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Influence of Buffer Ions and Divalent Cations on Coated Vesicle Disassembly and Reassembly

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Disruption of the coat of coated vesicles is accompanied by the release of clathrin and other proteins in soluble form. The ability of solubilized coated vesicle proteins to reassemble into empty coats is influenced by Mg^{2+}, Tris ion concentration, pH, and ionic strength. The proteins solubilized by 2 M urea spontaneously reassemble into empty coats following dialysis into isolation buffer (0.1 M MES–1 mM EGTA–1 mM $MgCl_2$–0.02% NaN_3, pH 6.8). Such reassembled coats have sedimentation properties similar to untreated coated vesicles. Clathrin is the predominant protein of reassembled coats; most of the other proteins present in native coated vesicles are absent. We have found that Mg^{2+} is important in the coat assembly reaction. At pH 8 in 0.01 M or 0.1 M Tris, coats dissociate; however, 10 mM $MgCl_2$ prevents dissociation. If the coats are first dissociated at pH 8 and then the $MgCl_2$ is raised to 10 mM, reassembly occurs. These results suggest that Mg^{2+} stabilizes the coat lattice and promotes reassembly. This hypothesis is supported by our observations that increasing Mg^{2+} (10 μM–10 mM) increases reassembly whereas chelation of Mg^{2+} by (EGTA) inhibits reassembly. Coats reassembled in low-Tris (0.01 M, pH 8) supernatants containing 10 mM $MgCl_2$ do not sediment, but upon dialysis into isolation buffer (pH 6.8), these coats become sedimentable. Nonsedimentable coats are noted also either when partially purified clathrin (peak I from Sepharose CL4B columns) is dialyzed into low-ionic-strength buffer or when peaks I and II are dialyzed into isolation buffer. Such nonsedimentable coats may represent intermediates in the assembly reaction which have normal morphology but lack some of the physical properties of native coats. We present a model suggesting that tightly intertwined antiparallel clathrin dimers form the edges of the coat lattice.

Key words: coated vesicles, coat dissembly, coat reassembly, coat dissociation, clathrin

Abbreviations used: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethyleneglycoltetraacetic acid; MES, 2-(N-morpholino)ethanesulfonic acid.

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Morphologic evidence has suggested that coated vesicles are the intracellular organelles responsible for receptor-mediated protein transport [1–3] and neuronal membrane recycling [4, 5]. Until recently, however, little was known concerning the structure and biochemical composition of coated vesicles. Pioneering structural studies by Kanaseki and Kadota [6] and Kadota and Kadota [7] have revealed that the coat consists of a lattice of interlocked hexagons and pentagons. The most striking image is that resulting from the assembly of 20 hexagons and 12 pentagons to form a spherical lattice. An elegant study by Crowther et al [8] supported these observations and demonstrated that there were other possible arrangements of hexagons and pentagons. Woods et al [9] made similar observations on coated vesicles from porcine brain and chicken oocyte. In addition they found that larger assemblies occurred in chicken oocytes [9]. A common observation of all of the preceding studies is that the coat lattice consists of rod-shaped particles which assemble into interlocked hexagons and pentagons. Ockleford [10] has proposed, on the basis of observations of coated vesicles in human placentas, that the sides of the hexagons and pentagons are composed of ridges of protein rather than rods.

Concurrent with these morphologic studies were investigations of the protein composition of purified coated vesicle preparations. Pearse [11] observed that porcine brain-coated vesicle preparations contained a single major protein (mol wt = 180,000 daltons), which she estimated made up greater than 80% of the total protein. Subsequently, she found that this protein, clathrin, was common to coated vesicles from a variety of sources [12]. Blitz et al [13] found that in addition to clathrin two other proteins were consistently found in significant amounts of brain coated vesicle preparations. Woods et al [9] also observed similar proteins in chicken oocyte and porcine brain coated vesicle preparations. Woodward and Roth [14] quantitated the amount of protein in the various bands observed in SDS gel electrophoretic patterns of porcine brain-coated vesicle preparations and found that clathrin, a 125,000-dalton protein, and a 55,000-dalton protein occurred with a mole ratio of approximately 2:1:2.

Pearse [11, 12] and Blitz et al [13] have argued that chlathrin is the sole structural protein of the coat lattice, since it is the major band on SDS gels. In support of this Blitz et al [13] found that clathrin was the major protein solubilized by 2 M urea. Partial confirmation of this hypothesis has come from studies by Woodward and Roth [14], Kartenbeck [15], Schook et al [16], and Keen et al [17], who have found that coats can reassemble spontaneously in solutions of solubilized coated vesicle proteins under appropriate conditions. However, it is still not certain that clathrin is the sole structural protein of the lattice or that it can catalyze its own self assembly. In this report we present further studies of the role of clathrin in coat structure.

METHODS

Isolation Procedure

Coated vesicles were prepared as described by Woodward and Roth [14]. Isolation buffer contained 0.1 M MES–1 mM $MgCl_2$ –1 mM EGTA–0.2% NaN_3 (pH 6.8). The purity of the preparations was monitored by electron microscopy of negatively stained samples or thin section images as described previously [14].

Electrophoresis Methods

SDS-polyacrylamide gel electrophoresis was performed using 10% slab gels according to Laemmli [18]. Gels were simultaneously fixed and stained in 30% isopropanol–10% acetic acid–0.05%. Coomassie blue R250 and destained in 10% isopropanol–10% acetic acid.

Protein Concentration

Protein concentrations were estimated from the amount of tryptophan fluorescence at 340 nm. Bovine serum albumin was used to construct a standard curve.

Centrifuation Procedures

Sucrose density grandient centrifugation. Coated vesicle preparations were analyzed on 10–30% (w/w) linear sucrose density gradients using a SW40 rotor. The gradients were centrifuged for 2 h at 57,000 g at 4°C. They were fractionated by upward displacement with 50% sucrose. Fractions were monitored by absorbance at 280 nm, electron microscopy, and SDS-polyacrylamide gel electrophoresis.

Airfuge centrifugation. Samples were centrifuged at room temperature for 20 min at 107,000 g in a Beckman Airfuge to separate reassembled coats from soluble proteins.

Sepharose CL4B chromatography. Coated vesicle proteins solubilized with 0.5 M Tris-isolation buffer (pH 7.0) [17] were chromatographed at room temperature on a Sepharose CL4B column (1.5 × 100 cm) in 0.5 M Tris-isolation buffer (pH 7.0) at 10 ml/h.

RESULTS

Purified coated vesicle preparations consisting of empty coats and coated vesicles contain many proteins [14]. We sought to determine which of these proteins are structural elements of the coat lattice by investigating in vitro reassembled coats.

Protein Composition and Structure of Reassembled Coats

Although a variety of reagents disrupt coat morphology and promote the release of clathrin, as well as other proteins [14], we observe only small differences between the resultant SDS-polyacrylamide gel patterns. Coated vesicles dissociated in 2 M urea, 0.25 M $MgCl_2$, or 10 mM Tris (pH 8) reassemble as empty coats after dialysis of the soluble proteins into isolation buffer [14]. These coats exhibit sizes and symmetries similar to those seen in undissociated coated vesicles [14]. In particular, empty coats with six-fold rotational symmetry are frequently observed (Fig. 1a). Isolated hexagons or pentagons, which could be intermediates in the reassembly reaction, are not evident. In high magnification, high-resolution images of negatively stained coats, the edges of the lattice often appear to have a helical substructure. This aspect of coat morphology is enhanced in Markham-rotated images (Fig. 1a).

To determine if reassembled coats have sedimentation properties similar to control coats, we purified reassembled coats on 10–30% (w/w) linear sucrose density gradients. Comparison of the sedimentation profile of empty coats reassembled from 2 M urea supernatants with that of undissociated preparations suggests that the two preparations have identical sedimentation characteristics (Fig. 2a). Both reassembled and untreated coats exhibited broad profiles on these gradients, the peak fractions having a sedimentation coefficient of approximately 200S. Coats were observed only at sucrose concentrations greater than 15% (w/w). The width of the peaks does not appear to be due to aggregation or association of the coats, since negatively stained images of the fractions did not contain clusters of coats. Rather, the broad sedimentation profile may be due to the size heterogeneity of the preparations.

Gradient fractions containing reassembled coats (lanes 7–14, Fig. 2b) appear to consist predominantly of clathrin when analyzed on SDS-polyacrylamide gels. The group of bands in the region of 90,000–125,000 daltons and 55,000 daltons, present in untreated preparations (lanes 6–14, Fig. 2c), are present in very much reduced amounts in

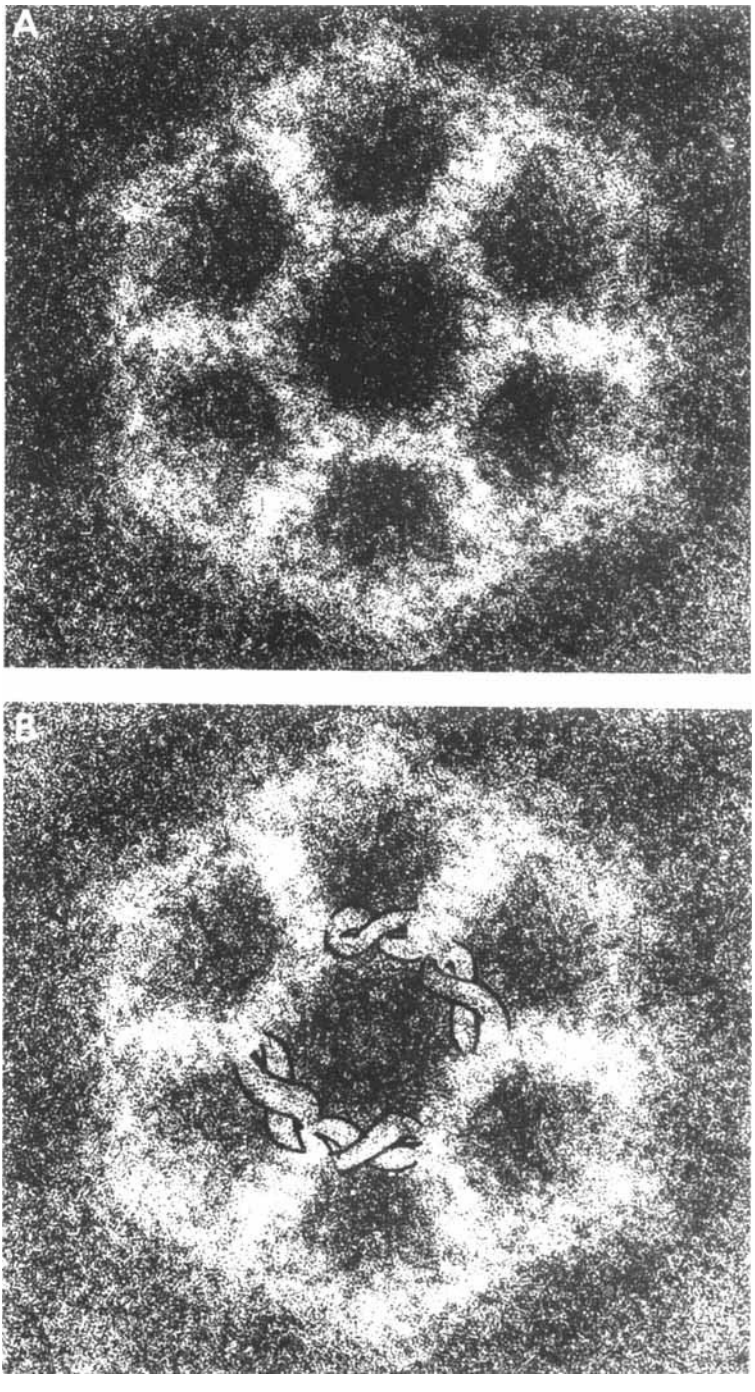

Fig. 1. Structure of the coat edge. A: Rotationally reinforced image of a reassembled coat with six-fold symmetry. We frequently observe such coats in both untreated and reassembled preparations. The lattice edges appear to be composed of intertwined rods. Negatively stained with uranyl acetate. × 1,200,000. B: To aid in visualizing the substructure of the lattice edges in A we have outlined the rods comprising several of the edges.

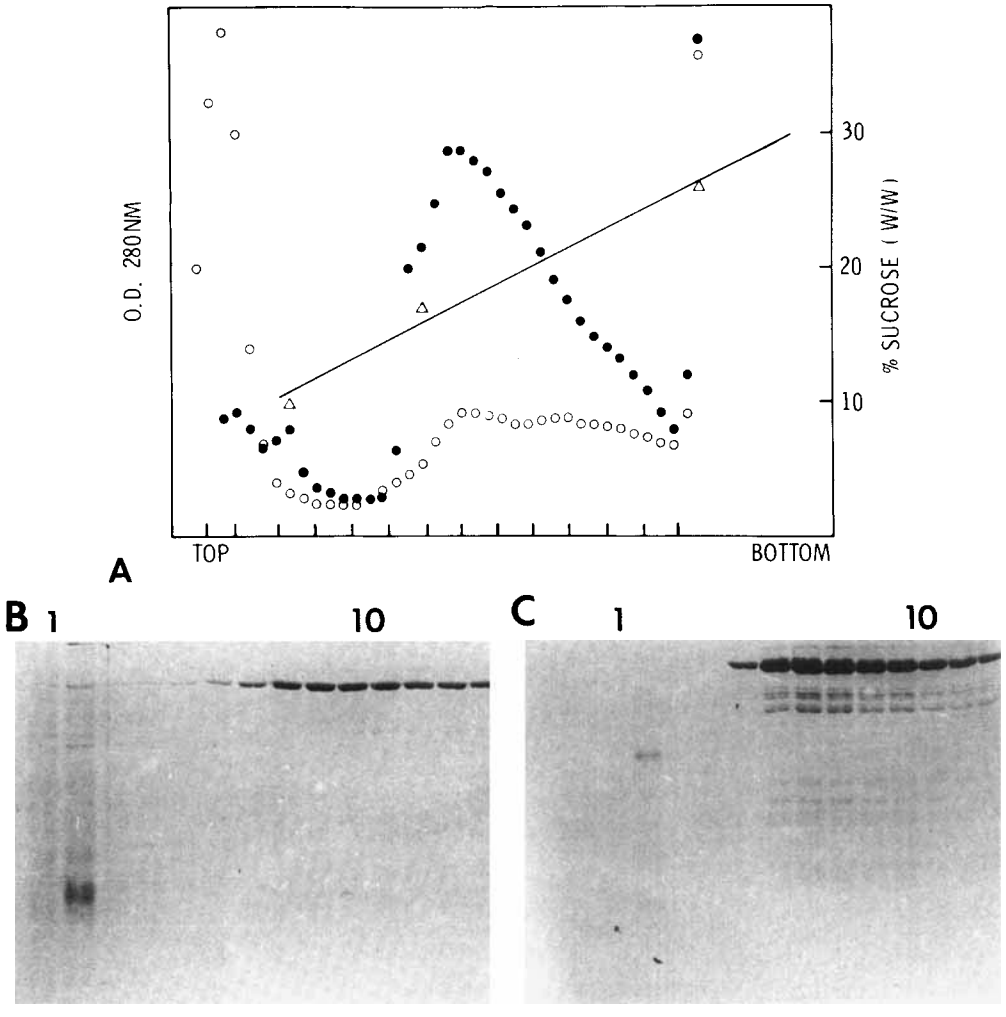

Fig. 2. A: Sedimentation profiles of reassembled coats (○) and untreated coated vesicles preparations (●). Aliquots of reassembled and native preparations were centrifuged in parallel on 10–30% (w/w) linear sucrose density gradients. Coats were observed between 15% and 26% (w/w) sucrose on both gradients. Most of the minor proteins remained at the top of the reassembled coat gradients, whereas they cosedimented with the native coats. The gradients were centrifuged for 2 h at 57,000g in a Beckman SW40 rotor. The hash marks on the abscissa indicate fractions analyzed by SDS-polyacrylmaide gel electrophoresis. B, C: Comparison of the proteins present in reassembled coats (B) and native preparations (C). Fractions of the reassembled and untreated gradients were analyzed by SDS-polyacylamide gel electrophoresis. Reassembled coats (lanes 7–14) appear to have fewer proteins than untreated coated vesicle preparations (lanes 6–14). In particular, the relative amounts of the 125,000-dalton proteins and 55,000-dalton proteins are markedly diminished in reassembled coats. Most of the minor bands (lanes 1 and 2) do not cosediment with reassembled coats.

reassembled coats. Many of the minor proteins present in the 2 M urea supernatant remain at the top of the gradient (lanes 1 and 2, Fig. 2b), whereas the majority of the clathrin is found in reassembled coats. These results suggest that clathrin is the predominant structural element of the coat.

$MgCl_2$ and Tris Effects on Reassembly

In some preliminary experiments using preparations dissociated at pH > 7.5 we observed that the $MgCl_2$ and Tris concentration of the dissociating buffer profoundly affected the reassembly equilibrium. Coated vesicles dissociated in low Tris (10 mM Tris-10 μM $MgCl_2$ – 1 mM EGTA, pH 8) or high Tris (100 mM Tris–10 μM $MgCl_2$ – 1 mM EGTA, pH 8.3) reassembled when dialyzed into isolation buffer (pH 6.5) containing 10 μM $MgCl_2$. However, if 10 mM $MgCl_2$ was present in the dissociating buffer, no dissociation was observed, which suggests to us that $MgCl_2$ stabilizes the coat and might promote reassembly. This possibility was tested by examination of the effect $MgCl_2$ has on reassembly at pH 8 or 8.3 and during dialysis into isolation buffer (pH 6.8).

Effect of $MgCl_2$ on Reassembly in 100 mM Tris (pH 8.3)

Reassembled coats form in preparations dissociated in high Tris at pH 8.3 if the $MgCl_2$ concentration is raised to 10 mM (Table I). The substantial increase in the amount of protein which sediments in the presence of 10 mM $MgCl_2$ (Table I) suggests that these coats sediment like native coats. At concentrations of $MgCl_2$ less than 10 mM, no coats are observed in the electron microscope and the majority of protein does not sediment, indicating that reassembly has not occurred. The material which does sediment at less than 10 mM $MgCl_2$ consists of vesicles and protein aggregates.

Since these results at pH 8.3 support the hypothesis that Mg^{2+} promoted reassembly, we next examined if increasing the $MgCl_2$ concentration shifted the reassembly equilibrium toward the assembled state at pH 6.8. After preparations dissociated in high Tris are dialyzed against isolation buffer (pH 6.8) containing 10 μM to 10 mM $MgCl_2$, reassembled coats, but not filamentous aggregates, are observed in the electron microscope. Interestingly, as the concentration of $MgCl_2$ is increased, more protein sediments, which suggests that more reassembly occurs as the concentration of $MgCl_2$ is raised (Table II).

Effect of $MgCl_2$ on Reassembly of 100 mM Tris (pH 8.3) Supernatants

We previously observed [14] that vesicle and aggregate free supernatants obtained following dissociation of coated vesicles with 2 M urea reassembled when dialyzed into isolation buffer at pH 6.8. This indicated that the 2 M urea supernatants contained all the proteins necessary for reassembly. Since supernatants from coated vesicles dissociated from both high and low Tris contained essentially the same proteins as 2 M urea supernatants as judged by SDS PAGE, we predicted they would also reassemble. However, when the high-Tris supernatants were tested for reassembly at pH 8 and 8.3, or following dialysis into isolation buffer at pH 6.8, no reassembly was observed (Table III). Raising the $MgCl_2$ concentration to 10mM did not result in reassembly as it did in noncentrifuged preparations (see Table II).

Effect of $MgCl_2$ on Reassembly of 10 mM Tris Supernatants

In contrast to the results of the previous experiment, reassembly did take place when the supernatants from coated vesicles dissociated in low Tris, instead of high Tris, were dialyzed against isolation buffer (pH 6.8) (Table IV). Coats reassemble at pH 8 (low Tris), in the presence of 10 mM $MgCl_2$, but these coats do not sediment under conditions which pellet control preparations. However, dialysis of each supernatant into isolation buffer (pH 6.8) containing 10 μM to 10 mM $MgCl_2$ resulted in the formation of coats which did sediment (Table IV). Despite the fact that the coats which reassembled at pH 8 (10 mM $MgCl_2$) did not sediment, they appear morphologically identical to those which did sediment at pH 6.8.

TABLE I. Effect of [$MgCl_2$] on Reassembly in 100 mM Tris (pH 8.3)

[$MgCl_2$]:	10 μM	100 μM	1 mM	10 mM	20 mM
Preparation I					
Coats present	–	–	–	+	+
% Protein sedimentable	26	35	31	75	nd
Preparation 2					
Coats present	–	–	–	+	
% Protein sedimentable	37	38	43	69	

Coated vesicles were dissociated with 100 mM Tris pH 8.3. (The buffer also contained either 100 mM MES–10 μM $MgCl_2$–1 mM EGTA or 10 M $MgCl_2$–1 mM EGTA.) Following dissociation aliquots were adjusted to 10 μM, 100 μM, 1 mM, 10 mM, or 20 mM $MgCl_2$ and then assayed by electron microscopy for the presence of coats and by centrifugation for sedimentable particles.

TABLE II. Effect of [$MgCl_2$] on Reassembly of Preparations Dissociated in 100 mM Tris (pH 8.3) and Then Dialyzed Into Isolation Buffer (pH 6.8)

[$MgCl_2$]:	10 μM	100 μM	1 mM	10 mM
Coats present	+	+	+	+
% Protein sedimentable	62	74	70	80

Coated vesicles were dissociated with 100 mM Tris (pH 8.3) as in Table I. Separate portions of the preparations were adjusted to 10 M, 100 μM, 1 mM, or 10 mM $MgCl_2$, dialyzed into isolation buffer at pH 6.8 containing the same $MgCl_2$ concentration, and then assayed for the presence of coats by electron microscopy and by centrifugation for sedimentable particles.

TABLE III. Effect of [$MgCl_2$] on Reassembly of 100 mM Tris (pH 8.3) Supernatants Dialyzed Into Isolation Buffer (pH 6.8)

[$MgCl_2$]:	10 μM	100 μM	1 mM
Coats present	–	–	–

Coated vesicles were dissociated by dialysis into 100 mM Tris (pH 8.3) containing 10 μM, 100 μM, or 1 mM $MgCl_2$. The dissociated preparations were centrifuged to remove vesicles and aggregates (Beckman Airfuge for 20 min, 107,000 g), then dialyzed into isolation buffer (pH 6.8) containing the same $MgCl_2$ concentrations, and assayed for the presence of coats by electron microscopy.

TABLE IV. Effect of [$MgCl_2$] on Reassembly of 10 mM Tris (pH 8) Supernatants

[$MgCl_2$]:	10 μM	100 μM	1 mM	10 mM
Coats present (pH 8)	–	–	–	+[a]
% Protein sedimentable	8	8	13	18
Coats present (pH 6.8)	+	+	+	+

A coated vesicle preparation was dissociated by dialysis into 10 mM Tris–10μM $MgCl_2$–1 mM EGTA (pH 8) and then centrifuged for 20 min at 107,000g in a Beckman Airfuge to remove vesicles and aggregates. Separate portions of the supernatant were brought to 10 μM, 100 μM, 1 mM or 10 mM $MgCl_2$ and assayed by negative staining for the presence of coats and by centrifugation for sedimentable particles. Then the aliquots were dialyzed into isolation buffer (pH 6.8) at the same $MgCl_2$ concentrations and assayed for the presence of coats by electron microscopy.

[a]Normal-looking coats were present but were nonsedimentable.

Effect of EGTA on Reassembly

If $MgCl_2$ promotes coat reassembly, it should be possible to inhibit its effect through the use of chelating agents. This possibility was tested by dialyzing preparations dissociated in 100 mM Tris–10 μM $MgCl_2$ (pH 8.3) into isolation buffer at pH 6.8 containing 100 μM to 10 mM EGTA (Table V). Although reassembly was observed at all concentrations of EGTA tested, less protein sedimented with increasing EGTA concentration. These results suggest that EGTA inhibited coat assembly and are consistent with our hypothesis that $MgCl_2$ promotes reassembly.

The divalent cation induction of coat reassembly appears to be specific for $MgCl_2$. For, when 10 mM $CaCl_2$, $MnCl_2$, $ZnCl_2$, or $FeCl_2$ was added to preparations dissociated in 10 mM or 100 mM Tris at pH 8, large aggregates of protein formed. Such aggregates were often in the form of filamentous bundles. Lower concentrations of these ions were not tested.

Effect of [Tris] on Reassembly

The ability of the 10 mM but not the 100 mM Tris supernatants to reassemble suggested that the physical properties of one or more proteins necessary for assembly may have been altered. To determine whether this inhibition was due to Tris or to increased ionic strength, a preparation was dissociated with low Tris and centrifuged, and portions of the supernatant were adjusted to low Tris, high Tris, or high NaCl (Table VI).

The presence of 1 mM $MgCl_2$ did not promote reassembly in any of the fractions at pH 8.0 (Table VI), and little protein sedimented upon centrifugation of the treated supernatants (Table VI). However, upon dialysis to pH 6.8 in isolation buffer, coats reassembled from both low Tris and 10 mM Tris–100 mM NaCl supernatants, but no coats were observed in the high-Tris supernatant (Table VI). This suggests that the effects on reassembly we observed were due to the Tris molecule rather than higher ionic strength.

Fractionation of Coated Vesicle Proteins

Our experiments with 2 M urea and low-Tris supernatants show that the proteins necessary for reassembly can be released in soluble form. However, these supernatants may well contain more proteins than those needed for reassembly. In order to determine which proteins are required for reassembly or are part of the coat lattice, we attempted to fractionate the 2M urea and low-Tris supernatants by chromatography on Agarose A-5M, but found that the columns did not separate the proteins (data not shown). This suggested to us that neither 2M urea nor low Tris completely disrupts the protein-protein interactions of the lattice. However, we were able to separate some of the coated vesicle proteins by chromatography on Sepharose CL-4B columns equilibrated with 0.5 M Tris-isolation buffer (pH 7) using the method of Keen et al [17].

Both we and Keen et al [17] find that unfractionated 0.5 M Tris supernatants reassemble when dialyzed into isolation buffer. However, unlike Keen et al [17] we found that such reassembled coats did sediment like control coats, whereas they observed that the coats did not sediment on sucrose gradients. The source of this difference is unknown to us.

Chromatography on Sepharose CL4B columns separates the proteins into two major peaks (Fig. 3a). Both we and Keen et al [17] find that the faster-eluting peak (peak I) contains clathrin and that peak II contains several proteins including the 95,000-

TABLE V. Effect of [EGTA] on Reassembly of Preparations Dissociated in 100 mM Tris (pH 8.3) and Then Dialyzed Into Isolation Buffer (pH 6.8)

[EGTA]:	100 μM	1 mM	10 mM
Coats present	+	+	+
% Protein sedimentable	72	68	46

Coated vesicles were dissociated in 100 mM Tris (pH 8.3) and then separate aliquots were dialyzed into isolation buffer containing 100 μM, 1 mM, or 10 mM EGTA at pH 6.8. Following dialysis the solutions were assayed for the presence of coats by electron microscopy and centrifugation for sedimentable particles.

TABLE VI. Effect of [Tris] on Reassembly

	10 mM Tris	100 mM Tris	10 mM Tris–100 mM NaCl
Coats present (pH 8)	–	–	–
% Protein sedimentable	13	16	nd
Coats present following dialysis to pH 6.8	+	–	+

A coated vesicle preparation was dissociated by dialysis into 10 mM Tris–10 μM $MgCl_2$–1 mM EGTA (pH 8) and then centrifuged 20 min at 107,000g to remove vesicles and aggregates. Separate portions of the supernatant were adjusted to 10 mM Tris–1 mM $MgCl_2$, 100 mM Tris–1 mM $MgCl_2$, or 10 mM Tris–100 mM NaCl–1 mM $MgCl_2$. These aliquots were assayed for the presence of coats by electron microscopy and for sedimentable particles by centrifugation in a Beckman Airfuge (20 min at 107,000g). The resultant supernatants were dialyzed into isolation buffer and then assayed for the presence of coats.

and 125,000-dalton proteins. However, unlike Keen et al [17] we find that peak I also contains two proteins with molecular weights near 30,000 daltons (Fig. 3b) in addition to clathrin.

We find, as did Keen et al [17], that neither peak I nor peak II alone reassembled when dialyzed into isolation buffer at pH 6.5, but that an equal mixture of peaks I and II did reassemble when dialyzed into isolation buffer (pH 6.5). However, the coats formed do not sediment. Peak I alone did reassemble when dialyzed against 10 mM MES–1 mM EDTA–1 mM $CaCl_2$ (pH 6.5), but it too did not sediment. Thus it appears that the ability of the proteins of peak I to reassemble is influenced by ionic strength and accessory proteins. That these coats did not sediment suggests that other factors necessary for coat stabilization were absent.

DISCUSSION

Pure preparations of coated vesicles contain a plethora of proteins which, after being solubilized by a variety of treatments, can reassemble into intact coats. This ability to reassemble has provided us with a major tool for examining which proteins are involved in coat structure, which proteins are important in regulating reassembly, and what ionic conditions are necessary for reassembly.

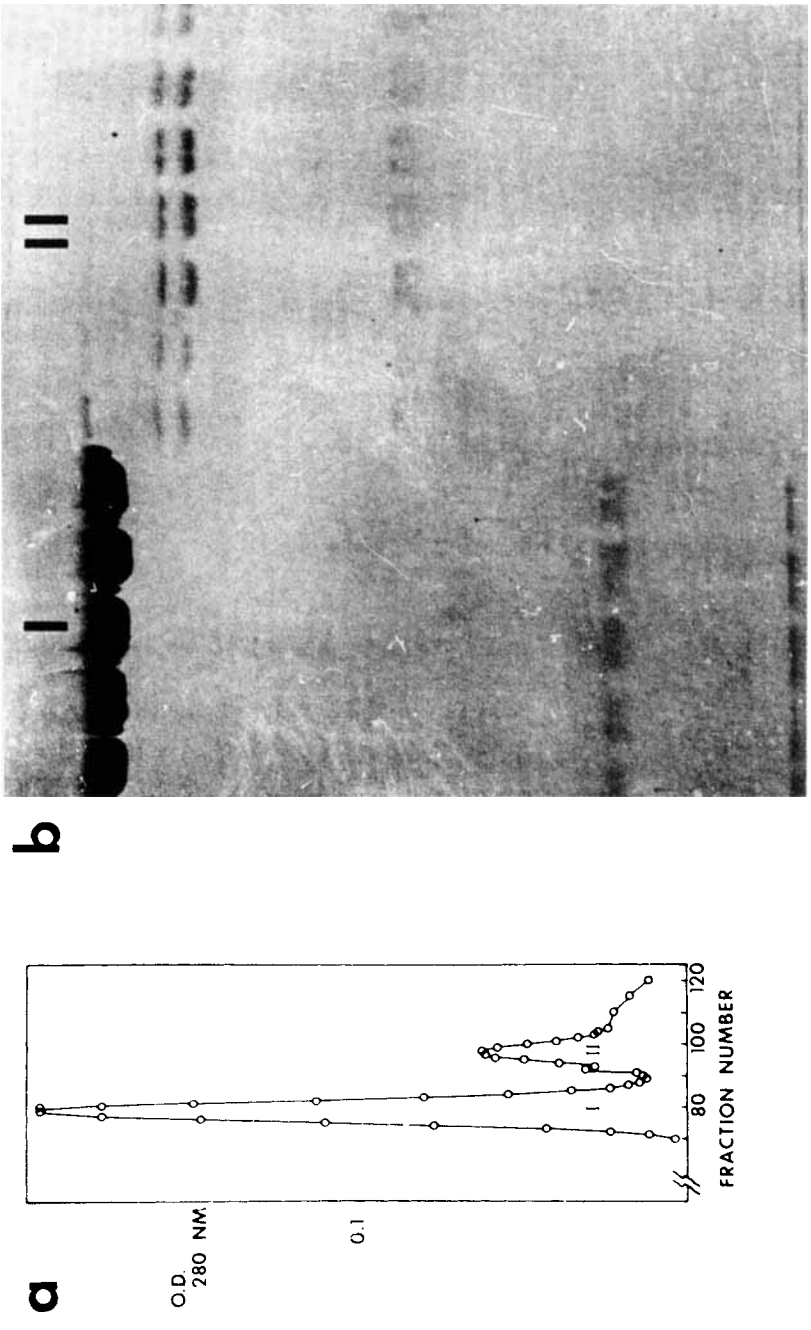

Fig. 3. Separation of coated vesicle proteins by chromatography on Sepharose CL4B. a: Elution profile of 0.5 M Tris-isolation buffer (pH 7.0) supernatants on Sepharose CL4B. Coated vesicle proteins solubilized by treatment with 0.5 M Tris-isolation buffer (pH 7.0) were chromatographed on a Sepharose CL4B column equilibrated with 0.5 M Tris-isolation buffer; b: SDS-polyacrylamide gel patterns of peaks I and II. Aliquots of peaks I and II were analyzed by SDS-polyacrylamide gel electrophoresis. Peak I consists primarily of clathrin. Peak II contains the 125,000-dalton proteins and the 55,000-dalton protein.

Proteins Involved in the Coat Lattice

We find that treatment of coated vesicles with 2 M urea, low Tris (10 mM, pH 8), and 0.5 M Tris-isolation buffer (pH 7) yields soluble protein preparations which reassemble into sedimentable coats when dialyzed against isolation buffer. These coats appear to have a morphology and size distribution similar to untreated preparations when examined in the electron microscope. Similar observations on the morphology of reassembled coats have been made by other investigators [15–17].

Reassembled coats, untreated coats, and coated vesicles have similar sedimentation properties on linear sucrose density gradients; however, their protein compositions are not identical. Coats reassembled from 2 M urea supernatants appear to consist predominantly of clathrin; however, other proteins are present. This observation tends to support the contention of others [11, 12, 15–17] that clathrin is the sole lattice protein. However, our observation that clathrin and the two 30,000-dalton proteins of peak I from Sepharose CL4B can reassemble into coats with normal morphology does not support the hypothesis that clathrin is the sole protein comprising the rods of the coat lattice. That the coats reassembled from peak I did not sediment was unfortunate, since it prevented our determining whether the two 30,000-dalton proteins cosedimented with reassembled coats. However, the two 30,000-dalton proteins are present in the reassembled coats following gradient purification of coats reassembled from 2 M urea supernatants. These observations strongly suggest that the two 30,000-dalton proteins are involved in the coat lattice. More detailed tests of this hypothesis are being presented in a separate communication [19].

$MgCl_2$ Effects on Reassembly

It is becoming increasingly clear from our work and that of others [16, 17] that the factors involved in promoting coat assembly are quite complex. Our results indicate that Mg^{2+} may be important in modulating the dissociation-reassembly equilibrium, since preparations dissociated in high or low Tris (pH 8) spontaneously reassemble at pH 8 in the presence of 10 mM $MgCl_2$, whereas lower concentrations of $MgCl_2$ are ineffective. The transition from no coats to coats occurs abruptly, which suggests that the process is cooperative and virtually all or none if sufficient Mg^{2+} is present. The apparent absence of partial coats is also consistent with this suggestion. When preparations dissociated with Tris (pH 8) are dialyzed into isolation buffer containing increasing concentrations of $MgCl_2$, more clathrin appears to reassemble into coats. This suggests that Mg^{2+} shifts the equilibrium toward reassembly and that it may stabilize the coat lattice. Such a role for Mg^{2+} is supported by our observation that increased chelation of Mg^{2+} increases the amount of nonsedimentable protein under reassembly conditions, and the observation of Schook et al [16] that chelation of Mg^{2+} with 6 mM EDTA or 5–10 mM adenosine triphosphate (ATP) interferes with reassembly.

The cation requirement appears to be specific for Mg^{2+}, since in our hands replacement of Mg^{2+} by 10 mM $CaCl_2$, $FeCl_2$, $ZnCl_2$, or $MnCl_2$ promotes the formation of filaments rather than coats. This is contrary to the report by Schook et al [16] that coats reassemble when partially purified clathrin preparations are dialyzed against isolation buffer (pH 6.8) containing 50–100 mM $CaCl_2$; however, the lower concentrations we used were not tested. Keen et al [17] observed that partially purified clathrin requires Ca^{2+} to reassemble but that this requirement is abolished if a hypothesized reassembly promotor protein(s) is present. Such apparent abolishment of the Ca^{2+} require-

ment suggests that the reassembly reaction is influenced by the presence of accessory proteins. Thus the varied effects of Ca^{2+} and Mg^{2+} observed by several workers may be due to differences in the protein composition of their preparations.

Tris Effects on Reassembly

The procedures used to prepare soluble coated vesicle proteins appear to affect the ability of such proteins to reassemble. Supernatants from preparations dissociated with low Tris (0.01 M, pH 8), 2 M urea, 0.25 M $MgCl_2$ [14], or 0.5 M Tris-isolation buffer (pH 7.0) reassemble when dialyzed against isolation buffer. In contrast, high-Tris (0.1 M, pH 8) supernatants do not reassemble when dialyzed against isolation buffer under the same conditions. That this effect is due to Tris and not to ionic strength is supported by our observation that replacement of Tris with NaCl results in supernatants from which coats will reassemble when dialyzed into isolation buffer. Whether Tris alters the properties of the soluble proteins sufficiently to either promote the sedimentation of an accessory protein necessary for reassembly or perturb protein-protein interactions necessary for reassembly is as yet unclear.

Reassembly Intermediates

In our studies of reassembly we have observed complete coats, but neither partial coats nor isolated hexagonal or pentagonal arrays were observed. This suggested that the reassembly reaction was essentially all or none; however, there is evidence which suggests that intermediate states do exist. When 10 mM $MgCl_2$ is present in low-Tris (10 mM, pH 8) supernatants, morphologically normal but nonsedimentable coats are observed. Following dialysis into isolation buffer (pH 6.8), these coats become sedimentable. Another instance in which nonsedimentable coats are observed occurs following dialysis of either partially purified clathrin (peak I from Sepharose CL4B columns) into low-ionic-strength buffer or peaks I and II into isolation buffer. Our hypothesis of reassembly intermediates is also supported by the work of Keen et al [17], who found that under certain ionic conditions coats reassemble but do not sediment. The existence of these intermediate states, which have normal morphology but are nonsedimentable, provides us with a tool for exploring how such factors as pH, divalent cations, ionic strength, and accessory proteins mediate and stabilize coat assembly. By determining how such factors effect the formation of intermediates in vitro, we hope to gain new insights as to how the reaction proceeds inside the cell.

Coat Structure

Kanaseki and Kadota [6] and Crowther et al [8] propose that the coat lattice consists of rods assembled into a spherical network of interlocking hexagons and pentagons. Crowther et al [8] postulated that the rods are comprised by clathrin dimers. However, no evidence was presented to show substructure in the rods. Our observations of Markham-rotated high-magnification images of both native and reassembled coats show a substructure in the rods consistent with a lattice edge made up of tightly intertwined molecules (Fig. 1).

Since the rods comprising the lattice are approximately 65Å in diameter and 165Å long [14], there is sufficient volume for a maximum of 4.5×10^5 daltons of protein based upon a partial specific volume of 0.735 cm^3/g. Thus there is enough volume for a clathrin dimer but not a trimer in each rod. Therefore, we propose that the rods are composed of intertwined antiparallel dimers of clathrin (Fig. 4). In this model, unlike that of

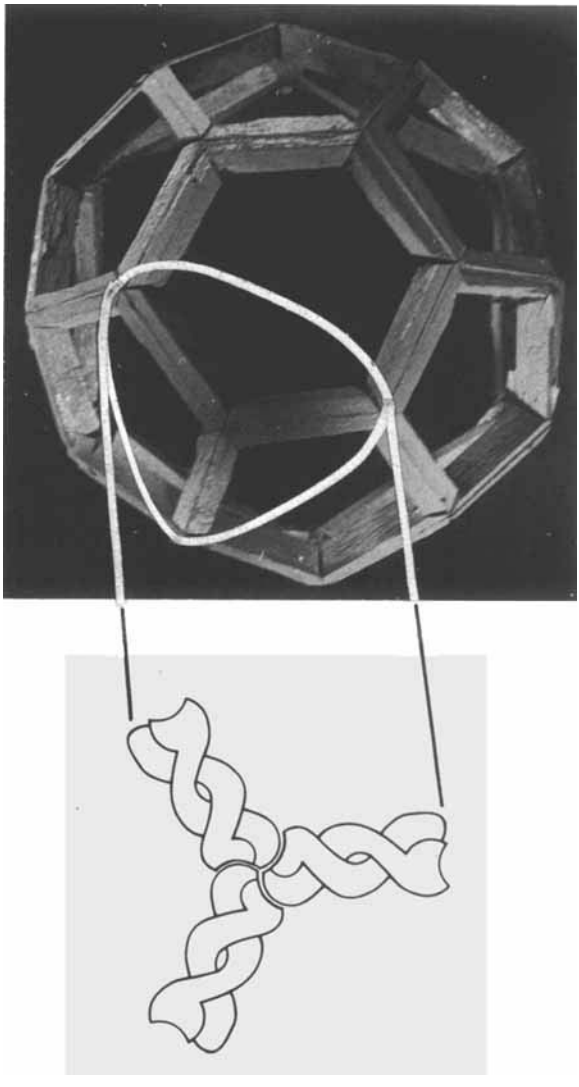

Fig. 4. Proposed structure for clathrin dimers and the coat vertices. We envision the lattice edge as consisting of an intertwined antiparallel clathrin dimer. Thus at each vertex six clathrin molecules interact. The head of one dimer interacts with the tail of an adjacent dimer. The proposed vertex structure is shown in relation to the entire coat lattice. The lattice structure shown is consistent with the six-fold symmetrical images we observe and is similar to that proposed by Crowther et al [8].

Crowther et al [8], a lattice vertex would involve three clathrin dimers in which the head of one dimer would interact with the tail of an adjacent dimer. In this organization only one class of clathrin-clathrin recognition sites is needed.

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