Coated Vesicle Isolation by Immunoadsorption on *Staphylococcus aureus* Cells

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ABSTRACT Porcine brain coated vesicles were isolated from crude fractions of tissue homogenates by affinity separation using anticlathrin-coated *Staphylococcus aureus* (Staph A) cells as a solid-phase immunoadsorbent. The specificity of the immunoadsorption was monitored by SDS PAGE analysis and by competitive ELISA assays. SDS PAGE of the material immunoadsorbed from a fraction of porcine brain smooth microsomes showed a selective enrichment in a 180,000 mol wt protein. In an ELISA assay, this protein competed effectively—in binding anticlathrin—with clathrin extracted from a coated vesicle preparation. When the immunoadsorbed fraction was examined by electron microscopy, coated vesicles and vesicle-free cages were found forming a quasicontinuous monolayer on the surface of the Staph A cells. Other particles were not adsorbed, and the controls were free of either clathrin cages or coated vesicles. Upon extensive dialysis (against MES buffer, pH 6.5), similar cages appeared on the surface of anticlathrin-coated Staph A cells reacted with extracted clathrin.

This study demonstrates that anticlathrin-coated Staph A cells can be used for the isolation and purification of a homogeneous population of coated vesicles. In addition, the ability of extracted clathrin to bind and to polymerize onto the Staph A cells raises the possibility of using this technique to further explore the conditions required for cage and/or vesicle reconstitution.

The ubiquitous presence of coated vesicles in eukaryotic cells has generated a great deal of interest and speculation concerning the role(s) these organelles play in cellular processes. Recent studies have shown that coated vesicles are involved in receptor-mediated endocytosis (2, 4, 14, 15, 17, 38, 40, 43), formation of primary lysosomes (13), membrane recycling (5, 18, 19), and intracellular vesicular transport (11–13, 36, 41).

The pertinent evidence has been derived either from morphological observations of cells actively engaged in one of the processes mentioned, or from cell fractionation studies. Both of these general approaches have technical limitations when applied to the study of coated vesicles because of the transient existence of these structures and the fragility of their clathrin coat. Besides, with these procedures, many important questions concerning the mechanisms involved in the assembly and/or disassembly of coated vesicles *in situ*, or the fate of the clathrin cage upon disassembly from the vesicle are virtually impossible to answer.

In an attempt to improve on available isolation procedures and to solve some pending problems concerning the formation and function of coated vesicles, we have developed a technique that uses anticlathrin antibody for the affinity separation of coated vesicles from crude fractions of tissue homogenates. In this report, we show that formaldehyde-fixed, heat-inactivated *Staphylococcus aureus* cells can be used as a solid phase immunoadsorbent for the isolation of coated vesicles. With this procedure, it is possible to: (*a*) increase the efficiency of separation, (*b*) shorten the time required to achieve it, (*c*) retain the intactness of the vesicles and their cages, and (*d*) improve the homogeneity of the preparation by eliminating or greatly reducing particulate contaminants, especially coatless vesicles.

MATERIALS AND METHODS

Materials

The following materials were used throughout this study: porcine brains (Copaco's Abattoir, Bloomfield, CT); starter culture of *S. aureus* (generous gift of Dr. D. Wall, Dept. of Anatomy, Johns Hopkins Medical School); human fibroblasts (subcultured by R. Blunden, Dept. Human Genetics Yale Medical School); MES (2[*N*-morpholino]ethane sulfonic acid), orthophenylenediamine, and horseradish peroxidase type IV (Sigma Chemical Co., St. Louis, MO); bovine serum albumin (Armour Pharamaceutical Co., Phoenix, AZ); Protein A (Pharmacia Fine Chemicals, Piscataway, NJ); FITC-goat antirabbit IgG (Miles Laboratories, Elkhart, ID); ^{125}I (New England Nuclear, Boston, MA); agarose low M_r (Bio-Rad Laboratories, Richmond, CA); Libro microtiter plates used in the ELISA assay

THE JOURNAL OF CELL BIOLOGY · VOLUME 92 MARCH 1982 846-857

(Flow Laboratories, Inc./Linbro Scientific, McLean, VA).

Preparation of Coated Vesicle Fractions

The coated vesicles used as test objects for the development of our procedure were isolated from frozen porcine brain tissue according to the method of Pearse (33). The tissue was removed from the animals upon slaughter (at the abattoir), and maintained in ice-cold saline during transport to the laboratory, where it was minced, frozen in liquid nitrogen, and stored at −70°C for no longer than 3 wk. Longer storage reduced the yield.

30 min before homogenization, 500 g of frozen porcine brain tissue were placed in 1,000 ml of MES buffer (0.1 M 2[*N*-morpholino]ethane sulphonic acid and the pH was brought to 6.5 by the addition of NaOH. The buffer also contained 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.02% Na azide). The thawed tissue was minced, placed in a Waring blender, and homogenized three times for 15 s each at maximum speed. The resulting homogenate (~1,500 ml) was filtered through bolting cloth and centrifuged at 20,000 *g* for 30 min using a JA 14 rotor and a Beckman Model J centrifuge (Beckman Instruments, Inc., Fullerton, CA).

After centrifugation, the supernate was carefully removed with a 50-ml plastic syringe, and recentrifuged at 100,000 *g* for 1 h in a Beckman L5-65 centrifuge, the instrument with which all subsequent separations were carried out (Beckman Instruments, Inc.). The pellets were suspended in 20 ml of MES buffer, pH 6.5, and 5-ml aliquots of the ensuing suspension were layered over a 5–60% sucrose gradient (30 ml) and centrifuged in a Beckman SW 27 rotor at 50,000 *g* for 120 min (Beckman Instruments, Inc.). The broadly dispersed band between 10 and 50% sucrose was collected, diluted fivefold with MES buffer, and concentrated by sedimentation at 100,000 *g* for 1 h. The ensuing pellet was resuspended in ~20 ml of the same buffer, and the suspension was layered over a 20–60% sucrose gradient (30 ml) and centrifuged for 20 h at 50,000 *g* (SW 27 rotor, Beckman Instruments, Inc.). The fine distinct band found at 50–55% sucrose at the end of this run was collected, diluted, and concentrated by pelleting as described above and characterized by electron microscopy and SDS PAGE as indicated below. Routinely, the final step of the Pearse procedure (33) (i.e., velocity sedimentation in a 5–30% sucrose density gradient for 1 h at 100,000 *g*) was skipped because, in our hands, it did not improve significantly either the purity or the yield of the coated-vesicle fractions.

A crude smooth microsomal fraction was obtained (as a pellet) by centrifuging for 1 h at 50,000 *g* the supernate of the first centrifugal step.

Extraction of Clathrin from Coated Vesicle Fractions

The following procedures were used to extract clathrin from coated-vesicle fractions (prepared as given above).

ALKALINE EXTRACTION (44): 0.1 M NaOH was added to the coated-vesicle preparation to a final pH of 8.5. The sample was stirred in an ice bath for ~1 h, and then centrifuged in a Ti 50 rotor for 90 min at 100,000 *g*. The amount of protein in the extract was estimated, and the types of proteins present were analyzed by SDS PAGE; a major protein band of 180,000 mol wt (the mobility recorded in the literature for clathrin) was detected by Coomassie Brilliant Blue (CBB) staining.

UREA EXTRACTION (6): 8 M urea was slowly added to the coated-vesicle preparation to a final concentration of 2 M. The sample was then incubated on ice for 1 h and centrifuged at 100,000 *g* for 90 min. As in the case of the alkaline extract, a predominant band at ~180,000 mol wt was detected by SDS PAGE.

Antibody Production

Anticlathrin antibody was produced by immunizing rabbits and chickens with antigen excised from SDS-polyacrylamide gels (32). ~300 μg of the alkaline extract of a coated-vesicle preparation was subjected to electrophoresis on a 5–10% SDS-slab gel. The clathrin band, identified by the procedure of Higgins and Dahmus (20) on unstained gels, was excised, suspended (by fragmentation) in 1 ml of PBS, and thoroughly mixed with an equal volume of Freund's complete adjuvant.

PRODUCTION OF ANTIBODIES IN RABBITS: Adult female rabbits were bled to obtain a supply of ~50 ml of control (preimmune) serum from each animal and were then given a series of dermal injections of antigen (prepared as given above) at multiple sites on their back. Each animal received the equivalent of 150 μg of clathrin band protein. At two-week intervals, three more injections of the same antigen were given by suspending 200 μg of protein in Freund's incomplete adjuvant. After ~8 weeks, the animals were bled and the sera tested for the presence of specific antibodies to clathrin by immunochemical techniques.

PRODUCTION OF ANTIBODIES IN CHICKENS: The same antigen was injected into the breast and both leg muscles of three chickens. Within three weeks of the booster injection, a positive response was detected.

Immunochemical Tests

OUCHTERLONY METHOD: Tests were performed on samples of rabbit and chicken antisera. The diffusion matrix was agarose (1%) in phosphate buffer (0.02 M), pH 7.4, containing NaCl (0.15 M), Triton X-100 (1%), polyethylene glycol (4%) and Na azide (0.02%). Immunodiffusion tests were carried out for 48 h (or more) at room temperature, and the precipitation of antigen-antibody complexes was promoted by subsequently transferring the plates to 4°C for at least 8 h. The plates were washed and then stained with CBB.

IMMUNE REPLICA TECHNIQUE: The specificity of the serum antibody against clathrin (referred to hereafter as anticlathrin) was also demonstrated by the immune replica technique of Showe et al. (39). An alkaline extract of a coated-vesicle fraction radioiodinated by the lactoperoxidase-glucose oxidase procedure (21) was analyzed by SDS PAGE. At the end of the run, the gel was swollen in deionized, distilled water for 5 min, then sandwiched between two plates of 1% agarose containing either preimmune (control) or immune serum, and incubated for ~8 h at room temperature in a humidified chamber. At the end of the incubation, the agarose plates were extensively washed first in 0.5 M NaCl containing 1% Triton X-100 (three changes over 8 h), then in 0.15 M NaCl containing the same amount of Triton X-100 (three changes over 24 h), and finally soaked in 0.15 M NaCl for ~8 h, rinsed in deionized, distilled water, air dried, and autoradiographed on x-ray film. Extensive washing is required for the effective elimination of nonspecific binding.

Immunoprecipitation of Cell Fractions with Staphylococcus Aureus Cells

PREPARATION OF STAPH A CELLS: Staph A cells, formalin-fixed and heat-inactivated by Kessler's procedure (25), give a stable adsorbent, readily usable for indirect immunoprecipitation of various antigens from heterogeneous solutions.

Staph A preparations, suspended at a concentration of 10% (wt:vol) in Tris-HCl buffer (50 mM), pH 7.4, containing NaCl (0.15 M), EDTA (5 mM) (referred to hereafter as NET buffer), remained active for at least 3 wk when stored at −70°C in 1-ml aliquots. Before being used, the thawed cells were washed five times by suspension in NET buffer supplemented with 5 mg/ml BSA and 0.5% Triton X-100, followed by pelleting in a Brinkmann Eppendorf Centrifuge (8,000 *g* for 2 min (Brinkmann Instruments, Inc., Westbury, NY). The initial concentration was reestablished at each resuspension. Extensive washing was needed to minimize or eliminate nonspecific binding to Staph A cells.

INCUBATION WITH CELL FRACTIONS: Immunoadsorption was carried out either (*a*) by first generating antigen-antibody complexes and then introducing the Staph A cells (25), or (*b*) by first coating the cells with antibody and then reacting them with antigen-bearing particles (31). The latter procedure was preferred because, in contrast to the former, it did not produce particulate aggregates. The immunoadsorption was carried out in two steps: first, 100 μl of a suspension of prewashed Staph A cells were treated with an equal volume of either purified immune or preimmune IgG (or immune or preimmune serum); after incubating the mixture for 1 h at 4°C, excess antibody (or serum) was removed by three cycles of pelleting (in a Microfuge) and resuspension (at constant volume) in supplemented NET buffer, as above. Binding of IgG (serum) to Staph A cells was monitored by measuring the OD_{280} of the antibody solution before and after exposure to the immunoadsorbent. In the second step, 100 μl of resuspended IgG-coated Staph A cells were reacted with 250 μl (~125 μg protein) of the cell fraction of interest. Nonadsorbed (as well as part of nonspecifically adsorbed) particles were removed by pelleting and resuspending the preparation twice in MES buffer containing 5 mg/ml BSA and twice in MES buffer alone. Triton X-100 was omitted from resuspension media throughout these steps, because it damaged the structure of the adsorbed particles.

The first supernate and the final pellet were collected and divided into aliquots for electron microscopy and biochemical analysis.

Elution of Immunoadsorbed Components

Samples (~0.1 ml) of immunoadsorbates prepared as above were suspended in 0.05 ml of 2% SDS-6M urea, sonicated in a Branson sonifier for 30 s (Branson Sonic Power Co., Danbury, CT), and then boiled in a water bath for 5 min. The Staph A cells were removed by centrifugation in an Brinkmann Eppendorf Centrifuge (2 min at 8,000 *g*) and then the supernatant was carefully collected and processed for SDS PAGE. The same elution procedure was applied to controls. The SDS-urea extraction did not remove detectable components from samples of Staph A cells processed as above.

SDS PAGE

Samples for SDS PAGE were treated as follows: (*a*) aliquots of cell fractions containing ~100 μg of protein were boiled 5 min in 1% SDS, dithiothreitol (DTT)

(20 mM), and EDTA (10 mM); (*b*) eluates of immunoprecipitates, already suspended in SDS and urea, were boiled for an additional 5 min in the presence of DTT (20 mM). All samples were subjected to electrophoresis for 9 h at 10 mAmp on gradient (5 to 10%) polyacrylamide slab gels (29). At the end of the runs, the gels were fixed and stained for 2 h in 50% methanol-10% acetic acid, 0.2% CBB, and then destained overnight in 25% methanol-10% acetic acid.

ELISA Assays

An enzyme-linked immunoadsorbent assay was developed from the procedure of Rennard et al. (35) as modified in by P. Yourchenko (personal communication), to quantitate clathrin in various cell fractions.

Aliquots (100 μl, ~20 μg protein/ml) of a clathrin-containing, alkaline extract of a coated-vesicle fraction prepared as above, or a comparable aliquot of a cell fraction to be tested, were adsorbed to the wells of microtiter plates (LIBRO). The latter were drained by inversion and washed three times with 0.04% Triton X-100 in PBS, pH 7.4, (PBS-T). Aliquots (100 μl) of rabbit anticlathrin serum were then added to the antigen-coated wells, incubated for 1 h at ~25°C, after which the plates were drained by inversion and washed at least three times with PBS-T. For competition assays, the anticlathrin aliquot was mixed with the sample of cell fraction to be tested immediately before being added to the clathrin-coated wells.

Protein A was conjugated to horseradish peroxidase (30); the conjugate was diluted 1:1,000 in PBS-T, and 100-μl aliquots were added to each antigen-antibody coated well. The plates were incubated for 1 h at ~25°C, then drained and washed as above. A 200-μl aliquot of a freshly prepared 4 mM solution of orthophenantroline (a peroxidase substrate) was added to each well, the reaction was allowed to proceed for 10 min at ~25°C and then stopped by adding 50 μl of 8 M H_2SO_4 to each well. The absorbance of the reaction product at 492 nm was recorded for the content of each well using a Beckman spectrophotometer or an automated densitometer (kindly provided by Dr. R. Rosenstein, Department of Pathology, Yale University).

Electron Microscopy

THIN SECTIONS: Cell fractions and immunoadsorbates of interest were fixed in suspension for 30 min in 1% glutaraldehyde in either 0.1 M MES buffer, pH 6.5, or 0.1 M cacodylate buffer, pH 7.4, and then pelleted by centrifugation for 20 min at 100,000 *g* in a SW 50.1 rotor (Beckman Instruments). The ensuing pellets were postfixed for 1 h in 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.4, and stained in block for ~2 h in uranyl acetate. After each step, the surface of the pellets was rinsed twice with the appropriate buffer. At the end of the procedure, the pellets were cut into orientable strips (to allow sectioning of their entire depth), and processed for electron microscopy.

Sections, cut with diamond knives on a Sorvall Ultramicrotome MT 2-B (DuPont Instruments, Newtown, CT), were stained with uranyl acetate followed by lead citrate and examined in a Siemens 101 or JEOL 100 CX electron microscope. To assess the degree of homogeneity of the preparations, sections of the pellets were examined systematically from top to bottom.

NEGATIVE STAINING: For negative staining, dilute samples of preparations of interest were deposited onto carbon films freshly cleaved from mica surfaces. Grids were stained with 2% uranyl acetate in H_2O and air dried.

Other Procedures

IgG PURIFICATION: IgG was purified from immune and nonimmune serum by ammonium sulfate precipitation followed by DEAE cellulose chromatography (27).

PROTEIN DETERMINATIONS: The Lowry assay was used for protein estimations with BSA in 0.1 M MES buffer as the standard (28).

Indirect Immunofluorescence of Human Fibroblasts

Cultured cells were fixed for 10 min at room temperature with 3% formaldehyde in PBS, pH 7.2, and permeabilized with Triton X-100 (0.05%) (3). Residual aldehydes were quenched by washing the preparations three times for 20 min each with 100 mM Tris-HCl, pH 7.1.

Immune and nonimmune serum were routinely diluted 1 to 20 with 20 mM phosphate buffer, pH 7.4, containing Triton X-100 (0.3%) and NaCl (0.5 M). The cells were overlayed with the corresponding serum, incubated for 2 h at 25°C in a humidified chamber, washed three times (20 min each) with the high salt buffer used to dilute the serum, and stained for 1 h at 25°C with fluorescein isothiocyanate- (FITC-) goat-antirabbit IgG diluted 1 to 20 with the same buffer.[1] After staining, the preparations were rinsed thoroughly with PBS and mounted in glycerol-phosphate buffer, pH 7.4. All micrographs were taken on Kodak Tri X-Pan Film, ASA 400, using a Zeiss photomicroscope II, provided with epifluorescence optics.

RESULTS

Coated Vesicle Fractions

MORPHOLOGY: The coated-vesicle fractions we isolated from pig brains by the procedure of Pearse (33) were monitored by electron microscopy using either negatively stained preparations (micrographs not shown) or thin sections of pellets (Fig. 1). Morphologically, the fractions were found to be relatively homogeneous. The bulk of the pellets were found to consist primarily of: (*a*) coated vesicles (~25%) with well-defined membranes, (*b*) vesicle-free cages (36%) and (*c*) caged structures of intermediate appearance (39%) that had a dense, inner core but lacked a clearly delineated membrane; these latter structures are probably grazing sections of coated vesicles (Fig. 1 *A*). The percentages given above varied from one preparation to another for reasons that remain to be understood. The diameter of coated vesicles ranged from 40 to 92 nm. Vesicles without cages, ranging in size from 35 to 72 nm, appeared as a minor component of the coated-vesicle fractions (Fig. 1 *A*). Moreover, a systematic survey of the preparations revealed the presence of larger coat-free vesicles and membrane fragments at the bottom of the pellets (Fig. 1 *B* and *C*). These membranous contaminants varied in amount from one preparation to another and in some cases accounted for a significant part of the mass of the entire cell fraction. Finally, the bottom layers of the pellets contained filamentous structures of indefinite length and ~20 nm diameter (Fig. 1 *C*); their nature and intracellular origin are unknown, but they are morphologically similar to structures previously described by Bloom et al. (7) and Ungewickell and Branton (42) in their coated-vesicle fractions.

PROTEIN COMPOSITION: The results obtained by SDS PAGE analysis of our solubilized coated-vesicle fractions are in agreement with those already recorded in the literature (24, 34, 44). The predominant component, identified as clathrin, bands at ~180,000 mol wt and accounts for ~45% of all stained components. Other proteins, detected at 100,000, 55,000 and 30,000 mol wt, account for ~15%, ~20%, and ~20%, respectively, of the total (Fig. 2).

The alkaline as well as the urea extracts of our fractions (see Materials and Methods) are preferentially enriched in the 180,000 mol wt species, the other bands being significantly reduced or absent (data not shown).

Production and Characterization of Anticlathrin Antibodies

We applied this fractionation procedure and SDS PAGE to obtain a preparation of clathrin that was used as an antigen to raise antibodies as given under Materials and Methods (Fig. 2 *B*).

IMMUNOCHEMICAL TESTS: The sera of the injected animals were tested for antibodies against clathrin by standard

[1] The diluted FITC/goat-antirabbit IgG was centrifuged for 2 min in a Brinkmann Eppendorf Centrifuge followed by filtration through a Millex filter unit (GS 0.22 μm) before use. This protocol proved to be helpful in eliminating aggregates, thereby reducing nonspecific staining.

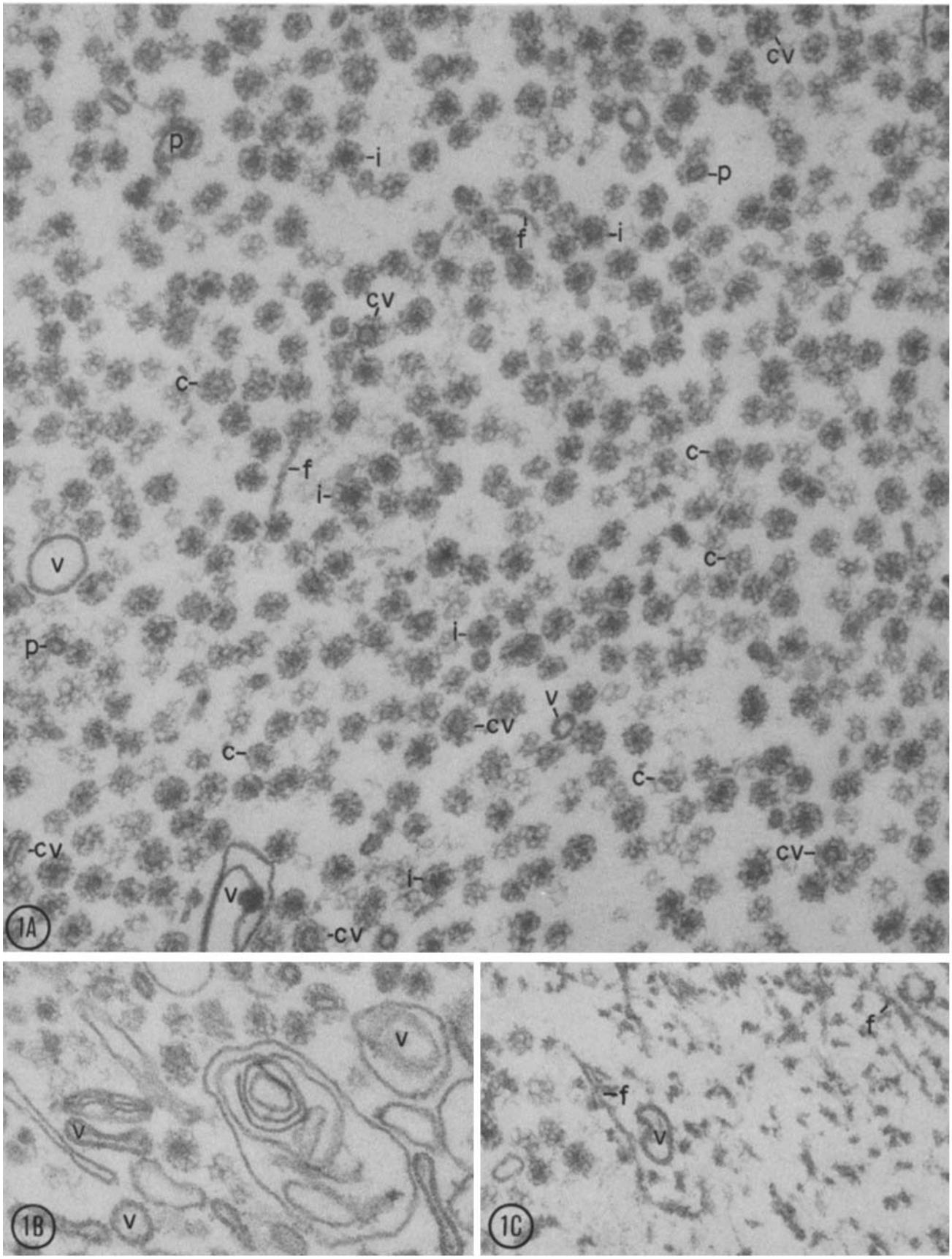


FIGURE 1 (*A*) shows a representative field from the middle layers of the pellet of a coated-vesicle preparation. The majority of the components are coated vesicles (*cv*), clathrin cages (*c*), and intermediate elements (*i*). Occasionally, vesicles which are partially coated with a clathrin cage (*p*) are observed. At this level in the pellet, small and large coat-free vesicles (*v*) are minor components of the fraction. × 86,000. (*B* and *C*) show the bottom of the pellet of the preparation shown in (*A*). At this level the coated-vesicle fraction is extensively contaminated by coat-free vesicles (*v*) and filamentous structures (*f*). × 172,000.

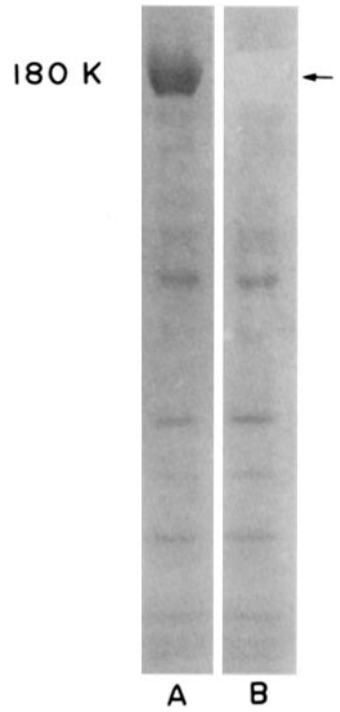


FIGURE 2 Analysis of a coated-vesicle fraction by SDS PAGE. Lane *A*: electrophoretogram of a ~50 μg protein sample of a coated-vesicle preparation; 5–10% gradient gel stained with Coomassie Blue. The prominent band at 180,000 mol wt is clathrin; other, less prominent bands appear at 100, 55, 32, and 30,000 mol wt. Lane *B*: an identical gel illustrating the site (arrow) of excision of the 180,000 mol wt band used as antigen for the production of anticlathrin (see Materials and Methods).

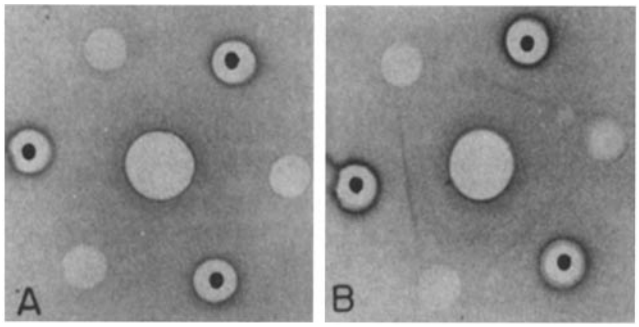


FIGURE 3 Double Immunodiffusion Tests: An alkaline extract of coated vesicles (5 μg of clathrin) was placed in the center well of both diffusion plates. In (*A*) the outer wells, marked by dots, contained 5 μl of preimmune rabbit serum and in (*B*) they contained 5 μl of rabbit anticlathrin. The precipitation lines were visualized by staining with Coomassie Blue.

immunochemical procedures. Double immunodiffusion tests revealed weak but well-defined single precipitation lines for immune (Fig. 3*B*) but not for preimmune (Fig. 3*A*) sera.

The specificity of the sera was further tested by the immune replica technique (Fig. 4) which revealed a definite interaction between the antisera and clathrin: a single band at ~180,000 mol wt interacted with antibodies present in both rabbit (Fig. 4*D*) and chicken (Fig. 4*B*) antiserum; no other bands were recognized by these antibodies. In both cases, the replica containing preimmune serum (Fig. 4*E* and *C*) was negative. Similar results were obtained with cruder brain fractions—i.e., the pellet obtained after centrifuging a crude extract of a brain homogenate at 50,000 *g* for 1 h (hereafter referred to as smooth microsomes): in immune replicas of the corresponding SDS PAGE electrophoretograms, the anticlathrin antisera did not react with bands other than 180,000 mol wt. On the basis of the results of ELISA assays, we estimated that the rabbit immune serum used in these experiments contained ~30 μg of anticlathrin per ml of serum.

IMMUNOCYTOCHEMICAL TESTS: Since our antibodies were raised against an SDS-denatured antigen, we checked their ability to bind to native clathrin on a type of specimen already tested and accepted as valid in the literature (i.e., cultured human fibroblasts). This is the cell type on which Anderson et al. (3) have demonstrated the existence of a characteristic punctate immunofluorescent pattern which is assumed to reveal the distribution of coated pits and coated vesicles on and close to the cell surface. Our immune serum, containing antibodies raised against denatured clathrin, revealed the same type of pattern (Fig. 5*B*) which was not present in controls (Fig. 5*A*).

On the basis of these tests, we concluded that the antibodies

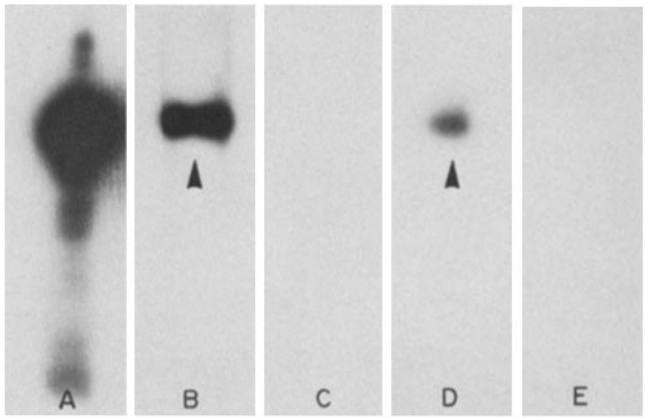


FIGURE 4 Immune replicas demonstrating the interaction between [^{125}I] clathrin and antisera. (*A*) is the autoradiogram of an SDS PAGE gel of a sample enriched in [^{125}I] clathrin; this material was used as a probe in the rest of the experiment. (*B*) and (*C*) are autoradiograms of immune replicas using chicken antiserum and chicken preimmune serum, respectively. (*D*) and (*E*) are immune replicas using rabbit antiserum and rabbit preimmune serum. A positive response to the immune sera was obtained only for the 180,000 mol wt band (clathrin) indicated by arrowheads. The autoradiograms were obtained after a 1 day exposure for the SDS PAGE of [^{125}I] clathrin (lane *A*), and after a 3-d exposure for the immune replicas (lanes *B* to *E*) using intensifying screens.

FIGURE 6 Smooth microsomal fraction obtained after subjecting a crude brain extract to centrifugation at 50,000 *g* for 1 h (see Materials and Methods). *A* is representative of tbe smooth microsomal fraction before immunoadsorption on anticlathrin-coated Staph A cells, and *B* is representative of the same fraction after immunoadsorption. Both fractions contain a large number of membrane-bounded vesicles of varied sizes. Coated vesicles (arrowheads) are a relatively minor component of this smooth microsomal fraction (*A*) and are rarely found therein after immunoadsorption (*B*). The corresponding electrophoretograms (not shown) demonstrated a band at 180,000 mol wt (clathrin) in *A* but not in *B*. × 64,000.

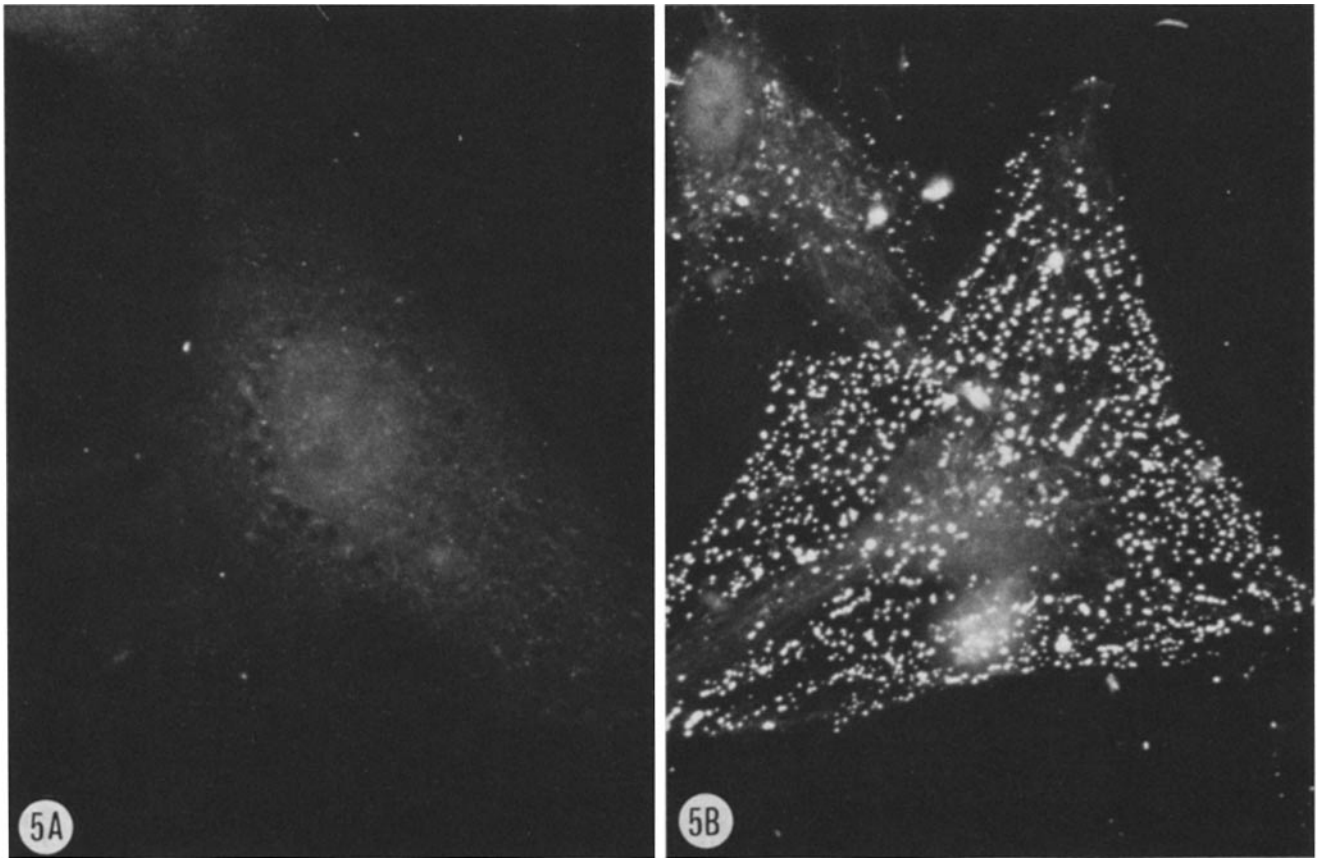
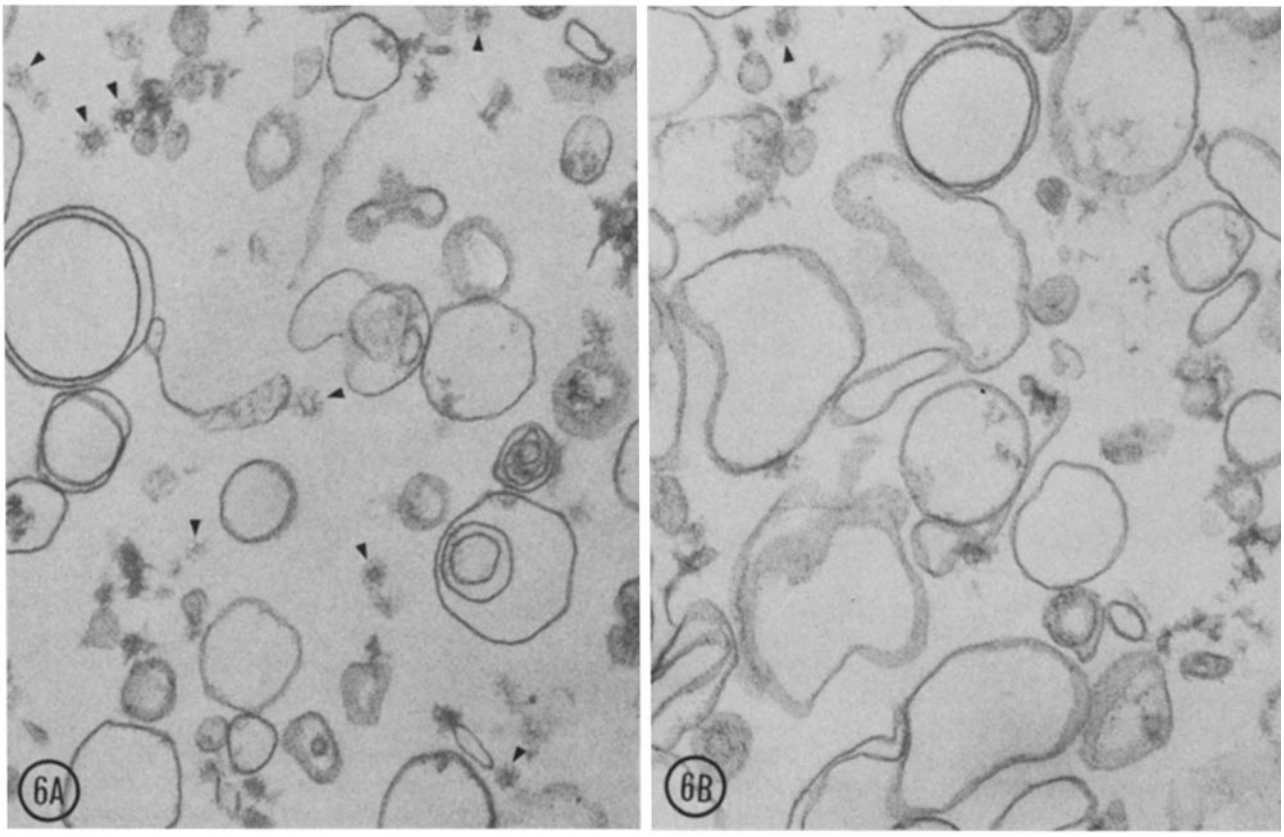


FIGURE 5 Indirect immunofluorescence tests were performed as described in Materials and Methods. *A* shows a fibroblast incubated with preimmune serum, and *B* shows a fibroblast incubated with clathrin-antiserum. Note the presence of a characteristic punctate pattern in *B* which is assumed to be due to staining of coated pits and coated vesicles on or near the cell surface. × 2,000.



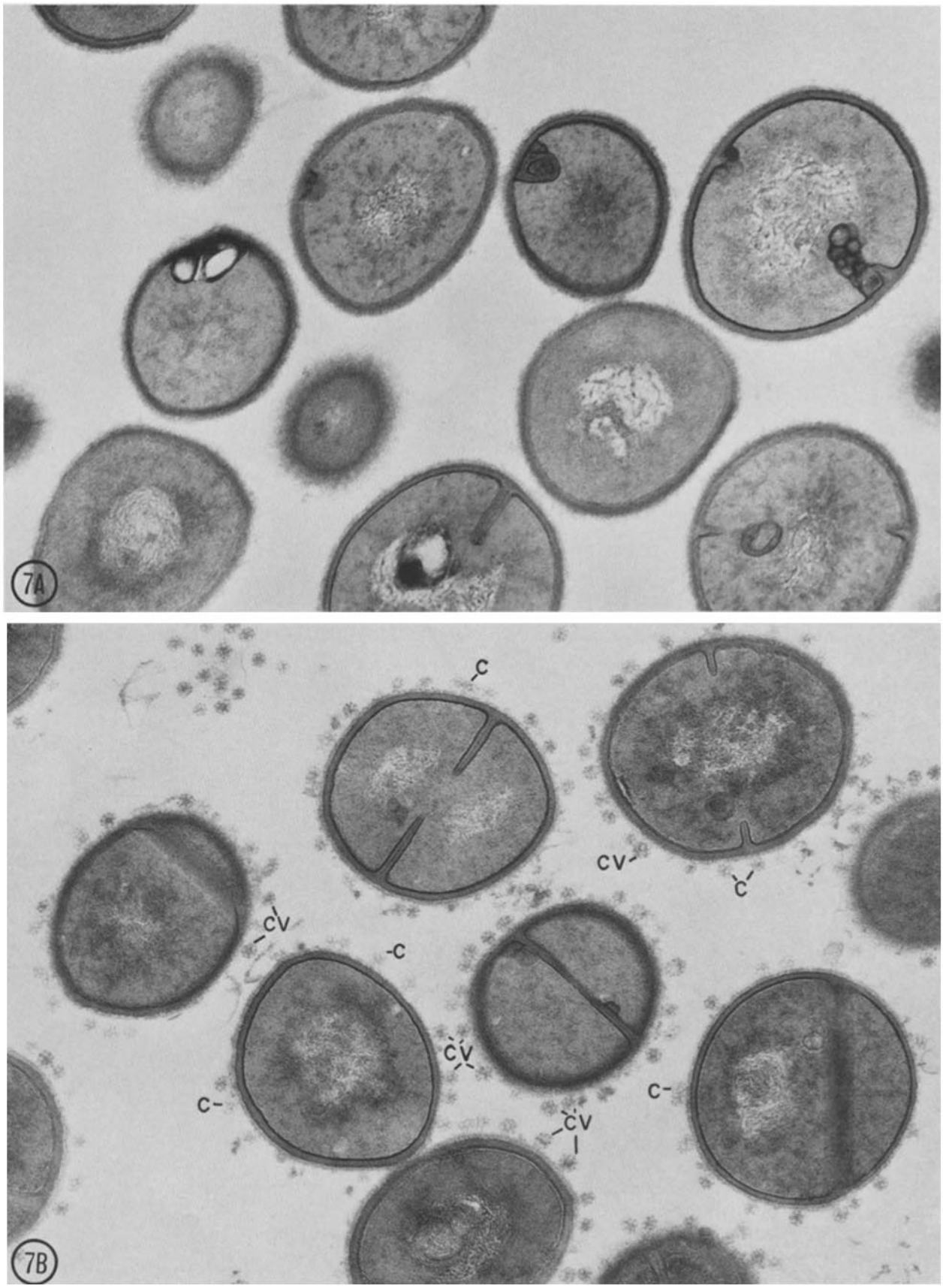


FIGURE 7 Smooth microsomal fraction immunoadsorbed to Staph A cells precoated with IgG, prepared from either preimmune (*A*) or immune (*B*) serum. Coated vesicles (*cv*) and clathrin cages (*c*) decorate the entire cell wall of the anticlathrin-coated Staph A cell (*B*), while the control preparation (*A*) is devoid of any cagelike structure. × 60,000.

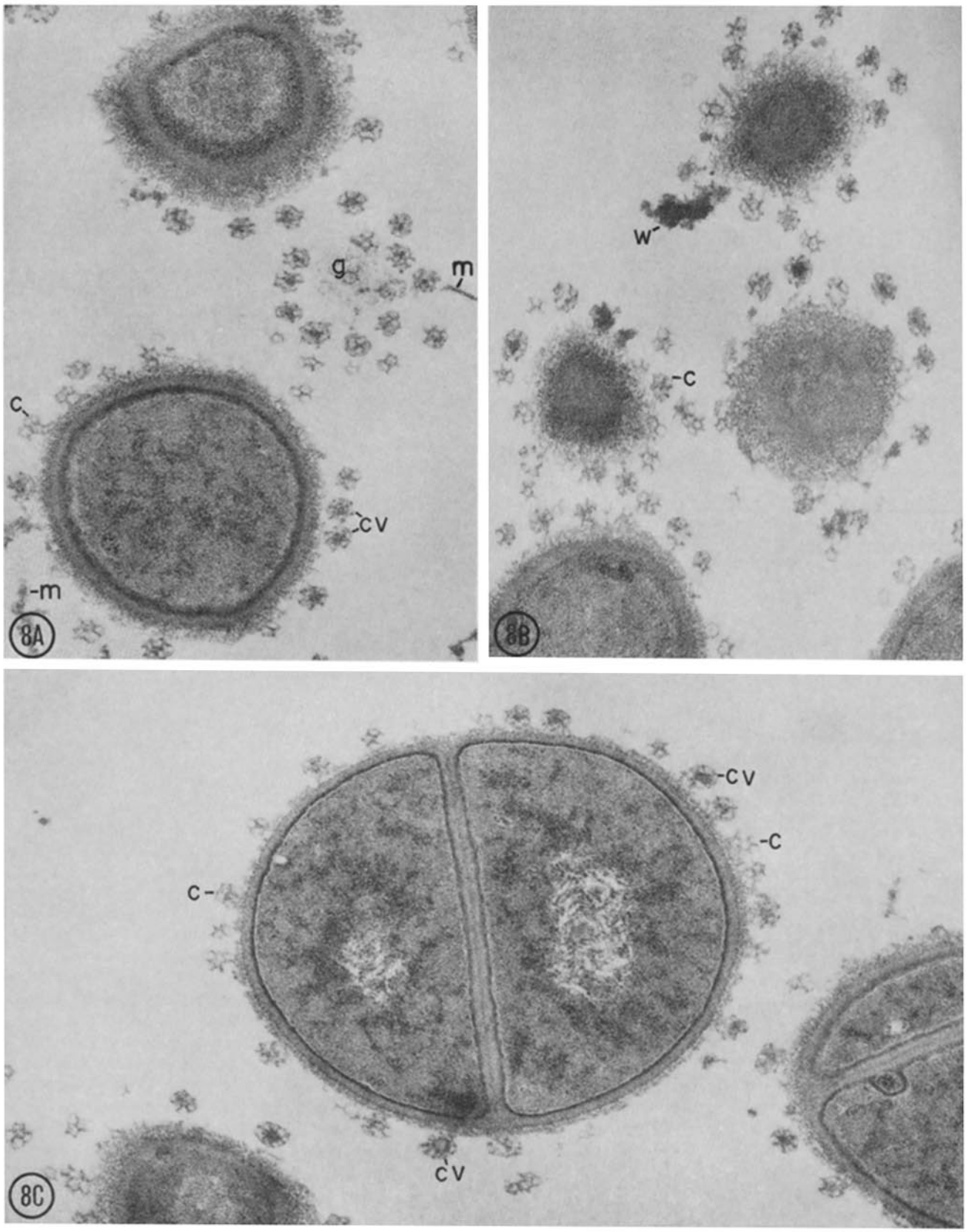


FIGURE 8 Preparations comparable to that in Fig. 7 *B* shown at higher magnification. Coated vesicles (*cv*) and clathrin cages (*c*) are distributed around the surface of the Staph A cells. Clathrin cages are more numerous than coated vesicles. 8*A* and *B* illustrate Staph A cells in normal as well as grazing sections; the latter give the erroneous impression that more than one layer of coated vesicles (*cv*) or cages (*c*) is adsorbed to the surface of the Staph A cells. The only recognizable contaminants observed after immunoadsorption are fragments of bacterial cell wall (*w*) (*B*), membrane fragments (*m* in *A*), and filamentous structures (not illustrated) of unknown origin. No coat-free vesicles are adsorbed to the Staph A cells. × 82,000.

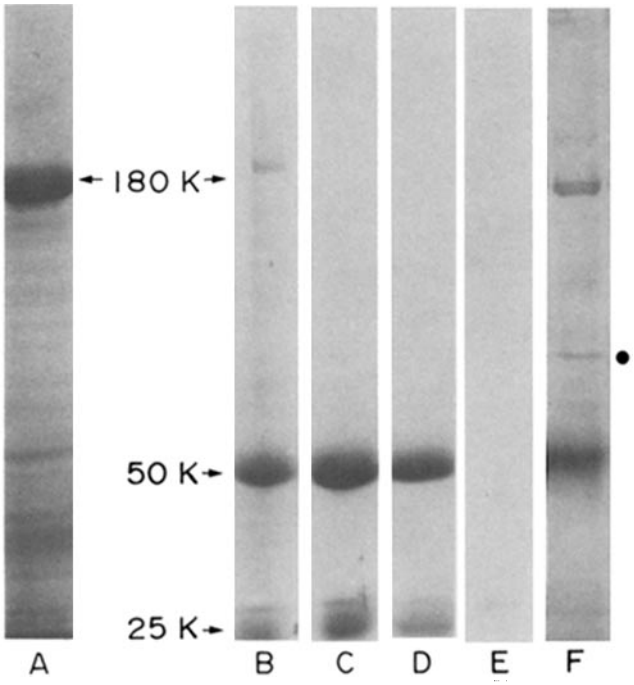


FIGURE 9 SDS PAGE analysis of immunoadsorbates prepared from a smooth microsomal fraction. Lane *A*: smooth microsomal fraction, before immunoadsorption. Lane *B*: eluate of immunoadsorbate on Staph A cells coated with (rabbit) anticlathrin IgG. Lane *C*: same as *B*, except that the Staph A cells were coated with (rabbit) nonimmune IgG. Lane *D*: eluate from Staph A cells reacted with immune (rabbit) IgG but not with smooth microsomes; the two bands detected in this eluate (and in lanes *B*, *C*, and *E*) at 50 and 25,000 mol wt are the heavy and light chains of IgG, respectively. Lane *E*: extract of Staph A cells boiled in elution buffer (exactly as for lanes *B*, *C*, *D*, and *F*); it contains a minor band in the 30,000 mol wt region which may represent protein A or another Staph A component; the same band can be recognized in lanes *B*, *C*, *D*, and *F*. Lane *F*: same as *B* except that after immunoadsorption (carried out in the presence of BSA) the Staph A cells were not washed as thoroughly as for lanes *B*, *C*, *D*, and *E*; the band at 68,000 mol wt is probably residual BSA. Clathrin is present in the immunoadsorbate only in lanes *B* and *F*, and is absent from the other (control) lanes.

we have raised can be used to detect native clathrin *in situ* or in cell fractions, and to immunoadsorb coated vesicles and other subcellular components that contain accessible clathrin.

Affinity Separation of Coated Vesicles from Purified Coated-Vesicle Fractions

Formaldehyde-fixed and heat-inactivated Staph A cells were first reacted with (rabbit) anticlathrin IgG, and then with a resuspended coated-vesicle fraction (similar to that illustrated in Fig. 1) as given under Materials and Methods. When the Staph A cells were recovered and examined by electron microscopy, they were found to be covered with a quasi-continuous corona of coated vesicles and vesicle-free cages (results not given). Coat-free vesicles were extremely rare. In control samples, in which the anticlathrin IgG was replaced by nonimmune rabbit IgG, attached particles were practically absent.

Affinity Separation of Coated Vesicles from Crude Fractions

MORPHOLOGICAL ASPECTS: The same protocol was followed in a series of experiments in which purified coated-vesicle fractions were replaced by cruder fractions, i.e., smooth microsomes. As illustrated by Fig. 6*A*, these fractions contained a variety of membrane-bounded vesicles, assumed to be derived from the endoplasmic reticulum, Golgi complex and plasmalemma of neuronal or glial cells. The fractions also contained readily identifiable coated vesicles which appeared as a minor component of the total population of vesicular and particulate elements. Staph A cells, reacted with anticlathrin IgG, preferentially immunoadsorbed coated vesicles from this heterogeneous mixture (compare Figs. 6*A* and 6*B*). In specimens processed for electron microscopy, anticlathrin-Staph A cell complexes were covered by a monolayer of coated vesicles and vesicle-free cages (Fig. 7*B*). The only other elements brought down by the Staph A-anticlathrin complexes were fibrillar structures that seemed to be attached to coated vesicles rather than directly to the immunoadsorbent (Fig. 8). Most of these fragments appeared to be derived from Staph A cell walls. Staph A cells complexed with preimmune IgG complexes were virtually free of adsorbed particles (Fig. 7*A*).

The first supernate derived after pelleting the Staph A with anticlathrin and immunoadsorbed particles was examined by electron microscopy and was found to contain a mixture of vesicles comparable to that of the starting preparation, except that coated vesicles were practically absent (Fig. 6*B*). This is in keeping with the results of ELISA assays (given below), which showed that 70% of the original clathrin content was immunoadsorbed onto the Staph A-anticlathrin complexes.

PROTEIN COMPOSITION: The immunoadsorbed complexes, eluted from Staph A cells by treatment with SDS-urea, were analyzed by SDS PAGE and found to contain a high molecular weight component with the mobility of clathrin (180,000 mol wt) (Fig. 9*B*); this band was absent when anticlathrin was replaced by nonimmune IgG (Fig. 9*C*). The two additional bands seen in lanes *B* and *C* at 50,000 mol wt and 25,000 mol wt are the heavy and light chains, respectively, of immune and preimmune IgG. The same bands appear in the electrophoretogram (lane *D*) of an eluate of Staph A cells reacted with immune IgG only (without exposure to coated vesicles). Only a negligible amount of a small mol wt protein was extracted from Staph A cells during the elution procedure (Fig. 9*E*). Eluted immunoadsorbates often contained BSA left behind from the immunoadsorption step (Fig. 9*F*); this residue was removable by extensive washing (compare 9*F* to 9*B*). In similar experiments, avian anticlathrin was also found to bind to Staph A cells, though less efficiently than the corresponding rabbit antibody (results not shown). The finding is of interest, since it is at variance with recorded data according to which avian IgG does not bind to protein A (26).[2]

ELISA ASSAY: An ELISA assay was used to quantitate the results of our separation procedure. A standard curve was obtained with clathrin (i.e., alkaline extract of a coated-vesicle fraction, see Materials and Methods) used as reference antigen (adsorbed to the solid-phase surface) as well as competing antigen for a constant amount of anticlathrin. The assay was linear for amounts of clathrin ranging from ~1 μg to ~0.1 ng (Fig. 10*A*). In Fig. 10*B* and *C* the reference antigen was the same as in 10*A*, but the competing antigens were a sample of a smooth microsomal fraction (either solubilized or nonsolubilized) in Fig. 10*B* and a sample of an eluted immunoadsor-

[2] Rabbit anticlathrin was preferred in our studies because it has an apparently higher affinity for its antigen, and it does not have the marked tendency to form aggregates shown by avian IgG.

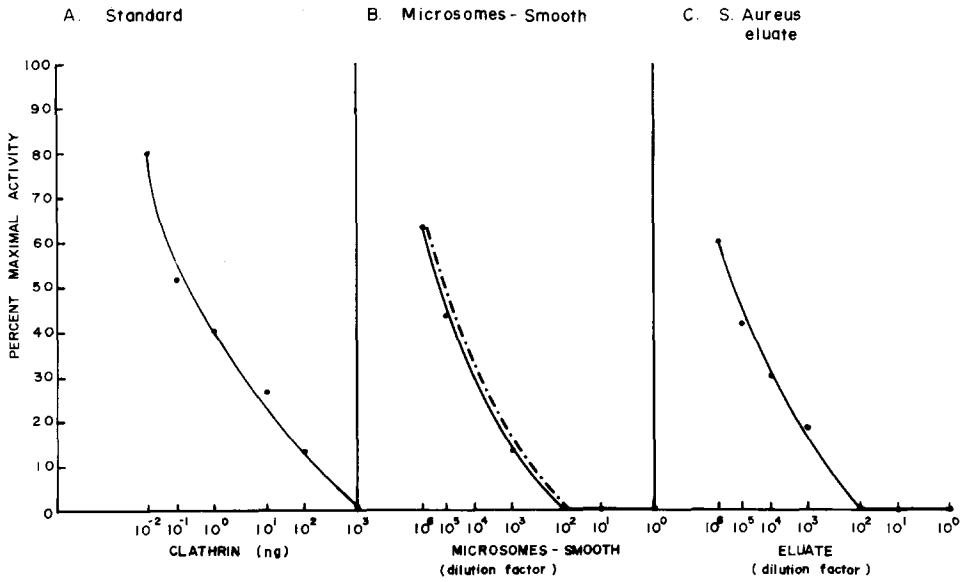


FIGURE 10 ELISA assays performed to determine the amount of clathrin in unknown samples (*B*) and (*C*) by means of a competition assay. The competing reagent was mixed with the antiserum immediately before being added to the clathrin-coated wells. After a 1-h incubation at room temperature, the nonadsorbed reagents were washed out of the wells; the protein A-HRP conjugate was added and incubated as described in Materials and Methods. HRP activity was determined and expressed relative to the maximal amount of HRP activity present in the precoated wells when no competing antigen was present. Appropriate antibody dilutions and optimal protein A-HRP conjugate concentration were established in preliminary experiments. (*A*) Standard Curve: represents the percent of HRP activity bound to the clathrin coated ELISA wells when various concentrations of clathrin were used as competing antigen. The assay was fairly linear for amounts of clathrin ranging from 1 ng to 1 μg. (*B*) Smooth Microsomal Fraction: percent of HRP activity bound in the presence of increasing amounts of a smooth microsomal fraction, with (——) or without (·—·—) prior solubilization in SDS and DTT. (*C*) Eluate: percent of HRP activity bound in the presence of material eluted with SDS and DTT from particles immunoadsorbed to anticlathrin-coated Staph A cells. Controls: when anticlathrin was omitted or replaced by nonimmune or preimmune serum, the assays gave uniformly low HRP activity values.

bate in Fig. 10*C*. By extrapolation from Fig. 10*A*, the amount of clathrin in the smooth microsomal fraction is ~15% of the total protein of this fraction and 70 to 80% of the clathrin content of the microsomal sample is recovered in the eluate.

The results indicate that anticlathrin-Staph A cell complexes can be used to isolate reasonably pure preparations of coated vesicles and vesicle-free cages in high yield from relatively crude fractions of brain homogenates; they also show that the clathrin distribution during the fractionation procedure can be followed quantitatively by ELISA assays.

CAGE ASSEMBLY OF ANTICLATHRIN-REACTED STAPH A CELLS: In another series of experiments, anticlathrin-coated Staph A cells were mixed with a urea extract containing clathrin prepared from a coated-vesicle fraction and dialyzed extensively against 0.1 M MES buffer, pH 6.5 (see Materials and Methods). When the Staph A cells were recovered by centrifugation and examined by electron microscopy, they were found to be covered by a discontinuous layer of clathrin cages (Fig. 11). Further experiments are needed to determine whether trimer formation (42) and repolymerization of such units into complete cages precedes immunoadsorption, or occurs around nucleation sites formed upon binding of clathrin or "triskelions" (42) to its insolubilized antibody.

DISCUSSION

Cell fractionation procedures in current use rely primarily on differences in general physical parameters (e.g. size, density, net surface charge, and solubility of surface components) to resolve tissue homogenates into distinct cell fractions (1, 8–10, 16, 37). Although the fractions obtained can be homogeneous in terms of a selected physical parameter (e.g. particle density in isopycnic centrifugation), they are not necessarily homogeneous in terms of particle biochemistry or function, even when prepared by elaborate fractionation procedures designed to attain physical homogeneity.

The results presented in this paper show that coated-vesicle fractions isolated by current procedures consist mostly of coated vesicles and vesicle-free cages, but are still heterogeneous in that they contain various vesicles and membrane fragments of unknown origin as contaminants. We have used such fractions to isolate clathrin (by SDS PAGE), and we have raised antibodies against the isolated clathrin in rabbits and chickens. The specificity of the antibodies was checked and confirmed by current immunochemical procedures, including immune replicas (39). In addition, the ability of these antibodies (raised against a denatured antigen) to recognize native or aldehyde-fixed clathrin was established by immunoadsorption of coated vesicles and by immunocytochemistry of cultured human fibroblasts.

The anticlathrin was insolubilized by interaction with Staph A cells, and the ability of the complex thus formed to bind coated vesicles and vesicle-free cages was tested by interaction with a "purified" coated vesicle fraction. Because the results were encouraging, the anticlathrin-Staph A complexes were used in an attempt to isolate coated vesicles and vesicle-free

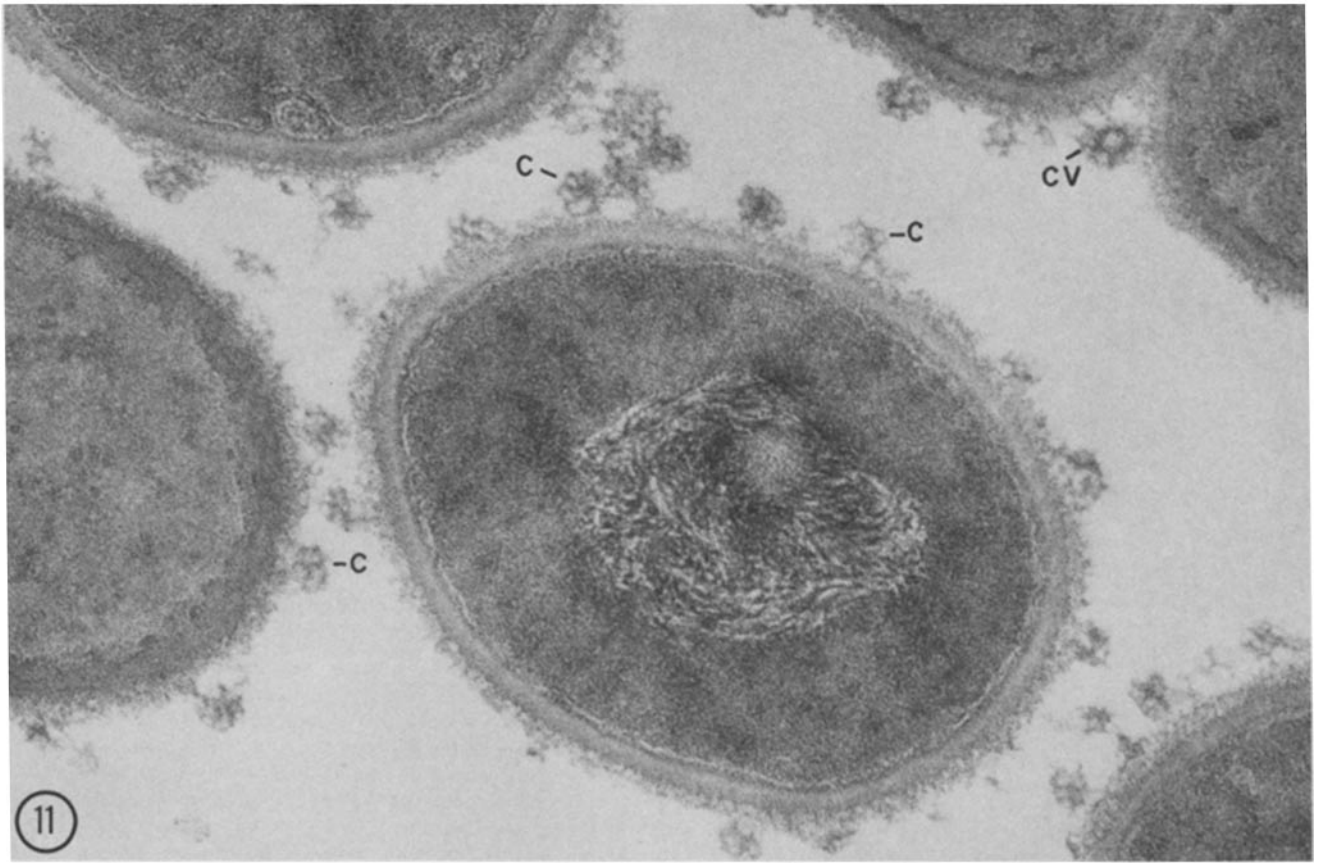


FIGURE 11 A preparation of clathrin (extracted from a coated vesicle fraction with urea) was added to Staph A cells preincubated with anticlathrin. The mixture was dialyzed for 3 d against MES buffer, i.e., under conditions conducive to repolymerization of clathrin cages. At the end of dialysis, samples were washed as described in Materials and Methods and the immunoadsorbent was processed for electron microscopy. Many clathrin cages (*c*) were found decorating the cell wall of the Staph A cells. A few coated vesicles (*cv*) were also recognized in the preparation. They may represent residual, uncoated vesicles still present in the extract. × 100,000.

cages from a crude smooth microsomal fraction prepared from pig brain homogenates. Coated vesicles are a minority component in this fraction: they represent <10% of the total particle population.

An enzyme-linked immunoassay (ELISA), developed and used to quantitate the removal of clathrin from the starting preparation, showed that the removal was efficient, and an electron microscope survey of the (Staph A plus anticlathrin plus bound particles) complexes revealed that the bound particles were either coated vesicles or vesicle-free cages that formed a discontinuous monolayer at the surface of the anticlathrin-coated Staph A cells; no other particle or vesicle of the starting preparation was bound to the immunoadsorbent. The absence of detectable clathrin in the nonimmunoadsorbed residue and its presence in the eluate of the immunoadsorbed material were confirmed by SDS PAGE.

The use of specific ligands, in this case anticlathrin, for the affinity separation of cell fractions has the salient advantage of obviating particulate contamination. Moreover, antibodies can be used to detect antigens common to various subcellular components which may have different physical properties but are biochemically and functionally related.

Immunoadsorption and immunoprecipitation were used by Kawajiri et al. (23) to subfractionate (rat) hepatic microsomes into vesicle populations bearing different antigens. The approach was adapted to an integrated structural-biochemical analysis of cell fractions by Ito and Palade (22) who used polyacrylamide beads coated with appropriate antibodies as immunoadsorbents. The beads were processed for transmission electron microscopy (TEM) to identify the immunoadsorbed particles by their morphology, and biochemical assays of adsorbed vs. nonadsorbed particles were carried out to characterize the separated vesicle populations enzymologically. With this procedure, it was possible to resolve hepatic Golgi fractions into vesicle populations with different enzymatic activities that are assumed to reflect the existence of biochemically differentiated domains within Golgi complexes *in situ*.

The procedure described in this paper follows the same experimental approach: it uses an insolubilized antibody as a specific ligand; it extends this approach to other subcellular components (coated vesicles and clathrin cages); and it provides a convenient, simplified variant (i.e., Staph A cells) for affinity separations. By virtue of the presence of protein A on their surface, Staph A cells bind the Fc segment of most antibodies with high affinity. Accordingly, it has the advantage that the critical ligand for affinity separation can be bound directly to the solid substrate (the Staph A cells), hence it is not necessary to insolubilize chemically a second antibody to the solid substrate. Moreover, removal of excess reagents by low-speed centrifugation can be effected rapidly and efficiently, and the processing of the specimens for microtomy and TEM can be carried out with less difficulty, because Staph A cells can be embedded and sectioned more readily than polyacrylamide beads. In principle, the same approach can be applied for the

isolation of any other subcellular component provided appropriate antibodies are available.

The immunoadsorption procedure described may prove especially useful in separating coated vesicles from the small amounts of starting material currently used in working with cultured cells. In such systems, separation by physical means is often inefficient. For coated vesicles and vesicle-free cages, the only reliable markers currently available are clathrin and the characteristic geodetic structure of the cages. The new separation procedure should make possible the isolation of homogeneous preparations in sufficient yield to permit a more extensive and reliable characterization of these ubiquitous structures than now available.

Finally, the ability of reconstituting clathrin cages at the surface of a solid immunoadsorbent opens the possibility of analyzing the reassembly process by step-wise addition of reactants. This approach may define the reagents (proteins) and the environmental conditions needed in the reassembly process.

We would like to acknowledge the excellent technical assistance provided by Bonnie Peng, Hans Stukenbrok, and Pam Ossorio throughout the course of this study. Also, special thanks are extended to Lynne Wootton and Cynthia Davis for secretarial assistance, and to all members of the Cell Biology Section who provided useful suggestions and critical discussions of the data.

This research was supported by research grant AM 17780 (to M. G. Farquhar) and GM 21714 (to G. E. Palade) and National Service Award GM 07562 from the National Institutes of Health.

Received for publication 8 June 1981, and in revised form 22 October 1981.

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