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ISOLATION AND PARTIAL CHARACTERIZATION OF THE BASAL CELL MEMBRANE OF HUMAN PLACENTAL TROPHOBLAST

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The function of the syncytiotrophoblast in maternal-fetal exchange is related to the properties of its microvillous (maternal-facing) and basal (fetal-facing) plasma membranes. We have previously reported the properties of the microvillous membrane (Smith, C.H., Nelson, D.M., King, B.F., Donohue, T.M., Ruzycki, S.M. and Kelley, L.K. (1977) Am. J. Obstet. Gynecol. 128, 190–196), and now describe the purification and partial characterization of the basal plasma membrane. Sonication and incubation with EDTA were used to isolate selectively the basal cell membrane. These steps were followed by a more conventional purification by centrifugation. The trophoblast was disrupted and its microvillous membrane and cytoplasmic contents were removed by sonication. The exposed basal cell membrane was selectively released from the underlying basal lamina by sonication in the presence of EDTA and further purified by discontinuous Ficoll gradient centrifugation. The material at the 4–10% Ficoll interface consisted of smooth membrane vesicles with internal microfilaments. It was 45-fold enriched in dihydroalprenolol binding activity and 11-fold enriched in ouabain binding activity. Other enzymatic analyses, including alkaline phosphatase, cytochrome-*c* oxidase, cytochrome-*c* reductase and galactosyl transferase indicated low contamination by other organelles. This procedure yields a preparation of relatively high purity which should be suitable for investigation of transport and other functions of the basal surface membrane of trophoblast. In principle, the purification procedures used may be applicable to other transporting epithelia.

Introduction

The syncytiotrophoblast of the human placenta is the site of exchange of nutrients between mother and fetus. Transfer of a substance requires its passage across the apical (microvillous) and the basal plasma membranes of this cell layer. We have previously characterized the isolated microvillous membrane of human placenta [1]. In our own laboratory and in others, this membrane has been shown to be the site of transport systems for glucose [2–5] and amino acids [3,5–7] as well as containing receptors for immunoglobulin G [8,9] transferrin [9–11] and insulin [3,12,13]. In other transporting epithelia such as those of the kidney [14–16] and intestine [17,18], the basolateral membrane has also been shown to be the site of transport and control mechanisms. It is highly likely that the placental basal cell membrane which faces the fetus is the site of transport mechanisms which regulate the transfer of nutrients between mother

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and fetus. An isolated preparation of basal plasma membrane would, therefore, greatly facilitate the study of transfer across the syncytium.

Basal or basal-lateral cell membranes have generally been isolated by homogenizing tissue or epithelial cells and separating the basal plasma membrane from the combined cell membranes by centrifugation [14–24]. In contrast, we have avoided a nonselective homogenization. In its place, we developed methods based on the structure of the tissue for selectively removing the overlying cell membranes and cytoplasm and isolating the exposed basal cell membrane. The basal cell membrane was then further purified by differential and gradient centrifugation. In light of other reports in kidney [15,19,20,25], intestine [18,21,22,26] and placenta [23,27–30], we selected ouabain binding and dihydroalprenolol binding as markers for basal cell membrane and alkaline phosphatase as a marker for apical membrane to aid in monitoring the isolation procedure.

A preliminary account of this work has been presented [52].

Materials and methods

Tissue homogenization

For comparison with purified fractions, whole villous tissue was homogenized in 10 vol. of 50 mM Tris-HCl/250 mM sucrose/1 mM EDTA (pH 7.4) at 4°C (Buffer 1). Homogenization was performed first using a Waring blendor operated for 0.5–3 min and secondly using a motor-driven Potter-Elvehjem homogenizer (clearance 0.005–0.007 inch) operated at 2100 rpm for eight strokes. This material was then filtered through six layers of gauze.

Basal plasma membrane preparation

Full-term placentas from normal vaginal deliveries or cesarean section were used. The placenta was placed on ice and cut into pieces approx. 10–15 cm in diameter. Decidua and the chorionic plate were removed using a microtome blade. The resulting villous tissue was washed with Earle's balanced salts solution [31] to remove blood and then ground with an electric meat grinder. The grinding process yielded 150–300 g of tissue in pieces of 1–5 mm diameter. This tissue was washed three times with 50 mM Tris-HCl buffer and collected on nylon mesh (100 μm pore size). The pH of all solutions used in preparation was adjusted to 7.4. Except where noted, the remaining steps were preformed at 4°C.

Tissue was subjected to initial sonication and stirring under conditions found to be optimal in preliminary experiments. 20-g portions of tissue were sonicated in 100 ml 50 mM Tris-HCl buffer. A Branson Model 350 sonicator was used with a 3/4-inch high-gain probe. The tissue was cooled in an ice-ethanol bath and sonicated at setting 9 (240 W) for 10 s. Under these conditions, the sonication medium reached a maximum temperature of 15–20°C. Sonicated tissue was collected on nylon mesh and washed three times with 5 mM Tris-HCl buffer. The sonicated tissue was then stirred in approx. 5 vol. of 5 mM Tris-HCl buffer for 30 min, washed once with the same buffer and collected on nylon mesh. By this point the tissue had lost most of its red color.

To remove basal cell membrane, the tissue (20 g/100 ml) was incubated 30 min at room temperature with occasional stirring in 10 mM EDTA adjusted to 300 mosM by the addition of 60 mM EDTA to 50 mM Tris/250 mM sucrose. This mixture was then cooled with an ice-ethanol bath and sonicated at setting 10 (250 W) for 20 s. After sonication, large pieces of remaining tissue were removed by filtration through six layers of gauze and the filtrate was centrifuged at $3300 \times g$ for 10 min (g forces are average values applied to the midpoint of the tube). The resulting supernatant was centrifuged at $81\,000 \times g$ for 40 min to yield a crude preparation of basal cell membrane which may be satisfactory for some purposes.

For further purification, the pellets were resuspended in buffer 1 by homogenization with a motor-driven Potter-Elvehjem homogenizer (clearance 0.004–0.006 inch) operated at 2100 rpm for 30 strokes and then stored overnight on ice. There was no significant loss in alkaline phosphatase activity, dihydroalprenolol binding or ouabain binding from this fraction during storage.

The resuspended material was applied to the top of a stepwise gradient of Ficoll in buffer 1. Steps of 4 and 10% Ficoll having densities of 1.05 and 1.07, respectively, were used. The gradients were centrifuged in a swinging bucket rotor at

90 000 × *g* for 2 h. Material at the 4–10% interface was collected and diluted approx. 5- to 10-fold with buffer 1. These samples were centrifuged at 81 000 × *g* for 40 min and the resulting pellets were resuspended in buffer 1 with eight strokes of a Potter-Elvehjem homogenizer (clearance 0.004–0.006 inch, 2100 rpm). The protein concentration in the homogenized samples was approx. 1–5 mg/ml.

Under certain circumstances, it may be desirable to perform the second sonication procedure in the absence of EDTA or to release basal membrane by homogenization carried out using the procedure described for whole tissue above.

Analytical measurements

Dihydroalprenolol binding [32] was measured for 15 min at 30°C in 10 nM [^{3}H]dihydroaprenolol followed by filtration onto two adjacent glass-fiber filters (Whatman GFC). Nonspecific binding was measured with 30 μM DL-propranolol. Ouabain binding [33] was measured by incubating 2.5–3 h at 37°C in 10 μM [^{3}H]ouabain/50 mM Tris-HCl/5 mM $MgCl_2$/5 mM H_3PO_4 (pH 7.4). Incubations were terminated by filtration into 0.45 μm mixed ester cellulose filters (type HAWP, Millipore Corp.). Filters were washed four times with 4-ml portions of ice-cold deionized water and dissolved in 2 ml cellosolve. Nonspecific binding was determined by addition of 1 mM ouabain.

Alkaline phosphatase [34] cytochrome *c* reductase [35] cytochrome *c* oxidase [36] and galactosyl transferase [37] were determined by previously established procedures. Protein was determined by the method of Lowry et al. [38] using bovine serum albumin as a standard.

Electron microscopy

Small pieces of intact placental villi, sonicated-stirred villi, and membrane pellets derived from the various Ficoll gradient interfaces were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1.5–2 h at 4°C, washed three times in cold 0.1 M cacodylate buffer, cut into small blocks, and post-fixed in 2% osmium tetroxide in 0.1 M *s*-collidine buffer. The tissues were subsequently stained en bloc with 0.5% aqueous uranyl acetate, dehydrated in a series of graded acetones, and embedded in an Epon-araldite epoxy resin mixture. Thin sections were cut on an ultramicrotome, picked up on uncoated copper grids, and stained with uranyl acetate and lead citrate prior to examination in a Philips 400 electron microscope.

Results

Effects of sonication and stirring

The success of the preparation of isolated syncytiotrophoblast basal plasma membrane depends upon the initial selective removal of apical membrane and cytoplasmic contents. We accomplished this by sonication and stirring in hypotonic medium. The efficacy of this process was determined by electron microscopy and the measurement of membrane markers for basal and apical membrane.

Fig. 1 illustrates part of an intact placental villus with microvilli on the apical surface (facing maternal blood) and basal cell membrane resting on the basal lamina and facing fetal capillaries. Fig. 2 illustrates a similar villus after sonication and stirring. The procedure resulted in villous tissue generally devoid of syncytiotrophoblast brush border and cytoplasm. The basal cell membrane of the trophoblast, however, remained adherent to the underlying trophoblastic basal lamina and the remainder of the villous core. The basal cell membrane was present as extensive sheets of membrane, small fragments, or as vesicles, and sometimes had small numbers of associated microfilaments. The fetal endothelial cells which remained in the villous core could often be identified because of their abundant intermediate filaments and were more resistant to sonication.

Approx. 80% of the alkaline phosphatase activity was removed from villous tisue by sonication (Table I) and an additional 10% was removed by vigorous stirring of the tissue in hypotonic buffer. Under the same conditions the loss of dihydroalprenolol-binding activity was only 34% during sonication and an additional 5% with stirring. Loss of ouabain-binding activity was nearly 2-fold greater than loss of dihydroalprenolol binding. Together, these treatments resulted in the increase in specific activity of ouabain binding and dihydroalprenolol binding associated with the tissue and a loss of specific activity of alkaline phosphatase. Sonication followed by hypotonic stirring

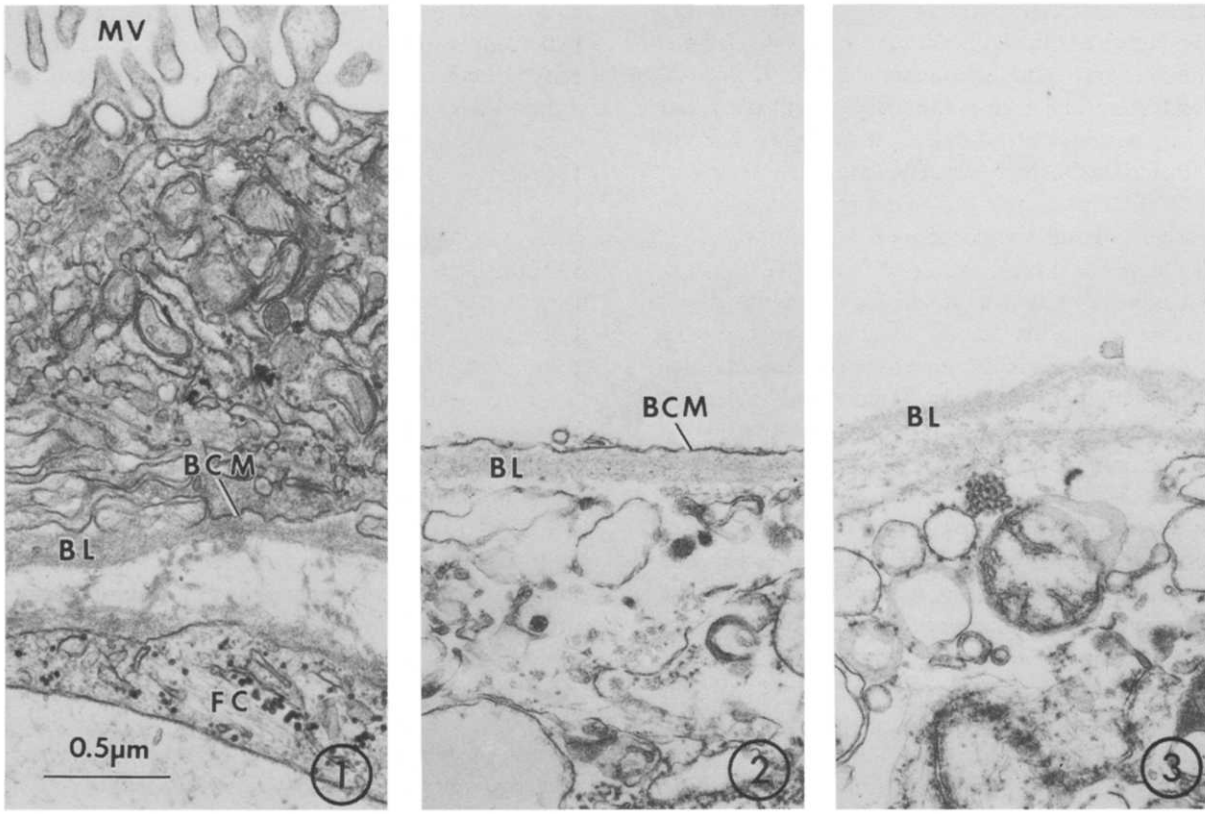


Fig. 1. Electron micrograph of an intact placental villus showing microvilli (MV) on the apical cell surface of the trophoblast and the basal cell membrane (BCM) resting on the basal lamina (BL). FC, fetal capillary. ×33000.

Fig. 2. Micrograph of villus after sonication and stirring. Basal cell membrane (BCM) remains adherent to basal lamina (BL). ×33,000.

Fig. 3. Micrograph of villus after second sonication step illustrating removal of basal cell membrane (BCM) and leaving the basal lamina (BL) on the external surface. ×33000.

TABLE I

VARIATION IN BINDING AND ENZYME ACTIVITY DURING SONICATION AND STIRRING

Placental villous tissue was subjected to sonication and then to stirring in a hypotonic medium. At each step portions were homogenized and analyzed. The table gives the fraction of original tissue activity remaining after each treatment and the specific activity at each stage. Values are mean ±S.E. with number of determinations in parentheses. Sonication and stirring selectively remove alkaline phosphatase, leaving dihydroalprenolol-binding and, to a lesser extent, ouabain-binding activity.

Sample	Dihydroalprenolol binding		Ouabain binding		Alkaline phosphatase	
	% remaining	fmol/mg protein	% remaining	pmol/mg protein	% remaining	μmol/min per mg protein
Original tissue	100	118 ± 10(5)	100	0.94 ± 0.095(10)	100	0.618 ± 0.182(8)
Sonicated tissue	66 ± 13(3)	397 ± 115(4)	38 ± 4.(9)	1.32 ± 0.16(9)	17 ± 2.(7)	0.475 ± 0.150(7)
Sonicated stirred tissue	61 ± 10(4)	531 ± 126(5)	35 ± 4(9)	1.54 ± 0.13(9)	10 ± 2(6)	0.351 ± 0.174(6)

is therefore an effective means for differentially removing the apical membrane and cytoplasmic contents from syncytium while leaving the basal membrane. The membranes isolated from tissue thus treated would be expected to be highly enriched in basal cell membrane.

Removal of basal cell membrane

To remove basal cell membrane from basal lamina and villous core material and prepare it for fractionation on the Ficoll gradient, the tissue was subjected to a second sonication procedure as detailed in the Materials and Methods section. The effectiveness of this procedure was determined by measurements of dihydroalprenolol binding and ouabain binding in material released from the tissue and by examination of the residual tissue by electron microscopy. The EDTA concentration and sonication conditions chosen gave an optimal yield and enrichment of basal cell membrane marker.

Electron-microscopic examination of villi after the second sonication step showed most of the basal cell membrane had been removed, leaving basal lamina on the external surface (Fig. 3). Most of the fetal capillary endothelium and other connective tissue components remained trapped within the basal lamina.

As an alternative, homogenization was used to remove basal cell membrane from basal lamina. This treatment gave similar yield and enrichment of basal cell membrane markers. Tissue that was subjected to homogenization was similar in appearance to that subjected to sonication, except that more of the connective tissue core components appeared to be removed.

Isolation of basal cell membrane

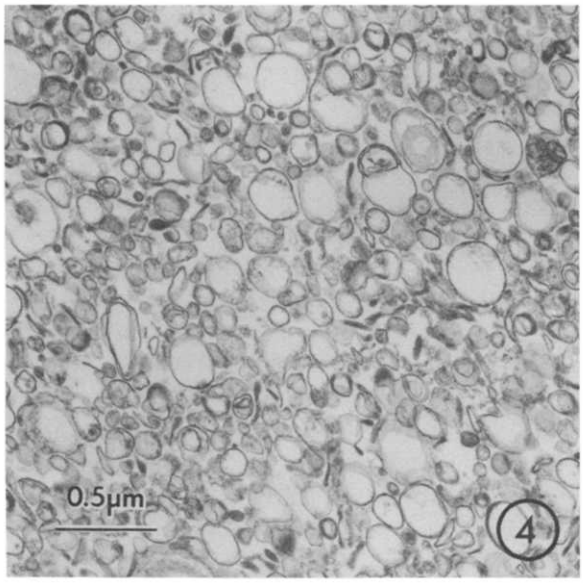
To determine the optimal conditions for purification of basal cell membrane on the Ficoll gradient, small Ficoll increments were used and the relative specific activity of markers determined (data not shown). These activities were similar after sonication or homogenization. Dihydroalprenolol binding enrichment reached a peak at 6–8% Ficoll, whereas ouabain binding was constant throughout. Cytochrome *c* oxidase activity in-

TABLE II

BINDING AND ENZYME ACTIVITIES OF INTERMEDIATE AND FINAL STAGES OF BASAL MEMBRANE PREPARATION

Activities were measured in five preparations. Values are mean ± S.E.

	Tissue homogenate	Crude basal cell membrane	Purified basal cell membrane	Microvillous membrane
Protein				
mg/100 g tissue	4044 ± 291	88 ± 9	6.3 ± 0.4	
% Tissue homogenate	100	2	0.2	
Dihydroalprenol binding				
fmol/mg	84 ± 6	2492 ± 215	3795 ± 300	67 ± 6
Relative activity		30	45	1
Ouabain binding				
pmol/mg	0.9 ± 0.1	8 ± 2	10 ± 2	2.7 ± 0.3
Relative activity		8	11	3
Alkaline phosphatase				
mol/min per mg	1.1 ± 0.2	0.96 ± 0.08	1.5 ± 0.3	
Relative activity		1	1	
Cytochrome *c* oxidase				
$min^{-1} \cdot mg^{-1}$	0.7 ± 0.1	2.6 ± 0.6	0.9 ± 0.1	
Relative activity		4	1	
Cytochrome *c* reductase				
mol/min per mg	40 ± 3	251 ± 14	192 ± 20	
Relative activity		6	5	
Galactosyl transferase				
nmol/min per mg	1.0 ± 0.1	2.2 ± 0.2	2.7 ± 0.4	
Relative activity		2	3	



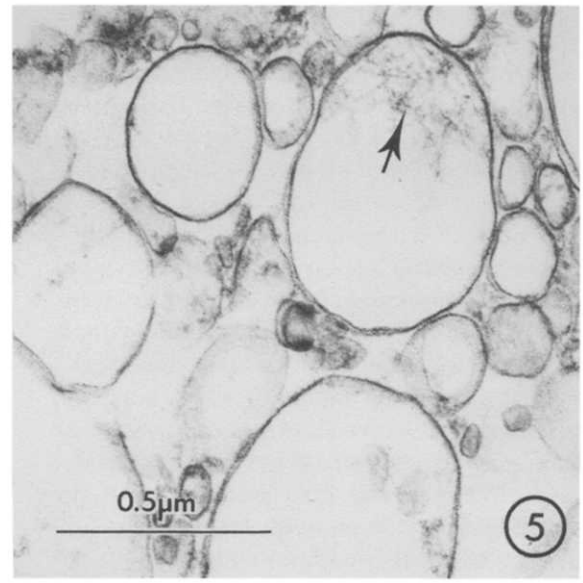


Fig. 4. Low-magnification micrograph of membrane vesicle fraction. ×26500.

Fig. 5. High-magnification micrograph of membrane vesicle fraction. Some vesicles contain microfilamentous material (arrow). ×57000.

creased as the density of Ficoll increased and alkaline phosphatase activity decreased somewhat. On this basis, a Ficoll interface of 4–10% was selected as appropriate for purification of basal plasma membrane.

The success of the overall purification procedure was determined by measuring the enrichment of basal cell membrane markers relative to markers of contaminating organelles. Table II lists the specific activities of these markers determined in whole tissue homogenate, the particulate fraction obtained after differential centrifugation (crude basal cell membrane), and in the final preparation of purified basal cell membrane. The recovery of protein and the highly enriched markers dihydroalprenolol and ouabain binding were studied after each step in the procedure. Total activity was never increased and losses were generally small (usually much less than 30%). Losses of dihydroalprenolol and ouabain binding paralleled losses of protein. We conclude that the enrichments observed reflect purification and are not substantially influenced by activation or unmasking of binding.

The enrichment of dihydroalprenolol-binding and ouabain-binding activities in the final basal cell membrane preparation was 45- and 11-fold, respectively, over whole-tissue homogenate. Cytochrome *c* oxidase, NADH cytochrome *c* reductase, alkaline phosphatase and galactosyl transferase were much less enriched in activity. In microvillous membrane, purified as described previously [1] dihydroalprenolol and ouabain binding were 1- and 3-fold those of the original whole-tissue homogenate.

Examination of fractions collected from the Ficoll gradient showed membrane vesicles about 0.1–1.0 μm in diameter (Fig. 4). At higher magnification (Fig. 5), it is seen that the vesicles sometimes contained moderately electron-dense microfilaments, suggesting these vesicles had an 'outside-out' orientation.

Discussion

The purification procedure for basal plasma membrane described in this paper utilizes a series of steps designed to isolate this membrane from a tissue of relatively diverse composition. Apical syncytiotrophoblast plasma membrane and cytoplasmic content are first removed by sonication and stirring, leaving the basal cell membrane ex-

posed on the external surface. The basal cell membrane is then released relatively selectively by a second sonication, carried out in the presence of EDTA and isolated as a crude preparation by high-speed centrifugation. The membrane is finally purified by Ficoll gradient centrifugation and collected as a pellet of vesicular smooth membrane of distinctive enzymatic composition.

This procedure yields a final preparation purified 45-fold with respect to dihydroalprenolol-binding activity and somewhat less with respect to ouabain binding. Several lines of evidence suggest that dihydroalprenolol binding, and to some extent ouabain binding, are appropriate markers to evaluate purification of basal syncytiotrophoblast membrane. (1) These markers are generally found in plasma membrane [15,19–22,25,26,33,39], yet, in agreement with data in the literature [29,33,40], we have found them to be not highly enriched in isolated microvillous membrane. However, in placenta they are enriched in a high-speed centrifugal pellet prepared after partial removal of apical membrane, suggesting their presence in the basal rather than microvillous membrane [29,30, 33]. (2) In other polarized epithelia such as those of the kidney [41] and intestine [26], adenylate cyclase with which the β-adrenergic receptor is associated has been demonstrated to be enriched in basal-lateral membrane and diminished in brush-border membrane. The same is true for ouabain binding (or $(Na^+ + K^+)$-ATPase activity) [15,18–22,25,26]. In the same epithelia, alkaline phosphatase is present largely or entirely in apical membrane [20,25,26], as it is in placenta [1,7,12,27,40]. (3) In the placenta, cyclic AMP and/or β-adrenergic stimulation is known to increase secretion of various hormones produced entirely or predominantly by the syncytium [42–47]. Thus, adenylate cyclase and the β-adrenergic receptor must be located there and must be present in basal plasma membrane.

These observations taken together strongly suggest that, while β-adrenergic receptor activity and ouabain binding may be found in other cell membranes within the villi as well, they are present in high concentrations in the basal plasma membrane and are suitable as markers of its purification. The lesser enrichment of ouabain binding relative to dihydroalprenolol binding may arise because the $(Na^+ + K^+)$-ATPase, a widespread plasma-membrane enzyme [48], is more widely distributed among cells of the villi than is β-adrenergic receptor activity.

The degree of microvillous membrane contamination in the final preparation is difficult to determine with certainty. Electron microscopy failed to demonstrate detectable microvilli in the final preparation. Studies from our own and other laboratories have demonstrated that alkaline phosphatase and 5′-nucleotidase are present in high concentration in the microvillous membrane [1,30,40]. Assuming that pure microvillous membrane is 15–25-fold enriched in alkaline phosphatase in relation to the tissue homogenate [1,12,40], the content of the final preparation indicates that no more than 5–10% of the protein could be microvillous membrane. The use of alkaline phosphatase and 5′-nucleotidase in detecting microvillous contamination of the basal cell membrane preparation is limited, however, by the potential presence of these enzymes in basal membrane both in the placenta and other organs [19,26,27] and the value given must be regarded as a maximum estimate of microvillous content.

Other possible sources of cell membrane are the small amount of residual cytotrophoblast present in the term placenta and the fetal capillary endothelial cells in the villous core. Significant contamination from the latter source is unlikely, since endothelium remains largely trapped within the basal lamina while basal membrane is released by sonication in the presence of EDTA. This sonication procedure is based on procedures using chelating agents to prepare syncytium from human placenta [49] and epithelium from rat intestine [50,51], and thus provides a simple, convenient means for selectively releasing basal membrane.

The degree of purification of basal cell membrane in this preparation is substantially greater than that of other preparations from syncytiotrophoblast [23,24,30]. The overall purification of marker enzymes for basal membrane and the apparent contamination by other organelles is generally comparable to that of basal-lateral membrane preparations of intestinal epithelium [18,21, 22] and in some respects is similar to that reported for renal tubular epithelium [15,19]. The purity of basal cell membrane preparation and its vesicular

nature indicate that it should be suitable for biochemial and physiologic studies. The stepwise procedures utilized may be generally applicable to the purification of basal or basal-lateral membrane from other transporting epithelia.

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