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Sequential preparation of highly purified microvillous and basal syncytiotrophoblast membranes in substantial yield from a single term human placenta: inhibition of microvillous alkaline phosphatase activity by EDTA

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Abstract

The human placental syncytiotrophoblast is a highly polarised epithelial layer responsible for regulating materno-fetal exchange. We here describe a novel procedure for isolating paired fractions of the maternal-facing and fetal-facing plasma membranes from this syncytium, from a single placenta, without the need for homogenisation procedures. This reduces the potential for contamination of these membrane fractions by intracellular membranes, or from plasma membranes from other cell types within the placenta. Microvillous membrane vesicles (MVM) were obtained by gentle stirring of dispersed villous tissue. The tissue sedimented at the end of this procedure was subjected to sequential ultrasonication to release the basal membrane (BM). Crude MVM was subsequently purified on a discontinuous sucrose gradient. Crude BM was further purifed using either discontinuous Ficoll or sucrose gradients. The Ficoll procedure, while producing a BM fraction extremely enriched in marker enzyme, resulted in unacceptably low protein recoveries and hence the sucrose gradient procedure was also adopted for BM. Yields for MVM and BM produced on sucrose density gradients approached 30 mg/100 g tissue. The MVM fraction was composed of vesicles of 232 ± 9 (S.E.) nm diameter of which nearly 90% were 'right side out'. These membranes were 37-fold enriched in the marker enzyme alkaline phosphatase. Purified BM vesicles were 317 ± 14 nm in diameter, also approximately 90% 'right side out' and over 40-fold enriched in dihydroalprenolol binding. Cross-contamination or contamination from intracellular membranes was negligible. MVM alkaline phosphatase activity was shown to be inhibitable in a dose-and time-dependent manner by EDTA present in the storage buffer.

Key words: Human placenta; Trophoblast; Microvillous membrane; Basal membrane; Alkaline phosphatase

1. Introduction

Procedures for isolating purified maternal- or fetal-facing plasma membranes from the human placental syncytiotrophoblast have utilised many differing preparative techniques. To date, the two most commonly used methods are (a) selective removal techniques such as agitation and sonication, and (b) differential centrifugation of tissue homogenates. Mechanical removal of the microvilli by gentle stirring of villous fragments [1] is now the basis of several commonly employed purification procedures [2–4], since it provides a high yield of relatively pure maternal-facing or microvillous membrane (MVM). Nevertheless, homogenisation procedures for MVM are also available [5,6]. The purity of the MVM fraction is usually related to the enrichment of the 'marker enzyme', alkaline phosphatase, which typically reaches values of about 25-fold relative to a crude placental homogenate.

The fetal-facing or basal membrane (BM) of the syncytiotrophoblast lies adjacent to the villous stroma and is separated from the fetal capillary endothelium by connective tissue and basal laminae. Initial attempts to isolate this membrane using homogenisation followed by differential centrifugation procedures resulted in moderate to severe contamination of the BM fraction with MVM and intracellular membrane com-

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ponents (e.g., endoplasmic reticulum, mitochondrial membranes, etc.). For example, Boyd et al. [7] used isopycnic rate zonal ultracentrifugation and found similar alkaline phosphatase activities in both their MVM and BM fractions. A method for preparing BM based on ultrasonication after the selective removal of MVM and cytoplasmic elements has now been widely adopted [8]. These workers used dihyroalprenolol binding as a surrogate marker for adenylate cyclase activity and showed a 45-fold enrichment in their purified BM fraction.

Recently, Illsley et al. [9] returned to differential centrifugation of homogenised placental tissue to purify MVM and BM from the same placenta. Their procedure resulted in high yields of moderately enriched membrane fractions, i.e., MVM enrichment of alkaline phosphatase activity (19.7-fold), and BM enrichment of adenylate cyclase activity (10.7-fold), were within published ranges. This study can be commended for addressing a very important complication inherent in homogenisation procedures – that of the inevitable contamination of syncytiotrophoblast membrane fractions not only with intracellular membranes, but also with plasma membranes from other cell types present in the original tissue. Placental macrophages (Hofbauer cells), fibroblasts and cytotrophoblasts, as well as endothelium, are all found in placental tissue in significant quantities. Illsley et al. [9] showed that contamination from these sources accounted for about one eighth of the total membrane protein in their final fractions.

We here report a new method, derived from established agitation procedures, which permits the sequential production of highly purified MVM and BM. The simple protocol allows purification of both plasma membrane fractions from a single normal or pathological placenta in sufficient quantities to allow comparative studies. BM fractions are generally prepared and stored in buffers containing EDTA in order to avoid the precipitation of non-microvillous membranes by calcium and magnesium [8,9]. During the course of this study we noted that alkaline phosphatase activity in MVM fractions stored in buffers containing EDTA were significantly lower than those stored in the absence of EDTA. This is of interest as traditionally the placental isoform of alkaline phosphatase was believed to be insensitive to EDTA (see Ref. [10] for review).

A preliminary account of some of these studies has been presented [11].

2. Materials and methods

2.1. Processing of placental tissue

A freshly delivered normal term human placenta obtained either from a vaginal delivery or an elective Caesarean section was placed immediately on ice. Villous tissue, from which the chorionic and basal plates had been removed with scissors, was excised in 1–2 cm^3 chunks and washed in ice-cold PBS to remove excess blood. Tissue pieces were then gently blotted, placed on a chilled glass plate and carefully teased with a spatula to disperse the villi. The villous tissue (100–120 g from a normal placenta) was weighed, resuspended in 5 volumes of ice-cold PBS and gently stirred for 30 min. The suspension was centrifuged at $300 \times g$ for 15 min and the supernatant decanted through a 250 μm pore size nylon mesh. The supernatant was subsequently used as the source of the MVM fraction while the sedimented tissue was further treated to release the BM fraction.

2.2. Purification of MVM

The procedure is based on methods previously described by our laboratory [4,12]. All operations were carried out at 4°C. The supernatant from the $300 \times g$ centrifugation was respun at $10\,000 \times g$ for 1 min to remove residual red cells and any remaining tissue debris. The resulting supernatant was re-centrifuged at $100\,000 \times g$ for 1 h to sediment the crude MVM pellet. This pellet, designated MP, was resuspended in a minimal volume of 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM phenylmethylsulphonyl fluoride (PMSF), using a Dounce homogeniser. The resuspended membranes were further purified by layering over a discontinuous gradient of 37% (w/w) sucrose, overlaid with 25% (w/w) sucrose, both prepared in the same buffer. Tubes were spun for 16 h at $80\,000 \times g$. The material above the 25% sucrose (M1) and the pellet at the bottom of the tube (M3) was discarded while the material at the interface of the two sucrose solutions (designated M2) was collected and diluted in three volumes of the Tris buffer, washed twice by centrifugation at $100\,000 \times g$ and finally resuspended in a 10 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM phenylmethylsulphonyl flouride (PMFS) and 0.02% sodium azide.

2.3. Preparation of BM

This part of the procedure is a modification of that described by Kelley et al. [8]. All operations were carried out at 4°C. The sedimented tissue from the initial low speed spin was washed three times with cold 50 mM Tris-HCl (pH 7.4), collected on a 250 μm pore size nylon mesh and divided into four equal portions. Each portion was sonicated in 100 ml of the same Tris buffer using a 3/4 inch high gain probe for 10 s at 240 W (Vibra-cell, Sonics and Materials, CT, USA). The suspensions were kept on ice. The sonication procedure selectively removes any remaining maternal-facing membrane.

Sonicated tissue was collected on the mesh and washed three times with 5 mM Tris-HCl (pH 7.4) and then stirred gently for 60 min in the same buffer. Tissue was then collected on the nylon mesh and washed again in the same buffer. This procedure disrupts and removes the intracellular components thus exposing BM.

25–30 g tissue portions were resuspended in about 100 ml of 50 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 250 mM sucrose and incubated for 30 min with occasional stirring. Portions were then sonicated twice for 20 s at 250 W to release the BM. Suspensions were strained through the nylon mesh and the supernatant centrifuged at $3300 \times g$ for 10 min to remove debris. The supernatant from this spin was re-centrifuged at $80000 \times g$ for 40 min to yield the BM pellet, which was resuspended, using a Dounce homogeniser, in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. This fraction was designated BP and was further purified either by centrifugation on a discontinuous gradient of 10% (w/v) Ficoll (Pharmacia) in the resuspension buffer overlaid with 4% (w/v) Ficoll (as described by Kelley et al. [8]), or using a discontinuous sucrose gradient of 40% (w/w) sucrose overlaid with 25% (w/w) sucrose also prepared in Tris buffer containing 1 mM EDTA. The sucrose concentrations were derived empirically from experiments to maximise the yield of B2 (data not shown). Ficoll gradient tubes were spun at $90000 \times g$ for 1 h [8]. Sucrose gradient tubes were spun for 16 h at $80000 \times g$. The material at the top of the tube (fraction B1) and that at the density gradient interfaces (B2) and at the bottom of the tube (B3) was collected, washed and resuspended in 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM EDTA, 1 mM PMFS and 0.02% sodium azide.

All membrane fractions were stored on ice prior to the enzyme assays before being aliquoted and stored at −30°C.

2.4. Enzyme analyses

Protein concentrations of the various membrane fractions were determined as described by Markwell et al. [13]. This is a modification of the procedure of Lowry et al. [14] employing 1% SDS in the alkaline tartrate reagent. Versatol (General Diagnostics) was used as the protein standard. This method is unaffected by the presence of up to 200 mM sucrose or 2.5 mM EDTA [13]. Membrane fractions were diluted between 50 and 500 times prior to being assayed. Alkaline phosphatase (MVM marker) was measured by the release of *p*-nitrophenol from *p*-nitrophenol phosphate in a carbonate/bicarbonate buffer (pH 10.5) at 37°C [15]. Binding of [^{3}H]dihyroalprenolol (New England Nuclear, BM marker) was measured in 50 mM Tris (pH 7.4), at a dihydroalprenolol concentration of 10 nM, following a 5 min incubation at room temperature. Membranes were separated by rapid vacuum filtration using cellulose nitrate filters (0.2 μm, Sartorius) and were washed with 10 ml of ice-cold PBS. Nonspecific binding was measured in the presence of 100 μM DL-propanolol (Sigma). Filters were dissolved in 3 ml of Filtersolve (Packard) and counted in a Packard 5400 beta-counter. Total ATPase activity was assayed as described by Wheeler and Whittam [16]. Succinate dehydrogenase (mitochondrial membrane marker) was assayed as described by Adams et al. [17] but without EDTA in the incubation medium. Aromatase activity (endoplasmic reticulum marker) was determined using the 'tritiated water' assay [18]. Spectrophotometric assays were performed using an Hitachi U2000 spectrophotometer. Enzyme specific activities were expressed as quantity of end-product produced per mg of membrane protein per min. Dihydroalprenolol binding was expressed as pmol bound per mg of membrane protein. Results are presented as mean ± S.E. for five or six preparations.

2.5. Vesicle ultrastructure

Samples of membrane fractions M2, BP and B2 were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 h. Samples were postfixed in 1% osmium tetroxide and 2% uranyl acetate, dehydrated and embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate and examined under a Phillips 200 transmission electron microscope.

2.6. Vesicle orientation

The binding of FITC-labelled concanavalin A to α-D-mannose residues present on the surfaces of the vesicles in the M2 and B2 fractions was measured as previously described [9]. Non-specific binding was determined in the presence of 0.5 M methyl α-D-mannopyranoside. Binding was measured in both the normal vesicle preparation and in vesicles that had been lysed by repeated freeze-thawing in liquid nitrogen.

3. Results

3.1. Membrane protein recoveries

Fig. 1 illustrates the protein recovery data from 11 preparations expressed as mg of membrane protein per 100 g of placental tissue. In six preparations the BM fractions B2 and B3 were obtained using the Ficoll gradient purification procedure [8] and in five preparations the sucrose density gradient methodology was used. Purification of the crude MVM pellet (MP) re-

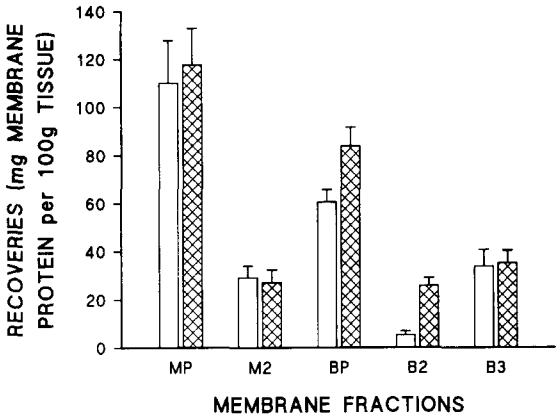

Fig. 1. Protein recoveries of crude and purified microvillous and basal membrane fractions. Recovery data is expressed as mg/100 g of villous tissue for the various membrane fractions (see text for details of abbreviations). Open bars are the recovery data from six placentae where the Ficoll density gradient purification procedure was used for BP while the hatched bars are from five preparations using the sucrose density gradient purification procedure.

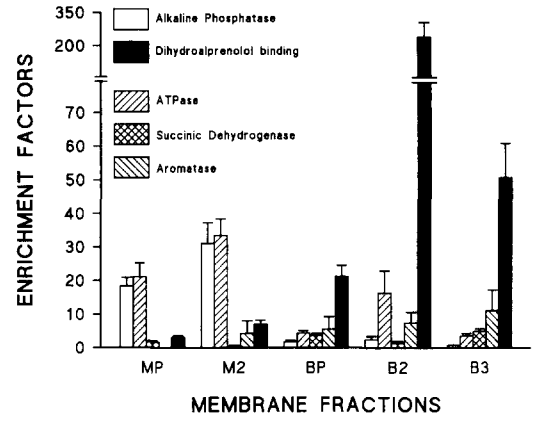

Fig. 2. Enzyme activities and [^{3}H]dihydroalprenolol binding activities in membrane fractions obtained using the Ficoll density gradient purifications of BM. Results are expressed as enrichment factors relative to an homogenate of villous tissue from the same placenta. Bars are means + S.E. for six placentae.

sulted in a mean reduction in protein recovery in M2 to about 26% of the MP value, i.e., from 110.1 ± 17.8 mg/100 g to 29.1 ± 4.9 mg/100 g. The Ficoll density gradient purification of the crude BM pellet (BP) resulted in a loss of over 91% of the protein content of the crude fraction, from 60.7 ± 4.8 mg/100 g to 5.2 ± 1.7 mg/100 g. This substantial loss of protein, although similar to that reported by Kelley et al. [8], made the procedure unsatisfactory in terms of the final yields. We therefore adopted a similar discontinuous sucrose density gradient procedure for BP as that used for MP, except that we empirically determined that the higher sucrose concentration needed to maximise the yield of B2 was 40% (w/w) (data not shown). Fig. 1 also shows the protein recovery data from this series of five preparations in which the BM fraction was purified on a discontinuous sucrose gradient. The B2 recovery of 25.8 ± 3.3 mg/100 g was comparable to the M2 recovery.

3.2. Enzyme analysis of membrane fractions

Table 1 gives the specific activities of alkaline phosphatase, ATPase, succinate dehydrogenase and aromatase and the dihydroalprenolol binding activity in the membrane fractions obtained from the six Ficoll gradient preparations. Values are expressed per mg of membrane protein. The enrichment factors relative to a villous tissue homogenate can be seen in Fig. 2. It can be seen that the alkaline phosphatase activity in MP is enriched over 18 times relative to the villous homogenate and this increases to over 31-fold following the sucrose gradient purification procedure. Alkaline phosphatase activities in the BM fractions were

Table 1
Characterization of MVM and BM fractions prepared using Ficoll gradient purification of BM

	Enzyme activities, or substrate bound, in membrane fractions				
	MP	M2	BP	B2	B3
Alkaline phosphatase (μg phenol/min per mg)	1398 ± 77	2361 ± 344	122 ± 31	154 ± 42	45 ± 12
Dihydroalprenolol binding (pmol bound/mg)	266 ± 36	575 ± 71	1757 ± 250	19213 ± 4512	3944 ± 474
ATPase (μg P_i/mg per min)	1032 ± 51	1697 ± 143	218 ± 7	745 ± 164	179 ± 17
Succinate dehydrogenase (μg INT/min per mg)	4 ± 1	1 ± 1	10 ± 1	3 ± 1	13 ± 1
Aromatase (nmol/min per mg)	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	0.7 ± 0.1

Enzyme activities are expressed as reaction product produced per min per mg of membrane protein. DHA binding is expressed as quantity bound per mg. Values are given as means ± S.E. from six placentae.

1–2-fold higher than that in the whole tissue homogenates. If the alkaline phosphatase activity in B2 was due solely to M2 contamination then M2 protein accounts for 6.5% of the total protein in B2. The DHA binding data in the two MVM fractions MP and M2 were 3- and 7-times the homogenate values respectively, whereas the BP fraction was over 21 fold enriched. The very high enrichment factors found in B2 reflects the high specific activity of the enzyme resulting from the low protein concentration, and probably reflects a membrane fractionation phenomenon [9]. B3 was 51-fold enriched in DHA binding. Total ATPase mirrored the alkaline phosphatase activity in the MVM fractions but was only 4-fold enriched in BP. None of the fractions revealed substantial succinate dehydrogenase activity and only moderate aromatase activity was detected in the BM fractions.

Since the full enzyme profiles had indicated the relative lack of contamination of BP with other membrane sources, in the experiments using the sucrose density gradient purification of BP, only alkaline phosphatase activity and DHA binding were examined. Table 2 illustrates the enrichment factors in MP, M2, BP, and B2 for these markers.

3.3. Effect of EDTA on alkaline phosphatase activity in M2

In some preliminary experiments in this study (data not shown) basal and microvillous membrane fractions had been resuspended, for convenience, in similar buffers containing 1 mM EDTA. Subsequent analysis of alkaline phosphatase activity had revealed lower activities in the M2 fraction when compared with those presented in Fig. 1 and Table 2. Hence a study was undertaken to determine the sensitivity of the alkaline phosphatase activity in M2 to increasing concentrations of EDTA. Fig. 3 illustrates the reduction in measured activity over time in the absence of EDTA or when the membrane fractions (all stored on ice) contained EDTA at a final concentration of 1, 5, 10 or 20 mM. The enzyme activity in the absence of EDTA is stable for at least 14 days under these conditions whereas significant reductions in activity within a few days were observed with fractions stored in the higher concentrations of EDTA.

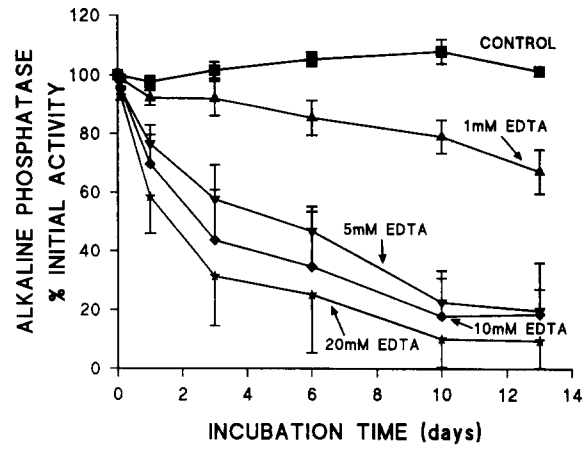

Fig. 3. Effect of EDTA on the activity of alkaline phosphatase in M2 fraction. Samples of M2 were incubated on ice for up to 14 days in storage buffers containing different concentrations of EDTA. The activity of the enzyme is expressed as a percentage of the zero time activity. Values are means ± S.E. from three incubations.

3.4. Ultrastructure of membrane fractions

Aliquots of M2, BP and sucrose-purified B2 from two representative experiments were prepared for EM. It can be seen from Fig. 4 that all three fractions contain predominantly vesicular structures with little evidence for membranes existing as sheets. The mean vesicle diameter (from two preparations) were, in the M2 fraction 232 ± 9 nm (S.E., $n = 126$), while vesicles in the B2 fraction were 317 ± 14 nm in diameter.

3.5. Concanavalin A binding studies

Vesicle orientation was measured in the M2 and B2 fractions from three fresh preparations. Results indicated that vesicles in the M2 fraction were 88.7 ± 2.7% 'right side out' while vesicles in the B2 fraction were 88.9 ± 6.4% 'right side out'.

4. Discussion

The human placental syncytiotrophoblast is a continuous epithelial barrier separating maternal blood from the fetal placental villous vasculature. The apical surface of the syncytium is in direct contact with mater-

Table 2
Comparison of alkaline phosphatase activity (MVM marker enzyme) and dihydroalprenolol (DHA) binding (BM marker) following membrane purification using sucrose density gradients

	Enrichment factors				
	MP	M2	BP	B2	B3
Alkaline phosphatase activity	18.1 ± 3.5	37.0 ± 9.5	1.8 ± 0.4	3.2 ± 0.6	0.6 ± 0.1
DHA binding	2.2 ± 0.6	6.1 ± 1.3	13.8 ± 4.2	40.6 ± 13.1	24.2 ± 14.4

Activities are expressed as enrichment factors relative to villous tissue homogenates. Values are given as means ± S.E. from five placentae.

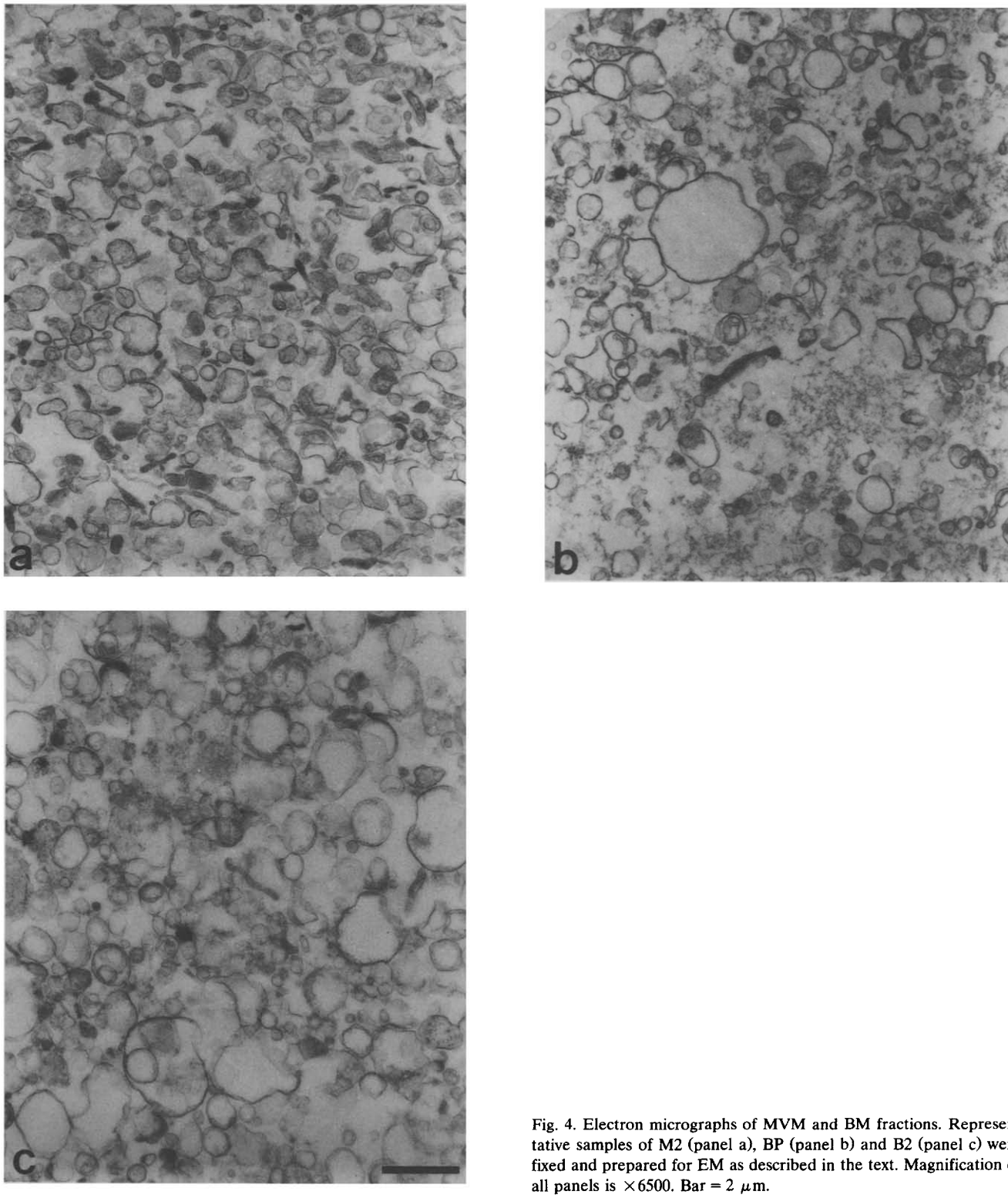

Fig. 4. Electron micrographs of MVM and BM fractions. Representative samples of M2 (panel a), BP (panel b) and B2 (panel c) were fixed and prepared for EM as described in the text. Magnification of all panels is ×6500. Bar = 2 μm.

nal blood in the placental intervillous space. All substances crossing the placenta in either direction must therefore diffuse or be transported across both this epithelial layer and the endothelial lining of the fetal vessels. Many methods are presently available to study trophoblast function, ranging from the ex vivo perfu-

sion of an isolated lobule of term placenta (which allows transplacental transfer to be studied, although the individual contributions of the two serial barriers cannot be determined), tissue slices and explants (which are mainly used for accumulation studies), to subcellular fractionation procedures. It has, however, become clear that an understanding of the role of this syncytium would benefit from the ability to produce highly purified membranes from both the maternal-facing and fetal-facing surfaces. In addition, a protocol for the production of 'paired' fractions from a single placenta might assist in the unravelling of many aspects of placental pathology.

The methods described here permit the preparation of highly purified 'paired' MVM and BM fractions, in high yield, from a single placenta without the need to homogenise the placental tissue either initially or after isolation of the MVM fraction. Yields of crude MVM and BM in excess of 100 mg/100 g of villous tissue can be routinely obtained, with sucrose density gradient purified fractions amounting to 25–30 mg/100 g. The procedure can therefore be readily applied to pre-term or pathological placentae which are usually only rarely available.

There are several important issues to be addressed in defining 'purity' and 'yield' in membrane fractions. The relevance of 'yield' data depends solely on the criteria used to define the preparation. Illsley et al. [9] have discussed the importance of recognising membrane heterogeneity and the problems associated with the final purification procedures for the BM fractions provide a good example of this issue. The original publication from Smith's laboratory [8] described a discontinuous Ficoll gradient procedure which resulted in a reduction in basal membrane protein of over 90%, while the dihydroalprenolol binding was only increased 1.5-fold. Dihydroalprenolol binding provides a measure of beta-adrenergic activity and, since these receptors are co-localised with adenylate cyclase, it is a useful surrogate assay of adenylate cyclase activity. Anatomical studies have shown that adenylate cyclase is specifically localised on the basal syncytiotrophoblast membrane [19]. In our hands the Ficoll procedures resulted in similar protein yields to those of Kelley et al. [8], i.e., we achieved 5.2 mg/100 g tissue, but with very high enzyme activities reflecting the low protein content of the fraction. This seemed to indicate a fractionation rather than a purification of BM. It is of interest to note that these authors have recently abandoned the Ficoll gradient purification stage [20,21]. We attempted to adapt the sucrose density methodology, used for the MVM, to increase the yield of B2 while significantly increasing the DHA binding enrichment factors. This procedure increased the B2 yield to 25.8 ± 3.3 mg/100 g, i.e., a nearly 5-fold increase in protein recovery which was associated with a nearly 3-fold DHA binding enrichment (see Table 2). The DHA enrichment factor in the sucrose density purified B2 of over 40-fold is within the upper range of published values.

There is still controversy in the literature concerning the localization of placental ATPase activities. Sodium/potassium, calcium and magnesium ATPases have been predominantly described as being MVM markers [3], (for review, see Ref. [22]) although Boyd et al. [7] suggested that Na^+/K^+-ATPase was mainly basal – despite the fact that their BM fraction had a similar alkaline phosphatase enrichment to their MVM fraction. Our data on total ATPase activity shows a similar M2 enrichment factor (33-fold) to that found for alkaline phosphatase (Fig. 2). B2, however, was only about half as enriched in ATPase activity as M2. Hence, although these results confirm that ATPase activity is present mainly in the MVM fraction, it is not as useful a marker as alkaline phosphatase for characterization of MVM fractions.

It was conceivable that the hypotonic stirring stage to disrupt and remove intracellular components prior to isolation of BM could result in small 'tails' of endoplasmic reticulum contaminating the preparation. We have therefore used a well established assay for P450-aromatase activity (which is located in high concentration in the microsomal fraction of placental tissue) to assess all membrane fractions for intracellular membrane contamination. There was negligible activity in the MVM fraction whereas the small enrichments observed in the basal fraction indicated that the above possibility was not, in fact, a serious problem. None of the membrane fractions were contaminated with mitochondrial membrane as demonstrated by the absence of succininic dehydrogenase activity.

The electron micrographs and the concanavalin A binding data indicate that the procedures described here produce homogeneous populations of vesicles which are almost all present in the correct orientation. The high percentage of 'right side out' vesicles (approaching 90% of the populations) probably reflect the absence of preparative homogenisation procedures.

The 37-fold enrichment factor for the MVM marker enzyme alkaline phosphatase produced by this methodology is one of the highest published values (see Ref. [9] for review). The isoenzyme of placental alkaline phosphatase which is produced at term is believed not to be sensitive to low concentrations of EDTA [4,23]. However, we here demonstrate a significant time and dose dependency for the inhibition of MVM alkaline phosphatase activity by EDTA (Fig. 3). Under the storage conditions employed, alkaline phosphatase activity in M2 was stable over a two week period. Some inhibition of enzyme activity was observed with time even at 1 mM EDTA, while activity was strongly inhibited after 6 days in the presence of 10 mM EDTA. This observation, demonstrating the sensitivity of the MVM

marker enzyme to an essential constituent of BM preparative buffers, has important implications for interpreting purification criteria for BM based on low alkaline phosphatase activity, particularly if the fractions are stored in the presence of high concentrations of EDTA.

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