

# Identification of Minor Components of Coated Vesicles by Use of Permeation Chromatography

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**ABSTRACT** Coated vesicles are thought to be vehicles for the intracellular transport of membranes. Clathrin is the major protein component of coated vesicles. Minor components of these organelles can be identified in highly purified preparations if they can be shown to copurify with clathrin. To show copurification we have made use of the relatively uniform diameter of coated vesicles (50–150 nm) to fractionate conventionally purified coated vesicles according to size on glass bead columns of 200-nm pore size. We have found that bovine brain coated vesicles prepared by the standard procedure of Pearse can be contaminated with large membrane fragments that are removed by permeation chromatography on such glass bead columns. Gel electrophoretic analysis of column fractions shows that only three major polypeptide chains, and a family of polypeptides with molecular weights close to 100,000 are always in constant ratio to clathrin, and are unique to fractions containing coated vesicles. Two other major polypeptides that appear to be components of coated vesicles are also present in other membrane fractions. We have also used permeation chromatography to monitor artifactual membrane trapping during vesicle isolation. Pure radiolabeled synaptic vesicle membranes were added to bovine brain tissue before homogenization. Considerable amounts of the added radioactivity could be recovered in the fractions conventionally pooled in the preparation of coated vesicles. After permeation chromatography, the radioactivity in the coated vesicle peak was reduced essentially to background.

Coated vesicles, first described by Roth and Porter (1), have been observed by electron microscopy in virtually all types of eucaryotic cells (see references 2 and 3 for review). They are thought to be involved in the uptake of adsorbed extracellular and plasma membrane proteins and in their subsequent delivery to lysosomes or other intracellular membrane compartments. In addition to their role in endocytosis, coated vesicles are probably also involved in the intracellular transport of membrane proteins from the endoplasmic reticulum to the Golgi apparatus, from the Golgi apparatus to the plasma membrane, and in membrane recycling. To serve as vehicles for each of these specific processes, signals must exist to enable internalization of the correct membrane components and to direct a coated vesicle to the correct intracellular membrane compartment.

Because many transported membrane components and destination signals are likely to be minor components of coated vesicles, their identification requires a preparation of these intracellular organelles that is highly pure. Pearse (4) has worked out an effective and widely used purification procedure,

which involves three sucrose density gradient steps. The purity of the final preparation has been assessed by electron microscopic examination. Gel electrophoretic analyses of these preparations (4–9) have established the presence of many minor polypeptides, in addition to a major 180,000 mol wt species that has been called clathrin (5). Unfortunately, the minor components reported by different authors vary quite widely, even when the coated vesicles are isolated from the same source. Although this variability can be explained in part by the differing amounts of material analyzed on gels, it is also in part attributable to the presence of small amounts of contaminating protein in the coated vesicle preparations.

In earlier work on cholinergic synaptic vesicle isolation we used a purification scheme that resulted in a >200-fold enrichment for acetylcholine (10). Despite this purification we found that the identification of proteins unique to synaptic vesicles required gel electrophoresis of every column fraction during the last purification step. The unique membrane proteins were those present in large amounts in the acetylcholine-rich fractions and nowhere else (11). Analogously, minor coated vesicle

components should copurify with clathrin, the major structural element of this organelle. The final step of the conventional coated vesicle purification is velocity sedimentation on sucrose density gradients. Because the sedimentation rate of organelles depends on both size and density, large, less dense, contaminating membrane vesicles could sediment at the same rate as the smaller, dense coated vesicles. Therefore, a demonstration of copurification during this step would not be sufficient for the identification of coated vesicle minor components. We chose instead to make use of the remarkably uniform diameter of coated vesicles in an additional purification step. Coated vesicles from the final velocity sedimentation were further fractionated according to size by permeation chromatography on controlled pore size glass bead columns.

By permeation chromatography we have been able to show that small amounts of other membranes can contaminate conventionally purified coated vesicles. Several of the minor components of coated vesicles can be attributed to these contaminants. Further, we can show that three of the major polypeptides and a family of polypeptides near 100,000 daltons, also observed by other workers, do indeed seem to belong to an organelle identical in size to coated vesicles.

Finally, in our attempts to localize cholinergic synaptic vesicle antigens (12) in coated vesicles isolated from marine ray electric organ (13) we have been concerned about the degree of artifactual trapping of exogenous membrane in the coated vesicle fractions. Here we demonstrate that trapping is indeed a problem but one that can be essentially eliminated by permeation chromatography.<sup>1</sup>

## MATERIALS AND METHODS

### Coated Vesicle Isolation

Coated vesicles were isolated from bovine brain essentially as described by Pearse (4) with minor modifications. All procedures were carried out at 4°C. Sucrose solutions were prepared (wt/vol) in isolation buffer, which consisted of 0.1 M 2-(*N*-morpholino)-ethanesulfonic acid, 1 mM EGTA, 0.5 mM MgCl<sub>2</sub>, and 0.02% sodium azide, pH 6.5.

150 g of bovine brain was homogenized in an equal volume of isolation buffer using three 10-s bursts in a Waring blender (Dynamics Corp. of America, New Hartford, Conn.). The homogenate was centrifuged at 20,000 *g* for 30 min; the resulting supernate was set aside on ice and the pellet was rehomogenized and centrifuged as before. The second supernate was pooled with the first, filtered through two layers of gauze, and centrifuged at 85,000 *g* for 1 h in a Beckman 45 Ti rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The 85,000-*g* pellet was resuspended in 7.5 ml of isolation buffer and layered onto a set of three sucrose gradients consisting of 4.5 ml each of 5, 10, 20, 30, 40, 50, and 60% sucrose. The gradients were centrifuged at 50,000 *g* for 2 h in a Beckman SW27 rotor. After centrifugation, the gradients contained a red region at the top, a middle white region, and a dense white band of material near the bottom. 2-ml fractions were collected and those fractions corresponding to the entire middle region (representing 29% of the protein on the gradient) were pooled, diluted threefold in isolation buffer, and concentrated by centrifugation at 100,000*g* for 1 h. The pellet was resuspended and layered onto three 20–60% sucrose gradients, poured in 5.9-ml steps of 20, 30, 40, 50, 55, and 60% sucrose. The gradients were centrifuged at 50,000*g* for 16 h in a Beckman SW27 rotor, and 2-ml fractions were collected. Although initially discontinuous, these density gradients were smooth after centrifugation as measured by refractometry. Fractions corresponding to the turbid band at ~50% sucrose were pooled, diluted threefold in isolation buffer, and concentrated by centrifugation at 100,000*g* for 1 h. The pellet was resuspended and loaded onto two 5–30% continuous sucrose gradients, which were then centrifuged for 1 h at 100,000*g* in a Beckman SW40Ti rotor. 16 0.78-ml fractions were collected.

All pellet resuspensions were carried out with a Potter-Elvehjem-type homogenizer (Kontes Co., Vineland, N. J.), using three loose-fitting-pestle strokes followed by three tight-fitting-pestle strokes. Sonication was not employed.

<sup>1</sup> A preliminary account of this work was presented at the ICN-UCLA Symposium on Cellular Recognition, March 1981 (Pfeffer, S. R., and R. B. Kelly. 1981. *J. Supramol. Struct.* 5(Suppl.):315 [Abstr.]).

### Permeation Chromatography on a Controlled Pore Glass Bead Column

The column was prepared by adding ~100 ml of CPG-10-2000 glass beads (mean pore diameter 221.5 nm, 80–120 mesh, lot no. 290; Electro-Nucleonics Inc., Fairfield, N. J.) to 1 liter, 1% (wt/vol) Carbowax (polyethyleneglycol), 20 M, Union Carbide Corp., New York). The mixture was swirled, allowed to settle five times, decanted, and rinsed five times with distilled water. The Carbowax-treated glass beads were then resuspended into 300-ml column buffer (0.2 M sucrose, 0.3 M NaCl, 10 mM HEPES, 10 mM EGTA, and 0.02% [wt/vol] sodium azide, pH 7.0). The bead slurry was degassed and packed into a column of dimensions 1.6 × 46 cm, which was strongly vibrated during packing to facilitate compacting. The sample was loaded onto the column in a volume of 1 ml, and was chromatographed at a rate of ~4 ml/h. 2-ml fractions were collected. Approximately 65% of the protein loaded onto the column was routinely recovered; we have found the yield to be poorer when the column is run in the absence of sucrose.

### Gel Electrophoresis

SDS PAGE was carried out as described by Laemmli (14) except that 30% glycerol was included in the stacking gel (Anderson et al. [15]). Gel samples were concentrated by acid precipitation, resuspended in sample buffer, and neutralized with 0.1 M NaOH, before loading onto the gel. Two-dimensional gel electrophoresis was carried out according to the procedure of O'Farrell (16).

### Electron Microscopy

Coated vesicles were prepared for electron microscopy by one of two procedures. Procedure *A* was that of Mello et al. (17), except that the primary fixative was prepared in isolation buffer and the secondary fixative was prepared in 0.1 M sodium cacodylate, pH 7.2. Procedure *B* involved primary fixation with 2% glutaraldehyde in isolation buffer, secondary fixation with 2% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.2, followed by *en bloc* staining with 1% uranyl acetate in 30 mM sodium barbital, 30 mM sodium acetate, pH 5.5. In each case, samples were dehydrated and embedded in Araldite. Thin, 60- to 90-nm sections were cut, stained with uranyl acetate and lead citrate, and examined with a Jeol-100B electron microscope. Procedure *B* resulted in darker staining of coated vesicles but facilitated visualization of large membrane contaminants.

### Other Methods

Protein was determined by Amido Schwarz staining (18) with bovine serum albumin as standard, and density by refractometry. Synaptic vesicles were purified from the electric organ of the marine ray, *Narcine brasiliensis*, by published procedures (10). Synaptic vesicles were radiolabeled by reaction with diazotized [<sup>125</sup>I]iodosulfanilic acid (New England Nuclear, Boston, Mass.) in the absence of detergent, as previously described (12).

## RESULTS

Coated vesicles were purified from 150 g of bovine brain essentially by the method of Pearse (see Materials and Methods). The coated vesicle pool from the third and final sucrose gradient step (~5 mg of protein) was concentrated by ultracentrifugation. 1 mg of this material was loaded onto a controlled pore glass column with an average pore size of 200 nm. Analysis of column fractions for protein revealed two peaks of material (Fig. 1, *upper panel*), one at the void volume and a second, containing ~85% of the protein, that was partially included. Absorbance at 280 nm was much greater than that predicted by the protein concentration, largely because of light scattering. The higher ratio of absorbance to protein in the excluded peak (Fig. 1, *upper panel*) suggested that this material was larger in size.

One-dimensional gel electrophoretic analysis of these column fractions (Fig. 1, *lower panel*) showed that the second, included peak contains nearly all of the coated vesicles, because it contained the bulk of the protein clathrin (*M*<sub>r</sub> 180,000), the major protein component of coated vesicles. Only a small amount of a polypeptide with the same mobility of clathrin is

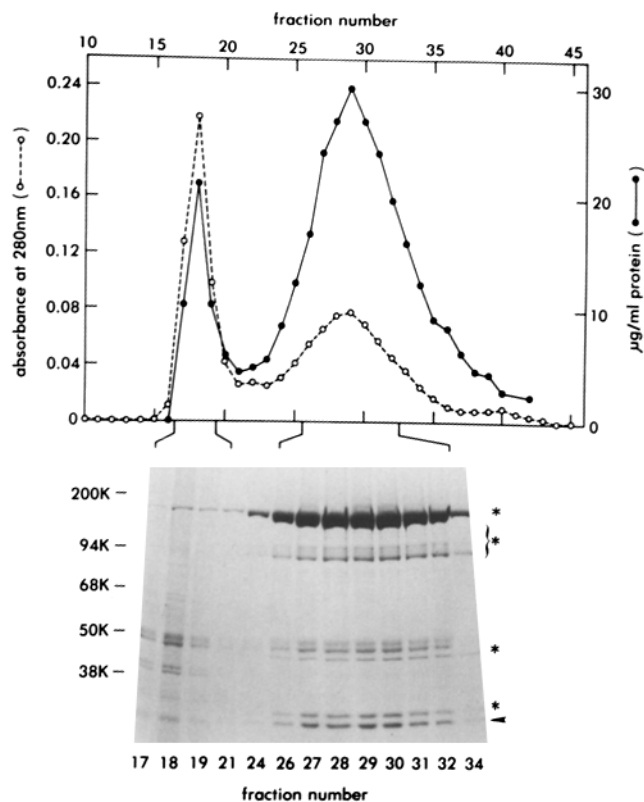


FIGURE 1 Controlled-pore glass column chromatography of conventionally purified bovine brain coated vesicles. (upper panel) 1 mg of coated vesicles purified through the third sucrose gradient were chromatographed as described in Materials and Methods. (●)  $\mu\text{g/ml}$  protein; (○) absorbance at 280 nm. 64% of the protein loaded onto the column was recovered. (lower panel) 0.4 ml of each fraction was concentrated by acid precipitation and run on a 9% polyacrylamide gel as described in Materials and Methods. Fraction numbers to which the gel lanes correspond are shown below the gel. Those polypeptides that appear to be unique to coated vesicles (see text) are designated (\*). The arrowhead indicates a polypeptide that has been shown to be unique to coated vesicles by two dimensional gel electrophoresis (see Fig. 2 and text). Molecular weight standards were run in a lane not shown at the left of the gel (myosin, 200,000; phosphorylase, 94,000; bovine serum albumin, 68,000; gamma globulin, 50,000; alcohol dehydrogenase, 38,000).

present in the first peak. Several polypeptides copurified with clathrin during this separation procedure: a family of one major and seven minor polypeptides in the molecular weight range of 100,000, a polypeptide triplet near 50,000 daltons, and two polypeptides of  $\sim 33,000$  and  $\sim 36,000$  daltons. The 100,000-dalton family, the fastest of the 50,000-dalton triplet, and the 36,000-dalton polypeptide (asterisks in Fig. 1, lower panel) are found in negligible amounts in the excluded peak and thus are considered unique to coated vesicles. Several polypeptides (for example, those of  $\sim 68,000$  and  $\sim 40,000$  daltons) are only found in the contaminating material excluded from the column.

Polypeptides with the same mobilities as the 33,000-dalton polypeptide and the larger two polypeptides of the 50,000-dalton triplet are detected in both column peaks. To determine whether these comigrating polypeptides are in fact identical, samples of material from the excluded peak and the included coated vesicle peak were subjected to two-dimensional gel electrophoresis. As can be seen in Fig. 2, the spots corresponding to the two larger 50,000-dalton polypeptides (a) are iden-

tical in both the included and excluded peaks; these two polypeptides are therefore common to both coated vesicles and other cellular membranes. The 33,000-dalton polypeptide is not a shared component, however. The excluded peak contains a spot (d) of the same molecular weight as the coated vesicle 33,000-dalton polypeptide (c) but with a different isoelectric point. Thus, two-dimensional gel electrophoresis shows that the excluded peak contains a 33,000-dalton polypeptide not present in the highly purified coated vesicles. It should be noted that in this preparation of coated vesicles the excluded material appears to contain a slightly higher proportion of coated vesicles than in Fig. 1. We believe that this is a consequence of slightly overloading the glass bead column. The trace of contaminating coated vesicles is useful, however, in showing the similarity in size of the more acidic 33,000-dalton component of coated vesicles and the corresponding polypeptide in the excluded material.

Because electron microscopy is conventionally used to judge the purity of coated vesicle preparations, we prepared thin-section electron micrographs of the coated vesicle preparation before and after separation on a controlled pore class column (Fig. 3). The fields were specifically chosen to show examples of the impurities. The starting material (Fig. 3 a) was typical of most published thin-section preparations (7, 8, 17). Coated structures clearly predominate and by visual inspection constitute  $>95\%$  of the structures observed. The excluded material (Fig. 3 b) was made up almost entirely of large, apparently

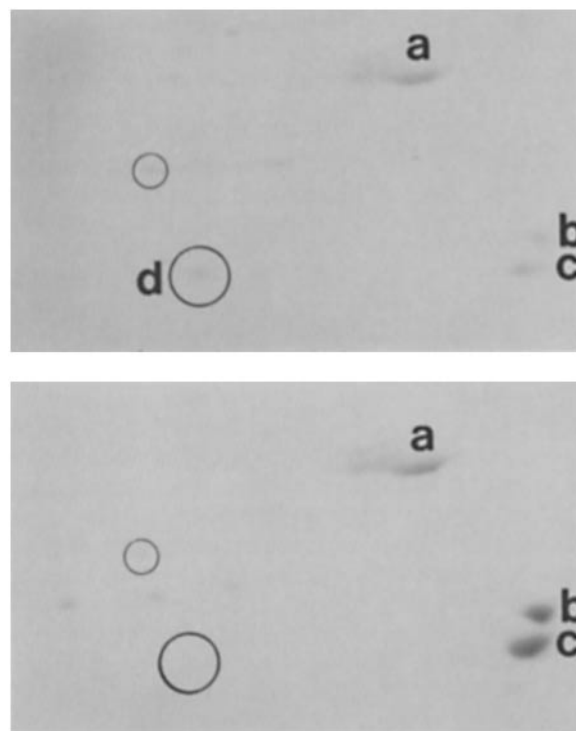


FIGURE 2 Two-dimensional gel electrophoresis of controlled-pore glass column fractions. (upper panel) Excluded peak material; (lower panel) included coated vesicle peak material. The acidic end of the gels is shown at the right. (a) 50,000-dalton doublet, common to coated vesicles and other membranes; (b) 36,000-dalton coated vesicle polypeptide; (c) 33,000-dalton coated vesicle polypeptide; (d) 33,000-dalton noncoated vesicle polypeptide. The small circle indicates a minor spot present on both gels that has been demarcated for orientation purposes. The large circles demarcate the position spot d would occupy if present on both gels.

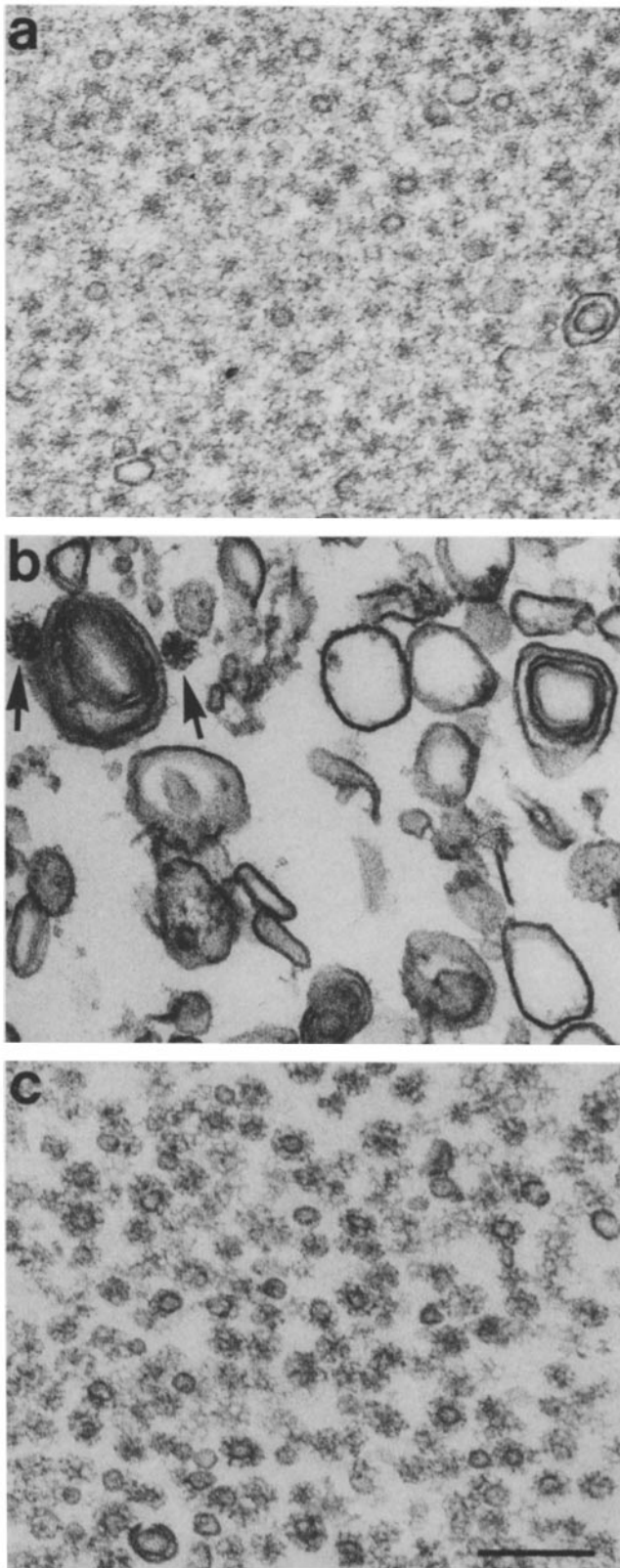


FIGURE 3 Electron micrographs of bovine brain coated vesicles before (a) and after (b and c) permeation chromatography. Panels b and c show material from the excluded and included peaks, respectively, of the controlled-pore glass column shown in Fig. 4 c. Arrows in panel b indicate occasionally observed coated vesicles. The samples for panels a and c were prepared for electron microscopy by procedure A (see Materials and Methods), the sample for panel b was prepared by procedure B. The sample shown in panel

random membrane vesicles with only rare examples of coated vesicles. Large membrane vesicles will scatter more light than the smaller coated vesicles and have a greater extinction coefficient (Fig. 1). The included peak (Fig. 3 c) was highly enriched in coated vesicles. Micrographs of the coated vesicle preparation after the controlled pore glass column (Fig. 3 c) were not noticeably different (>95% coated vesicles) from those before, even though contaminating material had been removed (Fig. 3 b).

A second way that we demonstrated the usefulness of the permeation chromatography step was by adding exogenous labeled membranes to the brain tissue before homogenization, and monitoring their fate during isolation of coated vesicles. For this experiment we chose to use cholinergic synaptic vesicles which have properties quite unlike those of the coated vesicles: a diameter of ~80 nm (19), and an equilibrium density in sucrose solutions (1.05 g/cm<sup>3</sup>) much lower than that for coated vesicles. These differences should minimize the probability of copurification. Of the radiolabeled synaptic vesicle membrane added before homogenization, 8.5% was present in the fractions of the first sucrose gradient conventionally pooled for coated vesicle isolations. Because this pool contained ~2% of the initial protein, the exogenous membrane displayed a fourfold enrichment during this step, as measured by the ratio of recovered radioactivity to protein. The pooled material was further fractionated by equilibrium centrifugation. Fig. 4 a shows the distribution of total protein and exogenously added radioactive synaptic vesicle membrane across the second sucrose gradient. In the Pearse procedure, the dense white band of material at ~50% sucrose is pooled for coated vesicles (fractions 10–12, Fig. 4 a). These fractions contained 1.4% of the radioactive synaptic vesicle membrane added before homogenization. The ratio of radioactivity to protein was reduced only ~1.2-fold relative to the pool from the first gradient. We conclude that coated vesicles purified only through sucrose gradient 2 of the Pearse procedure will most likely be contaminated with other cell membranes.

The concentrated pool of coated vesicles from the second sucrose gradient was further fractionated on a sucrose velocity gradient (Fig. 4 b), the third and final step of the Pearse purification. Only 10% of the radioactivity that had copurified with the coated vesicles on the second sucrose gradient remained with coated vesicles on the third sucrose gradient. Most of the radioactivity pelleted along with several major polypeptide contaminants (data not shown). Some radioactivity (0.16%) was still detected in the coated vesicle fractions from the third gradient, although the ratio of radioactivity to protein was reduced 3.3-fold further from its value for the second gradient pool. We found that this residual radioactivity could be essentially removed from the coated vesicles by pooling the fractions designated in Fig. 4 b, concentrating them, and running them on a controlled pore glass column as described before. As can be seen in Fig. 4 c, the bulk of the radioactivity present in the coated vesicle pool was excluded from the controlled pore glass column and separated from the bovine brain coated vesicles. Less than 0.01% of the initial radioactivity was recovered in the coated vesicle fractions. The ratio of radioactivity to protein in these fractions was at minimum four times less than that in the third gradient pool and over 17 times lower than that in the first gradient pool.

a was of higher protein concentration than those shown in panels b and c. Bar, 0.2  $\mu$ m.  $\times 78,000$ .

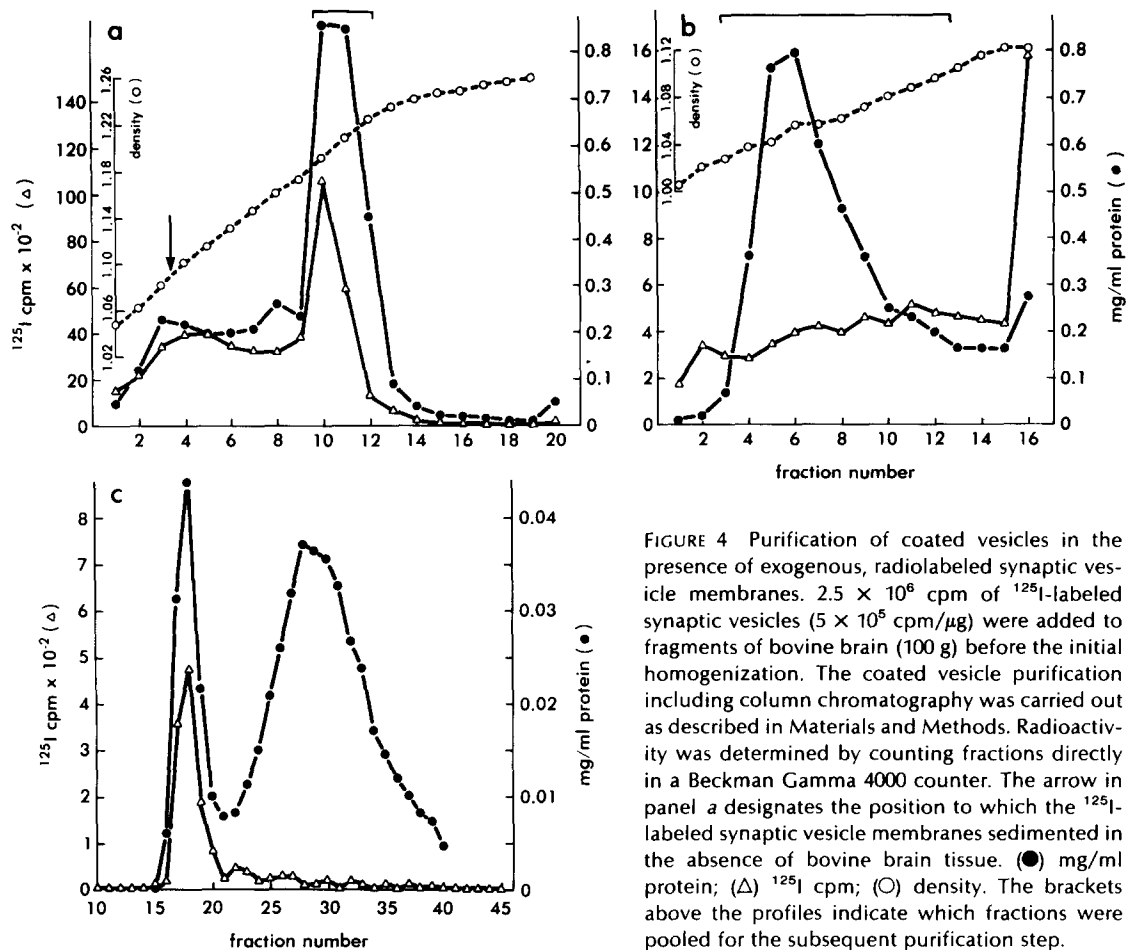


FIGURE 4 Purification of coated vesicles in the presence of exogenous, radiolabeled synaptic vesicle membranes.  $2.5 \times 10^8$  cpm of  $^{125}\text{I}$ -labeled synaptic vesicles ( $5 \times 10^5$  cpm/ $\mu\text{g}$ ) were added to fragments of bovine brain (100 g) before the initial homogenization. The coated vesicle purification including column chromatography was carried out as described in Materials and Methods. Radioactivity was determined by counting fractions directly in a Beckman Gamma 4000 counter. The arrow in panel a designates the position to which the  $^{125}\text{I}$ -labeled synaptic vesicle membranes sedimented in the absence of bovine brain tissue. (●) mg/ml protein; ( $\Delta$ )  $^{125}\text{I}$  cpm; (○) density. The brackets above the profiles indicate which fractions were pooled for the subsequent purification step.

## DISCUSSION

We have shown that the purity of conventionally purified coated vesicles can be improved by permeation chromatography. By using a glass bead column of pore size large enough to include coated vesicles, we could remove membrane contaminants present in the standard coated vesicle preparation. Although the initial coated vesicle preparation appeared to be at least 95% pure on the basis of morphological criteria, perhaps only 85% of the protein was contributed by coated vesicles. Electron micrographs of the coated vesicle preparations before and after permeation chromatography were not strikingly different, suggesting that morphology might not be a sensitive measure of purity.

Electron microscopy, although extremely useful in establishing the initial purification procedure (4), has some limitations as a criterion of purity. It cannot, for example, detect or quantify adsorbed soluble proteins. In addition, conditions optimal for the negative staining of coated vesicles may not reveal other kinds of membranes. Further, coated vesicles are often prepared for thin sectioning by pelleting. Large membrane contaminants can sediment faster than coated vesicles, resulting in a higher apparent purity in thin sections of some layers of the pellet.

Because glass bead columns fractionate particles according to size, the polypeptides that are found in association with clathrin during permeation chromatography are associated with a particle of average size and size range equal to that of coated vesicles. We believe that this is good evidence that they are minor components of coated vesicles. The minor compo-

nents fall into two categories, those that appear to be unique to coated vesicles (Figs. 1 and 2) and those that are apparently shared by the coated vesicles and the contaminating membranes. We have shown that the smallest member of the 50,000-dalton triplet is unique to coated vesicles, whereas the larger two are common to coated vesicles and other membranes. It is possible that the shared polypeptides are not true coated vesicle components but arise by the artifactual sticking of cytoplasmic proteins to all membranes during isolation. Alternatively, coated vesicles may specifically interact with certain polypeptides that are also found associated with other intracellular structures.

The polypeptides that copurify with clathrin during permeation chromatography have been detected by other workers in gels of coated vesicle preparations (4-9). Most of these preparations also contain peptides not present in coated vesicles obtained after glass bead column chromatography. Exact comparison is difficult however because of the variations in the amounts of protein analyzed on the gels. It is noteworthy that the 33,000- and 36,000-dalton coated vesicle polypeptides require chromatography in the presence of chaotropic salts for separation from clathrin during clathrin purification (Pfeffer, unpublished observations). These two polypeptides have been postulated to be part of a coated vesicle assembly unit (20).

Because clathrin makes up such a large fraction (63%) of the total protein mass of coated vesicles, electrophoresis conditions that give a sharp clathrin band yield barely detectable amounts of the minor components. Such components are best detected when the gel is heavily overloaded with clathrin, which then results in a distorted clathrin band such as that in Fig. 1 (*lower*

panel). It is encouraging that, even when the gels are massively overloaded as in Fig. 1, there are fewer minor components than in many other published preparations.

We had previously noted that antigens specific for cholinergic synaptic vesicles could be found in coated vesicles isolated from marine ray electric organ by conventional procedures (13). A weakness of this work was the possibility that synaptic vesicle membrane was artifactually trapped in coated vesicle fractions during the isolation procedure. To explore the likelihood of membrane trapping we monitored the extent to which an exogenously added membrane copurified with coated vesicles during their isolation from bovine brain. For these experiments we chose to add an exogenous membrane with physical properties quite unlike those of coated vesicles, thus decreasing the likelihood of their copurification. We used acetylcholine-containing synaptic vesicles, which had been purified to >90% homogeneity from the electric organ of a marine ray (10). Because synaptic vesicles have a very different equilibrium density in sucrose solutions than coated vesicles (see arrow in Fig. 4a), we did not expect them to migrate with the coated vesicles during the second sucrose equilibrium centrifugation (Fig. 4a). Our results indicate that the exogenously added membranes were interacting nonspecifically with some component present in the partially purified coated vesicle fractions. Some of this artifactual aggregate was removed by the third sucrose gradient, although both the second and third gradients together only decreased the ratio of exogenous membrane to protein by approximately fourfold from its value after the first gradient. The permeation chromatography step alone gave at least a fourfold purification of coated vesicles away from exogenous membranes, and resulted in removal of essentially all of the exogenous membrane from the coated vesicle fractions. These experiments do not rule out the additional possibility that clathrin coats form artifactually around a small fraction of synaptic vesicle membranes during the isolation procedure. Because of the large diameter of synaptic vesicles (80 nm), such structures would probably be excluded from the controlled pore glass column and would remain undetected. If such a process were occurring, however, it could not involve more than ~0.05% of the exogenous synaptic vesicle membranes, since this is the amount of label recovered in the excluded peak.

It has been estimated that there are ~100 molecules of clathrin per clathrin coat (21). Relative to this value for clathrin, the unique 36,000- and 50,000-dalton polypeptides, for example, are present at ~34 and ~17 copies per coated vesicle, respectively. Minor components that are present at perhaps one copy per coated vesicle would not be detected on gels such as that shown in Fig. 1, and would constitute a very small fraction of the total protein mass relative to the coated vesicle polypeptides detected on gels. Thus, removal of contaminating membranes that constitute 15% of the total protein is especially important when it comes to identifying this category of coated vesicle minor components.

It has been reported that sucrose density gradient purification results in destruction of coated vesicles (2), because of the nonisotonic dense sucrose solutions employed. It is possible that some content molecules and membrane components such as receptor molecules carried by coated vesicles may be lost during such isolation procedures. To avoid this difficulty we are currently isolating coated vesicles under isotonic conditions in conjunction with controlled pore glass column chromatography.

Coated vesicle preparations have been found to contain proteins of biological interest such as the  $\text{Ca}^{++}$ -ATPase (7), the low density lipoprotein receptor (17), the vesicular stomatitis virus (VSV) G protein (9), and calmodulin (22). As would be expected, most of these proteins are present as a very small fraction of the total protein. In light of the experiments reported here, the presence of activities such as these in morphologically homogeneous coated vesicle preparations is not sufficient to establish them as true components of coated vesicles. This is consistent with the observations of others; the coated vesicle fractions that contained the VSV G protein also contained the viral M protein, a cytoplasmic protein not thought to be a constituent of coated vesicles (9). Additional criteria must be met to establish that an activity is truly localized within a coated vesicle. For example, support for the presence of the low density lipoprotein receptor in coated vesicles came from the finding that 91% of the receptors had the correct orientation in the membranes of an adrenal cortex coated vesicle preparation (17). We believe that another useful criterion for the presence of a specific protein in coated vesicles is comigration with clathrin on glass bead columns.

#### ADDENDUM

After submission of this paper it was reported that the  $\text{Ca}^{++}$ -ATPase is not a coated vesicle component (Rubenstein, J. L. R., R. E. Fine, B. D. Luskey, and J. E. Rothman, 1981, *J. Cell Biol.* 89:357-361).

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