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Coated vesicles from human placenta carry ferritin, transferrin, and immunoglobulin G

(isotonic gradient purification/Triton X-100 extraction)

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ABSTRACT **Coated vesicles prepared by using isotonic gradients retain their contents. Ferritin, transferrin, and immunoglobulin G have been detected in human placental coated vesicles. Triton X-100 extraction of crude coated vesicles removes contaminating uncoated membranes, leaving coated particles and their associated proteins apparently intact; this provides an efficient means of preparing large quantities of coated vesicle proteins. The clathrin cages surround "cores" of structural protein containing polypeptides of 100,000 and 50,000 molecular weight.**

Coated pits, which invaginate to form coated vesicles, are believed to act as molecular filters, concentrating certain proteins while excluding others characteristic of the parent membrane (1). This is exemplified in the process of absorptive endocytosis, in which particular ligands bind to specific receptors and localize in coated pits, which rapidly form coated vesicles enclosing the ligands (2, 3). The placental syncytiotrophoblast plasma membrane, in contact with the maternal blood, displays many coated regions between the microvilli, and coated vesicles are abundant in the underlying cytoplasm (4). This membrane is rich in receptors for many maternal serum proteins, including transferrin receptor (5, 6) and immunoglobulin G (IgG) Fc receptor (7). Transferrin bound to its receptor has been observed to concentrate in the regions between the microvilli (8). Also, in the analogous systems of the rabbit yolk sac endoderm (9) and the baby rat gut (10) IgG has been shown to be taken up in coated vesicles.

Recently Booth and Wilson (11) demonstrated the presence of transferrin and transferrin receptor in coated vesicles purified from human placenta by sucrose density gradient centrifugation followed by treatment with wheat-germ agglutinin. However their preparations lacked IgG. Earlier work (12–14) suggested that sucrose density gradient centrifugation results in the partial disruption of coated vesicles, a loss of contents, and, to some degree, a loss of membrane and associated receptors. The purification procedures described here avoid the use of sucrose and make use of isotonic ^2H_2O and ^2H_2O/Ficoll gradients, as outlined earlier (14). The resulting preparations of coated vesicles are rich in contents, including ferritin, transferrin, and IgG and presumably their receptors.

METHODS

Isolation of Coated Vesicles. Placentae at term were obtained at the Maternity Hospital (Cambridge, England) and processed within 2 hr of delivery. The superficial membranes, blood vessels, and cord are removed and the tissue is cut up, teased out, and rinsed in ice-cold Tris·HCl-buffered saline, pH 7.2. Subsequent procedures are carried out at 0–4°C. The placental fragments are suspended in 3 vol of Hepes buffer (10 mM Hepes buffer, pH 7.2, containing 0.15 M NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3, and 0.2 mM phenylmethylsulfonyl fluoride), homogenized for 1 min at top speed in a Waring blender, and centrifuged at 1000 × *g* for 30 min. Pancreatic RNase (Worthington, 10 units/ml) is added to the supernatant, which is incubated at room temperature for 30 min and recentrifuged at 55,000 × *g* for 1 hr. The pellets (from one placenta) are resuspended in the above Hepes buffer, layered onto three 50-ml 10–90% ^2H_2O gradients (containing the Hepes buffer throughout) and centrifuged at 45,000 × *g* for 30 min. The total supernatants, excluding any very turbid fractions above the solid residues, are pooled and diluted 1:3, and the crude coated vesicles, contaminated mainly with smooth vesicles, are pelleted at 100,000 × *g* for 1 hr. The pellets are resuspended in the Hepes buffer containing 1% Triton X-100 to the same volume from which they were centrifuged and then left standing at room temperature for 30 min. The extracts are cleared at 10,000 × *g* for 10 min and then centrifuged at 100,000 × *g* for 1 hr. The pellets are resuspended in a small volume of the Hepes buffer containing 1% Triton X-100, layered onto a second 10–90% ^2H_2O gradient (12 ml) containing the Hepes buffer plus 1% Triton X-100 throughout, and centrifuged at 45,000 × *g* for 25 min. The pellet is discarded and the fractions richest in clathrin (monitored on a $NaDodSO_4$/polyacrylamide gel) are pooled, diluted 1:3 in the Hepes buffer, and centrifuged at 100,000 × *g* for 1 hr. The purified Triton X-100-extracted coated vesicles (TCVs) are resuspended in the Hepes buffer and stored at 0–4°C.

Alternatively, the crude coated vesicles from the first 10–90% ^2H_2O gradient are layered onto a gradient from 9% ^2H_2O/2% Ficoll to 90% ^2H_2O/20% Ficoll (the Ficoll was obtained from Sigma and extensively dialyzed against H_2O and freeze dried before use) containing the Hepes buffer throughout, and centrifuged at 80,000 × *g* for 16 hr. The coated vesicles sediment to the lowest region of the gradient and are washed and concentrated by centrifugation.

Protein concentration was estimated by the method of Lowry *et al.* (15), hexose content by an indole assay (16), and phospholipid content as described by McClare (17). $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out as before (12, 18).

Electron Microscopy. Generally, samples were negatively stained in unbuffered 1% uranyl acetate (19) on holey carbon films attached to specimen grids and were examined in a Philips EM301 microscope operating at 80 kV at a magnification of ×25,000 or ×45,000. TCVs on grids were washed with several drops of the Hepes buffer to remove any remaining Triton X-100 before staining. To disrupt the clathrin cages of TCVs, samples on grids were treated with seven successive drops of 6.7

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Abbreviation: TCV, Triton X-100-extracted coated vesicle.

parts 0.02 M Tris buffer (pH 7.5)/2 M urea/1 mM EDTA to 3.3 parts 0.01 M Mes buffer (pH 6.5). To visualize the ferritin associated with the TCVs, samples were stained with a solution of 1% glucose containing 0.05% uranyl acetate.

Antibody Staining of Gel Replicas. Proteins were transferred from small slab polyacrylamide gels to nitrocellulose sheets by electrophoresis (20). The "replicas" were washed first in 50 mM Tris·HCl buffer (pH 7.4)/150 mM NaCl/5 mM EDTA/0.25% gelatin/0.05% Nonidet P-40/0.02% NaN_3 and then incubated (2–12 hr) in appropriate sera diluted 1:100 in this buffer. The sera used were rabbit anti-human IgG (kindly provided by M. J. Hobart) and rabbit anti-transferrin (Miles). The replicas were rewashed and allowed to react (1 hr) with a solution (approximately 2×10^6 cpm/10 ml) of ^{125}I-labeled *Staphylococcus aureus* protein A (Pharmacia) (21). After extensive further washing the nitrocellulose sheets were autoradiographed.

Disruption and Reassembly. TCVs (approximately 7 mg/ml) were disrupted by dialysis in buffer D [0.02 M Tris·HCl, pH 7.5/1 mM EDTA/0.1% 2-mercaptoethanol/2 M urea (22)] containing 1 mM phenylmethylsulfonyl fluoride and pepstatin (Sigma) at 1 μg/ml. Samples were applied to a Bio-Gel A-15m column (1×150 cm) and eluted in the same buffer (23). Fractions from the column were reassembled by dialysis against 0.1 M Mes buffer (pH 6.5) containing 1 mM $MgCl_2$, 0.2 mM EDTA, and 0.02% NaN_3 (23, 24).

RESULTS

Coated Particles Extracted with Triton X-100 (TCVs). An electron micrograph of TCVs is shown in Fig. 1*A*. The structures exhibit the characteristic surface lattice of pentagons and hexagons associated with coated pits and coated vesicles. They range in size between about 500 Å and 1000 Å in diameter. Contaminants, including vesicles of uncoated membrane, are infrequently seen.

A typical analysis of a sample of TCVs by electrophoresis on a $NaDodSO_4$/polyacrylamide gel is shown in Fig. 2, lane a. Clathrin, the major 180,000 molecular weight polypeptide of coats (12), is the most prominent band. Apart from clathrin, other proteins characteristic of coated vesicles are shown (13), in particular those of 100,000 and 50,000 molecular weight and a doublet of clathrin light chains of about 32,000 molecular weight. Also visible is a fine background of many other species, including a polypeptide that migrates with the same mobility as IgG heavy chain. The same spectrum of polypeptides is observed in a sample of coated vesicles prepared on a ^2H_2O/Ficoll gradient. For comparison with the TCVs, electron micrographs of a ^2H_2O/Ficoll density gradient preparation are presented in Fig. 1 *B* and *C*, which shows the presence of vesicles inside coats of different sizes.

Generally the yield of coated particles, after Triton extraction, is between 7 and 10 mg of protein from one placenta. The preparation consists of about 75–80% protein, 20–25% lipid, and 1–2% carbohydrate.

One way of observing the material held within the clathrin cages is to apply samples of TCVs to electron microscope grids and to wash them with several drops of a urea-containing buffer, which disrupts the clathrin, before staining in 1% uranyl acetate. The resulting preparation (Fig. 1*D*) consists of "cores" of material surrounded by a "halo" of clathrin molecules and cage fragments. The cores look more like protein aggregates than vesicles, suggesting that the lipid bilayers of the original coated vesicles have been affected by the Triton treatment.

Ferritin Association with Placental Coated Vesicles. The most striking feature of these TCV and ^2H_2O/Ficoll coated vesicle preparations is their ferritin content. The peak clathrin-containing fractions of the final $^1H_2O/^2H_2O$ gradient of the TCV preparation are brown with ferritin, as are the final suspensions

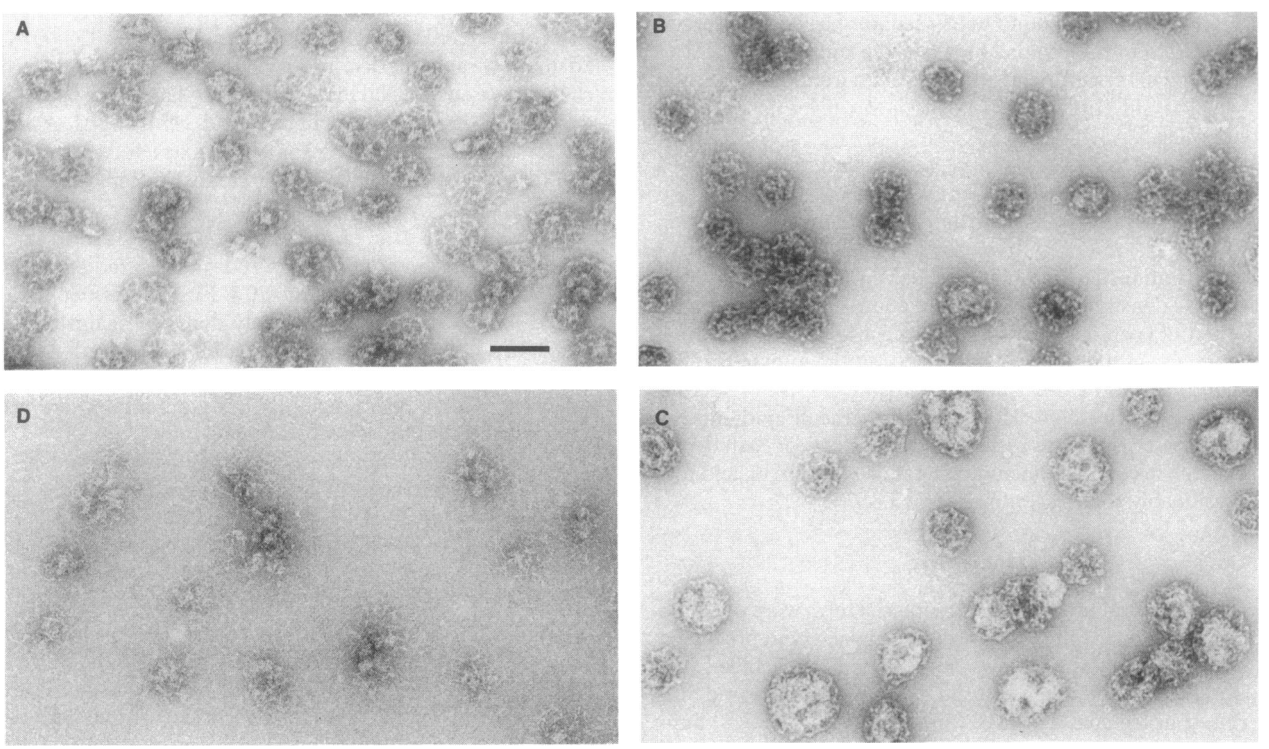

FIG. 1. Electron micrographs of coated vesicle preparations negatively stained in 1% uranyl acetate, shown at a magnification of ×75,000. Scale bar represents 1000 Å. (*A*) TCVs. (*B*) Coated vesicles purified by ^2H_2O/Ficoll density gradient centrifugation. (*C*) Coated vesicles purified as in *B* but which sedimented faster on the ^2H_2O gradient. (*D*) TCVs treated with urea-containing buffer before staining, showing internal "cores" surrounded by the disrupted clathrin.

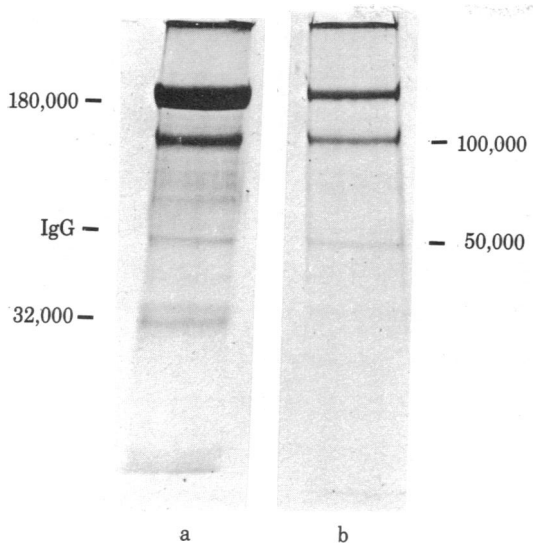

FIG. 2. Electrophoresis on a $NaDodSO_4$/polyacrylamide gel of a sample of TCV proteins (lane a) and, on the same gel (lane b), a sample of column-purified core proteins (see Fig. 5).

of purified particles. In contrast, exogenous ferritin added at the beginning of either purification procedure is removed. In order to see whether the ferritin is contained in the coated particles, samples of TCVs were applied to electron microscope grids and stained with several drops of a mixture of 1% glucose and 0.05% uranyl acetate. This allows both the particles and the ferritin molecules to be seen simultaneously, as shown in Fig. 3. Approximately 65% of the ferritin molecules appear to be associated with TCVs. About 40% of the TCVs encompass one or more (up to six) molecules of ferritin, while the remainder lack ferritin. Those associated with ferritin have an average of 1.4 ferritin molecules each. When the coated particles are disrupted by dialysis in buffer D (which contains 2 M urea) the bulk of the ferritin is released into solution.

IgG and Transferrin. IgG and transferrin, molecules involved in maternal–fetal transfer of passive immunity and iron, respectively, are also present in these preparations of TCVs. Both proteins were detected by using ^{125}I-labeled *S. aureus* protein A and appropriate antisera to stain nitrocellulose blots of samples of TCV polypeptides analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Fig. 4*A* demonstrates the presence of IgG heavy chain in samples of TCVs containing 15 and 30 μg of total protein. The original gel also contained marker human IgG at different loadings. The relative intensities of the bands on the autoradiograph suggest that the 30-μg TCV sample contains about 0.5 μg (or about 2% of the TCV protein) of IgG heavy chain. Thus the coated vesicles of one placenta might contain a total of about 150 μg of IgG. The extra labeled bands observed in the TCV samples may be due to some nonspecific binding of antibody to clathrin and the 100,000-dalton protein which, of necessity, are heavily overloaded on the gel. Similar nitrocellulose blots receiving identical washes but omitting the anti-human IgG serum were blank.

Fig. 4*B* demonstrates the presence of transferrin in the TCV preparation. The sample loaded on the gel was the supernatant of 200 μg of TCVs that had been disrupted in 2 M urea and centrifuged to remove the cores; 0.5 μg of marker transferrin was also applied to the gel. The relative intensities of the bands suggest that 200 μg of TCVs contain about 0.5 μg of transferrin (or 0.25% of TCV protein). This level of transferrin corresponds to about 25 μg of transferrin in the total coated vesicle population of one placenta. Again a control blot unstained with the anti-transferrin serum was blank.

Disruption and Reassembly of Coated Vesicle Components. To investigate the nature of the material within the coats, the TCVs were disrupted by dialysis in buffer D containing 1 mM phenylmethylsulfonyl fluoride and pepstatin at 1 μg/ml and fractionated by gel filtration on Bio-Gel A-15m. A typical column profile of absorbance at 280 nm and $NaDodSO_4$/polyacrylamide gel electrophoresis analysis of corresponding samples are presented in Fig. 5.

The first peak (I) to elute contains particles (Fig. 6*A*) corresponding to constituents of the cores seen previously by electron microscopy (Fig. 1*D*). These particles contain clathrin, 100,000-dalton polypeptide, and 50,000-dalton polypeptide, apparently in the ratio 2 (or more):2:1 as estimated by gel densitometry (see Fig. 2, lane b, for gel profile). The clathrin in this fraction seems to be in equilibrium with the clathrin trimers (triskelions), the bulk of which elute in peak II. Clathrin triskelions can be repolymerized to form empty cages at pH 6.5

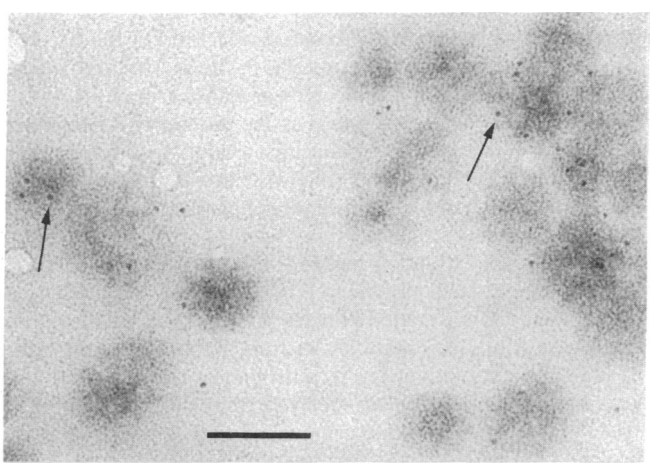

FIG. 3. TCV sample stained with a mixture of 1% glucose and 0.05% uranyl acetate, shown at a magnification of ×135,000. The iron cores of ferritin molecules appear as black dots, two of which are indicated by arrows. Some occur free in the background, but many are associated with the coated particles. Scale bar represents 1000 Å.

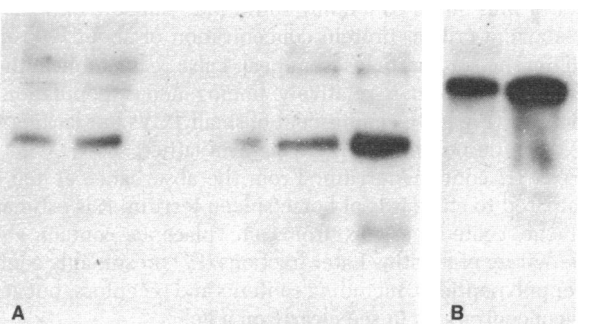

FIG. 4. (*A*) Autoradiograph of a nitrocellulose blot of proteins from a $NaDodSO_4$/polyacrylamide gel after staining with anti-human IgG antibody and ^{125}I-labeled *S. aureus* protein A. Lanes from left to right of the gel contained 15 and 30 μg of TCV, and 0.1, 0.25, 0.5, and 1 μg of human IgG, respectively. The higher molecular weight bands visible in the marker IgG represent contaminants frequently present in commercial preparations. (*B*) Autoradiograph of a nitrocellulose blot of proteins from a $NaDodSO_4$/polyacrylamide gel after staining with anti-transferrin antibody and ^{125}I-labeled *S. aureus* protein A. The left lane contained supernatant from 200 μg of urea-disrupted TCVs centrifuged to remove the cores. The right lane contained 0.5 μg of marker transferrin.

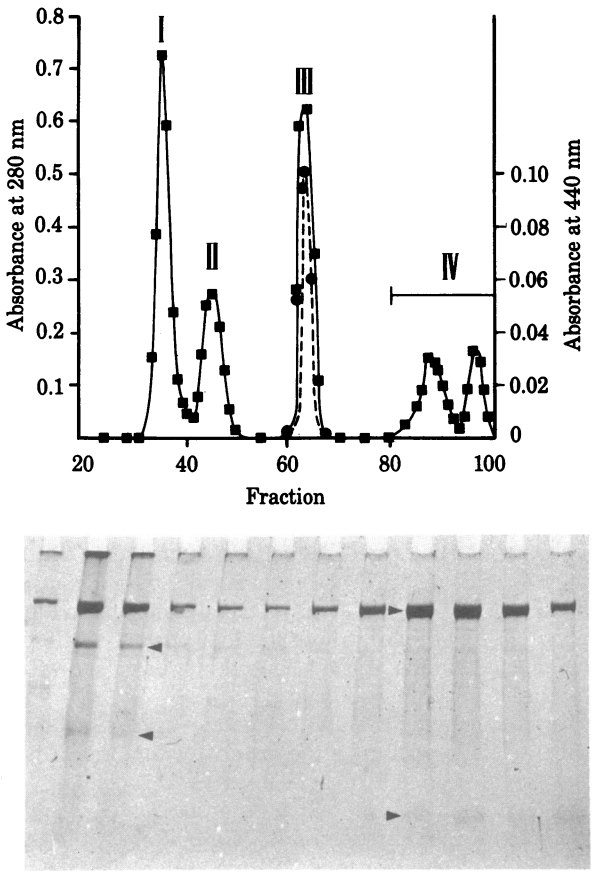

FIG. 5. (*Upper*) Typical profile of disrupted TCV proteins after gel filtration on Bio-Gel A-15m in buffer D. ■, Absorbance at 280 nm; ●, absorbance at 440 nm. Peak I contains core particles, peak II represents clathrin triskelions, and peak III contains ferritin. Fractions labeled IV include other polypeptides, some of which are presumably content and receptor molecules. (*Lower*) NaDodSO$_4$/polyacrylamide gel electrophoresis of samples across peaks I and II (between fractions 30 and 50) eluted from the gel filtration column (see *Upper*). The arrows indicate the characteristic polypeptides associated with coated vesicles—namely, clathrin plus light chains and core proteins of 100,000 and 50,000 molecular weight.

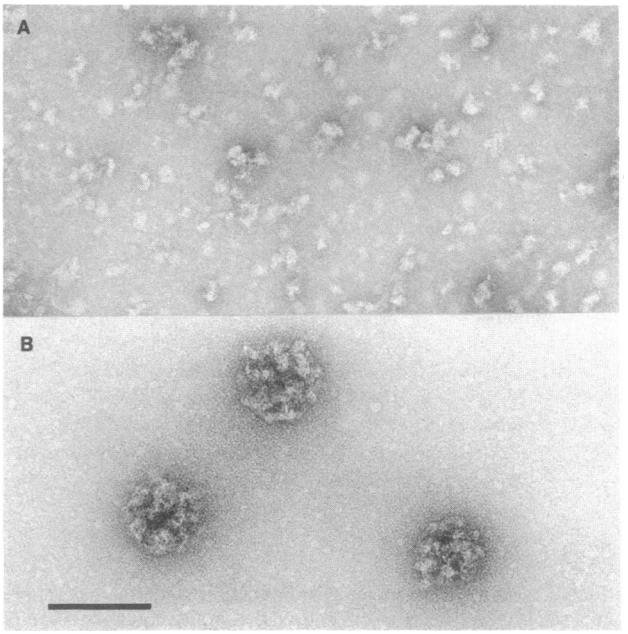

FIG. 6. (*A*) Core particles from peak I (Fig. 5) negatively stained in 1% uranyl acetate and magnified ×135,000. (*B*) Core particles as in *A* after reconstitution in 0.1 M Mes buffer (pH 6.5) containing 1 mM Mg^{2+} and 0.2 mM EDTA. The structures were negatively stained in 1% uranyl acetate and are magnified ×135,000. Scale bar represents 1000 Å.

in 0.1 M Mes buffer containing 1 mM Mg^{2+} and 0.2 mM EDTA (24) above a critical protein concentration of about 0.05 mg/ml (23). The core particles from peak I also reassemble in these conditions to form a relatively homogeneous population of structures (Fig. 6*B*), reminiscent of small TCVs but lacking the fine definition of the complete clathrin lattice.

Peak III contains ferritin. From the absorbance at 440 nm (compared to standards of horse spleen ferritin) it is estimated that the coated vesicles from one placenta contain about 200–300 μg of ferritin. Later fractions (IV) presumably contain other polypeptides, including contents and receptors, but at too low concentrations to see clearly on a gel.

DISCUSSION

Placental coated vesicles prepared by using isotonic gradients with or without Triton X-100 have contents. This is in contrast to the preparations obtained previously by means of sucrose density gradient centrifugation (12, 13). The functions of coated vesicles derived from human placenta are not all known. The organ contains several distinct cell types, in which coated vesicles are likely to be involved in a variety of processes, including secretion and lysosome formation. However, it is probable that many will contribute to the uptake of proteins from the maternal serum. The 40% of coats that have associated ferritin apparently contain an average of 1.4 molecules of ferritin per coat (distributed statistically between 1 and 6 molecules per coat), as judged by electron microscopy. This ferritin presumably acts as a vehicle for iron, which is required in increasing amounts [up to 4 mg per day (25)] by the fetus towards the end of pregnancy. Some ferritin may be synthesized in the placenta, but ferritin may also be taken up from the maternal serum. The most striking example of iron apparently being delivered in this way occurs during hemoglobin synthesis in the erythroblasts of bone marrow, where coated pits on the plasma membrane are packed with ferritin (26). In the placenta, the relative amounts of ferritin and transferrin estimated in these TCV preparations would suggest that ferritin could potentially transfer about 1000 times more iron than transferrin via coated vesicles. On the basis of the approximate quantities of proteins in the TCVs, there might be on average about 1–2 molecules of transferrin and 4 molecules of IgG per coated vesicle. Passive immunity is conferred on the fetus by transfer of certain classes of IgG from the mother across the placenta (27). The belief that this transfer occurs via coated vesicles, based on earlier morphological evidence (4, 9, 10), is supported here.

Triton X-100 extraction provides a rapid, efficient method for purifying coated particles (TCVs), possibly derived from both coated pits and coated vesicles. The method has also been used successfully to obtain TCVs from hybridoma cells [H6/31 (28)]. An alternative step to purifying placental coated vesicles from the crude pellet, derived from the initial 10–90% ^2H_2O gradient, might be to remove microvillar membranes by precipitation with wheat-germ agglutinin (11).

The TCVs prove to be a useful starting material for separating the protein components of the coated organelles. They appear to lack the lipid bilayer of vesicles, as previously observed by Woodward and Roth (29) in thin sections of coated vesicles treated with Triton X-100.

Disruption of the clathrin cages of the TCVs releases the content molecules originally bounded by the vesicles and also reveals core particles, which retain some bound clathrin in the conditions used. Recent work (30) suggests that the clathrin triskelions bind to the 100,000-dalton component of these cores, which can be extracted from the cytoplasmic surfaces of the vesicles. The core particles are capable of reassembly to fairly dense-looking structures, reminiscent of small TCVs, but presumably depleted in clathrin. Perhaps these proteins, underlying the clathrin cage, form the core of the molecular filter; binding specific receptors in the membrane but, by forming a close-packed structure, excluding others.

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1. Bretscher, M. S., Thomson, J. N. & Pearse, B. M. F. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4156–4159.
2. Roth, T. F. & Porter, K. R. (1964) *J. Cell Biol.* **20**, 313–332.
3. Goldstein, J. L., Anderson, R. G. W. & Brown, M. S. (1979) *Nature (London)* **279**, 679–685.
4. Ockleford, C. D. & Whyte, A. (1977) *J. Cell Sci.* **25**, 293–312.
5. Seligman, P. A., Schleicher, R. B. & Allen, R. H. (1979) *J. Biol. Chem.* **254**, 9943–9946.
6. Wada, H. G., Hass, P. E. & Sussman, H. H. (1979) *J. Biol. Chem.* **254**, 12629–12635.
7. McNabb, T., Koh, T. Y., Dorrington, K. J. & Painter, R. H. (1976) *J. Immunol.* **117**, 882–888.
8. King, B. F. (1976) *Anat. Rec.* **186**, 151–160.
9. Moxon, L. A. & Wild, A. E. (1976) *Cell Tissue Res.* **171**, 175–193.
10. Rodewald, R. (1973) *J. Cell Biol.* **58**, 189–211.
11. Booth, A. G. & Wilson, M. J. (1981) *Biochem. J.* **196**, 355–362.
12. Pearse, B. M. F. (1975) *J. Mol. Biol.* **97**, 93–98.
13. Pearse, B. M. F. (1978) *J. Mol. Biol.* **126**, 803–812.
14. Pearse, B. (1980) *Trends Biochem. Sci.* **5**, 131–134.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
16. Ashwell, G. (1957) *Methods Enzymol.* **3**, 79.
17. McClare, C. W. F. (1971) *Anal. Biochem.* **39**, 527–530.
18. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
19. Huxley, H. E. & Zubay, G. (1960) *J. Mol. Biol.* **2**, 10–18.
20. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
21. Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496.
22. Schook, W., Puszkin, S., Bloom, W., Ores, C. & Kochwa, S. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 116–120.
23. Crowther, R. A. & Pearse, B. M. F. (1981) *J. Cell Biol.*, in press.
24. Ungewickell, E. & Branton, D. (1981) *Nature (London)* **239**, 420–422.
25. Pribilla, W., Bothwell, T. H. & Finch, C. A. (1958) in *Iron in Clinical Medicine*, eds. Wallerstein, R. O. & Mettier, S. (Univ. of California Press, Berkeley, CA), pp. 58–64.
26. Fawcett, D. W. (1965) *J. Histochem. Cytochem.* **13**, 75–91.
27. Brambell, F. W. (1970) *Front. Biol.* **18.**
28. Pearson, T., Galfré, G., Ziegler, A. & Milstein, C. (1977) *Eur. J. Immunol.* **7**, 684–690.
29. Woodward, M. P. & Roth, T. F. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4394–4398.
30. Unanue, E. R., Ungewickell, E. & Branton, D. (1981) *Cell*, in press.