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Chapter 12

MEMBRANE VESICLES

PIERRE PROULX

Department of Biochemistry
University of Ottawa
Ottawa, Ontario K1H 8M5, Canada

I. INTRODUCTION

Membranes from certain types of epithelial cells can be isolated in relatively pure form using simple procedures. In the process of their isolation, they reseal to form closed vesicular structures capable of carrying out transport and other physiological functions. This is the case of the highly polarized epithelial cells of tissues such as intestine and kidney tubules, which can be used to prepare brush border and basolateral membrane vesicles. In the case of brush border membranes, because of adherence of cytoskeletal elements to the membrane, the vesicles obtained remain right side out. Such preparations offer advantages over whole-tissue or whole-cell preparations since they allow measurement of processes which are specific for a single membrane type, unaltered by influences from other parts of the cell. The physical properties of these membranes can vary to some extent, depending on, for example, diet, a diseased state, or such factors as the vitamin D status of the animal. Differentiation, development, and age also influence these parameters. The lipid and fatty acid compositions as well as the fluidity of the membranes will change under these

different conditions and such changes may in turn affect fluidity, transport, and enzyme activities of the membranes (Proulx, 1991).

Such membrane vesicles can take up lipids when incubated with lipid micelles or liposomes, a process which usually results in important compositional and fluidity changes within these membranes. Thus, the membrane vesicle can be a useful tool not only for kinetic studies of membrane functions under a particular set of conditions but also to establish structure–function relationships in membranes under varied conditions. In effect, brush border and basolateral membrane vesicles have been widely used to study the kinetics of transport and the conditions for uptake of a great variety of substances including ions, sugars, amino acids, peptides, bile salts, fatty acids, cholesterol, other steroids, and other lipids, as well as vitamins. The influence of membrane fluidity and lipid composition on some of these transport phenomena as well as on enzyme activities have been extensively examined using such vesicle preparations. These studies have been generally very useful for the further understanding of the functioning of enzymes and transport systems in membranes (Proulx, 1991). Currently, the preparation and uses of brush border and basolateral membrane preparations will be illustrated with some underlining of recent studies involving vitamin transport and effects of vitamin D status on the properties of these membranes.

II. METHODS

A. PREPARATION OF INTESTINAL LUMINAL MEMBRANE VESICLES

Brush border membranes from a number of species have been conveniently prepared by methods based on that reported by Kessler *et al.* (1978). In our case, female New Zealand white rabbits, weighing 4–6 lb and deprived of food for 12 hr prior to sacrificing were routinely used. The tissue from two to four animals is rinsed free of contents with ice-cold saline, everted, and further washed with saline. At this point it can be frozen and stored at −20°C or processed immediately. The epithelial cells are removed by scraping the luminal surface with a microscope slide and homogenizing the cells for 30 sec in a Waring blender using 15 vol of 0.05 *M* mannitol in 2 m*M* Tris–HCl, pH 7.4. After centrifugation for 10 min at 10,000*g*, the supernatant is kept and the pellet is resuspended in 7 vol of mannitol solution and homogenized for 1 min. Following centrifugation, this supernatant is pooled with the first and filtered through a cheesecloth. Solid $CaCl_2$ is added to the filtrate to a final concentration of 10 m*M* and after standing

in an ice bath with occasional stirring for 20 min, the suspension is centrifuged for 10 min at 4000*g*. The pellet is discarded and the supernatant, subjected to another centrifugation (40,000*g* for 15 min). The pellet obtained is resuspended in 100 m*M* mannitol–10 m*M* Hepes adjusted to pH 7.4 with Tris (4 ml/g of original scrapings) and is homogenized with a Potter glass/Teflon homogenizer by applying 10 strokes up and down at 1200 rev/min. After centrifugation of the homogenate at 40,000*g* for 15 min, the pellet is resuspended in mannitol/Hepes solution to yield 34 mg protein/ml. To guard against protease degradation, leupeptin (1 μg/ml), aprotinin (0.12 tyrosine inhibitor units/ml), and phenylmethylsulfonyl fluoride (30 μM) are added to all solutions used in the preparation. Vesiculation of the membranes and the absence of organelles such as mitochondria are verified by electron microscopy. The purity of the fraction can be further assessed by determining the increase in specific activity of the brush border marker enzymes, sucrase and alkaline phosphatase, and the lack of increase of specific activity for enzyme markers of other membrane or organelle fractions (Aubry *et al.*, 1986). In the isolation procedure for brush border membranes, the use of 10 m*M* Mg^{2+} instead of 10 m*M* Ca^{2+} for precipitating undesired organelles has been prescribed as a means of avoiding phospholipid breakdown by phospholipase A (Hauser *et al.*, 1980). However, in our experience, the use of Ca^{2+} does not significantly enhance phospholipid breakdown, at least when fresh tissue is used, and Mg^{2+} precipitation yields membranes more highly contaminated with basolateral membranes (Aubry *et al.*, 1986).

B. PREPARATION OF INTESTINAL BASOLATERAL MEMBRANE VESICLES

The procedure used is that of Scalera *et al.* (1980). Epithelial cells from one rabbit or two or three rats are isolated as indicated for the isolation of brush border membrane vesicles and suspended in 50 ml of buffer containing 250 m*M* sucrose, 10 m*M* triethanolamine hydrochloride (pH 7.6), and protease inhibitors. The suspension is homogenized for 3 min in a Waring blender and diluted 1 : 2 with sucrose buffer. This homogenate is centrifuged at 2500*g* for 15 min and the pellet is discarded. The supernatant is then centrifuged at 20,500*g* for 20 min. The resultant fluffy layer of the pellet is resuspended in 35 ml of sucrose buffer and homogenized in a glass/Teflon Potter homogenizer (20 strokes at 1200 rev/min). The membrane suspension (31.5 ml) is mixed with 3.5 ml of Percoll (final concentration 10%) by homogenizing as before with two or three strokes at 1200 rev/min. The suspension is then centrifuged at 48,000*g* for 1 hr. The Percoll gradient is fractionated from the top by pumping 60% sucrose to the

bottom of the centrifuge tube. After removal of the first 12 ml, basolateral membranes are found in a fraction comprising the next 3 ml. After pooling the fractions from several tubes, the basolateral membranes may be collected by centrifugation at 48,000*g* for 30 min. The membranes are obtained as a fluffy layer on top of a very solid Percoll pellet. These membranes are then be suspended in sucrose buffer. The vesiculation and purity of the fraction is assessed by electron microscopy and by determining the increase in specific activity of the basolateral marker enzyme, Na^+–K^+-ATPase, and the lack of enrichment of activities for marker enzymes of other organelle fractions (Hauser *et al.*, 1980).

C. TRANSPORT STUDIES

Isolated vesicles have been widely used for the study of transport studies. The method relies on the membrane filtration technique enabling measurements of uptake in the seconds to minutes range. For this purpose nitrocellulose filters with a pore size of 0.45 μm are very often used and apparati with a series of suction operated filtration ports are commercially available. For short intervals, the membrane suspension and the radiolabeled permeant solution can be added to separate parts of the filter, then mixed, and, after a given interval, inactivated by rapid dilution with unlabeled permeant and quick filtration, followed by washing of the filter membrane. The filter membranes can be efficiently counted directly by liquid scintillation in the presence of PCS (Amersham). Alternatively, as in the case of Ca^{2+} uptake, the reaction can be terminated by addition of an inhibitory ion such as La^{3+}, followed by filtration and washing (Merrill *et al.*, 1986). The normal functioning of membrane vesicles is often ascertained by testing whether they can carry out active processes such as Na^+ gradient-dependent transport in the case of brush border membranes. For example, when intestinal brush border membranes are incubated in the presence of labeled glucose and 150 m*M* NaSCN, a typical overshoot phenomenon is seen (Fig. 1). Again illustrating this point, a similar overshoot phenomenon is noticed when, for example, pyridoxine is incubated with rat kidney brush border membrane vesicles in the presence of a large initial gradient of NaSCN (Bowman *et al.*, 1990). As the gradient becomes dissipated, the rate of permeant entry falls to equilibrium levels. To distinguish between uptake by binding to the membrane and true transport into the intravesicular space, the effect of increasing osmolarity on the process is verified. For example, adding 300 m*M* cellobiose to intestinal brush border membranes very markedly decreases the uptake of glucose resulting from a shrinkage of the intracellular space (Fig. 1). Similarly, for example, the equilibrium uptake of thiamine by rat small intestinal basolateral membrane vesicles

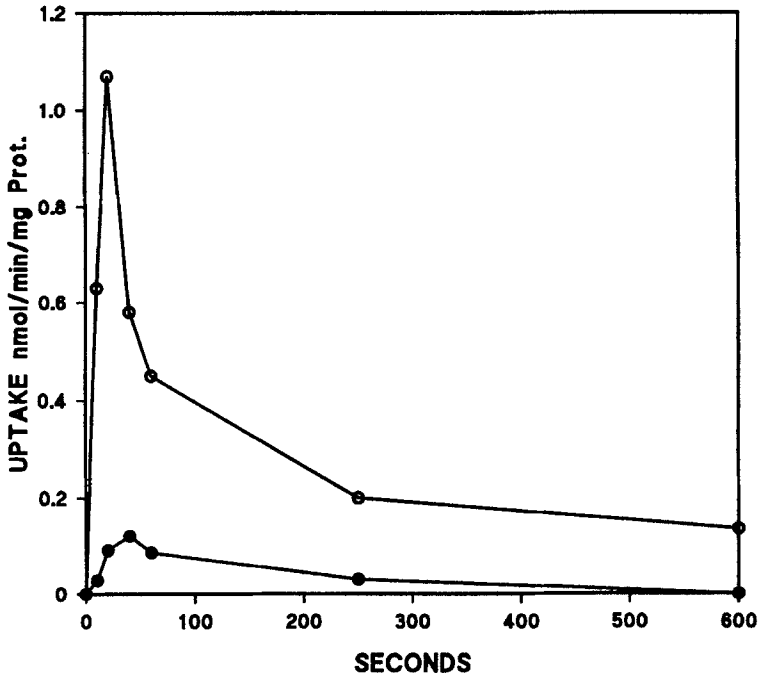

FIG. 1. D-Glucose uptake by intestinal brush border membranes and the effect of osmolarity. Brush border membranes (110 μg protein) were incubated for various times at 25°C with 200 μM [^{3}H]glucose, 150 mM NaSCN, 100 mM mannitol, 1 mM Hepes/Tris buffer, pH 7.5, in 40 μl. The vesicular uptake was terminated by a 25-fold dilution with ice-cold stop solution consisting of 100 mM mannitol, 150 mM NaCl, 0.2 mM phlorizin, and 55 $MgCl_2$ in 10 mM Hepes/Tris buffer, pH 7.5. The uptake was measured in the presence of 300 mM D-cellobiose (closed circles) or in its absence (open circles).

was decreased by increasing the osmolarity of the incubation medium with saccharose (Laforenza *et al.,* 1993).

Some recent studies on vitamin transport using membrane vesicles include those of vitamin B6 by rat kidney brush border membranes (Bowman *et al.,* 1990), ascorbic acid by teleost intestinal brush border membranes (Maffia *et al.,* 1993), biotin by human kidney brush border membranes (Baur and Baumgartner, 1992), pantothenate by human placental brush border membranes (Grassl, 1992), folate and riboflavin by rabbit intestinal brush border membranes (Said and Mohammadkhani, 1993a,b; Said *et al.,* 1993), and thiamine by rat small intestine basolateral membranes (Laforenza *et al.,* 1993). Bile acid transport in human placental, rat ileal, and rabbit small intestinal brush border membrane vesicles (Dumaswala *et al.,* 1993; Gong *et al.,* 1991; Kramer *et al.,* 1993) and the effect of vitamin D status

on Ca^{2+} uptake in chick intestinal basolateral, chick and pig intestinal brush border, and rabbit renal basolateral and brush border membrane vesicles have also been recently examined (Takito *et al.*, 1992; Kaune *et al.*, 1992; Boutiauy *et al.*, 1993). The use of membrane vesicles in these cases has helped elucidate mechanisms of transport, i.e, Na^+-gradient dependency or other requirements, kinetic parameters, etc. Results from such studies are more easily interpreted because they are attributable to the components of a single membrane type. Composite mechanisms resulting from interacting components of tissue or whole-cell preparations are more difficult to assess.

D. LIPID UPTAKE AND STRUCTURAL STUDIES

The lipid composition of membrane vesicles can be studied as a function of conditions displayed *in vivo* in relation to lipid dietary supplementation, vitamin D status, diabetes or other diseases, age, topography, etc. Such changes are characterized by alterations in the transition temperatures and fluidity of the membrane, as measured by fluorescence anisotropy, for example, and breaks in the Arrhenius plots of transport or enzyme activity data (Proulx, 1991). Other types of studies have indicated that the lipid composition of vesicular membranes can be altered directly by interacting isolated membranes with fatty acids, cholesterol, and complex lipids containing different polar headgroups (Proulx, 1991). The lipids are added either as micelles or liposomes and the treated membranes can be isolated by centrifugation and washed free of loosely adhering lipids with buffer or buffer-containing detergent (Proulx *et al.*, 1984). Filtration methods for such studies are to be avoided unless it can be established that the membrane filters used do not themselves markedly bind the lipid under study. Nitrocellulose filter membranes, for example, were found to be generally inappropriate for examining lipid uptake by membrane vesicles.

Such studies lend themselves to establishing structure–function relationships in membranes. For example, modifying the fatty acid composition of brush border membrane lipids by *in vitro* uptake of unsaturated fatty acids or their methyl esters decreases the fluorescence anisotropy of diphenylhexatriene-labeled brush border membranes (i.e., increases their fluidity, Fig. 2) while increasing their rate of Ca^{2+} uptake (Table I). In fact many investigations have dealt with attempts to establish relationships between lipid composition, fluidity, and enzyme or transport activities using membrane vesicles (Proulx, 1991). Recent studies typifying this approach are those of Ramsammy *et al.* (1993), using renal brush border and basolateral membranes isolated from normal and streptozotocin-induced diabetic rats, and

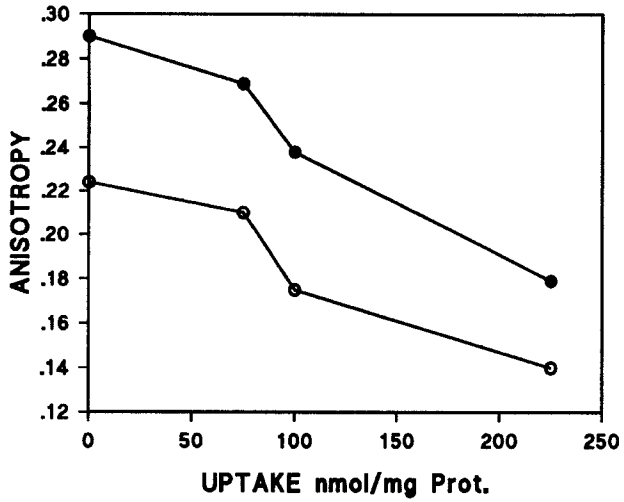

FIG. 2. The effect of methyloleate uptake on anisotropy of diphenylhexatriene-labeled brush border membranes. Conditions for methyloleate uptake and fluorescence anisotropy measurements were as described (Merrill *et al.*, 1987). The horizontal axis represents amounts of methyloleate taken up. Anisotropy values are reported for measurements at 20°C (closed circles) and 37°C (open circles).

Dudeja *et al.* (1991), using rat small intestine brush border membranes treated with benzyl alcohol.

III. DISCUSSION

Antipodal membranes of epithelial cells have been widely used for transport studies and recently an increasing number of investigations have dealt with mechanisms of vitamin and nutrient uptake using these preparations. With such preparations, however, rate measurements would be influenced by the presence of a substantial portion of vesicles, leaky to low molecular weight molecules. Accordingly it has been shown that actin, a marker of the cytosolic side, can be labeled by a number of reagents of molecular weights of up to 700 Da. Also studies comparing the compartmentation of radiolabeled sugars, inulin, and inulin carboxylic acid indicated that a maximum of 25% of the vesicles were sealed to small molecules (cf. references cited in Proulx, 1991). It is likely that a similar problem of leakiness exists with basolateral membrane vesicles. Nonetheless, results from many investigations based on kinetic and structural studies attest that the use of such vesicle preparations has greatly helped in elucidating mechanisms of

TABLE I

EFFECT OF METHYL OLEATE ON Ca^{2+} UPTAKE

Methyl oleate concentration (m*M*)	Lipid uptake (nmol/mg protein)	Rate of Ca^{2+} uptake (percentage of control)[a]
0.010	14 ± 1	118 ± 7
0.050	68 ± 4	124 ± 8
0.090	101 ± 4	133 ± 9
0.120	119 ± 8	143 ± 7
0.180	182 ± 10	156 ± 10
0.180[b]	220 ± 21	207 ± 29

[a] The control uptake was 7.5 ± 1.5 nmol/mg protein per 5 min. All methyloleate values represent incubations of 1 hr with membranes except for *b*, in which case incubations were for 4 hr. The results represent the mean ± SE from 6 to 20 determinations with membranes from three to five rabbits.

uptake in one or the other of the antipodal membranes and has increased our knowledge of how structural factors such as fluidity influence functional parameters.

In particular, the use of intestinal brush border membranes has offered much potential for the detailed investigation of absorptive phenomena. It is likely that the absorption of lipid nutrients including fat-soluble vitamins for example, which may intercalate transiently into the bilayer and affect the physical properties of the membrane, will concurrently influence the entry of other substances. Alternatively, interactions between nutrients in the gut might be expected to mutually influence absorption of these substances. Also, there may be competition between nutrients for the same transport system. Membrane vesicles are very much suited for studying this type of problem and their use has helped resolve, for example, the mechanism whereby lecithins inhibit cholesterol uptake (Merrill *et al.*, 1987). Again, the use of isolated brush order membranes has been conducive to a better understanding of the mechanisms of interaction between lipid micelles or lipid vesicles and the intestinal luminal membrane.

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