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Purification and Na^+ uptake by human placental microvillus membrane vesicles prepared by three different methods

Jocelyn D. Glazier [a], Carolyn J.P. Jones [b] and Colin P. Sibley [a,c]

Departments of [a] Child Health, [b] Pathology and [c] Physiological Sciences, University of Manchester, St Mary's Hospital, Manchester (U.K.)

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Three methods were used to prepare microvillus membrane vesicles from each of six human placentas. Two of these incorporated an agitation stage to preferentially remove microvilli and either Ca^{2+} (Method 1) or Mg^{2+} (Method 2) aggregation of non-microvillus membrane. The third method involved homogenisation of the tissue followed by Mg^{2+} aggregation of non-microvillus membrane (Method 3). Enrichment of alkaline phosphatase activity (27.6 ± 1.9, 25.3 ± 2.7) and ouabain binding (5.9 ± 2.6, 5.3 ± 2.2, respectively) was similar in vesicles prepared by Methods 1 and 2, respectively. Method 3 vesicles showed a significantly ($P < 0.01$) lower alkaline phosphatase enrichment (18.1 ± 1.2), but ouabain binding enrichment (6.3 ± 1.3) was not different and vesicle protein recovery (mg/g placenta) was 5-fold greater. Na^+ uptake in the presence of an outwardly directed proton gradient was significantly inhibited in all microvillus membrane vesicles by amiloride (0.5 mM). However, the amiloride sensitive component of Na^+ uptake was 3–6-fold greater in Method 3 vesicles than in Method 1 and 2 vesicles, and showed overshoot above equilibrium in the former but not the latter. Further experiments using the pH sensitive dye, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein suggested that the proton gradient dissipated faster from Method 1 than from Method 3 vesicles. Thus methodological differences can have a marked effect on transport processes in microvillus membrane vesicles prepared from the human placenta.

Introduction

The use of membrane vesicles isolated from the maternal facing plasma membrane of the syncytiotrophoblast of human placenta (microvillus membrane vesicles), has provided considerable information on a variety of trophoblast transport mechanisms [1–3]. Although methods for preparing placental vesicles are often based on that of Smith et al. [4], where microvilli are preferentially removed from syncytiotrophoblast by agitation and isolated by differential centrifugation, there are a number of refinements used. These include the addition of Ca^{2+} [5] and Mg^{2+} [6] treatments for aggregation of non-microvillus membrane and the use of zonal [7] and sucrose density centrifugation steps [8,9]. Alkaline phosphatase has been shown histochemically to be more abundant on

Abbreviations: Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; Mes, 4-morpholineethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein.

Correspondence: C.P. Sibley, Department of Child Health, St Mary's Hospital, Hathersage Road, Manchester, M13 0JH, U.K.

the microvillus plasma membrane than on the basal (fetal facing) plasma membrane of the syncytiotrophoblast [10] and has been commonly used as a marker enzyme for this fraction. With the methods described above [5–9], alkaline phosphatase enrichment in microvillus fractions has been reported to vary from 18- to 32-fold. Na^+/K^+-ATPase is also commonly used in these preparations as a marker enzyme for contamination of the microvillus membrane by the basal membrane, although there is, to our knowledge, no histochemical or other independent evidence that this enzyme is localised to the basal membrane; indeed in the guinea-pig placenta Na^+/K^+-ATPase is more abundant on the microvillus plasma membrane [11]. A range of Na^+/K^+-ATPase enrichments in microvillus plasma membrane fractions have been reported, from 12-fold [12] to 0.3-fold [9].

None of the methods [4–9] has ever been directly compared and it is therefore unclear whether the variations described above are the results of the different techniques used or of biological variation in the placentas. Moreover, it has not been directly shown whether the method of removing microvilli from the placenta by agitation before centrifugation is an improvement compared to the simpler homogenisation step used for preparation of microvillus membranes from other epithelia [13]. It is unclear what effects the different methods of preparation have on the transporters under investigation, although for example differences in Na^+ permeability between placental microvillus membrane vesicles from different laboratories can be inferred from data on Na^+-amino acid cotransport [5,14,15]. Also in studies on Na^+-H^+ exchange by placental microvillus membrane vesicles Chipperfield et al. [16] have reported marked differences in several aspects of Na^+ uptake (presence or absence of overshoot, degree of non-specific binding, sensitivity to amiloride and the proton ionophore FCCP) as compared with the similar study of Balkovetz et al. [17] who prepared their vesicles by a different method.

In this study we investigated purification of microvillus membrane vesicles prepared from the same placentas by three different methods, two of which have been widely used for placenta [5,6] and one similar to that used for kidney and intestine [13,18]. We also investigated the activity of the Na^+-H^+ exchanger in the three vesicle preparations.

Methods and Materials

Preparation of vesicles and purification

Six placentas obtained at term after vaginal delivery or elective casearian section were obtained and three 100 g segments of tissue taken after removal of the fetal membranes. The three methods used to prepare the vesicles from each segment were as follows.

(1) As described by Boyd and Lund [5] for the placenta (hereafter called Method 1) in which the 100 g segment was cut into approx. 3-g pieces and then washed three times by stirring for 1 min in 150 ml ice-cold 100 mM $CaCl_2$ followed by three further washes with 150 ml ice-cold buffered saline (150 mM NaCl, 4 mM KCl, 2 mM Tris-HCl, 2 mM $MgCl_2$ (pH 7.4 at 4°C)). The tissue was then teased out by scraping and minced with scissors and a small sample taken for enzyme activity and protein analysis before being stirred on ice in the buffered saline for 30 min. The solution was then passed through a 2 mm mesh sieve and the filtrate centrifuged at $800 \times g$ for 10 min; the supernatant was then spun at $10000 \times g$ for 20 min and finally the supernatant from this was spun at $110000 \times g$ for 30 min to pellet the microvillus membranes.

(2) As described by Booth et al. [6] for the placenta (hereafter called Method 2). This method is initially similar to Method 1 so that 3-g pieces of placenta from the 100 g segment were washed three times with 150 ml ice-cold 50 mM $CaCl_2$, then three times with 150 ml ice-cold 150 mM NaCl. The tissue was then teased out by scraping and minced with scissors and (after taking a small sample for enzyme activity and protein analysis) stirred on ice in 150 mM NaCl for 60 min after which the solution was passed through a 2 mm mesh sieve and the filtrate centrifuged at $800 \times g$ for 10 min. The supernatant was then centrifuged at $10000 \times g$ for 10 min, and the supernatant from this stage centrifuged at $90000 \times g$ for 30 min. The pellet from this spin was resuspended in 50 ml 10 mM mannitol, 2 mM Tris-HCl (pH 7.1 at 4°C); to this was added 10 mM $MgCl_2$ and the solution was stirred on ice for 10 min before being

centrifuged at 2200 × *g* for 12 min. Finally the supernatant from this was centrifuged at 15 000 × *g* for 30 min to re-pellet the microvillus membrane.

(3) Method 3 was similar to that described by Booth and Kenny [18] (hereafter called Method 3) for rabbit kidney, in which the 100 g segment was homogenised in 2.5 volumes ice-cold buffer (300 mM mannitol, 10 mM Hepes-Tris and 1 mM $MgSO_4$ (pH 7.4 at 4°C)). A sample of the homogenate was taken for enzyme activity and protein analysis and then 10 mM $MgCl_2$ was added to the remaining homogenate and the solution stirred on ice for 10 min. It was centrifuged at 2200 × *g* for 15 min and the supernatant from this was spun at 23 500 × *g* for 40 min to yield a pellet which was resuspended in the mannitol buffer and the Mg^{2+} treatment repeated followed by re-centrifugation as before to yield the microvillus membrane pellet.

All microvillus membrane pellets were resuspended in intravesicular buffer (see below) by passing them 20 times through a 25G syringe needle. Protein content of mince samples from Methods 1 and 2 (which were homogenised before assaying) and homogenate (Method 3) and of all vesicle fractions was measured by the method of Lowry et al. [19] using bovine serum albumin as standard, alkaline phosphatase activity was measured at pH 9.8 using *p*-nitrophenyl phosphate as a substrate [20] and ouabain binding, as a measure of Na^+/K^+-ATPase, was assayed using 10 nM [^{3}H]ouabain (Amersham International plc, Bucks., U.K.) as described by Kelley et al. [21].

Samples of pelleted vesicles from three placentas were prepared for electron microscopy by fixing them in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). These were post fixed in 1% osmium tetroxide, dehydrated in graded alcohols and propylene oxide and embedded in Taab resin (Taab Laboratories Equipment Ltd., Reading). Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips 301 electron microscope at 60 kV.

Na^+ uptake

Na^+ uptake into vesicles was measured at room temperature using ^{22}Na in the presence of a proton gradient. Thus the vesicle pellets were suspended in intravesicular buffer consisting of 25 mM Mes, 5 mM Tris, 149 mM KCl and 1 mM NaCl (pH 5.6), at a concentration of approx. 10 mg/ml. Uptake was initiated by adding 200 µl vesicle suspension to 900 µl of extravesicular buffer, consisting of 18 mM Hepes, 12 mM Tris, 149 mM KCl, 1 mM NaCl (pH 7.6) to which 2.5 µCi/ml ^{22}Na (Amersham) had been added. In separate incubations, uptake was measured as above except with the addition of 0.5 mM amiloride. At various time intervals after initiating uptake 100-µl samples of the incubate were taken and extravesicular ^{22}Na separated from intravesicular ^{22}Na using ion-exchange columns as described by Gasko et al. [22]. Disposable Pasteur pipettes were filled with Dowex 50W-X8 which was then equilibrated with the extravesicular buffer and kept at 4°C. The 100-µl samples of incubate were added directly to the columns which were then washed with 2.5 ml of the extravesicular buffer. The eluent, containing only vesicles and their associated ^{22}Na, was collected in counting tubes and counted in a gamma counter. Samples of radioactive buffer, without vesicles were also run through the columns and the eluent counted, so that 'column background' ^{22}Na cpm could be subtracted from the vesicle cpm. Also to identify membrane bound, as opposed to intravesicular ^{22}Na, further incubations were carried out in the presence of 1.8% Triton.

Vesicle orientation and pH measurements

Vesicles were prepared from a further three placentas using Methods 1 and 3. The orientation of these vesicles was then investigated by measuring alkaline phosphatase specific activity before and after permeabilising with 0.1% saponin.

To investigate the rapidity with which a proton gradient was dissipated from the vesicles in the absence of Na^+ the pH sensitive fluorescent indicator 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (Calbiochem; Cambridge Biosciences, Cambridge, U.K.) [23] was used. Vesicles (approx. 16 mg protein/ml suspended in the pH 5.6 Mes/Tris/KCl buffer described above without NaCl) were incubated for 18 h at 0°C with 100 µM BCECF as described by Cabrini et al. [9] for 6-carboxyfluorescein. Immediately prior to use 100 µl of the vesicles were washed three times with 1 ml of BCECF- and Na^+-free Mes/Tris/KCl (pH 5.6) buffer. Dye leakage from the loaded, washed

vesicles was found to be less than 2% in 30 min at room temperature. Experiments were begun by adding 10 μl of the washed vesicles to 2.0 ml of the Na^+-free Mes/Tris/KCl (pH 5.6) buffer in a stirred cuvette at room temperature in a Perkin Elmer LS-3 fluorimeter. Fluorescence (excitation 490 nm, emission 526 nm) was recorded for 1 to 2 min before the pH of the extravesicular medium was rapidly increased by addition of 10 μl of 2 M Tris. Fluorescence was then recorded for a further 2 to 3 min before addition of 20 μl Triton (20%) to disperse the vesicles and expose the dye to the external buffer. The fluorescence signal was calibrated by measuring the pH of the buffers before and then after the addition of 5-μl aliquots of 1 M HCl. This was continued until the fluorescence signal was less than that of the initial vesicle solution.

Chemicals and Statistics

All chemicals were obtained from Sigma Chemical Co (Poole, Dorset, U.K.) unless otherwise stated.

Data are presented in all cases as mean ± S.E.; statistical comparisons were made using the paired Student's '*t*'-test, *n* being the number of placentas.

Results

As shown in Table I, there was no significant difference between Methods 1 and 2 as regards protein recovery, alkaline phosphatase enrichments (which were similar to those reported previously [5,6]) or ouabain binding enrichment and both produced a vesicle fraction with greater alkaline phosphatase enrichment than Method 3. However, whereas the alkaline phoshatase enrichment of Method 3 vesicles was 67–72% of that achieved with Methods 1 and 2, the protein recovery was 5-fold greater. Ouabain binding enrichment was between 5–6-fold in all the vesicle fractions, there being no significant difference.

Under the electron microscope (Fig. 1), all the vesicle fractions were free from contamination with subcellular organelles and there were no obvious differences between them.

A typical time course for Na^+ uptake is shown in Fig. 2; uptake into vesicles prepared by all three methods was linear at least until 1 min, and this time point has been used for statistical comparison, as well as the 60 min uptake which approximated to the equilibrium value in all three. Membrane bound ^{22}Na as measured in the presence of Triton was always less than 8.0% of 60 min uptake, similar to that found previously for Method 1 vesicles by extrapolation to infinite osmolarity [16]; there were no significant differences between the three methods. Total (control) Na^+ uptake at 1 min was significantly higher into vesicles prepared by Method 3 as compared to Methods 1 and 2 (Table II). This was mainly due to a significantly ($P < 0.01$) higher amiloride-sensitive component, which was 1.52 ± 0.21

TABLE I

TOTAL PROTEIN CONTENT, ALKALINE PHOSPHATASE ACTIVITY (AP) AND OUABAIN BINDING ACTIVITY (OB) IN DIFFERENT FRACTIONS FROM HUMAN PLACENTA PREPARED BY THREE METHODS

Mean ± S.E., $n = 6$.

Fraction	Method	Protein recovery (mg/g placenta)	AP		OB	
			μmol/mg per min	enrichment	pmol per mg	enrichment
Mince	1	94.9 ±5.4	0.27±0.04	-	0.36±0.13	-
Mince	2	83.7 ±8.7 [c]	0.32±0.05	-	0.29±0.07	-
Homogenate	3	99.4 ±3.8	0.39±0.05	-	0.35±0.04	-
Vesicles	1	0.11±0.02	7.66±1.37	27.6±1.9	0.82±0.23	5.9±2.6
Vesicles	2	0.10±0.02	8.28±1.48	25.3±2.7	1.02±0.15	5.3±2.2
Vesicles	3	0.57±0.07 [b]	7.21±1.00	18.1±1.2 [a]	2.23±0.47	6.3±1.3

[a] $P < 0.05$ vs. Methods 1 and 2.
[b] $P < 0.01$ vs. Methods 1 and 2.
[c] $P < 0.05$ vs. Method 3.

TABLE II

Na^+ UPTAKE INTO MICROVILLUS MEMBRANE VESICLES WITH AN OUTWARDLY DIRECTED pH GRADIENT (AS Fig. 2) IN THE PRESENCE AND ABSENCE OF AMILORIDE (0.5 mM)

Mean ± S.E., $n = 6$.

Method	Na^+ uptake (nmol/mg protein)			
	1 min		60 min	
	control	amiloride	control	amiloride
1	0.40 ± 0.11	0.07 ± 0.04 [b]	1.36 ± 0.20	1.86 ± 0.23
2	0.60 ± 0.21	0.15 ± 0.11 [b]	1.66 ± 0.40	1.01 ± 0.34
3	1.83 ± 0.25 [c]	0.30 ± 0.07 [a,d]	1.60 ± 0.20	1.45 ± 0.43

[a] $P < 0.01$.
[b] $P < 0.05$ vs. respective 1 min control.
[c] $P < 0.01$ vs. Methods 1 and 2.
[d] $P < 0.05$ vs. Method 1.

nmol/mg protein for Method 3 at 1 min compared to 0.27 ± 0.12 nmol/mg protein and 0.52 ± 0.12 nmol/mg protein for Methods 1 and 2, respectively. The amiloride-insensitive component of Na^+ uptake was also significantly higher in Method 3 as compared to Method 1 (Table II). There were no significant differences between Methods 1 and 2 regarding Na^+ uptake at 1 min, and there were no significant differences between any Methods with respect to equilibrium (60 min) uptake (Table II).

It can be seen from Fig. 2 that Method 3 vesicle Na^+ uptake showed a marked overshoot above the equilibrium value which was not shown by Method 1 and 2 vesicles. This was a consistent result; mean maximal (control) uptakes for Methods 1 and 2 were 1.86 ± 0.28 and 2.10 ± 0.33 nmol/mg protein, respectively (not significantly different from equilibrium values in Table II, paired 't' test), whereas that for Method 3 was 3.14 ± 0.54 nmol/mg protein ($P < 0.01$ vs. equilibrium value in Table II, paired 't' test). This overshoot occurred at between 3 to 10 min and was abolished by amiloride (mean maximal value for Method 3 in the presence of amiloride was 1.86 ± 0.23 nmol/mg protein; c.f. Table II). In three further experiments, single alterations were made to Method 3 to try to abolish the overshoot: (1) $MgCl_2$ was replaced by $CaCl_2$, (2) tissue was stirred rather than homogenised, (3) tissue was initially homogenised in 150 mM saline buffer (c.f. Method 1) rather than in the mannitol, Hepes-Tris buffer. In all three experiments the overshoot persisted.

In the second series of experiments we found that the specific activity of alkaline phosphatase

TABLE III

MEASUREMENTS OF pH WITH BCECF IN MICROVILLUS MEMBRANE VESICLES: EFFECT OF ADDITION OF Tris AND OF Triton

The pH was measured in microvillus membrane vesicles suspended in 2 ml 25 mM Mes, 5 mM Tris, 149 mM KCl buffer (pH 5.6) (control), 0.5, 1 and 2 min after addition of 10 µl Tris (2 M) and after addition of 20 µl of Triton (20 %) to the external medium. Mean ± S.E. ($n = 3$).

Method	pH				
	Control	Addition of Tris			Addition of Triton
		0.5 min	1 min	2 min	
3	5.35 ± 0.08	6.13 ± 0.05	6.15 ± 0.05	6.24 ± 0.08	6.55 ± 0.05
1	5.41 ± 0.20	6.53 ± 0.06	6.57 ± 0.06	6.58 ± 0.05	6.64 ± 0.02

after treatment of the vesicles with saponin was 96.0 ± 4.0% and 95.0 ± 2.0% for Methods 1 and 3, respectively ($n = 3$), suggesting that there was no effect of the permeabilising agent.

The data from the pH measurements is shown in Table III. It can be seen that the initial pH of Method 1 and 3 vesicles was similar although somewhat lower than that of the suspending buffer,

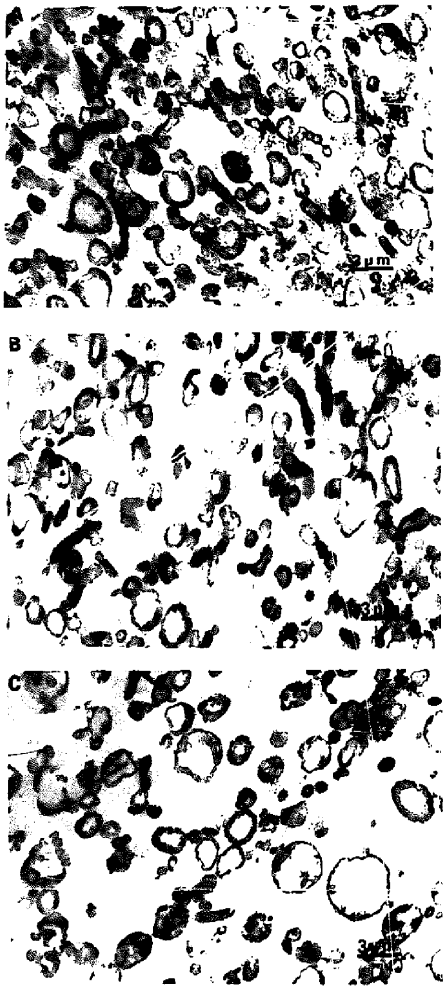

Fig. 1. Electron micrographs of vesicles prepared by Method 1 (A) Method 2 (B) and Method 3 (C). There are no subcellular organelles present and the three preparations appear to be very similar.

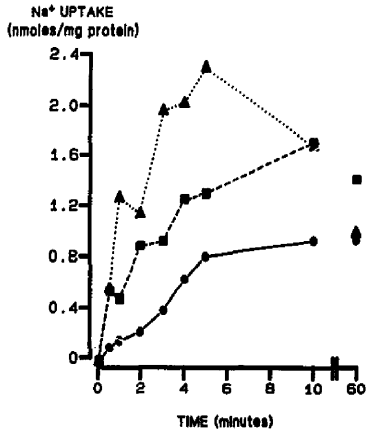

Fig. 2. Na^+ uptake, in the presence of a pH gradient, into microvillus vesicles prepared by Method 1 (●), Method 2 (■) and Method 3 (▲) from the same placenta. Vesicles (in 10 mg protein/ml) were suspended in 25 mM Mes, 5 mM Tris, 149 mM KCl and 1 mM NaCl (pH 5.6). Uptake was initiated by addition of 200 μl vesicle suspension to 0.9 ml of a solution containing 18 mM Hepes, 12 mM Tris, 149 mM KCl, 1 mM NaCl and 2.5 μCi/ml ^{22}Na, pH 7.6. At the times shown 100 μl samples were taken and applied to ion exchange columns for separation of vesicular from extravesicular ^{22}Na.

perhaps due to a Donnan effect as suggested for tracheal apical membrane vesicles [24]. Addition of 2 M Tris produced a 1.2 pH unit gradient between the inside of the vesicles and the external buffer (as measured after the addition of Triton) and the pH of the vesicles rapidly increased. However, the pH change in Method 3 vesicles was slower than that in Method 1 vesicles. In fact 2 min after the addition of Tris the pH of the Method 1 vesicles had equilibrated with the external medium in two of the three experiments, whereas none of the Method 3 vesicles had equilibrated at that time. In terms of proton concentration 99.4 ± 0.6% of the original gradient had dissipated from the Method 1 vesicles at 2 min, whereas 91.2 ± 4.2% had dissipated from the Method 3 vesicles.

Discussion

This study has shown marked differences between microvillus membrane vesicles prepared from the same placenta by different methods. The

differences were essentially between the vesicles prepared by the placenta-specific techniques incorporating agitation of the tissue to remove microvilli (Methods 1 and 2) and the more general method whereby tissue was homogenised and reliance placed on Mg^{2+} aggregation of non-microvillus membrane to ensure purification (Method 3).

Although the purification of placental microvillus membrane vesicles by Method 3 was not as great as that with Methods 1 and 2 as judged by alkaline phosphatase enrichment, it was still within the reported range [5–9] and neither ouabain binding enrichment, nor appearance under the electron microscope further suggested any marked difference in purity between the preparations. Together with the 5-fold greater protein recovery this might therefore make Method 3 particularly useful. The similarity of purification data for Methods 1 and 2 suggests little advantage in the latter, which is more time consuming; the alkaline phosphatase enrichments of these vesicles were comparatively high [5–9] and were similar to those obtained by others using density centrifugation steps [8,9].

The inhibition of Na^+ uptake in the presence of a proton gradient by amiloride confirmed the previous evidence of Na^+-H^+ exchange in the placental microvillus membrane [16,17]. However, Na^+-H^+ exchange (as calculated from the amiloride sensitive Na^+ uptake) appeared to be 3- to 6-fold greater in Method 3 vesicles as compared with Methods 1 and 2. This difference was not due to an increased total vesicle volume with Method 3, as equilibrium (60 min) uptake was similar for all three methods. We cannot exclude the possibility that a population of vesicles with a smaller average volume were produced by Method 3, increasing the surface to volume ratio, but there was no evidence of this from electron microscopy. Another possible explanation for the increased amiloride sensitive Na^+ uptake could be that Method 3 produces a greater proportion of inside-out oriented vesicles, exposing a regulatory pH-sensitive site normally at the cytoplasmic face. However, as alkaline phosphatase is normally located at the extracellular face of the membrane [25] and assuming that saponin would increase substrate availability to any enzyme located inside vesicles, its failure to have any effect on the measured specific activity of alkaline phosphatase suggests that the vast majority of Method 1 and 3 vesicles were right-side out. Both renal and intestinal brush-border membranes have previously been shown by freeze-fracture techniques to be mainly right-side out [26] and further studies with this technique could provide more information on the size as well as the orientation of the placental vesicles.

Our pH measurements demonstrated a similar rate of proton gradient dissipation as that previously reported for placental microvillus membrane vesicles by Cabrini et al. [9]. However, the proton gradient dissipated more rapidly from Method 1 than from Method 3 vesicles and a small proton gradient was still present across the latter membranes 2 min after its imposition. This could be at least part of the explanation of the increased Na^+-H^+ exchange activity of the Method 3 vesicles and of the overshoot of Na^+ uptake above equilibrium which was apparent only with these vesicles. In the other recent studies on Na^+-H^+ exchange in placental microvillus membrane vesicles, Balkovetz et al. [17] (vesicles prepared similarly to Method 2) found overshoot of Na^+ uptake, in the presence of a proton gradient, using a KCl-containing uptake buffer similar to our own. On the other hand Chipperfield et al. [16] reported similar results to ourselves in that they did not find overshoot into vesicles prepared by Method 1 when they used KCl in their uptake buffer. However, when they replaced KCl with mannitol they did find a reproducible overshoot. These authors [16] suggest that the proton gradient is rapidly dissipated by exchange of Cl^- for OH^- ions via an anion exchanger in the vesicles [27]. However, Cabrini et al. [9] have also reported an electrogenic proton transport mechanism in placental microvillus membrane vesicles the activity of which is increased by the presence of Cl^-. Therefore preparitive effects on the activity of either of these mechanisms could explain both the different results of Balkovetz et al. [17] and Chipperfield et al [16] as well as the differences between our Method 1 and Method 3 vesicles with regard to proton gradient dissipation and overshoot of Na^+ uptake.

In renal brush-border membranes it was found that overshoot of Na^+ uptake wa abolished by

using Ca^{2+} rather than Mg^{2+} during preparation of vesicles as a result of increased proton permeability [28]. However, we found that Ca^{2+} did not abolish overshoot in Method 3 vesicles and neither did use of NaCl rather than mannitol buffer as washing medium, nor did stirring rather than homogenising. Overshoot phenomena have also previously been reported in studies on neutral amino acid-Na^+ co-transport into placental microvillus membrane vesicles, but with marked differences in duration. Thus Ruzycki et al. [14] reported that an inward directed Na^+ gradient caused an overshoot of aminoisobutyric acid uptake above equilibrium into vesicles prepared by the method of Smith et al. [4]) which persisted for at least 120 min. Boyd and Lund [5] found that Na^+ gradient stimulated overshoot of L-proline uptake (into vesicles prepared by Method 1) was over within 3 min. Finally, the data of Cole [15] showed the Na^+ gradient stimulated overshoot of L-proline uptake (into vesicles prepared by a hybrid of Methods 1 and 2) persisting at least until 10 min. Thus the method of preparation may have effects on other transport mechanisms in placental vesicles.

In summary, although it has not been possible to elucidate the important factors in preparing placental microvillus membrane vesicles fractions which handle Na^+ and proton in different ways, this study has made it clear that this is due to methodological differences rather than biological variation. The simple explanation may be differential contamination of the microvillus membrane vesicle fractions with membrane from other sites, yet the large increase in the amiloride-sensitive component of Na^+ uptake in Method 3 compared with Methods 1 and 2 was accompanied by only a slight decrease in purification as judged by all criteria. As the microvillus plasma membrane of the syncytiotrophoblast is itself certainly heterogeneous as shown, for example, by the distribution of alkaline phosphatase [29], it could be that different regions of it are purified by the different methods. This may have functional significance and is worthy of further investigation.

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