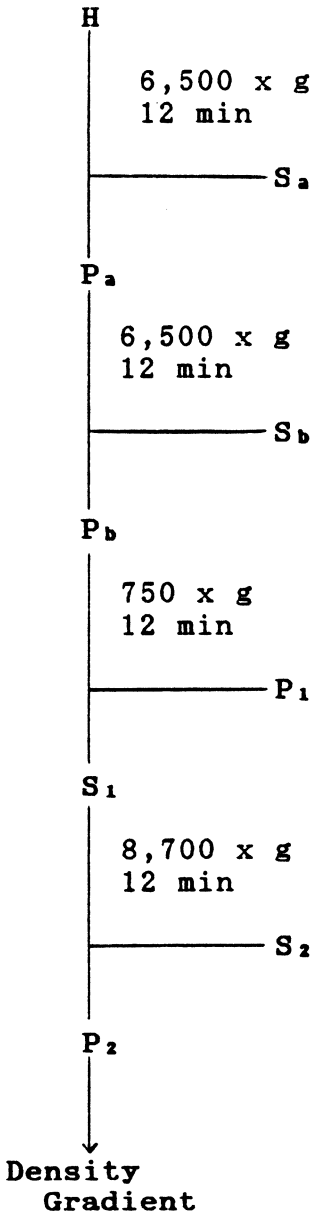

FIGURE 9. Stage 2 differential centrifugation scheme.

TABLE 3. ENRICHMENT AND PERCENT OF INITIAL ACTIVITY OF ALKALINE PHOSPHATASE, Na^+/K^+ ATPASE AND PROTEIN LEVEL OF STAGE 2 DIFFERENTIAL CENTRIFUGATION STEPS PREPARED WITH EITHER FRESH OR FROZEN TISSUE

Tissue[a] treatment	Differential[b] fraction	Component[c]	Percent of[d] initial	Enrichment[e]
Fresh	S_c	Protein	65.0	---
		Na^+/K^+ATPase	51.0	.8
		AP	50.0	.8
	P_1	Protein	24.0	---
		Na^+/K^+ATPase	38.0	1.6
		AP	16.5	.7
	P_2	Protein	3.4	---
		Na^+/K^+ATPase	7.7	2.3
		AP	3.9	1.1
Frozen	S_c	Protein	68.0	---
		Na^+/K^+ATPase	51.0	.8
		AP	80.0	1.2
	P_1	Protein	8.6	---
		Na^+/K^+ATPase	16.0	1.9
		AP	7.5	.9
	P_2	Protein	5.9	---
		Na^+/K^+ATPase	19.6	3.3
		AP	6.3	1.1

[a] Tissue treatment refers to the time post slaughter that the enterocytes were processed. Fresh indicates same day preparation while frozen indicates mucosal scrappings were flash frozen with liquid N_2 and stored at -80 C prior to use in membrane preparation.

[b] Membrane fractions resulting from differential centrifugation: Sc-combination of the supernatants which resulted from a 6500 x g centrifugation of the homogenate and a 6500 x g centrifugation of the resuspended pellet from the first wash step; P_1-resulted from 750 x g centrifugation of the resuspended pellet from the second wash steps; P_2-pellet resulting from 8700 x g centrifugation of the supernatant from P_1

[c] Protein, Na+/K+ATPase, and alkaline phosphatase components of the membrane fractions were analyzed so that enrichment of BLM and BBM could be monitored.

[d] Percent of initial determined by dividing the total amount or activity of each component found in a fraction by the total amount or activity of that component found in the initial homogenate.

[e] Enrichment represents specific activity of component in a membrane fraction divided by specific activity of that component in the homogenate.

The initial centrifugation at 750 x g resulted in a pellet (P_1) which contained 24.0% of the total protein, 38.0% of the total Na^+/K^+ATPase and 16.5% of the total alkaline phosphatase. The supernatant from P_1 was centrifuged at 8,700 x g to yield a pellet (P_2) containing 3.4% of the total protein, 7.7% of the total Na^+/K^+ATPase and 3.9% of the total alkaline phosphatase. The P_2 fraction was found to be adequate for further purification of BLM by density gradient centrifugation since the Na^+/K^+ATPase activity was enriched 2.3-fold and the cross contamination with alkaline phosphatase activity was reduced to 1.1-fold (table 3).

The P_2 fraction was applied to a discontinuous density gradient consisting of 31, 34, and 38% sucrose. The deletion of the 42% and 46% sucrose layer was a modification of the density gradients used in the stage 1 experiments. The 42 and 46% sucrose layers were not used in this experiment since neither of these bands showed enrichment of Na^+/K^+ATPase in the experiments of stage 1.

The membranes at the 31, 34, and 38% sucrose layers and the pellet at the bottom of the tube were enriched 10.4-fold, 8.9-fold, 7.4-fold and 1.7-fold in the Na^+/K^+ATPase activity, respectively, while alkaline phosphatase activity was enriched 11.3-fold, 3.1-fold,

3.1-fold, and .9-fold, respectively. Although the first band had the highest BLM enrichment it also had the highest BBM enrichment thus reducing its desirability for use in transport studies. The second band was clearly the best choice for use in transport studies since the Na^+/K^+ATPase was enriched 8.9-fold over the membranes of the homogenate and the BLM to BBM ratio was optimized, as indicated by the 2.9-fold enrichment of Na^+/K^+ATPase as compared to alkaline phosphatase. The Na^+/K^+ATPase activity of the first and third bands and the pellet were enriched .9-fold, 2.4-fold and 1.9-fold over alkaline phosphatase activity, respectively (table 4).

To maximize the number of experiments which could be accomplished per slaughtered animal, a procedure was developed to freeze the mucosal scrapings of the small intestine. The mucosal scrapings of the different intestinal segments were combined to form a homogeneous mixture which was divided into 3 to 4 g aliquots. These aliquots were placed in whirl pac bags and flash frozen in liquid nitrogen. Frozen bags were then placed in jars and sealed before being placed in the ultralow freezer at -80 C for long term storage. This step was instituted to minimize tissue damage due to freeze drying which is known to occur when samples are stored in plastic bags.

Table 4. ENRICHMENT OF NA⁺/K⁺ATPASE AND ALKALINE PHOSPHATASE IN THE MEMBRANE FRACTIONS OBTAINED FOLLOWING SUCROSE DENSITY GRADIENT CENTRIFUGATION OF PELLET 2, FROM STAGE 2 PREPARATION OF FRESH AND FROZEN TISSUE.

Tissue[a] treatment	Percentage[b] sucrose	Enrichment[c] Na⁺/K⁺ATPase	Enrichment[c] AP	Ratio[d] Na⁺/K⁺ATPase:AP
	31	10.4	11.3	.9
Fresh	34	8.9	3.1	2.9
	38	7.4	3.1	2.4
	Pellet	1.7	.9	1.9
	31	4.6	4.5	1.0
Frozen	34	11.1	2.6	4.3
	38	6.0	1.8	3.3
	Pellet	2.1	.8	2.6

[a] Tissue treatment refers to the time post slaughter that the enterocytes were processed. Fresh indicates same day preparation while frozen indicates mucosal scrapings were flash frozen with liquid N_2 and stored at -80 c prior to use in membrane preparation.

[b] Percentage of sucrose refers to the concentration of sucrose at each step of the gradient, pellet refers to membranes which migrated to the bottom of the gradient.

[c] Enrichment represents specific activity of an enzyme in a membrane fraction divided by specific activity of that enzyme in the homogenate.

[d] Enrichment of Na⁺/K⁺ATPase in a membrane fraction divided by enrichment of alkaline phosphatase in the same fraction.

The stored tissue showed no reduction in enzyme activities even after 3 mo in the ultralow freezer, but freezing did alter the distribution pattern of protein, Na^+/K^+ATPase, and alkaline phosphatase, during differential centrifugation.

The protein distribution in the wash step supernatants (S_c) was essentially unchanged but there was a shift of protein from the P_1 to the P_2 membrane fractions when frozen tissue was used. Pellet 1 was the result of a 750 x g centrifugation of the wash step pellet and P_2 the result of an 8,700 x g centrifugation of the supernatant from P_1. The Na^+/K^+ATPase levels in S_2 remained the same but a dramatic shift in activity occurred between P_1 and P_2, in fresh tissue preparations P_1 contained 38.0% and P_2 contained 7.7% of the total activity. In frozen tissue, P_1 contained only 16.0% of total activity and P_2 contained 19.6% of the total activity. The alkaline phosphatase activity in the S_c fraction shifted from 50.0% to 80.0% of the total activity while P_1 and P_2 changed from 16.5 to 7.5% and from 3.9 to 6.3%, respectively, with the use of frozen tissue preparations (Table 3). The result being a more favorable P_2 fraction to apply to the density gradient for BLM isolation due to the dramatic increase in

Na^+/K^+ATPase activity without a concurrent dramatic increase in protein and alkaline phosphatase activity. The Na^+/K^+ATPase enrichment of P_2 was increased from 2.3-fold to 3.3-fold with frozen tissue preparation.

The P_2 fraction from frozen tissue preparation was applied to a discontinuous density gradient consisting of 31, 34, 38% sucrose. The membranes at the 31, 34, 38% sucrose interfaces and the pellet were enriched 4.6-fold, 11.1-fold, 6.0-fold and 2.1-fold in Na^+/K^+ATPase activity, respectively, while AP activity was enriched 4.5-fold, 2.6-fold, 1.8-fold and .8-fold, respectively. This differentiation pattern was similar to that obtained when the P_2 fraction from fresh tissue was applied to the same type of discontinuous density gradient. The exception being the first band did not have the high AP activity which occurred when fresh tissue was used. The second band was the best suited for transport studies in both preparations but the enrichment of Na^+/K^+ATPase, 11.1-fold, and the Na^+/K^+ATPase:AP ratio, 4.3, was more favorable with frozen tissue than fresh tissue which had an 8.9-fold enrichment of Na^+/K^+ATPase and a Na^+/K^+ATPase:AP ratio of 2.9 (table 4).

The differences between the differential sedimentation pattern and density gradient distribution

for fresh and frozen tissue were probably due to differences in the membrane fragmentation. The freezing process may have weakened the plasma membranes in such a way that subjection to polytron homogenization shattered the cells in a pattern which differed from that achieved with fresh tissue. It appears as if the BLM and BBM from frozen tissue form small, less dense membranes vesicles which have a greater propensity to remain in their respective supernatant phases, as indicated by the increased alkaline phosphatase activity in S_c and a shift in Na^+/K^+ATPase to P_2. The decrease in AP in P_2 reduced the amount of AP applied to the density gradient. Thus, enhancing the enrichment of Na^+/K^+ATPase relative to AP in the resulting bands. Similar enhancement of isolation procedures upon freezing were reported by Crooker and Clark (1986). The favorable effects of freezing tissue prior to processing led to the adoption of freezing as standard procedure before BLM and BBM were isolated.

Stage 3 Once the procedure for the isolation of a membrane fraction suitable for evaluation of the transport capabilities of the BLM was developed, work was begun to develope a procedure for isolation of a fraction suitable for evaluation of BBM transport properties. To

minimize treatment differences between the preparation of BLM and BBM and to maximize the utilization of isolated mucosal tissue an attempt was made to utilize the BLM differential centrifugation scheme to isolate enriched brush border membranes. The logical starting point for BBM isolation was the supernatant fraction (S_c) from the wash steps since this fraction contained 80.0% of the total alkaline phosphatase activity (table 5).

Since a majority of the internal membranes and a large fraction of the Na^+/K^+ATPase activity remained in the pellets of the wash steps, isolation of BBMV was attempted with a single high speed centrifugation step. The centrifugation of S_c at 31,000 x g resulted in a pellet (P_d)(figure 10) enriched 3.6-fold in alkaline phosphatase activity, unfortunately this fraction was cross contaminated with a 3.7-fold enrichment in Na^+/K^+ATPase. This pellet was applied to a density gradient consisting of 31, 34, and 38% sucrose in an attempt to separate the alkaline phosphatase and Na^+/K^+ATPase activities of the fraction. As expected, from BLM isolation experiments, the greatest enrichment of BBM occurred at the 31% sucrose interface. The enrichment of alkaline phosphatase and Na^+/K^+ATPase activities were 7.7-fold and 4.2-fold, respectively

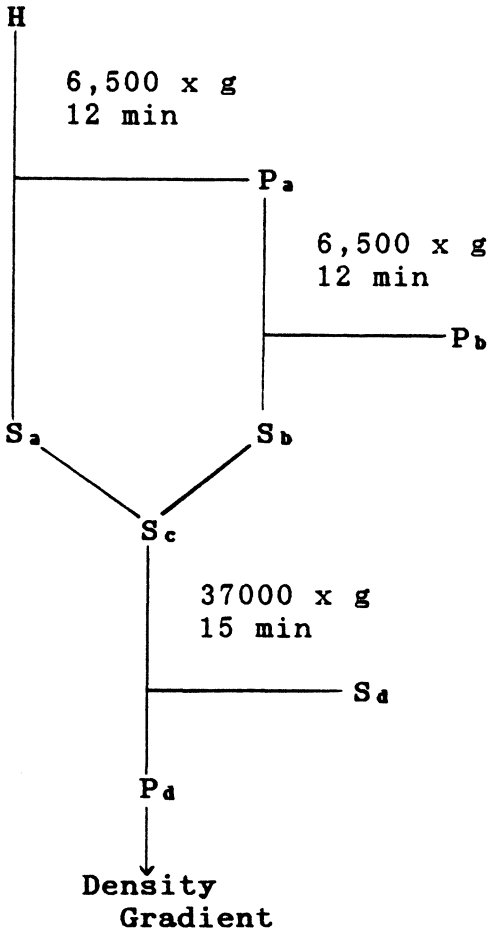

FIGURE 10. Stage 3 differential centrifugation scheme.

TABLE 5. ENRICHMENT AND PERCENT OF INITIAL ACTIVITY OF ALKALINE PHOSPHATASE, NA^+/K^+ ATPASE AND PROTEIN LEVEL OF STAGE 3 DIFFERENTIAL CENTRIFUGATION STEPS.

Differential[a] fraction	Component[b]	Percent of[c] initial	Enrichment[d]
	Protein	67.5	---
S_c	Na^+/K^+ATPase	54.0	.8
	AP	80.0	1.2
	Protein	63.5	---
S_a	Na^+/K^+ATPase	22.0	.3
	AP	75.5	1.2
	Protein	8.5	---
S_b	Na^+/K^+ATPase	26.0	3.0
	AP	8.5	1.0
	Protein	9.5	---
P_d	Na^+/K^+ATPase	35.6	3.7
	AP	34.0	3.6
	Protein	59.0	---
S_d	Na^+/K^+ATPase	13.4	.2
	AP	42.0	.7

[a] Membrane fractions resulting from differential centrifugation: Sc-combination of the supernatants S_a, which resulted from a 6500 x g centrifugation of the homogenate, and S_b, which resulted from a 6500 x g centrifugation of the resuspended pellet from the first wash step; P_d-resulted from 31000 x g centrifugation of S_c; S_d-resulting supernatant from P_d.

[b] Protein, Na+/K+ATPase, and alkaline phosphatase components of the membrane fractions were analyzed so that enrichment of BLM and BBM could be monitored.

[c] Percent of initial determined by dividing the total amount or activity of each component found in a fraction by the total amount or activity of that component found in the initial homogenate.

[d] Enrichment represents specific activity of component in a membrane fraction divided by specific activity of that component in the homogenate.

(table 6). While the enrichment of BBM was adequate for transport studies the cross contamination with BLM decreases the desirability of this fraction for characterizing the transport properties of the brush border membrane.

Upon evaluation of the individual wash steps, it was found that the first wash step yielded a supernatant containing 75.5% of the total alkaline phosphatase activity and 22.0% of the total Na^+/K^+ATPase activity, while the second wash yielded a supernatant containing 8.5% of the total alkaline phosphatase and 26.0% of the total Na^+/K^+ATPase activity (table 5). The negative contribution of the second wash step was evident since more BLM than BBM were contained in this fraction, therefore, diluting the enrichment attained in the initial wash step. The increase in Na^+/K^+ATPase activity of the second wash step may be explained by the dilution of Mg^{++} which occurred when the pellet from the first wash step was resuspended with transport buffer, thus allowing the BLM which were precipitated due to divalent cation cross-linking to become free in solution.

Stage 4. The findings of the stage 3 experiments prompted the development of a single wash step procedure.

Table 6. ENRICHMENT OF Na^+/K^+ATPASE AND ALKALINE PHOSPHATASE IN THE MEMBRANE FRACTIONS OBTAINED FOLLOWING SUCROSE DENSITY GRADIENT CENTRIFUGATION OF PELLET D FROM STAGE 3 PREPARATION.

Percentage[a] sucrose	Enrichment[b]		Ratio[c]
	Na^+/K^+ATPase	AP	AP:Na^+/K^+ATPase
31	4.2	7.7	1.8
34	7.6	5.0	.7
38	1.6	4.3	2.7
Pellet	3.4	5.3	1.9

[a] Percentage of sucrose refers to the concentration of sucrose at each step of the gradient, pellet refers to membranes which migrated to the bottom of the gradient.

[b] Enrichment represents specific activity of an enzyme in a membrane fraction divided by specific activity of that enzyme in the homogenate.

[c] Enrichment of alkaline phosphatase in a membrane fraction divided by enrichment of Na^+/K^+ATPase in the same fraction.

In this procedure the homogenate was centrifuged at 9,000 x g rather than at the 6,000 x g centrifugation used in the previous study in an effort to remove more of the Na^+/K^+ATPase activity from the supernatant (figure 11). The supernatant, Sa, resulting from this wash step was then centrifuged at 31,000 x g to yield a pellet, P_b, which was enriched 3.8-fold in alkaline phosphatase and 2.9-fold in Na^+/K^+ATPase (table 7). Applying P_b to the same density gradient used in the two wash step procedure yielded a membrane fraction at the 31% sucrose interface which was enriched 8.8-fold in alkaline phosphatase activity and 4.7-fold in Na^+/K^+ATPase activity (table 8). This was an improvement over the two-step wash procedure but the level of Na^+/K^+ATPase enrichment was such as to make this fraction unsuitable for characterization of the transport properties of BBM vesicles.

Stage 5. The cross-contamination with Na^+/K^+ATPase encountered in the stage 4 experiments lead to the use of a double Mg^{++} precipitation procedure which was a modification of Orsenigo et al. (1984) technique which used divalent precipitation to remove BLM from BBM after internal membranes had been removed by differential centrifugation. In this procedure the pellet formed from

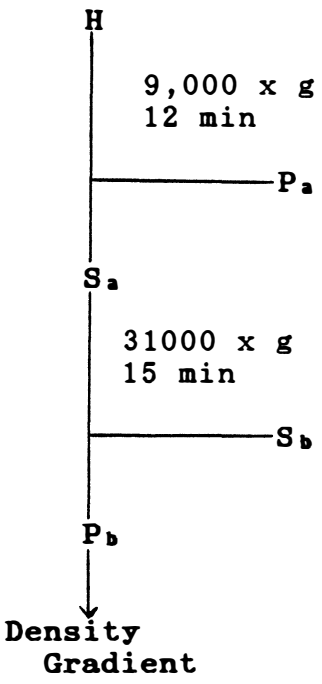

FIGURE 11. Stage 4 differential centrifugation scheme

TABLE 7. ENRICHMENT AND PERCENT OF INITIAL ACTIVITY OF ALKALINE PHOSPHATASE, NA^+/K^+ ATPASE AND PROTEIN LEVEL OF STAGE 4 DIFFERENTIAL CENTRIFUGATION STEPS.

Differential[a] fraction	Component[b]	Percent of[c] initial	Enrichment[d]
	Protein	61.3	---
S_a	Na^+/K^+ATPase	32.0	.5
	AP	67.0	1.1
	Protein	7.7	---
P_b	Na^+/K^+ATPase	22.0	2.9
	AP	29.3	3.8
	Protein	53.7	---
S_b	Na^+/K^+ATPase	5.1	.1
	AP	35.7	.7

[a] Membrane fractions resulting from differential centrifugation: S_a-resulted from a 9000 x g centrifugation of the homogenate, P_b-resulted from 31000 x g centrifugation of S_a; S_b-was resulting supernatant from P_b.

[b] Protein, Na+/K+ATPase, and alkaline phosphatase components of the membrane fractions were analyzed so that enrichment of BLM and BBM could be monitored.

[c] Percent of initial determined by dividing the total amount or activity of each component found in a fraction by the total amount or activity of that component found in the initial homogenate.

[d] Enrichment represents specific activity of component in a membrane fraction divided by specific activity of that component in the homogenate.

Table 8 ENRICHMENT OF Na^+/K^+ATPASE AND ALKALINE PHOSPHATASE IN THE MEMBRANE FRACTIONS OBTAINED FOLLOWING SUCROSE DENSITY GRADIENT CENTRIFUGATION OF PELLET B FROM STAGE 4 PREPARATION.

Percentage[a] sucrose	Enrichment[b]		Ratio[c]
	Na^+/K^+ATPase	AP	AP:Na^+/K^+ATPase.
31	4.7	8.8	1.9
34	6.2	5.3	.9
38	3.8	4.4	1.2
Pellet	3.5	2.8	.8

[a] Percentage of sucrose refers to the concentration of sucrose at each step of the gradient, pellet refers to membranes which migrated to the bottom of the gradient.

[b] Enrichment represents specific activity of an enzyme in a membrane fraction divided by specific activity of that enzyme in the homogenate.

[c] Enrichment of alkaline phosphatase in a membrane fraction divided by enrichment of Na^+/K^+ATPase in the same fraction.

a 31,000 x g centrifugation of the supernatant from the wash step was resuspended in mannitol-succinate buffer (Mg^{++} buffer) with 12 strokes of a tight fitting teflon glass homogenizer and incubated for 30 min. This step allows the Mg^{++} cation to form cross links between BLM thus facilitating their precipitation while allowing the BBM to remain suspended. Following the incubation period, the P_b fraction was centrifuged at 8,700 x g for 12 min to remove BLM (figure 12). The supernatant was then centrifuged at 31,000 x g for 15 min to form a pellet (P_d) which was enriched 4.8-fold in alkaline phosphatase activity and 2.5-fold in Na^+/K^+ATPase activity (table 9). The enrichment of the double precipitation procedure achieved by differential centrifugation was superior to either of the two previous techniques and showed the most promise for further purification by sucrose density gradient centrifugation.

The P_d fraction was applied to a sucrose gradient consisting of 28, 31, and 34% sucrose. The 28% sucrose layer was a modification of the previous sucrose gradients in an attempt to further separate the lower density BBM from the higher density BLM. The membrane fraction from the 28% sucrose band was enriched 10.1-fold in alkaline phosphatase activity and 2.1-fold in

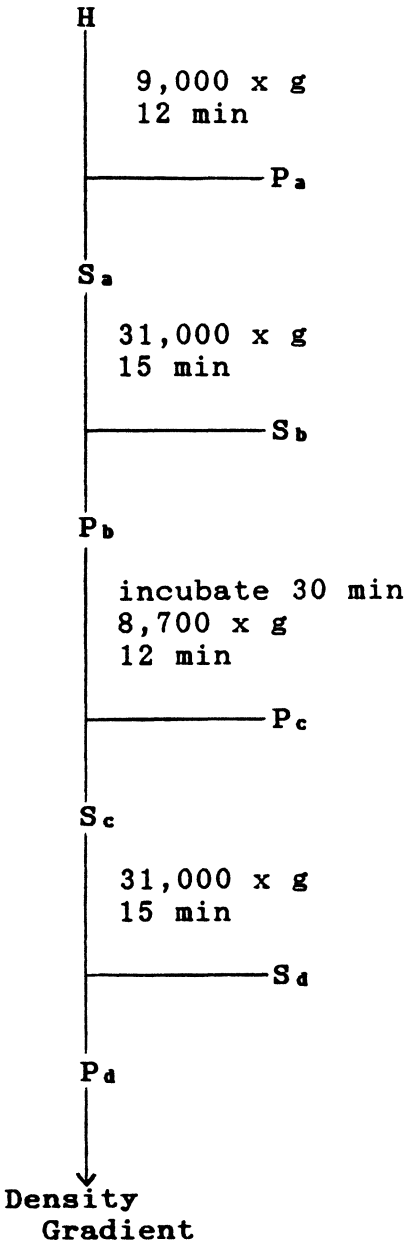

FIGURE 12. Stage 5 differential centrifugation scheme.

TABLE 9. ENRICHMENT AND PERCENT OF INITIAL ACTIVITY OF ALKALINE PHOSPHATASE, NA^+/K^+ ATPASE AND PROTEIN LEVEL OF STAGE 5 DIFFERENTIAL CENTRIFUGATION STEPS.

Differential[a] fraction	Component[b]	Percent of[c] initial	Enrichment[d]
	Protein	60.9	---
S_a	Na^+/K^+ATPase	32.1	.5
	AP	68.5	1.1
	Protein	53.6	---
S_b	Na^+/K^+ATPase	16.2	.3
	AP	38.5	.7
	Protein	2.8	---
P_c	Na^+/K^+ATPase	4.4	1.6
	AP	10.3	2.7
	Protein	2.1	---
P_d	Na^+/K^+ATPase	5.2	2.5
	AP	10.1	4.8
	Protein	4.1	---
S_d	Na^+/K^+ATPase	1.7	.4
	AP	4.9	1.2

[a] Membrane fractions resulting from differential centrifugation: S_a-resulted from a 9000 x g centrifugation of the homogenate, S_b-supernatant resulting from 31000 x g centrifugation of S_a; P_c-resulting pellet from a 8700 x g centrifugation of a resuspended P_b, pellet resulting from the 31000 x g centrifugation of S_a. P_d and S_d were the resulting pellet and supernatant, respectively, from a 31000 x g centrifugation of S_c, supernatant from P_c.

[b] Protein, Na^+/K^+ATPase, and alkaline phosphatase components of the membrane fractions were analyzed so that enrichment of BLM and BBM could be monitored.

[c] Percent of initial determined by dividing the total amount or activity of each component found in a fraction by the total amount or activity of that component found in the initial homogenate.

[d] Enrichment represents specific activity of component in a membrane fraction divided by specific activity of that component in the homogenate.

Na^+/K^+ATPase (table 10). The enzyme activities at the 31% sucrose band were similar for all three BBM isolation techniques suggesting that the membrane vesicles at this density were hybrids of BBM and BLM, since removal of a proportion of the BBM with a lower density sucrose band did little to affect the enrichment or the ratio of alkaline phosphatase to Na^+/K^+ATPase at the 31% sucrose interface. The BBM enrichment of the membrane fraction obtained from the 28% sucrose interface of the gradient was comparable to the enrichment values of BBM used in the literature for evaluation of BBM transport properties (Im et al., 1980; Ling et al., 1981; and Ganapathy and Leibaach, 1982).

The drawback to the procedure used to isolate BBM from the homogenized intestinal enterocyte was that the isolation was achieved by a differential centrifugation different from the one used to isolate BLM. Although it has of yet not been investigated completely, it appears the modification of the differential centrifugation scheme to a one wash step procedure would not be detrimental to the further fractionation of the sedimented membranes from the wash step to form a pellet enriched in Na^+/K^+ATPase. When the pellet from the one wash step procedure was subjected to the differential

Table 10. ENRICHMENT OF Na^+/K^+ATPASE AND ALKALINE PHOSPHATASE IN THE MEMBRANE FRACTIONS OBTAINED FOLLOWING SUCROSE DENSITY GRADIENT CENTRIFUGATION OF PELLET D FROM STAGE 5 PREPARATION.

Percentage[a] sucrose	Enrichment[b]		Ratio[c]
	Na^+/K^+ATPase	AP	AP:Na^+/K^+ATPase
28	2.1	10.1	4.8
31	5.6	7.3	1.3
34	3.5	6.5	1.9
Pellet	3.0	---	---

a Percentage of sucrose refers to the concentration of sucrose at each step of the gradient, pellet refers to membranes which migrated to the bottom of the gradient.

b Enrichment represents specific activity of an enzyme in a membrane fraction divided by specific activity of that enzyme in the homogenate.

c Enrichment of alkaline phosphatase in a membrane fraction divided by enrichment of Na^+/K^+ATPase in the same fraction.

centrifugation used in the two step wash procedure a final pellet (P_3) was enriched 3.2-fold in Na^+/K^+ATPase and 1.7-fold in alkaline phosphatase. This was comparable to the enrichment of 3.3-fold in Na^+/K^+ATPase and 1.1-fold in alkaline phosphatase found in the membrane fraction obtained by the two wash step procedure. Therefore, theoretically, application of P_3 from the one wash step procedure to the same density gradient used in the two wash step procedure would yield bands with Na^+/K^+ATPase enrichments similar to the two wash step procedure. The enrichments obtained using P_2 from the one wash step procedure may even exceed that of the two wash step procedure since this P_2 fraction contained 29.5% of the total Na^+/K^+ATPase activity, while the corresponding pellet in the two wash step procedure contained only 19.6% of the total Na^+/K^+ATPase activity. In any case enrichment of BBM and BLM suitable for transport characterization can be achieved, be it by separate differential schemes, even if density gradient centrifugation proves unable to enrich membranes of P_3 to a degree suitable for characterization of BLM transport.

Transport: The enrichment of enzyme markers is not a guarantee that the membrane fraction is suitable for measuring transport properties of the isolated membrane. The membrane must also be of vesicular nature as opposed to globular or membrane sheets. The vesicular nature of a membrane fraction can be demonstrated by showing that substrate transport is occurring into an osmotically active space. This is accomplished by monitoring the substrate uptake versus increasing osmolarity of the incubation buffer. The presumption is that the equilibrium uptake will be proportional to the intravesicular space and the intravesicular space will be determined by the osmolarity of the incubation buffer (Munck, 1966). By extrapolating the effects of osmolarity on uptake to infinite osmolarity, the level of substrate binding to the surface of the membrane vesicles can be determined (Faust et al. 1968; Eicholz et al. 1969). Since at infinite osmolarity there would be no intravesicular space, therefore all the substrate present would be due to surface binding.

The vesicular nature of the isolated BLM fraction was determined by measuring equilibrium D-glucose uptake versus increasing media osmolarity (figure 13). There was a linear decrease for both Na^+-dependent and

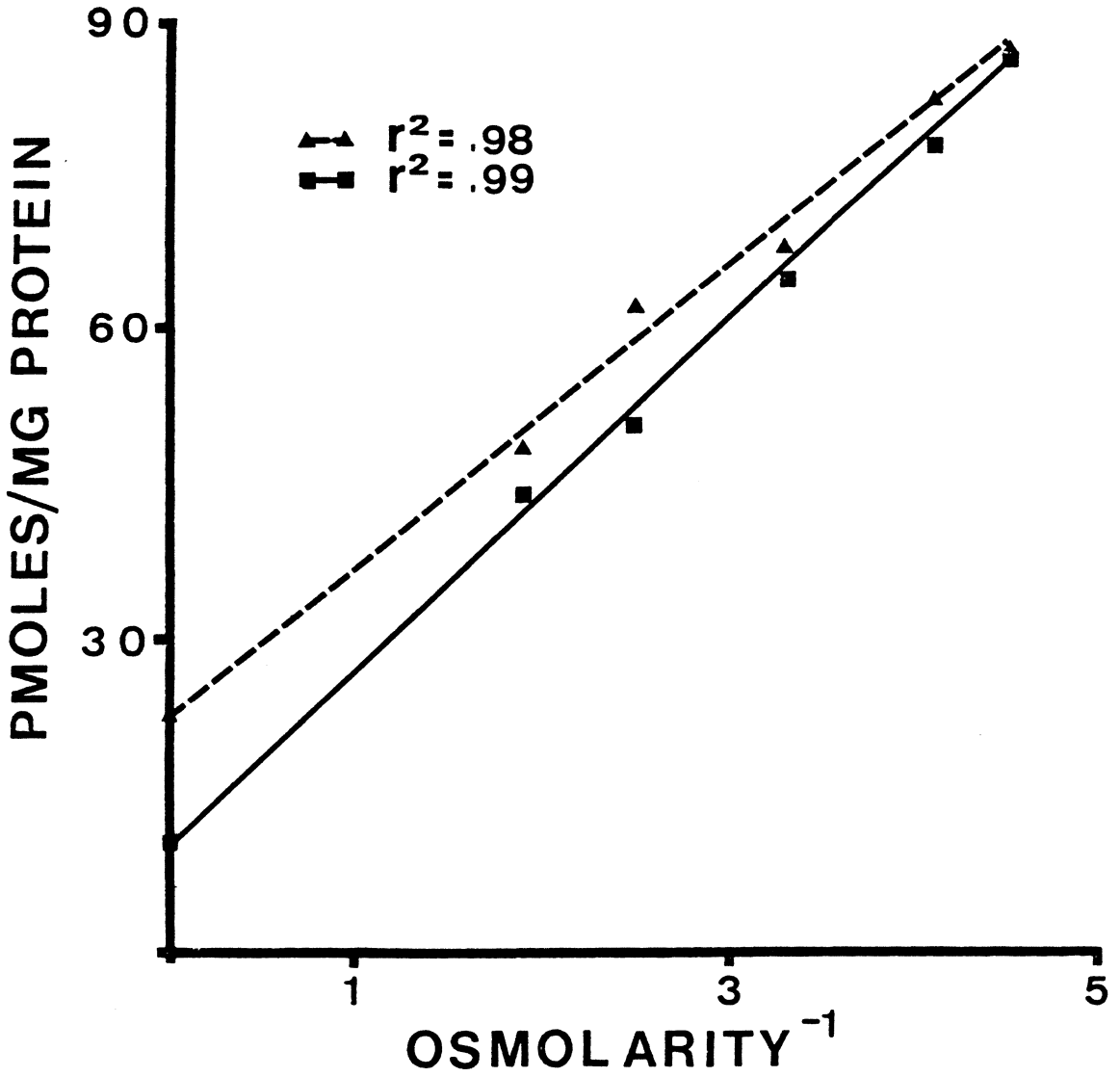

Figure 13. Effect of incubation buffer osmolarity on equilibrium (60 min) net flux of U-(^{14}C)-D-glucose (130 uM) in the presence of 100 mM NaSCN (■—■) or 100 mM KSCN (▲--▲). Data points are means of triplicate determinations.

Na^+independent D-glucose uptake with increasing osmolarity media. The Na^+-independent equilibrium concentration of D-glucose had a tendency to be higher than the Na^+-dependent D-glucose equilibrium values. An explanation for these higher values was found upon extrapolation to infinite osmolarity and the determination that the Na^+-independent measurements bound 19 pmoles of D-glucose per mg of protein while the Na+-dependent measurements bound 12 pmoles of D-glucose per mg of protein. This increased surface binding accounts for the tendency of the Na^+-independent measurements to have a higher equilibrium concentration of D-glucose than the Na^+-dependent measurements.

The reason for the increased surface binding in the Na^+-independent measurements is not known but one can postulate that it may be due to the presence of a Na^+-dependent transport system in the BLM. If this is the case the D-glucose molecules would bind to the Na^+-dependent transporter proteins on the vesicles surface but would not be transported due to the absence to Na^+ in the incubation media. In the Na^+-dependent measurement system this source of surface binding would not be present since Na^+ is contained in the incubation media.

In a preliminary study to evaluate the amino acid transport characteristics of the bovine BLM, a time course uptake of L-alanine was determined. The L-alanine transport of BLM showed no Na^+ overshoot but did have a Na^+-stimulation. The equilibrium L-alanine concentration was reached within 4 min in the presence of Na^+. The ratio of Na^+-dependent to Na^+-independent uptake at 4 min was 2.1 to 1 (figure 14). These results are consistent with the work of Mirchef et al. (1980) who demonstrated the presence of a Na^+-dependent L-alanine transport system in the BLM of the rat. Both the Na^+-dependent and the Na^+-independent systems resulted in the same L-alanine concentration at equilibrium, thus indicating there where no osmotic differences between the incubation buffers. These findings indicate the presence of a Na^+-dependent amino acid transporter in the BLM of the bovine since sodium stimulation is accepted as evidence for an active transport process (Hopfer, 1977).

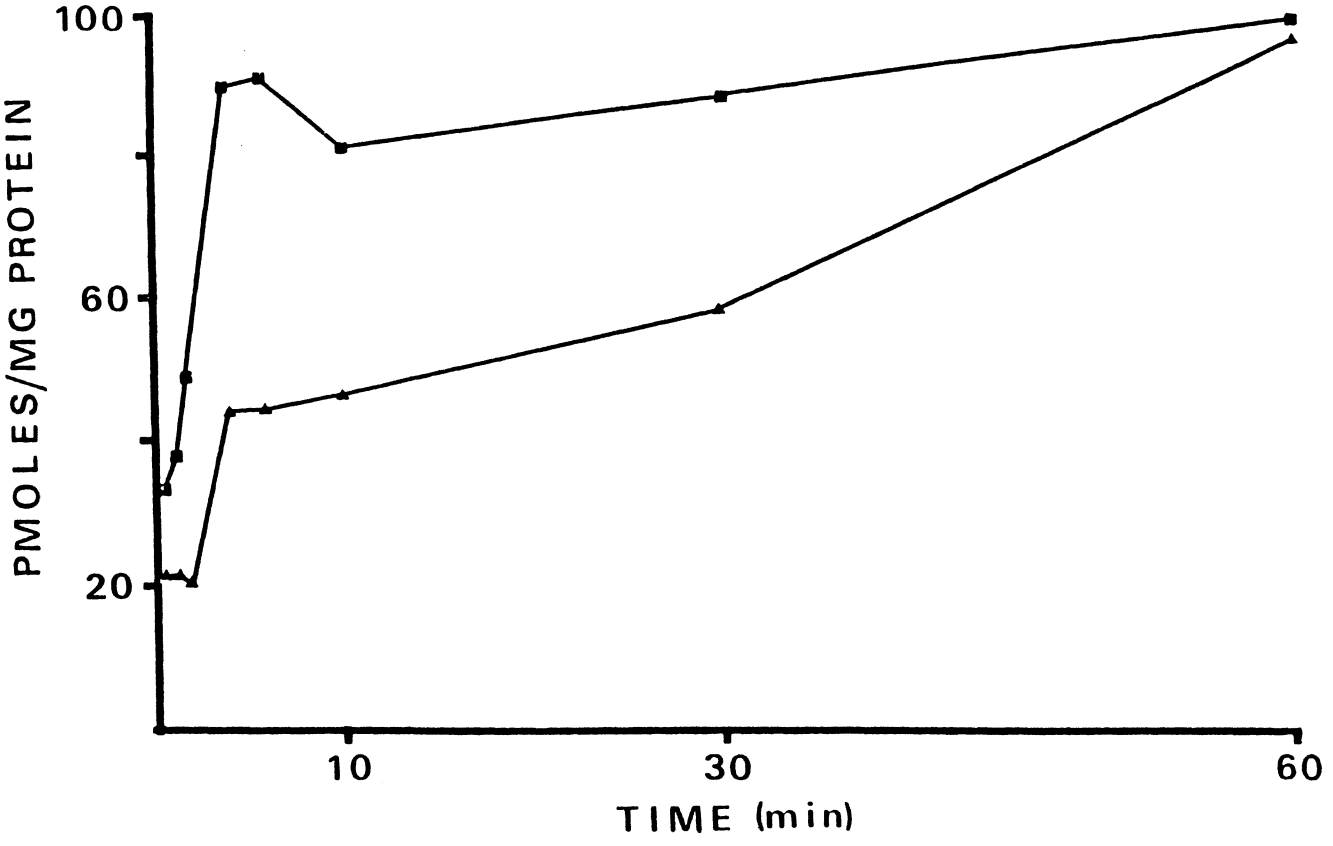

Figure 14. Time course uptake of U-(^{14}C)-L-alanine by B_2 of BLM isolation scheme in the presence of 100 mM NaSCN (■—■) or 100 mM KSCN (▲—▲).

SUMMARY

Brush border and basolateral membrane vesicles were isolated by subjecting homogenized mucosal cells from bovine small intestine to a divalent cation aggregation followed by a series of differential and density gradient centrifugations. Membrane marker enzyme assays were used to monitor the effectiveness of the fractionation procedure. Enrichments were determined by comparing the enzyme specific activities of the membrane fractions to the homogenate. Sodium-potassium adenosine triphosphatase and alkaline phosphatase served as the enzyme markers for the basolateral and bursh border membranes, respectively. Basolateral membrane vesicles enriched 11.1 fold were isolated from the interface of the 31 and 34 % sucrose bands of a discontinous sucrose gradient. Brush border membranes enriched 10.1 fold were isolated from the surface of the 28 % sucrose band of a discontinous sucrose gradient. The use of frozen rather than fresh mucosal tissue in the isolation procedures was found to enhance the purification of basolateral and brush border membrane fractions.

The transport capabilites of vesicles were demonstrated by incubating vesicles with radiolabeled

substrate, then separating the vesicles and transported substrate from the incubation buffer by filtration. Substrate uptakes were quantified by liquid scintillation counting. Basolateral membrane vesicles were observed to accumulate substrate into an osmotically active space and to have Na^+-dependent alanine transport capabilities. The use of basolateral and brush border membrane vesicles as tools to investigate nutrient uptake allows the investigator to manipulate both the extravesicular and intravesicular environments, thus making possible the evaluation of the complex interactions which are involved in nutrient transport mechanisms.

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APPENDIX

APPENDIX A

ISOLATION BUFFERS

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

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
MnCl2	197.9	.1	.01	.02	.04

pH adjusted to 7.4 with HCl

APPENDIX A
(cont.)

Mannitol-Transport Buffer

Compound	MW	mM	Grams .5 L	Grams 1 L	Grams 2 L
Mannitol	182.2	100	9.1	18.2	36.4
Hepes	238.3	20	2.4	4.8	9.5
MgCl2	203.1	2	.2	.4	.8

pH adjusted to 7.4 with 1M Tris

Gradient Buffer [2X]

Compound	MW	mM	Grams .5 L	Grams 1 L	Grams 2 L
Hepes	238.3	4	.95	1.91	3.82
MgCl2	203.1	4	.41	.82	1.63

pH adjusted to 7.4 with 1M Tris

Sucrose Gradient Solutions

Percent sucrose	Sucrose g	ml [2x] Gradient buffer	ml Total volume[1]
29	325.4	50	100
31	350.9	50	100
34	389.7	50	100
38	443.2	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

APPENDIX B

Na^+/K^+ ATPase ASSAY

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	.81	4.05	8.10

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.

APPENDIX C

ASSAY FOR INORGANIC PHOSPHOROUS

Ammonium Molybdate Solution

Compound	MW	[w/v]	Grams[1] .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

Stock Triton-X Solution

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

[1] brought to volume with double distilled water

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.

APPENDIX C
(cont.)

Calculations:

[ATPase] = (ug P_i /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

APPENDIX D

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)

APPENDIX E

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 F

TRANSPORT ASSAYS

Transport Buffer

Compound	MW	mM	g/l	2*[ ] g/l	4*[ ] g/l
NaSCN	81.1	100	8.11	16.22	32.4
$MgCl_2$	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

[1]Either NaSCN or KSCN are used but not both in same buffer

pH adjusted to 7.4 with HCl

Osmolarity Buffers

mM Mannitol	g/l	2*[ ] g/dl	4*[ ] Stock 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

APPENDIX F
(cont.)

Stopping Solution

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

Decision and calculations evolved with typical transport experiment:

1. Determine the number of time points to be evaluated, i.e. 6.
2. Decide upon the reaction volume to be used per time point, i.e. 120 ul.
3. Total volume should contain one half a reaction volume more than is actually needed, i.e. 60 ul.
4. Total reaction volume = 6.5 x 120 ul = 780 ul
5. One half of total reaction volume will be transport buffer, i.e. 390 ul.
6. The 390 ul remaining is made up equally of water, isotope and substrate, and membranes, 130 ul each.
7. Calculation of subtrate concentration is a multistep procedure which must take into consideration the radiolabled substrate.
8. Decide upon final concentration of substrate, i.e. 100 umolar glucose .

APPENDIX F
(cont.)

9. Total umoles glucose needed = 100 umoles/l x 780 ul x $1/10^6$ul = .078 umoles glucose

10. Total grams glucose needed = .078 umoles x 182.2 ug/umole = 14.06 ug glucose

11. Concentration of working glucose solution = 14.06 ug/130ul = .108 ug/ul = .108 mg/ml.

12. Determine mg glucose provided by 25 uCi of labeled glucose, note this glucose is dried in a vial which will contain working glucose solution:

    mg labeled glucose = 25 uCi x mmol/275 mCi x mCi/1000 uCi x 180.2 mg/mmole = .0164

13. If total volume of working solution is 1 ml then the amount of cold glucose needed equals .0916 (.108-.0164).

14. Thus 1 ml of a cold glucose solution, .0916 grams/l, will be added to vial containing hot glucose. Addition of 130 ul of this solution to reaction volume will result in a final glucose concentration of 100 uMolar

15. In summary total reaction volume, 780 ul, will be made up of 390 ul transport buffer, 130 ul isotope and substrate, 130 ul water and 130 ul membranes (1-3 mg protein/ml).

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PREPARATION OF BRUSH BORDER AND BASOLATERAL MEMBRANE VESICLES FROM BOVINE INTESTINE FOR NUTRIENT UPTAKE STUDIES

by

Jonathan W. Wilson

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Animal Science

(ABSTRACT)

Brush border and basolateral membrane vesicles were isolated by subjecting homogenized mucosal cells from bovine small intestine to a divalent cation aggregation followed by a series of differential and density gradient centrifugations. Membrane marker enzyme assays were used to monitor the effectiveness of the fractionation procedure. Enrichments were determined by comparing the enzyme specific activities of the membrane fractions to the homogenate. Sodium-potassium adenosine triphosphatase and alkaline phosphatase served as the enzyme markers for the basolateral and bursh border membranes, respectively. Basolateral membrane vesicles enriched 11.1 fold were isolated from the interface of the 31 and 34 % sucrose bands of a discontinous sucrose gradient. Brush border membranes enriched 10.1 fold were isolated from the surface of the 28 % sucrose band of a discontinous

sucrose gradient. The use of frozen rather than fresh mucosal tissue in the isolation procedures was found to enhance the purification of basolateral and brush border membrane fractions.

The transport capabilites of vesicles were demonstrated by incubating vesicles with radiolabeled substrate, then separating the vesicles and transported substrate from the incubation buffer by filtration. Substrate uptakes were quantified by liquid scintillation counting. Basolateral membrane vesicles were observed to accumulate substrate into an osmotically active space and to have Na^+-dependent alanine transport capabilities. The use of basolateral and brush border membrane vesicles as tools to investigate nutrient uptake allows the investigator to manipulate both the extravesicular and intravesicular environments, thus making possible the evaluation of the complex interactions which are involved in nutrient transport mechanisms.