Microvilli of the human term placenta

Isolation and subfractionation by centrifugation in sucrose density gradients

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Human placental microvilli were isolated and separated into two fractions by centrifugation in sucrose density gradients. Electron-microscopic morphology and morphometry, the distribution of enzymic activities and the results of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of proteins were used to assess the purity of the final preparations and to define their properties. The combined evidence strongly suggested that the preparations contained negligible material that was not plasma membrane. The two fractions of microvilli differed in buoyant density, protein composition, enzyme specific activities and microscopic appearance. Some of these differences were explained by the absence of internal structure in the microvilli of the lighter fraction.

The microvillus plasma membrane of the human placental syncytiotrophoblast is in direct contact with maternal blood and participates in many diverse functions which serve to maintain a suitable foetal environment. Nutrients and metabolites are exchanged across this surface membrane, and it is also involved in hormonal and immunological interactions between mother and foetus.

Investigation of the composition and function of the human placental villous membrane was stimulated by the discovery that a preparation of microvilli could be obtained by differential centrifugation of a saline extract of whole placental tissue (Smith *et al.*, 1974). Subsequently, studies on transport (Ruzycki *et al.*, 1978; Fant *et al.*, 1979), receptor–ligand interaction (Friedman *et al.*, 1977; Whitsett *et al.*, 1979; Loh *et al.*, 1980), immunological factors (Faulk *et al.*, 1978; McIntyre & Faulk, 1979) and other biochemical features (Smith *et al.*, 1977; Brown *et al.*, 1979; Kelley *et al.*, 1979) have been carried out on microvillus-rich fractions prepared in a similar way.

By modifying the original procedure, we have been able to prepare a fraction of microvillous membrane of greater purity than has been hitherto reported, and have separated the microvilli into two fractions which differ in their appearance, buoyant density, enzymic activities and protein composition.

Abbreviation used: SDS, sodium dodecyl sulphate.

In the present paper, some morphological and biochemical properties of these preparations are presented.

Experimental

Preparation of microvilli

Gross and microscopic abnormalities of the placenta, including the microvillous surface membrane of the syncytiotrophoblast, have been described in mothers who smoked cigarettes (Asmussen, 1977; Spira *et al.*, 1977; Naeye, 1978). Hence, only placentas from non-smoking mothers were used in the present study.

Placentas were collected within 30 min of vaginal delivery from mothers with uncomplicated full-term pregnancies. The membranes were removed and the villous tissue was cut into about 20 pieces. After washing with cold iso-osmotic (0.1 M) CaCl₂ and cold Krebs–Ringer saline (DeLuca & Cohen, 1964) to remove blood, 250 ml of cold iso-osmotic (0.15 M) NaCl solution was added and the mixture was shaken at 0°C for 30 min. The large pieces of placenta were removed and the saline extract was centrifuged at 1200 *g*_{av} for 10 min. All centrifugations were performed at 4°C. The supernatant was then centrifuged at 15 000 *g*_{av} for 5 min and the microvilli were sedimented by centrifugation of the second supernatant at 105 000 *g*_{av} for 60 min. The pellet of microvilli was rinsed with cold iso-osmotic

saline and resuspended in 5 ml of unbuffered 10% sucrose. The suspension was then centrifuged at $6000g_{av}$ for 10 min. The white supernatant (sample C, the microvillus-enriched fraction) was removed and the brownish pellet (sample B) was resuspended in 2 ml of 10% sucrose.

A homogenate of the placenta was prepared for comparison of enzyme activities. Approx. 3 g of washed villous tissue, scraped free of visible blood vessels, was homogenized gently in 25 ml of 10% sucrose in a Potter homogenizer with a motor-driven Teflon pestle. The homogenate was filtered through nylon mesh and the filtrate was then subjected to the same centrifugation procedure as the microvilli. Samples of the homogenate (H), the mitochondrial pellet (M, $15000g \times 5$ min pellet), the microsomal pellet (P, $105000g \times 60$ min pellet), and the supernatant (S) were retained. Pellets were resuspended in 2 ml of 10% sucrose and were used at once or stored at -20°C overnight. All sucrose concentrations are expressed as % (w/w).

Sucrose gradient centrifugation

Microvilli were further purified on a step gradient composed of 4 ml of 50%, 10 ml of 43%, 10 ml of 34%, 8 ml of 25% and 4 ml of 20% sucrose (unbuffered). A 1–2 ml portion of the microvillus-enriched fraction (sample C), containing about 10 mg of protein, was overlayed on to the gradient, which was centrifuged in a Beckman SW27 rotor at 25000 rev./min ($85000g_{av}$) for 18 h at 4°C . The bands at the 25%/34%-sucrose interface (sample C2) and at the 34%/43%-sucrose interface (sample C1) were collected by suction, diluted 2-fold with 0.15 M-NaCl and centrifuged at $105000g_{av}$ for 60 min. The pellets were resuspended in 10% sucrose.

Electron microscopy

Samples for electron microscopy were fixed in iso-osmotic buffered half-strength Karnovsky's fixative (Karnovsky, 1965), post-fixed in OsO_4 , dehydrated, and embedded in Epon 812. Thin sections were stained with lead citrate and uranyl acetate and examined with a Siemens 102 electron microscope.

Measurements of membrane thickness were made on micrographs of both whole cells and the isolated microvillus-enriched fractions. Thickness was measured only when the bilayer structure was clearly defined and, for the microvilli, only one measurement was made on any one membrane fragment. The proportions of vesicles and microvilli seen in the micrographs were also assessed. Phosphotungstic acid (2%, w/v), pH 7.2, was used for negative staining of microvillous membrane suspensions, which had been diluted to approx. 1 mg of protein/ml in 0.15 M-NaCl. Non-parametric methods were used for all statistical evaluations.

Samples were also treated with thiocarbonylhydrazide as described by Malick & Wilson (1975) and examined with an ISI Super IIIA scanning electron microscope.

Enzyme assays

All enzyme assays were performed at 37°C at a protein concentration which gave an activity within the linear range for the assay. Substrates for enzyme assays were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise indicated. Assays were usually performed in duplicate, with coefficients of variation of $\pm 5\%$ for continuous assays and $\pm 10\%$ for stopped assays. The storage properties of each enzyme were assessed both for the homogenate and for fraction C, and assays were performed at a time when little or no change in activity had occurred.

Alkaline phosphatase (EC 3.1.3.1) activity was measured by the method of Bowers & McComb (1966) as hydrolysis of *p*-nitrophenyl phosphate at pH 10.15. 5'-Nucleotidase (EC 3.1.3.5) was assayed as Ni^{2+} -sensitive Mn^{2+} -dependent phosphate release from AMP at pH 7.5 (Baginski *et al.*, 1977). γ -Glutamyl transpeptidase (EC 2.3.2.2) was determined, by using Beckman Enztrate reagents (Beckman Instruments, Fullerton, CA, U.S.A.), as hydrolysis of γ -glutamyl *p*-nitroanilide at pH 8.2 with glycylglycine as acceptor. Lactate dehydrogenase (EC 1.1.1.27) was assayed as the lactate-to-pyruvate reaction by using a single-vial reagent (Calbiochem, La Jolla, CA, U.S.A.) with or without 0.03% (w/v) sodium deoxycholate. Catalase activity (EC 1.11.1.6) was measured as described by Beers & Sizer (1952), and acid phosphatase (EC 3.1.3.2) was determined as tartrate (40 mM)- or fluoride (2 mM)-inhibited hydrolysis of *p*-nitrophenyl phosphate (Smith & Whitby, 1968) at pH 5.5. The method of Cooperstein & Lazarow (1951) was used for determination of cytochrome oxidase (EC 1.9.3.1), and NADH- and NADPH-cytochrome *c* reductase (EC 1.6.99.2 and 1.6.99.1) assays were performed by the method of Dallner *et al.* (1966). Glutamate-pyruvate transaminase (EC 2.6.1.2) was assayed with Roche Diagnostica reagents (F. Hoffman-La Roche, Schweizehalle, Switzerland).

Thiamin pyrophosphatase (EC 2.5.1.3) activity was measured as the difference between the activity of an untreated sample and a sample heated to 60°C for 20 min, under the buffer and substrate conditions of Cheetham *et al.* (1971). This method was used because thiamin pyrophosphate is also hydrolysed by alkaline phosphatase, which is very active in preparations of placental microvilli and is resistant to heating at 60°C for 20 min (Holmgren & Stigbrand, 1978). UDP-galactosyltransferase (EC 2.4.1.22) was assayed as described by Morré (1971),

but with pH 6.5 buffer to minimize non-specific release of galactose from UDP-galactose (Fleischer & Fleischer, 1970), and using 1 ml columns of Bio-Rad AG2-X8 anion-exchange resin to isolate the reaction product. Biosynthesis of oestrogens was assessed essentially by the method of Braselton *et al.* (1973). The reaction mixture contained 100 nmol (0.2 μ Ci) of [4- 14 C]testosterone (The Radiochemical Centre, Amersham, Bucks., U.K.) and 1.2 μ mol of NADPH in a final volume of 2 ml of 25 mM-sodium phosphate buffer, pH 7. The reaction was started by addition of enzyme and stopped after 10 min by shaking with 5 ml of dichloromethane. Steroids were extracted with a total of 15 ml of dichloromethane, which was then evaporated and the steroids separated by two-dimensional t.l.c. The silica-gel plates (60F₂₅₄; Merck, Darmstadt, Germany) were developed firstly in benzene/diethyl ether (5:4, v/v), and in the second dimension in methanol/chloroform (1:99, v/v). Spots were observed under u.v. light and by radioautography using Kodak XRP5 film (18 h exposure), and were identified by comparison with standards. The spots were scraped off the plates and the radioactivity was recovered by extraction with methanol/water (19:1, v/v). Radioactivity was measured in a Beckman liquid-scintillation spectrometer. The samples were dissolved in toluene (5 ml) containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenoxyloxazol-2-yl)benzene.

Chemical analyses

RNA and DNA were assayed by the ethidium bromide technique described by Le Pecq & Paoletti (1966). Lipids were extracted as described by Folch *et al.* (1957) and the solvent was evaporated. Cholesterol was assayed by dissolving the extract in 200 μ l of ethanol and using 50 μ l of this solution in an enzymic cholesterol assay (Abbot Laboratories, Pasadena, CA, U.S.A.). Total phospholipid was measured by the procedure of Baginski *et al.* (1967).

Protein concentration was measured by the method of Lowry *et al.* (1951), as modified by Markwell *et al.* (1978), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis

Slab gels containing 7.5% or 10% (w/v) acrylamide and 0.1% (w/v) SDS were made with the discontinuous Tris-buffer system of Weber & Osborn (1975). Samples containing approx. 0.1 mg of protein were heated at 100°C for 2 min with 0.5 vol. of overlay solution [0.3 M-Tris/HCl, pH 6.8, containing 3% (w/v) SDS, 3% (v/v) β -mercaptoethanol, 0.15% (w/v) Bromophenol Blue and 10% (v/v) glycerol]. This sample solution was layered directly on to the gel. Gels were run at 45 mA/gel until the tracking dye had moved 10 cm through the

separating gel (about 4 h). Gels were stained for protein with Coomassie Brilliant Blue G, and for carbohydrate with periodic acid/Schiff stain as described by Fairbanks *et al.* (1971). Molecular weights were determined with phosphorylase A (100 000 daltons), bovine serum albumin (68 000 daltons), ovalbumin (43 000 daltons) and myoglobin (17 500 daltons) as standards. Densitometer traces were obtained by using a Beckman 3600 spectrophotometer and gel-scanning accessory. Gels stained for carbohydrate were scanned at 525 nm, and Coomassie Blue-stained gels were scanned at 600 nm.

Results

Separation of fractions C1 and C2

When the microvillus-enriched fraction (sample C) was centrifuged on a step gradient of sucrose, two peaks of alkaline phosphatase activity and protein concentration were observed (Fig. 1). Fraction C1 banded at density 1.16–1.17 g/ml and fraction C2 at 1.12–1.13 g/ml. Similar separations were obtained whether sample C was sedimented or floated up through the step gradient, which indicated that the membranes differed in buoyant density. When fraction C1 and C2 were separately collected, made 10% in sucrose and re-centrifuged on step gradients, single bands were formed at the same density interface as was observed previously. Therefore fractions C1 and C2 were not interconvertible.

Electron microscopy

Fractions C1 and C2 could be clearly distinguished by their appearance in the electron microscope (Plates 1c and 1d). The microvilli of fraction C2, which had the lower buoyant density and higher specific activity of alkaline phosphatase, were much thinner and did not show the internal structure of the fraction C1 microvilli. Electron micrographs of pellet C showed that both types of microvillus were present before gradient fractionation. Microvilli resembling those seen in fraction C1 were observed in thin sections and scanning electron micrographs of whole placenta, whereas microvilli resembling those of fraction C2 were not.

Electron micrographs of placental tissue before and after saline extraction (Plates 2 and 3) showed that during extraction some areas of the syncytiotrophoblast were extensively damaged. The surface showed large invaginations and sloughing-off of membrane-bound cytoplasm, as well as loss of microvilli (Plate 3b). These areas were a potential source of contaminants in the microvillous preparations. However, many areas of the cell surface were relatively undamaged and numerous microvilli were present (Plate 3a). There were also areas, as

Fig. 1. Separation of fractions C1 and C2 by centrifugation in a step gradient of sucrose

Experimental details are given in the text. —, Alkaline phosphatase activity; ----, [protein]; □—□, density [% sucrose].

Table 1. Analysis of results of electron microscopy of negatively stained preparations of fractions C, C1 and C2

Experimental details are given in the text.

Fraction	Microvilli (%) (95% confidence limits)	Vesicles (%)	No. of microvilli + vesicles observed
C	72 (68–76)	28	493
C1	76 (72–81)	24	344
C2	89 (86–92)	11	393

described by Smith *et al.* (1974), where, after saline extraction, there were very few microvilli and the underlying cytoplasm showed little damage. In the fresh placenta, damaged and microvillus-free surfaces were observed, but were less extensive than after saline extraction. In the two placentas that were examined, there was considerable difference in the amount of damage that was observed both before and after saline extraction.

A major component of the saline extract of the placenta appeared as amorphous or slightly granular material on electron microscopy (Plate 1a). Most of this material was sedimented by the 6000g centrifugation step (Whitsett &

Lessard, 1978) and thus was separated from the microvilli.

Samples C, C1 and C2 consisted mainly of microvilli and vesicles in electron micrographs (Plates 1b, 1c and 1d). Very few contaminants could be identified. Rough endoplasmic reticulum, mitochondria and lysosomes were rarely seen. In pellet C1, a few double-membrane structures which may have been derived from mitochondria were present. Negative staining of suspensions of fractions C, C1 and C2 showed that many of the vesicles seen in thin section could have been cross-sections through the bulbous ends of microvilli. The proportion of vesicles found in the membrane preparations

EXPLANATION OF PLATE 1

*Electron micrographs of thin sections of fractions B (a), C (b), C1 (c) and C2 (d)
Magnifications $\times 44\,000$, $\times 29\,000$, $\times 40\,000$ and $\times 40\,000$ in (a)–(d) respectively.*

EXPLANATION OF PLATE 2

Electron micrograph of the syncytiotrophoblast before saline extraction
Magnification $\times 30\,000$. Insert shows the internal details of the microvilli (magnification $\times 65\,000$).

EXPLANATION OF PLATE 3

Electron micrographs of the syncytiotrophoblast after saline extraction

(a) A relatively undamaged area (magnification $\times 48,600$); (b) an area with more extensive damage showing loss of microvilli, and disruption of cytoplasmic components (magnification $\times 22,500$). M, mitochondria; N, endothelial cell nucleus.

is shown in Table 1. The vesicles could have been derived from plasma membrane or from internal cell membranes.

Measurement of membrane thickness

In micrographs of whole cells, the widths of plasma membrane (7.6 nm, 95% confidence limits 7.46–7.84) and internal membrane (5.8 nm, 95% confidence limits 5.44–5.97) were easily distinguishable. Membranes of Golgi, mitochondria, nuclei and endoplasmic reticulum all conformed in thickness to internal membranes; however, some lysosomal membranes approached plasma membrane in thickness.

Measurements on micrographs of the microvillous fractions allowed classification of the membranes as internal or plasma membrane (Table 2). Although the mean thickness of plasma membrane in fraction C2 was less than that in other fractions, this difference was not statistically significant. The classification of plasma membrane and internal membrane was not as clear-cut in fraction C2 as in the other fractions; therefore the lower limit of plasma-membrane content is shown, as well as the 'best estimate' values. Vesicles were over-represented in the thickness measurements because microvillous membranes were often not clearly defined. For example, in sample C (Table 2) 39 microvilli and 37 vesicles were measured in a micrograph that contained 67% microvilli. The proportion of internal membrane in each fraction was approx. 10%. All of the microvilli that contained a core structure were plasma membrane. In fraction C2, however, a small proportion of the microvillus-shaped membranes were, according to width, internal membranes.

Enzyme and chemical assays

The procedure routinely yielded 20–40 mg of microvillous protein/300–600 g of placenta. In Fig. 2, the specific activities of three enzymes commonly used as plasma-membrane markers are depicted,

namely alkaline phosphatase, 5'-nucleotidase and γ -glutamyl transpeptidase. The recovery of the total activities of these enzymes in the microvillous fractions was usually less than 5% of the activities present in the whole placenta as estimated from the activities observed in the placental homogenate. Alkaline phosphatase has been shown histochemically to be located almost exclusively on the plasma membrane of the normal human term placenta (Hoffman & Di Pietro, 1972; Jones & Fox, 1976). All plasma-membrane marker enzymes followed a similar distribution and co-purified with the microvilli, with an approx. 20-fold increase in specific activity from homogenate to fraction C. The purification ranged from 10-fold to 40-fold in different placentas. A substantial amount of plasma-membrane enzyme activity was lost in the B pellet (6–13%) along with contaminants (12–38% of total protein). The specific activities of plasma membrane enzymes were higher in fraction C2 than in C1 or C. The purification factor from fraction C to C2 averaged 1.5 and was occasionally as high as 2.5.

The enzymes in Fig. 3 are usually considered to be markers of intracellular components (Amar-Costesec *et al.*, 1974; Bramley & Ryan, 1978; Morré, 1979), but very little is known about their distribution in the human term placenta. The activities varied from placenta to placenta and were often undetectable in fractions C1 and C2. Lactate dehydrogenase, catalase, and glutamate-pyruvate transaminase and cytochrome oxidase were used as markers for cytoplasm, peroxisomes, and mitochondria, respectively. All were decreased in activity about 10-fold in the crude microvillous preparations as compared with the homogenate. Almost all lactate dehydrogenase activity was removed from the microvilli of fraction C by the gradient-centrifugation step. Some lactate dehydrogenase activity was detected when microvilli, which lacked demonstrable activity in the usual assay, were assayed in the presence of 0.03% (w/v) deoxycholate. These results suggest that the microvilli

Table 2. *Morphometric analysis of fractions C, C1 and C2*

Experimental details are given in the text. Numbers in parentheses for 'Microvilli' and 'Vesicles' are numbers of observations.

Fraction	Plasma-membrane thickness (nm) (95% confidence limits)	Composition (%)		Plasma membrane (%)		
		Microvilli	Vesicles	Microvilli	Vesicles	Total fraction (95% confidence limits)
C	7.5 (7.3–7.9)	67	33	100 (39)	76 (37)	92 (79–97)
C1	7.6 (7.1–7.9)	62	38	98 (54)	84 (58)	93 (83–98)
C2	7.3 (7.1–7.9)	81	19	94* (78)	78† (46)	91 (90–97)‡

* Limit 88%.

† Limit 72%.

‡ Limit 73%.

Fig. 2. Specific activities of putative plasma-membrane marker enzymes and concentrations of cholesterol and phospholipid in subfractions of the placental homogenate and the microvillous preparations

Data from the homogenate are on the left of each histogram and data from the microvillous preparations are on the right. The meaning of the abbreviations and the experimental details are given in the text.

contained cytoplasmic components. The sedimentation of the B pellet was effective in decreasing catalase, cytochrome oxidase and glutamate-pyruvate transaminase activities. Most of the catalase activity remaining was removed by the gradient step. Cytochrome oxidase and glutamate-pyruvate transaminase banded at a slightly greater sucrose density than did fraction C1 and, occasionally, small amounts of these activities were found in fraction C1. Electron microscopy showed, however, that mitochondrial contamination of fraction C1 was minimal.

Three possible endoplasmic-reticulum markers were assayed: NADH- and NADPH-cytochrome *c* reductase and RNA. The specific activities of the enzymes were about 2–4-fold less in the crude microvillous fraction than in the homogenate. These specific activities were decreased with the sedimentation of pellet B, and then further lowered during the gradient-centrifugation step. Specific activities in fractions C1 and C2 were decreased to one-fifth to one-tenth, or less, of that in the homogenate. The concentration of RNA, considered to be a marker of rough endoplasmic reticulum and cytosol, was found to be high in the microsomal fraction of the homogenate, but was low in the supernatant. It was decreased more than 3-fold in the microvillous fractions. Oestrogen

biosynthesis occurs primarily in the endoplasmic reticulum (Ryan, 1959). The percentage conversion of [14 C]testosterone into oestrone plus oestradiol averaged 4% in incubations with portions of the microsomal fraction of the homogenate, and was undetectable (less than 0.05%) in incubations with all of fractions C1 and C2 obtained from one placenta.

UDP-galactosyltransferase and thiamin pyrophosphatase have been used as Golgi-membrane markers by other workers, but are not always found exclusively on Golgi membranes (Morré *et al.*, 1969). The former activity was low in fraction C1 and absent from fraction C2, despite the fact that Golgi membranes and fraction C2 have similar isopycnic densities in sucrose gradients. Heat-sensitive thiamin pyrophosphatase activity followed a different distribution, being concentrated into the microvillous fractions. The activity was considerably decreased by the addition of NiCl_2 (3.2 mM) to the assay mixture. 5'-Nucleotidase is an Ni^{2+} -sensitive enzyme and is heat-sensitive and active at pH 8.2, therefore this plasma-membrane enzyme might have contributed to the observed thiamin pyrophosphatase activity. However, the Ni^{2+} -insensitive thiamin pyrophosphatase activity was also concentrated into the microvilli, although with a distribution different from that of the plasma-membrane marker enzymes.

Fig. 3. Specific activities of putative intracellular marker enzymes in subfractions of the placental homogenate and in the microvillous preparations

Data from the homogenate are on the left of each histogram and data from the microvillous preparations are on the right. Experimental details are given in the text. (a) Lactate dehydrogenase; (b) glutamate-pyruvate transaminase; (c) cytochrome oxidase; (d) NADH-cytochrome *c* reductase; (e) NADPH-cytochrome *c* reductase; (f) RNA concentration; (g) catalase; (h) tartrate-inhibited (—) and -resistant (---) acid phosphatase; (i) fluoride-inhibited acid phosphatase; (j) UDP-galactosyltransferase; (k) heat-sensitive thiamin pyrophosphatase; (l) heat-sensitive, Ni^{2+} -insensitive thiamin pyrophosphatase.

Tartrate-inhibited acid phosphatase, considered to be a lysosomal marker (Jones & Fox, 1976), was found to be distributed rather like the plasma-mem-

brane markers, whereas the tartrate-resistant activity was not. The latter activity was fairly evenly spread over all the fractions. The fluoride-inhibited activity

Fig. 4. Densitometer traces of gels after electrophoresis of fractions M (a), P (b), C (c) and B (d) in 10% polyacrylamide/SDS gels

The arrows mark bands characteristic of that particular fraction. Absorbance scale in (c) applies to all traces. Experimental details are given in the text.

was concentrated into the microvillous fractions and, as with thiamin pyrophosphatase activity, the distribution was different from that of the plasma-membrane markers.

The assignments of each of these enzyme activities to various intracellular locations, and therefore their use as markers of intracellular

contamination of the microvillous fractions, are supported by the results of assays performed on subfractions of the homogenate (Fig. 3). The cytosolic (lactate dehydrogenase) and mitochondrial (cytochrome oxidase, glutamate-pyruvate transaminase) markers used were distributed as expected. Glutamate-pyruvate transaminase is thought to be

Fig. 5. Densitometer traces of gels after electrophoresis of fractions C1 (a) and C2 (b) in 10%-polyacrylamide/SDS gels. Inserts show the results of scans of 7.5%-acrylamide gels in the 70 000–100 000-mol.wt. region. Bands characteristic of each fraction are marked with arrows. $\blacktriangle$, Bands staining strongly for carbohydrate; $\triangle$, bands staining weakly for carbohydrate. Experimental details are given in the text.

located in the cytosol and within the mitochondria (Lehninger, 1970). The distribution in homogenate subfractions of enzymic activities reputed to be associated with peroxisomes (catalase), lysosomes (acid phosphatase), endoplasmic reticulum (NADH- and NADPH-cytochrome *c* reductase) and Golgi membranes (thiamin pyrophosphatase, UDP-galactosyltransferase) was less easy to interpret. However, apart from the phosphatases, all showed a non-plasma-membrane distribution and all were decreased with purification of the microvilli. Acid phosphatase and thiamin pyrophosphatase activities are suspect as intracellular markers in placental microvillous fractions because of the likelihood of cross-reactions with the very active phosphatases that appear to be indigenous to the microvilli.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide gels showed that preparations of microvilli had many fewer proteins than did crude mitochondrial (M) or microsomal (P) preparations. It was possible to identify several major proteins in fractions M and P which were absent from, or very low in, the microvillous fractions (Fig. 4). Some proteins were seen in fraction C which were absent from fractions C1 and C2, indicating that they were either loosely bound constituents of the microvilli or impurities removed in the sucrose gradient step.

Electrophoretic patterns of fractions C1 and C2 were very similar, although there were distinct and reproducible differences between them. Proteins of mol.wts. approx. 45 000, 83 000, 97 000 and possibly 145 000 and 160 000 were more abundant in fraction C1, whereas for many placentas, proteins of mol.wt. 69 000, 78 000, 58 000 and possibly 18 000 and 90 000 were more abundant in fraction C2 (Fig. 5). Sample B contained at least three proteins which were present only in low amounts in the purified microvilli. The major band was a glycoprotein of mol.wt. 59 000, but proteins of 140 000, 160 000 and possibly 56 000 daltons were also characteristic of sample B.

Six strong and three weak bands were seen on gels stained by the periodic acid/Schiff procedure (Fig. 5). These bands were identical in position and relative intensity for each of the microvillus-rich fractions. Fractions M and P showed the same pattern of staining as the microvilli, although the intensity was much less. The only other glycoprotein seen in any of the gels was the 59 000-dalton protein of sample B.

Discussion

We have attempted to obtain preparations of syncytiotrophoblast microvillous membranes that

are free from contamination by other cellular components. Electron-microscopic morphology and morphometry, the distribution of enzymic activities and the results of SDS/polyacrylamide-gel electrophoresis of proteins were used to assess the purity of the final preparations and to define their properties. The combined evidence strongly suggests that the microvillous preparations isolated by sucrose-gradient centrifugation contained negligible material that was not plasma membrane. To our knowledge, the greatest purification of placental microvilli previously achieved is that by Whitsett & Lessard (1978), who reported an approx. 10-fold enrichment of plasma-membrane enzyme markers in their final preparations. The addition of a sucrose-gradient step significantly improved the purity of the microvillous preparations, especially those which were initially highly contaminated (P. Truman, unpublished work).

Conclusions derived from enzyme analyses about the purity of tissue subfractions rest on the assumptions that the enzyme used is a specific marker for a particular organelle and that its distribution and activity are not affected during sample preparation. In subfractions of the placental homogenate, the activities of lactate dehydrogenase, catalase, glutamate-pyruvate transaminase, cytochrome oxidase and NADH- and NADPH-cytochrome *c* reductase all appeared to be markers of intracellular components. All these activities were greatly decreased in the purified microvillous fractions. From the results of combined enzyme and electron-microscopic analyses of the subfractions of the placental homogenate, the concentrations of particular subcellular organelles in the microvillous fractions could be estimated. For instance, electron micrographs showed that less than 50% of fraction M was of mitochondrial origin. Since the total activities of glutamate-pyruvate transaminase and cytochrome oxidase in fraction C1 were less than 10% of those observed in the mitochondrial fraction, it could be estimated that mitochondrial contamination was less than 5%. The detection of low activity of mitochondrial enzymes in fraction C1 was consistent with the occasional observation of a double-membrane structure in electron micrographs of that fraction. When similar estimates were applied to the results of analyses of microsomal, peroxisomal and cytosolic marker enzymes in fractions C1 and C2, it was concluded that the extent of contamination by these components individually was negligible.

The vesicles seen in electron micrographs of the microvillus-rich samples could have been contaminants (plasma membrane or internal membrane), bulbous ends of microvilli, or microvilli whose structure had been disrupted. Membrane-thickness measurements were analysed to give an estimate of

contamination of the microvillous fractions by internal membranes. Although the numbers measured were insufficient to give a highly accurate proportion, the total contamination by internal membrane components was approx. 10% for each of the microvillous fractions. This estimate is subject to the disadvantage that the sample analysed was a small one and may not have been representative. However, for negative staining, a random sample of the microvillous suspension was taken. The negative-staining results for fraction C2 showed the proportion of vesicles to be close to the proportion of internal membrane structures, both approx. 10%. Fraction C1, however, contained significantly more vesicles than internal-membrane structures, which indicated that this fraction contained some plasma-membrane vesicles. All of these vesicles could have been derived from the plasma membrane of microvilli, but some may have arisen from non-villous areas of the placental surface. The presence of vesicles composed of non-villous plasma membrane in fraction C1, but not in fraction C2, might explain some of the differences in enzyme activities that were observed between the two fractions.

The membrane-thickness measurements were originally undertaken to help resolve the question of the nature of the components of the C2 fraction. One possibility was that the fraction C2 structures were Golgi membranes. Golgi membranes are often enzymically similar to plasma membrane (Morré *et al.*, 1969) and are known to have a buoyant density close to that of sample C2. However, measurement of Golgi membranes in micrographs of whole placenta confirmed that they are of similar thickness to other internal membranes. The membranes of fraction C2 were therefore not Golgi membranes. Assays of UDP-galactosyltransferase also indicated that contamination of fraction C2 by Golgi membranes was low.

The major differences between fractions C1 and C2 were the smaller cross-sectional area and absent internal structure of the microvilli of the C2 fraction. The latter finding was confirmed by the gel-electrophoresis results. The major proteins lacking in fraction C2 were the two with mol.wts. 45 000 and 97 000. Actin is the major component of intestinal microvilli (Bretscher & Weber, 1979) and, in placental microvilli, may correspond to the 45 000-dalton protein (Whitsett & Lessard, 1978; Kelley *et al.*, 1979). The 97 000-dalton protein could be villin (mol.wt. 95 000; Bretscher & Weber, 1979) or might correspond to the 110 000-dalton protein seen in SDS/polyacrylamide-gel electrophoretograms of intestinal microvilli by Matsudaira & Burgess (1979). These workers identified two other proteins as components of the microvillus core, of mol.wts. 95 000 and 68 000. In our system, a protein of

mol.wt. 83 000 was present in fraction C1 and, in lesser amount in fraction C2, which could correspond to the 95 000-dalton protein. A protein of 68 000 daltons would not have been resolved from proteins of similar molecular weight in our experiments.

The source of the microvilli sedimenting in fraction C2 has not been determined. They may be microvilli whose internal structure has been lost during the preparative procedure. The fact that no structures resembling fraction-C2 microvilli could be seen in the intact placenta argues for this interpretation. On the other hand, proteins were observed on SDS/polyacrylamide-gel electrophoretograms that were characteristic of fraction C2 and not of fraction C1. Hence, the fraction-C2 microvilli are not simply eviscerated remnants of fraction-C1 microvilli. The presence of a major contaminant in fraction C2 containing a few specific proteins in large quantities would explain these data; however, no such contaminant could be identified in electron micrographs. It is possible that the fraction-C2 microvilli represent a normal subpopulation, or that they reflect the patchy degeneration that is often present in the human term placenta (Fox, 1975).

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