# ISOLATION OF HOG RENAL BRUSH BORDER MEMBRANE VESICLES WITH APPLICATION TO THE STUDY OF CADMIUM NEPHROTOXICITY

by

Theresa Rose Leverone

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APPROVED:

D.R. Bevan, Chairman

M. L. Failla

R. E. Ebel

J. L. Hess

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To Kevin McAteer

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# Introduction

Interest in toxin-induced nephropathy has increased with the development of sensitive methods for detecting subtle effects of toxins on renal structure and function and as man faces increasing exposure to toxic compounds. Toxicants, which can include drugs as well as environmental contaminants, are most often transported to their site of action via the blood stream. Although the kidneys comprise only 0.4% of the body weight, they receive about 25% of the resting output of the heart. Since they receive such a large percentage of the cardiac output, the kidneys are quickly exposed to peak concentrations of chemicals in the blood. The blood is circulated to the nephron, the working unit of the kidney, and is filtered at the glomerulus. The glomerular filtrate is normally free of large molecular weight components, but nutrients and toxicants small enough to pass through the glomerulus will enter the tubule system of the nephron. Epithelial cells lining the proximal convoluted tubule, the first section of the tubule system, then selectively reabsorb nutrients from the luminal fluid. Toxicants in the luminal fluid may become concentrated as water, salt and other nutrients are reabsorbed by the epithelia. Thus, a non-toxic level of a chemical in the

plasma may attain a toxic concentration within the lumen of the nephron. Alternatively, toxicants in the luminal fluid may be actively reabsorbed by the proximal tubule epithelia, resulting in the intracellular concentration of the contaminants. The ability of kidney epithelial cells to concentrate make them particularly susceptible to damaging effects of circulating toxicants.

The plasma membrane of the tubular epithelial cells exhibits a distinct structural and functional polarity. The brush border surface of the epithelial cell faces the luminal fluid, while the basal-lateral surface faces the basement membrane, and is bathed by capillaries. Functional integrity of both membrane surfaces is required for nutrient reabsorption to occur. Either surface may be exposed to high concentrations of toxicants, by the processes described above, impairing renal epithelial function.

Examples of nephrotoxins, toxicants which adversely affect the kidney, are heavy metals, analgesics, antibiotics, anti-neoplastics and organochlorines ( Hook,et al.,1979; Schreiner and Maher, 1965 ). Nephrotoxicity may result from damage to the vascular, glomerular, or tubular components of the nephron. Tubular, or epithelial, damage is often characterized by the presence of glucose, amino acids, and other nutrients in the urine (Schreiner and Maher,1965;

Hook,et al.,1979). To ascertain the site and mechanism of action of nephrotoxins affecting nutrient reabsorption, methods have been devised to isolate each of the epithelial cell membrane surfaces as vesicles suitable for nutrient transport analysis. To date, no convenient method for simultaneous isolation of brush border and basal-lateral membrane vesicles under identical conditions has been acheived. In this project I have developed a method for the isolation of hog renal brush border and basal-lateral membrane vesicles. I have also investigated the effect of cadmium on the reabsorption of glucose by brush border membrane vesicles.

## Literature Review

Characteristics of the Brush Border and Basal-Lateral Membranes -The epithelial cell of the proximal convoluted tubule consists of two membrane surfaces which differ in their morphology, composition, biochemistry, and function. As shown in Fig. 1, the luminal, or brush border, surface has thousands of microvilli giving it a "feathery" appearance. These microvilli serve to increase the absorptive area of the kidney. This structure contrasts with the smooth infoldings of the contraluminal, or basal-lateral, surface. The extracellular surface of the microvilli is covered by a glycocalyx, and the intracellular surface is equipped with a well defined cytoskeleton. In contrast to the microvillar membrane, electron microscopy shows the basal-lateral membrane to be sharply delineated with neither glycocalyx nor a prominent cytoskeleton. Thus, the basal-lateral surface resembles the plasma membrane of other cell types.

Each of the epithelial cell surfaces also has specific enzymes associated with it. Table I lists renal epithelial membrane enzyme markers based on their relative enrichment in the membrane preparations. Many of the enzymes

Figure 1. Epithelial Cells of the Proximal Tubule

Shown are three cells with their microvillar surfaces facing the lumen and their basal surfaces resting on the tubular basement membrane. The tight junctions which couple these cells have been omitted.

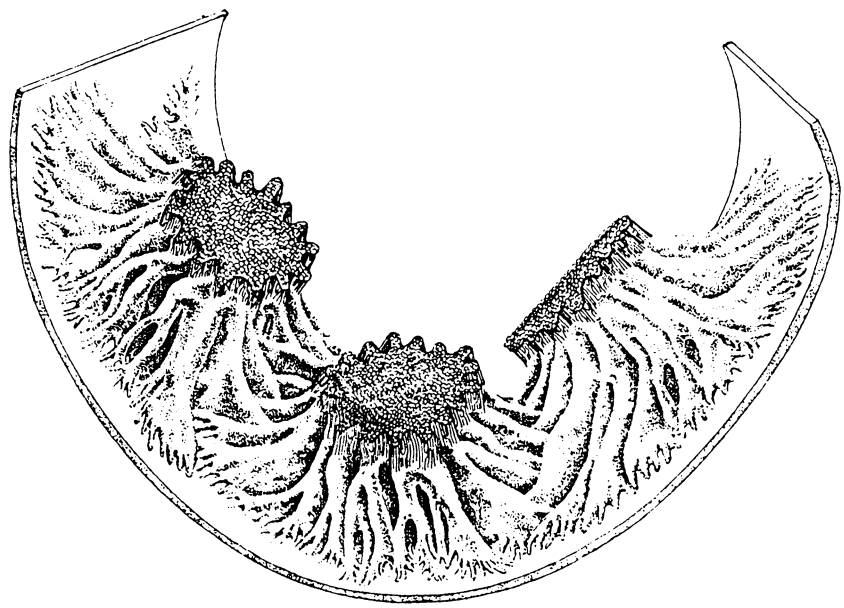

Figure 1. Epithelial Cells of the Proximal Tubule

Table I. Enzymes Associated With the Epithelial Cell Membrane

| Brush Border | Basal-Lateral |
|---|---|
| Disaccharidases ( maltase and trehalase)<br>5'-Nucleotidase<br>Aminopepridase(s),e.g., leucine aminopeptidase<br>Alkaline phosphatase<br>Gamma-glutamyl transferase<br>cAMP-dependent protein kinase<br>Carbonic anhydrase<br>$HCO_3$-dependent ATPase<br>Neutral endopeptidase<br>Dipeptidyl peptidase lV<br>Phosphodiesterase 1<br>Galactosyl (transferase) | $Na^+$-$K^+$-dependent ATPase<br>$Ca^{2+}$-$Mg^{2+}$-dependemt ATPase<br>Adenyl cyclase (PTH-stimulated) |

associated with the brush border are hydrolases ( disaccharidases, peptidases, phosphatases ) presumably involved in reabsorption of glucose, amino acids, and other nutrients from the luminal fluid. Gamma-glutamyl transpeptidase, for example, is thought to function in amino acid transport via a cycle of enzymatic reactions involving glutathione ( Meister 1973 ). A possible role of the disaccharidases in the transport of glucose has been proposed ( Sacktor,1968 ; Sacktor and Berger,1969) The precise roles of the other hydrolases are less well known, although other absorptive and secretory cell surfaces are rich in alkaline phosphatase and aminopeptidase.

The basal-lateral surface is especially rich in ($Na^+$-$K^+$)-ATPase, which plays an indirect role in the reabsorption process, as will be discussed in a later section.

Histochemical, immunocytochemical, and microdissection techniques were also used to determine the subcellular location of these enzymes. These methods have shown that the hydrolases are found predominantly in the brush border membrane, and ($Na^+$-$K^+$)-ATPase in the basal-lateral membrane, of the proximal convoluted tubule epithelia. However, the same studies also indicate that these enzymes can be found in other cell membranes of the nephron and that the distributions or enrichments of such "markers" may be

altered in different species. For example, alkaline phosphatase has been found to be tightly bound to the microvillus matrix of isolated brush border membranes (Thomas and Kinne,1972). Histochemical studies by Reale(1967) suggest that this enzyme may be present in the entire plasmalemma. Microdissection studies by Bonting,et al.,1958 point out that alkaline phosphatase is only twice as concentrated in the proximal tubule as in the distal tubule of man and is less active in the proximal tubule of man than in rat or dog. Similar observations are made by Quirk and Robinson (1972) concerning dissacharidases and aminopeptidases. The distribution of aminopeptidases is further complicated by the number of different enzymes which can act upon the same substrate. $(Na^+-K^+)$-ATPase has been reported to be present in both the brush border and basal-lateral membranes of intestine and kidney (Quigley and Gotterer,1972; Heidrich, et al.,1972; Liang and Sacktor,1972). A recent immunocytochemical study indicates that Gamma-glutamyl transpeptidase is located in both the brush border and basal-lateral membranes of rat (Spater,et al.,1982).

The question, then, of which enzyme(s) serves as the best marker for the brush border is a complicated one. Several different enzymes should be monitored to determine the purity of a membrane preparation.

In accordance with their structural, chemical, and biochemical differences, the brush border and basal-lateral membranes exhibit functional polarity. To be reabsorbed into the blood, nutrients must pass through both membranes. The types of transport systems, and their solute specificity, differ in the two surfaces. Table II gives a synopsis of these differences. Generally, the luminal membrane is rich in sodium cotransport systems, whereas the contraluminal membrane has predominantly simple facilitated diffusion systems.

Isolation Methods -The isolation of the luminal and contraluminal surfaces of the cellular envelope of epithelial cells is based on differences in composition. More specifically, they differ in buoyant density ( determined by the lipid to protein ratio), surface properties (such as surface charge density), and resistance to mechanical or osmotic disruption. The effect of the method of cellular disruption on the size and shape of the isolated plasma membrane is unique, because most other cellular organelles exist in a pre-determined size and shape. Different homogenization procedures can produce plasma membrane fragments of different sizes, allowing them to be separated from other organelles. Thus, the initial homogenization of the tissue represents a critical step in isolating plasma membranes.

Table II. Transport Systems in the Epithelial Cell Membrane

| Transport System | Brush Border Membrane | Basal-Lateral Membrane |
|---|---|---|
| Sodium cotransport systems | D-hexoses, neutral amino acids, acidic amino acids, glutamine, taurine, L-lactate, Krebs cycle intermediates, protons(exchange) inorganic phosphate, inorganic sulfate | Acidic amino acids |
| ATP-dependent transport | Protons | Sodium-potassium exchange, calcium |
| Simple facilitated diffusion systems | para-aminohippurate, N-Methylnicotinamide, amino acids | Neutral amino acids, D-glucose, 2-deoxy-D-glucose, para-aminohippurate, L-lactate, N'-methylnicotinamide, phosphate |

The brush border was the first of the two opposing membrane surfaces to be isolated.Early techniques took advantage of the rigidity of the brush border during homogenization as compared to the lability of the basal-lateral fragments. These methods, using multiple differential centrifugations followed by density gradients, rely on the original observation of Thuneberg and Rostgaard(1968) that the initial homogenization of the kidney cortex must be gentle to minimize the shearing of microvilli from the rest of the brush border membrane;the microvilli sediment with the microsomes. Large brush border fragments with attached microvilli are formed which differ enough from other organelles in sedimentation rate to allow their separation by sucrose density gradient centrifugation. Brush borders have been prepared in this way from rabbit ( Berger and Sacktor,1970; Aronson and Sacktor,1975), rat (Kinne and Kinne-Saffran,1969; Wilfong and Neville,1970) and human kidney (Scherberich,et al.,1974). Enzyme markers for brush border are enriched 15-20 fold over homogenate using these methods. These large intact brush border fragments, however, often also include parts of the lateral plasma membranes, which remain physically attached and cannot be removed (Berger and Sacktor,1970; Kinne and Kinne-Saffran,1969). They also contain the intervillous region of the membrane where pinocytosis can be initiated (Murer,1980).

The present method of choice for isolation of brush border membranes takes advantage of the high surface charge density of this membrane. The technique is called differential precipitation and is based on the observation that millimolar concentrations of divalent cations, such as $Ca^{2+}$ and $Mg^{2+}$, cause precipitation of mitochondria, lysosomes, basal-lateral plasma membranes, and microsomes. Luminal membranes remain in solution in the presence of high levels of the divalent cations (Kamath,et al.,1971; Schenkman and Cinti,1972). This phenomenon is due to the ability of the highly charged brush border membrane to compensate the charge of the divalent cation intramembraneally.The basal-lateral membranes and the intracellular organelles contain fewer negative charges and cation-mediated crosslinking or aggregation of the membranes occurs, with subsequent precipitation. Differential precipitation has been used to isolate brush border membranes from intestine and kidney of a variety of species (Booth and Kenny,1974; Malathi,et al.,1979; Kessler,et al.,1978). The membranes obtained by this method are microvillous vesicles, formed from individual microvilli with no intervillous membrane and no attached lateral plasma membrane. The method is very rapid and the vesicles seem well suited for transport studies However, the effects of the $Ca^{2+}$ or $Mg^{2+}$ ions on the brush border membrane structure and function is a matter of concern. These ions have been

shown to cause aggregation, phase separation, and fusion of phospholipid vesicles (Papahajopoulos et al,1979; Ohnishi and Ito,1974) and to affect the permeability and phase state of natural cell membranes (Manery,1966; Weller and Haug,1977; Breton et al.,1977).

Basal-lateral membranes from epithelial cells have also been isolated by differential centrifugation followed by separation on a density gradient (Ebel et al.,1971; Liang and Sacktor,1977; Mircheff et al.,1979). The introduction of Percoll for gradient centrifugation has facilitated the development of less cumbersome procedures for basal-lateral membrane isolation from intestine and kidney (Scalera,et al.,1980; Sacktor,et al.,1981; Inui,et al.,1981).

In order to ideally compare the transport properties of the luminal and contraluminal cell membranes, isolation of the two membrane surfaces should take place at the same time and under identical conditions. The sucrose gradient procedure of Liang and Sacktor(1977) nearly achieves such an isolation. A procedure utilizing free-flow electrophoresis is the only method thus far that truly effects simultaneous isolation under identical conditions. This method was first employed by Heidrich et al.(1972). In this procedure both size and charge differences of the two membranes influence their electrophoretic mobility. The less negative

contraluminal membranes actually migrate faster to the anode than the highly negative luminal membranes due to their different size and shape. Utilization of this technique is limited by the lack of the elaborate apparatus , the need for large amounts of tissue ( e.g., kidneys from 20 rats) and by the low yield.

Isolated Membrane Vesicles as Tools in Transport Studies - Many different approaches may be taken to investigate trans-epithelial transport in the kidney. While studies on the renal clearance of specific solutes by the intact animal have contributed greatly to the understanding of the overall renal physiology, elucidation of the mechanism of transport of various solutes requires a more direct approach. *In vivo* micropuncture and microperfusion techniques were developed to allow direct study of the transepithelial transport process in individual nephrons. These techniques, however, are limited to investigation of only those segments of the nephron which are readily accessible. For this reason, the technique of isolated perfused tubules was developed. This method allows the study of all the segments of the nephron and the manipulation of the external medium of the nephron, which is not as freely done in the *in vivo* microperfusion situation (Schafer, 1981). *In vitro* tissue slice techniques have also been used to study renal transport. Although these techniques do not

allow direct investigations on the epithilium itself, (Cross,1950), they have proved useful in investigating effects of nephrotoxins on organic acid or base transport (Hirsch,1976).

Isolated membrane vesicles have several advantages over the tissue and whole cell preparations for the study of transport processes. First, this technique eliminates potential interference of intracellular components that could metabolize or bind the transported solute. Second, both the intra- and extra-vesicular medium can be controlled, allowing the analysis of intracellular, as well as extracellular, factors on transport processes. Third, the ability to isolate both luminal and contraluminal membrane vesicles from polar epithelial cells allows the study of the transport processes unique to each membrane surface of the cells. Studies with isolated membrane vesicles should, however, be compared to the intact cell or tissue.

Membrane vesicles, isolated from bacteria, were found useful for the study of transport processes as early as 1960 (Kaback,1960). Later, similar studies with intestinal (Hopfer et al.,1973) and renal (Kinne et al.,1975), membranes were initiated. Uptake of solutes into membrane vesicles is classically studied by a rapid filtration

technique. Vesicles are incubated in the presence of radioactively labeled solute and then separated from the extravesicular medium by filtration. The radioactivity retained on the filter represents solute which has been transferred into the vesicles.

Interpretation of transport studies with isolated membrane vesicles can be affected by the orientation of the membranes and by whether there is binding of solute as well as uptake. Orientation of membrane vesicles refers to whether the extracellular surface faces the extravesicular medium (right -side out) or the intracellular surface faces the extravesicular medium ( in-side out). Both electron microscope freeze-fracture and immunological techniques have established that brush border vesicles isolated from intestine and kidney are predominantly (85%) oriented right-side out (Haase,et al.,1978). Basal-lateral membrane vesicles, however, seem to have a random orientation (Kinne,et al.,1978). The unusual predominance of the right-side out orientation of brush border vesicles could be due to several factors (Haase,et al.,1978) including 1)electrostatic repulsion and steric hindrance (Louvard,et al.,1976), 2)asymmetric distribution of the phospholipids (Zwaal,et al.,1973; Thompson,1976), and 3) the ability of the actin filament network to stabilize this configuration

of the membrane (Booth and Kenny,1976).

One way to distinguish between uptake and binding of solutes is to observe the osmotic sensitivity of the uptake. Increasing the osmolality of the medium outside the vesicle, by the addition of impermeant solutes, causes the vesicles to shrink. The amount of solute transported is dependent on the intravesicular space which is inversely proportional to the osmolarity of the medium (Beck and Sacktor,1975).

Epithelial Transport -Reabsorption of nutrients from the glomerular filtrate into the blood involves passage of the nutrient through both faces of the epithelial cell. The luminal membrane contains a large number of sodium cotransport systems, whereas the contraluminal membrane has predominantly simple facilitated diffusion systems. These transport carriers are specific for their solutes. In this way, the accumulation of each nutrient into the epithelial cell and its exit from the cell can be controlled. The glucose transport system will be the focus of this study since it represents one of the best understood carriers and because glucosuria is a common symptom of nephrotoxicity.

The passage of D-glucose across the luminal membrane into the epthelial cell occurs against a concentration gradient, by definition, an active transport process.

Studies on intact epithelia demonstrated that extracellular sodium and the ($Na^+$-$K^+$)-ATPase are essential for the transport of glucose and other solutes (Crane,1977; Schultz,1979; Ullrich,1980). Direct coupling between sodium and solute was not defined until experiments with isolated brush border vesicles were performed by Hopfer, et al., (1973) who demonstrated that the initial rate of uptake of D-glucose by intestinal brush border membrane vesicles is enhanced specifically by extracellular sodium. It is now established that one $Na^+$ ion is cotransported with every glucose molecule. Unlike most active transport processes, the energy source for glucose uptake is not derived from a direct coupling of the membrane carrier to metabolism. The energy is supplied indirectly by the $Na^+$ and $K^+$ dependent ATPase located predominantly in the contraluminal membrane. By extruding sodium from the cytoplasm, the ($Na^+$-$K^+$)-ATPase at the basal surface creates a chemical gradient at the luminal surface which favors $Na^+$ entry from the lumen. Glucose can then move uphill into the cell, driven by the $Na^+$ gradient (Crane,1962). This concept is not unlike Mitchell's chemiosmotic theory where proton gradients drive oxidative phosphorylation (Mitchell,1976). Other studies (Beck and Sacktor,1975; Murer and Hopfer,1974) indicate that the $Na^+$-dependent transport of D -glucose into brush border vesicles is an electrogenic process. These results suggest that both the sodium concentration gradient and an

electrical potential across the membrane contribute to net glucose flux.

Once in the cell, the glucose can move through the basal membrane by another carrier to the blood via "downhill" diffusion.The glucose carrier at the contraluminal membrane, therefore, must not be dependent on sodium since the ($Na^+$-$K^+$)-ATPase keeps the intracellular $Na^+$ concentration low. Studies with isolated basal-lateral vesicles show that glucose transport at this surface is, indeed, independent of $Na^+$ (Turner and Silverman, 1978).

Heavy Metal Nephrotoxicity -Heavy metals, particularly cadmium and mercury, are known to cause proximal tubule damage,as evidenced by increased excretion of proteins, amino acids, and glucose in the urine of exposed humans (Schreiner and Maher,1965). Rothstein, in 1959, was the first investigator to postulate that such metals exerted cellular toxicity via their interactions with membranes (Rothstein et al., 1959).He provided examples of how the permeability of membranes could be altered by the reaction of heavy metals with protein components of the membranes. Functional groups of proteins which are often modified by heavy metals include sulfhydryl, carboxyl, phosphoryl, imidazole, and amino groups. In addition to protein modification, heavy metals can also interact strongly with

the lipid components of membranes.

In the kidney, the effects of the interaction of heavy metals with membranes include changes in passive ion permeability, ion-transport ATPases, and $Na^+$-coupled transport (Pritchard,1979). Foulkes and associates (Foulkes,1971; Foulkes and Gieske,1973; Gieske and Foulkes,1974) have used *in vivo* techniques to describe the various effects of specific heavy metals (mercury, cadmium, uranium) on the luminal and peritubular uptake of acidic and neutral amino acids. Unfortunately, such studies cannot describe the mechanism whereby the metal adversely effects the transport process. A toxic compound could act on the transport process in four ways, by 1) affecting the carrier itself, 2) inhibiting the ($Na^+$-$K^+$)-ATPase needed to maintain the $Na^+$ gradient, 3) inhibiting oxidative phosphorylation, thereby decreasing the supply of ATP needed to maintain the sodium pump, and, 4) interacting with the lipid components of the membrane. Isolated membrane vesicles allow one to discriminate among these modes of action. For example,it has been seen that mercuric chloride, and other soluble mercury compounds, seem to inhibit the carrier for D-glucose in intestinal brush border vesicles (Klip et al.,1980; Miller, et al.,1980).

This thesis describes a procedure for simultaneously

isolating brush border and basal-lateral membranes under identical conditions. Simultaneous isolation will allow investigation and comparison of the toxic effects of metals, and other nephrotoxins, on the carriers in both membrane surfaces of the proximal tubule epithelial cell, as well as the effects on the $(Na^+-K^+)$-ATPase, located in the basal-lateral membrane.

In this study, cadmium toxicity is investigated. Although the industrial use of cadmium is under growing restraint, its continued contamination of the environment is inevitable since cadmium is a by-product of the large zinc ore industry. Longterm exposure results in cadmium accumulation in the kidneys. The metal appears to be stored as cadmium-metallothionein. The role of cadmium-metallothionein in the transport, renal absorption, and renal excretion of cadmium is being investigated (Foulkes, 1978; Foulkes, 1978b; Suzuki and Yamamura, 1979; Selenke and Foulkes, 1981; Nomiyama and Foulkes, 1977). The kidney is also the organ which exhibits the first adverse effects in man following long-term excessive exposure to cadmium (Nordberg 1976). The chronic effects include both glucosuria and amino aciduria. It has not been established if cadmium interacts with the carriers of these nutrients or inhibits their reabsorption by indirect means. This thesis presents some results concerning the effect of cadmium on

glucose uptake into brush border vesicles.

Literature Review Summary -The brush border and basal-lateral surfaces of the kidney proximal tubule epithelial cells differ in their structure, enzyme components, and transport processes. Once these two different membrane surfaces are separated and isolated as suspensions of membrane vesicles, they can be used to study the direct effects of nephrotoxins on transport processes of the nephron. Heavy metals, such as mercury and cadmium, are nephrotoxins of particular interest since they are believed to exert their toxicity by interacting with membrane components of kidney cells.

## Experimental Procedures

Materials - Percoll was obtained from Pharmacia Fine Chemicals (Uppsala,Sweden). Leucine-beta-Naphthylamide, gamma-N-glutamyl-beta-naphthylamide, beta -naphthylamine,and fluorescamine were from Sigma Chemical Company (St.Louis Missouri). Umbelliferone phosphate was obtained from Reasearch Organics (Cleveland,Ohio). Filters, HA 0.45um, 25mm, were from Millipore Corp. (Bedford,Massachusetts). D-[U-$^{14}$C]-glucose was from Amersham (Arlington Heights,Illinois),batches had specific activities of 270,283, and 296 mCi/mmol.

Enzyme Assays - Sodium and potassium dependent adenosine triphosphatase, ($Na^+$-$K^+$)-ATPase, was assayed by measuring the difference between phosphate released after incubation of enzyme with ATP plus $Mg^{2+}$,$Na^+$,and $K^+$ and with ATP plus $Mg^{2+}$ alone ( Quigley and Gotterer,1972). The $Na^+$,$K^+$,$Mg^{2+}$ ATPase assay mixture contained 1mM Tris/ATP,pH 7.0, 100mM NaCl, 10mM KCl, 3mM $MgCl_2$ and 10mM imidazole, pH 7.0.The $Mg^{2+}$ ATPase assay mixture contained 1mM Tris/ATP, pH 7.0, 3mM $MgCl_2$ and 10mM imidazole, pH 7.0. Samples were assayed in triplicate, and blanks in duplicate. For each assay,0.5 ml of reaction mixture was pre-incubated at 37°

for 10 minutes. At timed intervals, 10-30 ul of enzyme preparation containing about 50 to 100 ug of protein, were added to the reaction mixtures to initiate the reaction. After exactly 10 minutes of incubation at 37°C, 1.5mls of ice-cold 10% TCA was added to each sample to stop the reaction. The phosphate released was then quantified spectrophotmetrically by a phosphate assay (Chen,et al.,1956). To each sample, 2 mls of phosphate reagent,containing a 1:2:1:1 ratio of 6N $H_2SO_4$,$H_2O$, 2.5% ammonium molybdate,and 10% ascorbate, were added at timed intervals. A reduced phosphomolybdate complex is formed during incubation and can be quantified by absorbance at 820nm. All samples were incubated at 45°C, and absorbance at 820nm was read after 20 minutes of incubation.

Leucine aminopeptidase was assayed fluorometrically, using leucine-beta -naphthylamide as substrate(George and Kenny,1973). In a 3ml quartz cuvette, 10-50ul of enzyme preparation (1-6 mg/ml) were added to the substrate solution ( 0.2 mM leucinenaphthylamide in a 50mM mannitol, 2mM Tris-HCl buffer, pH 7.5) to a final volume of 2ml. Release of the product, beta-naphthylamine, was monitored continuously at room temperature with an Aminco-Bowman spectrofluorometer at an excitation wavelength of 335nm and emission wavelength of 415 nm. The quantity of product released was determined by relating the fluorescence change to a standard curve of

beta-naphthylamine. Care must be taken in handling this product since it is a suspected carcinogen.

Gamma-glutamyl transpeptidase was assayed similarly, using gamma-N-glutamyl -beta-naphthylamide as the donor amide and glycylglycine as acceptor peptide (George and Kenny,1973). The substrate solution contained 0.2mM gamma-N-glutamylbeta-naphthylamide, 3mM glycylglycine, 0.1M Tris-HCl, pH 8.5. The assay procedure was as described above for leucine aminopeptidase.

Alkaline phosphatase was also assayed fluorometrically using umbelliferone phosphate as substrate (Six,et al.,1974). The substrate solution contained 1mM umbelliferone phosphate in 0.5M Tris-HCl, pH 8.6. In a 3 ml quartz cuvette, 10-50ul of the enzyme preparation (1-6 mg/ml) were added to sufficient substrate solution to yield a final volume of 2ml. Release of the product, 7-hydroxycoumarin (umbelliferone),was monitored continuously at room temperature with an Aminco-Bowman spectrofluorometer using an excitation wavelength of 350nm and an emission wavelength of 453nm. The quantity of product formed was determined by relating the fluorescence change to a standard curve of 7-hydroxycoumarin.

Succinate-cytochrome c reductase was assayed by the

method of Tisdale (1967). The reaction mixture contained 0.1mM cytochrome C, 1uM KCN, and 0.01 mM succinic acid in a 50 mM potassium phosphate 1mM EDTA buffer, pH 7.4. The reaction was initiated by adding 10 ul of sample (about 4 mg/ml) to 1ml of reaction mixture in a 1.2 ml cuvette. The reduction of cytochrome C, indicated by an increase in absorbance at 550nm, was monitored continuously.

NADPH-cytochrome c reductase was assayed similarly. The reaction mixture contained 0.05 mM cytochrome C, 0.01% NADPH, and 0.01 mM KCN in a 50 mM potassium phosphate buffer, pH 7.7 (Mackler, 1967).

Acid phosphatase was assayed by continuously monitoring, at 300 nm, the release of salicylic acid from o-carboxyphenyl phosphate in an acidic buffer (Worthington Enzyme Manual).The reaction mixture contained 0.73 mM o-carboxyphenyl phosphate in a 0.15 M sodium acetate buffer, pH 5.0. In a 1.2 ml cuvette, 5- 10 ul of sample (10-20 mg/ml) were added to 1 ml of reaction mixture to initiate the reaction. The increase in absorbance at 300nm represented product released.

Protein and DNA determinations - Protein was determined fluorometrically by the method of Bohlen,et al., (1973) which is based on the interaction of fluorescamine with

primary amino groups. Lysozyme was used as the standard. DNA was determined by the method of Burton(1968), based on the reaction of diphenylamine with deoxyribose. Calf thymus DNA was used as the standard.

Electron Microscopy Membrane preparations were fixed in 2.5%glutaraldehyde, post-fixed with 1% osmium tetraoxide, and stained with 2% uranyl acetate,by a standard procedure. It was not necessary to mix these preparations with agar before post-fixing since the glutaraldehyde-fixed membranes formed a hard pellet upon centrifugation at 34,000 x g for 30 minutes.

Sucrose Gradient Method of Simultaneous Isolation of Brush Border and Basal-Lateral Membrane Vesicles - This procedure is based on the published method of Liang(1977), with some modification. A crude plasma membrane fraction was prepared from 10g of hog renal cortex by the procedure of Sacktor et al.,(1981). The tissue was minced in a "Virtis" homogenizer in 20 volumes of buffer containing 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, and was then homogenized by a Potter-Elvehjem homogenizer with a motor-driven pestle. The homogenate was centrifuged at 2,500 x g for 15 min. The pellet was discarded and the supernatant was centrifuged at 24,000 x g for 20 min. The fluffy upper portion of the pellet, representing the crude plasma

membrane fraction, was resuspended in the sucrose buffer and layered onto a 30 ml continuous, linear, sucrose gradient with the concentration ranging from 32.5% to 41.5% (w/w) sucrose, dissolved in 10 mM Tris-HCl, 1 mM EDTA buffer, pH 7.5 (buffer B). The gradient was centrifuged for 1 hr at 105,000 x g in a Beckman swinging bucket rotor (SW 27), and was allowed to stop without braking. Fractions were collected from the bottom of the tube while controlling the flow-rate with a ground-glass syringe. Each fraction was diluted with one-half volume of buffer B and centrifuged at 30,000 x g for 15 minutes to wash the membranes. The pellets were each resuspended in 1.5 ml of 2 mM Tris-HCl, pH 7.5, and were assayed for leucine aminopeptidase and ($Na^+$-$k^+$)-ATPase activity. Fractions representing basal-lateral and brush border membranes were pooled and washed as described above.

Percoll Method of Simultaneous Isolation of Brush Border and Basal-Lateral Membrane Vesicles - The development of the following procedure was based on published procedures of Scalera et al.,(1980),Sacktor et al.,(1981),and Inui et al.,(1981) who had designed methods for isolating basal-lateral membrane vesicles by utilizing the self-forming shallow gradient capabilities of Percoll. Percoll is composed of polyvinylpyrrolidone-coated silica particles which are able to form isotonic density gradients *in situ*.

It was noted that certain fractions of the gradient were high in brush border enzyme activity. The modified procedure described here succeeds in isolating both membrane fractions.

To insure a gentle homogenization, only kidneys from young hogs were used for this procedure, since the tissue from older pigs was found to be too tough. Kidneys were obtained from the Department of Food Science and Technology, VPI, Blacksburg and from Valleydale Meatpackers, Bristol, Va. The kidneys were stored on ice from the time of slaughter to the start of the preparation, which was approximately two and one-half hours for kidneys obtained from Bristol. A thin, outer-layer, of tissue was removed from a lateral face of one kidney and a thick slice of cortex,about 3-4 mm, was cut from this face. Nine grams of the renal cortex tissue was passed through a hand-operated tissue press equipped with a 1.5 mm pore sized screen ( EDCO Scientific). This device permits rapid mincing of the tissue. The minced tissue was homogenized in 5 volumes of cold buffer containing 0.25 M sucrose, 1mM EDTA, 10 mM Tris-HCl, pH 7.5 (buffer A) with 10 strokes of a Dounce homogenizer, using a loose-fitting pestle. The homogenate was briefly centrifuged by allowing the centrifuge to reach 2400 x g and then stopping it immediately. The pellet which resulted was discarded. This step allows the removal of cell

debris. The supernatant was centrifuged at 2400 x g for 15 minutes and the pellet was discarded. The supernatant and fluffy sediment were centrifuged at 20,500 x g for 20 min. The resulting supernatant and the hard pellet were discarded. The fluffy portion of the pellet, representing the crude plasma membrane preparation, was resuspended in buffer A with 10 strokes of a Potter-Elvehjem homogenizer with a motor-driven pestle. The membrane suspension was mixed with Percoll and buffer A using a Dounce homogenizer with a tight-fitting pestle to obtain 30 ml of a 13% (v/v) Percoll solution. The membrane-Percoll mixture was centrifuged in cellulose nitrate tubes with caps, using a Beckman 50.2Ti rotor at 48,000 x g for 30 minutes to form the first gradient. Fractions of 1 ml were collected from the top of the tubes using an ISCO density gradient fractionator and pumping a 50% sucrose solution into the bottom of the tube. Fractions were assayed for ($Na^+$-$K^+$)-ATPase and leucine aminopeptidase, marker enzymes for the basal-lateral and brush border membranes, respectively. Fractions were pooled based on these activities, diluted with buffer A, and centrifuged at 100,000 x g for 1 hour to remove Percoll. The consolidated, partially purified preparation of brush border membranes was mixed with Percoll and buffer A, using a Dounce homogenizer with a tight-fitting pestle, to obtain 30 ml of a 20% (v/v) Percoll solution. The crude basal-lateral preparation was similarly

resuspended in a final Percoll concentration of either 13% or 8.3% (v/v). The membrane-Percoll mixtures were centrifuged as described above to form the second gradients, fractions were collected, assayed, pooled, and washed as before. The final membrane preparations were resuspended in 100 mM mannitol, 10 mM Tris-Hepes, pH 7.4 and centrifuged at 48,000 x g for 30 minutes. The final pellets were resuspended in a minimum amount of the same buffer by passage through a fine needle (26 gauge) with a plastic syringe. Transport and enzyme assays were performed immediately.

Differential Precipitation Method of Brush Border Membrane Isolation - This is the current method of choice of most investigators for isolation of brush border membranes, due to the short preparation time and high purity resulting from its application. What follows is the method adopted by Malathi,et al., (1979).

Six grams of fresh pig renal cortex, obtained in the same manner as in the preceding procedure, were homogenized in 30 volumes (v/w) of cold 50 mM mannitol, 2 mM Tris-HCl, pH 7.0 ( buffer M) for 5 minutes in a Waring blendor with intermittent cooling on ice. A sample of the homogenate was saved for assay later. While stirring for 10 min., 1 M $CaCl_2$ was added dropwise to the homogenate to a final

concentration of 10 mM. The homogenate was then centrifuged at 3000 x g for 15 minutes and the pellet was discarded. The supernatant was centrifuged at 43,000 x g for 20 minutes. The pellet was resuspended in buffer M by passage through a fine needle with a plastic syringe, and the suspension was centrifuged again at 43,000 x g for 20 minutes. The final pellet was then resuspended with a syringe in 100 mM mannitol, 10 mM Tris-Hepes, pH 7.4. Transport and enzyme assays were performed immediately.

Glucose Uptake Assay - A rapid filtration technique for determining initial glucose uptake into vesicles in the presence of an imposed NaCl gradient was used, as described by Mamelok,et al.,(1981) with some modification. The sodium-dependent uptake reaction mixture contained a final concentration of 100 mM mannitol, 100 mM NaCl, 1 mM D-glucose containing 5 uCi/200 ul of reaction mixture of D-[$^{14}$C]-glucose in 10 mM Tris-Hepes, pH 7.4.

Membrane vesicles were prepared fresh and were resuspended in a buffer containing 100 mM mannitol, 10 mM Tris-Hepes, pH 7.4, at a final protein concentration of approximately 15 mg/ml. In separate tubes, 200 ul of reaction mixture and 100 ul of vesicles were pre-incubated at 25°C for 5 minutes. Glucose uptake was initiated by adding 70 ul of vesicles, approximately 1 mg of protein, to

the reaction mixture and starting a timer simultaneously. Aliquots of 20 ul were removed and added,at timed intervals, to about 2 ml of cold "stop" solution containing 150 mM NaCl, 0.2 mM phlorizin, 100 mM mannitol, and 10 mM Tris-Hepes, pH 7.4. The stop solution was held in the chilled wells of a filtration apparatus ( Hoefer Scientific). Exactly 1 minute after placing the vesicles in the wells, the solution was passed through the supporting filter by opening the well stopcock to the vacuum manifold. The filters were placed into scintillation vials and dried for 10 minutes in an 80°C oven. Scintillation fluid, 10 ml, was added to each vial and the radioactivity retained on the filters, representing glucose associated with the vesicles, was counted.

Effect of Heavy Metal Ions on Glucose Uptake - A study by Miller et al.,(1980) indicated that vesicles should be pre-incubated in the presence of metal ions before initiation of the glucose uptake assay. A five minute pre-incubation was found to be sufficient, showing no difference from longer incubations of up to 30 minutes. Citrate was added to all metal ion solutions in the ratio of 2 moles citrate/mole metal ion to insure the solubility of the metal ion. A 10 ul volume of an appropriate concentration of metal chloride solution was added to 0.1 ml of vesicles (15 mg/ml) to a final desired metal ion concentration. The Na-dependent

reaction mixture was also brought to the same metal ion concentration by addition of metal chloride solution, to prevent a possible change in the binding equilibrium of the metal ion upon dilution of the vesicle-metal preparation in the reaction mixture. The vesicle-metal ion preparation and metal-containing reaction mixture were pre-incubated for 5 minutes at 25°C. Glucose uptake was then assayed as described previously. Control vesicles and reaction mixture contained comparable amounts of citrate without metal ion.

Effect of Cadmium on Brush Border Enzyme Activity - As with the glucose uptake assay described above, vesicles were pre-incubated for 5 minutes with $Cd^{2+}$, then added to reaction mixtures which contained equivalent concentrations of the metal. In a 3 ml cuvette, 1.91 ml of substrate solution ( see procedures for leucine aminopeptidase, gamma-glutamyl transpeptidase and alkaline phosphatase) were mixed with 40 ul of an appropriate cadmium chloride solution, to a final desired metal ion concentration. The vesicle preparation, 0.2 ml containing 0.1 to 1.0 mg of protein, was pre-incubated with 4 ul of an appropriate cadmium chloride solution for 5-10 minutes at room temperature. To initiate the reaction, 50 ul of the vesicle suspension was added to the cuvette. Product formation was monitored as described previously.

# Results

Sucrose Gradient Method of Simultaneous Isolation of Brush Border and Basal-Lateral Membrane Vesicles -As dicussed in the Literature Review, simultaneous isolation of the opposing faces of the renal epithelial cell membrane has been previously achieved by the methods of free-flow electrophoresis and sucrose density gradient centrifugation. A sucrose gradient procedure was attempted in our experiments with little success. Fig.2 illustrates the profile of $(Na^+-K^+)$-ATPase and LAP activity found in the fractions collected from the gradient. Both activities were present in fractions 4-6, at the top or low density region of the gradient. $(Na^+-K^+)$-ATPase activity also showed a slight peak in fractions 10-12. According to the published procedure (Liang and Sacktor,1977), the brush border membranes are found at a low density, and the basal-lateral membranes at a high density, region of the sucrose gradient. Fractions 4-6, and 10-12, were pooled separately and washed to remove excess sucrose. The enrichment of marker enzyme activity in the final preparations is shown in Table III. The basal-lateral preparation did not show a significant enrichment of $(Na^+-K^+)$-ATPase. The brush border preparation showed a 4-fold enrichment of $(Na^+-K^+)$-ATPase, which was

Figure 2. Distribution of Enzyme Activities in Sucrose Gradient

A crude plasma membrane fraction was prepared from pig kidney cortex and centrifuged on a 31.5% to 41.5% sucrose gradient, as described in the Experimental Procedures. Fractions were collected from the bottom of the tube into 16 fractions of 2 ml and were assayed for enzyme activity. Fractions are numbered from the lowest density region of the gradient to the highest density region.

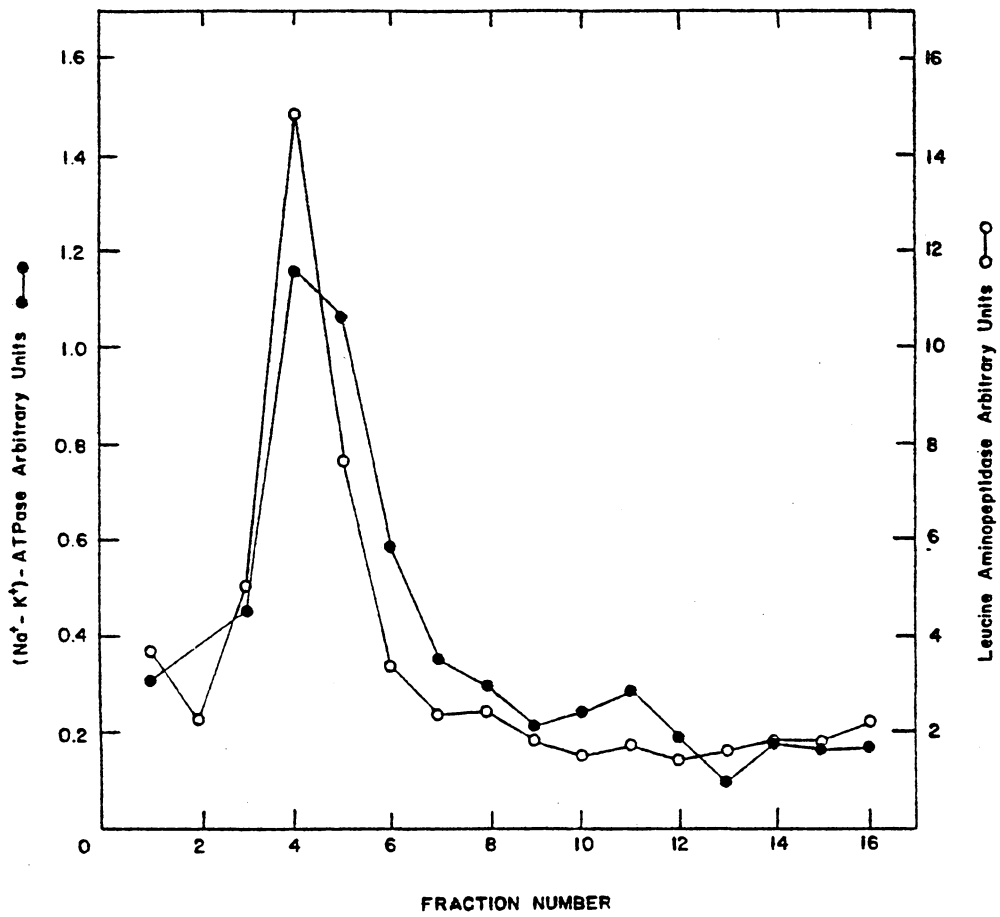
(Na⁺- K⁺)- ATPase Arbitrary Units
Leucine Aminopeptidase Arbitrary Units
FRACTION NUMBER
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
2
4
6
8
10
12
14
16

Figure 2.

Table III. Enrichment Factors for Marker Enzymes

Brush border and basal-lateral plasma membrane fractions were prepared from pig kidney cortex by the sucrose gradient method described in the Experimental Procedures. The enrichment factors are expressed as the ratio of the specific activities of the membrane fractions to that of the cortex homogenate. Only one preparation, representing the most successful of several attempts, is represented.

Table III. Enrichment Factors for Marker Enzymes

| | (Na-K)-ATPase | Leucine Amino-Peptidase |
|---|---|---|
| Brush Border | 4.0 | 6.0 |
| Basal-Lateral | 1.1 | 1.9 |

also seen by Liang and Sacktor (1977), and a 6-fold enrichment of LAP. Liang and Sacktor (1977) reported about 10-fold enrichment of LAP.

The brush border preparation shows an enrichment of marker enzyme activity almost comparable to the published values, but the basal-lateral preparation did not show an acceptable enrichment of ($Na^+$-$K^+$)-ATPase. For these reasons, this procedure was no longer used.

Percoll Method of Simultaneous Isolation of Brush Border and Basal-Lateral Membrane Vesicles - Fig. 3 illustrates the distribution pattern of leucine aminopeptidase, a marker enzyme for brush border membranes, and ($Na^+$-$K^+$)-ATPase, a marker enzyme for basal-lateral membranes, after centrifuging the crude plasma membrane preparation in 13% (v/v) Percoll medium. The distribution of gamma-glutamyl transpeptidase and of alkaline phosphatase followed a similar pattern as that of leucine aminopeptidase (not shown). The ($Na^+$-$K^+$)-ATPase profile exhibited a prominent peak between fractions 9 and 12. Two peaks of Leucine aminopeptidase activity were seen, one peak corresponding to that of the ($Na^+$-$K^+$)-ATPase, the other appearing in the higher density region between fractions 22 and 28. Fractions 9 through 12 were pooled as the basal-lateral membrane fraction and 22 through 28 as the brush

Figure 3. Distribution of Enzyme Activities in Percoll Gradient

A crude plasma membrane preparation was isolated from pig kidney cortex, suspended in 13% Percoll (v/v), and centrifuged as described in Experimental Procedures. Fractions were collected from the top of the gradient into 30 fractions of 1 ml and were assayed. Fractions are numbered from the top of the tube to the bottom.

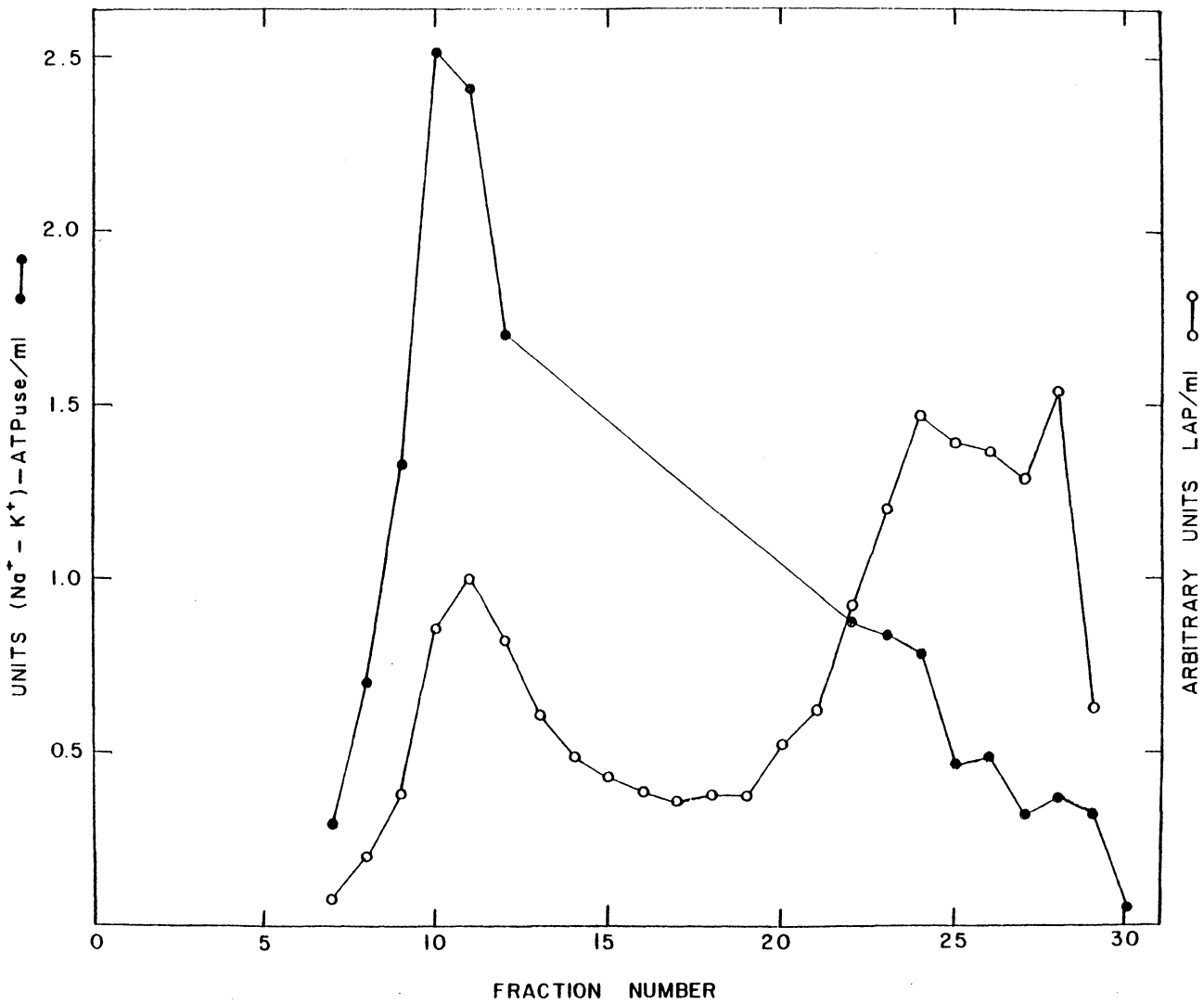
2.5
2.0
1.5
1.0
0.5
0
5
10
15
20
25
30
UNITS (Na+ - K+)-ATPuse/ml
ARBITRARY UNITS LAP/ml
FRACTION NUMBER

Figure 3.

border fraction. Table IV shows the relative enrichment of marker enzymes associated with the basal-lateral and brush border membranes. The values indicate the relative enrichment of activity over that of the homogenate in the fractions pooled from the first gradient, identified as the crude brush border and basal -lateral preparations.The crude basal-lateral preparation had substantial enrichment of ($Na^+$-$K^+$)-ATPase, but also appeared to be contaminated with leucine aminopeptidase (LAP) activity. The crude brush border preparation had enrichment of LAP, but it was only about 50% as high as the enrichment seen in the brush border membranes (BBMs) prepared by other methods (Malathi,et al.,1979; Liang and Sacktor,1977). The crude brush border also had enrichment of ($Na^+$-$K^+$)-ATPase.

In an attempt to improve the purity of the membranes, both preparations were centrifuged in a second Percoll gradient solution . The crude basal-lateral preparation was resuspended in 13% Percoll,and the crude brush border preparation was resuspended in 20% Percoll (v/v).

The basal-lateral membranes in the second gradient formed a single band with ($Na^+$-$K^+$)-ATPase activity present in fractions 9-12. These fractions were pooled and washed. The crude brush border preparation formed two distinct bands in the second gradient. The distribution of LAP activity in

Table IV. Enrichment Factors for Marker Enzymes

Brush Border and basal-lateral membrane vesicles were prepared from fresh pig kidney cortex by the Percoll Method described in the Experimental Procedures. The enrichment factors are expressed as the ratio of the specific activities of the membrane fractions to that of the cortex homogenate. The values are means $\pm$ standard deviation calculated from assays performed on five preparations.

Table IV. Enrichment Factors for Marker Enzymes

| | (Na-K)-ATPase | Leucine Amino-Peptidase | Succinate Cyt. C Reductase | Acid Phospha-tase | DNA |
|---|---|---|---|---|---|
| Crude Brush Border | 1.65 ± 0.07 | 5.2 ± 0.0 | | | |
| Crude Basal-Lateral | 8.2 ± 1.1 | 3.05 ± 0.07 | | | |
| Brush Border Band 1 | 5.0 ± 0.0 | 4.75 ± 0.07 | | | |
| Brush Border Band 2 | 0.93 ± 0.6 | 7.95 ± 2.2 | 3.0 ± 1.1 | 1.6 ± 0.86 | 0.60 |
| Basal-Lateral | 7.5 ± 3.4 | 3.2 ± 1.0 | 1.2 ± 1.5 | 1.3 ± 0.6 | 3.0 |

the 20% (v/v) Percoll gradient is shown in Fig. 4, with two peaks of activity corresponding to the two bands seen in the gradient. Fractions 8-13 and 21-27 were pooled separately and washed. The enrichment of marker enzymes in the basal-lateral preparation and in both pools of the brush border preparation are reported in Table IV. The higher density pool of the brush border preparation, band 2, exhibited higher LAP enrichment and less ($Na^+$-$K^+$)-ATPase than the crude preparation and band 1. This preparation represented the purest brush border membrane fraction obtained thus far by Percoll density gradient centrifugation. The second gradient had no effect on the marker enzyme activity of the crude basal-lateral preparation.

Figs. 5-8 are electron micrographs of the basal-lateral and brush border membranes isolated by this procedure. The basal-lateral membranes appear sharply delineated, with the membrane appearing as a trilaminar structure, typical of this type of plasma membrane. The brush border membranes exhibit a dark, thick coat and intact microvilli can be seen. Both preparations appeared to have mitochondrial contamination. Succinate-cytochrome c reductase activity indicated that the mitochondrial contamination in the brush border is significantly high, showing three-fold enrichment over the homogenate (Table IV). The basal-lateral membrane fraction also appeared to be contaminated by chromatin, as

Figure 4. Distribution of LAP Activity in 20% Percoll

A crude brush border membrane fraction was isolated by centrifuging a crude plasma membrane preparation in 13% Percoll and pooling the fractions high in LAP activity, low in ($Na^+$-$K^+$)-ATPase activity. The brush border membranes were then resuspended in 20% (v/v) Percoll and centrifuged as described in Experimental Procedures. 30 fractions of 1 ml were collected from the top of the gradient and assayed for LAP activity. The fractions are numbered from the top of the gradient to the bottom, that is from the lowest density to the highest.

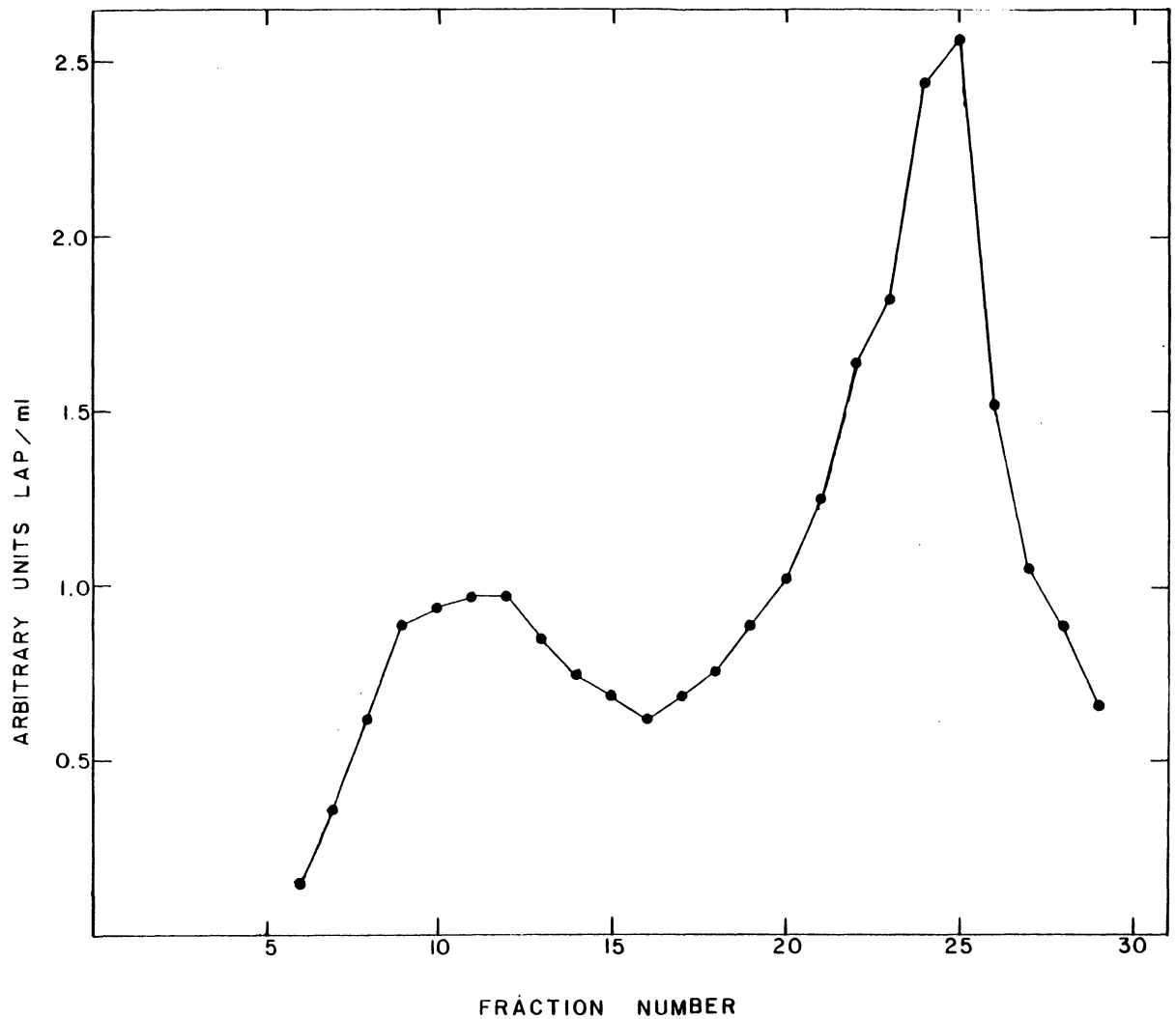
2.5
2.0
1.5
1.0
0.5
ARBITRARY UNITS LAP / ml
5
10
15
20
25
30
FRACTION NUMBER

Figure 4.

Figure 5. Electron Micrograph of Brush Border Preparation

Brush border membranes were isolated from pig kidney cortex by separating a crude plasma membrane preparation on two successive Percoll gradients, pooling fractions high in LAP activity and low in ($Na^+$-$K^+$)-ATPase. The final brush border membrane preparation was fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetraoxide, and stained with 2% uranyl acetate. Many closed vesicles of brush border membrane are seen (BB). Intact microvilli (MV) and mitochondria (MT) are also visible. (Magnification x 30,000).

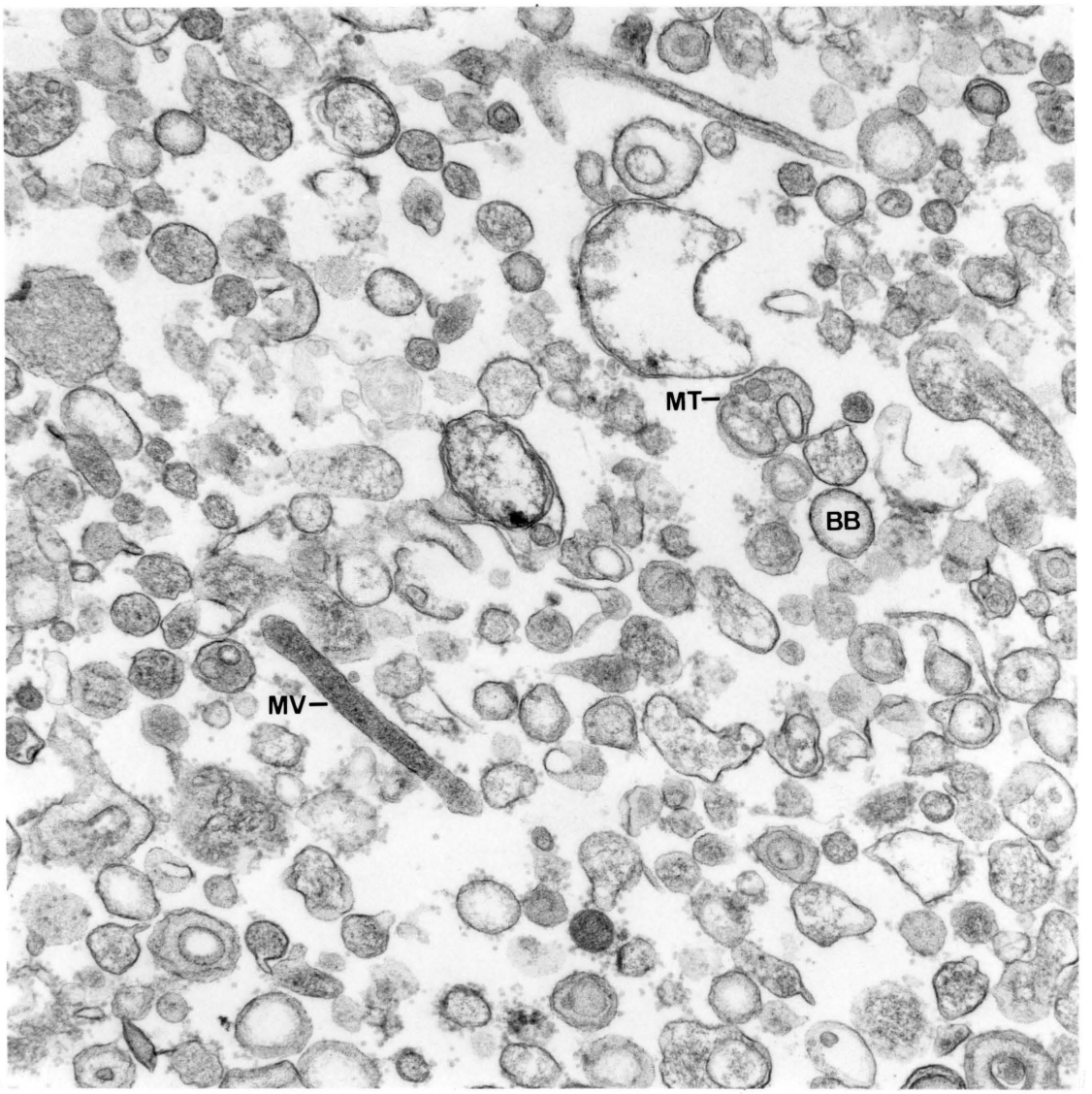


Figure 5.

Figure 6. Electron Micrograph of Brush Border Membranes

Membranes were prepared and stained as in Fig. 5. The dark-staining membrane coat, characteristic of brush border membranes is seen (BB). (Magnification x 100,000).

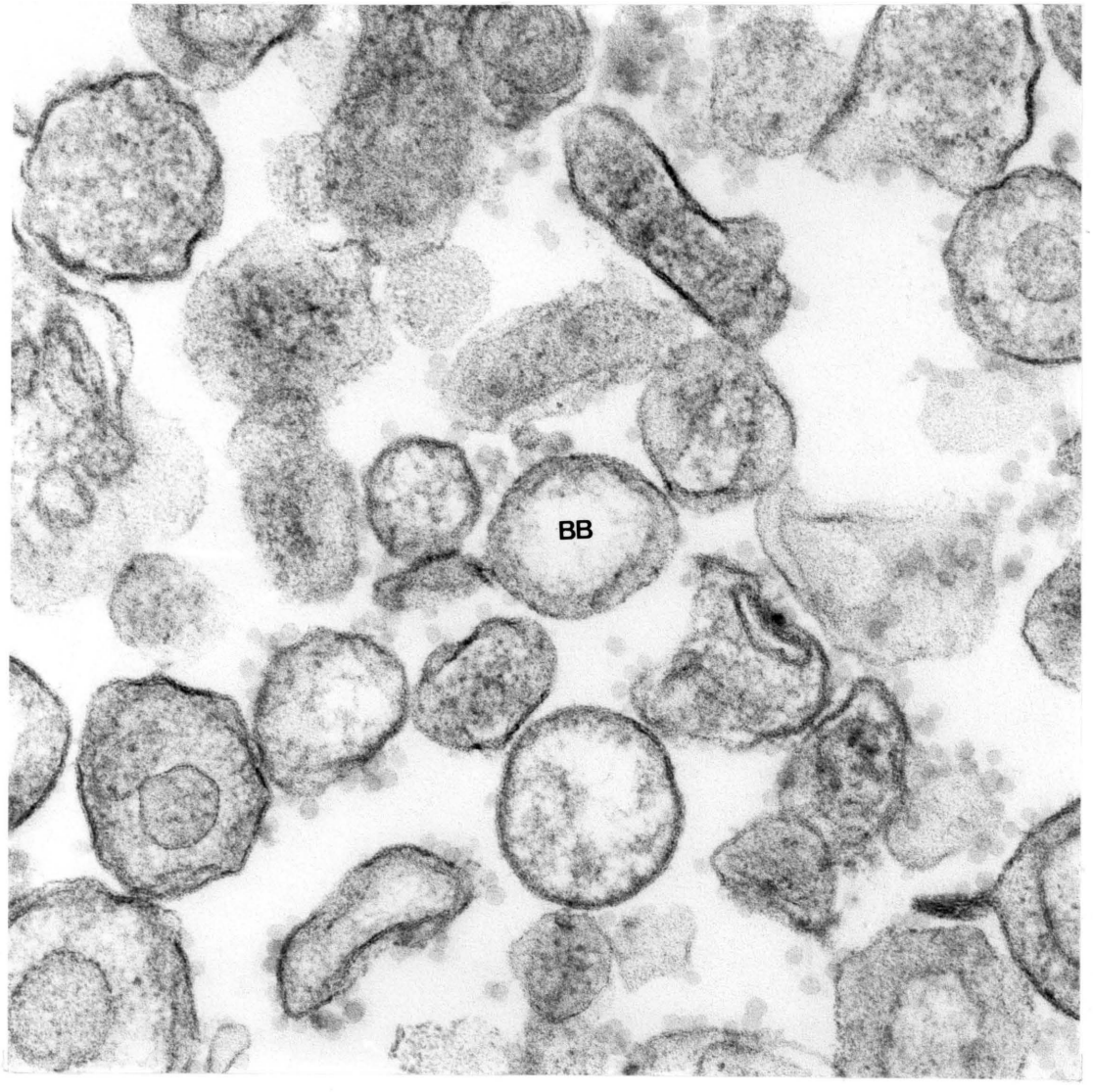


Figure 6.

Figure 7. Electron Micrograph of Basal-Lateral Membranes

Basal-lateral membrane vesicles were isolated from pig kidney cortex by separating a crude plasma membrane preparation on two successive Percoll gradients, pooling fractions high in ($Na^+$-$K^+$)-ATPase activity and low in LAP activity. The final basal-lateral membrane suspension was fixed and stained as described in Fig.5 and in the Experimental Procedures. Closed basal-lateral vesicles are seen (BL). Lysosomes (LY), mitochondria (MT) and chromatin (CH) are also visible. (Magnification x 30,000).

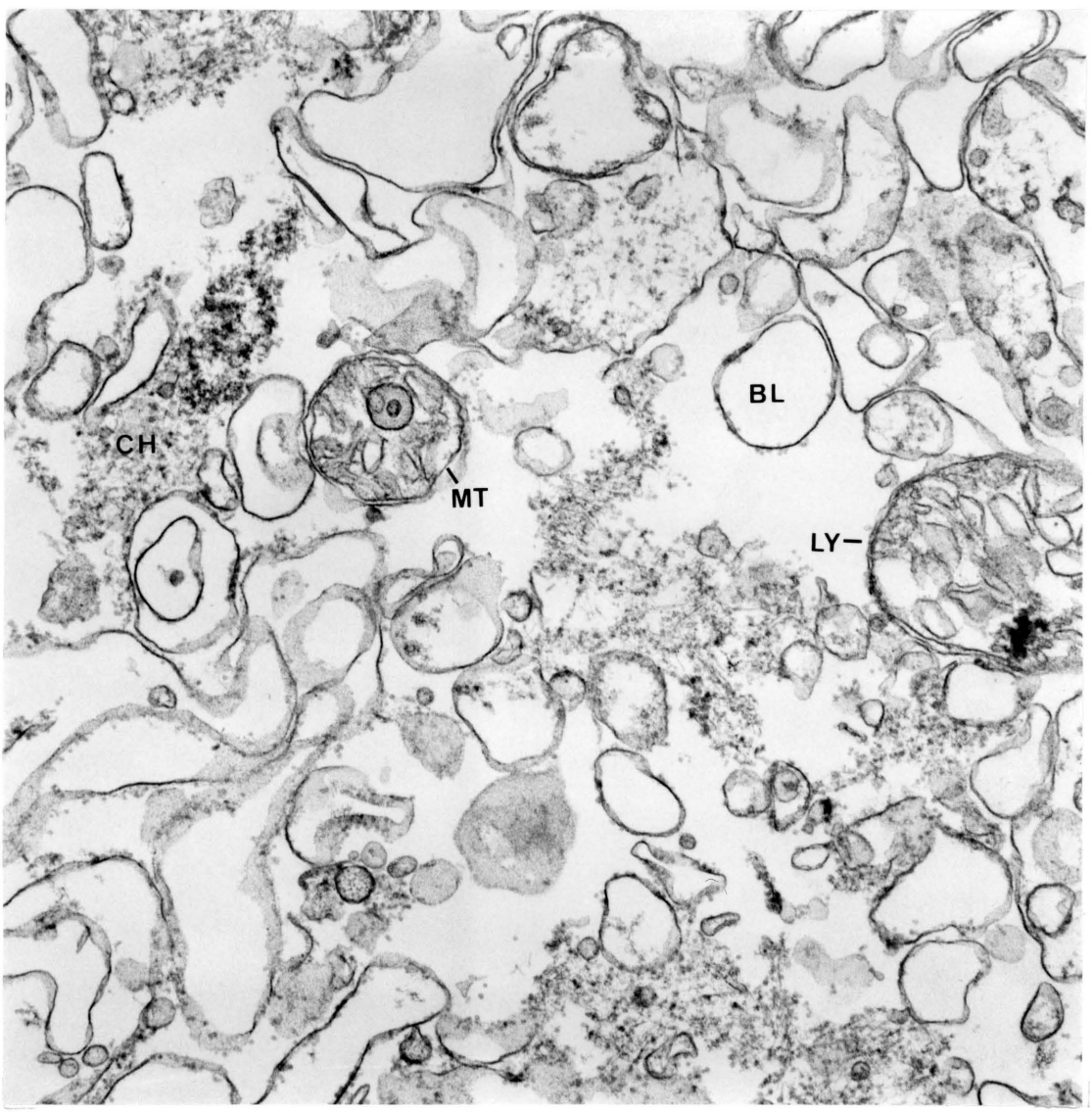


Figure 7.

Figure 8. Electron Micrograph of Basal-Lateral Membranes

Basal-lateral membranes were prepared and stained as described in Fig.7. The clearly defined, tri-laminar membrane, characteristic of basal-lateral membrane vesicles, is visible. (Magnification x 100,000).

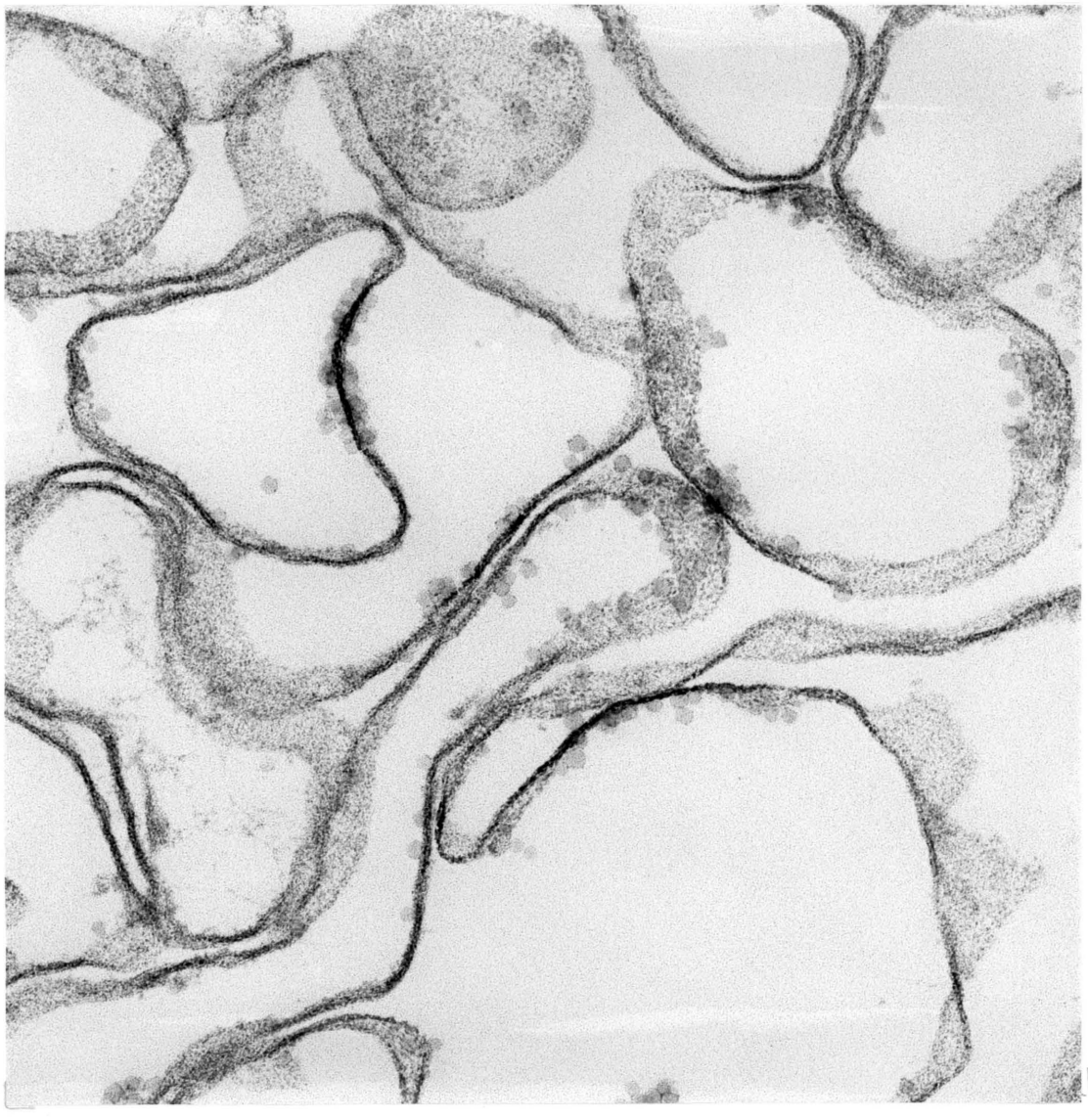

Figure 8.

observed by the electron microscope. This was confirmed by a DNA assay. This basal-lateral membrane preparation had a 3-fold enrichment of DNA over that of the homogenate (Table IV).

Further attempts were made to improve the purity of the membranes by altering the density gradients, applying an additional gradient or by collecting different fractions. The results of a final attempt to achieve improved purification are shown in Table V. The procedure used here differed from the previously described Percoll method in only two steps. First, lower density fractions (16-26) were collected from the second brush border gradient based on specific activity, rather than total activity, per fraction. Second,the crude basal-lateral preparation was centrifuged in 8.3% (v/v) Percoll, resulting in the appearance of two bands. The upper, or lower density, band was enriched 22-fold in DNA.

These alterations appeared to 1) increase LAP enrichment by 50% in the brush border, 2) increase $(Na^+K^+)$-ATPase activity 4-fold in the brush border fraction, 3) slightly increase $(Na^+-K^+)$-ATPase slightly in the basal-lateral fraction, and 4) increase LAP enrichment by four-fold in the basal-lateral fraction. Overall, these alterations did not improve the marker enzyme enrichment but

Table V. Enrichment Factors for Marker Enzymes

Brush border and basal-lateral membrane vesicles were prepared simultaneously from fresh pig cortex by the Percoll Method described in the Experimental Procedures, with the modifications described in the Results. Brush Border vesicles alone were prepared from fresh pig cortex by the $CaCl_2$ precipitation method also described in the Experimental Procedures. The enrichment factors are the ratio of the specific activities of the membrane fractions to that of the cortex homogenate. The values are the averages $\pm$ ranges calculated from assays performed on two preparations, except for the DNA assays which were performed on only one preparation. N.D. is not detectable.

Table V. Enrichment Factors For Marker Enzymes

| | Leucine Amino-Peptidase | Gamma-Glutamyl Transpeptidase | Alkaline Phosphatase | (Na-K)-ATPase | Succinate Cyt. C Reductase | NADPH Cyt. C Reductase | Acid Phosphatase | DNA |
|---|---|---|---|---|---|---|---|---|
| CaCl Brush Border | 13.0 ± 3.6 | 11.0 ± 1.3 | 10.8 ± 2.2 | 4.6 ± 0.4 | N.D. | N.D. | 1.65 ± 0.07 | 0.46 |
| Percoll Brush Border | 13.2 ± 3.2 | 5.6 ± 1.3 | 3.9 ± 1.6 | 4.0 ± 0.2 | 3.3 ± 2.0 | 1.2 ± 0.5 | 0.94 ± 0.08 | 0.60 |
| Percoll Basal-Lateral | 12.8 ± 2.0 | 7.0 ± 1.8 | 3.3 ± 0.4 | 17.3 ± 5.8 | 1.2 ± 0.6 | 1.2 ± 0.4 | 0.93 ± 0.24 | 0.62 |

did succeed in removing the DNA from the basal-lateral membrane fraction.

Brush border membranes were isolated by the $CaCl_2$ precipitation method at the same time,and from the same kidneys, as the Percoll preparations described above.The enrichment factors for the $CaCl_2$ brush border membrane preparation are included in Table V. LAP and ($Na^+$-$K^+$)-ATPase enrichments are comparable between the brush border membranes isolated by the different methods. The gamma-glutamyl transpeptidase and alkaline phosphatase enrichments, however, appear to be much lower in the membranes isolated by the Percoll method.

Enzyme assays and electron micrographs represent two criteria for determining the identity and purity of membrane fractions. Another criterion is the functional integrity of the membrane. The major function of the brush border membrane is the reabsorption of nutrients, such as glucose. The glucose uptake properties of the brush border membranes prepared by the Percoll vs.$CaCl_2$ techniques was very different. Results of transport studies using these vesicle preparations are shown in Fig.9. $Na^+$-dependent glucose uptake in the $CaCl_2$ prepared brush border membranes "overshoots" the equilibrium concentration. This phenomenom is characteristic of brush border membrane vesicles. The

Figure 9. Comparison of Glucose Uptake in Two Different Brush Border Preparations

Brush border membranes were prepared by a Percoll gradient method and by a $CaCl_2$ precipitation method from the same hog kidney. Both procedures are described in detail in the Experimental Procedures. Glucose uptake in the two different preparations was assayed twice. The results from both sets of assays are presented for comparison.

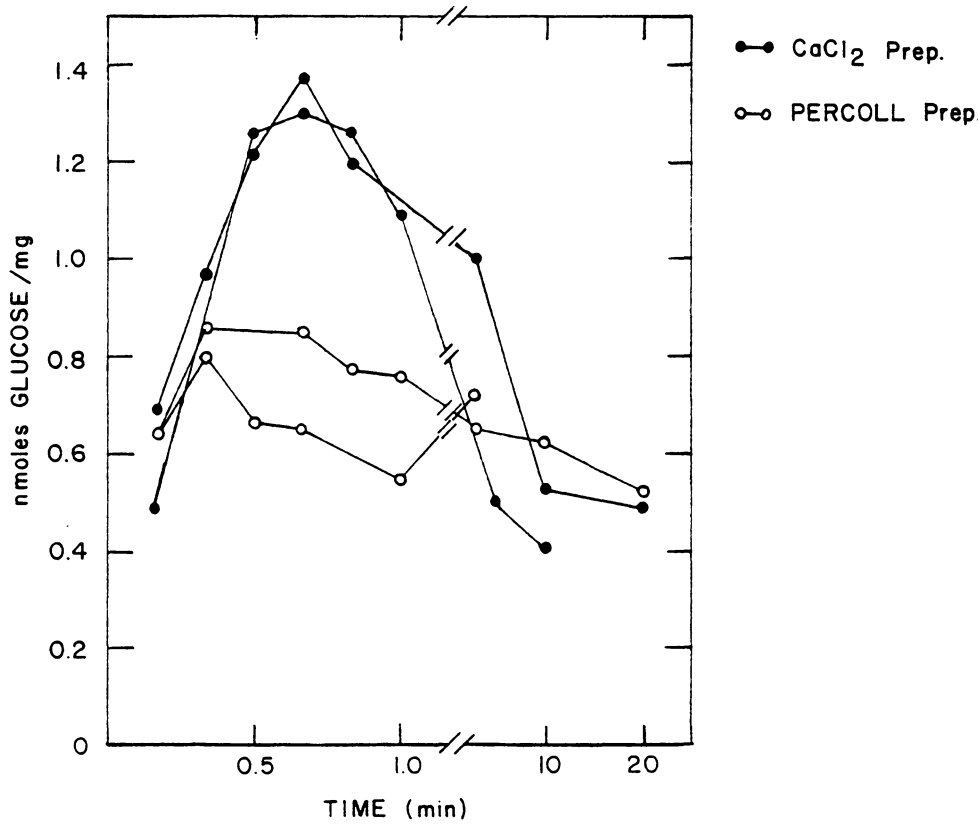
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
nmoles GLUCOSE/mg
0.5
1.0
10
20
TIME (min)
CaCl2 Prep.
PERCOLL Prep.

Figure 9.

Percoll prepared brush border membrane vesicles do not show a glucose uptake overshoot comparable to that of the $CaCl_2$ prepared membranes. For this reason, the $CaCl_2$ preparation was used for investigations of heavy metal effects on glucose uptake.

Glucose Uptake Into Brush Border Membrane Vesicles - Fresh hog kidney was often difficult obtain, and the combined time of collecting the tissue, preparing the vesicles, and performing the assays was quite long. The possibility of using frozen tissue in the preparation of the vesicles, or of freezing freshly prepared vesicles for assay later, was investigated. Fig. 10 illustrates the difference in the overshoot of glucose uptake between vesicles prepared from fresh tissue and from vesicles prepared from frozen-thawed tissue. The vesicles prepared from frozen tissue exhibited no overshoot of glucose uptake, indicating that they were not functionally intact.

The glucose uptake in freshly prepared vesicles, and in the same preparation after being frozen for one day, is compared in Fig. 11. Vesicles resuspended in mannitol buffer which had been frozen at -70°C for one day appeared to have a greatly reduced uptake of glucose. These comparisons were only made once, but the results discouraged any further investigations on determining effects of tissue storage.

Figure 10. Comparison of Glucose Uptake in Brush Border Membranes Prepared from Fresh Tissue and from Frozen Tissue

Brush border membranes were prepared by $CaCl_2$ precipitation from fresh pig kidney cortex and from the same tissue after freezing at -20°C for 20 hours. The glucose uptake in the two preparations is compared by subtracting the equilibrium value (20 min. uptake) of each preparation from all other values. This way only values which overshoot the equilibrium are shown and all other values are represented as 0 cpm. Both preparations had a protein content of 15 mg/ml.

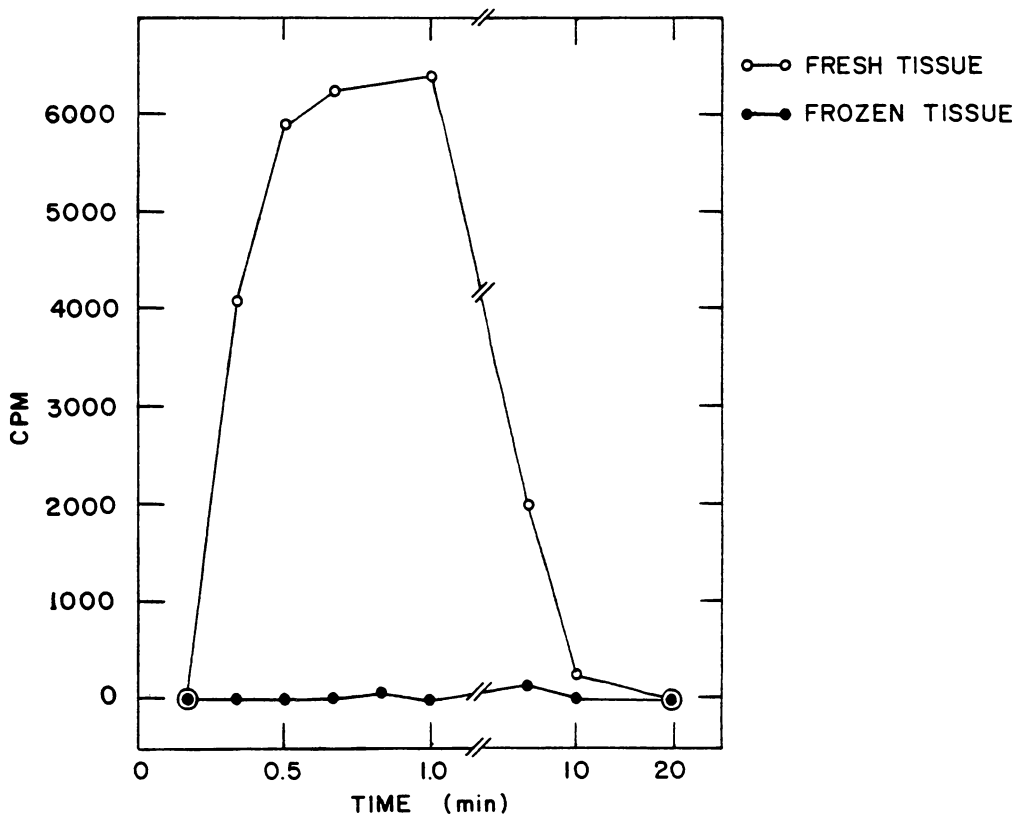
FRESH TISSUE
FROZEN TISSUE
CPM
6000
5000
4000
3000
2000
1000
0
0
0.5
1.0
10
20
TIME (min)

Figure 10.

Figure 11. Comparison of Glucose Uptake in Fresh and Frozen-Thawed Brush Border Membrane Vesicles

Brush border membranes were prepared by $CaCl_2$ precipitation and the glucose uptake was measured immediately and was measured again after the preparation had been frozen at -70°C for 20 hours. Only those values which overshoot the equilibrium are shown, and all other values are represented as 0 cpm.

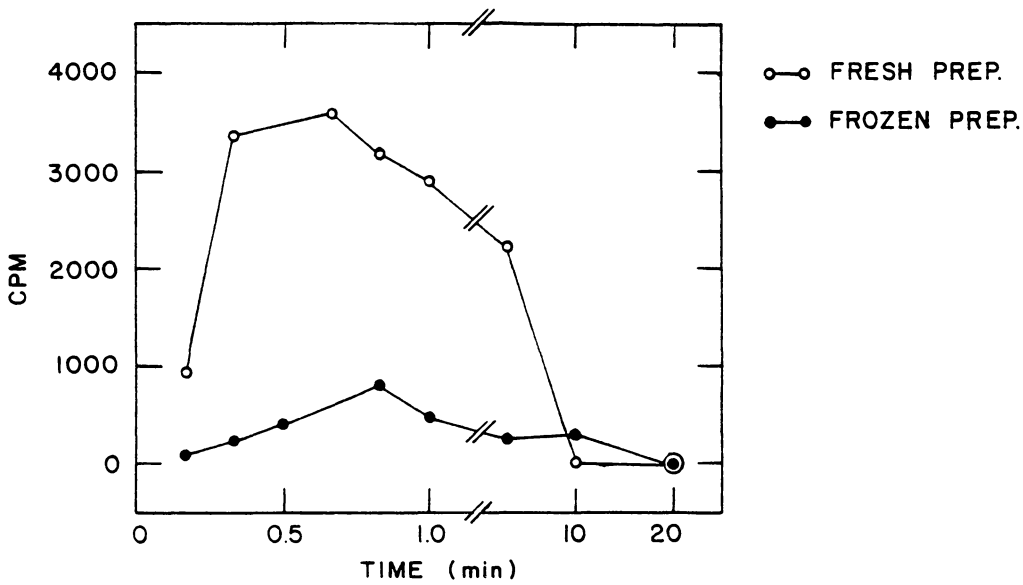
4000
3000
2000
1000
0
CPM
0
0.5
1.0
10
20
TIME (min)
FRESH PREP.
FROZEN PREP.

Figure 11.

Freshly prepared vesicles from fresh tissue were used for all of the succeeding studies.

Effect of Heavy Metals on Glucose Uptake - Initial results concerning the effects of cadmium on glucose uptake are shown in Fig.12. It appeared that 0.01 mM $Cd^{2+}$ had no effect on glucose uptake, whereas 0.1 and 1.0mM $Cd^{2+}$ had some inhibitory effect. The 10 and 20 min. uptake values indicated that 0.1 and 1.0 mM $Cd^{+2}$ may have reduced the glucose uptake equilibrium value. Later experiments, however, did not show this effect. Fig. 13 demonstrates that vesicles in the presence of cadmium reached the same equilibrium value for glucose uptake as did the control preparation. Again, 0.01 mM $Cd^{+2}$ had no effect on the uptake of glucose. The presence of 1mM $Cd^{+2}$ eliminated the overshoot. The 0.1 mM $Cd^{2+}$ seemed to have an inhibitory effect, but the range of values overlap those of the control. The values for glucose uptake after 50 sec. from initiation, where the highest uptake value occurred for most of the samples, along with the corresponding ranges for these values, are listed in Table VI. The value for the 0.1 mM $Cd^{2+}$ sample, again, seems much lower, but the upper range of this point overlaps with the lower range of the control value.

Considering both sets of data, the following

Figure 12. Effect of Cadmium on Glucose Uptake

Hog renal brush border membrane vesicles were prepared by $CaCl_2$ precipitation, and glucose uptake in the vesicles was assayed in the presence of different concentrations of cadmium chloride, as described in the Experimental Procedures. Each assay was performed twice on the same vesicle preparation and the average of the values of the two assays are presented.

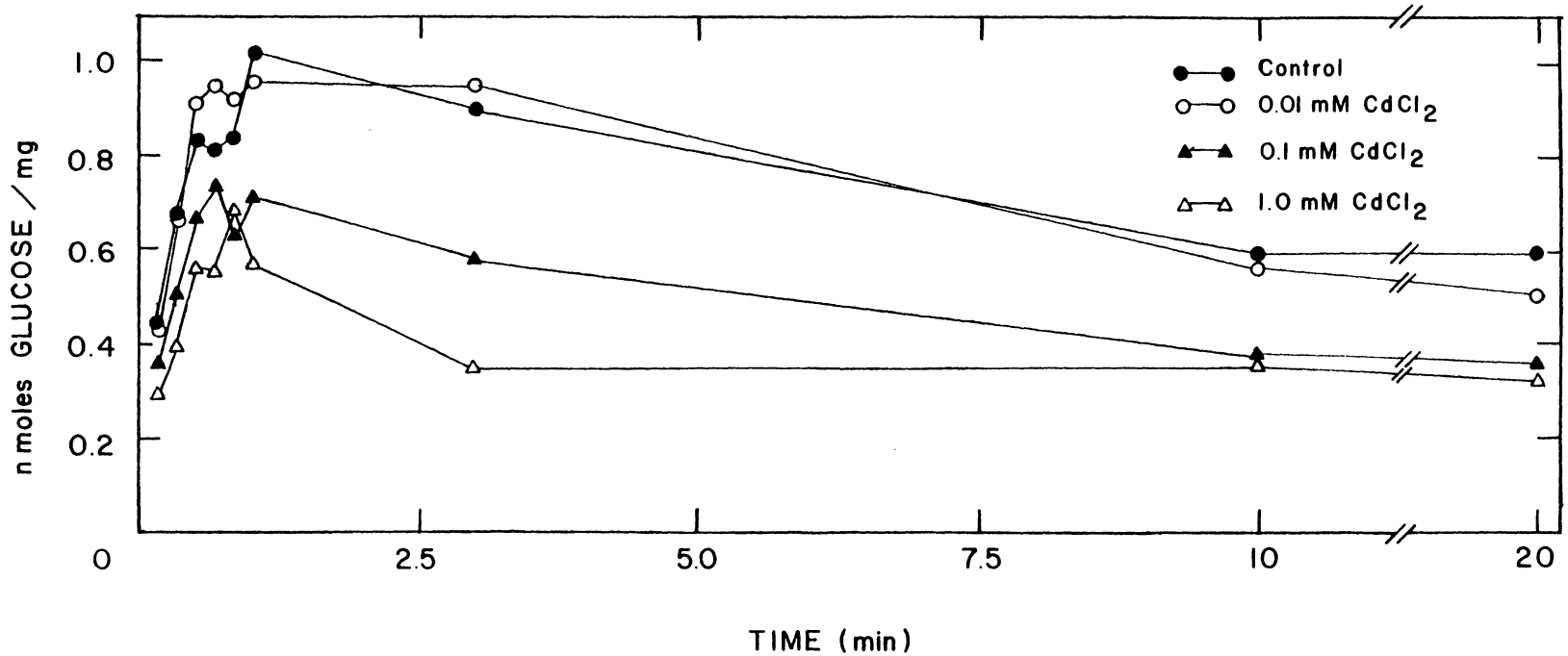
n moles GLUCOSE / mg
1.0
0.8
0.6
0.4
0.2
0
2.5
5.0
7.5
10
20
TIME (min)
Control
0.01 mM CdCl2
0.1 mM CdCl2
1.0 mM CdCl2

Figure 12.

Figure 13. Effect of Cadmium on Glucose Uptake

The experiment presented in Fig.12 was repeated on another brush border membrane vesicle suspension prepared in the same way.

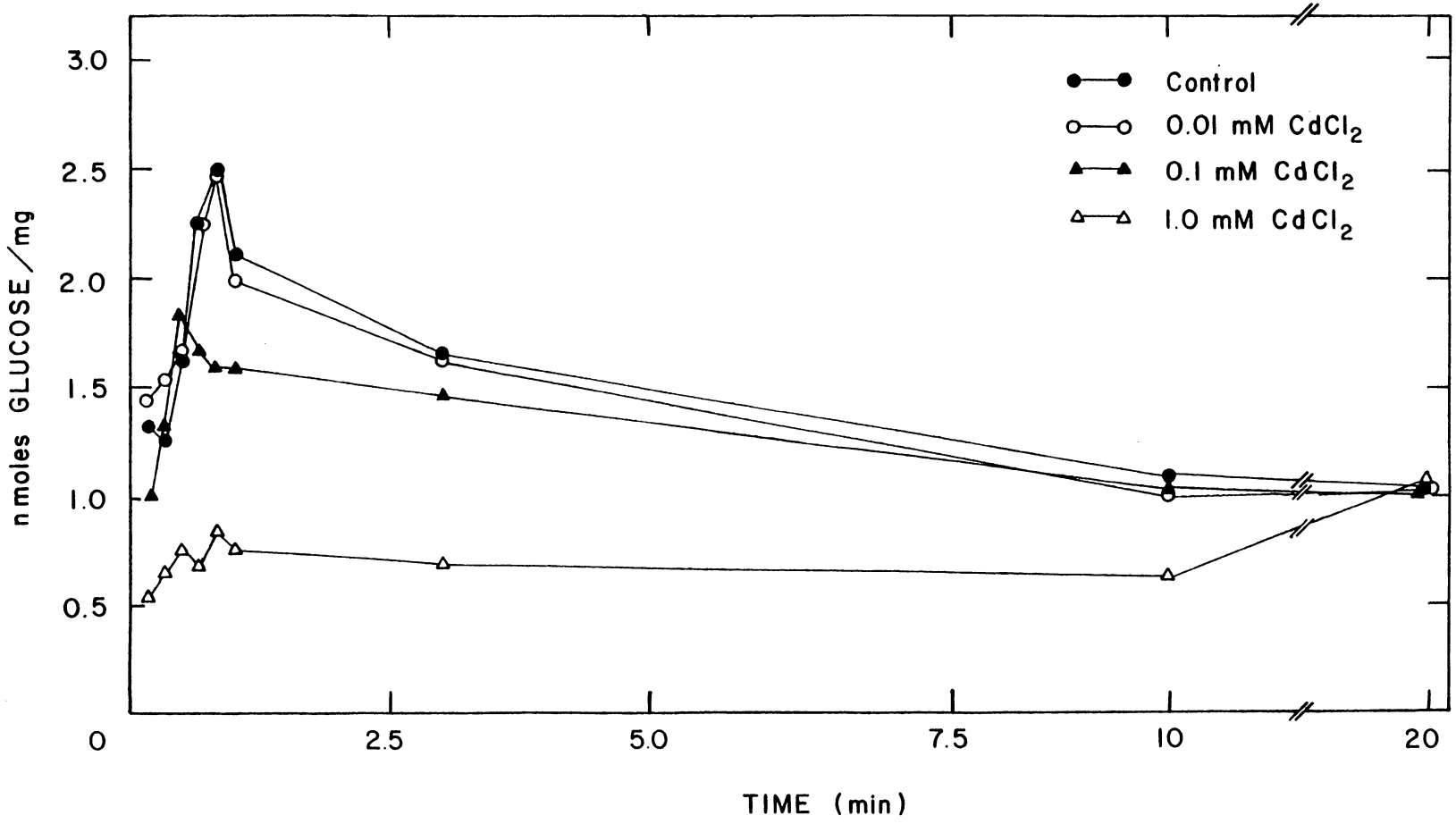
3.0
2.5
2.0
1.5
1.0
0.5
0
2.5
5.0
7.5
10
20
n moles GLUCOSE/mg
TIME (min)
Control
0.01 mM CdCl2
0.1 mM CdCl2
1.0 mM CdCl2

Figure 13.

Table VI. Effect of Metal Ions on Glucose Uptake

Brush border membrane vesicles were prepared from pig kidney cortex by the $CaCl_2$ precipitation method described in the Experimental Procedures. Glucose uptake was assayed as described in Experimental Procedures. The values obtained for glucose uptake after 50 sec. of incubation are listed. To discern effects on the $Na^+$-dependent overshoot of glucose uptake, the 20' equilibrium values were subtracted from the 50 sec. values and listed separately. All values are averages from two assays on the same membrane preparation $\pm$ the ranges for the values.

Table VI. Effect of Metal Ions on Glucose

| Sample | 50 sec. Glucose Uptake (nmol/mg) | 50 sec. Uptake Corrected For Equilibrium |
|---|---|---|
| Control | 2.5 ± 0.72 | 1.46 ± 0.74 |
| 0.01 mM $CdCl_2$ | 2.46 ± 0.25 | 1.44 ± 0.29 |
| 0.1 mM $CdCl_2$ | 1.58 ± 0.28 | 0.56 ± 0.29 |
| 1.0 mM $CdCl_2$ | 0.84 ± 0.09 | 0 |
| 0.1 mM $HgCl_2$ | 1.30 ± 0.39 | 0.23 ± 0.43 |
| 0.1 mM $ZnCl_2$ | 1.84 ± 0.27 | 0.84 ± 0.30 |
| 0.1 mM $CuCl_2$ | 3.18 ± 0.09 | 2.18 ± 0.10 |

conclusions are apparant: 1) 0.01 mM $Cd^{2+}$ has no effect on glucose uptake, 2) 1 mM $Cd^{2+}$ has a large inhibitory effect, and 3) 0.1 mM $Cd^{2+}$ may have a slight inhibitory effect.

To ascertain if the effect of cadmium on glucose uptake is specific for this metal, or is a general phenomenom seen with all metals, glucose uptake was monitored in the presence of 0.1 mM concentrations of zinc, cupric, and mercuric chloride. The results are shown in Fig.14, with the 50 sec. uptake values and the corresponding ranges for the values included in Table VI. Mercuric chloride causes a large reduction in glucose uptake at this concentration. Cupric chloride appears to enhance the uptake, but, again, the ranges of the values overlap with those of the control. The zinc chloride values also overlap those of the controls, discounting an inhibition of uptake. Considering these data, $Hg^{2+}$ has a much greater effect on glucose uptake than does $Cd^{2+}$. $Zn^{2+}$ and $Cu^{2+}$ appear to have little or no effect on glucose uptake at the concentrations used in these studies.

Effect of Cadmium on Brush Border Enzyme Activities - Table VII shows the effect of different concentrations of cadmium chloride on the specific activity of three different brush border enzymes. LAP was the enzyme most susceptible to the inhibitory effects of cadmium. LAP lost 90% of activity

Figure 14. Effect of Various Metal Ions on Glucose Uptake

Hog renal brush border membrane vesicles were prepared by $CaCl_2$ precipitation, and glucose uptake in the vesicles was assayed in the presence of mercuric, zinc or cupric chloride as described in the Experimental Procedures. Each assay was performed twice on the same vesicle preparation and the average of the values of the two assays are presented.

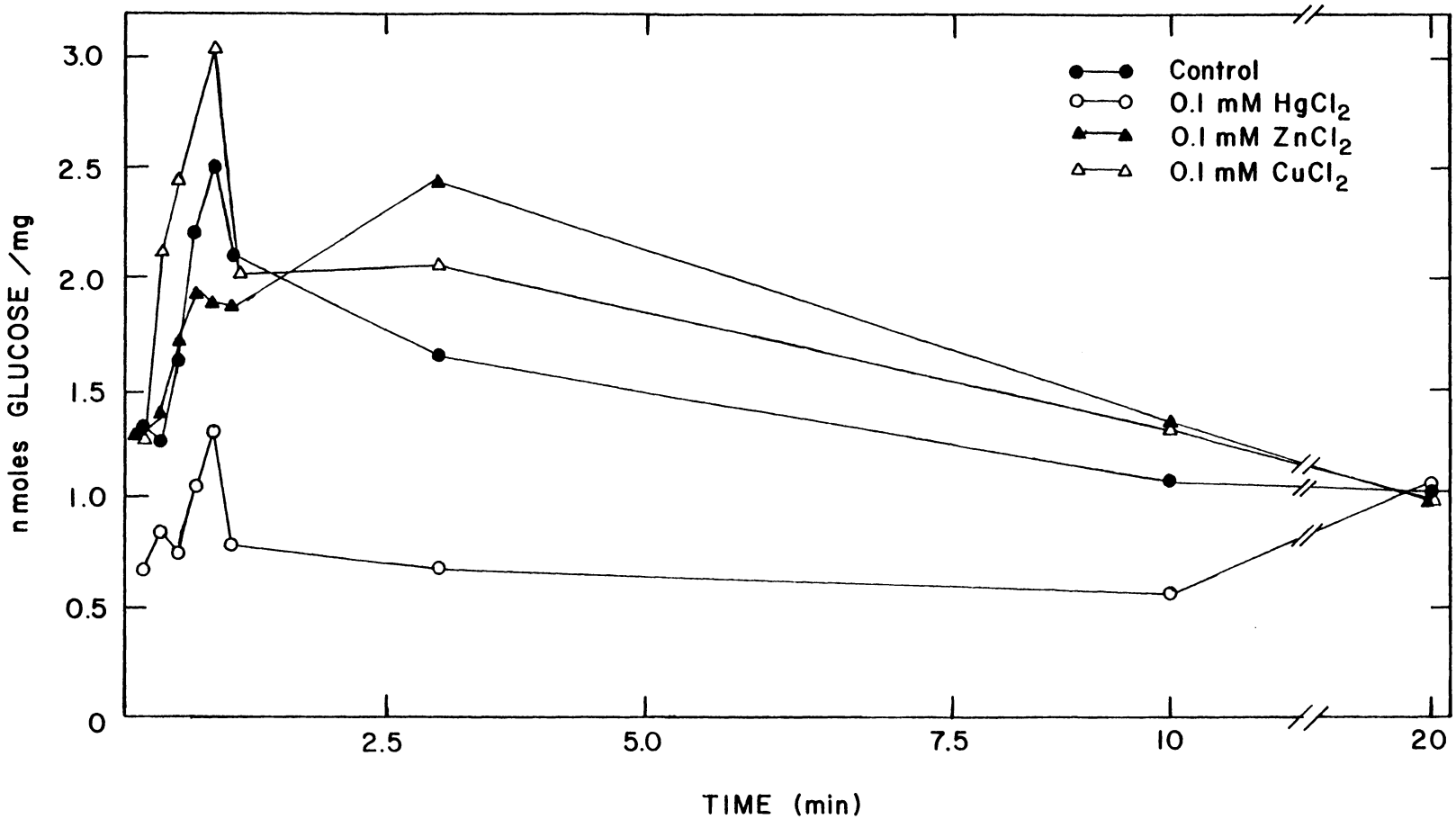
Control
0.1 mM $HgCl_2$
0.1 mM $ZnCl_2$
0.1 mM $CuCl_2$
n moles GLUCOSE /mg
3.0
2.5
2.0
1.5
1.0
0.5
0
2.5
5.0
7.5
10
20
TIME (min)

Figure 14.

Table VII. Effect of Cadmium on Enzyme Activities

Brush border vesicles were prepared from pig kidney cortex by the $CaCl_2$ precipitation method described in Experimental Procedures. Vesicles were assayed for enzyme activity in the presence of cadmium as described in the Experimental Procedures. Values were obtained from assays performed on the same vesicle preparation.

Table V11. Effect of Cadmium on Enzyme Activities

| | Leucine Amino-Peptidase (u/mg) | Gamma-Glutamyl Transpeptidase (u/mg) | Alkaline Phosphatase (u/mg) |
|---|---|---|---|
| Homogenate | 0.0158 | 0.0046 | 0.018 |
| Control Brush Border | 0.238 | 0.043 | 0.173 |
| 0.01 mM CdCl | 0.195 | 0.0408 | 0.104 |
| 0.1 mM CdCl | 0.026 | 0.032 | 0.141 |
| 1.0 mM CdCl | 0.0 | 0.0132 | 0.150 |

in the presence of 0.1 mM $Cd^{2+}$, and was completely inhibited in the presence of 1.0 mM $Cd^{2+}$. Gamma-glutamyl transpeptidase, however, lost only 20% of activity with 1.0 mM $Cd^{2+}$ present. Alkaline phosphatase activity appeared to show only very slight, if any, inhibition with even the highest concentration of $Cd^{2+}$ present. These results agree with observations made on the purified enzymes (Delange and Smith, 1971; Fernley, 1971). The specific activity of these enzymes in the homogenate is included to illustrate the enrichment of activity in the brush border membrane vesicles used for the transport studies.

# Discussion

Percoll Method of Simultaneous Isolation of Brush Border and Basal-Lateral Membrane Vesicles - A method is presented which appears to achieve simultaneous isolation of brush border and basal-lateral membrane vesicles. The results of the first procedure indicated an increase in the activity of the brush border marker enzyme, leucine aminopeptidase, of 6-10 fold over that of the homogenate, with a decrease in $(Na^+-K^+)$-ATPase activity. These enrichments are comparable to those reported by Smedt (1981) in pig kidney and Malathi et al. (1979) in rabbit kidney brush border membranes isolated by differential precipitation. LAP has been reported to be enriched by as much as 25-fold in rabbit kidney brush border membranes isolated by sucrose density gradients (George and Kenny, 1973), but this preparation also exhibited a 4-fold enrichment of $(Na^+-K^+)$-ATPase. The isolation method of Liang and Sacktor (1977), who used sucrose gradients to prepare both brush border and basal-lateral membranes, achieved 10-fold enrichment of two other brush border membrane marker enzymes in their preparation, but also had about 6-fold enrichment of $(Na^+-K^+)$-ATPase. Based on the enrichment of LAP and decrease in $(Na^+-K^+)$-ATPase, the brush border

membranes isolated by the method described in this thesis are comparable to those isolated by other methods, and are superior to the brush border membranes isolated by the simultaneous method of Liang and Sacktor(1977). These membranes did, however,appear to be contaminated with mitochondria, as evidenced by a small (3-fold) enrichment in succinate-cytochrome c reductase. This was a phenomenon not encounterd by other investigators. Although an in-depth electron microscopic survey was not done, the appearance of the brush border membranes is consistent with that seen by other investigators.

The basal-lateral membranes isolated by this procedure showed an average of 8-fold enrichment in ($Na^+$-$K^+$)-ATPase. Other investigators using Percoll to isolate basal-lateral membranes have seen 11-fold (Sacktor et al.,1981), and 21-fold (Inui et al.,1981) enrichments of ($Na^+$-$K^+$)-ATPase. Liang and Sacktor(1977),using sucrose density gradients for simultaneous isolation, obtained a 15-fold enrichment. The basal-lateral membranes isolated by the procedure described in this thesis also exhibited 3-fold enrichment of LAP and DNA. No other investigators saw this much enrichment of brush border membrane marker enzyme activity in their basal-lateral membrane preparations although Inui et al.(1981) did obtain a 2.2-fold enrichment of alkaline phosphatase. No data on DNA content is supplied by these investigators. The

BLMs isolated by this method appeared to have less enrichment of basal-lateral membrane marker enzyme activity and higher contamination by brush border membranes and DNA than those isolated by other researchers using the same or other techniques. The electron micrographs of this preparation, however, do resemble those reported by other investigators.

The modified procedure described in this text produced some interesting changes in the profile of these membranes (see page 59 for description of the modifications). The lower density gradient (8.3%),used to improve the crude basal-lateral preparation, effectively removed the DNA contamination from the basal-lateral membranes. This step resulted in an increase of about 45% in the enrichment of ($Na^+$-$K^+$)-ATPase, but also caused a 4-fold enrichment of LAP activity with alkaline phosphatase and gamma-glutamyl transpeptidase also enriched. The persistance of this high enrichment of brush border enzyme activity in the basal-lateral membranes, which look normal by electron microscopy, is not observed by other investigators and cannot be explained at this time. Further investigation is warranted.

The brush border membranes showed an increase in LAP enrichment and also in ($Na^+$-$K^+$)-ATPase. Four to five-fold enrichment of ($Na^+$-$K^+$)-ATPase is often seen in brush border

membranes prepared by procedures using sucrose density gradients (Berger and Sacktor,1970; Quirk and Robinson,1972; George and Kenny,1973). Four-fold enrichment of ($Na^+$-$k^+$)-ATPase has also been observed in brush border membranes isolated from human kidney by differential precipitation (Turner and Silverman, 1977). The brush border membranes prepared by CaCl precipitation from the same kidney as the membranes prepared by this Percoll procedure, exhibited similar LAP and ($Na^+$-$K^+$)-ATPase as those isolated by the Percoll procedure. The Percoll prepared membranes, however, differed markedly in gamma-glutamyl transpeptidase and alkaline phosphatase. Usually, alkaline phosphatase enhancement is comparable to that of the other brush border enzymes, but some investigators have reported large variations in alkaline phosphatase enrichment (Berger and Sacktor,1970). A comparison between different membrane preparations is shown in Table VIII.

In light of the questionable reliability of any of these enzymes as markers for brush border membranes, and of the possible effects that $Ca^{2+}$ may have on the activity of these membrane-associated enzymes, the Percoll prepared brush border membrane vesicles could be considered comparable in purity to the $CaCl_2$ prepared membranes. Comparison of tne glucose uptake characteristics of the two different preparations, however, indicate that the Percoll

Table VIII. Marker Enzyme Enrichments Reported for Membrane Preparations

The values shown are the relative enrichment of marker enzyme specific activity over that of the cortex homogenates. LAP = leucine aminopeptidase, GT = gamma-glutamyl transpeptidase, AP = alkaline phosphatase.

Table VII. Marker Enzyme Enrichments Reported for Membrane Preparations

| Membrane Fraction | Species | Isolation Method | ATPase Enrichment | BBM Enzyme Enrichment | Reference |
|---|---|---|---|---|---|
| Brush Border | Pig | Percoll | 0.93 | 7.95 (LAP) | Thesis |
| " " | Pig | $CaCl_2$ Precip. | 0.90 | 10 (LAP) | Smedt (1981) |
| " " | Rabbit | $CaCl_2$ Precip. | 0 | 9.4 (LAP) | Malathi (1979) |
| " " | Rabbit | Suc. Gradient | 4 | 25 (LAP) | George (1973) |
| " " | Rabbit | Suc. Gradient | 6.3 | 9.7 (GT) | Liang (1977) |
| Basal-Lateral | Pig | Percoll | 7.5 | 3.2 (LAP) | Thesis |
| " " | Rat | Percoll | 11.4 | 1.4 (GT) | Sacktor (1981) |
| " " | Rat | Percoll | 21.4 | 2.2 (AP) | Inui (1981) |
| " " | Rabbit | Suc. Gradient | 15 | 1.8 (GT) | Liang (1977) |

prepared brush border membranes may have impaired function. Enzymatic purity is, therefore,not always an indicator of functional integrity. It is not known at this time why the Percoll prepared membrane vesicles failed to accumulate glucose. Possible explanations could include: 1) $CaCl_2$ enhancement of glucose uptake, 2) $CaCl_2$ stabilization of the brush border membrane 3) impurity of the Percoll preparation. Further investigations on the effect, if any, of $CaCl_2$ on glucose uptake could help explain the difference in glucose uptake seen in these two preparations.

Effect of Freezing on Glucose Uptake - The use of freeze-thawed tissue to prepare BBMs resulted in the loss of the characteristic $Na^+$-dependent overshoot of glucose uptake. Freezing brush border membrane vesicles for assay the next day also resulted in an inability to take up the nutrient. Other investigators have reported similar effects of freezing on nutrient uptake into brush border membrane vesicles (Hittelman et al.,1981; Smedt and Kinne,1981). However, Turner and Silverman (1977) reported the presence of a glucose overshoot in brush border membranes prepared from a frozen-thawed crude plasma membrane preparation. Further investigation into the possibility of freezing membranes for assay later is needed before a final recommendation can be made.

Effect of Heavy Metals on Glucose Uptake and Enzyme Activity - As previously discussed in Results, cadmium affected the activity of LAP and AP in the brush border membranes in a manner similar to the effect of $Cd^{2+}$ on the isolated enzymes (Delange and Smith,1971; Fernley,1971).

Exposure to heavy metals has been implicated in proximal tubule damage, but to date, no investigations of heavy metal interaction with isolated proximal tubule brush border membranes have been reported. Studies with membranes isolated from intestine have shown that glucose uptake is inhibited by concentrations of HgCl as low as 0.01mM (Klip et al.,1980; Miller et al.,1980). The studies in this thesis show that renal brush border membranes are also very sensitive to $HgCl_2$ exposure. Cadmium, however, was less inhibitory than mercury. Other investigators have reported that glucose reabsorption in cadmium-exposed animals is not reduced as much as amino acid uptake (Gieske and Foulkes,1974). The role of accompanying ligands on the effect which heavy metals have on tubular reabsorption is also a factor to be considered in studies on the effects of these metals on membranes. Studies with intact animals have indicated that cadmium nephrotoxicity can be amplified by accompanying chelators,such as metallothionein and cysteine (Murakami and Webb,1981; Nordberg et al.,1975). Future investigations on metal toxicity using isolated membrane

vesicles could address this question.

## Summary

Simultaneous isolation of brush border and basal-lateral membrane vesicles from hog renal cortex was attempted by both sucrose and Percoll density gradient centrifugation. Successful isolation, based on the enrichment of marker enzymes, was not achieved with the sucrose density gradient procedure. A method utilizing the self-forming gradient capabilities of Percoll resulted in brush border and basal-lateral membrane fractions with 8 to 15-fold enrichment of marker enzyme activity. Based on the marker enzyme assays, the brush border membrane fraction exhibited no significant contamination by microsomes, lysosomes, or DNA, but did show a 3-fold enrichment in succinate cytochrome c reductase, a marker enzyme for mitochondria. This brush border fraction also exhibited 4-fold enrichment in $(Na^+-K^+)$-ATPase, a basal-lateral membrane marker enzyme which may also be present in the brush border. The basal-lateral membrane fraction exhibited no significant contamination by microsomes, lysosomes, mitochondria, or DNA. This fraction did, however, show enrichment of brush border marker enzyme activity. Electron micrographs indicated that both preparations have their characteristic appearances.

Glucose uptake into the brush border membrane vesicles prepared by the Percoll method was low, without the high overshoot characteristic of the brush border membranes prepared by $CaCl_2$ precipitation. This functional impairment in the Percoll-prepared membranes could be the result of contamination in the preparation, or perhaps it is the result of a need for $CaCl_2$ to stabilize the membrane. Another possibility is that high concentrations of $CaCl_2$ produce enhanced uptake in the $CaCl_2$ -prepared vesicles. Due to the low uptake in the Percoll-prepared membranes, brush border membranes prepared by $CaCl_2$ precipitation were used for studies of the effect of cadmium on glucose uptake.

It was found that brush border membrane vesicles used for uptake studies must be prepared from fresh tissue and cannot be frozen before use.

Cadmium was seen to have much less effect than mercury on glucose uptake. A concentration of 0.1 mM $CdCl_2$ reduced uptake slightly whereas the same concentration of mercury greatly reduced the uptake. A higher concentration of cadmium, 1 mM, effectively eliminated the glucose uptake overshoot. Concentrations of 0.1 mM of copper and zinc chloride had no effect on glucose uptake.

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# Appendix

Equation used to convert cpm to nmoles glucose :

[ (cpm - blank)/ efficiency]( 1/ 2.2 X $10^6$ dpm/uCi )
( nmol glu/ uCi ) ( 1/ % of total glu containing $^{14}$C-glu )
( 1 nmol/ 1 X $10^{-6}$ mMol).

Protein per aliquot :

x ml sample ( mg protein/ml ) ( 1/ 0.2 + x ml )
( 0.02 ml/ aliquot ).

# Epilogue

Based on enzyme activities, brush border and basal-lateral membrane vesicles were successfully isolated simultaneously by the Percoll method described in the text. Glucose uptake, however, was impaired in the brush border vesicles as compared to brush border vesicles prepared by CaCl precipitation. To determine if the difference in glucose uptake is due to impurity of the Percoll prepared vesicles, several experiments could be performed:

(1) Accurately determine how much $Ca^{2+}$ is associated with the CaCl -prepared membrane vesicles.

(2) Starting with Percoll-prepared brush border membranes, perform a CaCl precipitation and determine the effects such a procedure has on enzyme activity, glucose uptake, and amount of $Ca^{2+}$ associated with the membranes. If enzymatic purity is improved and $Ca^{2+}$ associated with the membranes is comparable to that of the CaCl prepared vesicles but glucose uptake is still impaired, then it can be postulated that the Percoll prepared vesicles have a unique disability, perhaps due to residual Percoll

particles.

If enzymatic and glucose uptake properties are improved, it can be assumed that the impairment was due to impurity of the preparation. Note, however, that $Ca^{2+}$ must have no effect on the enzymes tested in order to make this assumption

If enzymatic properties are not improved but glucose uptake is improved, then it would appear that $Ca^{2+}$ somehow enhances glucose uptake.

Further purification by varying the Percoll gradients is not very likely considering the efforts which have already been made and the amount of time which would be needed to pursue this avenue of research. By investigating the pattern of both the desired membranes and contaminating organelles in the gradients, however, one may be able to improve the isolation by altering the homogenization procedures. Again, this type of study could prove to be quite long.

Because of the interest in investigating metal ion effects on metal-free membrane vesicles, it might be of value to consider brush border membranes isolated by sucrose gradients. Many procedures have been published to

isolated these membranes using discontinous sucrose gradient or rate zonal centrifugation. Although only the brush border membranes can be purified at one time, these isolated vesicles would be metal-free and could be used to investigate the effects of metal ions on transportors in the brush border membrane.

Long-range studies on metal effects on nutrient reabsortion should most certainly include some research with the intact animal. Both injection and diet studies could be performed. Such studies have been done to investigate the metabolism of metals and effects of metals on nutrient reabsorption but no reports are yet published on correlating net reabsorption effects with effects on the transportors of the brush border membrane. After exposure of the animal to varying concentrations of metal for varying times, brush border membrane vesicles could be isolated and their nutrient transport characteristics measured. Eventually, several different transportors should be studied for a clearer picture of effects of metals on brush border membrane function.

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# Isolation of Hog Renal Brush Border Membrane Vesicles With Application to the Study of Cadmium Nephrotoxicity

by

Theresa R. Leverone

(ABSTRACT)

The epithelial cell of the kidney proximal convoluted tubule, like that of the small intestine, consists of two distinct plasma membrane surfaces referred to as the brush border, or luminal surface, and the basal-lateral, or contraluminal surface. Once isolated, membrane vesicles formed from the kidney epithelial cell can serve as models for the study of heavy metal nephrotoxin interaction with the two different plasma membrane surfaces. For ideal comparison between the two membranes, the isolation procedure should be identical for both. This has been accomplished by other investigators using differential centrifugation followed by free-flow electrophoresis or sucrose density centrifugation. Utilizing the self-forming gradient capabilities of Percoll, we have developed a less cumbersome procedure to effect simultaneous isolation of brush border and basal-lateral membrane vesicles. Brush border membrane vesicles isolated by this procedure exhibited 8 to 13-fold enrichment in marker enzyme

activity but appeared to have impaired glucose uptake activity. Since the Percoll-prepared vesicles seemed unsuitable for uptake studies, brush border membrane vesicles isolated by a differential precipitation method were used to investigate the effect of cadmium on glucose uptake. Of the concentrations of cadmium tested, 0.1 mM CdCl slightly inhibited glucose uptake whereas 1 mM CdCl greatly reduced glucose uptake in these vesicles.