Release of Clathrin from Coated Vesicles Dependent upon a Nucleoside Triphosphate and a Cytosol Fraction

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ABSTRACT Calf-brain coated vesicles were incubated with ATP and a cytosol fraction. As much as 90% of the clathrin was selectively released within 10 min at 37°C without detectable proteolysis. This uncoating process required the presence of both ATP and cytosol. Empty cages of clathrin could also be dissociated in a similar manner. A nonhydrolyzable analogue, 5'-adenylylimidodiphosphate (AMP-PNP), would not substitute for ATP. Clathrin was dissociated from coats in a form unable to reassemble into cages under standard conditions. These reactions may reflect a segment of a clathrin-coated vesicle cycle in which coats are removed from vesicles after budding.

Coated vesicles shuttle macromolecules between organelles, executing crucial steps in the sorting of proteins (1, 4, 5, 12–15). Clathrin (180,000 mol wt) and its tightly bound light chains (33,000 and 36,000 mol wt) are the major polypeptides of the cagelike polygonal coat. Under a variety of nonphysiological conditions (2, 7, 18, 21), coats dissociate into complexes of three clathrins and three light chains, termed triskelions because of their pinwheel configuration (8, 19). These trimers will spontaneously reform cages upon a return to more physiological conditions. All of these findings suggest that coats should be stable in the cytoplasm.

Despite their inherent stability, coats are lost or removed from coated vesicles within only a few minutes after budding, and probably before a demonstrable fusion event (1, 6, 20). Because clathrin spontaneously forms cages and membrane coats, the reverse process of uncoating that takes place in vivo would probably need to be energy-dependent and therefore enzyme catalyzed. Here we report that coated vesicles are rapidly and efficiently uncoated in vitro, provided both ATP and a high-speed supernatant fraction are supplied.

MATERIALS AND METHODS

Purification of Coated Vesicles and Clathrin Cages

Newborn calf brain was stored at −70°C. Coated vesicles were purified through three successive sucrose gradients according to the Pearse (11) procedure, except that the intervening pelleting step between the first and second gradients was omitted. Thus, the diffuse turbid zone in the middle of the first gradient was layered directly over a second continuous 40–60% (wt/vol) sucrose gradient (18 ml) and centrifuged in an SW27 rotor (Beckman Instruments, Inc., Fullerton, CA) at 26,000 rpm for 16–18 h. The final pellet of coated vesicles (harvested from the third gradient) was resuspended by homogenization in 1–2 ml of buffer A (0.1 M 2[*N*-Morpholino]ethane sulfonic acid [MES]-KOH [pH 6.5], 1 mM EGTA, 1 mM $MgCl_2$), centrifuged for 10 s in an Eppendorf 5412 centrifuge (Brinkmann Instruments, Inc., Westbury, NY) to remove any large aggregates, frozen in liquid nitrogen, and stored at −70°C at a protein concentration of 5–10 mg/ml.

For some experiments, coated vesicles (obtained from the third sucrose gradient) were further purified by electrophoresis in 0.15% agarose gels (16). 1–5 mg were applied in a continuous sample well of 10-cm length. Electrophoresis was performed in 50 mM MES pH 6.5 at 15 V for ~16 h. The coated vesicle band was located by its turbidity. To elute the coated vesicles, the band was excised and the agarose disrupted either with a Dounce homogenizer or by extrusion through a 16-gauge needle. Agarose was then removed by sedimentation in an Eppendorf 5412 centrifuge (Brinkmann Instruments) for 1 min. The coated vesicles in the supernatant were then pelleted (in an SW 50.1 rotor [Beckman Instruments] at 35,000 rpm for 60 min), resuspended in buffer A at a protein concentration of 1 to 2 mg/ml, frozen in liquid N_2, and stored at −70°C. The yield was ~10%.

Clathrin was purified from coated vesicles (obtained after the second sucrose gradient) according to Kirchhausen and Harrison (8), with slight modifications (17). In this way, ~10 mg of clathrin were purified from 1.5 kg of brain. As reported (8), the preparation consisted almost exclusively of clathrin and light chains. This "purified clathrin" was reassembled into empty cages by dialysis into 20 mM MES pH 6.2, 1 mM EDTA, 2 mM $CaCl_2$ for ~16 h at 4°C as described (8).

Preparation of Calf-Brain Cytosol

25 g of calf brain (stored frozen at −70°) was crushed into small pieces and mixed with 100 ml of 39 mM HEPES-KOH (pH 7.3), 74 mM KCl, 4.5 mM magnesium acetate, 0.8 mM dithiothreitol (DTT) (buffer B) at 4°C. After homogenization with 10 strokes of a motor-driven Teflon-glass homogenizer and centrifugation in a JA-14 rotor (Beckman Instruments) at 11,000 rpm for 15 min, the resulting supernatant was further centrifuged in a JA-17 rotor (Beckman Instruments) at 17,000 rpm for 25 min. Finally, this supernatant was centrifuged in a Beckman Instruments Type 30 rotor at 30,000 rpm for 90 min to remove any residual membranes. The supernatant of the final centrifugation, referred to as the cytosol fraction, was frozen in liquid N_2 and stored at −70°C until use. The protein concentration was ~4 mg/ml.

Incubations

Typically, ~10 μg of coated vesicles or cages (in ~15 μl buffer A) were incubated with 135 μl of cytosol and 40 μl of an ATP-regenerating system for 10

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min at 37°C. The ATP-regenerating system contained 7 mM Mg ATP, 35 mM sodium creatine phosphate, 25 U/ml of rabbit muscle creatine phosphokinase (Calbiochem-Behring Corp., San Diego, CA) dissolved in 20 mM HEPES-KOH (pH 7.3), 37 mM KCl, 2.2 mM Mg acetate, 0.4 mM DTT. After incubation, all samples were diluted to 0.2 ml with buffer B and centrifuged in a Beckman Instruments Airfuge (A 30/100 rotor) at 95,000 g_{av} for 10 min. The pellets were resuspended by pipetting in buffer B (10–40 μl) for analysis by electron microscopy, PAGE, or agarose gel electrophoresis. The exact conditions vary from experiment to experiment and are given in the appropriate figure legend.

SDS PAGE

10 percent SDS polyacrylamide slab gels (10 or 3.5 cm in length) were prepared, and samples were boiled in sample buffer and electrophoresed according to Laemmli (9). In some cases, for a more rapid analysis, a "minigel" procedure (8) was used. The gels were stained with Coomassie R-250 and were scanned with a densitometer. For quantitation, the amount of clathrin was determined from the area under the peak.

Electron Microscopy

Samples to be viewed under negative stain were adsorbed to carbon- and Formvar-coated copper grids (400 mesh) for 1 min at room temperature. After removing excess sample, the grids were then stained by adding several successive drops of 1% uranyl acetate over a period of 1 min. Protein concentration was determined as described by Bradford (3) using bovine serum albumin as a standard.

RESULTS

Conditions for ATP-dependent Dissociation of Coats

Coated vesicles were incubated alone or in combinations with ATP (and an ATP-regenerating system) and a cytosol (high-speed supernatant) fraction of calf brain at 37°C for 10 min. The mixtures were then centrifuged to pellet out the vesicles. Release of clathrin was followed by the appearance of this polypeptide chain in the supernatant, and its disappearance from the pellet (Fig. 1), and quantitated from densitometer tracings of Coomassie-stained SDS polyacrylamide gels. When incubation was carried out in the presence of cytosol, ATP, and an ATP-regenerating system (referred to as a complete incubation), 80–90% of the clathrin was released into the supernatant within 10 min. Clathrin was selectively released, as revealed by an examination of the polypeptide species of coated vesicles that remain in the pellet (Fig. 1). When either the cytosol fraction or ATP (and its regenerating system) were omitted (Fig. 1), only a small, background level of clathrin was released to the supernatant (see below). The ATP-regenerating system was included because the coated-vesicle preparation (after the third gradient) is contaminated with membranes having ATPase activity (16) but is unnecessary when either agarose-gel-purified coated vesicles or cages reconstituted from purified clathrin were used as substrates (see below). The extent of release depended upon the amount of cytosol protein added (Table I, Experiment *V*).

Release of clathrin does not appear to be accompanied by significant proteolysis. The molecular weight of clathrin is not detectably altered, and the sum of the clathrin recovered from the supernatant and the pellet is 99 ± 9% (SEM) of that added (six independent determinations). Also, none of the other coated-vesicle proteins or the polypeptide species contributed from the cytosol are noticeably altered in size or intensity.

Because clathrin coats are known to dissociate spontaneously on exposure to pH levels 7.5 and above (7, 8, 19), the pH dependence of spontaneous and ATP-dependent clathrin release was investigated (Fig. 2). No more than 15% of the clathrin was released during incubations with cytosol in the absence of ATP, even at pH 7.5. Release in the presence of ATP increased progressively between pH 6.5 and 7.5. The ATP-dependent release (the difference between the two curves shown in Fig. 2) was maximal between pH 7.0 and 7.3.

The source of the ATP-dependent, released clathrin could be either coated vesicles or empty cages present in the coated-vesicle preparation. To ascertain whether the release of clathrin resulted from the uncoating of vesicles, the pellets of incubations were analyzed by agarose gel electrophoresis and electron microscopy. The agarose gel will separate coated vesicles from released smooth vesicles (16). Membrane material in the gel was selectively visualized by staining with ANS, a hydrophobic fluorescent probe.

When coated vesicles (previously purified using a preparative agarose gel, and containing only 3% smooth vesicles) were incubated with cytosol but without ATP, an ANS-staining band migrating in the position of coated vesicles was observed (Fig. 3). But in the presence of both cytosol and ATP, the majority of membrane material now electrophoresed more slowly than intact coated vesicles (Fig. 3), in the position of vesicles depleted of clathrin by treatment with 0.5 M Tris, pH 7 (data not shown). This showed that the bulk of the membrane was affected by the incubation in an ATP-dependent manner.

Material eluted from these two bands, representing the major membrane products of the incubations, was viewed in the electron microscope after negative staining. The products of incubation with cytosol in the absence of ATP consisted entirely of coated vesicles and cages, indistinguishable from the starting material (Fig. 4*A*). In contrast, the products of incubation with cytosol and ATP (Fig. 4*B*) were mainly vesicles lacking a latticelike coat, similar in size to the vesicles present

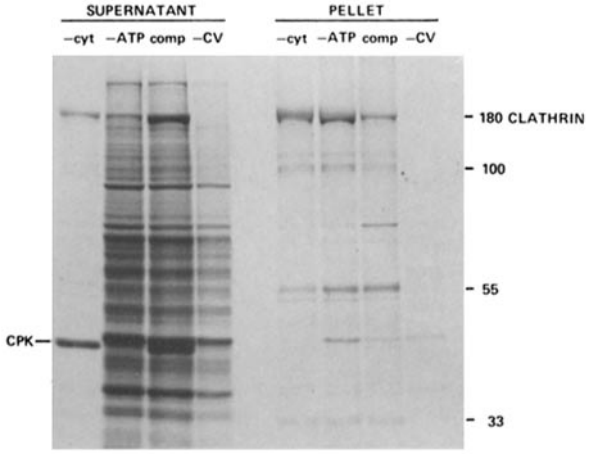


FIGURE 1 Release of clathrin into the supernatant upon incubation of coated vesicles with ATP and cytosol. Supernatant and pellet fractions were analyzed by SDS gel electrophoresis. *comp;* complete incubation. 80 μg of coated vesicles (in 15 μl of buffer A) were incubated with 135 μl of brain cytosol and 40 μl of the ATP-regenerating system at 37°C for 10 min. *-cyt;* cytosol was replaced by an equal volume of buffer B. *-ATP;* ATP and ATP-regenerating system were replaced by an equal volume of buffer B diluted twofold with H_2O. *-CV;* coated vesicles were replaced by buffer A and the ATP-regenerating system was replaced as described above. After incubation, the samples were centrifuged in a Beckman Instruments Airfuge at 95,000 g_{av} for 10 min. The pellets were resuspended in 50 μl of buffer B. 10% of the supernatant and pellet fractions were electrophoresed for all except the -CV incubation (in which 5% was electrophoresed). Molecular weight markers indicate the positions of known coated-vesicle proteins. *cpk;* creatine phosphokinase added with the ATP-regenerating system.

within cages (Fig. 4*A*). This indicates that the vesicles released during the ATP-dependent uncoating had not fused with each other extensively. The sample incubated with ATP and cytosol also contained vesicles with partial coats (Fig. 4*B*, large arrows). These might be intermediates in the uncoating process.

TABLE I

Requirements for Uncoating In Vitro

Experiment	Incubation conditions	Fraction of clathrin remaining in pellet*
I	No ATP	[1]
	2 μM ATP	0.75
	20 μM ATP	0.14
	2,000 μM ATP	0.09
	2,000 μM AMP	1.04
	2,000 μM ADP	0.19
	2,000 μM ADP, with hexokinase and glucose	1.08
II	Empty cages, no ATP	[1]
	Empty cages, 20 μM ATP	0.21
III	No ATP	[1]
	20 μM ATP	0.32
	20 μM AMP-PNP	1.04
IV	Untreated cytosol, no ATP	[1]
	Cytosol, precipitated with 70% saturated $(NH_4)_2SO_4$	0.13
	Cytosol, dialyzed against buffer B	0.15
	Cytosol, boiled	1.04
	Untreated cytosol plus EGTA (1.5 mM)	0.26
	Untreated cytosol plus $CaCl_2$ (3 mM)	0.22
	Untreated cytosol plus EDTA (17 mM)	0.75
V	No cytosol added	[1]
	20 μg cytosol protein	0.64
	40 μg cytosol protein	0.55
	80 μg cytosol protein	0.38
	160 μg cytosol protein	0.27
	280 μg cytosol protein	0.15

Exp. I: Incubations consisted of 5 μg of coated vesicles (purified by agarose gel electrophoresis after the third sucrose gradient) in 5 μl of buffer A, 50 μl of cytosol (200 μg protein), 2 mM $MgCl_2$ (in addition to the Mg^{++} in the cytosol) and the sodium salt of the nucleotide indicated at the stated concentration in a final volume of 60 μl. When included, yeast hexokinase (Boehringer Mannheim Biochemicals, Indianapolis, IN) and glucose were added at final concentrations of 60 U/ml and 5.5 mM, respectively. *Exp. II:* Empty cages were reconstituted from purified clathrin (see Materials and Methods). Incubation contained 10 μg of cages, 25 μl (100 μg) of cytosol, and 20 μM Na-ATP in a final volume of 45 μl of buffer B. *Exp. III:* Incubations consisted of 5 μg of coated vesicles (purified by agarose gel electrophoresis after the third sucrose gradient) in 10 μl of Buffer A, 70 μl (280 μg) of cytosol, 2 mM $MgCl_2$ (in addition to the Mg^{++} in the cytosol) and the sodium salt of the nucleotide indicated at the stated concentration in a final volume of 100 μl. *Exp. IV:* All incubations contained 20 μg of coated vesicles (purified through three sucrose gradients) in 2 μl of buffer A, 15 mM Mg-ATP, and 35 μl (140 μg) of a cytosol fraction (treated as described) in a final volume of 60 μl. The concentrations of EGTA, $CaCl_2$, and EDTA are the final concentrations in the incubation. The ammonium sulfate precipitate of cytosol was redissolved and dialyzed against buffer B before assay. Cytosol was boiled for 3 min and the precipitated protein was removed by centrifugation before assay. *Exp. V:* Incubations contained 30 μg of coated vesicles (purified through three sucrose gradients) in 10 μl of buffer B, 20 μl of the ATP-regenerating system, 2 mM $MgCl_2$ (in addition to the Mg^{++} in the cytosol), the indicated amount of cytosol (dialyzed against buffer B, protein concentration 4 mg/ml) and enough buffer B to make a final assay volume of 100 μl.

* Relative to a control incubation to which either ATP or cytosol was omitted, as indicated.

Similar results were obtained when samples of the whole incubation mixtures (rather than the eluted agarose gel bands) were analyzed by electron microscopy.

Several adenine nucleotides were tested for their effects upon

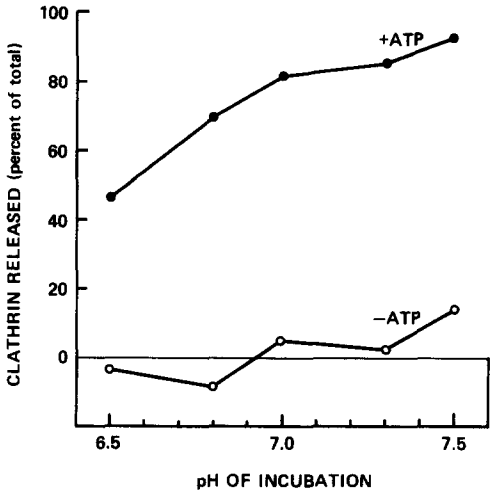


FIGURE 2 pH dependence of spontaneous and ATP-dependent release of clathrin. Incubations contained 25 μg of coated vesicles (in 10 μl of buffer A), 70 μl of cytosol (4 mg/ml) that had been dialyzed extensively against buffer B titrated to the indicated pH, and 20 μl of the ATP-regenerating system. After 10 min at 37°C, samples were diluted with 100 μl of buffer B at the indicated pH and centrifuged. The pellets were resuspended in 25 μl of buffer B and subjected to SDS gel electrophoresis. Densitometer tracings of the Coomassie-stained gels allowed comparison of the amount of clathrin in each pellet with the amount added to the incubation (electrophoresed in parallel). The percent of clathrin released into the supernatant was calculated from the values for the pellet as 100(1 − [clathrin remaining in pellet/total clathrin added]). Each data point represents the average of two independent experiments.

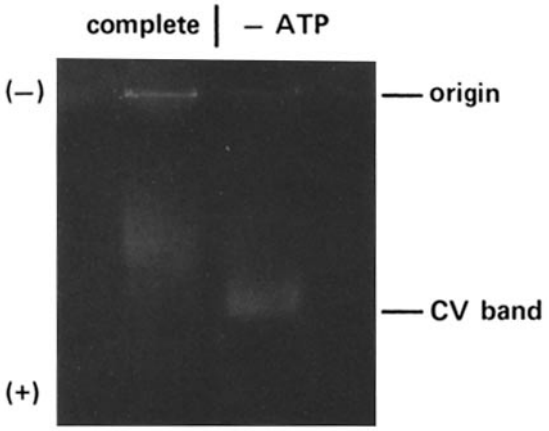


FIGURE 3 Analysis by agarose gel electrophoresis of sedimentable products of incubations. Coated vesicles (45 μg, previously purified by agarose gel electrophoresis, in 22.5 μl of buffer A) were incubated at 37°C for 10 min with 150 μl of cytosol in the presence (*complete*) or absence (−*ATP*) of 2 mM Na-ATP in a final volume of 180 μl. The ATP-regenerating system was not used. After centrifugation (95,000 × g_{av} for 10 min), the pellets were resuspended in 40 μl of buffer B. The suspensions were applied to a 0.15% agarose gel and then electrophoresed towards the anode, and subsequently stained with ANS as described (16). The gel was photographed with ultraviolet backlighting. The bands stained by ANS were also apparent as turbid regions. The origin and the position of authentic coated vesicles (run in parallel on the same gel) are indicated.

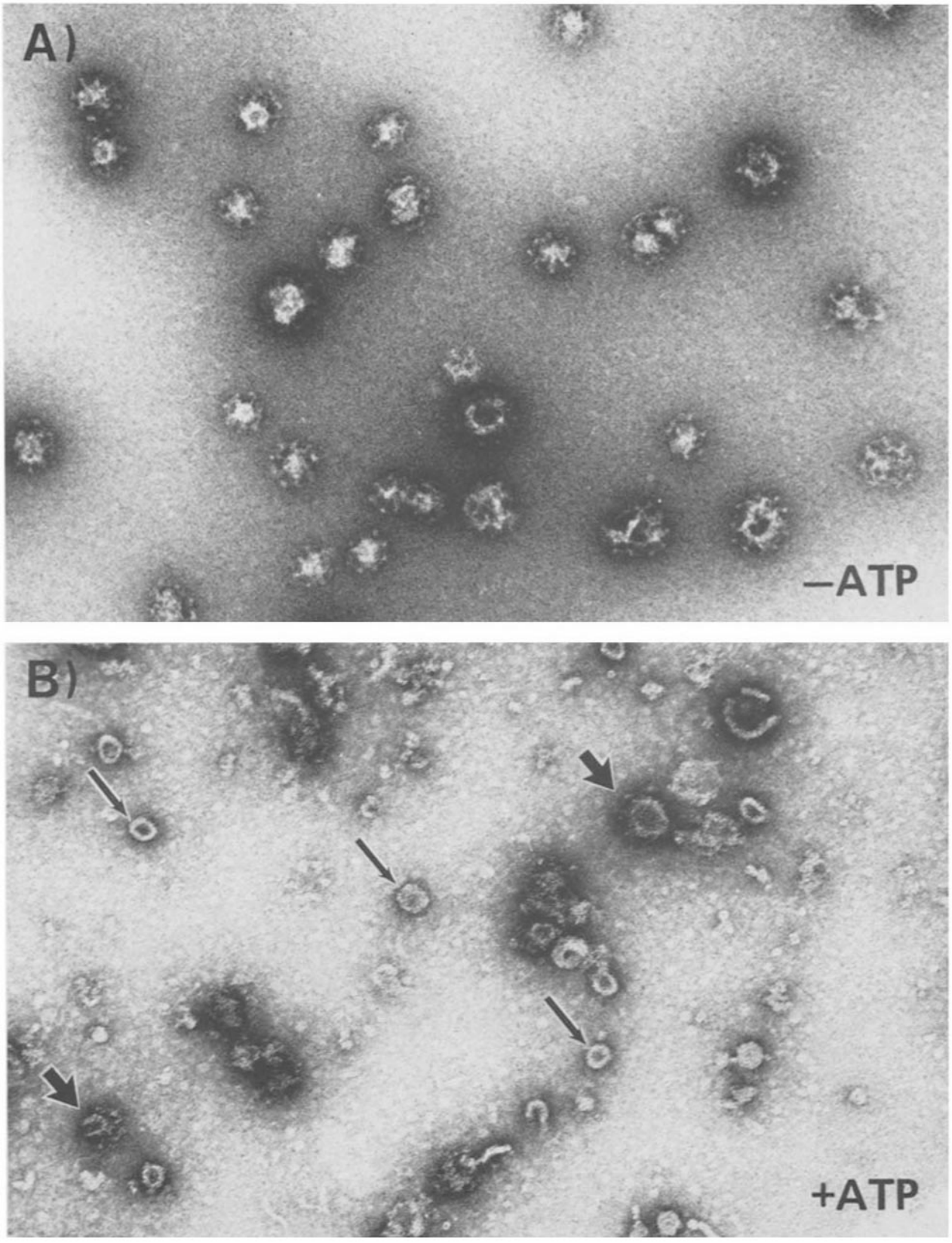


FIGURE 4 Electron micrographs of material eluted from the agarose gel depicted in Fig. 3. The major band (as judged by ANS staining and turbidity) of each lane in Fig. 3 was excised from the gel, and the agarose was disrupted by extrusion through a 16-gauge needle. This suspension was centrifuged in an Eppendorf 5412 centrifuge (Brinkmann Instruments) at 12,800 *g* for 1 min to remove the bulk of the agarose. The supernatant was negatively stained as described in Materials and Methods. (*A*) material eluted from the gel of the incubation (−ATP) in which ATP had been omitted. (*B*) material from the major band when a complete incubation (+ATP) was carried out. Thin arrows in *B* show examples of uncoated vesicles. Thick arrows show vesicles whose coats appear to have been only partially removed. × 110,000.

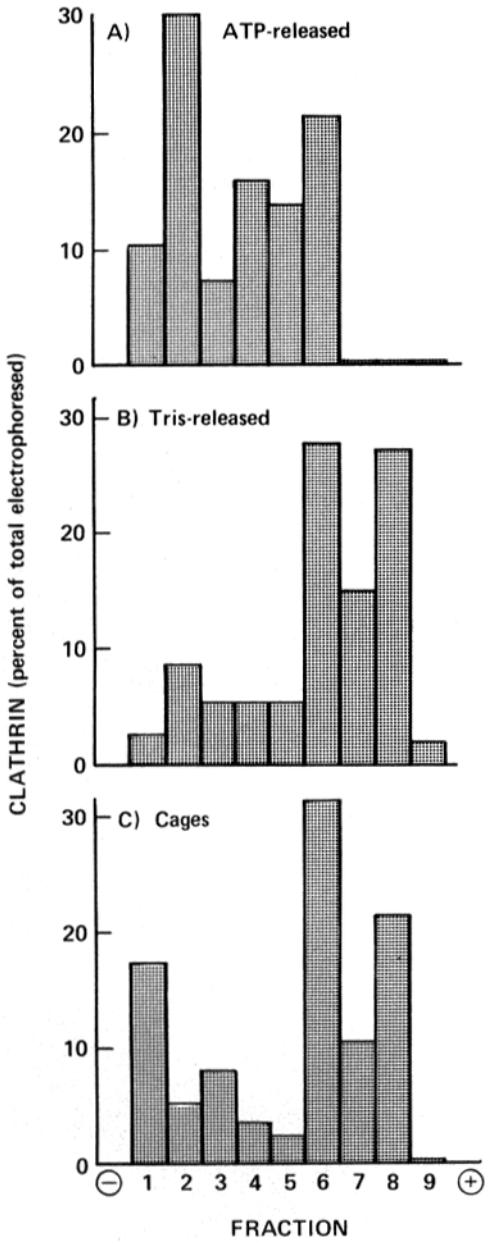


FIGURE 5 Agarose gel analysis of products formed upon dialysis against a standard assembly buffer of ATP-released (panel *A*) and Tris-released (panel *B*) clathrin preparations. For comparison, the distribution in the same gel of cages reconstituted from purified clathrin (panel *C*) is shown. (*A*) Clathrin was released with ATP and cytosol and chromatographed on a Bio Gel A (Bio-Rad Laboratories) 1.5m column (1.5 × 17 cm, equilibrated in buffer B) to remove most of the cytosol protein. The void volume fractions (containing the peak of clathrin) were pooled and concentrated by ammonium sulfate (70% of saturation) precipitation. After 30 min at 4°C, the precipitate was collected (25,000 rpm for 10 min in a SW 50.1 rotor) and resuspended in 0.5 M Tris-HCl (pH 7.0) at about 1 mg/ml protein. (*B*) Clathrin was released with Tris by incubating 1.3 mg of coated vesicles (150 μl) with 1.0 ml of cytosol and 0.23 ml of 3 M Tris HCl (pH 7.0) for 10 min at 37°C, and the supernatant was then treated in the same manner as the ATP-released supernatant. The resuspended ammonium sulphate precipitates were dialyzed (at 4°C) first against 0.5 M Tris-HCl (pH 7) and then against an assembly buffer (20 mM MES-KOH (pH 6.2), 1 mM EDTA, 2 mM $CaCl_2$ [8]). Samples (60 μl) of the dialysates were then electrophoresed in parallel on the same 0.15% agarose gel, as described (16). Fractions (obtained by slicing across all three lanes at once with a razor blade) of the agarose gel were subject to SDS gel electrophoresis, thus permitting the content of clathrin in each fraction to be determined from densitometer scans of the stained SDS gel of that fraction. The distribution of clathrin among the agarose gel fractions is presented, expressed as a percent of the total clathrin applied to the agarose gel. The origin is included in Fraction 1. Electrophoresis is to the right. The dip in the profiles in fraction 7 is an artefact due to the crude method of slicing of the agarose gel, resulting from an unusually thin slice.

the release of clathrin from agarose gel-purified coated vesicles (Table I). In each case the amount of clathrin remaining in the pellet after incubation was measured and expressed as a fraction of the amount found in the pellet in a parallel, control incubation in which no nucleotide had been added. None of these experiments used the ATP-regenerating system. Experiment I shows that as little as 2 μM ATP (about four molecules of ATP per clathrin) produced a measurable release of clathrin, and release was nearly complete by 20 μM ATP. The nonhydrolyzable analogue AMP-PNP did not substitute for ATP (Experiment *III*), although in one experiment (not shown) some release was observed. AMP was totally ineffective. Surprisingly, ADP addition did result in uncoating. However, this effect was abolished when excess hexokinase and glucose were also added. It thus appears that the ADP had been converted to ATP in the crude cytosol and that ADP itself is without effect. Therefore, the crude nature of the cytosol fraction precludes a meaningful investigation of nucleoside triphosphate specificity.

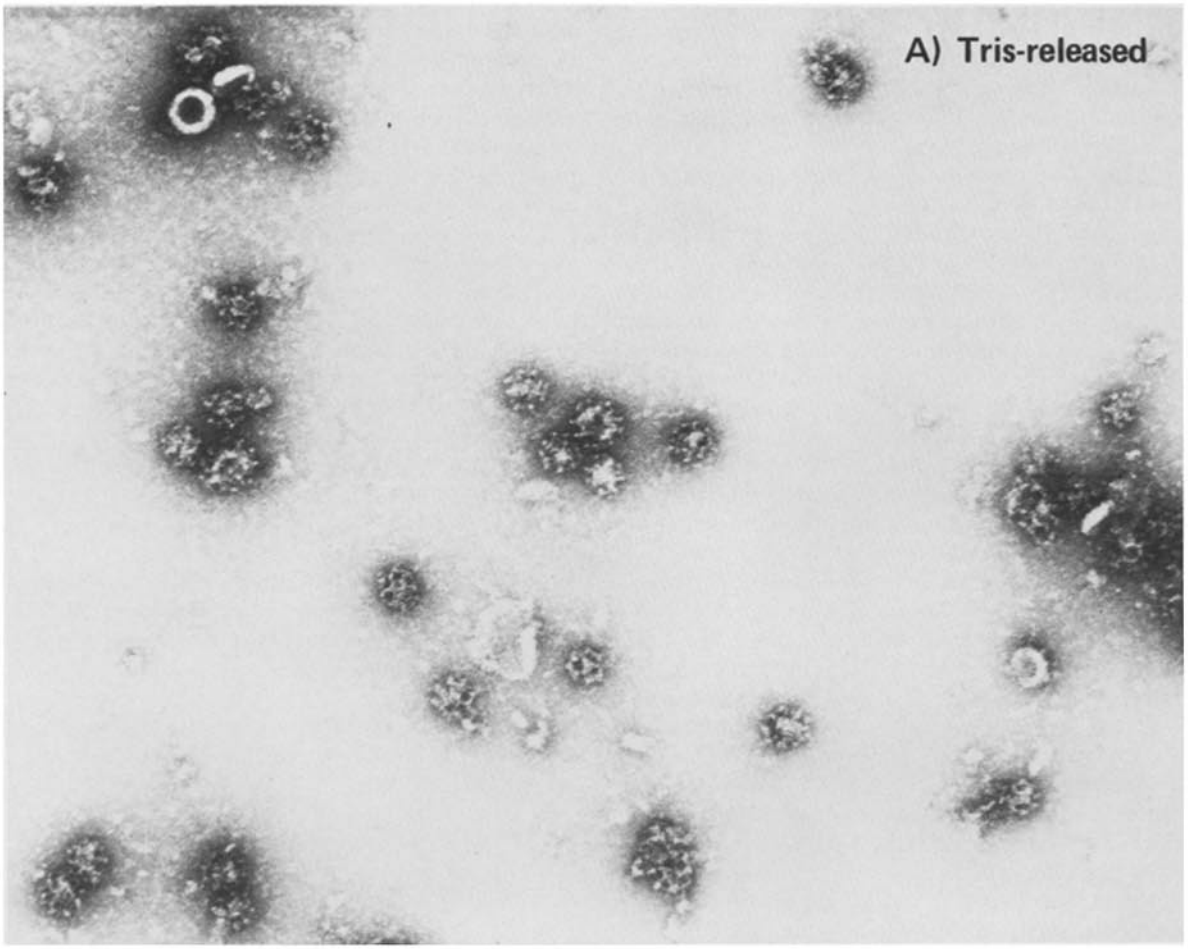
Table I (Experiment *II*) also reveals that empty cages reconstituted from purified clathrin are dissociated in an ATP-dependent fashion. Therefore, membranes are not absolutely required for this process. However, Table I measures only the extent and not the rate of uncoating.

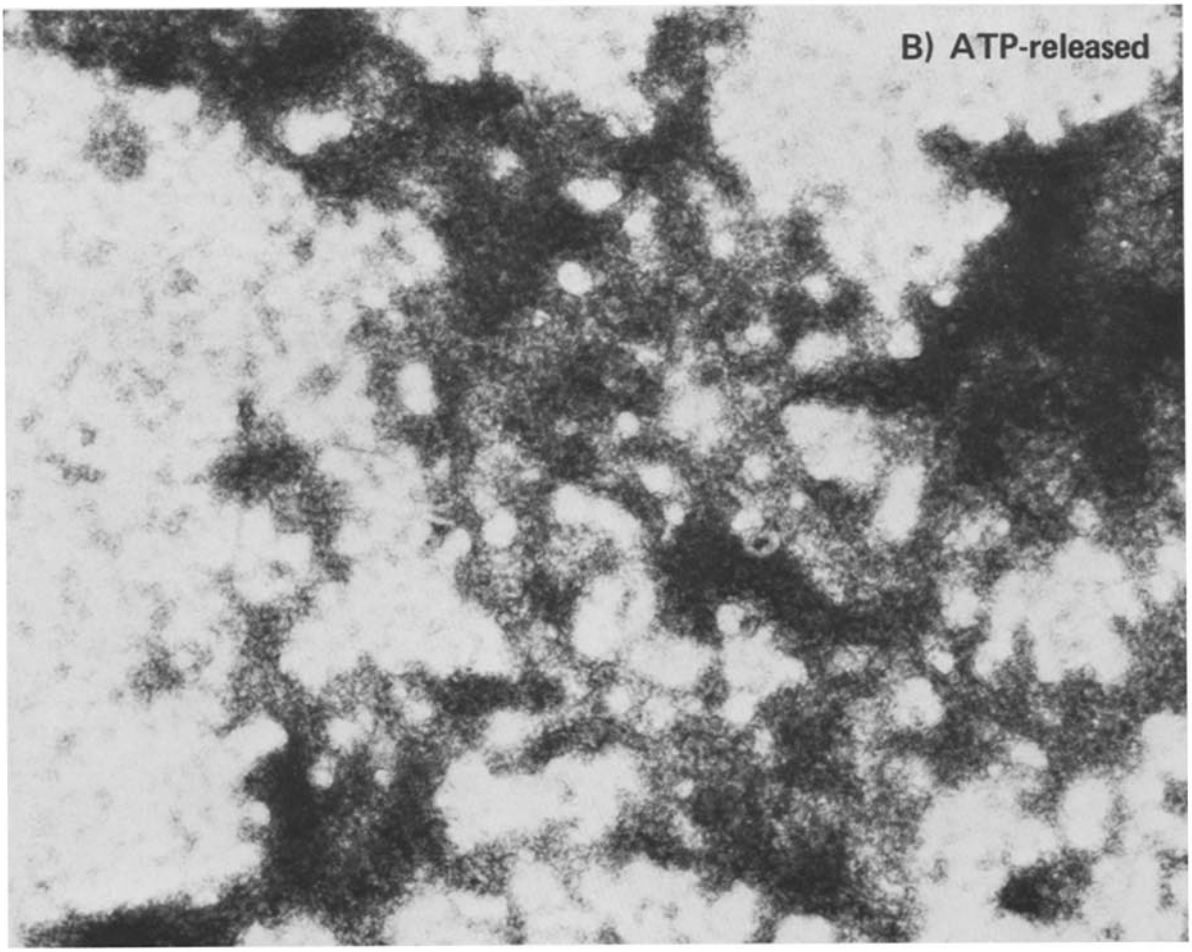
Several treatments and additions to the cytosol fraction were also explored (Table I, Experiment *III*). An active component of the cytosol fraction was precipitated with 70% ammonium sulphate and remained active after dialysis for 16 h at 4°C against buffer B. However, all activity was lost upon boiling. Because brain cytosol is a rich source of calmodulin and because this polypeptide has been reported in coated-vesicle preparations (10), possible effects of Ca^{++} and EGTA on ATP-dependent uncoating were sought. None were found. Excess EDTA, however, markedly limited the extent of uncoating. Moreover, pure calmodulin (the gift of Dr. H. Schulman, Stanford University) did not replace cytosol (not shown).

Properties of the Released Clathrin

When supernatants from standard incubations (containing ATP-released clathrin) were recentrifuged at 130,000 g_{av} for 30

FIGURE 6 Electron micrographs of Tris-released (*A*) and ATP-released (*B*) clathrin-containing preparations after dialysis against an assembly buffer (20 mM MES pH 6.2, 1 mM EDTA, 2 mM $CaCl_2$). The samples studied here are the same as those electrophoresed in the agarose gel in Fig. 5. The dialysates were negatively stained (Materials and Methods). Aggregates such as that shown in *B* were very unevenly distributed on the grid, as expected from their large size. × 116,000.





min, <3% of the released clathrin could be pelleted. Therefore, clathrin in the supernatant is not a residue of intact coated vesicles or cages but must consist instead of fragments or subunits of the coats. A preliminary analysis by gel filtration and electron microscopy suggests that ATP-released clathrin is present in oligomeric complexes approximately the size of triskelions (data not shown).

Clathrin released from coated vesicles by incubation with ATP and cytosol was tested for its ability to reassemble into cages after dialysis into 20 mM MES (pH 6.2), 2 mM $CaCl_2$, 1 mM EDTA, an established condition (8). For comparison, clathrin released from coated vesicles via 0.5 M Tris treatment (in the presence of cytosol) was prepared and tested in parallel. To remove the bulk of the cytosol protein, supernatants containing the released clathrins were first passed through Bio Gel A-1.5m columns (Bio-Rad Laboratories, Richmond, CA) before dialysis of the void volumes into the assembly buffer. The whole dialysates were subjected to agarose gel electrophoresis (Fig. 5). Most of the clathrin present in the dialysed, Tris-released preparation (Fig. 5 *B*) electrophoresed in the same position as cages reconstituted from purified clathrin (Fig. 5 *C*). (The dip in the peaks in *fraction 7* is an artefact due to variation in the thickness of the slices of the agarose gel.) In contrast, the clathrin present in the dialysate of the ATP-released sample (Fig. 5 *A*) electrophoresed more slowly, with little or none in the band characteristic of cages. The dialysates were also examined by electron microscopy (Fig. 6). Cages were readily apparent in the dialysate of the Tris-released preparation (Fig. 6 *A*) but absent from the dialysate of the ATP-released material. Instead, large aggregates (presumably of clathrin and similar to those observed after dialysis of elastase-digested clathrin [8, 17]) were observed in both the dialysate (Fig. 6 *B*) and the major agarose gel band (not shown) of the dialyzed, ATP-released material.

DISCUSSION

Clathrin is released from coated vesicles in a soluble form, provided both ATP and a high-speed supernatant fraction are supplied (Fig. 1). This release results from the uncoating of vesicles when coated vesicles are used (Figs. 2 and 3) but can also occur in the absence of vesicle membranes when reconstituted, empty cages are used (Table I). About 3 μg of crude cytosol protein is needed for the ATP-dependent release of 1 μg of clathrin, as can be calculated from Table I, Experiment *V*. This is much less than the ratio of cytosol protein to clathrin in intact cells.

It seems highly unlikely that this release results from nonspecific proteolysis, for several reasons: (*a*) The process depends upon a nucleoside triphosphate. (*b*) Release is selective for clathrin, is extensive, and is complete within 10 min. (*c*) Despite an extensive release, significant proteolysis of clathrin or its associated light chains (Figs. 1 and 4) cannot be detected (either as a net loss of material or as a decrease in apparent molecular weight). The light chains are known to be especially sensitive to proteolysis (8, 17). (*d*) Even when clathrin and its light chains are purposefully and extensively cleaved with protease, cages do not dissociate; in fact, protease-digested cages will reform spontaneously if they are disrupted (17).

Clathrin released from coats in the ATP-dependent manner seems to have lost its ability to form regular cages under at least some standard conditions (Figs. 5 and 6). Conceivably, the modification responsible for this is an artefactual one with a trivial explanation. Alternatively, this may represent the accumulation of a modified intermediate that is primed for incorporation into budding coated vesicles. If so, then it should prove possible to reverse the modification and to restore the capacity of clathrin to self-assemble.

The biological significance of the cytosol and nucleoside triphosphate-dependent uncoating process that takes place in vitro is, of course, not yet clear. So far, its properties parallel those expected of the uncoating segment of a clathrin-coated vesicle cycle (15). If this reconstituted reaction turns out to use a distinct enzymatic mechanism that can be elucidated with a purified cytosol factor, then its physiological importance will be evident.

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