Assembled and Unassembled Pools of Clathrin: A Quantitative Study Using an Enzyme Immunoassay

BRUNO GOUD, CHRISTIAN HUET,* and DANIEL LOUVARD*
*Immunocytochemistry Unit, Department of Immunology, and * Membrane Biology Unit, Department of Molecular Biology, Pasteur Institute, 75724 Paris Cedex 15, France*

ABSTRACT Using polyclonal antibodies raised against clathrin, we have developed an enzyme-linked immunoassay that can specifically measure the quantity of clathrin in crude cell extracts. We found that the quantity (weight percent of total protein) of clathrin was similar in cell types that exhibit large differences in their levels of endocytosis and exocytosis (lymphoid cells, 0.11%; liver cells, 0.07%, fibroblasts, 0.18%; myeloma cells, 0.16%). However, the quantity of clathrin was found to be significantly higher in brain cortex (0.75%).

Cellular clathrin was separated by high-speed centrifugation into two fractions: an unassembled form present in high-speed supernatants and an assembled form (clathrin coats) present in the pellets. We show that the fraction of clathrin in the unassembled state varies considerably depending on the cell type studied (14% in brain cortex to 70% in lymphocytes).

Our data support the view that the amount of clathrin (relative to total cell protein) in eucaryotic cells is not related to the extent of receptor-mediated endocytosis and intracellular membrane traffic. However, the fraction of assembled clathrin seems to be higher in endocytically and/or exocytically active cells.

Clathrin is the main constituent of the polyhedral protein lattice that forms the coat of coated pits and coated vesicles (1). Considerable data has shown that coated pits and coated vesicles are involved in many cellular processes, including receptor-mediated endocytosis (for a review, see reference 2), exocytosis of newly synthesized proteins (3), and plasma membrane recycling (for a review, see reference 4). However, an important question still unresolved concerns the molecular mechanism(s) underlying the fission and fusion events that occur during the vesicular transport.

Many arguments support the view that the fission and fusion events are related to a cycle of assembly-disassembly of clathrin coats. In vitro, clathrin coats dissociate reversibly into triskelions, flexible, three-armed structures comprising three clathrin heavy chains (180,000 mol wt) associated with three light chains (30,000–40,000 mol wt depending on the tissue and species) (5, 6). Triskelions are able to bind to stripped coated vesicles reforming clathrin cages (7). In vivo, coated vesicles arising from coated pits during receptor-mediated endocytosis appear to shed their clathrin coats rapidly before fusing with endosomes (8, 9). It is likely that the coats are removed enzymatically from the coated vesicles (10) and that the disassembled components return to the plasma membrane to be reassembled.

However, the question of whether a pool of free triskelions exists is still being debated (11, 12). Immunocytochemical studies that use polyclonal anticlathrin antibodies have revealed clathrin associated with coated pits or coated vesicles with very little background staining in the nonmembranous regions of the cytoplasm. This has been interpreted to indicate the absence of clathrin within the cytoplasm (13–16). On the other hand, Louvard et al. (16) have recently produced a monoclonal antibody that recognizes an epitope present on the clathrin heavy chain. This monoclonal antibody gives diffuse staining throughout the cytoplasm, which suggests the presence of a "soluble" pool of clathrin.

In an effort to determine whether or not an intracellular pool of unassembled clathrin exists, we developed an enzyme immunoassay in which crude cell lysates (containing clathrin) inhibit the binding of anticlathrin antibodies to immobilized clathrin. We separated clathrin pools into their assembled and unassembled forms by centrifugation. Our results indicate that although the amount of clathrin is relatively constant from one cell type to another (0.1–0.2% of cellular proteins), with the notable exception of brain cortex (0.7%), the ratio of assembled to unassembled clathrin varies greatly. A positive

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correlation exists between the size of the clathrin assembled pool and the extent of cellular activities mediated by coated structures.

MATERIALS AND METHODS

Animals: Male 5–8-mo-old rats of the Fischer 344 strain were used. They were reared in specific pathogen-free conditions in the breeding center of the Pasteur Institute (Dr. J-L. Guénet).

Cells and Tissues: Macrophage-depleted lymphoid cells were prepared from rat cervical and mesenteric lymph nodes as previously described (17). Mouse myeloma IgG-secreting cell line X63Ag8 (18) was grown in Dulbecco modified Eagle's medium (Boehringer GmbH, Mannheim, Federal Republic of Germany [FRG]) supplemented with 1 mM sodium pyruvate (Flow Laboratories, Irvine, U. K.), 50 IU/ml penicillin and 50 μg/ml streptomycin (Flow Laboratories), and 10% fetal calf serum (Boehringer) in an atmosphere of 90% air and 10% CO_2. Cells were harvested during exponential growth. Vero cells (monkey kidney fibroblasts) were grown in monolayer in Dulbecco's modified Eagle's medium (Seromed, Münich, FRG), supplemented with 25 mM glucose (Sigma Chemical Co., St. Louis, MO), penicillin-streptomycin, and 10% fetal calf serum (Seromed) in an atmosphere of 90% air and 10% CO_2. Cells were seeded 24 h before the experiments and were just confluent when they were removed from the petri dishes. Brains and livers were taken from freshly killed rats. Sheep erythrocytes were purchased from Institut Pasteur Production (Paris, France). They were kept at 4°C in Alsever.

Preparation and Fractionation of Cell Lysates and Tissue Homogenates: Before lysis, lymphoid cells and myeloma cells were washed four times in PBS (10 mM potassium phosphate buffer, pH 7.4, 0.15 M NaCl). Fibroblasts were removed from petri dishes with 0.05% trypsin, 0.02% EDTA (Boehringer) and washed once in PBS containing 10% fetal calf serum and three times in PBS. Cells were counted in a hemocytometer. The cell viability, as tested by trypan blue dye exclusion, was >90% in all of our experiments. Lymphoid cells ($5–7 \times 10^8$ cells), myeloma cells ($4–7 \times 10^7$ cells), and Vero cells ($3–6 \times 10^7$ cells) were lysed in 2–3 ml of a buffer (Pearse's buffer A) consisting of 0.1 M 2-(*N*-morpholino) ethane sulfonic acid, 1.0 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3 (wt/vol), 0.005% phenylmethylsulfonyl fluoride, pH 6.5, and containing 0.2% Triton X-100 (vol/vol) (Merck AG, Darmstadt, FRG). Cell suspensions were left for 10 min at room temperature and 20 min at 4°C. Nuclei were removed by centrifugation at 1,000 *g* for 5 min at 4°C. The cell lysates were then divided in two fractions. In order to dissociate clathrin coats, we adjusted 0.5 ml of cell lysates to pH 9.5 with 15–20 μl of 1 M Na_2CO_3. After 30–45 min at 4°C, these fractions were centrifuged for 1 h at 100,000 *g* at 4°C in a TST 55.5 rotor (Kontron, Zurich, Switzerland). The supernatants were collected and neutralized to pH 7.4 with 100 μl of 1 M phosphate buffer, pH 7.4, or 25 μl of 1 M KH_2PO_4. Total clathrin was measured in these fractions. The remaining cell lysates (1.5–2.5 ml) were centrifuged for 1 h at 100,000 *g* at 4°C. The supernatants were collected, the pH was raised to 9.5, and neutralization was performed as described above. Clathrin measured in these high-speed supernatants is referred to in the text as unassembled clathrin. The corresponding pellets were resuspended in 0.5 ml of cold buffer A containing 0.2% Triton X-100 using a Dounce homogenizer, adjusted to pH 9.5, and recentrifuged for 1 h at 100,000 *g*. The supernatants were then collected and neutralized. Clathrin measured in these fractions is referred to as assembled clathrin. In each supernatant, the protein concentration was estimated using the Bio-Rad (Bradford) protein assay (Bio-Rad Laboratories, Munich, FRG) with bovine IgG (Miles Laboratories, Elkhart, IN) as standard.

Sheep erythrocytes (10^{10} cells) were washed twice in PBS and lysed in 5 ml of buffer A containing 0.2% Triton X-100 as described above. After centrifugation for 5 min at 1,000 *g*, the protein concentration was determined by using the method of Lowry et al. (19) with bovine IgG as the standard. 2 ml (20 mg/ml) of the lysate was then adjusted to pH 9.5, centrifuged at 100,000 *g*, and neutralized with KH_2PO_4 as described above.

Rat brain cortex (after removal of brain stem, cerebellum, and meninges) and livers were washed several times with a large volume of cold buffer A. In some experiments, livers were perfused through the whole circulation or through the portal vein with PBS, 1 mM EDTA, pH 7.0. Livers and brain cortex were then homogenized with a Waring blender (four times for 10 s each time at full speed) in ~100 and 10 ml, respectively, of cold buffer A containing 0.2% Triton X-100. Homogenates were centrifuged for 10 min at 1,500 *g* to remove large aggregates. The pellets were washed once in the same volume of cold buffer A containing 0.2% Triton X-100 and the two supernatants were pooled. The protein concentration was adjusted to 5–20 mg/ml. Tissue homogenates were then fractionated as described above. We have summarized in Fig. 1 the entire experimental procedure.

Purification and Coated Vesicles: Coated vesicles were isolated from calf brains following the method first developed by Pearse (1) as modified by Keen et al. (20) and using D_2O-Ficoll gradients instead of sucrose gradients (21). The final purification was achieved by gel filtration through a Sephacryl S-1000 (Pharmacia Fine Chemicals, Uppsala, Sweden) column preequilibrated with buffer A. The fractions corresponding to the second peak of elution (22) were pooled and concentrated. By electron microscopy using negative staining methods, these fractions were seen to contain nearly pure coated vesicles (<5% of smooth vesicles). Coated vesicles were kept at 4°C in the same buffer until use.

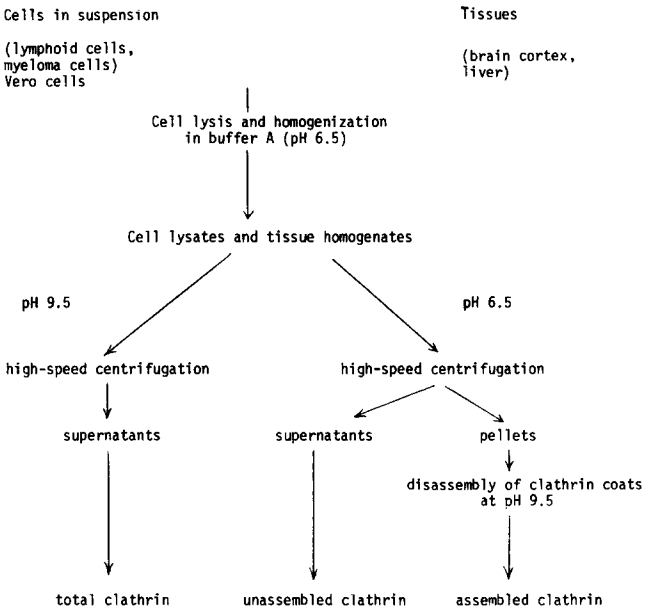

FIGURE 1 Preparation of cell and tissue fractions.

Purification of Clathrin Triskelions: Coated vesicles purified as described above were dialysed overnight at 4°C against 10 mM Tris-HCl, pH 7.5. Coated vesicles were then centrifuged for 1 h at 100,000 *g*. The analysis of the supernatant by SDS PAGE (23) revealed one band ~180,000 mol wt and two bands around ~30,000 mol wt. Protein concentration was estimated using a specific absorptivity $E|^{1\%}_{1cm}$ of 11.9 at 280 nm for clathrin triskelions (7). Aliquots of purified clathrin were kept at −80°C until use.

Enzyme Immunoassay: Clathrin present in cell or tissue extracts was quantitated using an enzyme-linked immunoadsorbent assay. To perform this assay, we have used a crude rabbit immune serum raised against empty clathrin cages prepared from pig brain coated vesicles (16). In control experiments, a pool of normal rabbit sera was used instead of the immune serum. Polystyrene flat-bottom microtiter plates (Nunc, Denmark) were coated with purified clathrin. Coating was carried out for 2 h at 37°C and overnight at 4°C in 0.1 M sodium carbonate buffer, pH 9.5 (1.5 μg/ml clathrin, 50 μl/well). The cells were then washed five times with PBS supplemented with 0.1% Tween 20 (Merck) and free-binding sites were saturated in the same buffer containing 0.4% BSA (fraction V, Industrie Biologique Française, Villeneuve-la-Garenne, France) (PBS-Tween-BSA buffer) for 30 min at 4°C. Cell lysates or tissue homogenates (1–20 mg/ml) were serially diluted in PBS-Tween-BSA (dilutions: 1/5 up to 1/640), preincubated for 1 h at 37°C, and overnight at 4°C, with rabbit anticlathrin immune serum (final dilution: 1/3, 200) and then added onto the plates for 3 h at 4°C (60 μl/well). A standard curve with purified clathrin diluted in buffer A containing 0.2% Triton X-100 and 0.4% BSA was carried out simultaneously for each experiment. A stock solution of clathrin was raised to pH 9.5 with 1 M Na_2CO_3, neutralized with 1 M phosphate buffer, pH 7.4, or 1 M KH_2PO_4, diluted in PBS-Tween-BSA, and preincubated with rabbit anticlathrin immune serum under the same conditions as cell lysates. To determine 100% antibody binding, we added immune serum to dilutions of buffer A containing 0.2% Triton X-100 and 0.4% BSA (previously raised to pH 9.5 and neutralized) in PBS-Tween-BSA. To measure nonspecific binding, we performed control experiments using a pool of normal rabbit sera (final dilution: 1/3,200) instead of the immune serum. The microtiter plates were washed six times with PBS-Tween, the wells were filled with β-galactosidase-linked affinity-purified horse anti-rabbit IgG (a gift from Dr. L. Leclercq, Institut Pasteur), diluted in PBS-Tween-BSA, and incubated for 2 h at 4°C. After six additional washings with PBS-Tween, the enzymatic reaction was allowed to take place at 37°C in 0.1 M phosphate buffer, pH 7.0, containing 10^{-3} M $MgSO_4$, 2×10^{-4} M $MnSO_4$, 2×10^{-3} M magnesium titriplex (Merck),

0.1 M 2-mercaptoethanol, and 2.5×10^{-3} M *p*-nitrophenyl β-D-galactopyranoside (Sigma Chemical Co.), according to Guesdon et al. (24). After the yellow color of the reaction product developed (usually 4–6 h at 37°C), the reaction was stopped by adding 2 M Na_2CO_3 and the absorbance was measured at 414 nm in a Titertek Multiskan Photometer (Flow Laboratories).

We calculated the concentration of clathrin present in cell lysates or tissue homogenates at 50% inhibition by referring to the concentration of purified clathrin giving the same inhibition. Knowing the total protein content, we calculated the absolute amount of clathrin in the different fractions of cell lysates or tissue homogenates. The percentage of unassembled clathrin was given by the formula:

percent unassembled = (amount of clathrin in high-speed supernatants)/

(amount of clathrin in high speed supernatants + amount of clathrin in

corresponding pellets) × 100.

To estimate the clathrin recovery during the fractionation procedure, we calculated the amount of clathrin in the total clathrin fraction (with the protein content of this fraction taken as 100%). The yield of the fractionation was given by the formula:

percent recovery

= (amount of clathrin recovered in high-speed supernatants

+ pellets)/(amount of total clathrin) × 100.

RESULTS

The polyclonal rabbit anticlathrin immune serum used in these experiments has been shown to bind to clathrin coats in situ and to clathrin heavy chains and its associated light chains blotted on nitrocellulose (16). Binding to immobilized native triskelions is now reported.

Standard Curve with Purified Clathrin

In preliminary experiments, we were able to show that binding of the rabbit anticlathrin immune serum to clathrin immobilized on microtiter plates was efficiently inhibited by the addition of serial dilutions of purified clathrin. This observation allowed us to develop a quantitative immunoassay (see Materials and Methods). The purified clathrin, under the conditions of our assay, should be present in solution as triskelions (6, 25) and should not reassociate into baskets (see section below). Fig. 2*A* shows a typical curve of inhibition with purified clathrin. 50% binding (or 50% inhibition) was usually obtained by preincubating a dilution of immune serum (1/3, 200) with 0.3–0.5 μg/ml of clathrin.

In control experiments, the binding of a pool of normal rabbit sera was found to be <10% of the binding of anticlathrin immune serum at the same dilution. We also tested the ability of unrelated antigen to interfere with the specific binding. Concentrations up to 0.5 mg/ml of mouse actin, mouse myosin, pig and mouse tubulin, rabbit IgG, or pig thyroglobulin produced no significant inhibition. Finally, when a sheep erythrocyte lysate was used, no inhibition was observed. This observation is consistent with the hypothesis that this cell type lacks clathrin (<0.01% of proteins in cell lysate). It is known that mature erythrocytes do not perform either endocytosis or intracellular membrane transport (26, 27), two cellular mechanisms thought to require the participation of clathrin.

Inhibition Curves with Cell and Tissue Extracts

The binding of anticlathrin immune serum was inhibited by extracts obtained from either tissue culture cells or organs. The cell extracts were prepared in a buffer (buffer A) known to stabilize clathrin coats in vitro. Clathrin coats were then efficiently disassembled into triskelions (see Table II) by raising the pH of the extracts to 9.5, centrifuging them at 100,000 *g*, and neutralizing the supernatant to pH 7.4. Next, the immune serum was added to serial dilutions of these supernatants. Fig. 2*B* shows a typical curve of inhibition obtained with a rat liver extract. The slope of this curve was parallel to that obtained with purified clathrin, a result consistent with the inhibition of binding being due to clathrin in the extract.

To confirm the specificity of the immunoassay, Vero or X63Ag8 cell extracts were depleted of their clathrin content using an immunoprecipitation procedure. For this purpose, an aliquot of the cell extracts was incubated with an excess of affinity-purified rabbit anticlathrin antibodies. The immunocomplexes were subsequently precipitated using sheep anti-rabbit IgG. The removal of the excess of anticlathrin antibodies was monitored by incubating the cell extracts on microtiter plates coated with clathrin. Using a cell lysate depleted in clathrin by this procedure, we observed no inhibition of binding to immobilized clathrin (data not shown).

In other sets of experiments, we added purified clathrin to the extracts before performing the immunoassay. In the experiment shown in Fig. 2*B*, clathrin was added to a liver extract to a final concentration of 10 μg/ml. The resultant clathrin level was found almost equal to the sum of endogenous and exogenous (added) clathrin. This indicates that in our experimental conditions, the amount of measurable clath-

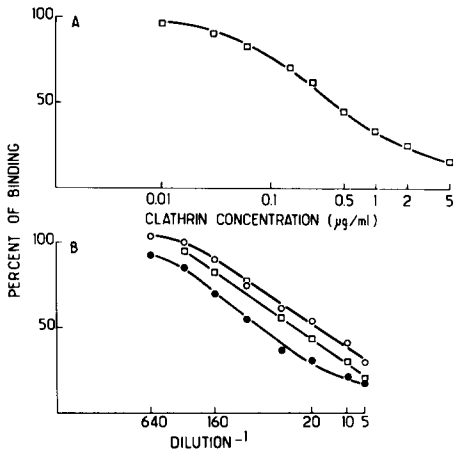

FIGURE 2 (*A*) Standard curve with purified clathrin. A solution of purified clathrin (25 μg/ml) in stabilizing buffer A containing 0.2% Triton X-100 and 4 mg/ml BSA (previously adjusted to pH 9.5 and neutralized) was serially diluted in PBS-Tween-BSA. Rabbit anti-clathrin immune serum (final dilution: 1/3, 200) was added to each dilution, incubated for 1 h at 37°C and overnight at 4°C. The inhibition of binding was then measured by enzyme-linked immunosorbent assay as described in Materials and Methods. 50% inhibition of binding corresponded to 0.38 μg/ml of purified clathrin in this experiment. (*B*) Inhibition curve with a rat liver extract. Rat liver extract (10 mg/ml) was prepared as described in Fig. 1. The inhibition of binding of the rabbit anti-clathrin immune serum was measured with (●) or without (○) addition of 10 μg/ml of purified clathrin (exogenous clathrin). A standard curve was constructed in a parallel experiment (□), using purified clathrin, and in this case 50% inhibition of binding was obtained with 0.35 μg/ml purified clathrin. 50% inhibition of binding was obtained at the dilutions 1/56 (●) and 1/17 (○) of liver extracts, which corresponded to 0.6 μg/ml and 0.2 μg/ml of clathrin respectively. The difference between these two values (0.4 μg/ml) is due to added exogenous clathrin (0.35 μg/ml).

rin was not affected by other cellular proteins. It is worth mentioning that we have been able to measure clathrin in cell or tissue extracts that contained up to 4–5 mg/ml of total cellular protein. Therefore, given that 0.3–0.5 μg/ml of purified clathrin produced 50% inhibition of the binding of immune serum (Fig. 2*A*), it should be possible to detect clathrin in an extract when it makes up only 0.01% of the total protein. The experiment shown in Fig. 2*B* also indicates that clathrin is stable during the whole experimental procedure. We cannot rule out that some proteolytic degradation occurred; however, it did not quantitatively affect the antigen-antibody reaction.

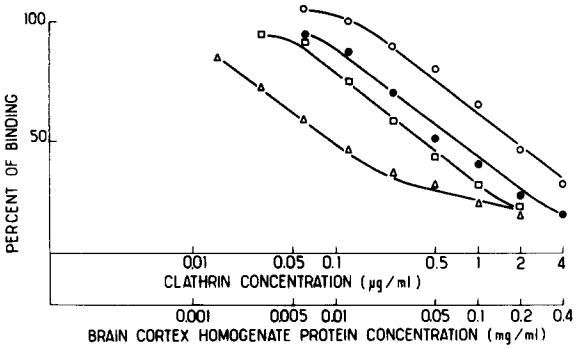

FIGURE 3 Quantification of clathrin in rat brain cortex. A rat brain cortex extract was prepared and fractionated as described in Fig. 1. 50% inhibition was obtained for 0.057 mg/ml of "total" (●), 0.175 mg/ml of "high-speed supernatant" (○) and 0.0094 mg/ml of "pellet" (△) fractions respectively. By referring to the standard curve of inhibition with purified clathrin (□), we have calculated the concentration of clathrin in these fractions and the absolute amount of clathrin per mg of protein. In this experiment, the percentage of unassembled clathrin was found to be 24%. Clathrin recovery was 110%.

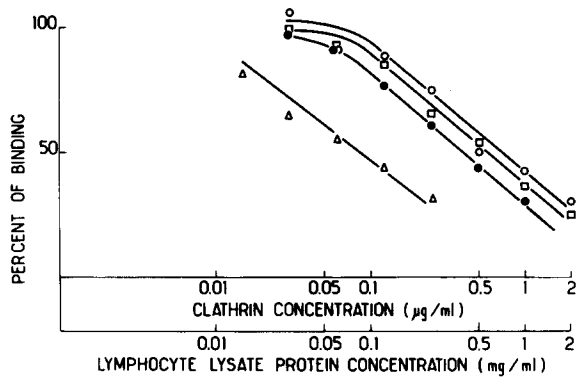

FIGURE 4 Quantification of clathrin in rat lymphocytes. A rat lymphocyte extract was prepared and fractionated as described in Fig. 1. 50% inhibition was obtained with 0.4 mg/ml of "total" (●), 0.7 mg/ml of "high-speed supernatant" (○) and 0.082 mg/ml of "pellet" (△) fractions respectively. By referring to the standard curve of inhibition with purified clathrin (□), we have calculated the concentration of clathrin in these fractions and the absolute amount of clathrin per mg of protein. In this experiment, the percentage of unassembled clathrin was found to be 72%. Clathrin recovery was 87%.

Quantification of Total, Assembled, and Unassembled Clathrin in Cell and Tissue Extracts

Total, assembled, and unassembled clathrin was quantitated in rat lymphoid cells, Vero cells, X63Ag8 myeloma cells, rat liver, and rat brain cortex. Figs. 3 and 4 give typical titration curves that we obtained with rat brain cortex and rat lymphocytes. We then measured the ratio of clathrin in an assembled and unassembled state using the centrifugation procedure. We found that most of the clathrin was in an assembled form in rat brain cortex extracts (86%), whereas only 30% was in the assembled form in lymphocytes (Table I). In these experiments, the yield of clathrin was 110% (brain cortex) and 87% (rat lymphocytes). Similar experiments were performed with Vero cells, myeloma cells, and rat livers. We summarize in Table I the results we obtained. We observed that the relative abundance of clathrin with respect to total cellular protein is similar in Vero cells (0.18%), myeloma cells (0.16%), rat liver cells (0.07%), and lymphocytes (0.11%). The amount of total clathrin was found to be significantly higher (0.75%) in brain cortex. From these values, we also calculated the average number of triskelions per cell, assuming a molecular weight of 640,000 for a clathrin triskelion. This number ranged from 2.8×10^4 in lymphoid cells to 4.8×10^5 in myeloma cells.

Additional Control Experiments

The following experiments were performed to further validate our experimental procedure.

EFFECTIVENESS OF THE FRACTIONATION PROCEDURE: Clathrin pools were separated by centrifugation (Fig. 1). We assumed that unassembled clathrin would be recovered in the supernatants whereas clathrin coats would be recovered in the corresponding pellets. As the clathrin quantification assay requires that clathrin triskelions be free in solution, the pellet that contained clathrin coats, once isolated, was exposed to a pH of 9.5 to dissociate clathrin coats into triskelions. Triskelions were subsequently separated from an insoluble residue by centrifugation, and the super-

TABLE I

Clathrin Content and Distribution of Clathrin Pools in Various Cell Types

Cell extract	Clathrin (% of cellular proteins)	Triskelions per cell	Unassembled clathrin	Assembled/unassembled
			%	
Rat brain cortex	0.75 ± 0.1*	ND	14 ± 8	6.0
Rat livers (perfused)	0.07 ± 0.0	ND	35 ± 3‡	1.8
X63Ag8	0.16 ± 0.07	$3.8 \times 10^5 \pm 1.25 \times 10^5$	45 ± 5	1.2
Vero cells	0.18 ± 0.02	$4.8 \times 10^5 \pm 10^5$	59 ± 5	0.7
Rat lymphoid cells	0.11 ± 0.03	$2.8 \times 10^4 \pm 0.9 \times 10^4$	70 ± 2	0.42
Sheep erythrocytes	Undetectable	—	ND	ND

ND, not determined.

* Means ± 1 SD of three or four experiments.

‡ Unperfused livers were used for the measurements of assembled and unassembled clathrin to avoid a possible redistribution of clathrin pools induced by perfusion.

natant was recovered and adjusted to pH 7.4. To quantitate unassembled clathrin in the same experimental conditions, we also adjusted the pH of the first supernatant to pH 9.5 and then neutralized it.

The following experiments were designed to test the validity of this procedure. First, we centrifuged at 100,000 *g* a solution of purified clathrin (10 μg/ml) in buffer A containing 0.2% Triton and 4 mg/ml BSA (previously adjusted to pH 9.5 and neutralized). Under these conditions, no significant amount of triskelions was found in the pellet. Then we centrifuged freshly prepared coated vesicles and centrifuged them at 100,000 *g* in buffer A containing 0.2% Triton and 4 mg/ml BSA (Fig. 5). Two concentrations of coated vesicles were tested (20 and 2 μg/ml), corresponding to the range of concentrations of assembled pools of clathrin calculated to be in extracts from brain cortex and lymphocytes, respectively. As shown in Fig. 5, >90% of clathrin from coated vesicles could be recovered as a pellet. The above results demonstrate the stability of clathrin coats during our experimental procedures. Moreover, these results indicate the efficiency of a centrifugation to separate quantitatively unassembled from assembled clathrin.

EFFICIENCY OF CLATHRIN DISASSEMBLY BY HIGH PH: It is known that high pH leads to a nearly complete dissociation of clathrin coats into triskelions (20). To confirm this, we analyzed the pellet resulting from centrifugation at pH 9.5 of a fibroblast extract. We treated this pellet at pH 9.5 and then centrifuged the sample and measured the quantity of clathrin in the supernatant and pellet fractions. As shown in Table II, the amount of re-extracted clathrin represented only 2% of that found after the first centrifugation. We found also that the residual pellet contained no significant amount of clathrin.

Similar experiments were performed with the residual pellet resulting from the extraction of the assembled pool of clathrin of brain cortex. These experiments also indicate that a negligible amount of clathrin was lost in the residual pellets (Table II). These results have also been confirmed by immunoblotting of the residual pellet (data not shown).

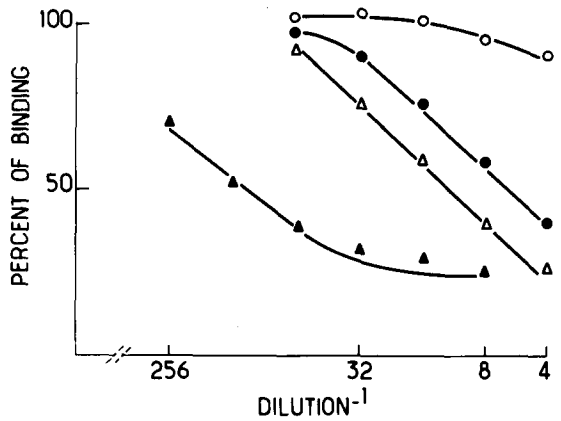

FIGURE 5 Stability of purified coated vesicles. Purified coated vesicles (20 μg/ml [△, ▲] and 2 μg/ml [○, ●]) in 1 ml of stabilizing buffer (0.2% Triton containing 4 mg/ml BSA) were centrifuged for 1 h at 100,000 g. 0.95 ml of the supernatants were removed, adjusted to pH 9.5 and neutralized (▲, ●). The remaining 0.05 ml was brought to 0.5 ml of buffer (0.2% Triton, 4 mg/ml BSA), adjusted to pH 9.5 and neutralized (△, ○). These fractions were then tested for clathrin content. 91% of clathrin molecules were present in the "pellet" fraction (▲). The amount of clathrin molecules in the high-speed supernatant corresponding to a 2-μg/ml concentration of coated vesicles (○) was not measurable.

EFFECT OF CELL LYSIS PROCEDURE ON THE RECOVERY OF UNASSEMBLED CLATHRIN: In order to lyse the cells efficiently, we routinely added 0.2% Triton X-100 to buffer A. Triton X-100 up to a concentration of 1% does not dissociate clathrin from coated vesicles (28). Under these cell lysis conditions, we might be isolating assembled clathrin in the form of complete cages (derived from coated vesicles) and partial cages (derived from coated pits) (29). Pearse has reported that some of the coated particles isolated in the presence of Triton X-100 from human placenta are possibly derived from both coated pits and coated vesicles (21). To determine whether in our conditions Triton X-100 may favor the dissociation of incomplete cages, we either broke X63Ag8 myeloma cells in the absence of Triton X-100 with a Dounce homogenizer or homogenized liver with a Waring blender, and fractionated this cell extract. As shown in Table III, almost the same amount of unassembled clathrin was found, whatever the mode of preparation of the cell extract.

LACK OF CLATHRIN REASSOCIATION IN CELL OR TISSUE EXTRACTS: We tested the possibility that a part of the unassembled clathrin could be reassembled during the experimental procedures. For this purpose, 10 μg/ml of purified clathrin (triskelions) was added to a rat brain cortex extract. We then fractionated this extract as described in Fig. 1. Under these conditions, unassembled clathrin now represented 50% of the total clathrin, whereas in the control

TABLE II

Efficiency of Clathrin Coat Disassembly at High pH

Cell extract	First supernatant	Second supernatant	Pellet
	μg	μg	μg
Vero cells*	26.5	0.4 (2)§	ND
Rat brain cortex‡	39	5.1 (11)	1

ND, not detectable.

* 2.5. ml (4.1 mg/ml) of a Vero cells extract was adjusted to pH 9.5 with Na_2CO_3 and centrifuged for 1 h at 100,000 g. The pellet was resuspended in 0.5 ml of buffer A, 0.2% Triton (pH 6.5) and clathrin was re-extracted at pH 9.5. The total amount of clathrin was estimated in the first (column 1) and second (column 2) supernatants. The residual pellet was also analyzed for clathrin content (column 3).

‡ 0.5 ml of a rat brain cortex "pellet" fraction (Fig. 1) was centrifuged for 1 h at 100,000 g. We then re-extracted clathrin from the pellet as described above and estimated clathrin content in the first and second supernatants as well as in residual pellet.

§ Numbers in parentheses indicate the percentage of the first supernatant.

TABLE III

Effect of Cell Lysis Procedure on Recovery of Total and Unassembled Clathrin

		Clathrin	
Lysis procedure	Cell extract	% of cellular proteins	% of unassembled clathrin
Mechanical breaking alone	Myeloma cells	0.20	45
	Liver	0.10	42
In presence of Triton X-100	Myeloma cells	0.22	51
	Liver	0.07	37

X63Ag8 myeloma cells were lysed in buffer A containing Triton X-100 (0.2%) or in the same buffer without Triton X-100 with a Dounce homogeneizer (100 strokes, on ice). Livers were homogenized with a Waring blender in buffer A containing Triton X-100 (0.2%) or without detergent. Cell lysates were obtained and each subcellular fraction was analyzed for clathrin content as usual.

experiment only 16% of clathrin was found in an unassembled form. We reasoned that most of the added clathrin was recovered in the high-speed supernatant (unassembled form), suggesting that no reassociation of added triskelions had occurred during the experiment.

CLATHRIN HEAVY CHAINS AND ASSOCIATED LIGHT CHAINS ARE BOTH PRESENT IN ASSEMBLED AND UNASSEMBLED CLATHRIN POOLS: One could argue that clathrin heavy chains and associated light chains may not be stoichiometrically represented in the assembled and unassembled pools of clathrin. Since our antiserum recognizes both, we tested this possibility by immunoblotting analysis using affinity-purified rabbit anticlathrin antibodies (as previously described in reference 16). These experiments were carried out using total assembled and unassembled fractions from brain and lymphocyte extracts. In all cases, heavy and light chains were easily identified. Furthermore, the ratio of heavy to light chains appeared to be similar (data not shown). Therefore, we conclude that each clathrin pool contains both polypeptides.

DISCUSSION

The aim of this study was to determine the clathrin content and the distribution of assembled and unassembled clathrin pools within various cell types. For this purpose, we have developed a competitive solid-phase enzyme immunoassay that can measure clathrin in cell lysates. A fractionation procedure was also designed to separate the pools of assembled and unassembled clathrin.

As illustrated in Fig. 2*B*, cell lysates inhibited the binding of the rabbit anticlathrin immune serum to immobilized clathrin in a specific way. The inhibition of binding was found to be nearly total, which indicates that similar antigenic determinants are recognized on clathrin molecules present in cell lysates and on immobilized clathrin. In all cell types so far tested, these antigenic determinants were present on both clathrin heavy chains and associated light chains as demonstrated by immunoblotting (reference 16 and unpublished results). In addition, the curves of inhibition were found to be parallel, which suggests that the anticlathrin antibodies have the same affinity for clathrin present in all cell extracts tested. These antibodies were found to cross-react efficiently with clathrin from chicken, rodents, and other mammalian cells including dog, bovine, and human cell lines (16). Therefore, the assay we have developed could be applied to a wide variety of cells or tissues from various origins.

One important question, still debated, concerns the existence or the nonexistence of unassembled clathrin inside the cells (11, 12). The results we report here strongly suggest that in fact a measurable fraction of clathrin is present in an unassembled form (Table I). These data provide further independent evidence for the presence of unassembled clathrin in the cytoplasm, as we previously suggested using a monoclonal antibody reacting with an epitope unavailable in clathrin coats but accessible in triskelions (16). It is unlikely that this cytosolic pool could be generated during our experimental procedures, since in using purified coated vesicles, as a model system, we found that only a small amount of clathrin dissociated during the whole procedure (Fig. 3). It also seems unlikely that the unassembled pool of clathrin could result from the dissociation of incomplete cages (for example, from coated pits) after the solubilization of associated membranes with Triton X-100 because we found a similar amount of clathrin in the high-speed supernatants after mechanical breaking of the myeloma cells or livers prepared in the same stabilizing buffer (Table III), a treatment that probably preserves the association of coated structures with membranes (30).

We have been able to estimate the clathrin content in various cell types and tissues. As summarized in Table I, it appears that the percentage of clathrin in eucaryotic cells is relatively constant. Our data indicate that clathrin represents ~0.1–0.2% of total cellular proteins. The slightly lower value obtained for perfused liver may be due to the fact that hepatocytes contain a significant amount of protein destined for secretion, in addition to their cellular proteins. A remarkable exception, however, is the brain cortex, in which clathrin represents 0.7% of total cellular proteins. The significance of this result is currently unknown.

The cell types we have chosen in these experiments exhibit large differences in the cellular activities that are thought to require the participation of clathrin. In brain cells, coated vesicles play a major role in the retrieval of excess cell surface membrane in presynaptic neurons (31). In fibroblasts and liver cells, clathrin, organized as coated pits and coated vesicles, has been shown to be involved in receptor-mediated endocytosis of various ligands (for a review, see reference 2). In myeloma cells, a major function of coated vesicles could be to mediate plasma membrane retrieval following the secretory process of immunoglobulins (17, 32). In this context, that cells contain a constant level of clathrin confirms that the degree of expression of clathrin is not directly related to a particular cellular function as first suggested by Pearse (33). Perhaps the most striking data concern normal lymphoid cells, because these cells display low secretory and endocytic activities yet contain as much total clathrin as other cells. For example, we have calculated that normal rat lymphoid cells internalize ~0.3 nl/h per 10^6 cells of fluid medium (34). This represents 106 times less than mouse L fibroblasts (35) or 364 times less than rat hepatocytes (36).

It has been suggested (8, 9) that coated pits and vesicles form in vivo by a cycle of assembly-disassembly of clathrin coats that has been shown to occur in vitro (5–7, 10, 20, 25, 28, 37). Furthermore, whereas the concentration of clathrin (relative to other proteins) is similar in most of the cell types we studied, the ratio of assembled to unassembled clathrin varies greatly (Table I). It is striking that brain contains the highest level of assembled clathrin and lymphoid cells the lowest. This raises the possibility that a positive correlation may exist between the size of the assembled pool and the intracellular membrane transport activity of the cell. As a consequence, an activated lymphocyte could recruit clathrin from its unassembled pool to form coated pits and/or coated vesicles when the membrane activity increases, for instance, during differentiation. Salisbury et al. have indeed observed a recruitment of clathrin coats during the capping of surface IgG induced by multivalent anti-Ig antibodies on lymphoblastoid cells (38). Note also that in the secreting myeloma cells there is only 45% of unassembled clathrin compared with 70% in the lymphoid cells (Table I). In cultured fibroblasts, Lubinski and Huet (39) have recently shown that various ligands can induce accumulation of coated vesicles, independently from coated pits, and Larkin et al. (40) reported that in fibroblasts depleted in potassium, there is a dramatic inhibition of low-density lipoprotein uptake, associated with

a disappearance of immunoreactive clathrin coats at the cell surface. In each of these examples, it is likely that the size of the unassembled clathrin pool is changing with the physiological state of the cell. That is, the amount of unassembled clathrin will be decreased with increased membrane transport and, conversely, will increase when membrane transport is arrested.

In conclusion, the results of experiments we report in this paper favor the existence of at least two intracellular pools of clathrin. The enzyme immunoassay that we have developed to quantify these two pools of clathrin should provide a useful tool to analyze the state of clathrin in response to changes in cellular demands for intracellular membrane transport.

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