SIMULTANEOUS ISOLATION AND CHARACTERIZATION
OF BRUSH BORDER AND BASOLATERAL MEMBRANE VESICLES
FROM BOVINE SMALL INTESTINE

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ABSTRACT

Purified brush border and basolateral membranes were isolated from homogenized intestinal enterocytes of Holstein steers by divalent cation precipitation followed by differential and sucrose density gradient centrifugation. Alkaline phosphatase and Na/K adenosine triphosphatase served as marker enzymes for the brush border and basolateral membranes, respectively. The brush border and basolateral membrane fractions were enriched 5.1- and 10.1-fold, respectively, over the cellular homogenate. Electron micrographs, obtained with transmission electron microscopy, confirmed the vesicular nature of the membranes and revealed that basolateral membrane vesicles generally were smaller and more irregular in shape than brush border membrane vesicles. The vesicular nature of isolated membrane preparations was confirmed with osmotic activity experiments. Enrichment of brush border and basolateral membrane fractions compared to the initial homogenate and the vesicular configuration of both preparations indicate that the isolated brush border and basolateral membrane preparations were suitable models for evaluating nutrient transport properties of bovine small intestine. The number of transport experiments possible per animal using the membrane vesicle technique is many times more efficient than some other in vitro techniques (i.e., intestinal rings or everted sacs).

(Key Words: Brush Border, Basolateral, Bovidae, Vesicles, Small Intestine, Enterocytes.)


Introduction

The basis for the majority of information on the mechanisms of nutrient transport has arisen through the use of in vitro techniques. These in vitro techniques have included intestinal rings, everted sacs, ligated intestinal segments and isolated mucosal cells (Fisher and Parsons, 1949; Agar et al., 1954; Wilson and Wiseman, 1959; Kimmich, 1975; Phillips et al., 1976). These techniques have limitations in that cellular metabolism is occurring and that the experimenter cannot regulate the intracellular substrate and electrolyte concentration gradients. The recent use of isolated membrane vesicles has overcome some of these problems and has given researchers the ability to characterize nutrient transport systems at both the luminal and serosal side of the mucosal lining.

Isolated brush border (BB) and basolateral (BL) membrane vesicles have been used to evaluate the transport properties of the small intestine and kidney of several nonruminant species (Douglas et al., 1972; Hopfer et al., 1973; Im et al., 1980; Ganapathy et al., 1981; Ling et al., 1981). Only recently have the transport properties of intestinal BB tissue of ruminant species been evaluated with the membrane vesicle technique (Kaunitz and Wright, 1984; Moe et al., 1985; Crooker and Clark, 1986). Isolation of intestinal BL membranes from ruminant species has not been reported to date. Characterization of

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the transport properties of both BB and BL membranes will aid our understanding of the mechanisms involved in transport of organic solutes across intestinal epithelia, from the lumen to the circulation.

These experiments were conducted to develop a simple and rapid procedure for the isolation of BL membrane vesicles in conjunction with BB membrane vesicles utilizing the same starting tissue.

Materials and Methods

Animals. Holstein steers weighing an average of 400 kg were used as the tissue donors for the experiments. Steers were fed to gain .9 kg/d. The diet (as-fed basis) consisted of 30% ground corn, 30% orchardgrass hay, 13.3% soybean meal, 5% molasses, .42% defluorinated rock phosphate, .78% limestone and .5% trace mineral salt and was available until 12 h before slaughter. A total of 14 steers were used to develop various aspects of the vesicle isolation procedures.

Tissue Preparation. Steers were mechanically stunned and then were killed by exsanguination. Viscera were removed from steers within about 12 min of stunning. The small intestine was cut free from the mesentery beginning 2 m distal of the pyloric valve and proceeding distally to 2 m proximal of the ileocecal junction. The intestine was cut into 1-m segments and flushed free of digesta with buffer containing 300 mM mannitol and 12 mM tris base; pH was adjusted to 7.4 with HCl (mannitol buffer).

Tissue was maintained at 4°C throughout the procedure unless otherwise specified. The intestinal segments then were everted and incubated in buffer containing 1 mg hyaluronidase/ml, 1 mg bovine serum albumin/ml, 120 mM NaCl, 20 mM tris base, 1 mM MgCl2 and 3 mM K2HPO4 (pH was adjusted to 7.4 with HCl) for 20 min at 37°C. Following incubation, the intestinal segments were placed in a porcelain pan seated on a bed of crushed ice. The mucosal lining then was harvested by scraping the intestinal segments with a glass slide. The use of hyaluronidase incubation with the isolated intestinal segments was implemented to separate mucus from the luminal lining and to facilitate the harvesting of enterocytes (A. J. Moe, personal communication). Residual hyaluronidase was removed from enterocytes by twice resuspending cells in equal volumes of mannitol buffer and centrifuging at 4,500 × g for 12 min. All centrifugations were performed at 4°C in refrigerated centrifuges. Isolated enterocytes were divided into 3- to 4-g aliquots, placed in whirl-pac bags and flash-frozen in liquid N2. Enterocytes were stored at -80°C in a ultralow temperature freezer for future use.

Marker Enzymes. Alkaline phosphatase (EC 3.1.3.1) was the marker for BB membranes. Activity of alkaline phosphatase was determined with an enzyme assay kit. Activity was expressed on a per milligram of protein basis.

Sodium-potassium adenosine triphosphatase (EC 3.6.1.3) (Na/K ATPase) was the marker for BL membranes. The assay used to measure Na/K ATPase activity was the procedure of Pugita et al. (1971). Liberated orthophosphate concentration was determined spectrophotometrically by the method of Eibel and Lands (1969). All samples were analyzed in triplicate. The activity of Na/K ATPase was obtained by subtracting ouabain-sensitive ATPase activity from total ATPase activity, which was measured in the absence of ouabain. Activity of Na/K ATPase was expressed on a microgram of orthophosphate per milligram of protein basis. Protein was assayed using a Coomassie Blue reagent (Bradford, 1976).

Tissue Fractionation. The enterocytes were suspended at a concentration of 3 g of tissue per 24 ml of buffer containing 5 mM MgCl2, 150 mM mannitol, 10 mM tris base, 30 mM succinate, 5 mM potassium phosphate and .1 mM MnCl2; pH was adjusted to 7.4 with NaOH (mannitol-succinate buffer) and homogenized with a polytron, equipped with a 20-mm-diameter probe, for 15 s at 13,200 rpm. For each preparation, 36 g of mucosal tissue were used. The 36 g of tissue was a composite of 12 g of tissue from three different steers. The BB and BL membranes then were isolated by differential and density gradient centrifugations (Figure 1). The homogenate was incubated for 30 min with gentle stirring to allow Mg++ aggregation of internal (i.e., endoplasmic reticulum, lysosomes and mitochondria) and BL membranes (Schmitz et al., 1975; Kessler et al., 1978). After incubation, the homogenate was centrifuged at 8,700 × g for 12 min. Through the use of marker enzymes, the resulting pellet (P2) was found to contain a majority of the BL mem-
Figure 1. Scheme for the isolation of bovine brush border and basolateral membrane vesicles. S, P and B correspond to supernatant fluids, pellets and bands, respectively. The letter and number subscripts differentiated membrane fractions of the brush border and basolateral membrane isolation schemes, respectively.

Brush border membranes, whereas BB membranes remained primarily in the supernatant fluid (S\textsubscript{a}). Brush border membranes were enriched further by applying the double precipitation technique of Orsenigo et al. (1985). This technique consisted of first harvesting BB membranes from S\textsubscript{a} by centrifuging at 3,100 × g for 15 min. The resulting pellet (P\textsubscript{b}) then was resuspended in mannitol-succinate buffer with 12 strokes of a teflon-glass homogenizer and was incubated for 30 min with mild agitation. This second Mg\textsuperscript{2+} precipitation facilitated the removal of BL membrane contamination. The suspension was centrifuged at 8,700 × g for 12 min, yielding the BL membrane fraction in the pellet (P\textsubscript{c}).

The BB membrane fraction (P\textsubscript{d}) was resuspended in mannitol-transport buffer (to a protein concentration of 2 to 3 mg protein/ml) and applied to a sucrose gradient of 27% and 31% sucrose (wt/wt). Sucrose solutions were prepared with buffer containing 4 mM MgCl\textsubscript{2} and 4 mM HEPES (pH 7.4, adjusted with NH\textsubscript{4}OH). Approximately 5 ml of membrane suspension were applied to density gradients made up of 3.5 ml 27% sucrose solution layered on 3.5 ml 31% sucrose solution and centrifuged at 105,000 × g for 90 min in an IEC model M-60 ultracentrifuge. The resulting bands then were collected by aspirating the membrane band and underlying sucrose layer. Bands were placed either in cryovials and frozen in liquid N\textsubscript{2} or diluted to 40 ml with mannitol-transport buffer and centrifuged at 105,000 × g for 60 min. The resulting washed pellets were resuspended in mannitol transport buffer and used for either marker enzyme analysis or transport experiments. The membranes originally frozen in liquid N\textsubscript{2} were stored at -80°C in an ultralow temperature freezer for future transport experiments.

Transport Assay. Transport experiments were conducted with the micro-filtration techniques of Murer et al. (1974) and Kimmich (1975). Prior to the start of the transport assay, two to three cryovials of sucrose-membrane suspension were removed from -80°C storage, thawed in warm tap water and then suspended in 10 volumes of mannitol-transport buffer and centrifuged for 60 min at 105,000 × g to wash sucrose from the membranes. The pellet was resuspended in a volume of mannitol-transport buffer that yielded 2 to 3 mg protein/ml.

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\textsuperscript{9}Kontes Scientific Glassware, Vineland, NJ.
\textsuperscript{10}International Equipment Company, Needham Heights, MA.
\textsuperscript{11}Nunc cryovials, Vangard International Inc., Neptune, NJ.
The composition of the intravesicular space was a function of the buffer used for the resuspension of the membranes. For the purposes of the present experiments, a Na-free buffer was required so that a Na gradient could be formed when the vesicles were added to the reaction mixture containing Na. Mannitol-transport buffer was a suitable buffer for this purpose.

Transport was initiated by the addition of 70 μl of transport buffer and 105 μl of methionine solution to the vessel and incubating at 37°C. Transport was initiated by the addition of 70 μl of membrane solution to the reaction vessel. The transport buffer was prepared so that the final methionine concentration would be 1 mM, including 2.5 μCi of L-[35S]-methionine13 (specific activity 1,151 Ci/mmol) per reaction vessel as the radiotracer.

Reactions were terminated after 60 min by removing 100-μl aliquots of reaction mixture, placing it on a .45-μm nitrocellulose filter14 and applying vacuum. Filters then were washed with three 5-ml aliquots of ice-cold 150 mM KCl solution. Three 100-μl aliquots were filtered for each reaction vessel with four reaction vessels per treatment. The filters were air-dried and placed in 20-ml scintillation vials and mixed with 9 ml of Ecoscint scintillation fluid15. Radioactivity was determined by liquid scintillation counting16. Nonspecific radioactivity retention on the membrane filters was determined by applying 30 pmol of substrate retention by vesicles.

Microscopy. Both fresh and frozen mucosal scrapings were evaluated with a differential interference contrast microscope17. After obtaining a sample of fresh mucosal scrapings, a subsample was placed in a whirl pac bag and flash-frozen in liquid N2. Frozen tissue was thawed at room temperature. Wet mounts of tissue from fresh and frozen origin were fitted with coverslips and placed under an oil immersion lens for evaluation. Total magnification was 1,250.

Electron Microscopy. Brush border and BL membrane vesicle preparations that had been stored at -80°C were separated from sucrose solution and resuspended in mannitol transport buffer as previously described. The suspension was centrifuged at 31,000 x g for 15 min. The resulting pellet was fixed in a mixture of 5.0% glutaraldehyde, 3.0% formaldehyde and 2.5% picric acid, buffered with 1 M sodium cacodylate at pH 7.4. It was washed in buffer and post-fixed for 1 h in 1.0% osmium tetroxide in the same vehicle. The post-fixed pellet was washed again then dehydrated in increasingly concentrated ethanol solutions and embedded in epoxy resin. Ultrathin sections were cut18 and stained with lead citrate (Reynolds, 1963) and uranyl acetate (Watson, 1958) and examined in a JEOL 100CX-II transmission electron microscope19.

Results and Discussion

The technique utilized in the simultaneous isolation of BB and BL membranes from the same initial homogenate relied on the differences in the surface charge densities that exist between BB and BL membranes (Schmitz et al., 1975; Kessler et al., 1978). The BB membranes have a surface charge density that allows for the incorporation of both of the positive charges of divalent cations, such as Mg++. This property minimizes the aggregation of BB membrane fragments that would result from Mg++ cross-linking. In contrast, the BL membrane surface charge density can accommodate only one of the positive charges of a divalent cation, thus facilitating cross-linking between two fragments and subsequent precipitation of BL membrane fragments. This difference in surface membrane properties allowed for separation of BL from BB membrane fractions by differential centrifugation (Figure 1). The resulting membrane fractions were purified further with the use of discontinuous sucrose gradients.

The use of a sucrose gradient consisting of 27 and 31% sucrose bands proved most suitable for
both BB and BL membrane isolation. Several other sucrose gradients were evaluated, including the higher-density sucrose gradients recommended by Moe et al. (1985) and Crooker and Clark (1986). Membrane vesicles were prepared with deliberate homogenization of the enterocytes with a polytron followed by a resuspension of each subsequent pellet with a teflon-glass homogenizer. This process resulted in the most repeatable preparation of vesicles. Different homogenization procedures are likely to result in vesicles of different sizes and, therefore, different buoyant densities, which would result in optimal separation on different sucrose gradients.

The application of the membrane fractions from differential centrifugation to the 27% and 31% sucrose gradient and centrifugation at 105,000 × g for 90 min resulted in the formation of two bands, one between the interface of the buffer and the 27% sucrose layer and another at the interface of the 27 and 31% sucrose layers. The third fraction formed was a pellet at the bottom of the 31% sucrose layer. When the BB membrane fraction (P2) was applied to the sucrose density gradient, the three membrane fractions from top to bottom were referred to as B1, B2 and B3; the fractions resulting from centrifugation of the BL membrane fraction (P3) were referred to as B1, B2 and B3.

The middle band (B2) of the BB sucrose density gradient was found to have the highest BB enrichment (5.1-fold for alkaline phosphatase; Table 1). This enrichment is similar to that achieved by Moe et al. (1985) but is slightly lower than that of Crooker and Clark (1986). The fraction at the interface between the buffer and the 27% sucrose layer (B1) of the BL membrane density gradient was the most suitable for use as a model for the BL membrane in transport studies. This fraction had a 10.1-fold Na/K ATPase enrichment and only a .5-fold alkaline phosphatase cross-contamination (Table 1). This high ratio of Na/K ATPase:alkaline phosphatase, 20.2:1, indicates that transport measurements by vesicles of B1 membrane fraction represent substrate uptake via BL membranes.

In addition to being enriched with the marker enzyme, the membranes also must be of a vesicular nature, as opposed to globular or membrane sheets. The vesicular nature of the membrane preparations was confirmed by both visual and experimental appraisals. Electron micrographs obtained with transmission electron microscopy showed that both BL and BB membrane preparations were of vesicular orientation (Figure 2). The BL membrane vesicles appeared to be consistently smaller and more irregular in shape and size than the BB membrane vesicles. This is similar to results obtained by Fugita et al. (1972) using isolated BB and BL membrane vesicles from rat intestines. The higher magnification of the membrane preparations revealed that the vesicles were formed by a well-preserved trilaminar plasma membrane. There appears to be a rougher outer texture (possibly microvilli) in the BB membrane preparation. Neither BB nor BL membrane preparations had any recognizable organelle contamination (i.e., mitochondria).

The vesicular nature of the membrane preparations also was tested experimentally by monitoring the osmotic responsiveness of the membrane preparation. The assumption made with this technique was that equilibrium uptake by vesicles would be proportional to the intravesicular space and that the intravesicular space will be determined by the osmolality of the incubation buffer (Munck, 1966). Therefore, by monitoring the equilibrium accumulation of a substrate at various osmolalities by a membrane preparation, one can determine the configuration of that membrane preparation. If uptake decreases with increasing osmolality of incubation
buffer, then the membrane preparation is considered to be at least partially vesicular. If uptake is not affected by osmolality, the preparation is considered to be nonvesicular.

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

Substrate accumulation was plotted against inverse osmolality to allow for the extrapolation of uptake data to infinite osmolality, Y-intercept, in order to predict the level of substrate that was present due to surface binding but not due to uptake into the vesicle. It is assumed that at infinite osmolality there would be no intravesicular space (Faust et al., 1968; Eicholz et al., 1969). The BB and BL membrane surface binding, after 60 min of incubation, was determined to be 276 and 222 pmoles of methionine/mg of protein,
fresh tissue. Freeze-fracturing makes the use of homogenization techniques, such as N₂-cavitation, impractical when frozen tissue is being used.

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

By using these membrane vesicles, investigators will be able to gain information leading to a better understanding of fundamental aspects of nutrient absorption and transport by ruminants. Furthermore, alterations in mucosal tissue function as a result of dietary or other factors might be explored. In the present paper, methionine was the substrate used to evaluate the quality of membrane preparation. Membrane vesicles prepared by the above procedures could be utilized to study the absorption and transport of numerous nutrients. By collecting enterocytes from discrete locations along the small intestine, all of the above could be evaluated with reference to site of intestinal activity. The diversity of applications for these membrane vesicles makes this attractive as an alternative method for evaluating nutrient absorption and transport.

**Literature Cited**


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