

Solubilization of Proteins from Bovine Brain Coated Vesicles by Protein Perturbants and Triton X-100

BERTRAM WIEDENMANN,* KAREN LAWLEY,* CHRISTINE GRUND,‡ and DANIEL BRANTON*

**Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138; and †Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg 1, Federal Republic of Germany. Dr. Wiedenmann is currently at the Institute of Cell and Tumor Biology in Heidelberg.*

ABSTRACT To identify integral and peripheral membrane proteins, highly purified coated vesicles from bovine brain were exposed to solutions of various pH, ionic strength, and concentrations of the nonionic detergent Triton X-100.

At pH 10.0 or above most major proteins were liberated, but four minor polypeptides sedimented with the vesicles. From quantitative analysis of phospholipids in the pellet and extract, we determined that at a pH of up to 12 all phospholipids could be recovered in the pellet. Electron microscopic examination of coated vesicles at pH 12.0 showed all vesicles devoid of coat structures.

Treatment with high ionic strength solutions (0–1.0 M KCl) at pH 6.5–8.5 also liberated all major proteins, except tubulin, which remained sedimentable.

The addition of Triton X-100 to coated vesicles or to stripped vesicles from which 90% of the clathrin had been removed resulted in the release of four distinct polypeptides of approximate M_r 38,000, 29,000, 24,000 and 10,000. The 38,000-D polypeptide ($pK \sim 5.0$), which represents ~50% of the protein liberated by Triton X-100, appears to be a glycoprotein on the basis of its reaction with periodic acid–Schiff reagent. Extraction of 90% of the clathrin followed by extraction of 90% of the phospholipids with Triton X-100 produced a protein residue that remained sedimentable and consisted of structures that appeared to be shrunken stripped vesicles. Together our data indicate that most of the major polypeptides of brain coated vesicles behave as peripheral membrane proteins and at least four polypeptides behave as integral membrane proteins. By use of a monoclonal antibody, we have identified one of these polypeptides (38,000 mol wt) as a marker for a subpopulation of calf brain coated vesicles.

Coated vesicles consist of a membrane surrounded by a polyhedral protein lattice (7) whose assembly unit is a triskelion consisting of three clathrin heavy chains (180,000 D per subunit) and three clathrin light chains (33,000 to 36,000 D per subunit) (11, 24). The triskelion can be dissociated from the coated vesicle by biochemical manipulations that include altered pH, ionic changes, and low concentrations of urea (9, 16, 27). After removal of the triskelions, the major remaining protein constituents of bovine brain coated vesicles include various polypeptides of ~100,000–115,000 mol wt subunits (which we refer to as 100,000-D polypeptides), a 50,000 D polypeptide subunit, and tubulin (10, 18, 26). Pearse (17)

used the nonionic detergent Triton X-100 during all purification steps of coated vesicles. This method generated coated particles that apparently also retained their major polypeptide composition. Furthermore, removal of some triskelions with 2 M urea yielded particles that consisted mainly of polypeptides of 100,000 and 50,000 mol wt. Keen et al. (9) showed that high ionic strength treatments (0.5 M Tris HCl) of coated vesicles at pH 7.0 removed the triskelions together with many of the 100,000-D polypeptides, some 55,000-D polypeptides (now recognized as 50,000-D polypeptides distinct from the 55,000-D tubulin doublet), and ~10% of the phospholipids. Despite some additional evidence that the 100,000 polypep-

tides provide the binding site for clathrin (6, 23), very little is known about the molecular organization of the membrane proteins of bovine brain coated vesicles. In this paper we present evidence that all major membrane proteins appear to be peripheral membrane proteins, whereas four distinct components have integral membrane protein characteristics. At least one of these components (38,000 mol wt) is associated with only ~20% of the total population of calf brain coated vesicles.

MATERIALS AND METHODS

Materials

All standard chemical reagents used were of the highest purity available commercially. Calf brains were obtained from a local slaughterhouse, transported to the laboratory on ice, and used immediately.

Methods

COATED VESICLE ISOLATION: Coated vesicles were isolated on a D₂O-ficoll gradient, and gel filtration was then performed as described previously (26). Stripped vesicles were prepared by the method of Unanue et al. (23) with some modifications. Coated vesicles suspended in 100 mM 2-(*N*-morpholino)ethane sulfonic acid (MES),¹ pH 6.5, 1.0 mM EGTA, 0.5 mM MgCl₂, 0.02% NaN₃, and 0.005% phenylmethylsulfonyl fluoride (buffer A) were diluted 100-fold in stripping buffer. Stripping buffer consisted of 5 mM Tris pH 8.5, 50 mM sucrose, and 0.5 mM EGTA, degassed for 20 min and bubbled with nitrogen for 10 min. Where necessary, the pH of the coated vesicle stripping buffer mixture was adjusted to 8.5 with NaOH. After 1 h of incubation at room temperature, the stripped vesicles were centrifuged for 1.5 h at 160,000 g.

EXTRACTION PROCEDURES: For extraction, coated vesicles in buffer A were resuspended after ultracentrifugation in 10 mM buffer (either MES or TRIS-HCl as specified) at a concentration of 0.2 mg/ml and further incubated in individual 5-ml samples as indicated in the figure legends. After incubation, sedimentable material in pellets was separated from extracted or solubilized material in the supernatant by sedimentation for 2 h at 110,000 g. To avoid contamination of the supernatant with slowly sedimentable material, the top 4 ml of the supernatant was carefully removed and the remaining supernatant (~1 ml) was discarded. Analyses were performed on the 4 ml of sampled supernatant and the pellet. Because of variable loss in the ~1 ml of discarded supernatant, the combined contents of supernatant and pellet did not equal the contents of the starting material.

ELECTROPHORESIS: We performed SDS PAGE by the procedure of Laemmli (13) using a 6–15% linear gradient gel system. The gels were stained with either Coomassie Blue R250 or silver according to Eschenbruch and Burk (4). The amount of protein in individual bands was quantified by colorimetric measurement of the Coomassie Blue stain eluted from the gel with 25% pyridine as described by Fenner et al. (5).

We performed two-dimensional gel electrophoresis of coated vesicles by procedures described by O'Farrell (15) using a wide range pH gradient in the first dimension and a 10% polyacrylamide gel with SDS in the second dimension. Immunoblots of these two-dimensional gels were prepared as described by Batteiger et al. (2) using 0.05% Tween in phosphate buffered saline, pH 7.4, to block nonspecific nitrocellulose binding sites. Details of the production and characterization of the monoclonal, IgG antibody (Y 38.1.1) to the 38,000-D polypeptide used for our immunoblots and immunoelectron microscopy will be described elsewhere.²

PROTEIN AND PHOSPHOLIPID DETERMINATION: We measured protein concentrations either by the method of Lowry et al. (14) or by that of Bradford (3). We estimated phospholipid by assaying inorganic phosphorus according to Bartlett (1). Phosphoproteins did not contribute significantly to the phosphorus measurements: when >90% of the total vesicle protein (including the 50,000-D protein, which is the most heavily phosphorylated protein) was released from the vesicles by treatment at pH 12, the inorganic phosphorus detected by our measurements remained pellet associated.

ELECTRON MICROSCOPY: We prepared coated and stripped vesicles for negative staining by first floating a carbon film formed on newly split mica onto a solution of ~30 µg/ml vesicle protein for ~1 min, after which we transferred the film onto a drop of 2% uranylacetate for 1 min (25). The film

¹ Abbreviation used in this paper: MES, 2-(*N*-morpholino)ethane sulfonic acid.

² Wiedenmann, B., C. Kuhn, C. Grund, and W. W. Franke. Manuscript submitted for publication.

was placed on a 400 mesh copper grid, blotted, dried, and examined in a Phillips EM 301 electron microscope at 60 kV.

Coated vesicles for immunoelectron microscopy were fixed with 2% formaldehyde in phosphate buffered saline, pH 6.8, washed in this buffer, and concentrated by ultracentrifugation. The pellets were frozen, cut into 5-µm cryostat sections, and processed as described by Kartenbeck et al. (8). The monoclonal antibodies used to stain the cryosections will be characterized by Weidenmann et al. elsewhere.²

RESULTS

Lipid-to-Protein Ratio in Coated Vesicles

Centrifugation in a D₂O-Ficoll density gradient followed by exclusion chromatography on a Sephacryl S-1000 column yields fractions that contain at least 95% coated vesicles (26) with 76 ± 14 nmol phospholipid inorganic phosphorus/mg protein (average of 10 independent coated vesicle preparations). Because this phospholipid/protein ratio is considerably less than previously reported for coated vesicles isolated by procedures that did not use gel filtration (reference 16, 410 nmol P/mg protein; reference 20, 150 nmol P/mg protein), we determined the lipid content of our partially purified coated vesicles before they had passed through the S-1000 column, as well as of pooled fractions I, II, and III (Fig. 1a) from the column. The material applied to the column contained 157 nmol P/mg protein whereas the pooled fractions I, II (coated vesicles), and III contained, respectively, 524, 76, and 293 nmol P/mg protein (average values for four independent preparations). (Note that the pooled fractions in Fig. 1a do not sample all of the applied material and that the peak heights are the sum of light absorbance due to protein mass and light scatter due to vesicular material.) Chromatography on Sephacryl S-1000 clearly separated two lipid-rich vesicle pools from the coated vesicles (pool II) and thus accounted for the relatively low lipid content of our coated vesicles. To exclude the possibility that we underestimated the phospholipid/protein ratio by isolating a large number of empty clathrin cages, we counted the number of empty cages seen in negatively stained samples from our purified coated vesicle peak. Less than 2% of the vesicles were empty cages.

Analysis of the protein composition of the S-1000 column fractions as judged by SDS PAGE (Fig. 1b) shows that a protein of *M*_r 55,000, probably tubulin, represents the major protein constituent in the first peak. The third peak contains the typical protein composition of coated vesicles observed in the second peak but also contains a 250,000-D polypeptide that is absent from purified coated vesicles. Silver-stained gels (Fig. 1b, right) emphasized the presence of several low molecular weight polypeptides barely seen after Coomassie Blue staining (Fig. 1b, left). The silver staining intensity is apparently not a linear function of protein concentration, and an *M*_r 38,000 polypeptide that was barely detectable in Coomassie Blue-stained gels of coated vesicles (Fig. 1b, arrows) became particularly prominent.

Solubilization of Coated Vesicles with Alkali

Reduction in ionic strength and an increase in pH have been used to preferentially release peripheral membrane polypeptides while retaining lipid-associated, integral membrane proteins (22). Coated vesicles isolated at pH 6.5 in buffer containing 100 mM MES (buffer A) were resuspended in 10 mM MES pH 6.5 and exposed to various alkaline pH's on ice, then immediately centrifuged at 160,000 g for 1.5 h. Almost all coated vesicle proteins remained sedimentable at

a

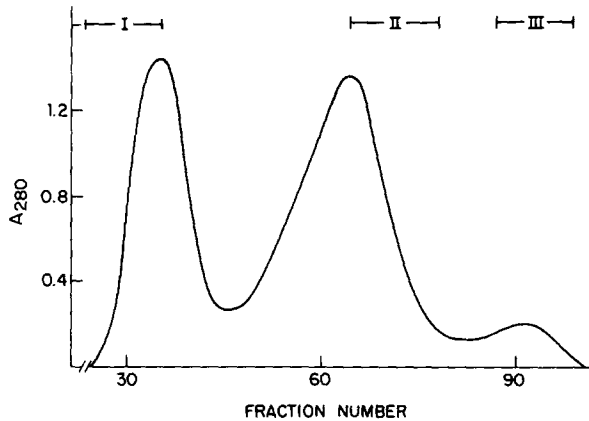
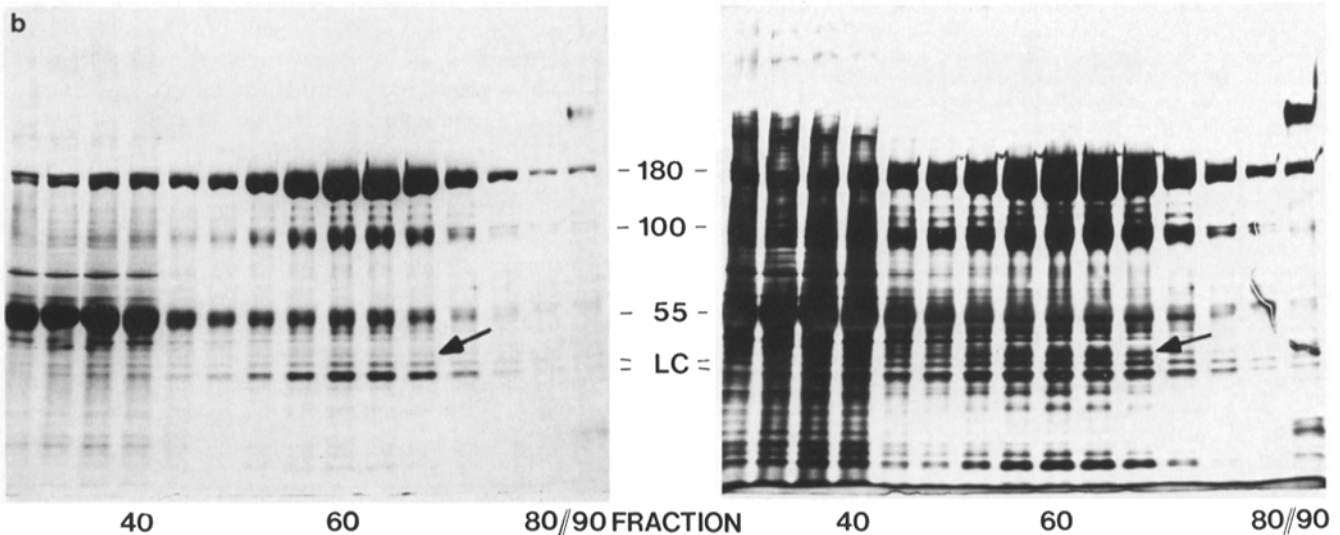


FIGURE 1 (a) Elution profile of D_2O -Ficoll gradient-purified coated vesicles applied to Sephacryl S-1000 gel filtration. The first peak contains contaminating membranes, the second coated vesicles, and the third small membrane contaminants. Fractions (3.5 ml) within the bars designated I, II, and III were pooled and subjected to inorganic phosphorus determination as described in Materials and Methods. (b) SDS PAGE of membrane fractions during coated vesicle purification by gel filtration stained with Coomassie Blue (left) and silver (right). In this and all other figures the relative molecular weights are indicated $\times 10^{-3}$, and LC refers to the clathrin light chains (24). Equal volumes of every fourth fraction were taken and analyzed. The last fraction was concentrated by centrifugation before electrophoresis. Arrows point to a polypeptide of M_r 38,000.



pH 6.5 in 10 mM MES (Fig. 2a). Raising the pH to 7.0 resulted in the loss of some triskelions and further increases in pH (pH 8–10) liberated more of the triskelions as well as some of the 100,000-D polypeptides and the 50,000-D polypeptide (Fig. 2a). At pH 11 tubulin was solubilized, and at pH 12 all major proteins of the coated vesicles were released into the supernatant. Careful inspection of membranous residue showed that four faintly stained polypeptides, M_r ~38,000, 29,000, 24,000, and 10,000, remained associated with the pellet even at pH 11 and 12 (Fig. 2a, arrowheads).

To exclude the possible presence of some minor aggregates that were not sedimentable during the standard sedimentation procedure, in several experiments the supernatant was subjected to an additional centrifugation, which was followed by SDS PAGE. Centrifugation for 1.5 h at 160,000 g did not sediment any additional proteins (data not shown).

Protein determinations (14) showed that 95% of the protein was routinely recovered in the pellet at pH 6.5, but only 9% was recovered in the pellet at pH 12. To exclude the possibility that this protein release could be attributed to lipid hydrolysis and the consequent disintegration of sedimentable mem-

branes, the amount of inorganic phosphorus in the pellet and supernatant was determined. Upon appropriate titration (bolus addition of NaOH at 0°C as described in the legend to Fig. 2) followed by immediate centrifugation, all phospholipids could be recovered in the pellet.

In parallel, pellets and supernatants at given pH's were negatively stained and examined by electron microscopy. To avoid staining artifacts, all samples were adjusted to pH 6.5 before staining. We observed intact coated vesicles in the pellet at pH 6.5 (Fig. 2b), whereas at pH 12 (Fig. 2c) all coats were lost and some membrane material appeared to be fused or aggregated.

Effect of KCl on Coated Vesicles

Coated vesicles were exposed to various concentrations of KCl at pH's ranging from 6.5 to 8.5. At pH 8.5 (Fig. 3a) some 100,000-D polypeptides and some of the 50,000-D polypeptides together with triskelions were solubilized without the addition of KCl (Figs. 2a and 3a). However, KCl concentrations >0.12 M caused an additional release of the 100,000-

and 50,000-D polypeptides. The addition of KCl at pH 7.5 or 6.5 resulted in a qualitatively identical solubilization pattern, but higher concentrations of KCl were required to achieve comparable protein release than at pH 8.5 (not shown). Again, most of the tubulin remained sedimentable,

whereas the 50,000-D polypeptide together with the 100,000-D polypeptides were partly released. When Tris-HCl was used in place of equivalent molarities of KCl, an almost identical pattern of polypeptides was released (not shown). With increasing Tris molarity, equivalent percentages of the 100,000-

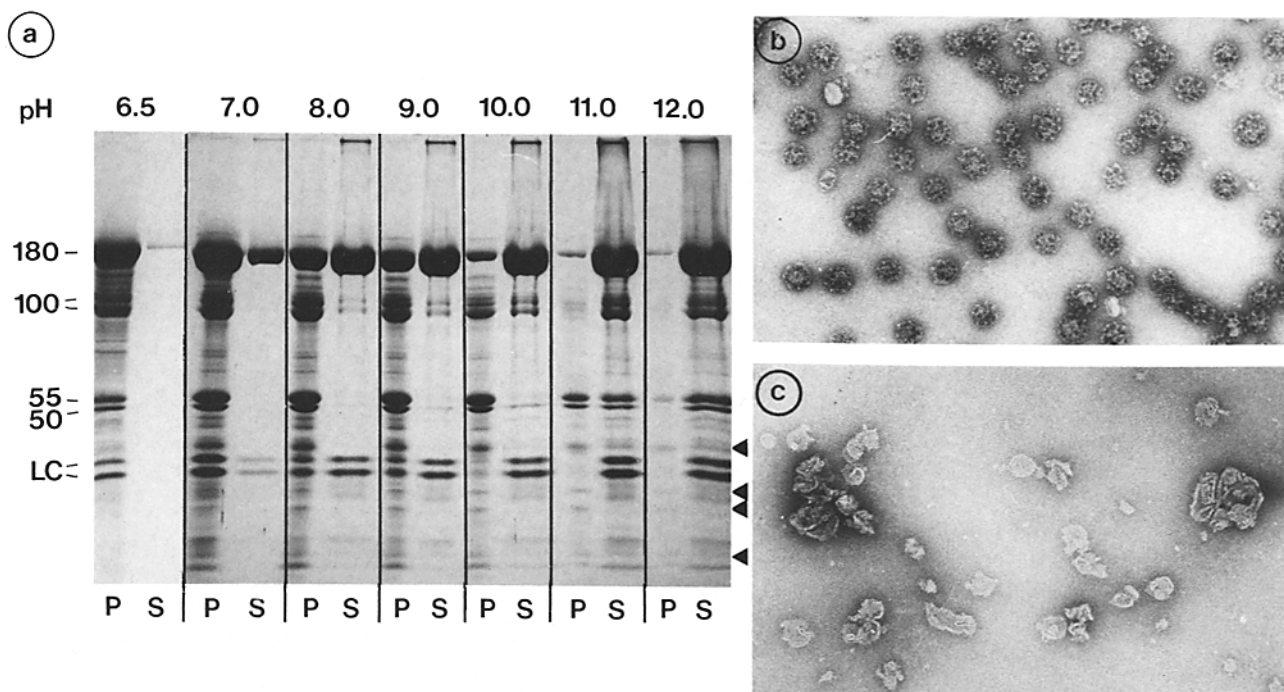


FIGURE 2 Release of membrane polypeptides at alkaline pH. Coated vesicles were dialyzed overnight against 10 mM MES pH 6.5, 1 mM EGTA, 0.5 mM MgCl₂, 0.02% NaN₃, 0.005% phenylmethylsulfonyl fluoride, and 5-ml aliquots (0.2 mg protein/ml) were adjusted, while agitated on ice, to the required pH by the addition of a single bolus of 0.5 or 1.0 N NaOH. The volume of NaOH needed to bring the pH to the required level was determined in preliminary experiments, but the final pH achieved was checked after NaOH addition for each experiment. Thereafter, the vesicles were immediately centrifuged as described in Materials and Methods. (a) The pellets (P) and supernatants (S) were examined by 6–15% gradient SDS PAGE and stained with Coomassie Blue. Arrow heads: M, 38,000, 29,000, 24,000, and 10,000 D. (b and c) Electron micrographs of negatively stained pellets at pH 6.5 (b) and pH 12.0 (c). × 50,000.

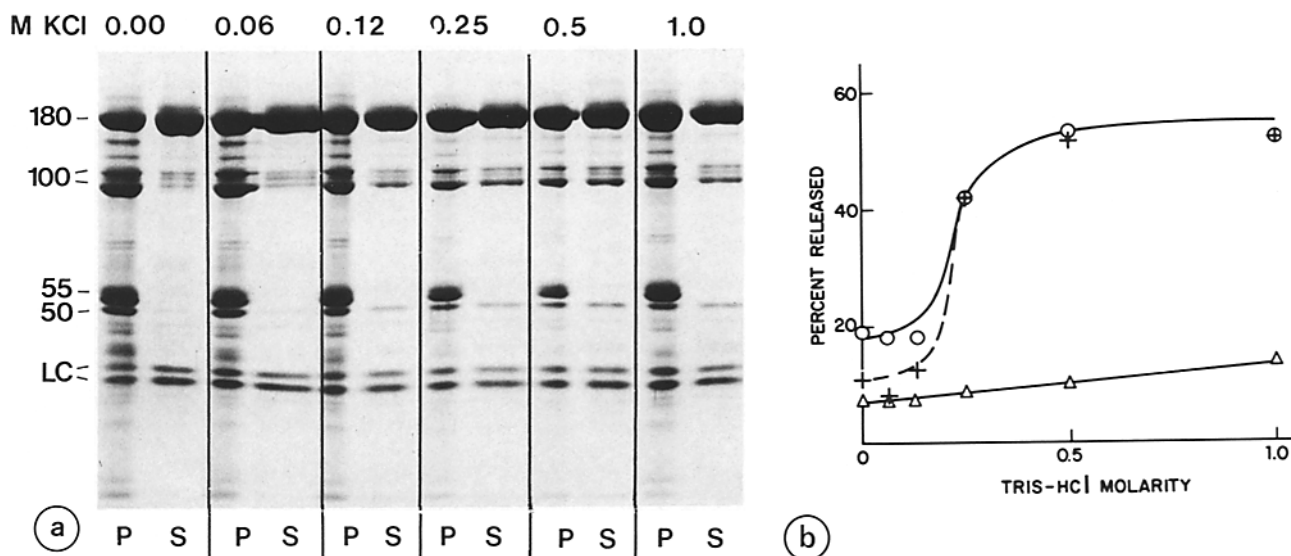


FIGURE 3 Release of membrane polypeptides (a) by KCl at pH 8.5 or (b) Tris at pH 7.5. Packed coated vesicles were resuspended in (a) 10 mM Tris-HCl pH 8.5 or (b) pH 7.5, 1.0 mM EGTA, 0.5 mM MgCl₂, 0.2% NaN₃, 0.005% phenylmethylsulfonyl fluoride to a final concentration of 0.2 mg/ml, and an equal volume of buffer containing various concentrations of (a) KCl or (b) Tris was added. Coated vesicles were incubated for 1 h at room temperature and then centrifuged as described in Materials and Methods. (a) Pellets (P) and supernatants (S) from a KCl extraction were analyzed by SDS PAGE. (b) The fraction of the 100,000 (+), the 50,000 (O), and the tubulin (Δ) polypeptides released to the supernatant after incubation in Tris-HCl.

and the 50,000-D polypeptides were released (Fig. 3*b*). Under all conditions, no phospholipid was found in the supernatant.

Effect of Triton X-100 on Coated Vesicles and Stripped Vesicles

To remove contaminating smooth membranes, Pearse (17) purified coated vesicles in the presence of Triton X-100 in 0.15 M NaCl. Although coated vesicle structures are recovered by this procedure, we found that the addition of 0.05% Triton X-100 (Triton/protein, 2.5:1 gram/gram) caused 65% of the phospholipids of highly purified coated vesicles to be released into the supernatant. Higher Triton X-100 concentrations (up to 1.0%, Triton/protein, 50:1 gram/gram) did not extract more phospholipid. With 0.05% Triton X-100, the polypeptides of approximate M_r 38,000, 29,000, 23,000, and 10,000, corresponding to the proteins recovered in the pellet after alkali extraction, were liberated (Fig. 4). Higher Triton X-100 concentrations extracted no additional polypeptides.

The 38,000-D polypeptide appeared to be the major Triton X-100-solubilized protein as seen in Coomassie Blue-stained gels (Fig. 4, lane 2) and in silver-stained gels (not shown). A

monoclonal antibody to this polypeptide² clearly demonstrated the presence of the M_r 38,000 polypeptide in the supernatant of detergent-extracted vesicles and in the pellets of alkali-extracted vesicles (Fig. 4, lanes 3-7 and 3'-7'). The monoclonal antibody detected two isoelectric variants of this polypeptide (Fig. 5), both rather acidic (pH 4-5). Periodic acid-Schiff treatment of heavily loaded gels showed two polypeptides faintly stained as glycoproteins (Fig. 6). Of these, the 38,000-D polypeptide was detergent extractable but not alkali extractable. (The 48,000-D polypeptide was not extracted under any of the conditions we used and was difficult to visualize in Coomassie Blue-stained gels because of overlapping stain from other more prominent proteins.) We observed no additional periodic acid-Schiff-staining material in gels of unstripped coated vesicles.

The M_r 38,000 polypeptide appeared to be specifically enriched in coated vesicles, but pooled fractions I (Fig. 1*b*) from the S-1000 column also contain this polypeptide, albeit in lesser amounts. To determine whether the 38,000-D polypeptide was truly associated with coated vesicles rather than with contaminating vesicles that co-purified with our coated vesicles, we examined electron micrographs of our prepara-

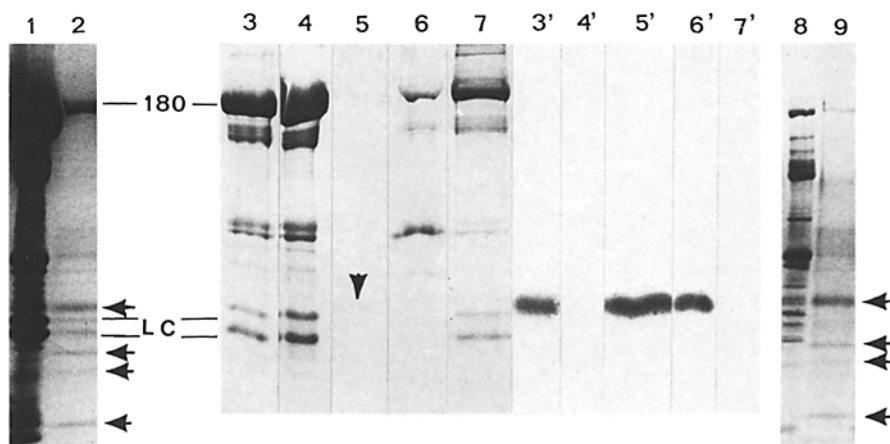


FIGURE 4 Release of membrane polypeptides by Triton X-100 or alkali. Purified coated vesicles in 10 mM MES were extracted by incubation in MES plus 0.1% Triton X-100, pH 6.5, or MES plus NaOH, pH 12, for 30 min at room temperature and then centrifuged as described in Materials and Methods. The pellets and supernatants were analyzed by SDS PAGE and stained with Coomassie Blue (lanes 1-7 and 8 and 9) or transferred to nitrocellulose and stained using monoclonal antibody Y38.1.1 (lanes 3'-7'). Lane 1, pellet of coated vesicles after Triton X-100 extraction, heavily overloaded; lane 2, corresponding Triton extracted supernatant showing that 38,000-, 29,000-, 23,000-, and 10,000-D polypeptides (arrowheads) were extracted. Lanes 3 and 3', coated vesicles before treatment; lanes 4 and 4', coated vesicle pellet after extraction with Triton X-100; lanes 5 and 5', supernatant corresponding to 4 and 4' (arrowhead denotes the 38,000-D polypeptide weakly stained with Coomassie Blue); lanes 6 and 6', coated vesicle pellet after extraction at pH 12; lanes 7 and 7', supernatant corresponding to 5 and 5'. Lane 8, pellet of purified stripped vesicles incubated in 10 mM Tris-HCl, pH 7.5, plus 0.5% Triton X-100; lane 9, corresponding supernatant.

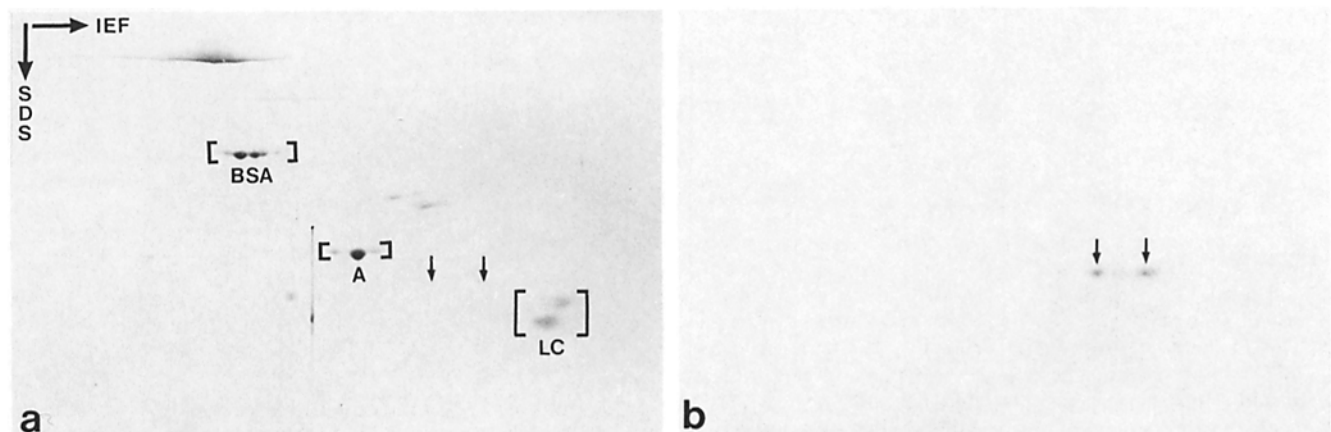


FIGURE 5 Two-dimensional electrophoresis and immunoblotting of purified coated vesicles using monoclonal antibody Y38.1. IEF, isoelectric focusing. (a) Electrophoretogram stained with Coomassie Blue. Reference proteins co-electrophoresed with the coated vesicles: bovine serum albumin (BSA), actin (A), and clathrin light chains (LC). (b) Corresponding blot on nitrocellulose stained with antibody Y38.1.1. The two isoelectric variants of the 38,000-D polypeptide are indicated by arrows.

tions that were labeled with the monoclonal antibody to the $M_r \sim 38,000$ polypeptide. A negative control with a cytokeratin monoclonal antibody showed <2% labeled vesicles (Fig. 7a); a positive control with a monoclonal anticlathrin antibody showed ~90% vesicles labeled (Fig. 7b), whereas the result with the anti- $M_r \sim 38,000$ antigen showed ~17% of the vesicles labeled (all vesicles bearing more than three gold particles were considered labeled). Correcting for the ~90% labeling efficiency observed with coated vesicles, we estimate ~20% of our vesicles contained the 38,000-D antigen.

To determine if the integrity of Triton X-100-extracted coated vesicles depended on the protein interactions of the assembled coat, we subjected vesicles stripped of their clathrin coats to detergent treatment. The stripped vesicles (see Materials and Methods) contained between 8 and 11% of the original clathrin, 51% of the 100,000-D polypeptides, and >42% of the other major polypeptides. Phosphorous determinations showed 191 nmol of inorganic phosphorus/mg protein. When stripped vesicles were treated with 0.5% Triton X-100 (Fig. 4, lanes 8 and 9) all of the major proteins remained sedimentable (Fig. 4, lane 8), and, as with coated vesicles, four polypeptides of approximate $M_r \sim 38,000$, 29,000, 23,000, and 10,000 remained in the supernatant. (Triton concentrations between 0.05 and 1.0% extracted the same polypeptides but only the results at 0.5% are shown.) But in contrast to results from the coated vesicles, incubating the stripped vesicles in Triton X-100 at all concentrations $\geq 0.05\%$ left 90% of the lipid inorganic phosphorous in the supernatant after centrifugation at 160,000 g for 1.5 h.

DISCUSSION

Our results show that most of the major polypeptides of purified coated vesicles were non-selectively solubilized under alkaline, low ionic strength conditions (Fig. 2a). If the para-

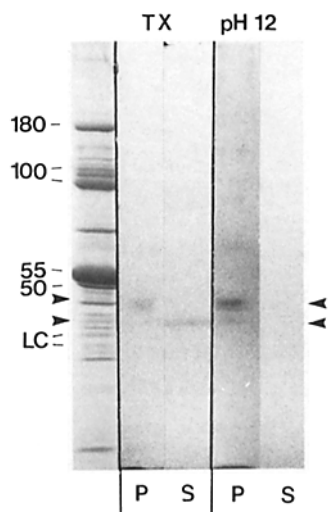
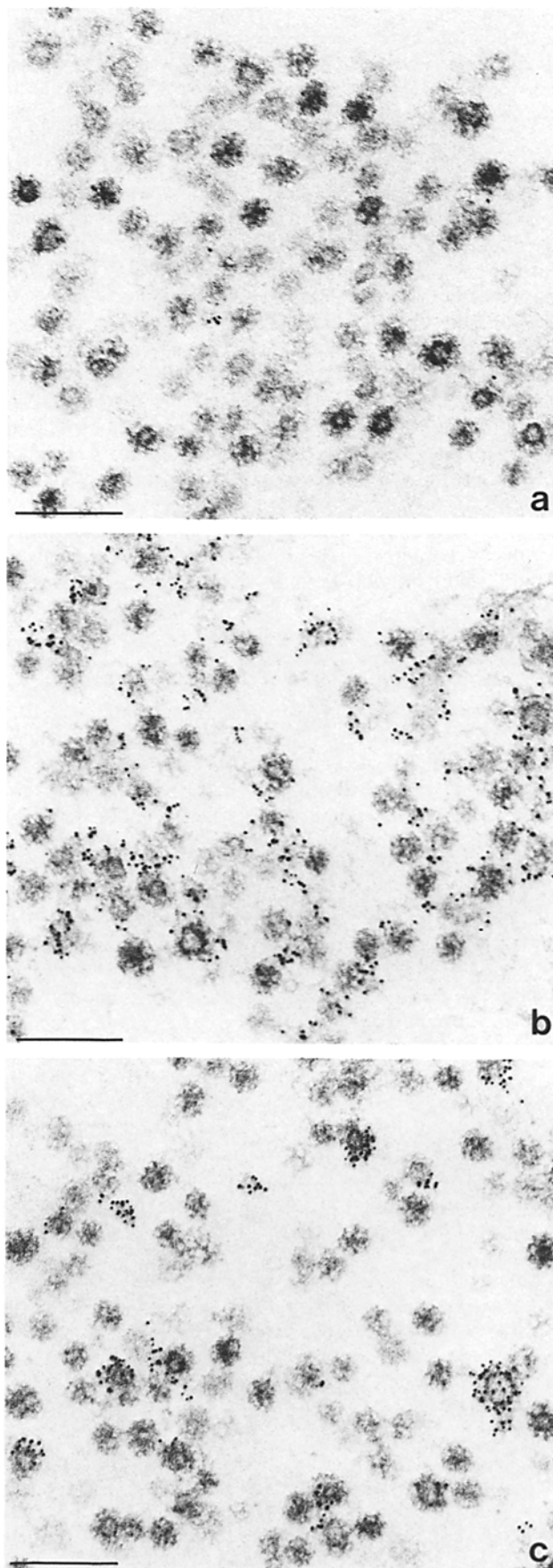


FIGURE 6 Glycoproteins in SDS polyacrylamide gels of stripped vesicles after incubation with 0.1% Triton X-100 (TX) or after incubation at pH 12 as described in Fig. 4, followed by sedimentation as described in Materials and Methods. Pellets (P) and supernatants (S) were stained with periodic acid-Schiff reagent. Coomassie Blue-stained portion of the same slab gel shows stripped vesicles, partially extracted with alkali (left lane), for reference. Arrowheads point to the polypeptides of $M_r \sim 48,000$ and $\sim 38,000$ that were stained.

FIGURE 7 Electron micrographs of immunostained coated vesicles. Sections were reacted with a mouse monoclonal first antibody to



(a) cytokeratin, (b) clathrin, and (c) the 38,000-D polypeptide of coated vesicles. The second antibody to mouse was linked to colloidal gold. See Materials and Methods for details. $\times 80,000$.

digm developed from studies of other membranes, such as the erythrocyte plasma membrane (21), is used, these findings indicate that the solubilized proteins are peripheral in nature. The released proteins make up >90% of the total coated vesicle protein, whereas the membrane residue retains all of the vesicle phospholipids. Although we do not know the state of the released proteins, electron microscopic examination and additional centrifugation of the supernatants after alkaline treatments suggest that these proteins were not aggregated. Furthermore, studies in several laboratories (9, 17, 18) show that the liberated proteins can be separated under mild alkaline conditions by gel filtration.

Except for tubulin, all of the major alkali-solubilized polypeptides were also solubilized, but poorly, by high ionic strength treatments at pH's between 6.5 and 8.5 (Fig. 3). The salt insolubility of tubulin is puzzling. The tubulin may be bound to lipid, as observed by Kumar et al. (12), but it remained sedimentable even after removal of 90% of the lipid and clathrin.

In contrast to the major protein components, only minor polypeptides were solely pellet associated (Fig. 2a) together with all phospholipids, even at pH 12. The proteins of M_r 38,000, 29,000, 24,000, and 10,000 were released into the supernatant after the addition of a non-ionic detergent (Fig. 4). This solubilization pattern was identical for stripped as well as coated vesicles and indicates that these polypeptides, which accounted for very little of the total Coomassie Blue staining pattern, should be classified among the integral membrane components of the vesicles we isolated. Because the 48,000-D polypeptide visualized by periodic acid-Schiff staining (Fig. 6) was not solubilized in either alkali or detergent, its status remains ambiguous.

Although only ~17% of our vesicles were labeled by our anti-38,000-D polypeptide, this amount of labeling is significantly greater than our background labeling in the absence of first antibody or in the presence of a control anticytokeratin antibody. We therefore believe that the 38,000-D antigen is contained in a subpopulation of the bovine brain coated vesicles. Recent experiments indicate that both of the isoelectric variants of the 38,000-D integral membrane antigen are specifically enriched in presynaptic vesicles and are found in brain- but not other bovine tissue-coated vesicles.² The coated vesicles bearing this 38,000-D polypeptide may represent those coated vesicles that are involved in recycling membrane and protein added to the cell surface by synaptic vesicle fusion. Pfeffer and Kelly (19) also noted the enrichment of a 38,000-D polypeptide in coated vesicles carrying secretory vesicle antigens.

Although they are always present in our highly purified coated vesicle preparations, it is conceivable that the 29,000-, 24,000-, and 10,000-D polypeptides are contained in contaminating vesicles rather than in coated vesicles. Even the 38,000-D polypeptide, which is clearly associated with coated vesicles, is not found in all coated vesicles. Although some fraction of the coated vesicle proteins may not bind Coomassie Blue in proportion to its mass, the apparent paucity of integral proteins that could serve as anchorage for the

dominant peripheral components is striking. Although current concepts of coated pit and coated vesicle function imply associations between transmembrane components and the cytoplasmic facing peripheral components, self-associations between peripheral components may, even in the absence of clathrin and the lipid bilayer (e.g., stripped vesicles, Fig. 4), be more important to the integrity of the pits and vesicles than are associations that anchor these peripheral components to integral membrane proteins.

This work was supported by National Institutes of Health grant GM31579.

Received for publication 12 March 1984, and in revised form 12 March 1985.

REFERENCES

- Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466-467.
- Batteiger, B., W. J. Newhall V, and R. B. Jones. 1982. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. *J. Immunol. Methods.* 55:297-307.
- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* 72:248-254.
- Eschenbruch, M., and R. R. Burk. 1982. Experimentally improved reliability of ultra-sensitive silver staining of protein in polyacrylamide gels. *Anal. Biochem.* 125:96-99.
- Fenner, C., R. R. Traut, D. T. Mason, and J. Wikman-Coffelt. 1975. Quantification of Coomassie blue stained proteins in polyacrylamide gels based on analyses of eluted dyes. *Anal. Biochem.* 63:595-602.
- Hanspal, M., E. Luna, and D. Branton. 1984. The association of clathrin fragments with coated vesicle membranes. *J. Biol. Chem.* 259:11075-11082.
- Kanaseki, T., and J. Kadota. 1969. The "vesicle in a basket." *J. Cell Biol.* 42:202-220.
- Kartenbeck, J., K. Schwechheimer, R. Moll, and W. W. Franke. 1984. Attachment of vimentin filaments to desmosomal plaques in human meningioma cells and arachnoidal tissue. *J. Cell Biol.* 98:1072-1081.
- Keen, J. H., M. C. Willingham, and I. H. Pastan. 1979. Clathrin-coated vesicles: isolation, dissociation and factor-dependent reassociation of clathrin baskets. *Cell.* 16:303-312.
- Kelly, W. G., A. Passaniti, J. W. Woods, J. L. Daiss, and T. F. Roth. 1983. Tubulin is a molecular component of coated vesicles. *J. Cell Biol.* 97:1191-1199.
- Kirchhausen, T., and S. C. Harrison. 1981. Protein organization in clathrin trimers. *Cell.* 23:755-761.
- Kumar, N., R. Blumenthal, M. Henkart, J. N. Weinstein, and R. D. Klausner. 1982. Aggregation and calcium-induced fusion of phosphatidyl-choline vesicle-tubulin complexes. *J. Biol. Chem.* 257:15137-15144.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
- Pearse, B. M. F. 1975. Coated vesicles from pig brain: purification and biochemical characterization. *J. Mol. Biol.* 97:93-98.
- Pearse, B. M. F. 1982. Coated vesicles from human placenta carry ferritin, transferrin, and immunoglobulin G. *Proc. Natl. Acad. Sci. USA.* 79:451-455.
- Pfeffer, S. R., D. G. Drubin, and R. B. Kelly. 1983. Identification of three coated vesicle components as α - and β -tubulin linked to a phosphorylated 50,000-dalton polypeptide. *J. Cell Biol.* 97:40-47.
- Pfeffer, S. R., and R. B. Kelly. 1984. Coated vesicles carrying secretory vesicle antigens contain unique proteins. *J. Cell Biol.* 99 (No. 4, Pt. 2):3a.
- Rubenstein, J. L. R., R. E. Fine, B. D. Luskey, and J. E. Rothman. 1981. Purification of coated vesicles by agarose gel electrophoresis. *J. Cell Biol.* 89:357-361.
- Singer, S. J. 1971. The molecular organization of biological membranes. In *Structure and Function of Biological Membranes*. L. I. Rothfield, editor. Academic Press, Inc., New York. 146-222.
- Steck, T. L., and J. Yu. 1973. Selective solubilization of proteins from red cell membranes by protein perturbants. *J. Supramol. Struct.* 1:220-232.
- Unanue, E. R., E. Ungewickell, and D. Branton. 1981. The binding of clathrin triskelions to membranes from coated vesicles. *Cell.* 26:439-446.
- Ungewickell, E., and D. Branton. 1981. Assembly units of clathrin coats. *Nature (Lond.)* 289:420-422.
- Valentine, R. C., B. M. Shapiro, and E. R. Stadtman. 1968. Regulation of glutamine synthetase XII—electron microscopy of the enzyme from *Escherichia coli*. *Biochemistry.* 7:2143-2154.
- Wiedenmann, B., and L. T. Mimmis. 1983. Tubulin is a major constituent of bovine brain coated vesicles. *Biochim. Biophys. Res. Commun.* 115:303-311.
- Woodward, M. P., and T. F. Roth. 1979. Influence of buffer ions and divalent cations on coated vesicle disassembly and reassembly. *J. Supramol. Struct.* 11:237-250.