Isolation of Intestinal Brush-Border Membranes

This unit describes how to prepare isolated brush-border membrane vesicles from the small intestine of rat, pig, and cow. The procedure is based upon cation precipitation which causes intracellular organelles to coalesce and takes ~3 hr to complete (Hopfer et al., 1973). Membranes isolated in this way can be used for the identification of integral membrane proteins found at the apical pole of the intestinal epithelium or for transport assays because the microvilli spontaneously form sealed membrane vesicles. The technique does not require much specialized equipment apart from a high-speed centrifuge and a Polytron homogenizer. The enrichment of apical membrane proteins is about twenty-fold and will be a product primarily of the enterocyte (absorptive cell) population which makes up ~80% of the cells. There will also be a small contribution from endocrine and mucus-secreting cells.

The Basic Protocol describes isolation of brush-border membranes from rat, while Alternate Protocols 1 and 2 describe isolation of the same material from pig and cow intestines, respectively. A Support Protocol describes methods for assessing the enrichment of the membrane fraction.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: Maintain all solutions at 4°C during preparations. Increased stability of rat membranes has been reported by including phospholipase inhibitors in the initial homogenization medium (Maenz et al., 1991), and the inhibitors should also be used in the initial homogenization of pig and cow membranes.

ISOLATION OF INTESTINAL BRUSH-BORDER MEMBRANES FROM RAT

BASIC PROTOCOL

This protocol provides the procedure for taking mucosal scrapings from the small intestine and isolating the apical (brush-border) membranes (see Fig. 3.21.1) from the epithelial cells with minimal contamination of intracellular or basolateral membranes.

Materials

Anesthetic (e.g., sodium pentobarbital; MTC Pharmaceuticals, Ontario, Canada)
Rats (e.g., male Sprague-Dawley rats, ~300 g)
Isotonic saline (0.9% w/v NaCl) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), ice-cold
Water, ice cold
Rat collection solution A (see recipe)
Formaldehyde
100 mM $MgCl_2$
Rat homogenization solution B (see recipe)
Final suspension medium (see recipe)

1-ml syringe with 26-G needle
Surgical instruments for laproscopic surgery: scissors and forceps
30 × 20–cm Plexiglas or other flat plate, chilled on ice
Glass microscope slides, chilled on ice

Contributed by Chris I Cheeseman and Debbie O'Neill
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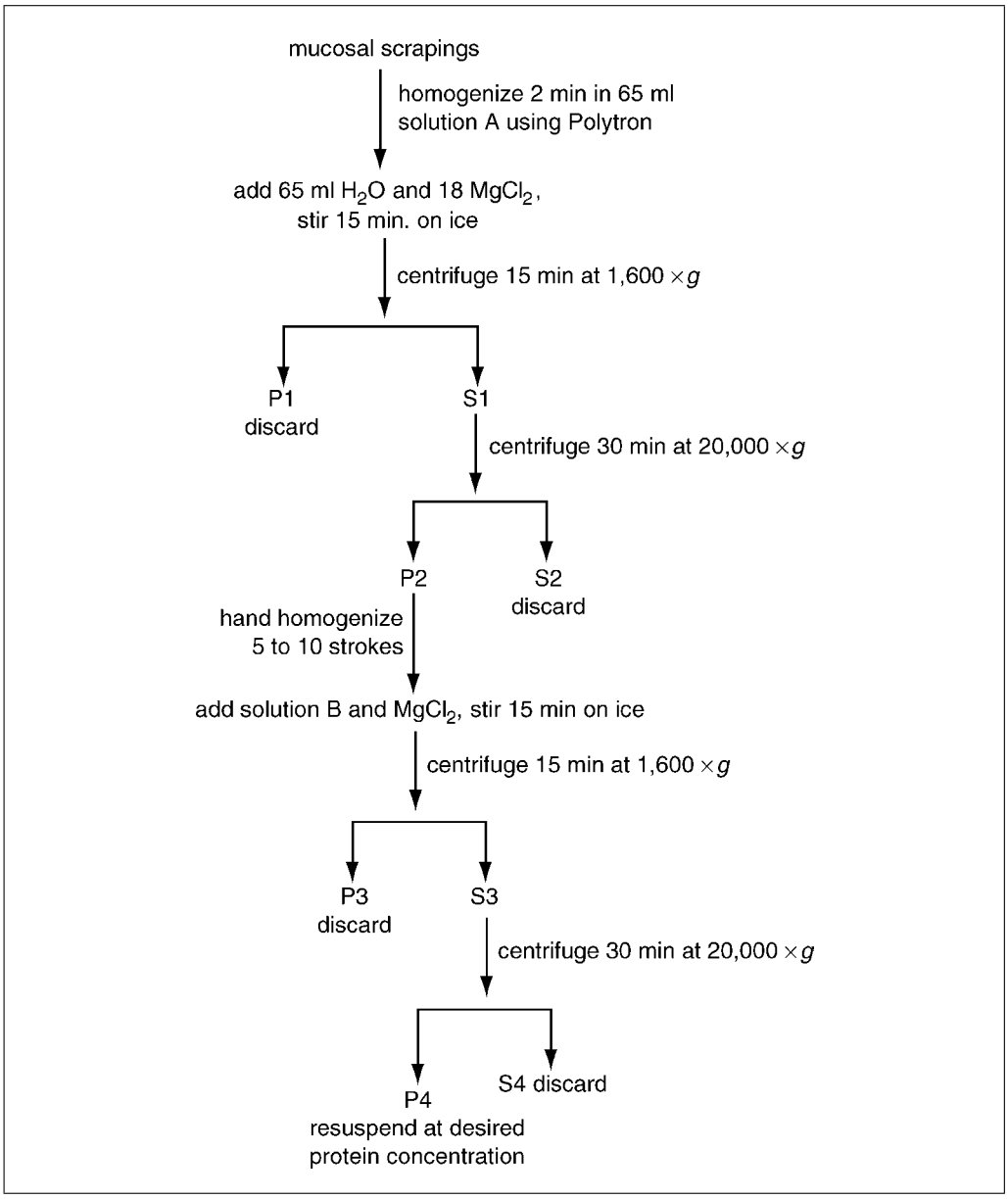

Figure 3.21.1 Flow chart for procedures to isolate rat intestinal brush-border membranes. Pig and bovine preparations differ in the details (see text).

100- and 250- ml beakers
Polytron homogenizer (Brinkmann) or equivalent
Magnetic stirrer and stir bar
50-ml polycarbonate centrifuge tube
High-speed centrifuge capable of generating 20,000 × *g* and handling samples up to 50 ml
15-ml hand held glass homogenizer
Additional materials for determination of protein concentration (*APPENDIX 3H*) and fixing, sectioning, and staining tissue for light microscopy (optional)

Prepare rat intestinal mucosa

1. Anesthetize each rat using sodium pentobarbital, 45 mg/kg body weight, for intraperitoneal injection with a 1-ml syringe with 26-G needle.

   The strain, weight, and sex of the rats will vary, depending on the objectives of a particular study.

The authors use Somnotol (sodium pentobarbital), but the choice of anesthesia will depend upon the jurisdiction and local animal regulations.

If the animals are fasted overnight there will be less lumenal content and the remaining material can be removed more easily. However, it must be remembered that fasting may change the expression of surface enzymes and integral membrane proteins.

2. Expose the small intestine by laproscopic surgery, allowing for blood flow to be maintained.

   Rat intestine is very sensitive to anoxia, and membranes are best prepared from tissue that has the blood flow interrupted as late as possible.

3. Identify the region of interest (small intestine) and make small incisions at both ends of the required section (normally no longer than 30 cm).

   The best way to remove the epithelium from the underlying tissues (muscle, neuronal, and connective tissue) is to take mucosal scrapings (see below).

4. Flush the lumen gently, in situ, with ~15 to 30 ml ice-cold isotonic saline containing 0.1 mM PMSF.

   It is necessary to cool the tissue in this way while maintaining the blood flow in order to reduce stress and avoid possible reinternalization of membrane proteins. It is essential to have the protease inhibitor present at this initial step or the resulting isolated membranes will degrade very rapidly. It is also essential that cooling proceed rapidly and that no delays occur once this procedure has started.

5. Remove the section of intestine from the animal and place it on an ice-cold plate.

   A piece of Plexiglas, sitting on ice, will serve this purpose very well.

   The rat is killed, after the dissection with an anesthetic overdose.

6. Open up the intestine along its antimesenteric border, i.e., along the surface opposite to the blood and nerve supply.

7. Lay this sheet of tissue flat, but avoid touching the exposed mucosal surface.

Collect and homogenize mucosal cells

8. Scrape off the mucosa using an ice-cold glass slide held at a 45° angle.

   This is best done using short strokes.

9. Shake the collected cells into a 100-ml beaker containing 65 ml of collection solution A. Proceed along the length of the sheet of intestine.

   This procedure takes practice to achieve the degree of harvesting required. A light pressure and a few strokes will result in harvesting only the villus tips. More pressure and a greater number of strokes will remove cells from further down the villi towards the crypts.

10. Determine the extent of harvesting the epithelium by fixing the remaining tissue overnight with formaldehyde, sectioning (with basic paraffin sectioning technique), staining with hematoxylin and eosin, and examining by conventional light microscopy.

    Microscopic examination need not be performed for every preparation but is useful when establishing the technique. Only the villi should be harvested without cells being taken from the crypts.

11. Homogenize the cell suspension from step 9 using a Polytron homogenizer 2 min at setting 5, on ice.

    For enrichment assays (see Support Protocol) hold back a 1-ml sample of this initial homogenate.

Precipitate intracellular and basolateral membranes

12. Add 65 ml ice-cold double distilled water and 18 ml of 100 mM $MgCl_2$ to the homogenate. Keep this solution on ice for 15 min and gently stir continuously using a stir bar and a magnetic stirrer.

    The magnesium will cause a large proportion of the intracellular and basolateral membranes to aggregate.

13. Pellet these membranes by centrifuging the solution for 15 min at 1600 × *g*, 4°C.

14. Collect the supernatant (S1 in Fig. 3.21.1) in a 50-ml centrifuge tube. Discard the pellet (P1). Centrifuge S1 30 min at 20,000 × *g*, 4°C. Discard the resulting supernatant (S2).

15. Homogenize the pellet (P2) with a hand-held 15-ml glass homogenizer in 2 to 3 ml homogenization solution B, using 10 strokes.

16. Add up to 30 ml homogenization solution B and repeat the magnesium precipitation step by adding 4 ml of 100 mM $MgCl_2$ to the suspension and stirring on ice for another 15 min.

17. Pellet the aggregated membranes by centrifuging 15 min at 1,600 × *g*, 4°C. Collect the supernatant (S3) and discard the pellet (P3).

Isolate brush-border membranes

18. Pellet the brush-border membranes in S3 by centrifuging this supernatant 30 min at 20,000 × *g*, 4°C.

19. Finally, resuspend the resulting pellet, P4, in the required final resuspension medium and determine the protein concentration using standard protocols (see *APPENDIX 3H*).

    These isolated brush-border membranes spontaneously form vesicles with a trapped volume normally of about 1.5 to 2.0 μl per mg protein.

    If these vesicles are to be used for rapid filtration flux studies a maximum protein concentration of 8 mg/ml is recommended or the filters clog, lengthening filtration time and making flux assays inaccurate.

20. Proceed to the Support Protocol to analyze membrane enrichment.

    Membranes can now used for enzyme assays, flux studies with rapid filtration, or for protein identification with (western) immunoblotting.

SUPPORT PROTOCOL

ASSESSMENT OF MEMBRANE ENRICHMENT

There are several methods which can be used to determine the degree of enrichment of the resulting membrane preparation, most of which rely on enzyme assays. Sucrase-isomaltase and alkaline phosphatase are both enzymes associated with the intestinal brush-border membrane. Assays for these enzymes can be used to determine the level of enrichment of the isolated membranes (Fredricksen and Wirsen, 1956; Tsuboi et al., 1985). Normally, the degree of enrichment is about fifteen- to twenty-fold compared to the original homogenate.

ALTERNATE PROTOCOL 1

ISOLATION OF INTESTINAL BRUSH-BORDER MEMBRANES FROM PIG

This is a modification of the procedure employed for rat tissue. It requires slightly different solutions and takes account of the greater yield. Pig BBM vesicles are ideal for use in rapid filtration uptake assays because they are very robust and give very reproducible data.

Additional Materials *(also see Basic Protocol)*

Pig intestine (freshly isolated from slaughter or animal house)
Pig and cow collection solution A, ice cold
Pig and cow homogenization solution B

25-G needle
10-ml syringe
−70°C freezer

Isolate membranes

1. Flush the intestinal lumen of freshly isolated pig intestine pieces (30 cm long) with 60 to 70 ml ice-cold isotonic saline containing 0.1 mM PMSF.

   Intestine can be collected at the slaughter or animal house and must be chilled immediately. Ideally it should be sectioned and flushed, with mucosal scrapings taken on site and kept in ice-cold saline until they can be used. If mucosal scrapings cannot be prepared immediately, flush out the lumenal contents of the intestinal segments with ice-cold saline and keep the tissue on ice in the saline for no more than 2 hours.

   Note that the flow chart in Figure 3.21.1 does not fully match the isolation steps for pig or cow.

2. Open up the intestine along its antimesenteric border, i.e., along the surface opposite to the blood and nerve supply and lay it flat on an ice-cold plate or piece of Plexiglas.

Collect and homogenize mucosal cells

3. Take mucosal scrapings, as for rat tissue (Basic Protocol, step 8), using a microscope slide held at 45° angle.

4. Collect the scrapings in ice-cold pig and cow collection solution A, at a ratio of ∼20 ml solution for each gram of tissue.

5. Homogenize the suspension from step 4 with a Polytron homogenizer 90 sec at setting 5, on ice.

Remove intracellular and basolateral membranes

6. Centrifuge this suspension (in 50-ml tubes) 15 min at 1,600 × *g*, 4°C. Collect the supernatant (S1) and discard the pellet (P1).

7. Add the 100 mM $MgCl_2$ to S1 bringing the magnesium concentration to a final concentration of 10 mM and stir 15 to 20 min on ice.

8. Centrifuge the suspension 15 min at 1,600 × *g*, 4°C.

9. Collect the supernatant (S2) discarding the pellet (P2). Centrifuge S2 30 min at 20,000 × *g*, 4°C. Retain pellet (P3). Discard supernatant (S3).

10. To resuspend P3 effectively, add 1 ml of ice-cold pig and cow homogenization solution B and draw the solution back and forth 5 to 10 times through a 25-G needle on a 10-ml syringe.

    At this stage aliquots of this samples can be frozen up to 4 months at −70°C with little deterioration.

Isolate brush-border membranes

11. Dilute the 1 ml of P3 suspension to 35 ml with pig and cow homogenization solution B and centrifuge 30 min at 20,000 × *g*, 4°C. Retain P4 and discard S4.

12. Resuspend P4 in a small volume of homogenization solution B, measure the protein concentration (see *APPENDIX 3H*) and then dilute to the required protein concentration in final suspension medium.

    The desired concentration for rapid filtration assays is 6 to 8 mg/ml.

ISOLATION OF INTESTINAL BRUSH-BORDER MEMBRANES FROM COW

Clearly, the tissue cannot be flushed *in situ* as with smaller species but, tissue samples store well for subsequent membrane isolation.

Additional Materials *(also see Basic Protocol)*

Cow intestine
Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 0.1 mM PMSF and 0.1 mM aprotonin
Pig and cow collection solution A (see recipe)
Pig and cow homogenization solution B (see recipe)
Aluminum foil or plastic vials suitable for freezing
Liquid nitrogen or −70°C freezer
25-G needle
10-ml syringe

Collect intestinal tissue

1. Cut the intestine into 10-cm lengths. Flush pieces of intestine with 30 ml ice-cold PBS containing 0.1 mM PMSF and 0.1 mM aprotonin. Place in flask containing ice-cold flushing solution and transport rapidly to preparation site.

   Tissue will keep for a couple of hours on ice.

2. Blot the pieces and wrap in aluminum foil before dropping into liquid nitrogen for storage.

   Alternatively mucosal scrapings can be prepared as below and then placed in plastic vials for rapid freezing under liquid nitrogen. These will store well under liquid nitrogen for several weeks. If they are subsequently kept at −70°C they will only keep for 1 week.

Isolate membranes

3. Thaw samples on ice and collect mucosal scrapings as in Basic Protocol, step 8. Add 1 g of mucosal scrapings to 20 ml of pig and cow collection solution A.
4. Homogenize the material with a Polytron homogenizer 2 min at setting 5, on ice.
5. Then centrifuge the suspension for 15 min at 1,600 × *g*, 4°C. Retain supernatant (S1) and discard the pellet (P1).

Precipitate intracellular and basolateral membranes

6. Add stock 100 mM $MgCl_2$ to a final concentration of 10 mM $MgCl_2$. Stir the solution slowly on ice for 15 to 20 min.
7. Centrifuge 15 min at 1,600 × g, 4°C. Collect S2 and discard P2.
8. Centrifuge S2 30 min at 20,000 × *g*, 4°C, and retain P3 while discarding S3.

Homogenize brush-border membranes

9. Wash P3 with 1 ml ice-cold pig and cow homogenization solution B and mix by drawing back and forth through a 25-G needle on a 10-ml syringe five to ten times.

   At this stage aliquots of this sample can be frozen up to 4 months at −70°C with little deterioration.

Isolate brush-border vesicles

10. Dilute the 1 ml of P3 suspension to 35 ml in pig and cow homogenization solution B and centrifuge 30 min at 20,000 × g, 4°C. Retain P4 and discard S4.

11. Resuspend P4 in a small volume of pig and cow homogenization solution B, measure the protein concentration (see *APPENDIX 3H*) and then dilute to the required protein concentration using final suspension medium.

For rapid filtration assays, 6 to 8 mg protein/ml is the desired concentration for rapid filtration assays.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Final suspension medium

Using solution C (see recipe), add components, including protease inhibitors, buffers at the appropriate pH, and chemicals to provide the ionic composition that will be required for the experiment to be performed. Store up to 1 week at 4°C.

The composition of this solution will determine what the vesicles contain because this is the point at which they seal and equilibrate. Mannitol is often used as the osmolyte so that the effects of different extravesicular cations can be tested on transport processes; however, the disadvantage of using the sugar is that the ionic strength is not maintained across the membrane.

Pig and cow collection solution A

For 500 ml: 50 mM mannitol
2 mM Tris base, pH 7.4
Store up to 1 month at 4°C
Add 200 µl of 250 mM PMSF just before use (0.1 mM. final)

Pig and cow homogenization solution B

30 ml 500 mM mannitol (300 mM final)
5 ml of 500 mM HEPES (50 mM final), adjusted to pH 7.4 with Tris base
H_2O to 50 ml
Store up to 1 month at 4°C

Rat collection solution A

300 mM mannitol
5 mM EGTA
12 mM Tris·Cl
Adjust to pH 7.4 with 5 M NaOH
0.1 mM PMSF (add just before use; see *APPENDIX 2A* for stock solution recipe)
Store up to 1 month at 4°C

Rat homogenization solution B

150 mM mannitol
2.5 mM EGTA
6 mM Tris·Cl
Adjust to pH 7.4 with 5 M NaOH
0.05 mM PMSF (added just before use)
Store up to 1 month at 4°C

Solution C

300 mM mannitol
5 mM Tris·Cl, pH 7.4
Store up to 1 month at 4°C

COMMENTARY

Background Information

Polarized epithelial cells, such as those of the small intestine, have two distinct membrane domains. The basolateral membrane (BLM) bounds the sides and basal surface of the epithelial cells while the apical surface is often referred to as the brush-border membrane (BBM). This name comes from the appearance of this surface as bristles of a brush under a low-power light microscope. These bristles are formed by the plasma membrane having numerous uniform projections called microvilli, which have a very complex cytoskeletal structure in their core (Fig. 3.21.2A and 3.21.2B). A key feature of polarized cells is their asymmetrical expression of membrane proteins which allows them to mediate vectorial transport of numerous solutes and ions. In the case of the small intestine, amino acid and hexose absorption is achieved by the expression in the BBM of proteins like SGLT1 (sodium-coupled glucose transporter) and GLUT5 (a facilitated fructose transporter). These proteins are not expressed in the BLM; instead we find GLUT2, a low-affinity glucose and fructose facilitated transporter, and sodium/potassium ATPase, which pumps sodium out of the cell into the blood stream. This differential expression then promotes the uptake of fructose and the coupled entry of glucose and sodium, followed by the exit of these hexoses via GLUT2 and the pumping out of sodium by the ATPase. The end result is the absorption of hexoses and sodium from the lumen into the blood stream.

The isolation of BBMs then allows one, using a variety of biochemical and molecular techniques, to determine the expression of proteins at the apical surface of the epithelial cells. The intestine is a very plastic tissue, varying the expression of numerous proteins in response to environmental conditions, e.g., starvation, alterations in diet, and some disease states like diabetes. In addition, there is evidence for regulatory processes in which transport proteins are rapidly inserted or removed from the BBM. Isolated BBMs permit quantification of the degree of changes in protein expression and also allow for measurement of the time course of expression or turnover. The major technique for determining transporter protein expression would be immunoblotting (*UNIT 6.2*) with densitometric scanning (*UNIT 6.3*) which allows for at least semiquantitative analysis. Confirmation of the identity of proteins expressed in the BBM can also be achieved by using mass spectrometry. In addition to the embedded membrane proteins, the intestine also expresses a number of surface enzymes which are anchored by a single membrane-spanning domain (Hauser and Semenza, 1983). Their activity can be assayed using isolated BBMs and a variety of substrates depending upon the enzyme to be assayed, e.g., sucrase-isomaltase (Tsuboi et al., 1985).

The second major use of these isolated BBMs is for transport studies. During the homogenization processes the individual microvilli are sheared off from the cells producing tubes of plasma membrane containing microtubules. The majority of these individual microvilli then seal at their base by the fusion of the lipid bilayer around the cytoskeleton. During the final centrifugation the suspension medium equilibrates with the intramicrovillus space giving vesicles containing a solution of known composition. The trapped volume of these isolated BBM vesicles is usually in the range of 1 to 2 μl per mg membrane protein. If these vesicles are then suspended in a medium containing a radiolabeled substrate of known concentration, incubated for a few seconds and then separated from the medium by filtration, it is possible to measure the functional activity of transport proteins expressed in the BBM.

Critical Parameters and Troubleshooting

This protocol is based upon the principle of divalent cation precipitation (calcium or magnesium) and can be used to isolate BBMs from a variety of different species (Hopfer et al., 1973). The initial description is for rat small intestine with later protocols indicating modifications for pig and cow. It must be recognized that the intestinal epithelium is heterogeneous, with ~20% of the cells being non-epithelial, including endocrine cells, mucus secreting goblet cells, and immunoreactive Paneth cells. Thus, any preparation will at best have a contribution of 80% from intestinal epithelial cells (enterocytes). Potential contamination from basolateral and intracellular membranes must also be allowed for.

Rat BBM vesicles are quite labile and are best used fresh, although they can be frozen under liquid nitrogen for up to a week or so. Pig BBMs are far more robust and will store well under liquid nitrogen for extended periods. Bovine material can be stored frozen as tissue, scrapings, or fully isolated

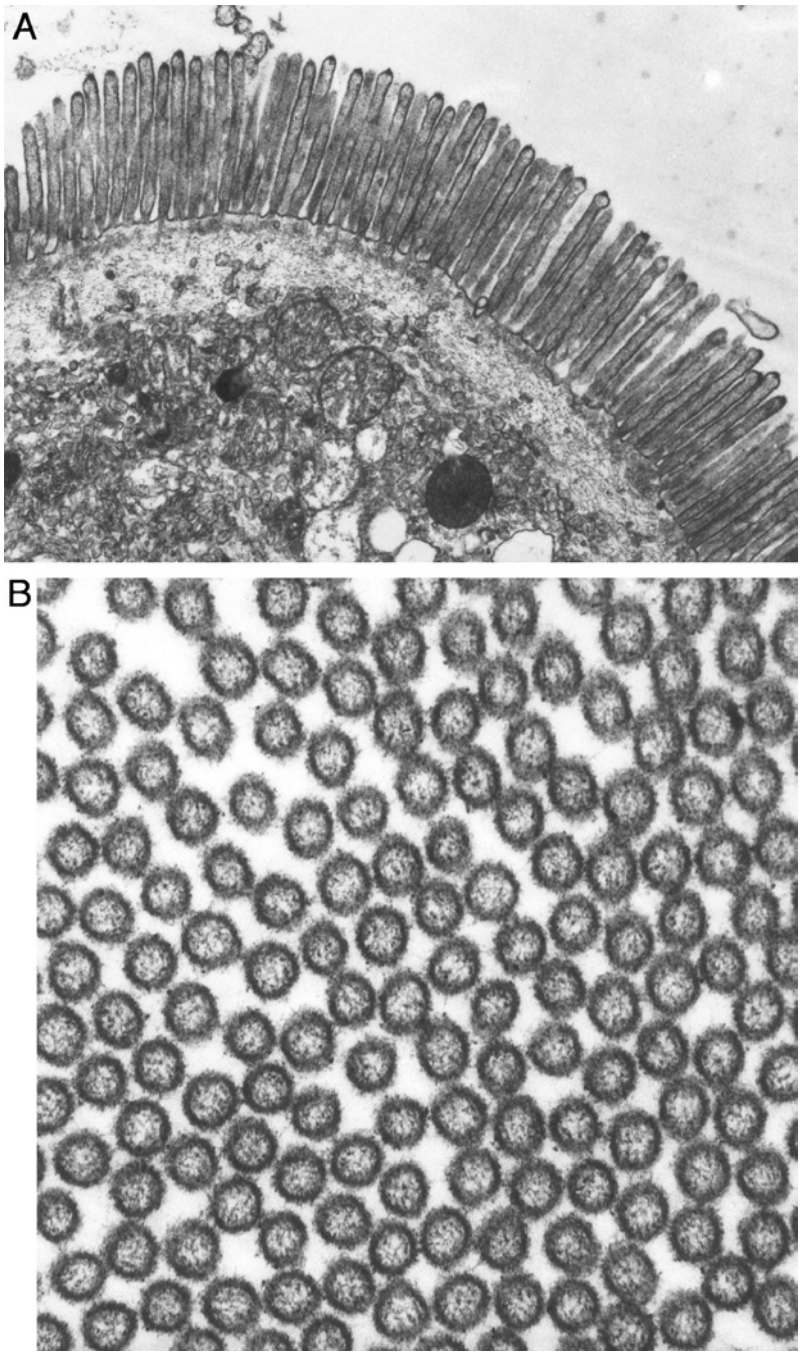

Figure 3.21.2 (**A**) Low-power electron micrograph of isolated rat enterocytes (intestinal epithelial cells) showing highly specialized apical membrane organized into microvilli. (**B**) Cross-section electron micrograph of intestinal microvilli showing plasma membrane surrounding a core of structural filaments.

membranes. It should be noted that a new protocol has been reported using polyethylene glycol (PEG) which may produce vesicles with a better phospholipid profile than is obtained with the divalent cation method (Prabhu and Balasubramanian, 2001). This may be a consequence of the possible stimulation of cation-dependent phospholipases when using the divalent cation precipitation method, and it may be avoided using PEG precipitation.

The isolated membranes are sheared-off microvilli, which will seal spontaneously at their bases forming vesicles that are almost invariably oriented outside out (Hearn et al., 1981); thus, they can be used for rapid uptake experiments. They usually trap ~1 to 2 μl per

mg protein, and so even with a suspension of 8 to 10 mg protein/ml the total trapped volume is negligible compared to the extravesicular suspension volume. The sealing process and equilibration of intravesicular medium with the suspension medium takes place during the final centrifugation of S3. Thus, in these protocols the vesicles will contain an isotonic mannitol medium. If a different composition is required, an additional step is required in which P4 is resuspended in 10 ml of the medium of choice, mixed by repeated flushing through a plastic pipet tip, and then given a final 15-min centrifugation at 20,000 × *g*, 4°C. Discard the supernatant and resuspend in the same solution using a minimal volume (~ 500 µl) to allow for final dilution to the required protein concentration. If the yield cannot give 8 to 10 mg protein/ml for flux assays it is possible to go as low as 5 mg/ml. However, the transport assays will not be as reliable.

When designing an uptake experiment where the composition of the intravesicular medium may be different from that of the uptake medium, it is necessary to take into account the ratio of intravesicle medium versus uptake medium to determine the actual final concentration of substrates and electrolytes. Normally, the ratio of vesicle suspension to uptake medium is in the range of two to five parts uptake medium to one part vesicles.

Anticipated Results

The yield of isolated BBMs varies significantly among species. Three 30-cm pieces of rat jejunum will yield ~8 to 10 mg membrane protein. The ileum will give a slightly lower yield, but there is ~70 cm available in the adult animal. The BBMs can be suspended to the desired protein concentration depending upon whether they are to be used for vesicle transport assays, enzyme assays, or immunoblotting. Normally, for transport assays 8 to 10 mg protein would be resuspended in 1 ml incubation medium. A higher concentration will clog the filters used in the assay.

One 30-cm segment of pig small intestine will generate up to 5 to 6 mg isolated BBM membrane protein.

One 30-cm segment of cow small intestine will generate up to 5 to 6 mg isolated BBM protein.

Time Considerations

Total preparation time from harvesting of the tissue to the final suspension of purified material takes 4 to 5 hr. Ideally, preparation should be commenced at the start of the day and the experiments with the isolated membranes can then be performed in the afternoon.

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Contributed by Chris I Cheeseman and Debbie O'Neill
University of Alberta
Edmonton, Alberta Canada