

Coated vesicles: Characterization, selective dissociation, and reassembly

(clathrin/urea/gel electrophoresis)

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ABSTRACT Sodium dodecyl sulfate/polyacrylamide gels of coated vesicles from porcine brain (mean 76% coated vesicles) show three major proteins (180,000, 125,000, and 55,000 daltons) that account for 73% of the total protein. Preparations consisting predominantly of coats (65%) have less of the 55,000-dalton protein. Clathrin (180,000 daltons) comprises 40% of the protein of a coated vesicle. Conditions of 2 M urea, 0.25 M MgCl₂, or pH 7.5 disrupt the coat and solubilize clathrin. Solubilized clathrin reforms coat structures after dilution of urea or MgCl₂. High-pH-solubilized clathrin reassembles after dialysis against buffer at pH 6.5 containing dithiothreitol (5 mM). Reassembled coats are predominantly clathrin.

Coated vesicles, which occur in all eukaryotic cells, possess a lattice-like network that appears as radial spokes (165 Å) extending from the vesicle into the cytoplasm. Their function is generally not known; however, in developing oocytes (1) and fibroblasts (2) coated vesicles mediate the selective uptake of proteins. In neurons, the vesicles may recycle membrane after synaptic transmission (3, 4). Bretscher (5) and Pearse (6) have suggested that coated vesicles are involved in recycling membrane lipids.

Elucidating the function of coated vesicles is, in part, dependent on determining their structure and molecular composition. Prior studies (6-9) suggested that clathrin (molecular weight = 180,000) comprised 70-90% of the total protein. Blitz *et al.* (8, 9) observed a Ca²⁺-ATPase activity in their coated vesicle preparations. Both groups suggested that clathrin makes up the lattice-like coat. Recently presented evidence from our laboratory (10) indicates that three proteins represent important structural elements of coated vesicles from dissimilar sources. Preliminary reports of our dissociation experiments have appeared elsewhere (11).

METHODS

Isolation of Coated Vesicles. Pig brains, either fresh or frozen, were homogenized with 2 vol of isolation buffer [0.1 M 2-(*N*-morpholino)propanesulfonic acid (Mes)/0.5 mM MgCl₂/1 mM ethylene glycol bis(β -aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA)/0.02% NaN₃, pH 6.5] for 1 min at top speed in a Waring Blendor. This homogenate was centrifuged for 4.8 \times 10⁵ g-min (16,000 \times g) and then the supernatant was re-centrifuged for 4.2 \times 10⁶ g-min (70,000 \times g) to obtain a pellet of crude coated vesicles. The pellet was resuspended in 40% (wt/wt) sucrose in isolation buffer and centrifuged for 1.5 \times 10⁸ g-min (125,000 \times g) to remove membranes, and the pellet was then resuspended in 48% (wt/wt) sucrose and centrifuged for 5.0 \times 10⁸ g-min (254,300 \times g) to give a pellet that was >90% coated vesicles.

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Further purification, when necessary, was achieved by resuspending the 48% pellet in 30% (wt/wt) sucrose and centrifuging this solution through a 40-45-50-55% (wt/wt) sucrose gradient (96,300 \times g, 48 hr). Coated vesicles and coats formed a sharp band at a density of 52% sucrose, which was collected, diluted 1:8 with isolation buffer, and centrifuged for 6 \times 10⁶ g-min. The final pellet was stored at 4°C.

Coated Vesicle Assay. Coated vesicles were examined in the electron microscope following negative staining with uranyl acetate.

Some coated vesicle preparations were fixed with 2.5% (vol/vol) glutaraldehyde in isolation buffer for 30 minutes followed by 2% OsO₄ buffered with 0.1 M cacodylate/5 mM CaCl₂ (pH 7.2) while others were fixed according to Kanaseki and Kadota (12). Preparations were stained *en bloc* with 2% aqueous uranyl acetate, dehydrated, and embedded in either Epon (13) or 10% Epon/20% Araldite/70% dodeceny succinic anhydride (14). Sections were stained with uranyl acetate and lead citrate and examined in a Hitachi HU-12 electron microscope.

Protein Concentration. Protein concentration was estimated from the amount of tryptophan fluorescence at 340 nm. All samples were made 0.1% in sodium dodecyl sulfate (NaDodSO₄) prior to assaying. Bovine serum albumin was used to construct a standard curve.

Dissociation Experiments. Aliquots of coated vesicle preparations were incubated for 60 min at room temperature in one of the following buffers: (i) isolation buffer; (ii) isolation buffer (pH 8.2); (iii) isolation buffer with 2 M urea; or (iv) isolation buffer with 1% Triton X-100. The solutions were then centrifuged to remove vesicles and large aggregates (3 \times 10⁷ g-min). For the dissociation experiments shown in Fig. 2a the starting solutions (2.5 ml) contained 7.5 mg of protein. After centrifugation the supernatant volume was 2.5 ml, and the pellets were suspended in 1 ml of isolation buffer. Aliquots of the resulting supernatants were examined after negative staining with uranyl acetate. Pellets from the various treatments were fixed and embedded as described above. Supernatants and pellets were subjected to NaDodSO₄ gel electrophoresis (15, 16).

RESULTS

Purity of Coated Vesicle Preparations. Our procedures yield a population of coated vesicles and empty coats (Fig. 1 a-c). Coated vesicles have an electron-transparent core in negatively stained preparations (the vesicle) that obscures the surrounding lattice-like array of short, thin filaments (Fig. 1b). Often more of the coat structure is seen because the core is reduced in diameter or incomplete. Empty coats are highly ordered lattices of hexagons and pentagons whose edges are about

Abbreviation: NaDodSO₄, sodium dodecyl sulfate.

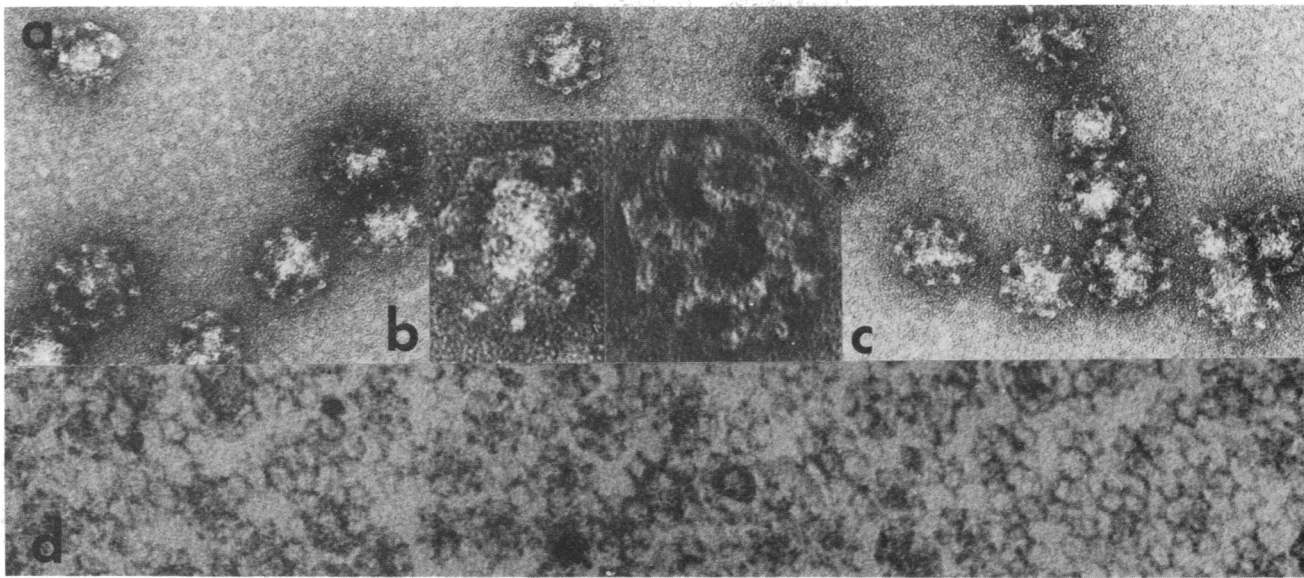


FIG. 1. Porcine brain coated vesicles. (a) Preparation negatively stained with uranyl acetate. ($\times 150,000$.) (b) A coated vesicle. Note that part of the coat lattice is obscured by the vesicle. ($\times 300,000$.) (c) An "empty coat." Close examination of the central hexagon reveals the bottom portion of the coat. ($\times 315,000$.) (d) A thin section of coated vesicles stained with uranyl acetate and lead citrate. ($\times 150,000$.)

65 Å wide by 165 Å long (Fig. 1c). Ninety-eight to 100% of the particles in our preparations are coated vesicles or coats, of which coats can comprise from 16 to 65%. In thin section images, greater than 98% of the particles are coats or coated vesicles (Fig. 1d).

Proteins of Coated Vesicles. NaDodSO₄/polyacrylamide gel patterns of our final preparations are complex (Table 1 and Fig. 2a). On the basis of densitometric analysis of the gel patterns from five preparations (mean 23.6% coats) we estimate that clathrin comprises approximately 46% of the total protein, while the proteins with molecular weights 125,000 and 55,000 each comprise about 14% of the total protein (Table 1). Clathrin accounts for about 40% by weight of the protein of a coated vesicle (Table 1). The mole ratio of the three major peaks in a coated vesicle is 1.8:1:2.2 (Table 1). In a sixth preparation with a substantially higher percentage of coats (65%), clathrin comprised approximately 65% of the total protein, the 125,000-dalton region 12%, and the 55,000-dalton protein 3% (Fig. 2a lane 2 and 2b control).

Dissociation of the Coat. Several reagents appeared to perturb coat morphology (Table 2). Buffer at pH ≥ 7.5 , 0.25 M MgCl₂, 2 M urea, and 1% Triton X-100 were chosen for further study.

Control preparations incubated at room temperature for 60 min in isolation buffer have normal morphologies. Following centrifugation the supernatants contained approximately 5% of the total protein (Fig. 2a).

Triton altered neither the coat nor the vesicle morphology in negatively stained images. Thin sections of the pellets contained coats and coated vesicles with less distinct edges than controls and no bilaminar membrane images were noted (Fig. 3a). Triton supernatants contained approximately 5% of the total protein and gave an electrophoretic pattern nearly identical to the control supernatant (Fig. 2a). Treatment of coated vesicles with 2 M urea and 1% Triton resulted in the disappearance of vesicles. Triton treatment of preparations consisting of 76% coated vesicles solubilized some of the 125,000-dalton protein, but none of the 55,000-dalton protein (data not shown).

At pH 7.5, dissociation is incomplete after 2 hr at room temperature and uncoated vesicles and many large aggregates

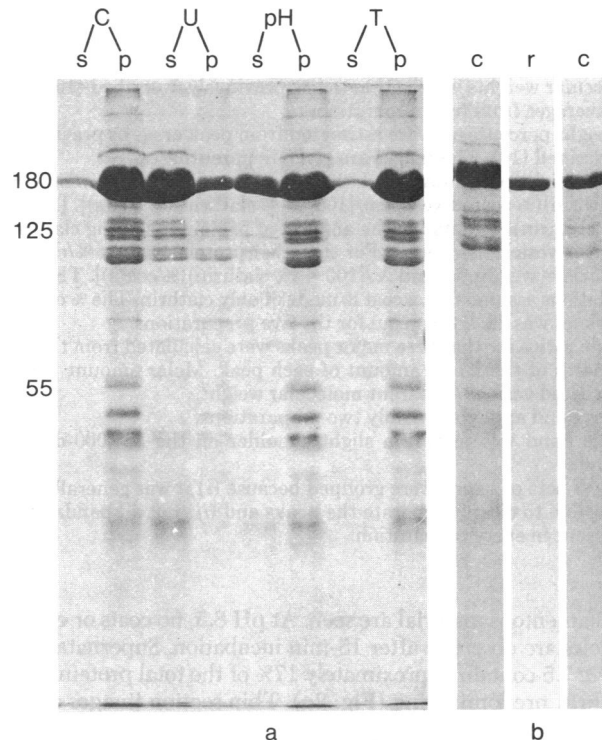


FIG. 2. NaDodSO₄/polyacrylamide gel patterns of (a) coated vesicle preparations treated with dissociating agents and (b) reassembled coats. In a, C = control, U = urea, pH = pH 8.2, T = Triton X-100, s = supernatant, and p = pellet. Equal sample volumes from the supernatants (40 μ l) and pellets (10 μ l) were analyzed with the exception of the urea pellet (40 μ l). The control pellet sample contained approximately 75 μ g of protein. In b, c = control and r = reassembled coats. After centrifugation on 10–30% linear sucrose gradients (6×10^6 g·min, Spinco SW 40 rotor) the control and reassembled coat preparations were fractionated and aliquots from the peak tubes were analyzed. The control preparation used in these experiments is the same as in a. The left-hand control slot contains approximately 35 μ g of protein, the reassembled coat slot 15 μ g, and the right-hand control slot 20 μ g. For a and b electrophoresis was performed according to Laemmli (16) on 3% stacking/10% separating polyacrylamide gels containing 0.1% NaDodSO₄. Molecular weights $\times 10^{-3}$ are given on the left.

Table 1. Coated vesicle proteins: Molecular weights and weight percentages

Molecular weight $\times 10^{-3}$	Mean wt %*	Wt %/ coated vesicle†	Mole ratio‡
300	0.4 (0-1.0) [§]		
180	46.2 (41.3-52.2)	39.6	1.8
140	1.1 (0-5.6) [¶]		
125	13.5 (11.4-19.5)	15.1	1.0
105	8.92 (6.8-10.5)		
96			
80			
70			
58			
55	13.7 (9.7-17.9)	15.4	2.2
48	16.5 (12.3-22.5)		
42			
39			
37			
31			
26			
25			
21			
20			
18			

NaDodSO₄ electrophoresis was performed on 5% and 7.5% polyacrylamide gels according to Maizel (15) or on 7.5% polyacrylamide gels with 0.188 M Tris/0.188 M/glycine/0.1% NaDodSO₄ (pH 8.3). Molecular weights were estimated as previously described (10) and are averages from five preparations.

* Weight percentages were estimated from peak areas as previously described (10). The ranges are given in parentheses.

† The weight percentage of clathrin per coated vesicle = (weight % clathrin)(% coated vesicles)/[100 - (% clathrin)(% coats)]. [100 - (% clathrin)(% coats)] is the amount of protein including clathrin that is vesicle associated. For the other proteins weight %/coated vesicle = weight % band X/[100 - (% clathrin)(% coats)]. The calculations assume that a coat is made of only clathrin. The % coated vesicles was 76.4, the mean for the five preparations.

‡ Mole ratios for the three major peaks were calculated from the estimates of the molar amount of each peak. Molar amount = (wt %/coated vesicle)/apparent molecular weight.

§ This band appeared in only two preparations.

¶ This band was usually a slight shoulder on the 125,000-dalton band.

|| These sets of bands were grouped because (i) it was generally not possible to cleanly separate the peaks and (ii) not all bands were present in every preparation.

of filamentous material are seen. At pH 8.5, no coats or coated vesicles are observed after 15-min incubation. Supernatants at pH \geq 7.5 contain approximately 17% of the total protein, with clathrin predominating (Fig. 2a). Thin section images of the pellets contain many filamentous aggregates.

Incubation of coated vesicle preparations in 2 M urea results in the solubilization of approximately 32% of the total protein and the disruption of coat structure. On NaDodSO₄ gels of urea supernatants, clathrin is the major protein (Fig. 2a). After disruption of the coat, filamentous aggregates appear in solution. Similar aggregates are observed in thin section images of urea pellets (Fig. 3b). In contrast to the pH pellet, the urea pellet is difficult to resuspend in isolation buffer. The addition of 2% NaDodSO₄ and 5% 2-mercaptoethanol slightly increases solubilization. Our experiments with 0.25 M MgCl₂ gave results qualitatively similar to those obtained with urea.

Reassociation Experiments. In our initial experiments the urea- and MgCl₂-treated preparations were diluted with isolation buffer prior to centrifugation. Because the supernatants contained prominent clathrin bands and the pellets contained

Table 2. Influence of various agents on the structure of coated vesicles

Agent	Concentration	Effect
Colchicine	1 mg/ml	None
Dithiothreitol	100 mM	None
2-Mercaptoethanol	0.6 M	Aggregates
KCl	3 M	None
KI, KBr, CsCl	\geq 0.6 M	Removes coat
PO ₄	0.4 M	None
CaCl ₂ , MgCl ₂	\geq 0.25 M	Removes coat
Cytochalasin B	25 μ g/ml	None
Triton X-100	1.0%	None
Room temperature (24 hr)		None
ATP	10 mM	None
pH	\geq 7.5	Removes coat
4°C (12 weeks)		None
Urea	\geq 2 M	Removes coat

The effect of an agent was monitored by electron microscopy. Those agents that disrupted ultrastructure were effective within 15 min.

coats and coated vesicles, we initially interpreted these data as indicating that these agents only partially dissociated the coat lattice. However, upon seeing the preliminary reassociation results of Schook *et al.* (17) we realized that we were treating the vesicles so as to favor reassociation.

Prior to centrifugation, urea- or Mg²⁺-treated preparations reassemble after dilution of either reagent. After centrifugation, supernatants were concentrated by vacuum dialysis and assayed by negative staining. No coats were present, but filamentous aggregates and particles with a diameter of approximately 130 Å were present (Fig. 3c). The solutions contained coats following a 5- to 10-fold dilution of either reagent (Fig. 3d-f). Reassembly after urea treatment occurred in the presence of 50 mM EDTA, but lowering the pH below 5 inhibited reassembly. Reassembly occurred at either 4°C or room temperature and was rapid (less than 5 min).

Preparations dissociated at pH 7.5 did not form coats when the pH was returned to 6.8. However, coats were present when the pH was returned to 6.8 by dialysis (2 hr at room temperature, then overnight at 4°C in isolation buffer containing 5 mM dithiothreitol).

Reassociated coats have morphologies similar to those of coats seen in untreated preparations but have a wider variety of lattice configurations. Solutions containing reassembled coats also contain two classes of much smaller particles. One type has a diameter of about 130 Å with a hollow center, while the second appears to be a dimer of 50-Å by 100-Å subunits. However, these may be different views of the same particle.

Sedimentation profiles of reassembled preparations on 10-30% (wt/wt) linear sucrose gradients are identical to control profiles except that they also contain a peak at the top of the gradient containing unreassembled clathrin and nearly all of the minor bands (data not shown). Comparison of the control and reassembled coat peaks by NaDodSO₄ gel electrophoresis suggests that reassembled coats consist primarily of clathrin (Fig. 2b). There was no obvious stoichiometry between clathrin and any of the minor bands present.

DISCUSSION

Morphology. Our isolation method yields coated vesicles and empty coats virtually free of contaminating membranes. The variation in vesicle morphology seen in negatively stained preparations and the frequent lack of a bilaminar membrane in thin section specimens is consistent with a disorganization or loss of vesicle material during the isolation procedure.

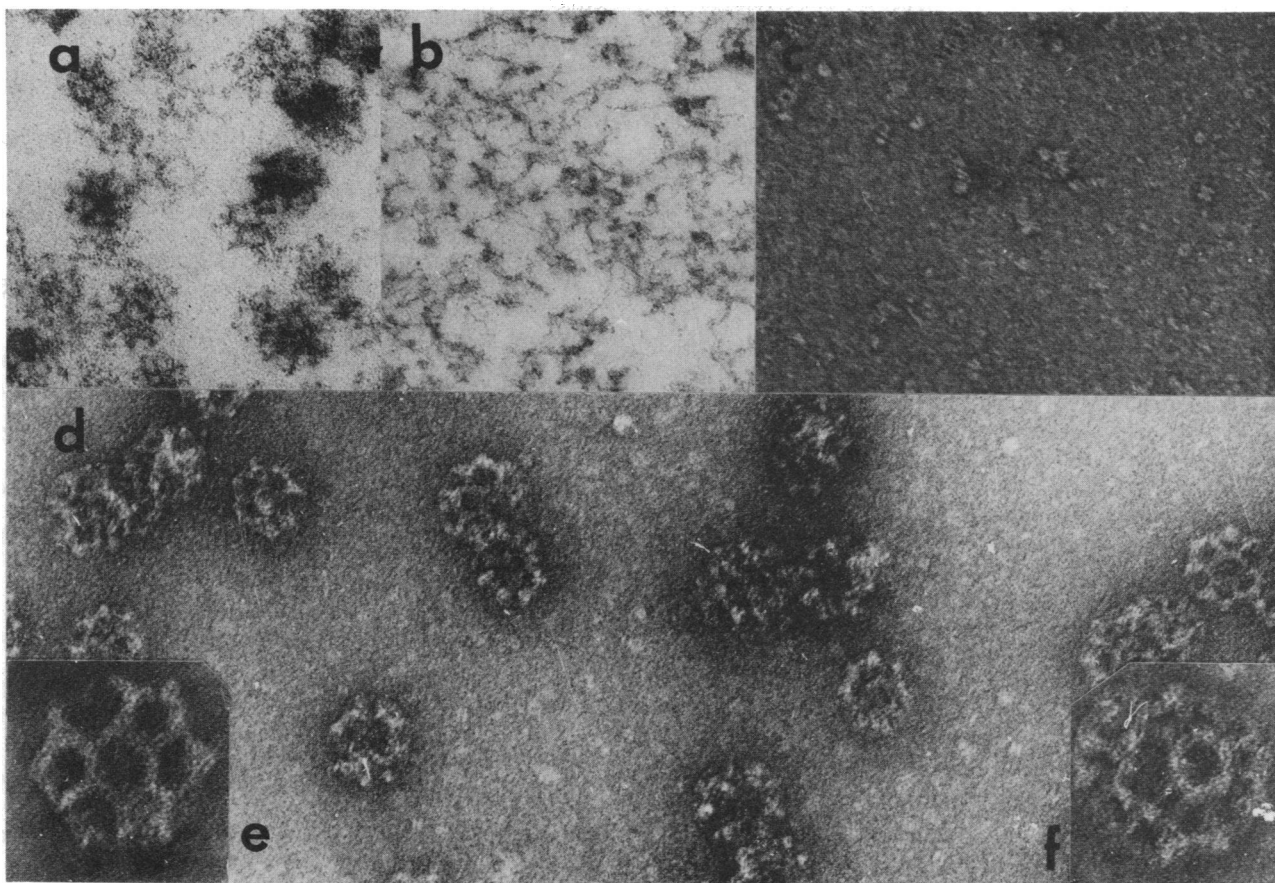


FIG. 3. Coated vesicle preparations after dissociation and reassembly. (a) Pellet from 1% Triton X-100 treatment. ($\times 150,000$.) (b) Pellet from 2 M urea-treated preparation. ($\times 82,000$.) (c) The 0.25 M $MgCl_2$ supernatant prior to dilution. ($\times 200,000$.) (d-f) The 2 M urea supernatant after dilution. Reassociated coats appear to have normal morphologies. (d, $\times 150,000$; e and f, $\times 300,000$.)

Earlier workers (6-9) observed that preparations contained coated vesicles and coats but have overlooked this when discussing the protein composition of coated vesicles. We find that coats comprise from 16 to 65% of our various preparations. Our examination of Pearse's micrographs (7) indicates that her preparations are approximately 70% coats, in agreement with an estimate made by Crowther *et al.* (18). Our examination of the micrographs of Blitz *et al.* (9) indicates that their preparations are approximately 28% coats.

Protein Composition. Many proteins copurify with coated vesicles and coats as judged by stainable bands on Na-DodSO₄/polyacrylamide gels. Variation in the number of visible bands depends on the gel concentration, electrophoresis buffer, and amount of protein on the gel. However, variations in the amount of the major proteins present appear to depend on the relative percentages of coats and coated vesicles in a preparation. Preparations containing approximately 24% coats have higher percentages of the 125,000- and 55,000-dalton proteins than preparations with approximately 65% coats. This observation is consistent with the data presented by Pearse (6, 7) showing low percentages of proteins in the 125,000- and 55,000-dalton regions in preparations consisting predominantly of coats. Conversely, Blitz *et al.* (8, 9) have observed major bands at 100,000 and 60,000 daltons in preparations that were predominantly coated vesicles. The decrease in the 55,000-dalton protein with increasing concentration of coats suggests that this protein is vesicle associated. The change in the 125,000-dalton protein is not as great; however, this may reflect its ability to interact with both clathrin and the vesicle surface.

Pearse (6, 7) and Blitz *et al.* (8, 9) argue that clathrin comprises $\geq 70\%$ of the protein of a coated vesicle because clathrin accounts for $>70\%$ of the protein in their preparations. However, because coated vesicle preparations consist of coated vesicles and coats, only a fraction of the total clathrin is vesicle associated. Our calculations (Table 1) indicate that clathrin comprises about 40% of the protein of a coated vesicle. Using Pearse's 70% clathrin value (7) and the observation by Crowther *et al.* (18) and ourselves that her preparations are 30% coated vesicles, we arrive at a value of 43% clathrin per coated vesicle. Comparison of the gel pattern of Blitz *et al.* (9) with our gels run to give similar densities suggests that clathrin comprises approximately 50% of the total protein. If this estimate is accurate and their preparation is 72% coated vesicles, their vesicles are also 42% clathrin. These calculations assume that clathrin is the only protein of the coat lattice.

Dissociation and Reassembly of the Coat. Pearse (6, 7) and Blitz *et al.* (8, 9) have suggested that clathrin is the sole coat protein. In a previous study we hypothesized that clathrin, a 125,000-dalton protein, and a 55,000-dalton protein were important to coat and coated vesicle structure. Our studies with dissociating agents suggest that the 55,000-dalton protein is vesicle associated. Reassembled coats consist almost entirely of clathrin and no constant stoichiometry between clathrin and any of the other proteins present in reassembled coats is evident. This suggests that the lattice portion of the coat is predominantly clathrin. The role of the 125,000-dalton protein is unclear; however, it may serve to join the coat lattice to the vesicle surface. If this is the case then some of the 125,000-dalton protein could remain associated with the coat lattice during the

preparative procedure. Our present evidence is consistent with such a possibility.

Our results with Triton X-100 suggest that when the vesicle is surrounded by a coat lattice the vesicle proteins are resistant to Triton solubilization. However, some perturbation seems to occur, because bilaminar membranes are not seen in those structures that appear to contain a central vesicle.

Disruption of coat structures is accompanied by the appearance of large filamentous aggregates. Then, when urea- or Mg^{2+} -treated preparations are diluted, coats reassemble and filaments disappear, suggesting that dissociated clathrin may be able to reversibly form such filaments. The inability of pH ≥ 7.5 -treated preparations to reassemble without dialysis suggests that pH-dissociated clathrin is different from that treated with urea of Mg^{2+} . Divalent cations are not required for reassembly, which suggests that coat formation is not controlled by the Ca^{2+} -ATPase activity associated with coated vesicle preparations (8, 9).

The variety of lattice configurations present following reassembly suggests that the reaction has several favorable end points. Coat lattices will also form around vesicles, suggesting the presence of coat-vesicle association sites; perhaps as complexes of the 125,000- and 55,000-dalton proteins. Reassembly of coats in solutions consisting predominantly of clathrin suggests it may self-assemble without the aid of other proteins. The apparent absence of stoichiometric amounts of either of the two other major bands in reassembled coats supports this conclusion. However, verification of these conclusions awaits solutions of highly purified clathrin.

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