BLOOM, WILLIAM SAMPSON

BRAIN CLATHRIN: ULTRASTRUCTURAL, BIOCHEMICAL AND IMMUNOLOGICAL STUDIES

City University of New York PH.D. 1980

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BRAIN CLATHRIN: ULTRASTRUCTURAL, BIOCHEMICAL AND IMMUNOLOGICAL STUDIES

by

WILLIAM SAMPSON BLOOM

A dissertation submitted to the Graduate Faculty of Biomedical Sciences, in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New York

1980

Dedicated to my family, with love.

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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FOREWORD

Among the many cellular events awaiting elucidation, endocytosis is the process whose molecular nature is least understood. Morphologically, a coated vesicle is the organelle believed to play a pivotal role in endocytosis; an advance in understanding endocytosis could be accomplished if coated vesicles were fractionated, the coat components separated, the proteins identified and properties characterized. At the time this study was undertaken, steps in the enrichment of coated vesicles and identification of their components had been reported. It was felt, therefore, that the isolation and characterization of clathrin--the major element of the coat--was the logical next step and an attainable goal. Clathrin was purified to a high degree and its ultrastructural features analyzed. The finding that clathrin formed baskets or cages *in vitro* convinced researchers that clathrin is the main structural protein of the coat. The next step was to specifically localize clathrin in cells. Its antigenicity permitted antibodies to be prepared and purified. In this way, clathrin was visualized in cells as lattices giving a distinct pattern. The fact that antibodies to brain clathrin recognized clathrin from other cells suggested that it is phylogenetically well preserved, widespread, and essential for cell functions. It is hoped that the studies herein described will effect a better understanding of the endocytotic process in particular and its contribution to cell functions in general.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF CHARTS & FIGURES

INTRODUCTION

1. Receptor-mediated Endocytosis:

The first observations of coated vesicles were made with the electron microscope by Porter (1), Fawcett (2) and Gray (3), during the 1960's, at a time when electron-microscopic techniques dealing with the tissue specimen, as well as with the instrument *per se*, were becoming highly refined. These coated vesicles were assigned various names such as "bristle-like" (4), "complex" (5), "alveolate" (6), or "vesicles in a basket" (7). Fortunately, all of these different names now refer to a single morphological entity: the coated vesicle.

The first role attributed to coated vesicles, that of selective, receptor-mediated uptake of macromolecules, was proposed by Roth and Porter (4), while working with mosquito oocytes. From studies by Telfer (8), such oocytes were known to take up selectively certain yolk proteins. During the resting state, the cortex of the oocyte was covered by many unoriented villi. Within the border of the microvilli were many pit-like invaginations which had bristle-like spikes (coats) on their cytoplasmic sides. Roth and Porter found that after incubating the eggs with the yolk proteins, large numbers of coated invaginations and coated vesicles formed containing the yolk proteins.

Such change in morphology was absent in controls. These workers hypothesized that the yolk proteins were bound specifically to a receptor site on the membrane surface that had a juxtaposed protein coat underneath on the cytoplasmic side of the membrane. Once receptor-ligand binding took place, the membrane invaginated and pinched off, forming coated, micropinocytotic vesicles.

Later work gave support to this hypothesis (9-16). Rodewald (17) looked at the jejunal epithelial lining in 10-day-old rats. These young rats were known to acquire passive immunity by absorbing protective IgG from mother's milk through the gut. The mechanism of absorbing intact protein was studied at the electron microscope level in this work. It was found that a ferritin-labelled IgG was selectively pinocytosed into the cell via coated invaginations at the base of the microvilli. The endocytosed IgG was transported via coated vesicles to the basolateral part of the cell and then exocytosed into the intercellular space. This uptake, intracellular transport, and exocytosis to the intercellular space was accomplished through a coated vesicle system. Control chick, ferritin-labelled IgG, ferritin-labelled bovine serum albumin (BSA), and free ferritin neither entered the cells nor the intracellular space, nor did adult rat jejunum absorb rat ferritin-IgG. Disappearance in the adult of a large number of coated invaginations at the base of the microvilli coincided with the loss of

passive immunity. Thus it apppeared that one of the basic cellular mechanisms for pinocytosis involved coated-membrane uptake of receptor and ligand, with the formation of coated vesicles.

2. Golgi-associated Coated Vesicles:

Recently, a considerable number of papers have shown association between coated vesicles, the Golgi[1] cisternae, and the GERL[2] system in different cell types. The Golgi and GERL consistently contained coated membranes which appeared to pinch off and form coated vesicles. Studies have shown that some of these Golgi/GERL-derived coated vesicles contained lysosomal hydrolases destined to fuse with lysosomes. The Golgi/GERL-derived vesicles also fused with surface membranes in what was intepreted as involvement in the turnover of plasmalemma. Both of these functions relate to the concept of membrane translocation within the cell (18-22).

In their study of the fine structure of the hepatocyte, Bruni and Porter found many small coated vesicles (750 Å) associated with the Golgi apparatus (1). Two general types of proteins were identified morphologically in the Golgi: lysosomal-like enzymes, and proteins to be secreted

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[1] Membranous cisternae usually located in a perinuclear position representing the first step of a synthesizing process for polypeptides, their storage and eventual distribution to other areas of the cytoplasm.

[2] Vesicular system related to Golgi cisternae located further into the cytoplasm serving as a conversion site for products arriving from the Golgi or from the plasma membrane.

from the hepatocyte. Coated Golgi membrane pinched off from the cisternae, formed coated vesicles, and eventually fused to form multi-vesicular bodies, then lysosomes specifically containing the lysosomal enzymes. The other smooth vesicles budding off from the Golgi cisternae contained the secretory proteins. Thus a role for coated vesicles in *segregating* the proteins of the Golgi cisternae was hypothesized.

Friend and Farquhar studied the morphology of protein reabsorption in the rat vas deferens (15). They found two populations of coated vesicles in the epithelial-lumenal cell. Large coated vesicles, 1,000 Å in diameter, were involved in uptake of horseradish peroxidase from the lumen into the cell. The protein was transported by the coated vesicles to lysosomes (multi-vesicular bodies). The other population of coated vesicles, averaging 750 Å in diameter, was associated with the Golgi apparatus. They contained reaction product when treated with thiamine pyrophosphate, Na β-glycerophosphate and cytidine 5'-monophosphate respectively, indicating the presence of hydrolytic, Golgi-derived enzymes. Finally, these smaller coated vesicles were observed fusing with the lysosomes. Ten minutes after the introduction of peroxidase into the vas deferens lumen, not only was the tracer found specifically in the large coated vesicles, but also there was a dramatic increase in the number of Golgi-associated coated vesicles which did not contain tracer. After 20-40 minutes, the lysosomes were engorged

with tracer, and the smaller, tracer-less, Golgi-derived coated vesicles were in the apical cytoplasm fusing with the luminal membrane. It was theorized that the Golgi-derived coated vesicles not only transported proteolytic enzymes to the lysosomes, but also replaced lumenal membrane lost to the endocytotic large coated vesicles.

Recently, elegant work on the Golgi apparatus and the GERL system by Novikoff and Jahen *et al.*, showed a close association between the GERL, coated vesicles and lysosomes (23, 24). The GERL-associated coated vesicles were of the smaller variety (750 Å). Their hypothesized role was the transport of acid hydrolases and other functional substances to newly formed lysosomes. Secretory products present in the GERL could be transported to the cells' peripheries through a coated vesicle system. Jaeken (25) discussed a wide range of functions for coated vesicles in the regenerating hepatocyte. He felt that there was a coordinated movement of membrane from the Golgi/GERL coated region(s) to lysosomes and from the plasma membrane via transfer tubules to multi-vesicular bodies. This organized movement of membrane appeared mediated through coated vesicles.

3. Recycling of Vesicles in Brain Nerve-endings:

Various studies implicated coated vesicles in the replenishing of synaptic vesicles in presynaptic neuronal

endings. Birks (26) and Ceccarreli (27) showed that after stimulation of neuronal elements there was depletion of synaptic vesicles, concomitant with transmitter release. Birks found a depletion of synaptic vesicles in the superior cervical ganglion of cats after stimulation at a frequency of 20 per second for one minute or one per second for 20 minutes. Since there was no increase in surface area of the presynaptic membrane, the disappearance of synaptic vesicle membrane could not be accounted for. Ceccarrelli did similar work on the frog sartorious neuromuscular junction. He stimulated the muscle at 2 Hz per second for 4-9 hours which resulted in the disappearance of synaptic vesicles and a concomitant swelling of the nerve terminals. It was hypothesized that synaptic vesicles reformed locally from plasmalemma since more transmitter was being released than the depletion of synaptic vesicles could account for (28).

Direct morphological evidence for local synaptic vesicle reformation in neuromuscular junction was obtained by Heuser and Reese (29). Stimulated frog muscle at the physiological levels of 10 Hz for 1-15 minutes showed synaptic-ending buttons depleted of vesicles with a simultaneous increase in membrane surface area at the plasmalemma and the internal cisternae of the neuronal bouton. Recovery from stimulation consisted of a decrease in the plasmalemmal and cisternal surface areas back to their original states and

the formation of synaptic vesicles, probably from the membranous cisternae. The recovery process, monitored by sequential addition of horseradish peroxidase, showed the tracer transported from the extracellular space to coated vesicles, to smooth membranous cisternae, and finally to plain synaptic vesicles. Tracer was not placed directly into synaptic vesicles, arguing against the theory of transient vesicular attachment and immediate synaptic vesicle reformation without actual fusion (30). The total amount of membrane in the synaptic vesicle pool, plus the plasmalemmal membrane, plus the coated vesicles and cisternal membrane were constant. An increase of one membrane compartment resulted in a decrease of another; *i.e.*, when the synaptic vesicles were depleted, the cisternal and plasmalemmal membrane surfaces and the population of coated vesicles increased. Thus, coated vesicles appear to function as the membrane retrieval system in the rat neuromuscular junction (31).

Further work by Heuser and Reese (32, 33) showed, through freeze fracturing, that vesicle fusion and movement within the plasmalemma could be monitored by observing the dense particles imbedded in the synaptic vesicle membrane. Upon release of transmitter, the dense particles fused with the plasma membrane. The dense particles moved laterally in the membrane and then were retrieved back into the cytoplasm at the junction where coated vesicles were known to form.

It was hypothesized that the coat protein recognized the dense particle as a specific marker for synaptic vesicle membrane, and retrieved the membrane back for recycling.

Involvement of coated vesicles in synaptic vesicle recycling was found to extend beyond cholinergic or peripheral synapses. The turtle retina (34), rat synaptosomal preparations (35), and Mauther cells of vertebrate cerebellum (36) all have a similar pattern of synaptic vesicle depletion, surface membrane increase, and finally a recycling of membrane via a coated vesicle mechanism. Coated vesicles also are involved in the recycling of synaptic vesicle membrane in the post-ganglionic synapses of the autonomic nervous system (37). Occurrence of coated vesicles in various stimulated neuronal systems points to a basic role for coated vesicles in the normal nervous system function of many different species (Fig. 1).

4. Synaptogenesis:

Rat brain coated vesicles were implicated in the process of synaptogenesis from studies performed on cell cultures and rat brain slices. Altman (38) observed the formation of synapses in brain slices in successively older rats starting at birth and continuing until an age of 21 days. During this period, the rat brain rapidly developed synaptic connections. The actively developing young brain tissue (up to 7 days old) had numerous coated vesicles in

Figure 1. Thin section electron micrograph of a bovine brain synaptosomal preparation--This figure shows 3 synaptosomes with plain vesicles (PV), coated vesicles (CV), mitochondria (M), and cisternae (C). Two coated vesicles apparently in process of formation can be visualized. Magnification: 120,000 x.

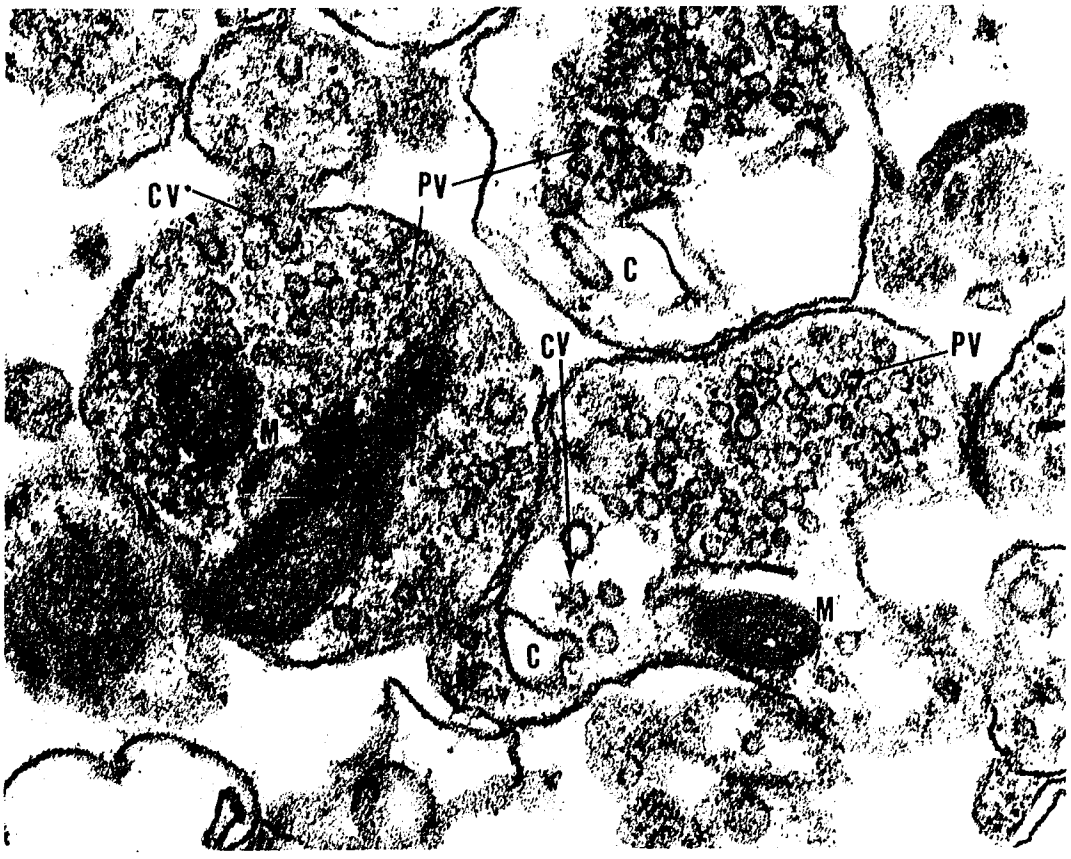


FIGURE I

the perinuclear region and in association with the Golgi apparatus of Purkinje and granule cells in the cerebellar cortex. Both of these cells formed numerous dendritic (post-synaptic) connections. Some coated vesicles seen at this stage of development in the vicinity of the cell membrane, were opened into the extracellular space and resembled flask-shaped pinocytosomes. Significantly, those flask-shaped coated invaginations were situated opposite cell processes with which synaptic connections would later develop in the older animal. Parallel fibers were situated inside the invaginated coated membrane of the developing Purkinje dendrites. Altman felt that the coated membrane formed attachment sites for the parallel fibers in what would later become a synaptic connection. The morphology suggested a process in which coated membranes budded off from the Golgi apparatus (of granule and Purkinje cells) and moved outward toward the cell membrane in what would later be the dendritic, post-synaptic element. This post-synaptic specialization would attract and attach axonic connections for future developing synapses.

Rees, Bunge and Bunge studied synaptogenesis in a culture of rat superior cervical ganglion cells and spinal cord cells (39). The spinal cord cell (pre-synaptic element) grew out and contacted the ganglion cell (post-synaptic element) and a synapse developed. It was found that the first synaptic developmental events were in the Golgi appa-

ratus of the ganglion cell. There was hypertrophy of the Golgi with formation of many coated pieces of membrane pinching off from the cisternae. A large number of coated vesicles accumulated under the surface membrane opposite the pre-synaptic connection site. Coated pieces of membrane with dense material (which later would constitute the post-synaptic density) fused with the membrane surface. Direction of membrane flow from the Golgi via coated vesicles to the plasmalemma was determined by tracer/labelling studies. Only after the post-synaptic dense material was inserted (via coated vesicles) into the membrane surface did pre-synaptic differentiation begin. These studies of coated vesicles in the process of synapse formation suggested that the coated membrane in the post-synaptic element was involved in the specialization of this area for neurotransmission. In both cases, membrane moved from the Golgi cisternae to the cell surface via coated vesicles.

5. Biochemical Characterization:

The biochemical investigation of the protein and lipid composition of coated vesicles began with the studies of Pearse who adapted the subcellular fractionation method of Kanaseki and Kadota (7) to obtain a subfraction enriched with coated vesicles. The enriched fraction of coated vesicles was morphologically homogeneous enough to start meaningful biochemical analysis. By weight, the enriched fractions of coated vesicles contained 3 times more proteins

than lipids. The lipid composition consisted of 43% phosphatidyl choline; 30% phosphotidyl ethanolamine; and 12% spingomyelin. In sodium dodecyl sulfate gels about half of the total protein present migrated to an electrophoretic zone equivalent to a molecular weight of 180,000. This protein, which appeared to be the major protein of coated vesicles, was named "clathrin," after the Greek word *"clathri,"* meaning latticework (40).

It was reported that protein composition of coated vesicles from different tissues and species was remarkably similar (41). Sodium dodecyl sulfate gel electrophoretic patterns and peptide maps from varying coated vesicle preparations showed striking homologies. Coated vesicles from rat, pig, rabbit, and beef brains, bullock adrenal medulla, chicken oocytes (42-45), and a lymphoma cell line (41) all contained a similar major polypeptide band representing over 60% of the total protein population and migrating to a 180,000 molecular weight zone, clathrin's reported molecular weight. About 10% migrated to a 100,000 molecular weight zone; another 10% to a 55,000 molecular weight zone (46). Researchers felt that clathrin was the sole protein of the coat surrounding the vesicle, while the 100,000 and 55,000 molecular weight proteins were integral parts of the vesicle membrane *per se*. The role of the last two, in effect, would serve to anchor clathrin's basket-like assembly to the vesicle membranes. These assumptions were based on experi-

ments in which certain buffers dissociated clathrin molecules from coated vesicles while the other two proteins always pelleted with the vesicle membrane (47, 48).

The striking homology of sodium dodecyl sulfate gel banding patterns between coated vesicles from different sources correlated with their similar morphologies (40, 41). Coated vesicles' diameters from varying sources consistently ranged between 600-1,000 Å. The vesicle membranes' diameters held within the basket-like protein shells ranged from 300-600 Å. Electron micrographs of negatively stained clathrin, and tangential, thin sections showed the coat shell to be made of pentagons and hexagons arranged in polyhedral, lattice-like arrays. The coats forming closed shells around vesicles contained at least 12 pentagons, ensuring sufficient curvature for closure. The number of hexagons varied depending on the size of the baskets formed. On cross section, the edges of the polygons appeared as spokes radiating from the central vesicle, and were 150 Å in length. Thickness of the edges of the polygons was approximately 50 Å. This morphology was remarkably constant across species and tissue types (40-45).

Antibodies elicited by crude coated vesicle extracts consisting of 80-90% clathrin were raised to estimate the amount of clathrin in various tissues by a radioimmunoassay. Also, the antibodies were used to probe the degree of homology of coat protein structures from different sources. The

coated vesicles from rat brain competed almost as well as the calf brain coated vesicles, indicating considerable immunochemical homology between species. The coated vesicles from calf adrenal medulla and parotid gland competed immunologically to a lesser extent, indicating immunochemical differences between tissues. An immunoprecipitate assay showed that clathrin composed of 0.4% of the soluble protein in a brain supernatant, and 0.2% in a human diploid fibroblast supernatant (49).

AIMS OF THIS STUDY

1. General Considerations:

At present, all the information available points to an important role for clathrin and coated vesicles in the economy of cells. Little is known of the mechanism governing membrane turnover, movement of vesicular organelles and compartmentalization of compounds within the cytoplasm. Such mechanisms undoubtedly are complex, and probably involve interrelated mechanochemical protein systems. Although the complete role of clathrin, free or associated with vesicles, remains to be elucidated, it seems this protein interacts with the plasma membrane, with the internal cisternae system, and possibly with the cytoskeletal proteins during cellular events.

Morphological observations are strong evidence for the involvement of clathrin with cytoplasmic organelles. The visualization of coat protein surrounding just that part of the membrane which is invaginating or fusing, and nowhere else along the plasmalemma, indicates a distinct specificity of structure and function. Since the coat of a variety of vesicles is similar in appearance in tissues ranging from mammals to plants, it is apparent that the protein forming basket structures is conserved phylogenetically because of its biological importance.

Biochemical determinations also point to a basic role for all clathrins. The homology of sodium dodecyl sulfate gel polypeptide banding patterns and of peptide maps from coated vesicle preparations across tissues and species is striking.

Although the coated vesicle pathway has been recognized for years as being potentially significant, little work has been done specifically on the characterization of the proteins involved. Understanding its properties could explain how important, yet poorly defined cellular processes take place such as endocytosis, membrane turnover, and transport of macromolecules through the cytoplasm. The biochemical and biophysical characterization of the components of coated vesicles is viewed as crucial to an understanding of special cellular events.

2. Specific Considerations:

This study of the coated vesicle protein was designed to attain three main objectives. The first objective was to purify the major constituent of the protein coat. Because of the importance of membrane recycling, neurotransmitter uptake and the coated vesicles in nerve endings, brain tissue was selected as the source for the preparations of clathrin. As a rule, isolation of proteins from brain is difficult due to its complexity and the amount of lipids present. Adequate research techniques exist for the subcellular fractionation of organelles from brain tissue.

Homogenization followed by differential centrifugation (separation by differences in size, density, and shape) and density gradient centrifugation (separation by buoyant density) result in the preparation of enriched fractions of nuclei, mitochondria, synaptosomes, plasma membrane fragments, microsomes, myelin, synaptic vesicles and soluble, non-particulate cytoplasmic constituents (59). Using such fractions, enrichment of proteins associated with any particular organelles can be achieved.

The literature describes procedures for obtaining enriched, crude coated vesicles based on such techniques. Due to its large molecular weight, compared to other coated vesicle constituents, clathrin figured to be a relatively simple protein for separation by column chromatography. Standard biochemical techniques for protein isolation were to be explored in an attempt to purify the protein species.

The second objective was to characterize clathrin *in vitro*. Could clathrin form reversibly basket or cage structures *in vitro* and, if so, what biochemical and biophysical conditions favored such assemblies? Such information would be important to establish if clathrin is the only protein of the coat and also to understand coated-vesicle dynamics. Parameters such as dependance of clathrin on the ionic environment, nucleotides, reassociation with membranes, viscometry and turbidity of the protein polymers also could be determined.

The third objective was clathrin's immunological localization. Antibodies to the purified coat protein could help localize it, estimate its content. and its distribution in cells and tissues. Immunocytochemical techniques such as indirect immunofluorescence could show its localization by light microscopy. Peroxidase/anti-peroxidase staining could be used both on the light and electron microscopic levels for the ultrastructural localization of clathrin in particular tissue preparations. With these objectives in mind, my efforts developed as hereinafter described.

EXPERIMENTAL PROCEDURES

1. Isolation of Coated Vesicles from Bovine Brain:

Bovine brains were delivered in ice from a local slaughterhouse. The brains were cleaned of meninges and the gray matter separated from the white matter by suction. For the preparation of coated vesicles, the gray matter from 3 bovine brains was homogenized by three 10-second bursts at 1-minute intervals using a Waring blender at top speed, with 400 ml of 0.1 M Tris-HCl, pH 7, containing 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 0.02% sodium azide. The homogenate was centrifuged at 20,000 x g for 30 minutes, the supernatant decanted, and further centrifuged at 100,000 x g for 60 minutes. The crude, coated vesicle pellet was extracted by 10-12 strokes of a Teflon glass homogenizer with 50-100 ml of 0.02 M Tris-HCl, pH 7.5, containing 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.02% sodium azide, and left standing for 16 hours. The crude coated vesicle extract was centrifuged at 100,000 x g for 60 minutes to remove membrane fragments.

The supernatant was made 30% saturated in ammonium sulfate by addition of solid crystals and the pellet obtained after centrifugation at 15,000 x g resuspended and dialyzed against 20 mM Tris-HCl, pH 7.5, 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.02% sodium azide. When necessary, the protein preparation was stored frozen at -20°C in 10% sucrose, or used for further purification.

2. Chromatography on Sepharose 4B:

The crude clathrin solution was dialyzed overnight against the buffer used for column chromatography. A volume of 10 ml protein containing approximately 8-10 mg/ml was layered gently on top of a Sepharose 4B column, 2.5 x 90 cm. The column was run with the aid of a peristaltic pump at 12 ml/hour. Eluting fractions were read by absorbance at 280 nm; the protein peaks pooled, concentrated by 50% ammonium sulfate precipitation and dialyzed against the appropriate buffer. They were stored frozen as indicated previously or used for further purification.

3. DEAE/Ion-exchange Chromatography:

Fibers of DEAE-cellulose (Whatman DE·52) were swollen in the appropriate running buffer and loaded into a column of 1.5 x 25 cm. Protein dialyzed against the buffer used for column equilibration was loaded into the column and allowed to adsorb at a rate of 10 ml/hour. The unadsorbed material was allowed to elute from the column by running with the same buffer and the adsorbed protein eluted with a continuous gradient of 0-0.6 M KCl dissolved in the same buffer. A flow of 10 ml/hour was produced with the aid of a peristaltic pump of a gradient built from a 2-chamber mixer with constant agitation. Fraction volumes of 2-3 ml were collected, read at 280 nm and samples of representative parts of the peaks run on polyacrylamide gels for the determination of their protein composition.

4. Sodium Dodecyl Sulfate Acrylamide Gel Electrophoresis:

Single cylindrical gels of 7.5% polyacrylamide were prepared. Protein samples (25-100 µg) were loaded on top of the gels and an electrical current applied for about 16 hours until the tracking dye reached the gel bottom. Proteins were solubilized in 1% sodium dodecyl sulfate and reduced by 0.1% 2-mercaptoethanol before application to the gel.

Slab gels of sodium dodecyl sulfate acrylamide 5-20% were prepared with the aid of a peristaltic pump and a double-chamber gradient mixer. Gel lanes were loaded with 10-25 µg of protein and run under an electrical current for 16 hours at room temperature. The gels were fixed in 45% methanol, 7% acetic acid for 2 hours at 37°C, stained with 0.5% Coomassie blue, and destained by diffusion in 7% acetic acid, 5% methanol (55).

5. Protein Determination:

Total proteins were estimated by the Lowry method (56) using bovine serum albumin as the standard. Samples were prepared in triplicate and the absorbance read at 750 nm in a Beckman spectrophotometer.

6. Electron Microscopy:

For negative staining electron microscopy, appropriate amounts of protein in solution were mixed with buffers of various compositions and pH's and aliquots (4-5 µg of total protein) placed on Formar-coated grids, left for 20 seconds,

drained, stained for 2 minutes with 1% uranyl acetate, air-dried and examined in a JEOL 100 B electron microscope at 80 Kv.

For trasmission microscopy, pellets of vesicles, membranes or other proteins were fixed in 2.0% gluteraldehyde, 0.1 M sodium phosphate buffer or cacodylate buffer, pH 7.4, for 1 hour at 4°C. They were washed in the phosphate or cacodylate buffer overnight, stained with 1% osmium tetroxide, washed, dehydrated with ethanol and embedded in Epon. Thin sections were cut, placed on grids, stained with uranyl acetate and lead citrate, and observed in a Jeol 100 B electron microscope at 100 Kv.

7. Determination of Binding of Nucleotides and Divalent Cations:

For binding of ATP, equilibrium dialysis was used. Aliquots of stock adenosine 5' triphosphate, tetrasodium salt (ATP, New England Nuclear) [8-^{14}C] were dried in a glass vial and dissolved in 500 µl of 20 mM Tris, pH 7.5. Appropriate volumes were withdrawn from a stock solution of 10 mM cold ATP and diluted in Tris buffer. The ATP mixture concentrations, radioactive plus non-radioactive, were adjusted to a range of 1-100 µM. Each side of the dialysis chamber contained equal volumes of fluid with the ATP mixture added to the opposite chamber side of the protein. The protein chamber contained 0.8 ml of purified clathrin and a total amount of protein of 1.8 mg. The solutions were introduced or withdrawn through an orifice leading to each

chamber by means of a micropipette. Cuvettes were allowed to equilibrate in the cold room with gentle shaking. Aliquots of 50 µl were dissolved in 5 ml of aquasol solution and radioactivity measured with a model 2405 Packard scintillation counter.

For determination of Ca^{++} binding, the protein, after incubation with a mixture of ^{40}Ca and ^{45}Ca, adjusted to a final concentration of 50 µM, was loaded in a Sepharose 4B column to determine if the protein peak contained ^{45}Ca bound. The $^{40}Ca/^{45}Ca$ stock solution was prepared as follows: 5 µl of ^{45}Ca (new England Nuclear 4-30 Ci/g) in a volume of 4 ml of 20 mM Tris-HCl, pH 7.2, containing 10 mM $^{40}Ca^{2+}$. A protein volume of 0.6 ml (1.8 mg protein) was incubated at room temperature for 1 hour with 6 µl of the $^{40}Ca/^{45}Ca^{++}$ mixture. The protein-calcium solution was loaded onto a 1.5 x 30 cm column packed with Sephadex G 100 equilibrated with 20 mM Tris-HCl, pH 7.2. The column ran at 1 drop every 10-12 seconds. Fractions of 1 ml were collected in tubes. Protein concentration was monitored by the method of Lowry, and radioactivity measured in a Packard Model 2405 scintillation counter.

8. Sucrose Gradient Centrifugation:

Discontinuous gradients of 5 ml each of 10, 20, 30, 40, 50, 55, and 60% sucrose dissolved in various buffers were prepared. Approximately 1-2 ml of clathrin assembled as baskets at pH 6.5 in 20 mM Tris-HCl at a concentration of

3-4 mg/ml were layered gently on top of the gradients, and centrifuged in a swinging bucket rotor SW 27 at 27,000 rpm in a Beckman ultracentrifuge for 16 hours at 4°C. Gradients were fractionated with the aid of a peristaltic pump into 1 ml aliquots, and protein read by absorbance at 280 nm.

9. Turbidimetric Determinations:

Clathrin's turbidity was measured at 425 nm using a Beckman Model 25 recording spectrophotometer at room temperature. Clathrin in solution was pumped continuously through a microflow cuvette using a peristaltic pump (Buchler Instruments) from a reservoir of about 3-4 ml of fluid agitated gently with the aid of a magnetic bar and electromagnetic stirrer, wherein a combination electrode monitored pH changes. The pH was adjusted by addition of calibrated volumes of 0.5 M MES buffer, pH 6.0, or 0.5 M Tris-HCl, pH 7.5. At selected pH values, samples were withdrawn for viscosity measurements, if necessary, or for electron microscopic analysis by negative staining in order to correlate turbidity changes with other parameters or with the ultrastructural organization of clathrin.

10. Viscometric Determination:

An Oswald viscometer with a capillary flow time of 335 seconds for distilled water was used. Viscometric determinations were performed in a constant temperature bath at 25°C. At each pH change, a sample was removed for electron microscopy. Protein aggregates that occasionally formed as a

result of the passage of clathrin through the capillary tube were removed by centrifugation at 37,000 x g for 10 minutes to prevent clogging of the viscometer. The values showing viscosity changes of clathrin as a function of concentration were obtained by gradual dilution and clarification by centrifugation at each determination point. The solution of clathrin used for this purpose was free of urea and filaments.

11. Double-gel Immunodiffusion:

Antibody/antigen activities were followed by the formation of immunoprecipitates in agarose medium. A 0.85% agarose gel was made 0.05 M in barbital buffer solution, pH 8.6 (10.31 gm Na barbital, 6.8 gm Na acetate, 1.86 gm barbital, and 0.01% Na azide in a volume of 1 L). The gel was made by heating the suspension in a boiling water bath until the agarose-indubiose-A 45 powder dissolved using gentle stirring. The solution was divided into vials with 11 ml aliquots, cooled and stored at 4°C until needed.

For immunological formation of precipitates in agarose gels, a tube containing solidified agarose was warmed by immersion in water at 90°C and the soft agarose spread on microscope slides and allowed to harden in a humidified chamber at room temperature. Wells were bored and the suitable proteins loaded in volumes of 25 µl. Reactions were allowed to proceed for 24-72 hours at room temperature and the excess protein allowed to diffuse from the gel by immersion in 0.9% NaCl solution for 24 hours, followed by

immersion in distilled water for 16 hours. Gels were dried, stained and destained as explained in the next section.

12. Immunoelectrophoresis:

Immunoelectrophoresis was performed in 1% agarose dissolved in 0.1 M veronal buffer (0.05 M diethyl barbituric acid, 0.05 M sodium diethylbarbiturate, 0.05 M sodium acetate), pH 8.6. Ten ml of the melted agarose-indubiose A 45 was layered in each side of the immunoframe containing 3 glass slides (25 mm x 75 mm). The antigens were placed in the wells (10 µl) and were electrophoresed for 120 minutes at 10 mA/frame. The antibodies were then placed in the trough and allowed to diffuse overnight at room temperature in a humidified container. The frames were stained in 0.1% amido black 10 B and destained by diffusion in a solution of methyl alcohol:glacial acetic acid:water (45:5:50 v/v). Additionally, specificity of antibodies elicited by clathrin was determined by slicing acrylamide gels after resolution of clathrin from accompanying proteins by disc gel polyacrylamide electrophoresis. The slices were placed in rows, embedded in 0.85% agarose, wells bored between 2 rows and filled with apprpriate antisera. After the precipitin lines were formed, the acrylamide gel slices were removed gently by suction and the agarose gels stained and destained as described above. Parallel acrylamide gels were processed for the localization of clathrin bands as described in the section on disc gel electrophoresis.

13. Raising of Antibodies:

White New Zealand rabbits were immunized with clathrin. Three different types of preparation were used in separate animals for injection. The first preparation consisted of 1 mg of highly purified clathrin emulsified in complete Freund's adjuvant. Typically, 0.5 ml of a clathrin solution (2 mg/ml) was emulsifed with 0.5 ml of complete Freund's adjuvant by repetitive passage through a 20-gauge, double-connected needle syringe tip. The second preparation consisted of Lytron particles coated with clathrin. The Lytron particles were coated by incubating 20 mg of Lytron with 2 mg/ml solution of highly purified clathrin in 20 mM Tris-HCl, pH 7.2, at room temperature for 10-20 minutes. The Lytron particles were spun down at 20,000 x g for 15 minutes, and the pellet resuspended in 0.5-1.0 ml of 20 mM Tris, pH 7.2. This suspension was injected as such into the subcutaneous sites along the back of the rabbit. The third preparation used to elicit antibodies in rabbits consisted of clathrin polypeptides resolved on cylindrical sodium dodecyl sulfate polyacrylamide containing approximately 75 µg of purified clathrin. A parallel, unsliced gel, used for control, was fixed and stained for clathrin visualization to indicate the position of clathrin's band migration in the unstained gels. The gel slices with clathrin were emulsified in an equal volume of complete Freund's adjuvant and used for injection. Initial immunizations with all preparations were done at multiple subcutaneous sites in the neck and on the back of the animal.

One month following the initial injections, a small bleeding from the ear vein was performed; the blood was allowed to clot; and the serum was separated by centrifugation. When positive reaction for immunoreactivity was obtained, rabbits were bled from an ear artery or by intracardiac puncture under mild anesthesia, using 0.25 ml of Nembutal per Kg. Animals were boosted and bled 10 days later. Sera were not pooled, and samples were stored in small aliquots at -20°C.

14. Affinity Chromatography--Coupling of Protein to Gel:

Highly purified clathrin was bound covalently to a determined amount of CNBr Sepharose 4B. Protein solutions were at a concentration of 8.5 mg/ml. Three grams of CnBr-Sepharose 4B powder were weighed out and placed in a beaker. The powder was swollen into a gel by adding 50 ml of 1 mM HCl with stirring. The swollen gel solution was placed on a sintered glass filter and with gentle suction washed with 500 ml of 1 mM HCl. The protein for coupling, after overnight dialysis, against 0.1 M $NaHCO_3$--0.5 M NaCl--pH 8.3 (coupling buffer), was mixed with the gel suspension and placed in large test tubes with screw caps. The protein-gel mixture was gently mixed for 2 hours at room temperature. Uncoupled excess protein was removed by washing with coupling buffer to gel on sintered glass filter. Remaining uncoupled groups were blocked by suspending the protein coupled gel in 1 M ethanolamine for 2 hours. The blocking reagent and

excess uncoupled ligand were washed away with buffer solutions on a glass filter. The final product was washed alternately with low and high pH of acetate and borate buffer solutions respectively 4-5 times (acetate buffer = 0.1 M, pH 4.0, and 0.1 M borate buffer, pH 8.0, each with 1 M NaCl). The protein-gel conjugate was suspended in sodium phosphate buffer 0.2 M, pH 7.5 and stored at 4°C.

Affinity chromatography was performed at room temperature in columns (1 x 10 cm) at at flow rate of 12 ml/hour. Forty-to-50 mg of rabbit IgG containing anti-clathrin were chromatographed each time. Effluent IgG was monitored in a microflow cuvette using a recording Beckman spectrophotometer. Elution of specific anti-clathrin antibodies was obtained with 0.1 M glycine, 0.5 M NaCl, pH 2.8, and the eluted protein peak, collected in a single tube, was rapidly brought to pH 7.5 with 1 M Tris-HCl buffer, pH 8.5. This neutralized solution was dialyzed at 4°C against 0.02 M phosphate saline buffer, pH 7.5, for 24 hours and after addition of 5% bovine serum, was stored frozen at -20°C.

15. Preparation of Cerebellum Cultures:

Brains were removed using sterile techniques from 2-3-day-old Wistar/Furth rats. The pups were rinsed with methanol, decapitated, and brains removed. While soaking in phosphate-buffered saline in a large petri dish, the brains were cleansed of the non-cerebellar choroid and large pieces of meninges. The crebella were separated, each chopped into 4

pieces, and transferred to a large sterile vial. A solution of Trypsin (5-7 ml) consisting of 2.5 mg Trypsin and 10 ml of OU-1 [200 ml of Earles B.S.S. (1X) Ca^{++}- and Mg^{++}-free (from Gibco-Riccult) with 0.2% glucose and 0.3% bovine serum albumin with 2 ml of 3.8% $MgSO_4$ $7H_2O$] was added to the vial and incubated at 35°C with shaking. After 15 minutes, 7 ml of a 1-to-10 diluted DNAse solution (30 ml of OU-1 with 1.2 mg of DNAse, 15.6 mg soybean trypsin inhibitor with 0.3 ml of $MgSO_4$) were added to the mixture, spun up to 1,000 RPM in a bench top centrifuge and then turned off. The supernatant was decanted and 1-1.5 ml of concentrated DNAse solution was added. The tissue was sheared 10 times by trituration through a siliconized pipette. Tissue clumps were allowed to settle and the clump-free layer transferred to a 10 ml sterile conical tube. This procedure was repeated twice with the remaining tissue. The milky suspension was diluted to approximately 4 ml with concentrated DNAse solution, underlayered with 2 ml of 4% bovine serum albumin in Earles BSS, and centrifuged for 7 minutes at 1,200 RPM. The number of cells remaining in the upper layer was calculated. Fifty ul with $3x10^5$ cells were added to each coverslip coated with polylysine in individual wells of Limbro multi-well plates. Finally, 0.4 ml of culture medium (Eagle's minimal essential medium 10% fetal calf serum, 24.5 mM KCl, 0.6% glucose) was added to each well. After 24-48 hours, the culture medium was changed, with 8×10^{-5} M FUdR being added to the medium.

16. Immunofluorescent Staining:

Live cells from rat cerebellar and secondary fibroblast cultures grown on coverslips were stained for clathrin localization. The cells on coverslips were rinsed with phosphate-buffered saline and balanced on 8-mm-diameter pedestals in humidified petri dishes. Two types of fixation were used: a) 100% methanol was added to each coverslip and the specimens placed in a freezer at -20°C for 5-7 minutes; b) 10% formaldehyde was added for 15 minutes at room temperature, drained from the coverslip, and replaced by 0.05% Triton for 15 minutes, at room temperature. All samples were washed with phosphate-buffer saline for 15 minutes. The antisera, diluted with 5% fetal calf serum in phosphate-buffered saline, were added to coverslips in 50 µl vol. for 30 minutes at room temperature. Coverslips were rinsed through beakers of phosphate-buffered saline. Bound immunoglobulins were detected by further incubation for 30 minutes with rhodamine-conjugated goat anti-rabbit IgG. After staining, cultures were post-fixed with 5% acetic acid in ethanol for 10 minutes at room temperature and rinsed through beakers of phosphate buffered saline. Coverslips were mounted in 50% glycerol in phosphate buffered saline and sealed with nail varnish. Slides were stored at 4°C and remained in good condition for several weeks.

Staining of cerebellar sections with either rhodamine or fluorescein-conjugated antibodies followed the same basic procedure described above except that the layering of antisera

and their subsequent rinsing took 45 minutes to 1 hour to ensure tissue penetration. No post-fixation was used on brain slices. Slides were examined under phase-contrast and epifluorescent illumination on a Zeiss standard microscope. Photographs were taken either with Ektachrome (Kodak) high-speed color slide film developed at ASA 400 or with Tri-X black and white film, ASA 400.

17. Peroxidase/Antiperoxidase Staining:

White, Wistar rats, weighing 200-300 gm were used. Brain tissue was removed for the localization of clathrin in the nervous system. Rats were anesthesized with 0.5-1.0 ml of equithesin. An incision was made under the diaphragm to expose the body cavity. Ribs were cut along the sides of the thorax, the rib cage lifted, and the heart exposed while still beating. Perfusion was carried out with the aid of a peristaltic pump using 4% formaldehyde, and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2.

The perfusion fixative was prepared as follows: formaldehyde powder (40 gm) was added to 500 ml of distilled water at 60°C with stirring. Approximately 2-3 ml of 4% NaOH was added and stirring continued until the solution cleared. The formaldehyde solution was then cooled to 4°C. A volume of 500 ml of 0.2 M sodium phosphate buffer, pH 7.4, was added to a final molarity of 0.1 M sodium phosphate. The perfusion cannula tip was quickly placed in the left ventrical after nipping the right atrium. Pump speed was turned

up to ensure rapid perfusion. After 1 minute, perfusion rate was reduced, continuing for 5 more minutes. Brains were excised and soaked in perfusion medium for 1 to 2 hours in the cold room, and prepared for sectioning.

A saggital slice of brain, about 5 cm thick, was made with a razor blade, glued to a plastic vibratome specimen holder, and mounted on the vibratome stage. Ice cold 0.1 M phosphate buffer, pH 7.4, was used as the fluid for the brain-cutting process. The buffer was kept cold by a flow through refrigerant system. After smoothing over the tissue surface with a few thick sections, the stage was raised by 20 mm after each slice to give 20 mm-thick sections. Sections floated in individual wells filled with the same 0.1 M phosphate buffer. Each experiment used approximately 32 slices. Cut sections were rinsed twice for 5 minutes in 0.1 M Tris-HCl, pH 7.6, with 1 N saline, transferred to 0.25% Triton in 0.1 M Tris-HCl for 15 minutes, and rinsed twice in 1% Tris-saline for 5 minutes each time.

A preparation of normal goat serum made 1% in Tris-saline was then used to incubate tissue slides for 30 minutes. Antisera samples and control (pre-immune serum) were diluted 500 times with 1% goat serum in 100 mM Tris buffer. The antisera used were: a) anti-clathrin antiserum; b) an IgG fraction from anti-clathrin antiserum; c) affinity column purified antibodies. Tissue slices immersed in the appropriate antibody solutions were incubated overnight in the cold using a humidified chamber. Gentle mixing was used.

Tissue slices, warmed to room temperature, were rinsed twice with 1% goat serum Tris saline for 8 minutes, and then incubated in 50 times diluted goat anti-rabbit IgG for 30 minutes. Two more rinses of 8 minutes each were performed after a 30-minute incubation with peroxidase anti-peroxidase. Excess peroxidase anti-peroxidase was washed by a diaminobenzidine solution (22 mg of crystals in 175 ml of Tris buffer). Afterwards, 40 ml of 30% hydrogen peroxide was added with rapid stirring. Tissue samples, incubated in this solution for 6 minutes, turned brown when viewed against the white background of the incubation wells. Samples were then rinsed twice with distilled water. For light microscopy, slices were spread out on glass slides coated with a thin layer of albumin, partially air dried, and completely dehydrated by rinsing for 8 minutes each in 100% methanol and xylene. Tissue was covered with a coverslip and sealed. Slides were viewed under phase and Nemarsky optics.

RESULTS

1. Purification:

Purification of clathrin was started by its extraction from crude coated vesicle fractions. The contents of one such fraction examined by electron microscopy is shown in Figures 2a & 2b. This fraction, obtained through a modification of Pearce's procedure (40, [see Flow Chart]), is composed of coated vesicles, empty protein cages, and membrane fragments. The method of Schook *et al.* (51), used for the isolation of brain α-actinin, was adapted to yield a protein extract from the coated vesicle fraction. On gel electrophoresis (Fig. 3a), the major polypeptide (approximately 70%) banded with an electrophoretic mobility of 180,000 molecular weight. Six other proteins appeared in smaller but significant amounts. Most prominent were: a) a 55,000 molecular weight species (5-10% of total protein) which slab gel electrophoresis resolved as a doublet comigrating with the α and β subunits of the tubulin dimer (W. Schook, personal communication); b) a 45,000 molecular weight band (5-10% of total protein) which comigrated with rabbit skeletal muscle actin (kindly supplied by E. Hua). Less prominent bands consisted of a doublet with an electrophoretic mobility of 28,000 and 30,000 molecular weight, a band at 100,000 molecular weight and traces of a protein heavier than clathrin with a molecular weight of 250,000-300,000.

Figure 2a. Thin section electron micrograph of a crude coated vesicle fraction--Clathrin cages or baskets (B), coated vesicles (CV). This fraction was used to extract and purify clathrin as detailed in Methods. Magnification: 75,000 x.

Figure 2b. Higher magnification of the same preparation in Fig. 2a--Here, coated vesicles are distinguishable from empty baskets by the circular vesicle membrane surrounded by a clathrin coat. Empty baskets (B). Magnification: 150,000 x.

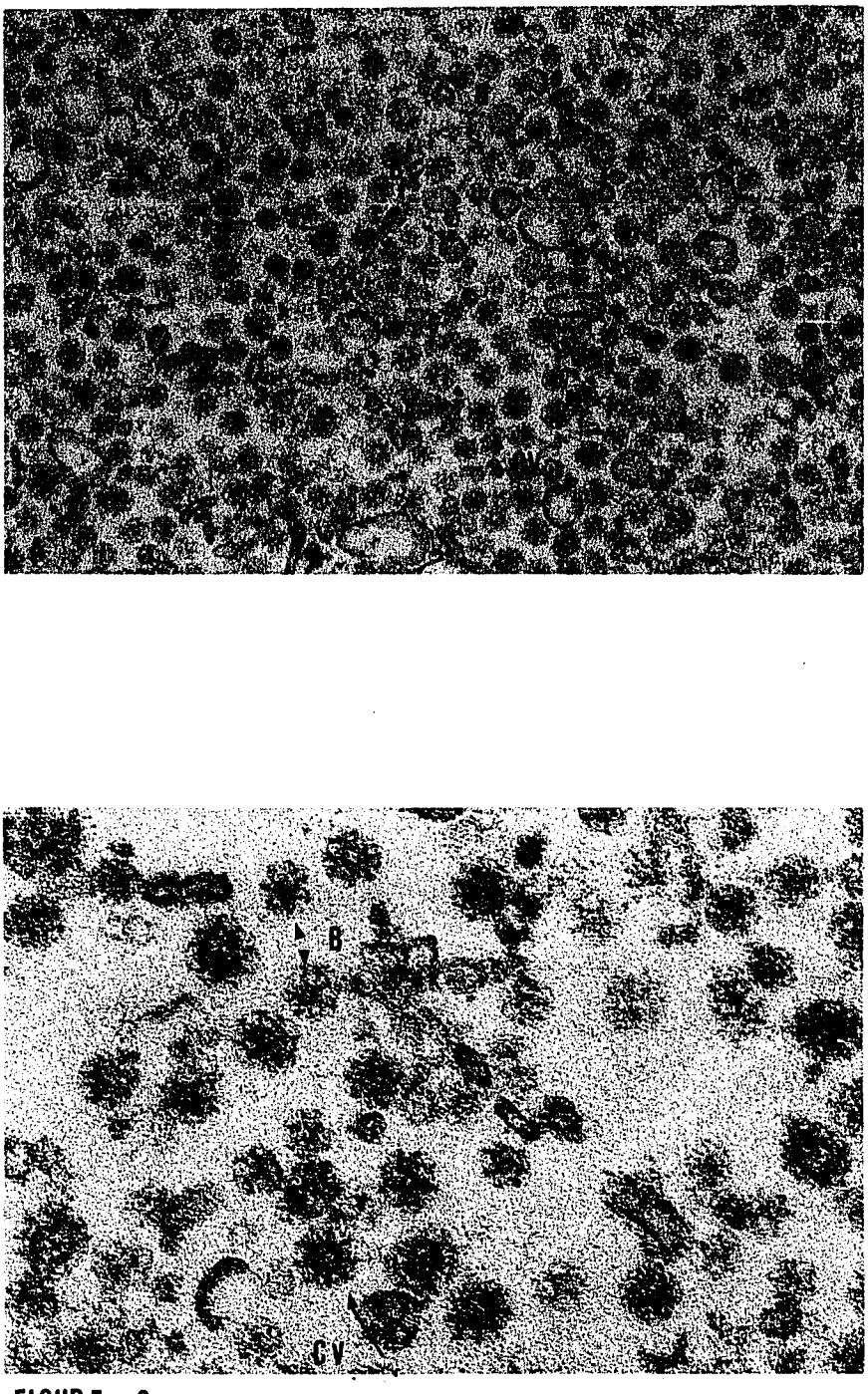
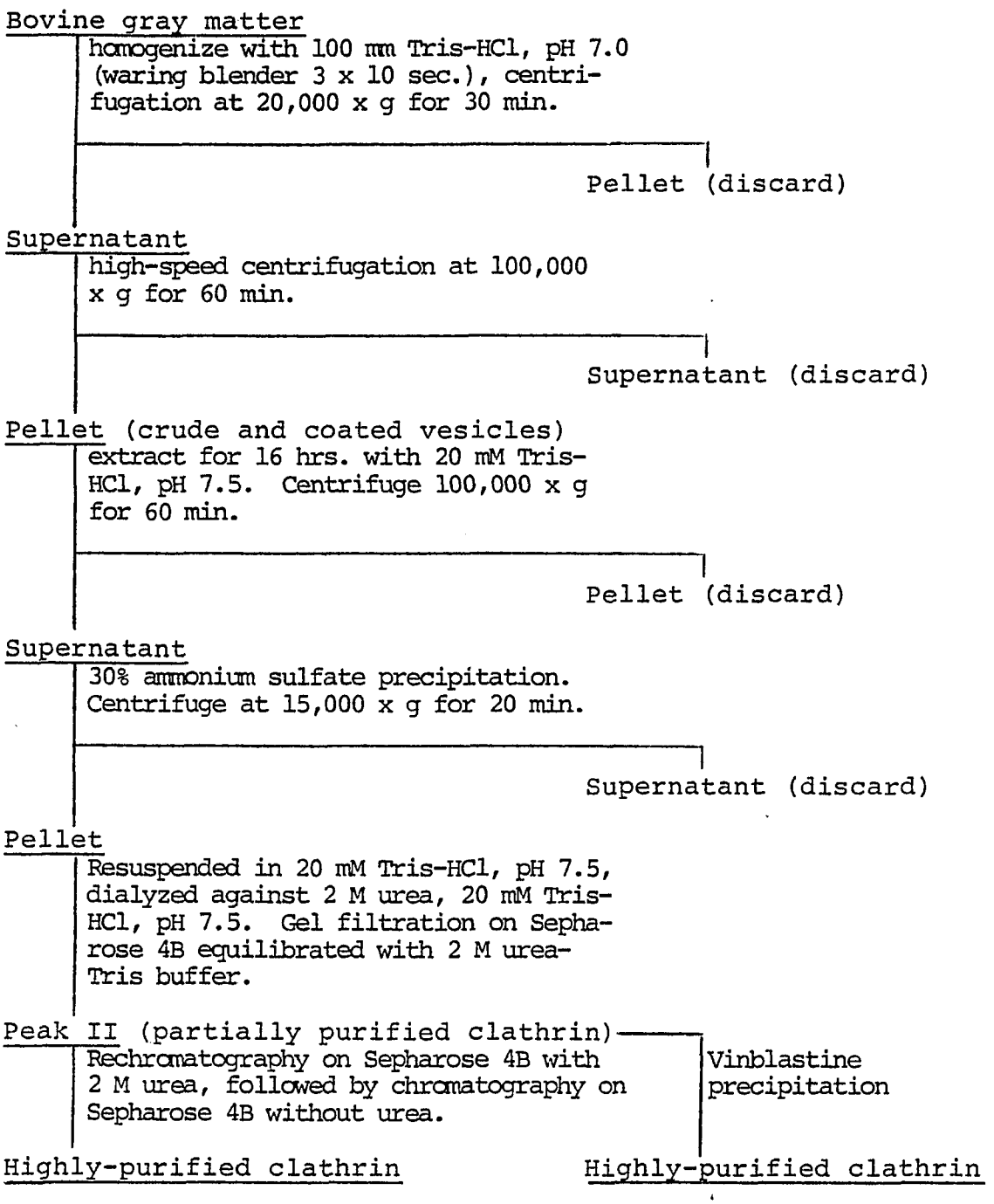


FIGURE 2

FLOW CHART OF CLATHRIN'S PURIFICATION

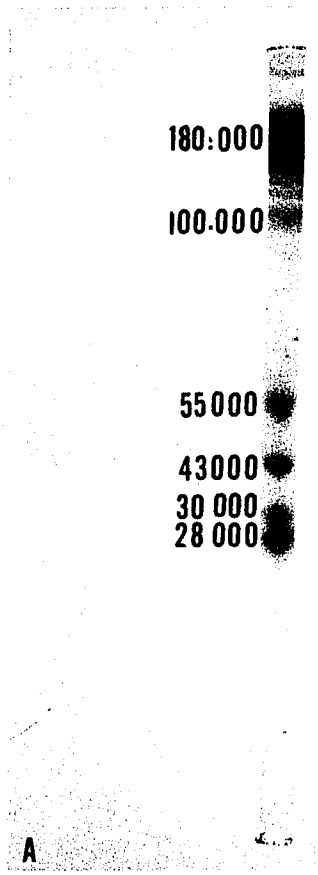


The coated vesicle extract was salted out with 30% ammonium sulfate, and the precipitate resuspended in and dialyzed against the buffer used in the chromatographic column. As such, it was applied to various bead gel columns in an attempt to isolate the 180,000 molecular weight species from accompanying components. The first gel-buffer system used was Sepharose 4B suspended in 20 mM Tris-HCl, pH 7.5. Only one peak eluted from the column and the elution pattern is shown in Figure 3b. The inset, showing the protein's composition in cylindrical acrylamide gels, indicates that clathrin eluted together with the other polypeptides. Also, negative results were obtained using an anion-exchange resin consisting of DEAE-Sephadex A-50 and elution with a continuous salt gradient of 0-0.6 M KCl (Fig. 4a). Clathrin and accompanying proteins eluted from the column as one peak at a KCl concentration of 0.15-0.25 M KCl.

A partial separation was achieved upon the addition of 2 M urea to the 20 mM Tris-HCl elution buffer, pH 7.5. Figure 4b shows the elution pattern of the protein fractions when 2 M urea is present in the buffer and the inset shows the protein composition of each peak, various organelles and aggregated proteins. The content of the peaks was examined by electron microscopy. Each peak was centrifuged at 105,000 x g for 60 minutes and the pellets fixed for transmission electron microscopy. The pellet of the first peak consisted mostly of membrane fragments (Fig. 5a). The

Figure 3a. Sodium dodecyl sulfate polyacrylamide electrophoresis on cylindrical gels--Single polyacrylamide gel (A) of 7.5% acrylamide loaded with 100 µg of a crude clathrin obtained from a coated vesicle fraction. Estimated molecular weights of the polypeptides are indicated. Heavily stained band with an electrophoretic mobility of 180,000 daltons represents clathrin. Other accompanying bands are: tubulin, molecular weight 55,000; actin, 43,000; and a doublet, probably representing tropomyosin, molecular weights 30,000 and 28,000. A weakly stained band above clathrin can be observed with an electrophoretic mobility of 250,000-300,000. A weakly stained, slightly diffused band with a mobility of 100,000 daltons also can be seen. It may represent brain α-actinin.

Figure 3b. Sepharose 4B column chromatography--The elution pattern of a crude clathrin extract from coated vesicles is illustrated. The protein composition is shown in Fig. 3a. Approximately 100 mg of total protein were loaded on a Sepharose 4B column 2.5 x 80 cm equilibrated with 20 mM Tris-HCl, pH 7.5. Flow rate was approximately 15 ml per hour. Protein concentration was monitored by adsorption at 280 nm. Inset shows protein composition of aliquots of fractions numbers 80, 100 and 150 belonging to the eluted peak, run on sodium dodecyl sulfate acrylamide gels. Data shows no chromatographic separation of clathrin's accompanying proteins.


180:000
100.000
55000
43000
30 000
28 000
A

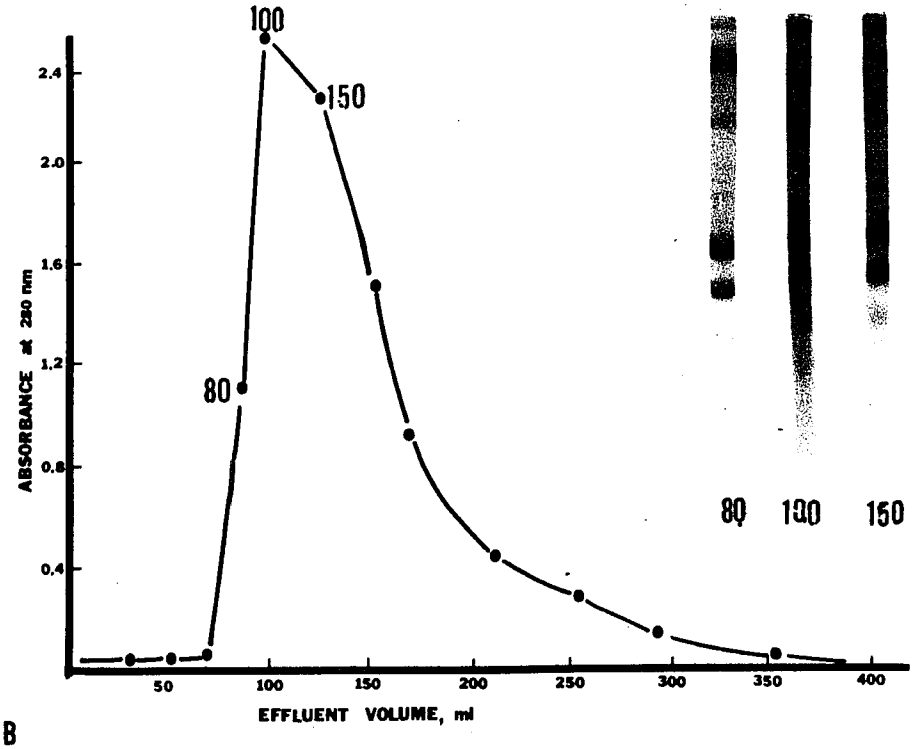
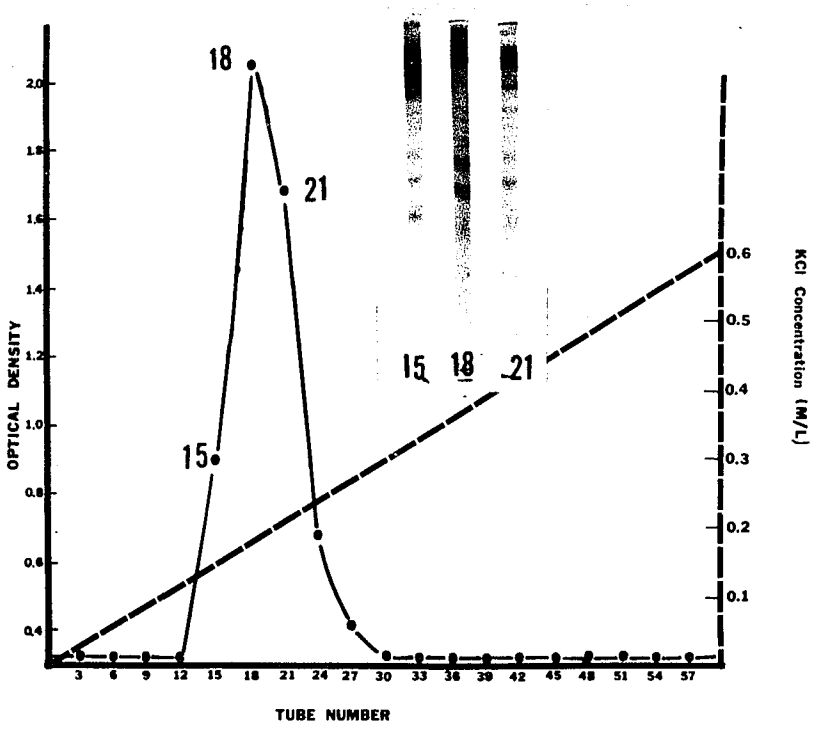

100
150
80
2.4
2.0
1.6
1.2
0.8
0.4
ABSORBANCE at 280 nm
50
100
150
200
250
300
350
400
EFFLUENT VOLUME, ml
80
100
150
B

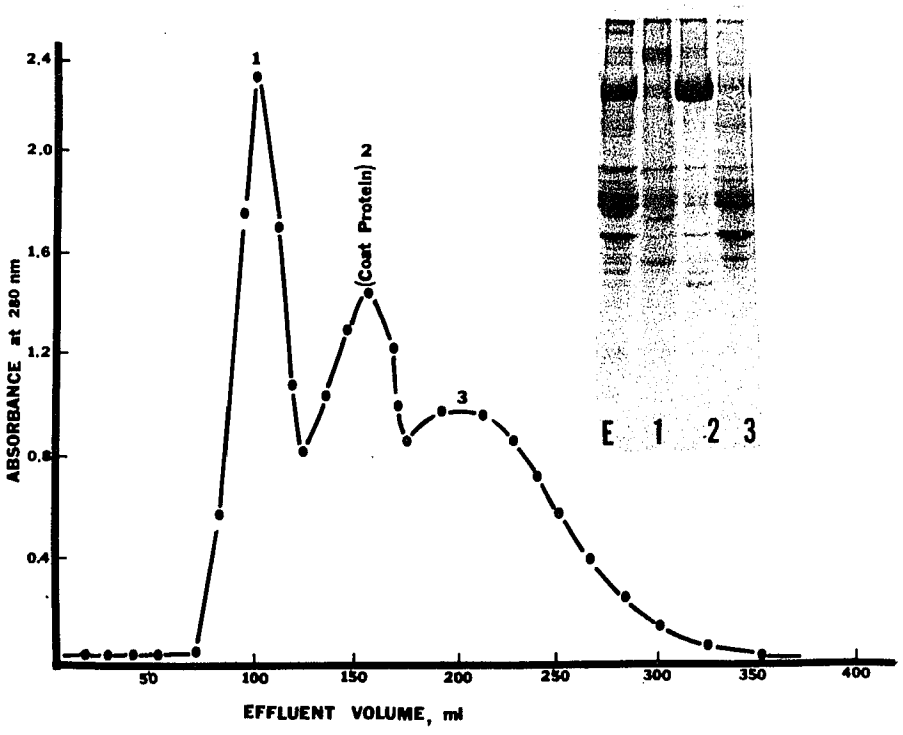
FIGURE 3

Figure 4a. Elution pattern of a crude clathrin extract of 50 mg total protein loaded onto an anion exchange column 1.5 x 30 cm of DEAE Sephadex A-50 and eluted with 180 ml of a continuous gradient of 0-0.6 M potassium chloride--Each fraction volume was 3 ml. Inset shows protein composition resolved on sodium dodecyl sulfate cylindrical gels of samples from fractions 15, 18 and 21. Dashed line denotes the KCl concentration of the gradient. One protein peak eluted between 0.15 M and 0.25 M KCl with a polypeptide composition similar to the preparation loaded onto the column (Fig. 3a).

Figure 4b. Elution pattern of 100 mg of crude clathrin extract loaded onto a Sepharose 4B column 2.5 x 80 cm equilibrated with 2 M urea, 20 mM Tris-HCl buffer, pH 7.5--Three overlapping peaks eluted. Inset shows the protein composition of the numbered samples from each peak on sodium dodecyl sulfate acrylamide slab gel. Gel #2 illustrates enrichment of clathrin eluting in the second peak, compared with the crude clathrin extract loaded onto the column (E gel).


18
21
15
15 18 21
OPTICAL DENSITY
KCl Concentration (M/L)
TUBE NUMBER

A


1
2
(Coat Protein)
3
E 1 2 3
ABSORBANCE at 280 nm
EFFLUENT VOLUME, ml

B

FIGURE 4

pellet of the second peak (Fig. 5b) consisted of empty baskets and amorphous aggregates of protein, but devoid of membranes. The pellet of the third peak contained amorphous material without membrane fragments or baskets (Fig. 5c).

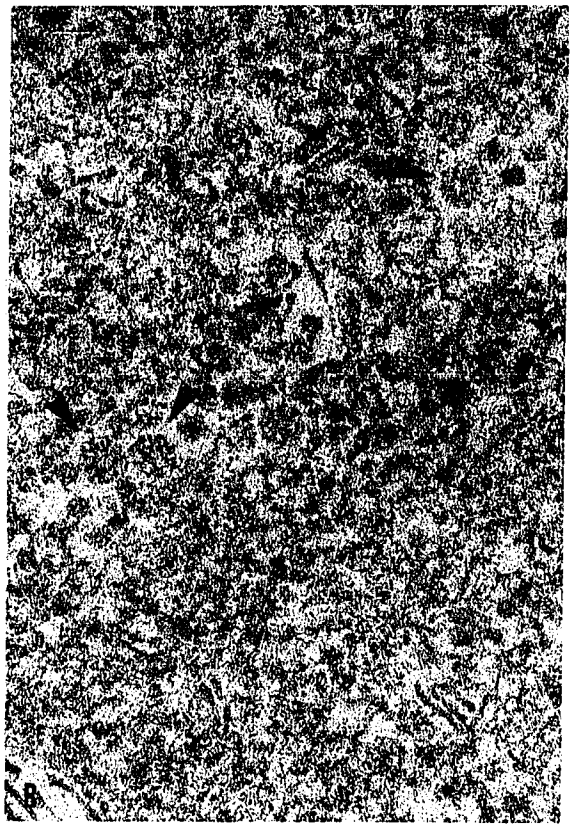
The coat protein peak (peak 2) by gel densitometry was about 70% clathrin. Rechromatography of this clathrin-enriched fraction on a similar Sepharose 4B column and elution with the Tris-HCl, 2 M urea buffer resulted in a similar elution pattern as before, except that the first peak was reduced greatly. This second run yielded clathrin about 80% purified (Fig. 5d). When this clathrin fraction was rechromatographed again on a Sepharose 4B column but this time eluted with 20 mM Tris-HCl buffer, pH 7.5, (without urea), 2 peaks emerged (Fig. 6a). The first peak contained varied amounts of clathrin and most of the other proteins. The second peak contained clathrin and only a small amount of a low molecular weight doublet. By gel densitometry, the band of clathrin represented 95-98% of the total protein with the doublet contributing 2-5%. Figure 6b illustrates clathrin from three main steps of its purification: gel #1 illustrates a typical crude clathrin preparation; gel #2 illustrates a typical partially purified clathrin (approximately 70% pure) which has been run through a 2M urea-Sepharose 4B column (we call this fraction partially purified clathrin); gel #3 illustrates a highly purified clathrin preparation after the last column used in the isolation procedure.

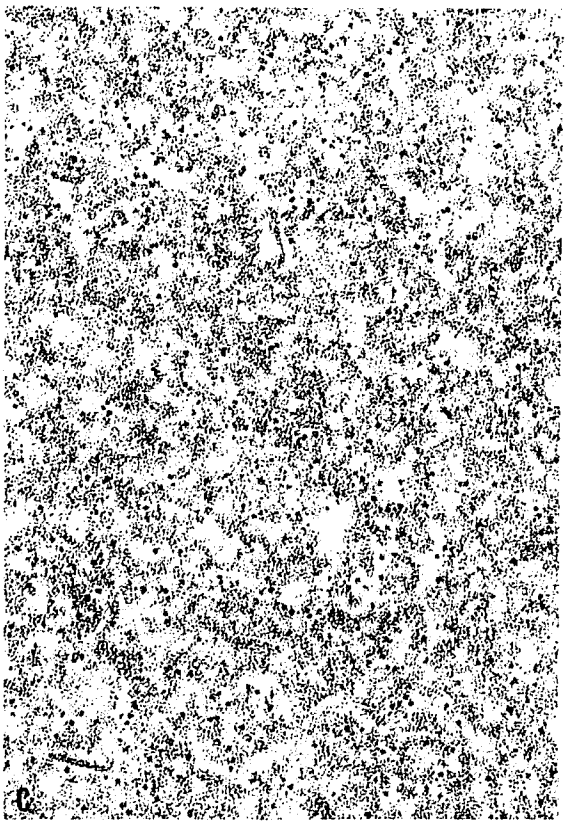
Figure 5a. Thin section electron micrograph of material pelleted at high speed (105,000 x g for 60 minutes) of peak I (illustrated in Fig. 4b)--Membrane fragments are observed while coated vesicles or baskets are absent. Membranes (M). Magnification: 120,000 x

Figure 5b. Thin section electron micrograph of material pelleted from peak II (shown in Fig. 4b)--Sediment contains some baskets, denoted by arrows, few membrane fragments and aggregated clathrin. The supernatant of this peak was highly enriched with clathrin. Magnification: 120,000 x

Figure 5c. Electron micrograph of the material sedimented from the third peak of the Sepharose 4B column--This peak contains mostly amorphous material. The supernatant contained trace amounts of clathrin. Magnification: 120,000 x

Figure 5d. Sodium dodecyl sulfate cylindrical gels of 7.5% acrylamide loaded with clathrin purified by a second chromatographic run on a Sepharose 4B column (essentially as described in Fig. 4b)--Gel #1 (from left) = 10 µg of protein; Gel #2, 25 µg; Gel #3 50 µg; Gel #4, 100 µg of protein. Overloading amounts of clathrin show traces of a heavy molecular weight polypeptide and four other polypeptides similar to those shown in Fig. 3a.





1 2 3 4

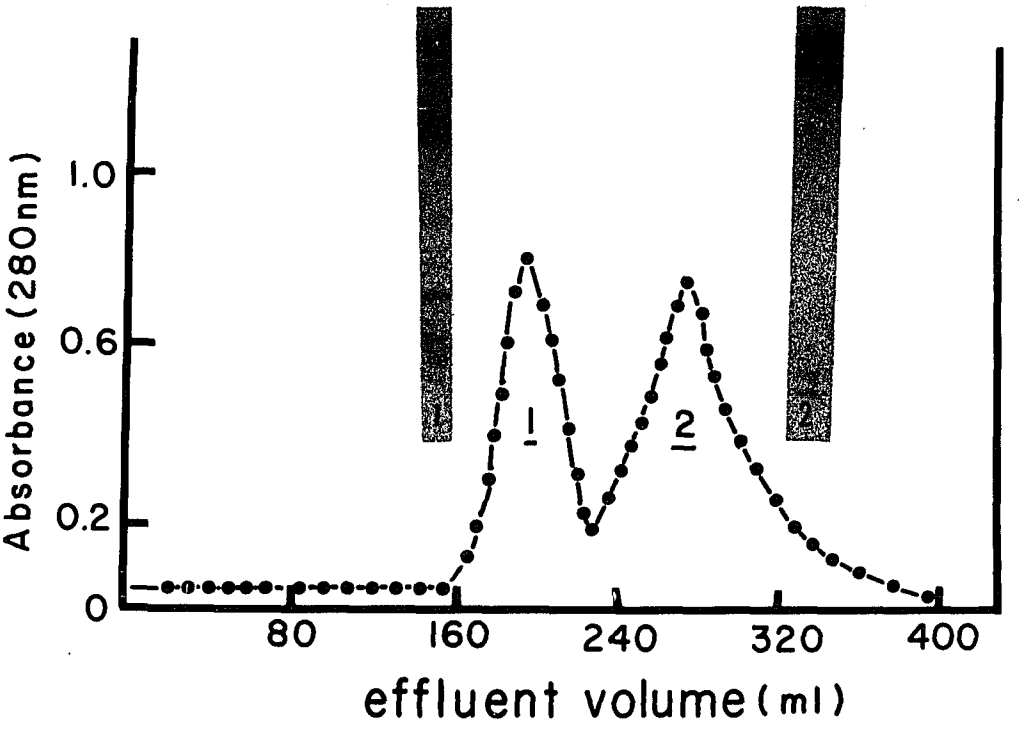
D

FIGURE 5

Figure 6a. Elution pattern of clathrin from peak II--This Sepharose 4B column (2.5 x 80 cm) was equilibrated in a 20 mM Tris buffer, pH 7.5, without urea. The material loaded onto the column was an ammonium sulfate concentrated clathrin from peak II (as shown in Fig. 4b). A representative fraction of each peak was run on sodium dodecyl sulfate slab gel electrophoresis as illustrated in inset. Peak I showed small amounts of clathrin and larger amounts of clathrin's contaminating proteins. Peak II was essentially mono-dispersed clathrin with the doublet of molecular weight 30,000 and 28,000.

Figure 6b. Cylindrical 7.5% acrylamide gels with 0.1% sodium dodecyl sulfate--Gel #1 was loaded with 100 µg of a crude coated extract. Gel #2 contains 100 µg of partially purified clathrin obtained from the chromatographic procedure on Sepharose 4B using 2 M urea buffer. Gel #3 illustrates 100 µg of highly purified clathrin.

Figure 6c. Clathrin precipitation by vinblastine sulfate--This figure shows sodium dodecyl sulfate acrylamide gels loaded with clathrin precipitated by increasing concentrations of vinblastine. Gel #1 shows the material precipitated by 0.1 mM vinblastine added to a crude clathrin preparation. Gel #2 shows the precipitate formed by increasing the concentrations of vinblastine to 0.2 mM. Gel #3 shows the third precipitate formed by increasing the concentrations of vinblastine to 0.3 mM. Gel #4 shows the final precipitate formed after increasing the concentrations of vinblastine to .5 mM.


Absorbance (280nm)
1.0
0.6
0.2
0
80
160
240
320
400
effluent volume (ml)
1
2
1
2

A

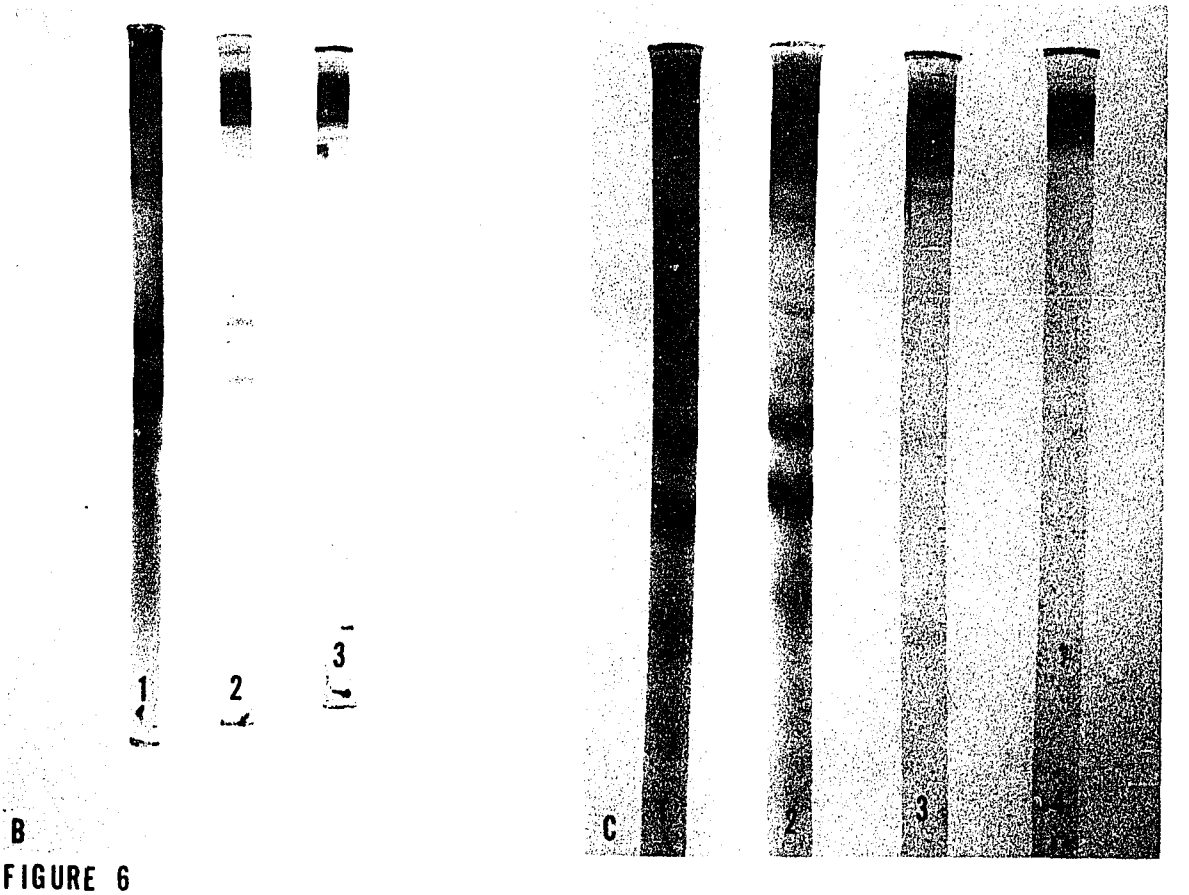

1
2
3
B
C
1
2
3

FIGURE 6

The crude clathrin preparation can be enriched further in clathrin by the precipitation of contaminating cytoskeletal protein, mostly actin and tubulin, with up to 1 mM of vinblastine sulfate. Tubulin and actin were made preferentially insoluble by this vinca-alkaloid reagent. Figure 6c shows sodium dodecyl sulfate acrylamide gels of the proteins precipitated by vinblastine after sequential addition of vinblastine to a crude clathrin preparation. Gel #1 shows the protein composition of a precipitate produced by the addition of 0.1 mM vinblastine. The supernatant was made 0.2 mM with vinblastine. Gel #2 illustrates the polypeptide composition of the precipitate formed. The supernatant then was made 0.3 mM with vinblastine. The protein precipitated is shown in gel #3. The precipitate consisted of highly purified clathrin. When the supernatant was made 0.5 mM vinblastine, more clathrin precipitated.

2. Basket Assembly:

Highly purified clathrin was used to elucidate its assembly/disassembly characteristics. Since coated vesicles were stable to fixation and isolation procedures, it was felt that *in vitro* reassembly of clathrin into coats should be feasible. Parameters such as pH, ionic strength, and temperature were used at various levels in an attempt to find the conditions affecting clathrin's polymerization characteristics.

The effect of hydrogen ion concentration was analyzed. Clathrin preparations were viewed by uranyl acetate staining in the electron microscope. Regardless of the buffer used (Table 1), at pH 7.2-8.0, clathrin molecules were dispersed, rod-like shapes, forming amorphous aggregates. No structures resembling the coat of coated vesicles were observed (Figs. 7a & 7b).

Since coated vesicles were isolated initially using a buffer composed of 0.1 M MES, pH 6.5, this buffer was used in an attempt to reproduce conditions by which coated vesicles were found stable. Figure 7c shows the dramatic effect produced on clathrin by lowering the pH from 7.5 to 6.5. Clathrin formed empty baskets or cages morphologically similar to the coated vesicles isolated directly from tissue, except that these *in vitro* cages contained no membrane. The basic units of pentagons and hexagons arranged as cages or baskets was evident. The dimensions of these baskets (600-1,000 Å in diameter) were within the range of coated vesicles reported in the literature (7, 40). Over the pH range 6.0-6.8 for all buffers tested, baskets were the predominant structure formed. Often varying amounts of amorphous aggregates were present. With clathrin at a concentration of 1 mg/ml or greater, some aggregates were present along with large numbers of baskets. Baskets formed at a clathrin concentration between 0.4-0.6 mg/ml were adequate in number for electron microscope observation and with the most minimal

TABLE 1.

EFFECTS OF pH ON BASKETS WITH DIFFERENT BUFFERS

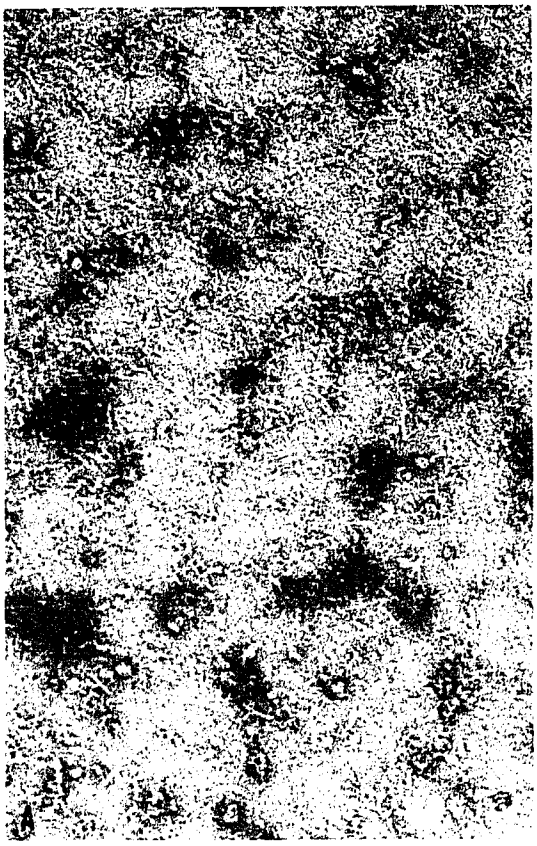
		Amorphous Aggregates	Baskets
Tris Maleate	pH 6.2-6.8	-	+
	pH 6.8-7.2	±	±
	pH 7.2-7.6	+	-
Cacodylate	pH 6.2-6.8	-	+
	pH 6.8-7.2	±*	±
	pH 7.2-7.6	+	-
MES	pH 5.5-7.0	-	+
	pH 7.2-7.5	+	-
Tris	pH 7.0-8.5	+*	-
Glycine	pH 3.0	+	-

* Filamentous structures were present.

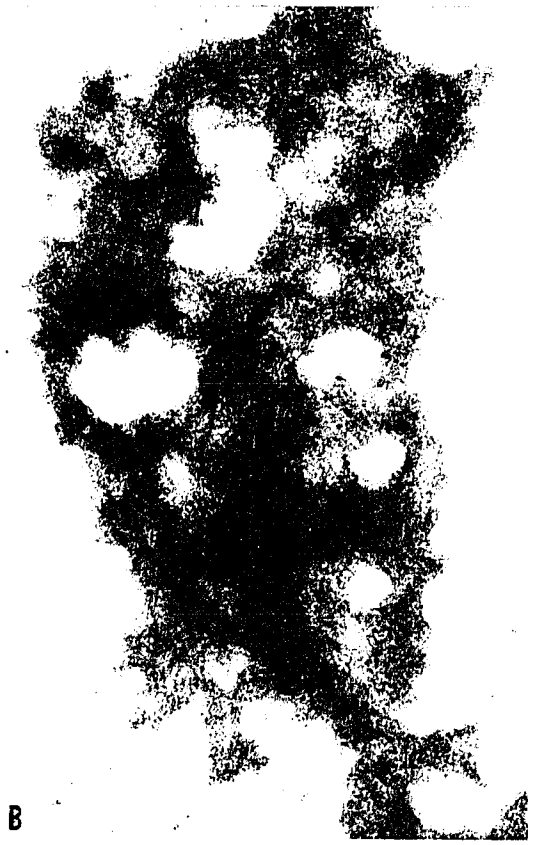
Figure 7a. A negatively stained preparation of clathrin suspended in Tris-HCl buffer, pH 7.5--In this preparation, we observe clathrin molecules of rod shape and some aggregates. Magnification: 120,000 x

Figure 7b. This figure illustrates an amorphous aggregate of clathrin that forms at pH 7.5. Magnification: 120,000 x

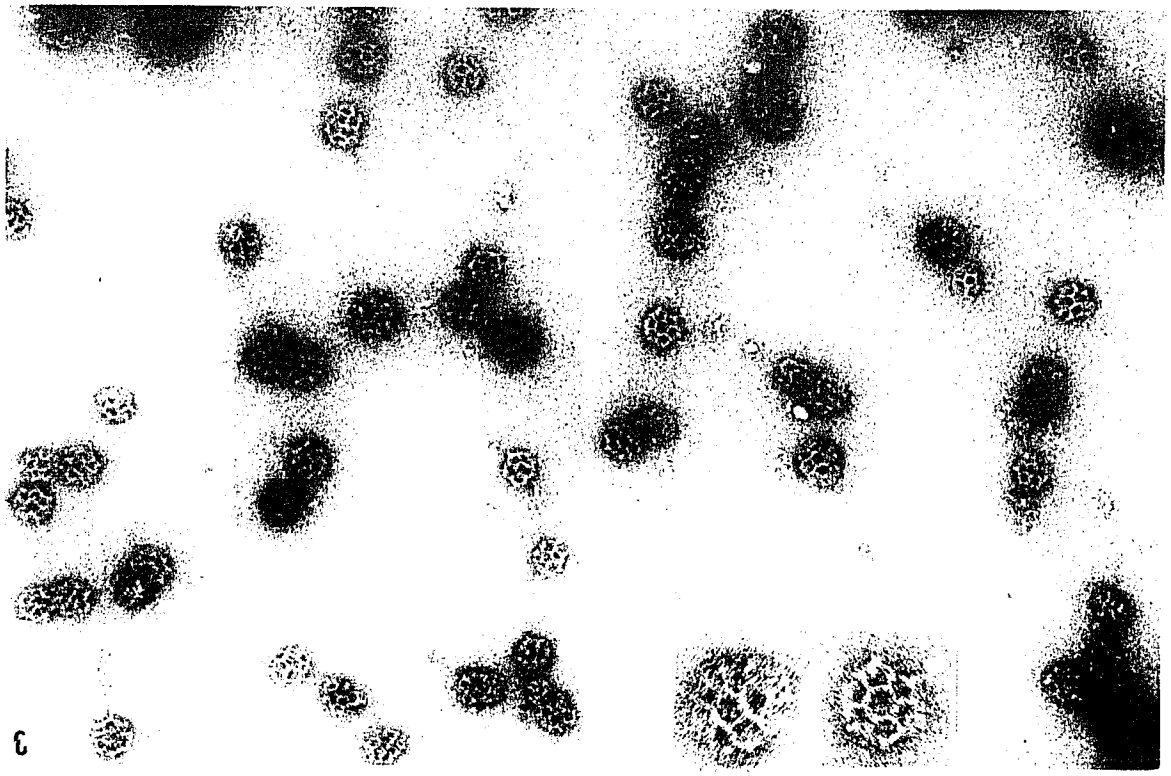
Figure 7c. This picture shows the unique formations of clathrin assembled when the pH is reduced from 7.5 to 6.5. These structures were negatively stained and are apparently formed by polygonal and hexagonal units joined together forming an icosahedral structure. Magnification: 120,000 x Inset magnification: 250,000 x



A



B



C

FIGURE 7

amount of aggregates, as judged from scanning the grids. Baskets appeared with clathrin concentrations as low as 0.2 mg/ml. In the pH range 6.9-7.1, both baskets and depolymerized clathrin were present over all concentrations tested. This pH range appeared to be a zone of transition between two forms of clathrin since neither structure was predominant.

The effect of various ionic conditions on basket formation was investigated. The results are summarized in Table 2. Neither calcium nor magnesium ions were required for basket formation since EGTA and EDTA 0.1-1.0 mM had no effect on assembly of cage structures (Fig. 8a). However, concentrations of $CaCl_2$ above 200 mM and KCl above 500 mM completely inhibited basket formation (Fig. 8b). ATP inhibited basket formation at 10 mM, but below 5 mM there was no effect. Basket formation was prevented by 2 M urea, the concentration used to dissociate clathrin from membrane prior to Sepharose 4B column chromatography.

3. Stability of Baskets:

Sedimentation in discontinuous sucrose gradients was used to investigate the effect of ionic conditions on preformed basket structures. The gradients consisted of discontinuous layers of 10, 20, 30, 40, 50, 55 and 60% sucrose dissolved in 0.1 M MES, pH 6.5, the buffer system favoring basket structures. Total protein concentrations were monitored by absorbance at 280 nm (Fig. 9a). Under control

TABLE 2.

EFFECTS OF IONIC CONDITIONS ON BASKET FORMATION

		Amorphous Aggregates	Baskets
$CaCl_2$	EGTA	-	+
	10 mM	-	+
	50 mM	-	+
	100 mM	-	+
	200 mM	+	-
$MgCl_2$	EDTA	-	+
	10 mM	-	+
KCl	50 mM	-	+
	100 mM	-	+
	200 mM	-	+
	500 mM	+	-
ATP	0.1 mM	-	+
	1 mM	-	+
	5 mM	-	+
	10 mM	+	-
Urea	2.0 M	-	-
SDS*	2.0%	-	-

* Sodium Dodecyl Sulfate

Figure 8a. Electron micrograph of negatively stained clathrin--Clathrin is assembled as baskets in the presence of 1 mM EGTA.

Figure 8b. This figure illustrates the absence of baskets in the presence of 500 mM KCl.

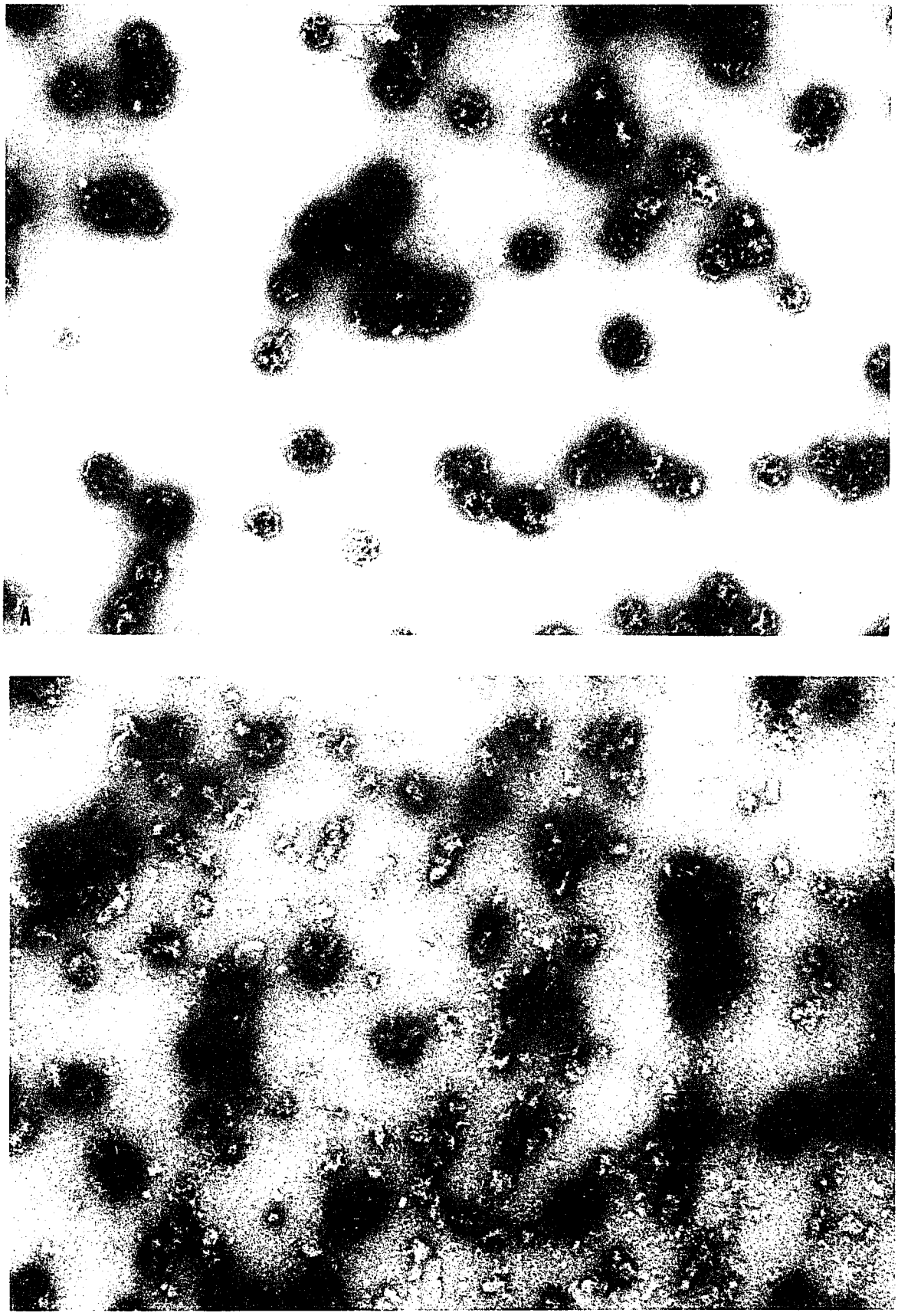


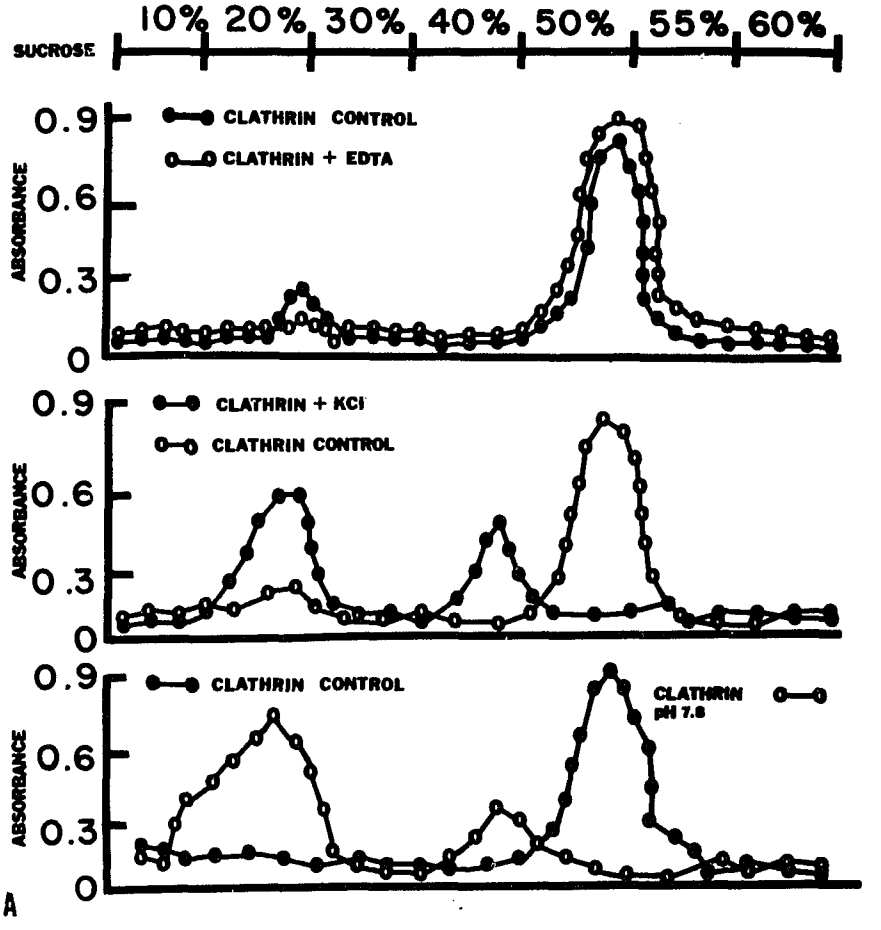
FIGURE 8

conditions (*i.e.*, 0.1 M MES, pH 6.5) protein structures banded at the 50-55% interface. The presence of 2.5 mM EDTA in all sucrose layers had no effect on this pattern. Sucrose gradients prepared in 0.5 M KCl or 20 mM Tris, pH 7.8 (Fig. 9a, middle graph), showed markedly different sedimentation patterns. Most protein absorbance in these gradients was at the 20-30% interface, with smaller amounts at the 40-50% interface. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that protein absorbance was due to a 180,000 molecular weight species (Fig. 9b).

Examination by negative staining electron microscopy was performed to analyze the nature of the assembly of the clathrin molecules from the gradients. The electron micrographs showed that baskets were present in the control, EDTA and 5 mM ATP containing gradients only at the 50-55% interface (called the "basket zone," Figs. 10a & 10b). The protein sedimenting at the 20-30% interface in both the 0.5 M KCl and 20 mM Tris, pH 7.8, sucrose gradients appeared polymerized either as filaments of various widths (Figs. 10c & 10d) or aggregated. The protein added to the 0.5 M KCl or Tris-HCl, pH 7.5, gradient and equilibrating at the 40-50% region also showed clathrin aggregates (Figs. 11a & 11b). No baskets could be found. Pellets of protein sedimenting in the basket zone and also at the 40-50% interface of the 0.5 M KCl sucrose gradient were fixed with gluteraldehyde and viewed by positive transmission electron microscopy

Figure 9a. Centrifugation of clathrin on sucrose gradients--Absorbance was measured at 280 nm, while fractions were being withdrawn from the centrifuge tube. The top figure shows clathrin assemblies sedimenting and equilibrating at the 50-55% sucrose interface. Both the control and EDTA sucrose gradients show similar sedimentation patterns. The middle graph was obtained with clathrin on a sucrose gradient containing 0.5 M KCl. The lower graph shows the effect of sedimenting clathrin as baskets or cages in a sucrose gradient with a Tris-HCl buffer of pH 7.8.

Figure 9b. The sodium dodecyl sulfate gel electrophoresis of representative samples separated from sucrose gradients--In all fractions clathrin was protein present. Gel #1: clathrin from the basket zone (50-55% interface); Gel #2: protein sedimenting at the 20-30% interface of the KCL containing sucrose gradient; Gel #3: protein sedimenting at the 40-50% sucrose interface; Gel #4: protein of the 20-30% sucrose at pH 7.5; Gel #5: control clathrin used to load the sucrose gradients.


SUCROSE
10%
20%
30%
40%
50%
55%
60%
ABSORBANCE
0.9
0.6
0.3
0
CLATHRIN CONTROL
CLATHRIN + EDTA
CLATHRIN + KCl
CLATHRIN CONTROL
CLATHRIN CONTROL
CLATHRIN
pH 7.8
A

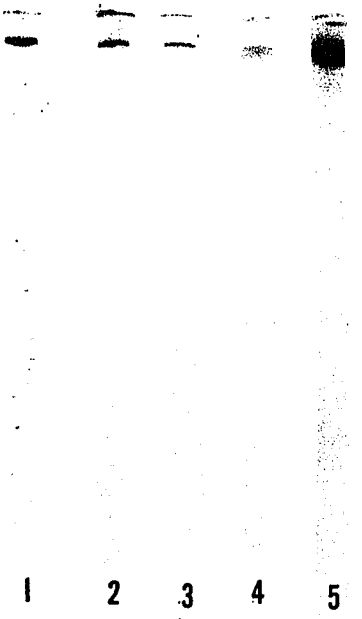

1
2
3
4
5
B

FIGURE 9

Figure 10a. A negatively stained preparation of the material sedimented in the 50-55% sucrose interface or "basket zone"--Baskets are intact and of similar shape to those formed typically by clathrin.

Figure 10b. Baskets sedimented to the 50-55% sucrose interface in the presence of EDTA.

Figure 10c. Filaments formed by clathrin equilibrated at the 20-30% sucrose interface in the presence of 0.5 M potassium chloride.

Figure 10d. Filaments formed at the 20-30% sucrose interface in buffer pH 7.5.

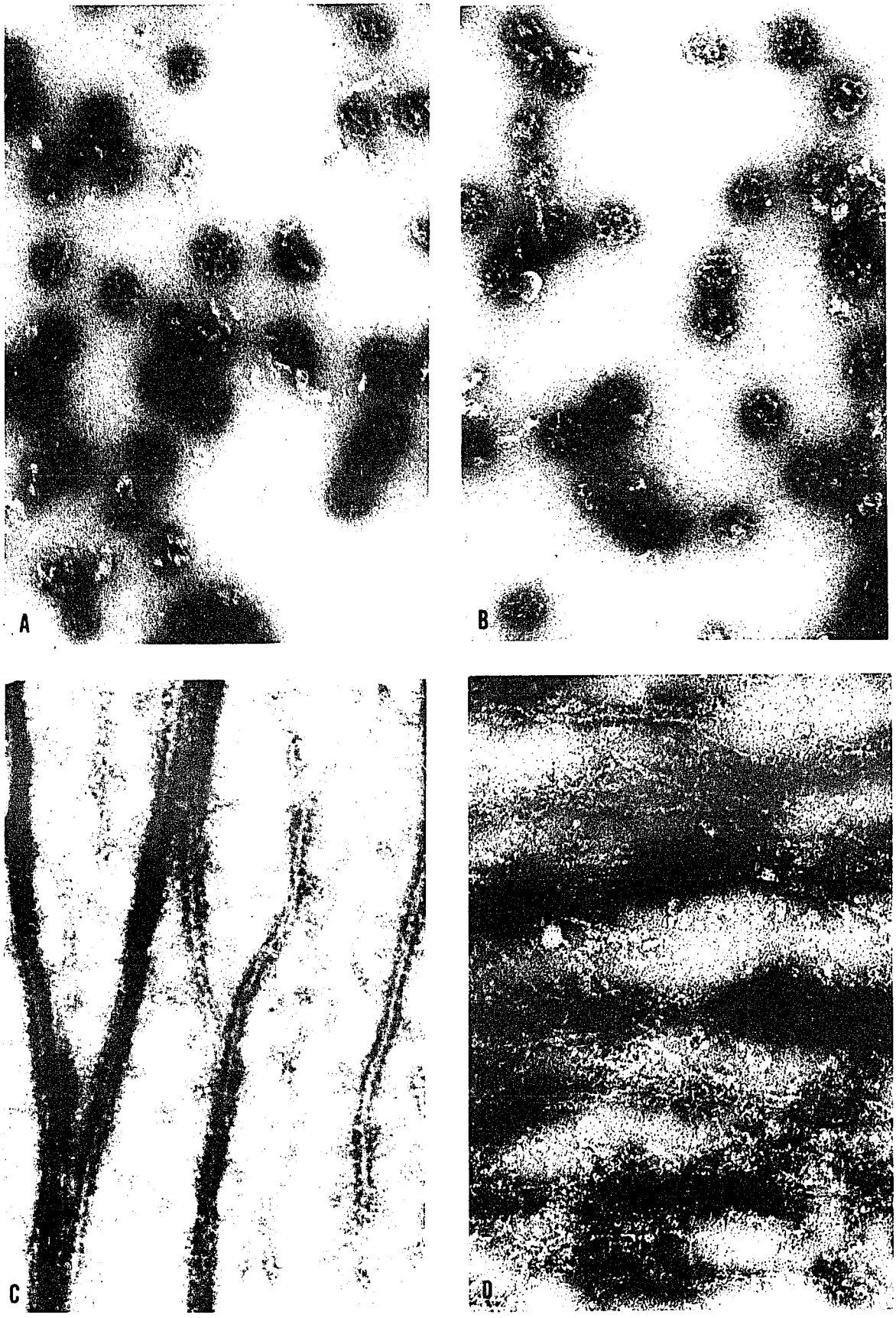


FIGURE 10

Figure 11a. Negatively stained material sedimented at the 40-50% sucrose interface of a gradient containing 0.5 M KCl--Clathrin appears in aggregated form.

Figure 11b. Negatively stained material sedimented at a 40-50% sucrose interface in buffer pH 7.5--Strings of aggregated clathrin are found.

Figure 11c. Thin section electron micrograph of high-speed centrifuged pellet of clathrin formed at the basket zone of a sucrose gradient, pH 6.5.

Figure 11d. Thin section, high-speed centrifuged material from clathrin sedimented at the 40-50% sucrose interface showing an amorphous state of the clathrin molecules having been dissociated by 0.5 M potassium chloride present in the gradient.

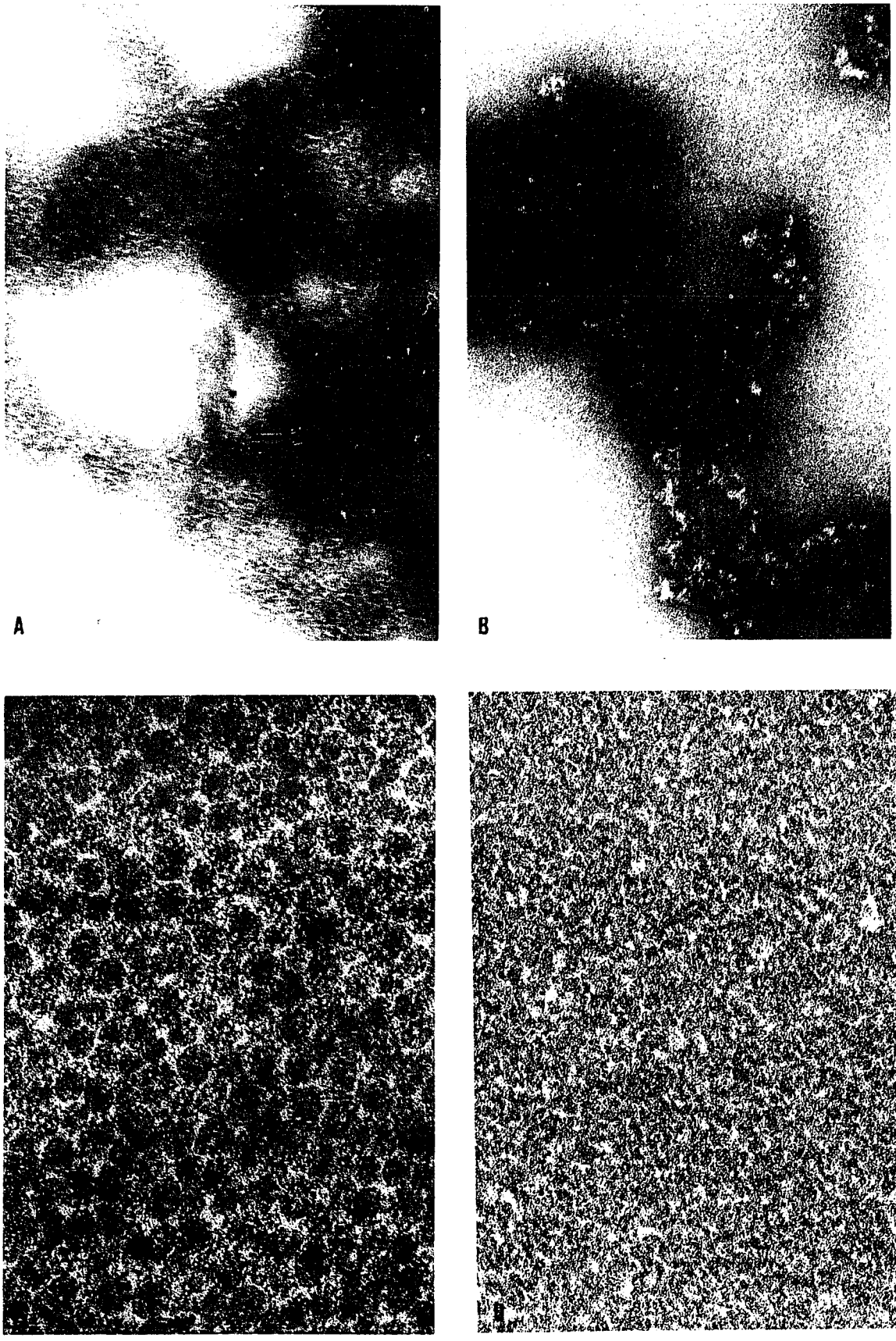


FIGURE 11

(Figs. 11c & 11d). The clathrin control showed the typical hexagonal and pentagonal units forming basket structures. The 0.5 M KCl-treated protein showed amorphous, nondescript material which lacked the basket structures. It was apparent from these experiments that the pre-formed baskets were sensitive to various ionic conditions. High pH (7.8) and KCl concentration (0.5 M) caused the baskets to break down and/or dissociate into filaments and aggregates. This change in clathrin assembly was reflected by the differences noted in sedimentation rates.

4. Effect of Cytoskeletal-disrupting Reagents on Clathrin Structures:

Clathrin obtained by vinblastine purification was tested for its ability to form baskets at pH 6.5. Figure 12a illustrates perfectly assembled baskets. Vinblastine at a concentration of 0.5 mM induced clathrin to precipitate at pH 7.2. When the precipitate was examined by negative stain at the electron microscope, a large network of filamentous material exhibiting extensive cross-linking was observed (Fig. 12b).

When the pH of clathrin was adjusted to 6.5 in the presence of a microtubular depolymerizing compound (colchicine) at a concentration of 250 μM, filamentous bundles were formed with attached clusters of baskets (Fig. 13a). In the presence of 10 μM cytochalasin B, a concentration that effectively disrupts microfilaments of actin, clathrin

Figure 12a. Clathrin molecules remaining in solution after addition of 0.5 mM vinblastine showed perfectly assembled baskets after adjusting the pH to 6.5. Magnification: 120,000 x

Figure 12b. A network of cross-linked microfilaments formed by clathrin precipitated by 0.5 mM vinblastine at pH 7.2. Magnification: 120,000 x

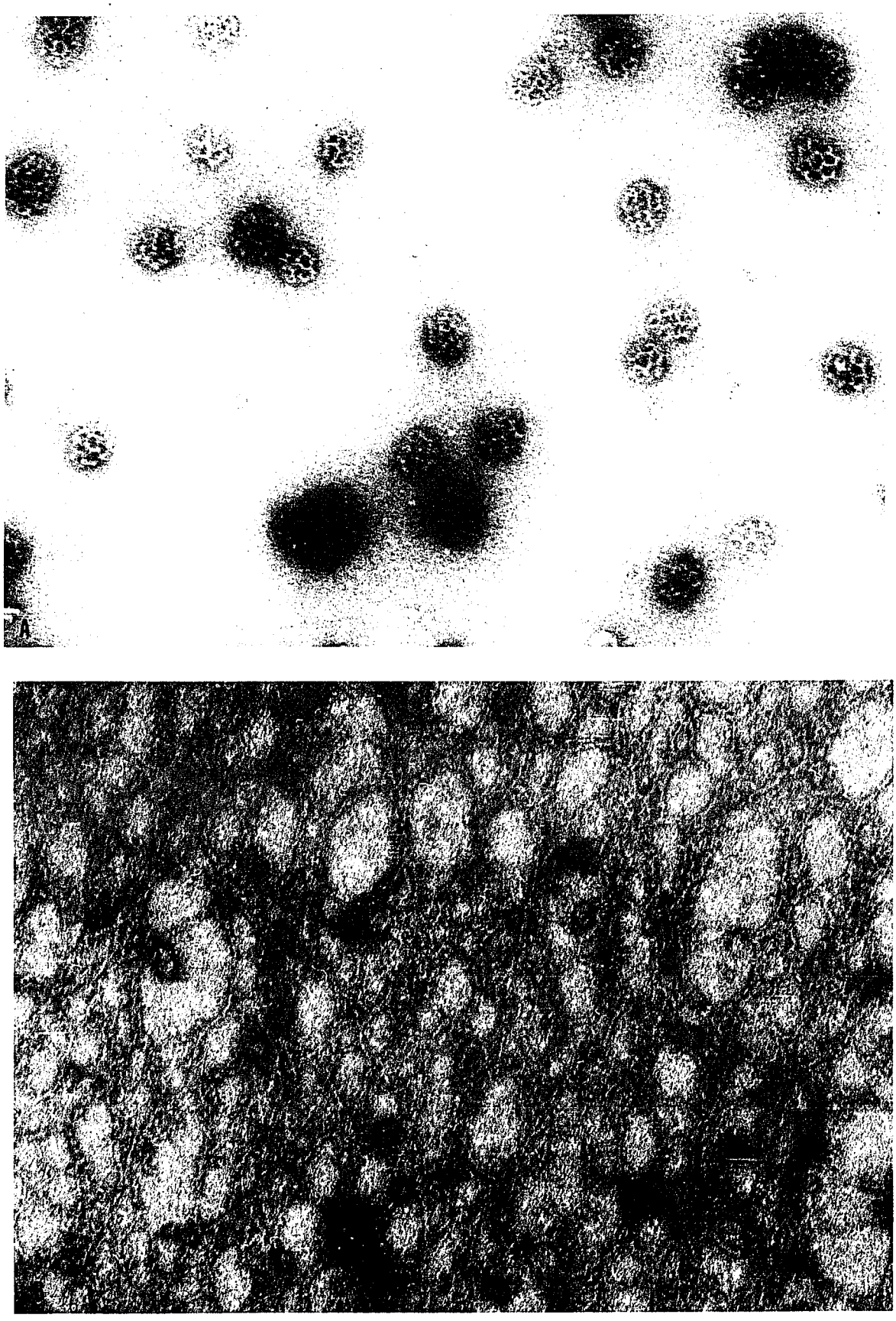


FIGURE 12

Figure 13a. Electron micrographs of negatively stained clathrin preparations formed in the presence of a) 0.25 MM colchicine--Clusters of baskets attached to filament bundles are observed, pH 6.5.

Figure 13b. Typical microfilament bundles formed by clathrin at pH 6.5 in the presence of 10 μM cytochalasin-B.

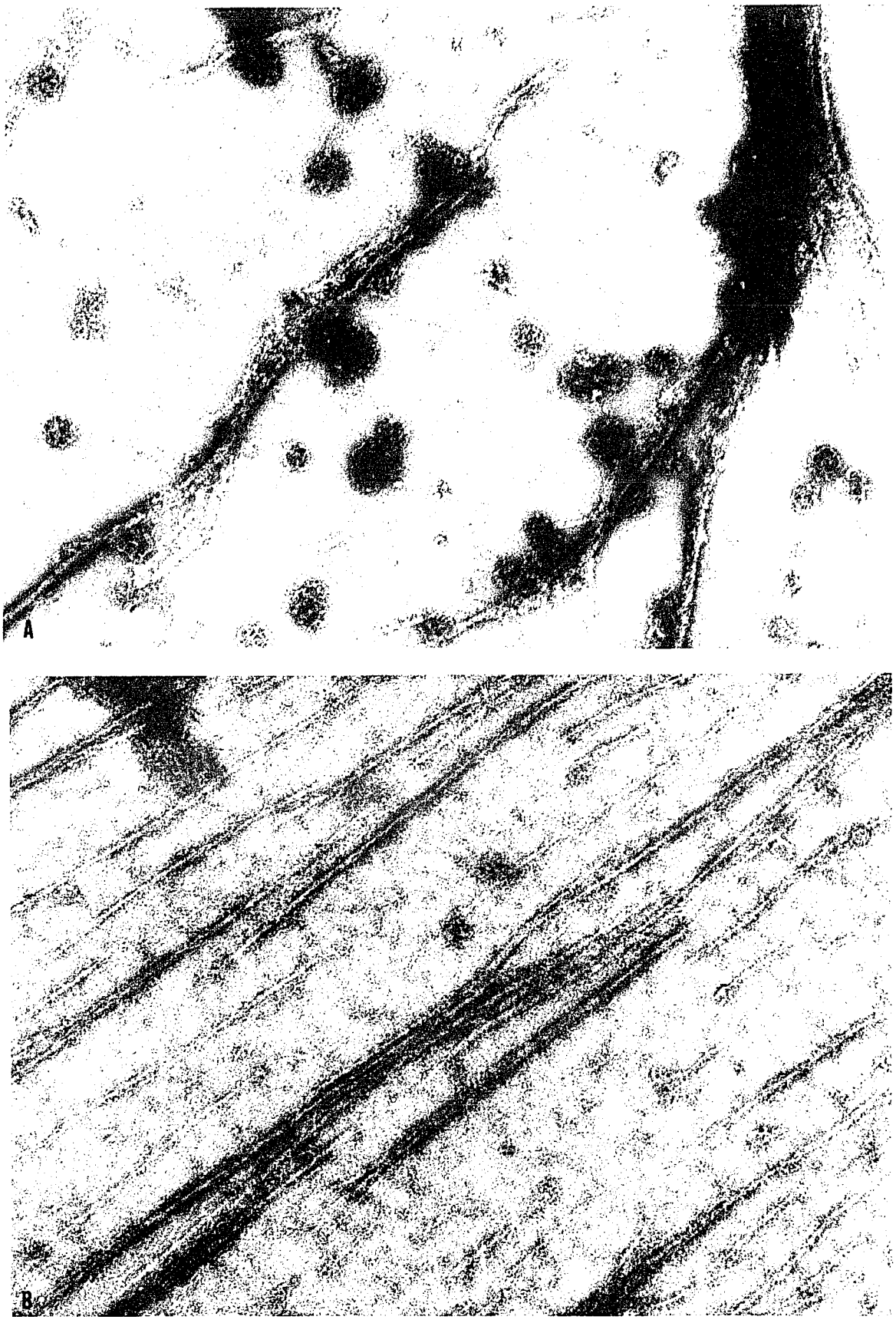


FIGURE 13

formed baskets and linear bundles when the pH was adjusted to 6.5. In Table 3, the overall effect of various drugs is summarized. None of these drugs prevented clathrin from forming baskets at pH 6.5. Furthermore, drugs that inhibit microtubule polymerization (vinblastine or colchicine), drugs that depolymerize actin microfilaments (cytochalasin-B), and drugs that stabilize actin in the filament form phalloidin), cause clathrin to form filamentous bundles (Fig. 13b).

5. Determination of Binding of Cations and Nucleotides by Clathrin:

To determine if purified clathrin possessed binding affinity for calcium and nucleotides, experiments were performed by incubating purified clathrin with a mixture of Ca^{40} and Ca^{45} (Fig. 14) and with a mixture of ATP and ATP C^{14}. After incubation with the calcium mixture, the samples were loaded on top of chromatographic columns containg Sephadex G-100, eluted, and the pattern of elution determined. It was found that clathrin did not show binding of calcium as manifested by the lack of radioactivity in the clathrin peak eluted from the column. The control protein was troponin, which is known to bind calcium., This troponin complex from muscle showed a radioactive peak eluting with the protein.

A highly purified clathrin preparation, when tested for ATP binding using equilibrium dialysis, gave negative results. The number of counts on the protein side of the equilibrium dialysis chamber was always equal to or less than the number of counts on the buffer side, indicating the absence of binding.

TABLE 3.

EFFECTS OF PURIFIED REAGENTS AND DRUGS ON CLATHRIN AT pH 6.5

		Filaments	Baskets
0.1 mM	VB	+*	+
1 mM	VB	+	+
0.1 mM	Cytochalasin-B	-	+
1 mM	Cytochalasin-B	+	+
0.1 mM	Cytochalasin-D	+	+
1 mM	Cytochalasin-D	+	+
0.1 mM	Colchicine	-	+
1 mM	Colchicine	+	+

* pH 7.0

Figure 14a. Determination of Ca binding--A muscle troponin preparation was incubated with a mixture of Ca^{40} and Ca^{45}. The cation/protein mixture was run through a Sephadex G-100 column and the elution pattern is illustrated. "X--X" represents mg/ml of total protein. ".__." represented cpm/0.2 ml. Bound calcium emerged on the protein peak.

Figure 14b. Similar determination as decribed in 14a, using a purified clathrin preparation--The protein peak emerged without Ca^{++}. X--X clathrin .__. Ca^{45} expressed as cpm/0.2 ml.

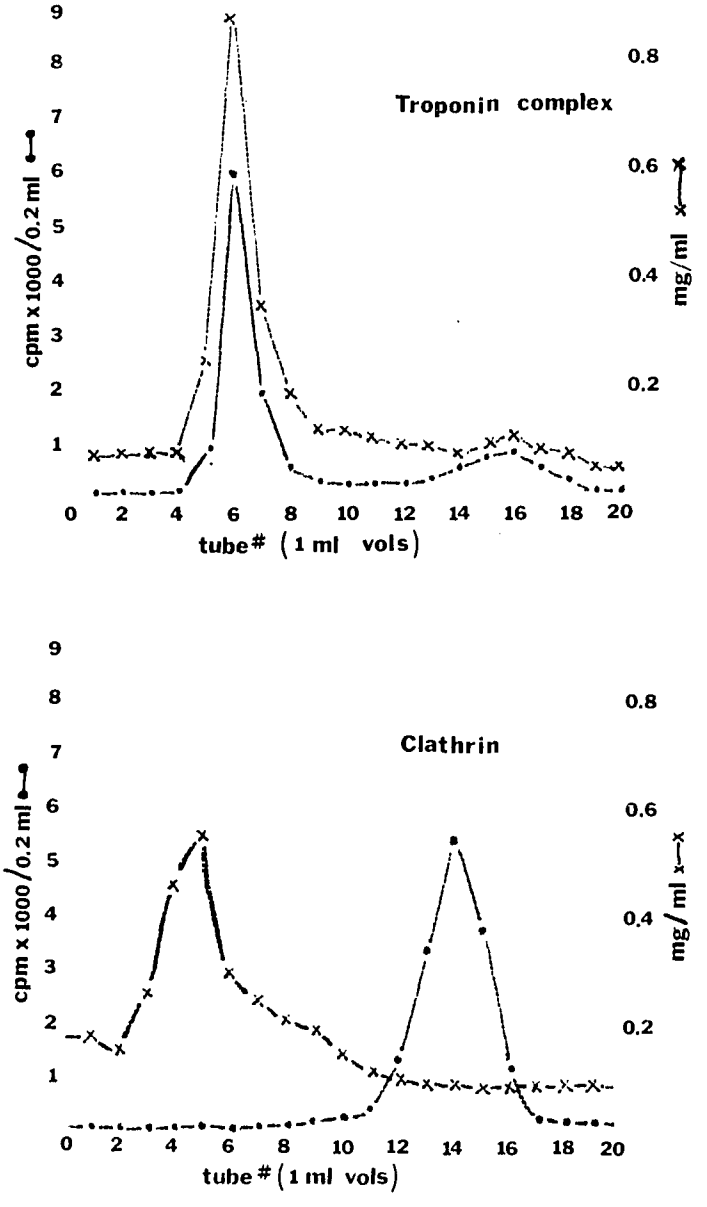

Troponin complex
cpm x 1000/0.2 ml
mg/ml
tube# (1 ml vols)
Clathrin
cpm x 1000/0.2 ml
mg/ml
tube # (1 ml vols)

FIGURE 14

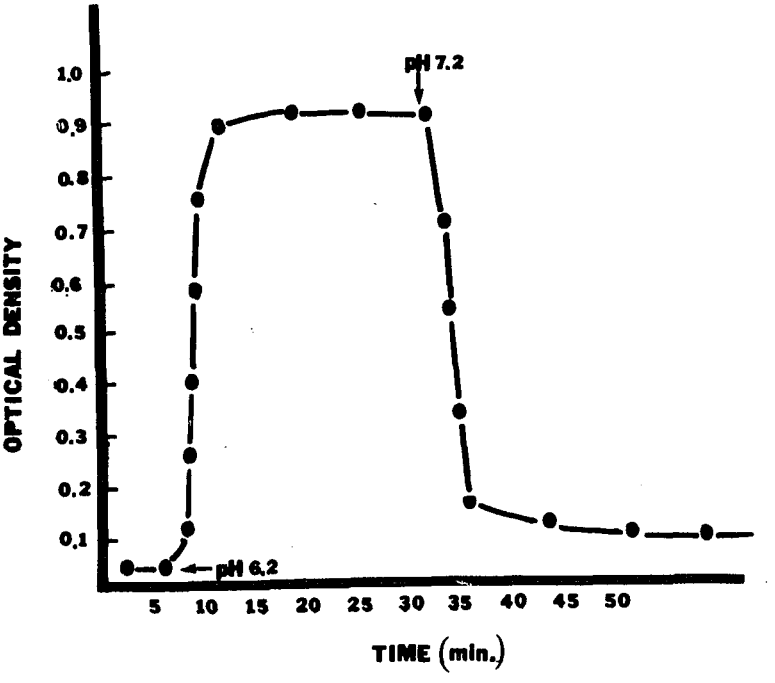
Control for nucleotide binding was myosin, and it showed 1.6 molecules of ATP bound per molecule of myosin. Under the conditions of experimental design, clathrin apparently did not exhibit binding affinity for calcium or ATP.

6. Biophysical Characterization:

The turbidimetric changes found with purified clathrin solutions were monitored by use of a recording spectrophotometer at 425 nm. When the pH was lowered from 7.5 to 6.8 or 6.0, turbidity increased (Fig. 15a). The turbidity change was rapid and reached a plateau value within one minute over a protein concentration range of 1.0-4.0 mg/ml. Raising the pH above 7.2, turbidity decreased rapidly to control values. When the pH was lowered again, turbidity increased (Fig. 15B). To determine the assemblies responsible for such changes, clathrin in solution at various pH's was examined by negative staining electron microscopy. Well-assembled clathrin baskets always were present in optically turbid solutions (pH 6.0-6.8). The possible contribution of contaminating tubulin to increments of turbidity by microtuble assembly was investigated. Colchicine, at a concentration which would inhibit microtubule assembly, had no effect on the turbidimetric changes (Fig. 15d). Furthermore, the supernatant from a vinblastine-precipitated clathrin solution had the same turbidity profile as a control clathrin preparation. It was apparent that clathrin's assemblies were responsible for the increase in optical absorption.

Figure 15a. Changes in optical density by adjustment of pH of a purified clathrin preparation--An increase in turbidity is observed when the pH is adjusted to 6.2.

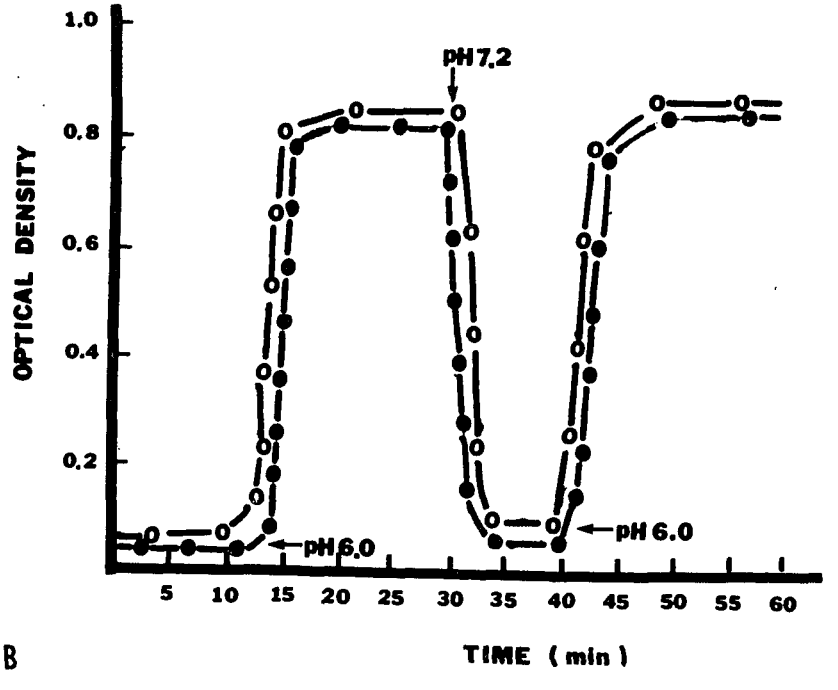
Figure 15b. Similar determination of turbidity as illustrated in 14a, in the presence of 250 μM colchicine. This figure illustrates the reversibility of turbidity by a sequential adjustment of pH from 7.2 to 6.5, and vice versa.



A

o—o +0.25 mM Colchicine

●—● No Colchcine



B

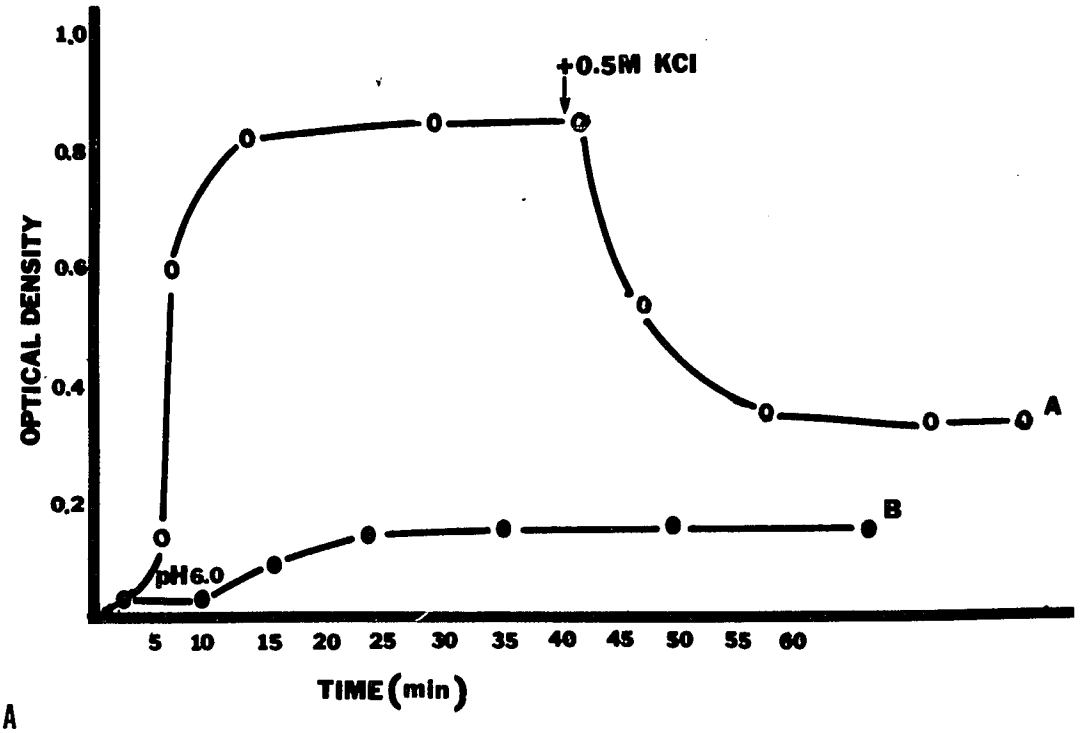
FIGURE 15

Figure 16a shows the effect of 0.5 M KCl on turbidity. When KCl was present, before basket assembly occurred, there were relatively small turbidity changes upon lowering or raising the pH. The addition of KCl after basket assembly had occurred caused turbidity to drop (Fig. 16a).

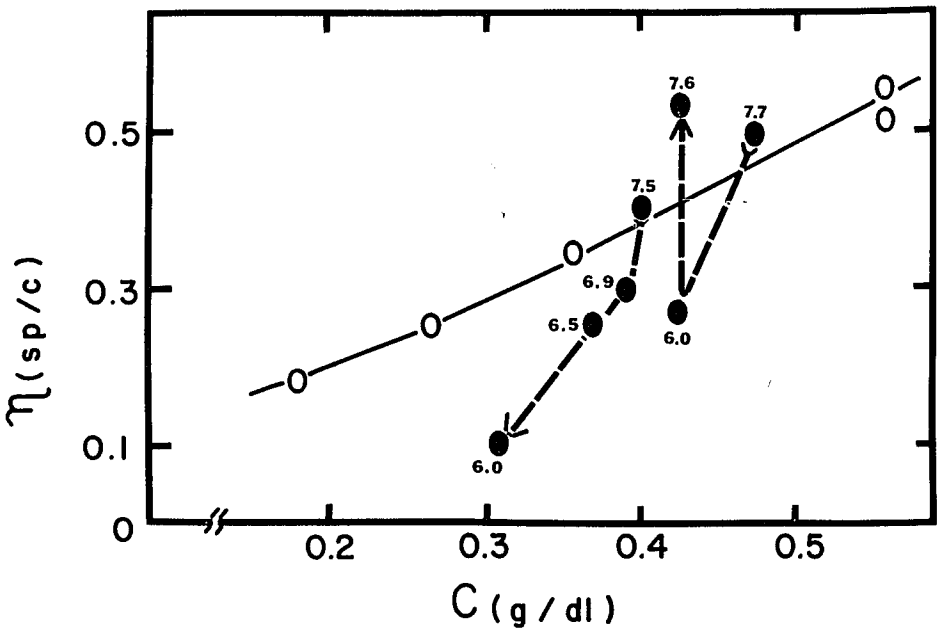
Viscometric measurements of clathrin were done in order to establish if there was a correlation between reversible basket formation and changes in viscosity. The reduced specific viscosity of clathrin in solution as a function of concentration is illustrated in Figure 16b by the line connecting the open circles. Reference to the reduced specific viscosity obtained at various protein concentrations established that a modification in viscosity also occurred by changes in pH. The results obtained by adjusting the pH of two different preparations of clathrin at various starting protein concentrations are shown in Figure 16b (dark circles and dotted lines connecting the various pH values). In all instances, a drop in viscosity was observed when the solution's pH was decreased. Viscosity returned to the expected value after its pH was raised. Viscosity changes were evident as soon as the sample was transferred from the pH meter to the viscometer and a measurement made, usually 1-2 minutes after adjusting the pH. Morphological correlation to the viscometric changes also were monitored by electron microscopy. Low ph, low viscosity solution showed many baskets, while high pH, high viscosity solutions

Figure 16a. Similar determination of turbidity as shown in Figs. 15a and 15b, but illustrating the effect of KCl on clathrin: (A) in the absence of KCl turbidity increases when the pH is adjusted to 6.0. Addition of 0.5 M KCl (at arrow) produces a decrease in turbidity. The effect obtained when the pH is altered in the presence of 0.5 M KCl (B). A very small change in turbidity is observed.

Figure 16b. A plot of specific viscosity as a function of concentration. A normalized curve joined by the open circle denotes the changes in specific viscosity at different protein concentrations. The dark circles joined by the dashed line indicate a given preparation of clathrin whose pH was changed. In one sample, the pH 7.7 value of viscosity is indicated and the drop in viscosity occurs below the normalized line when the pH is reduced to 6.0. Viscosity increased above the normalized line when the pH was returned to 7.6. Another clathrin preparation illustrates viscosity changes at intermediate points in the adjustment of pH: 7.5, 6.9, 6.5, and 6.


1.0
0.8
0.6
0.4
0.2
OPTICAL DENSITY
+0.5M KCl
A
B
pH6.0
5 10 15 20 25 30 35 40 45 50 55 60
TIME (min)

A


0.5
0.3
0.1
0
η (sp/c)
7.6
7.7
7.5
6.9
6.5
6.0
6.0
0.2
0.3
0.4
0.5
C (g/dl)

B

FIGURE 16

revealed a mixture of dispersed protein molecules, amorphous aggregates and bundles of filaments, but no baskets.

7. Immunological Characterization of Clathrin:

Characterization of the antibody--The antiserum raised against purified clathrin was characterized by standard and special immunological techniques. Double gel immunodiffusion was used to detect a precipitating antibody/antigen complex. The antiserum was tested with clathrin preparations of varying purity. Figure 17a shows the heavy immunoprecipitin line formed between clathrin antiserum and a partially purified clathrin preparation. There is a light, broad immunoprecipitin band formed with peak 1 of a Sepharose 4B column. Immunoelectrophoresis was performed using a crude clathrin preparation and also highly purified clathrin. As shown in Figure 17b, one precipitin lined formed between each antigen and the antiserum. Each precipitin band formed at equal distances from the loading well. The results from these determinations suggest that the antibody was elicited by one protein antigen, and that the antigen probably was clathrin.

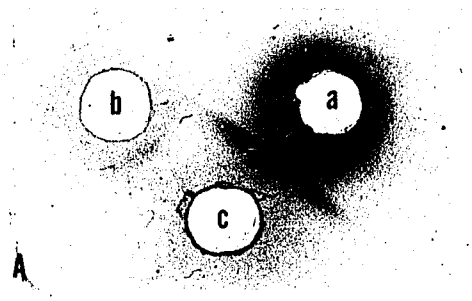
Immunoprecipitates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. For this, the anti-clathrin serum was incubated at serial dilutions up to 1:8 with a partially purified clathrin preparation. The immunoprecipitates were spun down at low speed and the pellets run on sodium dodecyl sulfate cylindrical gels. The pattern in Figure 17c shows that clathrin was precipitated

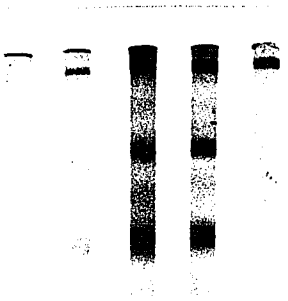
Figure 17a. Double gel immunodiffusion illustrating the immunoprecipitin bands formed between anti-clathrin serum and a purified clathrin preparation: a) well loaded with 25 µl of partially purified clathrin; b) well loaded with 25 µl of a Sepharose 4B column (membrane containing fraction); c) well containing 25 µl of rabbit antiserum to clathrin.

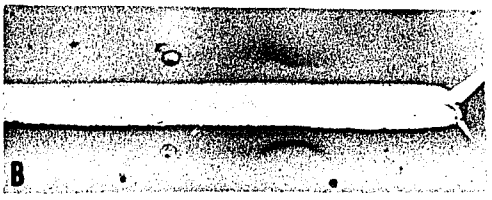
Figure 17b. Immunoelectrophoresis in 0.5% agarose--The upper, small well contains 5 µl of crude clathrin extract (2. mg/ml); the lower, small well contains 5 µl of purified clathrin (1 mg/ml); the trough contains clathrin antiserum.

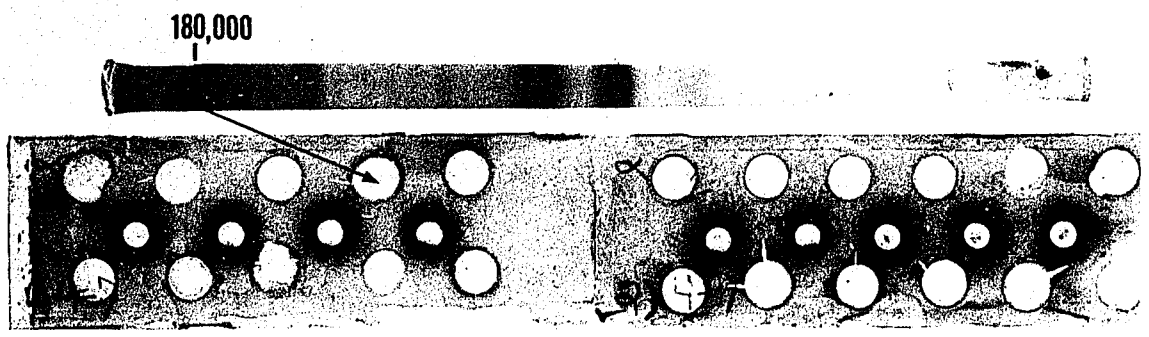
Figure 17c. Sodium dodecyl sulfate acrylamide gel electrophoresis of immunoprecipitate formed by clathrin and increased concentrations of antiserum--Gel #1 indicates the absence of polypeptides in a pellet formed by purified clathrin and preimmune rabbit serum; Gel #2: immunoprecipitate formed by purified clathrin with rabbit antiserum diluted 1:4; Gel #3: immunoprecipitate formed by clathrin and its antiserum diluted 1:2; Gel #4: immunoprecipitate formed by clathrin and undiluted rabbit antiserum; Gel #5: shows the clathrin migration standard.

Figure 17d. A crude preparation of clathrin resolved by sodium dodecyl sulfate acrylamide gel electrophoresis is in the horizontal cylindrical gel--A parallel gel ran, was sliced, mounted on microscope slides, surrounded by agarose and loaded with antibodies to agarose in the center well. The slice indicated by arrow #4 is reactive with the antiserum.









D

FIGURE 17

along with proteins migrating to a molecular weight zone corresponding to the heavy polypeptide chains of the IgG molecule. The minor bands present probably are due to other serum proteins co-precipitating along with the immune complex. No protein pellet formed when pre-immune serum was used with clathrin.

Further characterization was performed by use of a method developed in Dr. Puszkin's laboratory (50). The partially purified antigen (crude clathrin extract) was loaded on sodium dodecyl sulfate polyacrylamide gels and electrophoresed. The gel was sliced; the slices were aligned on a glass slide, embedded in agar, incubated in a humidity chamber, and allowed to diffuse from the acrylamide gel into the agar medium in order to react with antiserum. This technique permits an antibody to react with its specific antigen even from a protein mixture after its components have been separated by gel electrophoresis. Figure 17d shows immunoprecipitin bands which formed with a polypeptide that diffused from acrylamide slices corresponding to the molecular weight zone of 180,000. No other immunoprecipitin lines were evident. This data suggested that the antiserum reacted with clathrin, and apparently not with other accompanying proteins in the clathrin preparation.

Small amounts of highly purified clathrin were tested after a brief chymotryptic digestion. When clathrin was assembled as baskets, this enzyme rapidly hydrolyzed the smaller accompanying proteins at pH 6.5. The clathrin poly-

peptide and the basket structure remained intact. By high speed centrifugation, a pellet of packed baskets was sedimented and analyzed. Even with gross overloading onto slab gels, the protein appeared virtually as homogeneous clathrin (Fig. 18a). By immunodiffusion (Fig. 18b), there was a line of identity between this highly purified antigen and a crude clathrin preparation. Figure 18c shows anti-clathrin reactivity with an extract containing clathrin obtained from rat brain. Figure 18d shows reactivity with a similar extract prepared from bovine adrenal medulla.

For the subcellular localization of clathrin in cells and tissues using indirect staining techniques, the antibodies were purified by affinity chromatography. Such preparations are desirable when using immunocytochemical techniques where nonspecific background staining with whole antiserum may be high, and endogenous antibodies (usually anti-nuclear) may be present in the rabbits' blood circulation. A highly purified clathrin (Fig. 19a) was covalently bound to CNBr-Sepharose 4B. Upon addition of 6-8 ml of a 10-12 mg/ml DEAE-purified anti-clathrin IgG fraction to the column, the elution profile illustrated in Figure 19b was obtained. The non-absorbing IgG molecules formed the large, front-running protein peak. After addition of a 0.1 M glycine-0.5 M NaCl solution, pH 2.8, a sharp, symmetrical, protein peak eluted from the column. This IgG fraction (3-5 mg of total protein) was immunoreactive against clathrin, whereas, the front-running peak was not. A precipitin line formed when using

Figure 18a. Further characterization of the antiserum elicited by clathrin--This shows increasing concentrations of chymotryptic digested clathrin on slab gel electrophoresis. In clathrin which is undigested, the doublet polypeptides are weakly stained (arrows).

Figure 18b. The reactivity of the antiserum with non-digested clathrin and digested clathrin--Well #1 contains 25 µl of crude clathrin extract; well #2 contained 25 µl of chymotriptic treated clathrin; well #3 contains 25 µl antiserum.

Figure 18c. Double gel immunodiffusion--Well #1: highly purified clathrin; Well #2: rat brain extract containing clathrin; Well #3: the antiserum to clathrin.

Figure 18d. Well #1: antiserum to clathrin; Well #2: a crude preparation of clathrin obtained from bovine adrenal medulla.

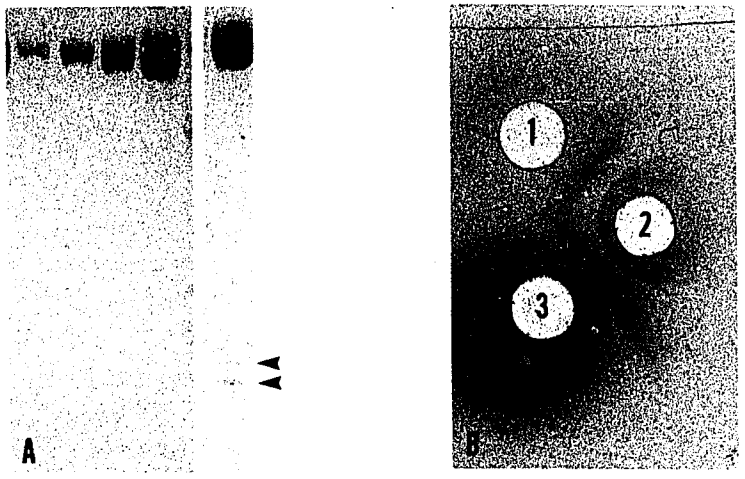
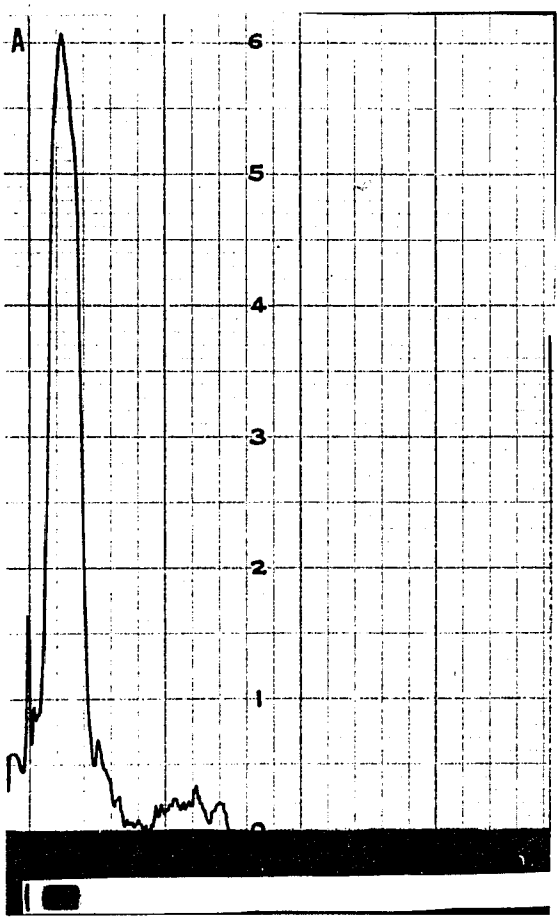
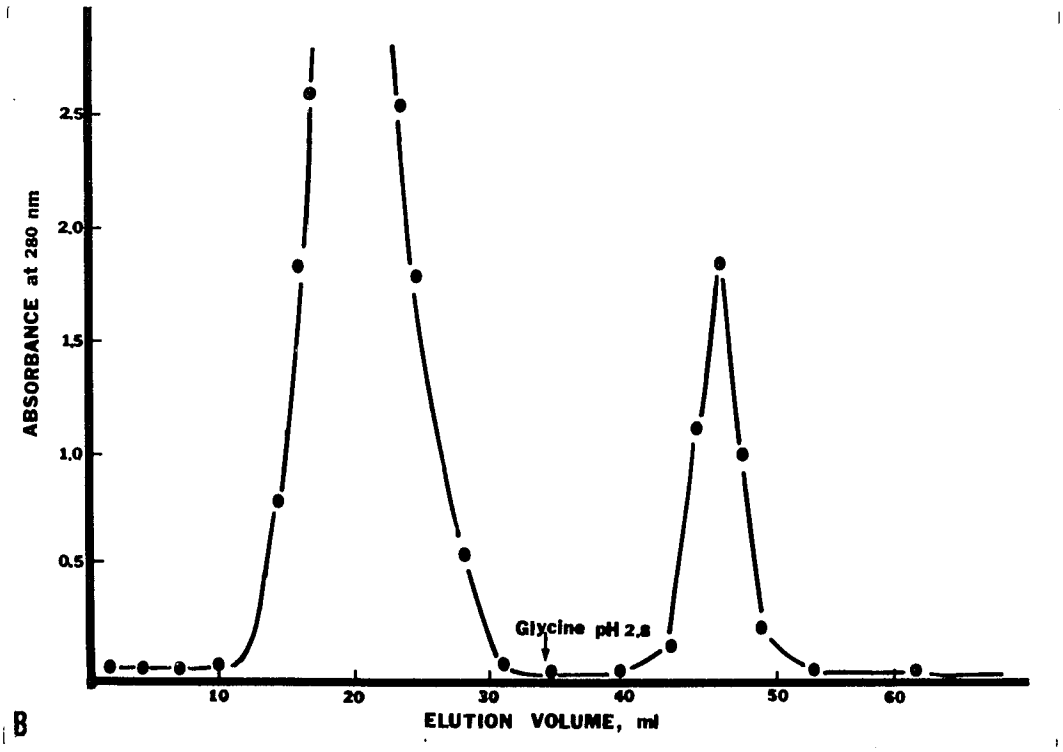


FIGURE 18

Figure 19a. Sodium dodecyl sulfate densitometry of a gel of highly purified clathrin--This protein was covalently bound to cyanogen bromide Sepharose 4B in order to isolate the specific IgG molecules reacting with clathrin.

Figure 19b. Elution pattern of an affinity chromatography column using cyanogen bromide Sepharose 4B conjugated with clathrin--Approximately 10 ml of beads were loaded into a small column of 1 x 10 cm containing the coupled clathrin to Sepharose 4B.


A
6
5
4
3
2
1
0


ABSORBANCE at 280 nm
2.5
2.0
1.5
1.0
0.5
Glycine pH 2.8
10
20
30
40
50
60
ELUTION VOLUME, ml

B

FIGURE 19

the affinity chromatography-purified antibody solution. It was lighter than that obtained either with the clathrin antiserum or its IgG fraction. This may have been due to the selective elution of low-affinity IgG molecules from the affinity column, while the high-affinity IgG molecules remained bound to the clathrin-coupled Sepharose beads.

8. Immunolocalization:

Various cells were stained with anti-clathrin antibodies. In all instances, the antiserum and the affinity column-purified IgG molecules gave identical results. Both pre-immune serum and clathrin-adsorbed antibodies showed no immunofluorescent staining patterns. As a consequence, all the fluorescent patterns described below are attributed to their immunoreactivity for clathrin.

Three different types of fibroblasts were tested: a) low-passage rat fibroblasts; b) a rat fibroblast cell line; c) and normal human diploid fibroblasts. All cell lines gave similar immunofluorescent staining patterns. Table 4 shows the results obtained using various fixation techniques. Fixation by paraformaldehyde or benzoquinone followed by either methanol or 0.05% Triton gave a distinct staining pattern of discrete, bright dots, many of them in linear arrays parallel to the long axis of the fibroblasts (Figs. 20a & 20c). Often, the linear arrangement of dots appeared parallel to and oriented along the microfilamentous stress fibers in the fibroblast cytoplasm. There was also intense,

TABLE 4.

IMMUNOFLUORESCENT STAINING

	Fluorescent Dots in Fibroblasts		Cytoplasmic Staining in Cerebella Cultures
	Human	Rat	
Methanol	-	-	++
Paraformaldehyde & Methanol	++	++	++
Methanol & Paraformaldehyde	±	±	++
Paraformaldehyde & Triton	+++	++	++
Paraformaldehyde	-	-	-
Benzoquinone	-	-	-
Benzoquinone & Methanol	++	++	++
Benzoquinone & Triton	++		++

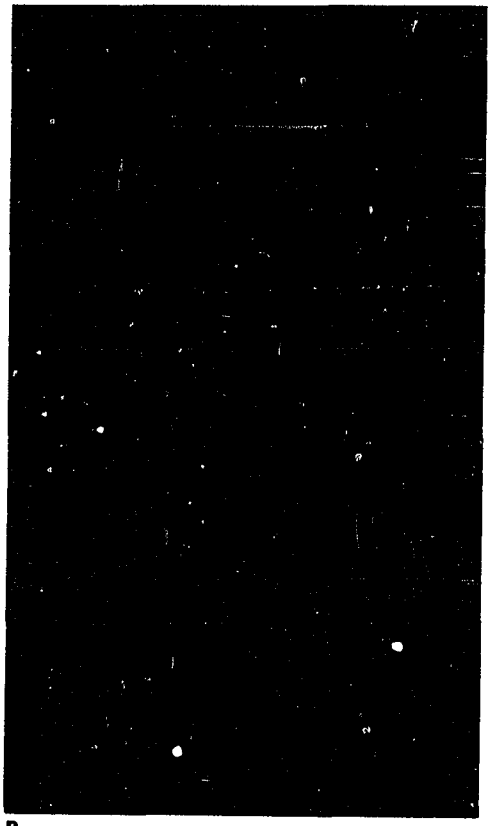
Figure 20a. Typical staining pattern of cultured rat fibroblasts stained with clathrin antibodies--Dots are arranged in linear arrays, seemingly parallel to the long axis of the cell. Perinuclear staining also is apparent. Magnification: 1,500 x

Figure 20b. Fibroblast control, using a clathrin antiserum absorbed with purified clathrin--Low background levels of staining are present. Magnification: 1,500 x

Figure 20c. Fibroblasts similar to those shown in Fig. 20a, indicating the alignment of dots parallel to the long axis of the cell. Magnification: 1,500 x

Figure 20d. Phase contrast of the same cell shown in Fig. 20c, showing the actin stress fibers apparently aligned along the long axis of the cell. Magnification: 1,500 x

A



B

C

D

FIGURE 20

dotted, non-aligned, perinuclear staining with both the antisera and the affinity-purified antibodies. Pre-immune serum, normal rabbit serum and clathrin-adsorbed antibodies gave low levels of background staining. When permeabilizing agents *i.e.*, methanol or Triton X-100 were omitted from the protocol, few discrete areas of dotted fluorescence were visualized. Methanol fixation alone resulted in a diffuse pattern of fluorescence.

In cerebellar granule cell cultures, intense immunofluorescent staining was observed after varying fixation conditions (Table 4). Both the clathrin antiserum and the affinity column-purified antibodies gave intense staining of granule cell cytoplasm and in the processes of the long cytoplasmic projections (Fig. 21a). The neuronal processes presented a discontinuous fluorescent staining pattern that correlated with varicosities observed by phase contrast (Figs. 21a, 21b & 22a). Nuclear staining was absent. The other supporting cells growing in the culture, such as astrocytes, had lower levels of staining seen only at very low dilutions of antisera.

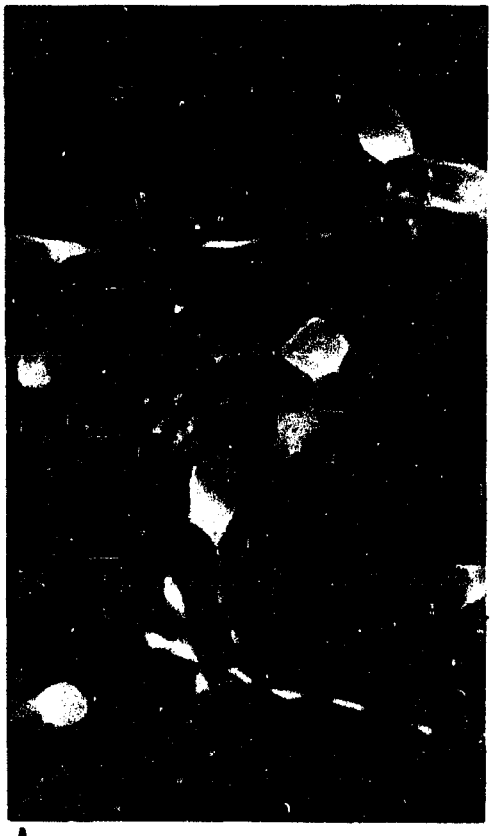
Thin, frozen sections of cerebellum from 5-7-day-old rat and adult mouse brains were stained for immunofluoresence using clathrin antiserum and the affinity column purified IgG. Figures 22c and 22d show bright cytoplasmic staining of the granular layer. The fluorescence is cytoplasmic, with the unstained granule cell nuclei appearing dark. The cytoarchitecture of the 7-day-old rat cerebellum

Figure 21a. Immunofluorescent staining using clathrin antibodies on cerebellar cell cultures--The cytoplasm of the granule cells show intense staining and stained varicosities are visible along the processes. Magnification: 1,500 x

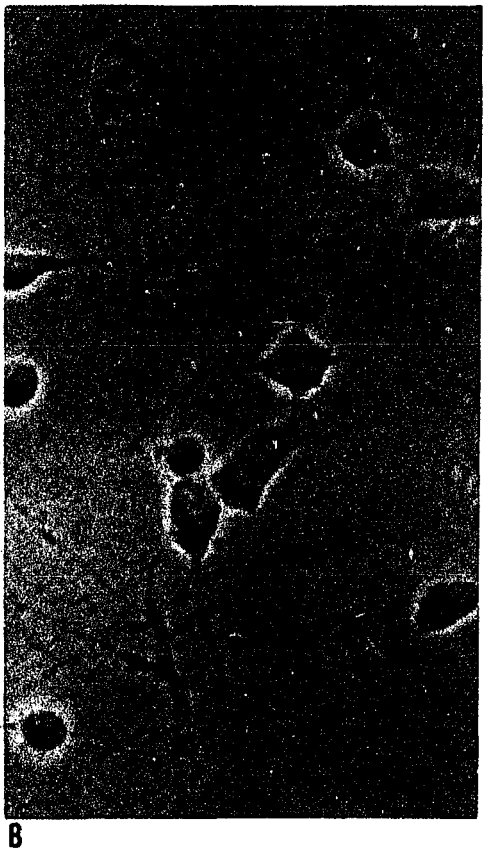
Figure 21b. The same culture and cells viewed by phase contrast indicating the limits of the cells and the projections and varicosities. Magnification: 1,500 x

Figure 21c. Similar to Fig. 21a, showing a different plain focus of a cluster of granule cells. Magnification: 1,500 x

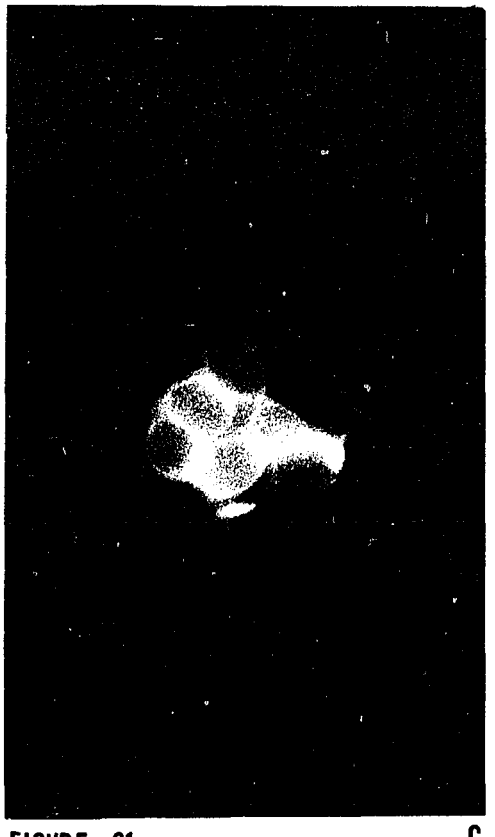
Figure 21d. Same as Fig. 21c, except viewed by phase contrast. Magnification: 1,500 x



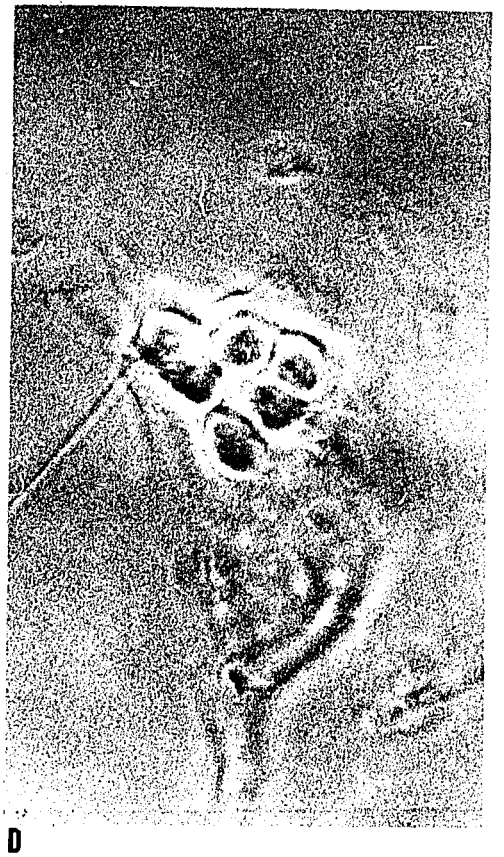
A



B



C



D

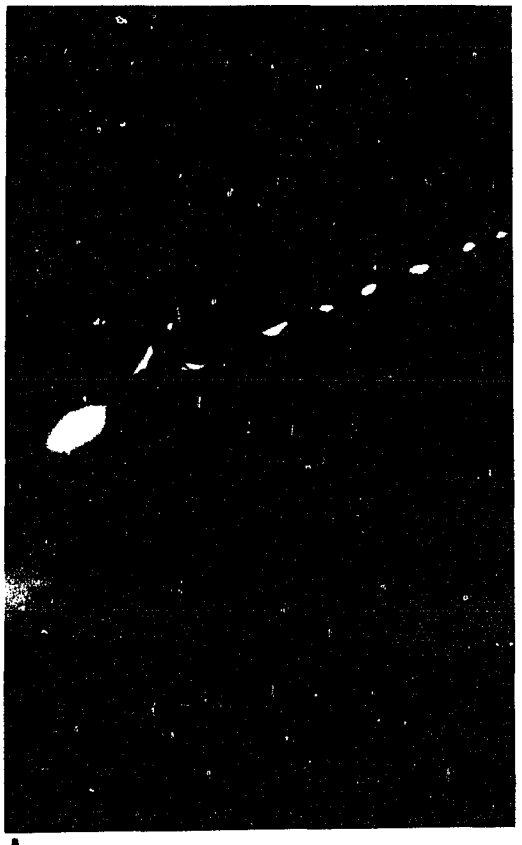
FIGURE 21

Figure 22a. Similar to Fig. 21a, showing a cytoplasmic projection from the granule cell cultures indicating clearly the intense varicosities stained along the projection of the cells--Dots indicate a local concentration of clathrin in this area. Magnification: 1,500 x

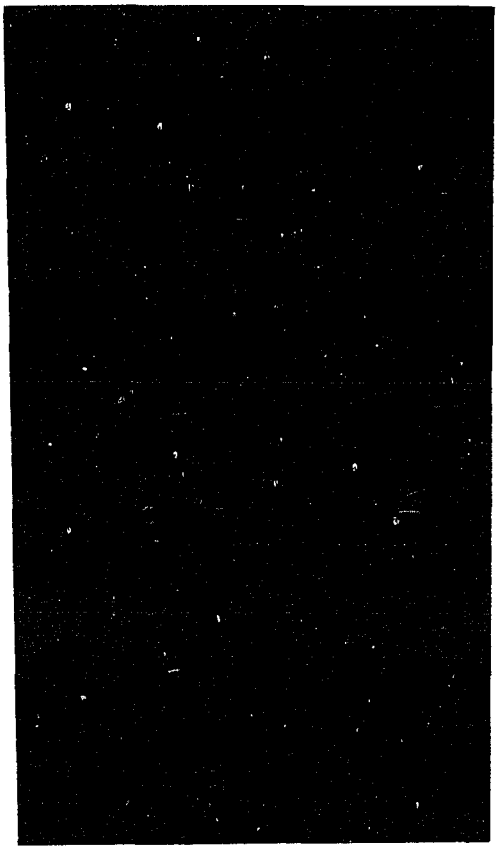
Figure 22b. Control using absorbed anti-clathrin antibodies.

Figure 22c. Cerebellar section from 7-day-old rat illustrating immunofluoresence in the developing internal granular layer--The fiber tract shows no staining. Magnification: 300 x

Figure 22d. Cerebellar section from adult mouse stained using affinity-purified antibodies to clathrin--The immunofluoresence is localized to the granule cell cytoplasm, Purkinje and molecular layers. The cytoarchitecture is typical of developed cerebellum. An unstained fiber tract is present. Magnification: 300 x



A



B

C

D

FIGURE 22

(Fig. 22c) was not yet fully developed. The maturing internal granular layer showed strong anti-clathrin staining. A fiber tract running transversely across the field was not stained. The adult mouse cerebellar section (Fig. 22d) had a well defined cytoarchitecture typical of matured cerebellum. The immunofluoresence appeared in the granular layer (cytoplasmic stain with the dark, unstained nuclei standing out), the Purkinje, and molecular layers. An unstained fiber tract was present. (All the controls using pre-immune serum, normal rabbit serum, or clathrin-adsorbed antiserum showed low levels of nondescript background staining.) Apparently, clathrin is present in both growing and already-matured cerebellar tissue.

9. Peroxidase/Anti-peroxidase Staining Technique:

Utilizing the method of Sternberger and Pickel (see Methods), indirect immunoperoxidase staining was attempted using adult rat cerebral cortex. Immunochemical reactions were carried out using the clathrin immune serum, an IgG fraction of this serum, and also the affinity column purified material, all diluted 1:500. The control used was normal rabbit serum. Black precipitate was seen (in experimental tissue sections) in discrete locations around the membrane of all bodies and axon hillocks. The nuclei were unstained (Fig. 23a). Control normal rabbit serum showed diffuse nuclear staining (Fig. 23b) due to the presence of anti-nuclear antibodies which are found in approximately 50% of all rabbits (Pickel, personal communication). There was a lack of delineation of cell body and axon

Figure 23a. Adult rat cerebral cortex stained using clathrin antibodies with the peroxidase/anti-peroxidase technique--The stain, blackened precipitates, is seen to be localized in discrete areas around the cell bodies and along axon hillocks as indicated by arrows. The nuclei (N) are unstained. Magnification: 1,200 x

Figure 23b. Control experiment of Fig. 23a, using normal rabbit serum for staining--The diffuse nuclear staining represents anti-nuclear antibodies non-specifically found in many rabbits. Note the absence of dilineation of cell body and axon hillock membranes as seen in Fig. 23a. Magnification: 900 x

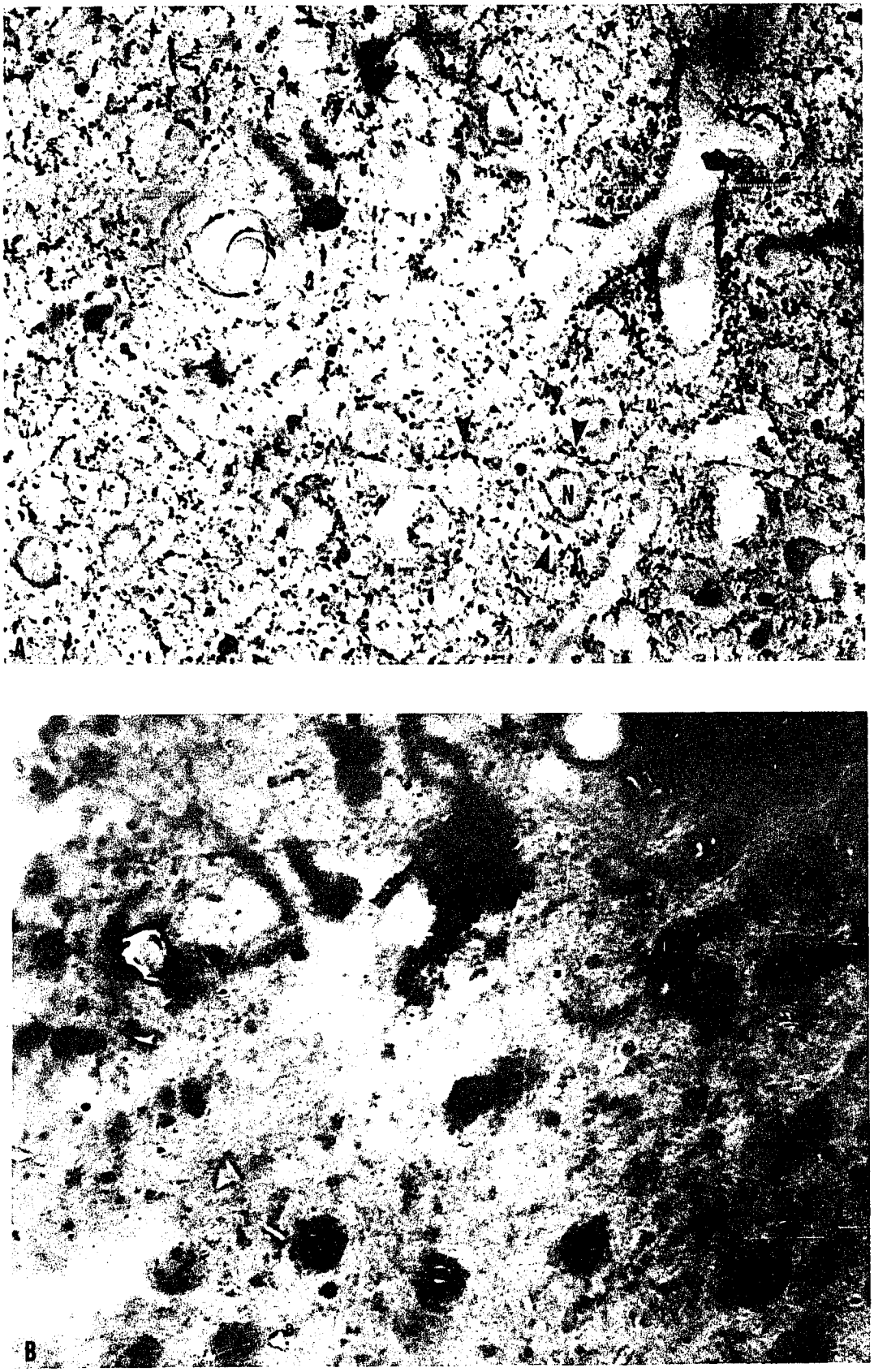


FIGURE 23

hillock membrane in the control sections as compared to the sections stained with anti-clathrin antibodies. The difference, as confirmed using Nemarsky optics, was dramatic.

DISCUSSION

The method devised for the preparation of a subcellular fraction enriched with coated vesicles is similar to that used to prepare a microsomal fraction (59). With suitable variations, a considerable increase of well preserved coated vesicles was obtained by the utilization of a Tris-HCl buffer, pH 7.0, at the homogenization step of the brain gray matter. It was apparent under our experimental conditions, that clathrin neither disassembled nor dissociated from the vesicle membrane. The next task was to dissociate and separate clathrin from the membrane of vesicles and other plasma membrane fragments. The following steps were devised based on the assumption that clathrin is neither part of nor inserted into the lipid bilayer of membrane but rather bound to one or more of its constituents. As such, clathrin would dissociate either with the use of high salt concentration buffers at pH's above 7, or with low ionic strength buffers containing disssociating reagents.

Buffers with high salt concentration were ineffective in extracting clathrin because they fragmented and emulsified the existing membranes with concomitant solubilization of a large number of membrane-associated components. Consequently, a mostly heterogeneous preparation was obtained. To avoid this, a solution containing 20 mM Tris-HCl, pH 7.5

was devised to dissociate clathrin assemblies from the crude coated vesicle pellet with minimal membrane fragmentation or lipid emulsification.

To separate clathrin from remaining proteins and membrane fragments, gel filtration chromatography was used. When the clathrin extract was gel-filtered on Sepharose 4B equilibrated with 20 mM Tris-HCl buffer, pH 7.2, a single peak emerged with some trailing behind showing the same heterogeneity of the protein mixture loaded originally.

When purification of the clathrin extract was attempted using ion-exchange chromatography on DEAE-Sephadex A50 and elution by a linear gradient of potassium chloride, lipidic material appeared in the wash fraction and a heterogeneous protein peak emerged between 0.2-0.4 M potassium chloride. It consisted of the same protein mixture loaded initially on the column. A loss of clathrin occurred, apparently tightly bound to the resin beads. Attempts to dissociate and elute it with urea and/or with washes of 0.5 M sodium hydroxide did not produce a protein eluate. It was apparent that aggregation with or irreversible denaturation of clathrin had occurred. In view of these results, ion exchange chromatography was not pursued at this step.

This suggested the need for a reagent in the buffer possessing the properties of reducing protein/protein interaction, as well as protein/membrane affinity. Urea, known to possess such properties, was tested. The addition of 2 M

urea to the Tris-HCl buffer proved effective in fractionating clathrin from other components. The use of urea in the gel filtration buffer resulted in the elution of 3 partially overlapping peaks, with clathrin highly enriched in peak 2. This indicated that the continuous presence of urea was required to maintain the molecules of clathrin dissociated from membrane fragments and from accompanying proteins during the molecular sieve filtration through Sepharose beads.

The remaining contaminants were proteins that comigrated on sodium dodecyl sulfate gel electrophoresis with known components of the cytoskeleton. They included a 100,000 molecular weight polypeptide which, most likely, was α-actinin, a protein recently isolated from crude coated vesicles; from tubulin, which on slab sodium dodecyl sulfate gels resolved into a doublet of α and β subunits; actin, the protein of thin microfilaments; and tropomyosin, a regulatory protein of contractility. The high speed pellet from each peak of the 2 M urea Sepharose column, viewed by electron microscopy, revealed that gel filtration succeeded in removing membranes and amorphous proteinaceous material from the clathrin peak.

Further attempts to purify peak II were carried out. It was concentrated, resuspended, dialyzed and rechromatographed as before on a Sepharose 4B column equilibrated with the same buffer. As expected, the first peak (membranes) was reduced largely, and the second peak (clathrin) was predominant. There remained only traces of tubulin, actin and a doublet whose relative concentration apparently had increased.

To separate aggregates from monodispersed clathrin formed during dialysis (to remove urea and other salts), clathrin was gel-filtered through a third Sepharose 4B column equilibrated with a Tris-HCl buffer, pH 7.5, without urea, but with 1 mM 2-mercaptoethanol. Of the two peaks eluted, the first emerged in the void volume and contained a small amount of clathrin bound to most of the contaminating proteins. The second peak consisted mostly of clathrin accompanied by a small amount of the low molecular weight doublet. There seemed to be a significant affinity between these proteins. Studies in progress suggest that removal of the doublet protein by proteolysis under conditions which do not affect clathrin molecules, inhibits clathrin's capacity to reform baskets or cages at pH 6.0-6.8 (52).

Vinblastine precipitation of contaminating actin and tubulin was tested by an alternate procedure. Reports in the literature have shown that vinblastine, an inhibitor of microtubule assembly, was able to precipitate tubulin and actin when actin was one of the contaminating proteins (53). At a concentration of 0.1-0.5 μM, vinblastine precipitated contaminating proteins and some clathrin. The remaining clathrin was mostly homogeneous. The material prepared by vinblastine precipitation was adequate for biochemical and biophysical characterization.

Cages or baskets formed by the highly purified clathrin indicated that this capacity was retained by the clathrin

molecule. Clathrin preparations of low and high purity (60-80% or 95% respectively) produced cages or baskets at pH 6.5 that remained intact when sedimented at high speed. The cage structures were identical to those observed in preparations of crude coated vesicles and appeared with hexagonal or pentagonal subunits, with each side of a pentagon or hexagon representing the length of a molecule of clathrin. If correct, the estimated molecule length ranged between 110 and 140 Å. It is likely that the molecular thickness of clathrin is due to 2 clathrin molecules attached lengthwise or interwoven (54). Partially polymerized clathrin molecules filling the background surface of a Formvar-coated grid appeared as dimers, trimers or tetramers in geometrical arrangements of triangles, squares, pentagons or hexagons. Protein assemblies of these types are difficult to visualize with sufficient detail to ascertain their precise configurations or arrangements. They usually flatten on the plastic surface coating the grid and do not acquire enough negative stain contrast.

At present, we know with certainty that the basket- or cage-like configurations assembled as a function of pH. At a pH lower than 7.0, the surrounding proton change probably favors clathrin to remain attached at the end of its molecules favoring the basket lattice and its closure. When the pH is above 7.0, clathrin's molecules dissociate, disrupting the cage structure as well as the hexagonal or pentagonal units.

In addition to pH, ionic strength influenced the configuration of clathrin assemblies. Clathrin, in the presence of 0.5 M KCl, at pH 6.5, did not form cages with the distinct appearance typical of those formed in the absence of salt. Clathrin molecules in the presence of salt and low pH (6-6.5), formed aggregates or clusters of nondescript shapes and sizes. Obviously, the presence of ionized salt affected the charge distribution along the clathrin molecules and, consequently, the interaction at the sites responsible for basket formation.

To determine conditions that affected the stability and shape of clathrin baskets, they were subjected to centrifugal forces and their sedimentation parameters analyzed on sucrose gradients where pH, salts and other compounds could be added to predetermined values or concentrations. Clathrin pre-assembled as baskets or cages sedimented on discontinuous sucrose gradients of pH 6.6, between the 50 and 55% sucrose interface. A small amount equilibrated between the 20 and 30% interface. Sucrose gradients with 0.5 M potassium chloride showed about 50% of clathrin sedimenting between the 40 and 50% interface. The remainder was found at the 20 to 30% sucrose interface. The rationale for this experiment was to test the stability of preformed baskets to the same ionic conditions which had been shown to prevent their assembly. Apparently, the ionic environment provided by 0.5 M KCl overcame the protein/protein interaction between the clathrin molecules in the baskets. The clathrin molecules

rearranged into 2 populations: one of high bouyant density (aggregated), and the other of a fine cross-linked filamentous reticulum of light buoyancy.

As mentioned above, it was apparent that pre-formed baskets introduced into a proton environment unfavorable to basket formation reversed the assemblies into structures similar to those produced by the sucrose/salt gradients. As before, 2 populations of clathrin were noted: amorphous aggregates and filaments. This emphasized the apparent plasticity of clathrin in response to its ionic environment.

It is interesting to note that, in our hands, clathrin was insensitive to divalent cations. Chelating reagents such as EGTA and EDTA neither affected the sedimentation pattern, nor clathrin basket morphology. This finding correlated well with the lack of calcium binding exhibited by clathrin of high purity. Since the preparations of clathrin required extensive treatment with 2 M urea, the possibility exists that clathrin indeed may be regulated in its function by calcium, but lost during purification. However, from a purely biophysical point of view, clathrin does not require divalent cations to assemble into basket configurations.

Turbidity levels exhibited by clathrin in solution changed as a function of its assembled structures. Solutions of clathrin, which at pH 7.2-7.5 were optically clear, were turbid at pH 6.-6.5. Samples for electron microscopy taken

before and after pH change corroborated that no baskets existed above pH 7. It was apparent that turbidity changes correlated with the various clathrin configurations. This cycle of pH change was repeated several times with similar results. This fact illustrates the dynamic nature of clathrin in its configurations.

Some of the preparations tested were of varied purities and among the proteins that contaminated clathrin was the doublet of 30,000 molecular weight, actin and tubulin. To exclude the possibility that tubulin could be forming microtubules by changes in pH producing the turbidity, a solution of clathrin was made 0.25 mM with colchicine. At this concentration, colchicine inhibited turbidity due to the polymerization of microtubules, but did not alter clathrin's turbidity values. Therefore, turbidity changes were most likely due to clathrin assembly of its characteristic structures. The dissociating activity of high salt concentrations, such as 0.5 M KCl, was followed turbidimetrically. Addition of 0.5 M KCl produced a decrease in turbidity. By electron microscopy it was obvious that clathrin baskets had disassembled. This supports the postulate that ionic forces control and modulate clathrin configurations.

The viscosity of clathrin solutions was studied. It is known that actin, tubulin and actomyosin molecules polymerize. Their viscosity increases due to the resistence of flow through a capillary tube. Solutions of clathrin assembled as baskets offered low or reduced viscosity. Solutions of

clathrin at pH 7.5--where filamentous formations or aggregates are formed--exhibited higher viscosity. The graphed curve of protein versus concentration was found to follow typical protein viscosity patterns.

It was concluded that clathrin configurations were unique. Clathrin possessed characteristics of assembly without similarities to any other proteins reported to date. Actin, upon polymerization, undergoes increased birefringency and viscosity. Depolymerized actin, as G-actin molecules, exhibit drastically reduced viscosity. Tubulin preparations and other non-cytoskeletal proteins such as IgG, fibrinogen, etc., exhibit similar properties. Thus clathrin stands alone as a protein with distinct turbidity and viscosity given by its particular configurations.

Having obtained a highly purified clathrin, we proceeded to analyze its immunochemical properties. If specific antibodies were to be elicited, we could study cross reactivity with clathrin from other tissues and establish their localization in order to obtain insight as to their role in cellular functions. In our hands, the injection of purified clathrin assembled as baskets before emulsification with Freund's adjuvant yielded the most consistent and immediate antibody response.

The antibodies reacted with various clathrin preparations and in all instances one immunoprecipitin band was visualized. Nevertheless, the purity of the elicited anti-

bodies also was analyzed by immunoelectrophoresis, which combines electrophoresis plus immunodiffusion. Using this procedure, a single arc was formed. These determinations were indicative that a single antibody had been elicited by clathrin injection. It remained to be determined if this antibody was reacting with clathrin molecules or with other small contaminating proteins still bound to clathrin.

Further testing was carried out by adsorption of purified clathrin from solution with a clathrin antiserum. Depolymerized clathrin precipitated from solution with its own antibody, forming an insoluble complex. This complex, when dissolved and resolved on sodium dodecyl sulfate acrylamide gels, revealed IgG molecules and a heavy polypeptide band comigrating with clathrin. This indicated that the antibodies recognized clathrin molecules in a solution and were capable of aggregating and precipitating them. To demonstrate direct immunological reaction between clathrin's 180,000 dalton polypeptide and the antibody, the clathrin molecules resolved from other proteins on sodium dodecyl sulfate polyacrylamide gels were used for direct reaction with the antibody. Since the protein diffusing from the sliced gels and belonging to the 180,000-dalton zone was the only one that gave an immunoprecipitin reaction, it was considered conclusive enough that antibody molecules recognized antigenic determinants in clathrin.

To maximize the specificity of the antibodies reacting with clathrin in tissue, the specific immunoglobulin molecules

formed by clathrin were separated by affinity chromatography. The purified IgG molecules showed reactivity to clathrin similar to those in the serum.

Since the antigen was prepared from bovine brain and clathrin's localization were to be attempted in a variety of culture cells and tissues of non-bovine origin, a crude clathrin extract from rat brain was tested for immunoreaction with the antibodies. The affinity-purified antibodies as well as the whole antiserum reacted with this preparation. Similar results using whole antiserum elicited by a crude clathrin preparation against human lung fibroblasts recently were reported (49).

This study indicated that clathrin was an ubiquitous protein whose antigenic determinants were preserved phylogenetically. As our antibodies were being characterized, it was reported that antibodies to coated-vesicle extracts obtained from pig brain tested in rat fibroblasts gave a characteristic stain pattern (60). Encouraged by these results, the affinity-purified clathrin antibodies were used in cultured rat and human fibroblasts. Characteristic patterns of fluorescent dots were observed distributed throughout the cytoplasm. It is possible that the stained dot pattern obtained represented lattices of clathrin situated below the membrane attached to its cytoplasmic side. The Triton X 100 treatment probably dissolved the bilipid layer and exposed the lattice to binding by the antibodies' molecules. Also it is possible that an amount of lattices of

clathrin having been washed, left the ones remaining attached somehow to the cytoskeletal protein fixed and insolubilized as stress fibers of actin and realted proteins, such as α-actinin or tropomyosin, by the fixation reagents. A recent report showed clearly that clathrin lattices were indeed in different stages of assembly, under the membrane, and laterally attached to cytoskeletal actin stress fibers (58). The immunofluorescent staining found with our antibodies, therefore can be attributed (with confidence) to clathrin.

The localized clathrin is considered to be cytoplasmic since membrane solubilization was needed to obtain fluorescent staining. Two previous works on receptor-mediated endocytosis in the fibroblast showed that after ligand binding, there was a rapid clustering of surface ligand-receptor complexes into a dotted pattern virtually identical to that shown in this study for clathrin (61, 62). Also, it is significant that different ligand-receptor complexes formed the same dotted pattern along the surface of the fibroblast (60). This data, when combined with information derived from our study, indicates that clathrin localized into discrete areas under the plasmalemma could function as a generalized mode of entry into the cell for receptor-bound macromolecules.

When cells showed the stained dots aligned in parallel arrays, they clearly were correlated by phase contrast with actin stress fibers, thus suggesting a close interaction between actin and clathrin. In support of this, there was a

previous report from Dr. Puszkin's laboratory showing a binding affinity between clathrin and actin as determined on the surfaces of polystyrene particles *in vitro* (57). This interaction was even stronger when α-actinin was present and bound to clathrin. Molecules of α-actinin have been found in fibroblasts, in their membranes, and along actin stress fibers in a staggered arrangement (63). Recent evidence indicated that the doublet seen in association with clathrin is identical to brain tropomyosin (52). Considering brain tropomyosin's affinity for clathrin--it copurifies with clathrin after several chromatographic procedures--and the known affinity of tropomyosin for actin molecules (51), an *in vivo* relationship of clathrin with cytoskeletal proteins is feasible and of potential significance.

Of great interest was the intense perinuclear array of fluorescent staining in the region which could be expected to have the Golgi cisternae and the GERL. In many cell types, coated vesicles have been observed in this region (64), either budding from or fusing with the Golgi cisternae and the GERL. Ultrastructural studies eventually will establish how closely associated clathrin is with the Golgi and/or the GERL system(s).

The predominant neuron of the cerebellum is the granule cell, which survives well in cultures (65). The strong cytoplasmic neuronal staining seen with anti-clathrin in culture and the staining of the developing and mature cere-

bellar granule cell layers in tissue sections may be related to the extensive formation and growth of dendrites by the granule cells (65). Growing dendrites in developing neuronal tissue are rich in coated vesicles (66). The clear localization of clathrin inside the granule cell cytoplasm and in its processes is one advantage of using dissociated cell cultures for this work. Coated vesicles have been described specifically in the cytoplasmic processes growing out from developing granule cells in culture. Also, the varicosities described contained many vesicles and specialized segments of membrane thought to be involved in future synaptic development (66). The fact that this study has localized clathrin to such areas is of interest. Evidently developing cerebellar granule cells produce large amounts of clathrin, which probably are involved in synaptogenesis.

The localization of clathrin to mature rat cerebral cortex points to a role for this protein in neuronal function. The literature has provided evidence for coated vesicle involvement in the recycling of synaptic vesicles at central synapses (67). Finding discrete areas of peroxidase precipitate localized around neuronal cell bodies and along the axons is consistent with its hypothesized role in synaptic functions. The staining probably represents discrete synaptic connection with large amounts of clathrin present within.

It should be emphasized that not all endocytotic or pinocytotic processes are mediated by assembly of clathrin into coated vesicles. In many cells, particularly endothelial cells, the large amount of endocytotic vesicles observed are probably responsible for the uptake of metabolites from the lumen to the basal membrane. However, these vesicles do not seem to exhibit a typical coat or lattice-like formation of coated vesicles. In such a case, pinocytotic in origin, plain vesicles could be responsible for the uptake of metabolites not requiring a specific receptor for their binding to the membrane. The close correspondence of clathrin on the cytoplasmic side of the membrane with invaginations may indicate that a stimuli produced by the ligand-receptor complex is expressed through the membrane inducing clathrin molecules to polymerize. If clathrin's ability to respond to pH can be extrapolated to its function in the cell, it is possible that alteration of local pH values in the cytoplasmic side of the membrane occurs, stimulating clathrin to assemble into typical structures, with eventual formation of a coated pit, then a coated invagination, and finally a coated vesicle. If this be the case, a small variation of pH could be the trigger for many important events.

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