

Subpopulations of Liver Coated Vesicles Resolved by Preparative Agarose Gel Electrophoresis

Nancy L. Kedersha, Diane F. Hill, Kathryn E. Kronquist, and Leonard H. Rome

Department of Biological Chemistry and the Mental Retardation Research Center, UCLA School of Medicine, Los Angeles, California 90024

Abstract. Rat liver clathrin coated vesicles (CVs) were separated into several distinct subpopulations using non-sieving concentrations of agarose, which allowed the separation of species differing primarily in surface charge. Using preparative agarose electrophoresis (Kedersha, N. L., and L. H. Rome, 1986, *Anal. Biochem.*, in press), the CVs were recovered and analyzed for differences in morphology, coat protein composition, and stripped vesicle protein composition. Coat proteins from different populations appeared identical on SDS PAGE, and triskelions stripped from the different populations showed the same mobility on the agarose gel, suggesting that the mobility differences observed in intact CVs were due to differences in the surface charge of underlying vesicles rather than to variations in their clathrin coats. Several non-coat polypeptides appeared to segregate exclusively with different populations as resolved by two-dimensional electrophoresis. Stripped CVs also exhibited

considerable heterogeneity when analyzed by Western blotting: the fast-migrating population was enriched in the mannose 6-phosphate receptor, secretory acetylcholine esterase, and an M_r 195,000 glycoprotein. The slow-migrating population of CVs was enriched in the asialoglycoprotein receptor, and it appeared to contain all detectable concanavalin A-binding polypeptides as well as the bulk of detectable WGA-binding proteins. When CVs were prepared from ^{125}I -asialoorosomucoid-perfused rat liver, ligand was found in the slow-migrating CVs, suggesting that these were endocytic in origin. Morphological differences were also observed: the fast-migrating population was enriched in smaller CVs, whereas the slow-migrating population exhibited an enrichment in larger CVs. As liver consists largely of hepatocytes, these subpopulations appear to originate from the same cell type and probably represent CVs of different intracellular origin and destination.

THE intracellular transport of both secretory and endocytic macromolecules involves several organelles and organelle systems. Secretory proteins are directed to the rough endoplasmic reticulum by a signal sequence, which in many proteins is recognized by a cytoplasmic ribonucleoprotein complex termed signal recognition particle (33). The interaction between this complex and the signal sequence provides the mechanism whereby the cell is able to distinguish between secretory and nonsecretory proteins as well as to segregate those destined for export by inserting them into the lumen of the rough endoplasmic reticulum. Mechanisms which mediate further sorting and transport events are less well characterized. Considerable evidence suggests that clathrin coated vesicles (CVs)¹ are involved in such processes (for reviews see references 10, 26). Coated vesicles also mediate the endocytosis of numerous receptor-ligand complexes (12, 15). Presumably, within different populations of CVs there exist sorting signals that direct in-

dividual vesicles to target organelles. It has been suggested that CVs contain "adaptor" molecules which determine the contents and destination of individual CVs (26). The identification of such molecules or other sorting signals requires that subpopulations of coated vesicles be resolved and characterized.

Several studies have been directed along these lines. Pfeffer and Kelly have used antibodies to select subpopulations of brain CVs containing specific transmembrane synaptic glycoproteins (27) and have identified two possible adaptor molecules of M_r 38,000, and 29,000. Weidenmann et al. used protein solubilization techniques to identify integral membrane molecules as candidates for adaptor molecules in brain CVs (34, 35), and found four such species of M_r 38,000, 29,000, 24,000, and 10,000. Helmy et al. (18) have used a density shift technique to separate liver CVs containing secretory acetylcholine esterase (AChE) from the remaining (presumably endocytic or recycling) CVs; the authors found no appreciable protein differences between the two populations.

Using a preparative modification of an agarose gel electrophoretic technique previously described (29), we have separated liver CVs into discrete subpopulations. Analysis of

1. *Abbreviations used in this paper:* AChE, acetylcholine esterase; ASGP, asialoglycoprotein; ASOR, asialoorosomucoid; CV, coated vesicle; MES, 2-(*N*-morpholino-)ethane sulfonic acid; M6P, mannose 6-phosphate; TBS, Tris-buffered saline; WGA, wheat germ agglutinin.

these subpopulations indicates that all electrophoretically resolved species possess identical clathrin coat proteins, however, numerous other polypeptide differences are apparent between the populations. Morphological differences were observed between the populations; the faster CVs were smaller and more stable than the slower species. Finally, endocytic and exocytic markers of CV content (or "cargo") proteins (28) are resolved by this technique, suggesting that exocytic and endocytic CVs differ in surface charge and can be separated accordingly.

Materials and Methods

2-(*N*-Morpholino)-ethane sulfonic acid (MES) was obtained from Research Organics, Inc., Cleveland, OH. Ultrapure sucrose, used in all gradients, was obtained from Bethesda Research Laboratories, Gaithersburg, MD. Nitrocellulose, molecular weight standards, conjugated horseradish peroxidase-goat anti-rabbit IgG and conjugated horseradish peroxidase-wheat germ agglutinin (WGA) lectin were obtained from Bio-Rad Laboratories, Richmond, CA. Polyvinylpyrrolidone, concanavalin A, horseradish peroxidase, Ficoll 70, and EGTA were obtained from Sigma Chemical Co., St. Louis, MO. "Isogel" agarose was obtained from FMC Bio Products, Rockland, ME. The "minigel" apparatus for polyacrylamide gels was purchased from Idea Scientific, Corvallis, OR. Ampholines were purchased from Serva Fine Biochemicals Inc., Garden City Park, NY. Rabbit anti-asialo-orosomucoid (ASOR) antibodies were a generous gift from Dr. Alan Schwartz (Boston, MA). The rabbit anti-mannose 6-phosphate (M6P) receptor antibodies were generously provided by Robert P. Searles of our laboratory. Adult Sprague-Dawley rats were obtained from our own breeding colony. Pure ASOR was a gift from Dr. James Paulson (University of California at Los Angeles). The iodination reagent Iodogen was purchased from Pierce Chemical Co., Rockford, IL, and carrier-free Na¹²⁵I was from

Amersham Corp., Arlington Heights, IL. PD-10 columns were from Pharmacia Fine Chemicals, Piscataway, NJ.

Coated Vesicle Purification

Coated vesicles were isolated by a modification of the method of Blitz et al. (2). Briefly, fresh rat livers were homogenized in 4 vol of 0.09 M MES, 0.01 M sodium phosphate, 1 mM EGTA, 1.5 mM magnesium chloride, 0.02% sodium azide, pH 6.5 (MES buffer) containing 0.25 M sucrose. This homogenate was centrifuged for 30 min at 12,000 g, and the supernatant further centrifuged for 120 min at 80,000 g to obtain a crude microsomal fraction. The microsomes were resuspended in MES buffer containing 6.25% sucrose and 6.25% Ficoll 70, and centrifuged at 40,000 g for 40 min. The supernatant was diluted fourfold in MES buffer and recentrifuged at 80,000 g for 2 h to obtain crude CVs. This step removed large amounts of membranous material and greatly improved the purity of the final CVs obtained. Crude CVs were further purified by two additional sucrose gradients. First, they were applied to a discontinuous gradient consisting of 8 ml of 5% sucrose in MES buffer layered over 5 ml each 10/20/30/40% sucrose in MES (wt/vol), in a 35-ml ultracentrifuge tube, and centrifuged for 50 min at 25,000 rpm in a Beckman SW 28 rotor. The material between the 5/10 and 30/40 interfaces (excluding the 30/40 interface material) was pooled, diluted threefold with MES buffer, and centrifuged for 120 min at 80,000 g. Pellets were resuspended in MES buffer and layered over a second set of discontinuous sucrose gradients, consisted of 6.0 ml each 30/40/45/50/55% sucrose (wt/vol) over a 2-ml cushion of 60% sucrose prepared in SW 28 ultracentrifuge tubes. These gradients were centrifuged at 25,000 rpm for 16 h. CVs were recovered from the 45/50 and 50/55 interfaces, pooled, and then diluted and concentrated as above. Despite reports of instability of brain CVs in concentrated sucrose solutions (23), the liver CVs prepared using this scheme were entirely coated and homogeneous by negative staining and electron microscopy.

CVs were subjected to agarose gel electrophoresis (29) and recovered using a preparative modification of the technique (Kedersha N. L., and L. H. Rome, 1986, *Anal. Biochem.*, in press) within 1 wk of purifi-

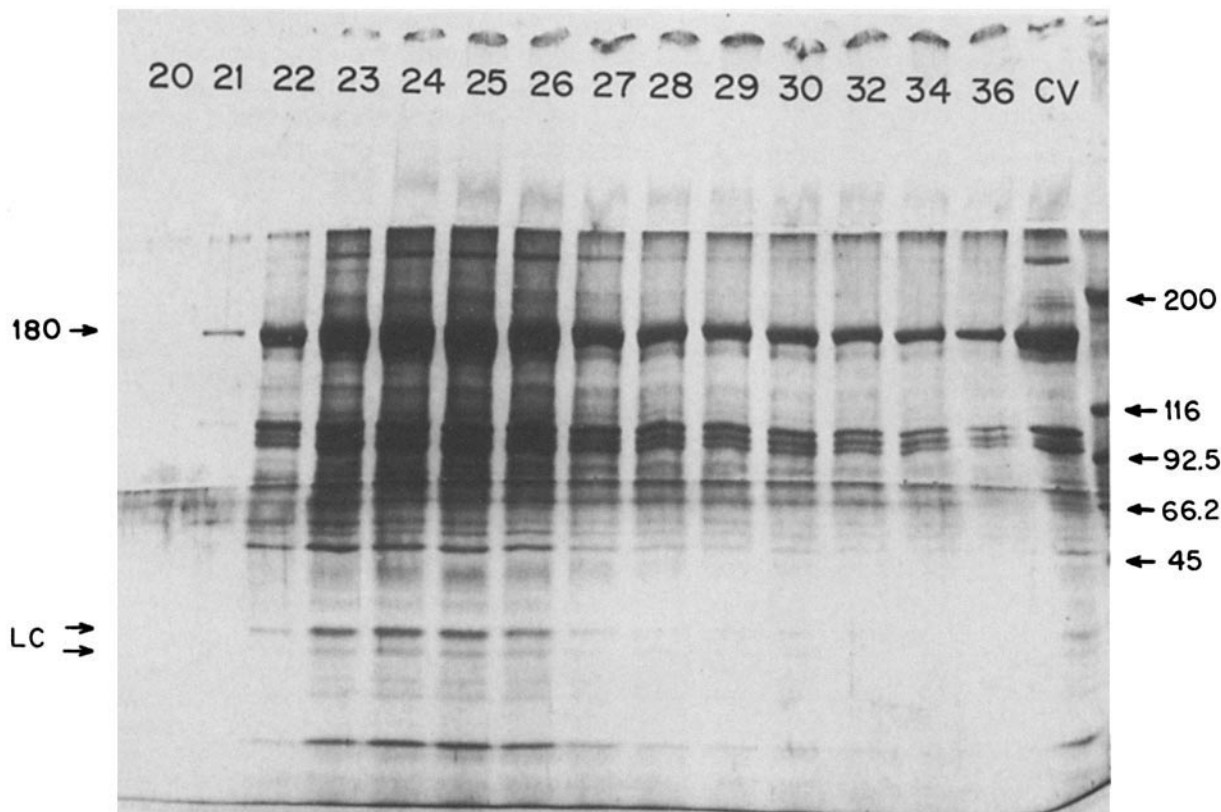


Figure 1. Silver-stained SDS PAGE of agarose-fractionated CVs. Equal volumes were taken from consecutive CV fractions eluted from the preparative agarose gel, treated with SDS and 2-mercaptoethanol, and subjected to SDS PAGE. The gel contained an upper separating gel of 8% acrylamide and a lower resolving gel of 12% acrylamide. Numbers at the top indicate the fraction numbers; the fastest CVs have the lowest numbers as they were eluted first from the gel. The lane denoted CV contains unfractionated CVs. The molecular masses of standard marker proteins ($\times 10^{-3}$) are indicated on the right. LC denotes position of the CV light chains.

cation. Briefly, the modification entailed the addition of a second well cut electrophoretically downstream of the sample lane (towards the anode). Throughout the run, this second well was continuously flushed with 50 mM MES buffer, pH 6.5, at a flow rate of 12 ml/h and collected in 20-min fractions using a fraction collector. A standard horizontal agarose electrophoresis apparatus was used and small holes were drilled into the cover through which the flushing tubes were inserted into the second eluting lane. An agarose concentration of 0.15% was routinely used, and electrophoresis was performed at 4°C using 20 mA constant current. Buffer was continuously recirculated, and the typical run time was 28 h. Immediately after the agarose electrophoresis, aliquots of the eluted CVs were analyzed by SDS PAGE. Fractions were pooled, concentrated by ultracentrifugation, and resuspended in MES buffer.

Iodination of ASOR

ASOR was radiolabeled with ^{125}I by a modification of the procedure of Markwell and Fox (22). Briefly, 0.1 ml (1 mg/ml in CHCl_3) Iodogen was evaporated in a 12 × 75-mm glass tube and 1 mg ASOR was added in a total volume of 0.18 ml in phosphate-buffered saline (PBS). This was followed by the addition of 2 mCi ^{125}I . After 30 min on ice, 0.3 ml PBS and 0.5 ml of 1 mg/ml (each) dithiothreitol and NaI was added to the reaction mixture and the total volume was applied to a PD 10 column previously equilibrated in PBS. The reaction tube was rinsed with an additional 1.5 ml PBS and added to the column; the effluent was discarded. Finally, 3.5 ml PBS was added to the column and the effluent collected. Typically, the specific activity was 2.5×10^6 cpm/ μg ASOR. Radiolabeled glycoprotein was aliquoted into small tubes and stored frozen.

Isolated Liver Perfusion

Isolated livers from 18-h-fasted rats were perfused with ^{125}I -ASOR according to the method of Dunn et al. (9). After 10 min of perfusion at 18°C, ^{125}I -ASOR was added directly to 75 ml of perfusate (final concentration of ASOR was 1.5 $\mu\text{g}/\text{ml}$).

PAGE

One-dimensional polyacrylamide slab gels were prepared with SDS using the buffer system of Laemmli (20). Isoelectric focusing was performed using the method of O'Farrell (24) with the following modifications: samples were dissolved in dissociation buffer containing 1% SDS, 8% wt/vol Nonidet P-40, 5% (vol/vol) 2-mercaptoethanol, 2% (wt/vol) pH 3–10 ampholytes, and 9 M urea. The gels contained 8 M urea, 2% (wt/vol) Nonidet P-40, 4% acrylamide, 2% pH 3–10 ampholytes, 0.8% pH 5–7 ampholytes, 0.2% pH 2.5–4 ampholytes, and 10 mM each lysine and arginine. The anolyte was 0.01 M phosphoric acid; the catholyte was 0.055 M NaOH. The second dimension SDS polyacrylamide gradient gels, made according to Laemmli (20), were linear 5–15% polyacrylamide gradients containing 2.7% bis-acrylamide.

AChEase Assay

AChEase activity was measured using the method of Johnson and Russell (19) with the following changes. A 10-mM Tris buffer (pH 7) was used instead of a 100-mM sodium phosphate buffer. The reaction was incubated in 0.5 ml centrifuge tubes in a total volume of 0.02 ml and was terminated

with 2 ml glycine/HCl introduced directly into the reaction tubes placed within scintillation vials. Subsequently, 10 ml of scintillation fluid were added to each vial.

Western Blots and Lectin Analyses

CV proteins were separated on SDS polyacrylamide gels and transferred to nitrocellulose sheets as described previously (32). For immunostaining, the blots were incubated for at least 2 h in Tris-buffered saline (TBS buffer; 10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 1% bovine serum albumin and 1% powdered non-fat milk. The blots were repeatedly washed in TBS buffer, then incubated for 2 h in TBS containing 2% bovine serum albumin and either 2% anti-M6P receptor antibodies or 0.2% antiasialoglycoprotein (ASGP) receptor antibodies. After thorough washing, the blots were incubated for 1 h in a 1:200 dilution of horseradish peroxidase-goat anti-rabbit antibodies, washed and developed using the 4-chloro-1-naphthol reagent as described (17). Concanavalin A staining was performed as described by Hawkes (17). The WGA staining technique of Bartles and Hubbard (1) was used, substituting a horseradish peroxidase-conjugated form of the lectin for the iodinated species, followed by development using 4-chloro-1-naphthol.

Electron Microscopy

CVs were fixed in 5% glutaraldehyde in MES buffer for 30 min at room temperature and applied to carbon-coated copper grids. Free aldehyde was reduced by a 10-min incubation in 10 mM sodium borohydride. The grids were washed with distilled water and stained with 1.0% uranyl acetate, dried, and viewed on a Hatachi HS 50 electron microscope. Sizing determinations were performed as follows: photographs were enlarged in order to obtain an overall magnification of 62,000 ×. Particles were sized by fitting each coat structure within a circular template. Partially coated vesicles were sized only if the coats were minimally damaged; otherwise they were counted but not sized.

Other Procedures

Protein was determined using the Bio-Rad protein assay; all samples were incubated in 50 mM NaOH for 30 min before the assay in order to improve the detection of membrane proteins. Polyacrylamide gels were routinely stained using the silver stain technique of Merrill et al. (21). Iodinations of CVs were performed using Iodogen (Pierce Chemical Co.) as described above with the following exceptions: iodinations were performed in MES buffer containing 12 mM Tris, either with or without 1.0% 3-[(3-cholamidopropyl)-dimethylammonio]propane-sulfonate (CHAPS) detergent. Free iodide was removed on PD-10 columns equilibrated with MES buffer containing 0.5% Nonidet P-40.

Results

CVs Are Resolved into Discrete Subpopulations by Agarose Gel Electrophoresis

Highly purified (greater than 98% when examined by electron microscope using negative stain) liver CVs were subjected to agarose gel electrophoresis and found to migrate as

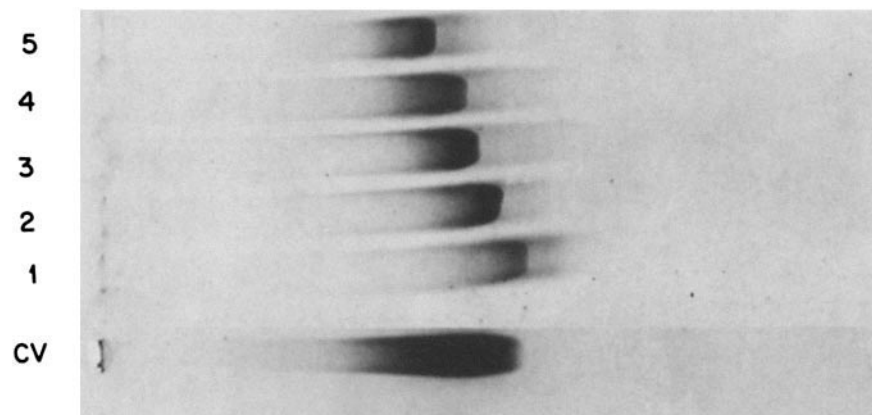


Figure 2. CVs separated by preparative agarose gel electrophoresis. CVs that were separated by preparative agarose gel electrophoresis were concentrated and applied to a second analytical agarose gel. The gel was fixed and stained with Coomassie Brilliant Blue. (Lane 1) pool 1; (lane 2) pool 2; (lane 3) pool 4; (lane 4) pool 6; (lane 5) pool 8. Lane CV contains purified CVs unfractionated by preparative agarose gel electrophoresis, showing the broad distribution of total CVs. Anode is to the right.

a single broad band (see Fig. 2, lane CV). We separated and recovered the CVs using a preparative gel run under the same conditions, and collected the eluted CVs into 4-ml fractions which were analyzed in terms of protein composition by SDS PAGE (Fig. 1). All fractions were dominated by the expected coated vesicle polypeptides: clathrin heavy chains (180 kD), the multiple 100-kD protein species and clathrin light chains (34 and 36 kD). No differences in polypeptide composition were immediately apparent (Fig. 1).

To determine whether this broadness reflected an actual separation of CV subspecies or simply spreading due to diffusion, the 20–25 individual CV fractions were consolidated into eight pooled fractions, concentrated by ultracentrifugation (100,000 *g* for 2 h), adjusted to equal protein concentration, and applied to an analytical agarose gel, which was electrophoresed and then stained to visualize the migration positions of the CVs. The various fractions were found to migrate to their original relative positions (Fig. 2), suggesting the early- and late-eluting CVs were, in fact, electrophoretically distinct species. This is not immediately apparent from viewing the stained gel of unfractionated CVs (Fig. 2, lane CV) because as originally isolated, the CV sub-

populations are not present in equal amounts. When equal quantities of the fractionated CVs are separated on a second agarose gel, the CV heterogeneity with regard to electrophoretic mobility becomes more obvious.

Size Heterogeneity and Stability of Fractionated CVs

Aliquots of the alternate pooled fractions were adsorbed onto carbon-coated copper grids, fixed with glutaraldehyde, and negatively stained with uranyl acetate. Examination by electron microscopy (Fig. 3) revealed that some of the slow-migrating CVs appeared to be less stable, possessing coats which often seemed to be slightly damaged or partly breached (Fig. 3, C and D), whereas the fast-migrating CVs appeared completely intact (Fig. 4). This selective damage may have occurred either during the final (postelectrophoresis) centrifugation step or by exposure to uranyl acetate used in the negative stain procedure. Although the vesicles eluting early and late appeared, upon visual examination, to be similar in size, when the diameters of CVs in pools 1, 3, 5, and 7 were measured, a significant size gradient was detected. Despite the non-sieving conditions used in the

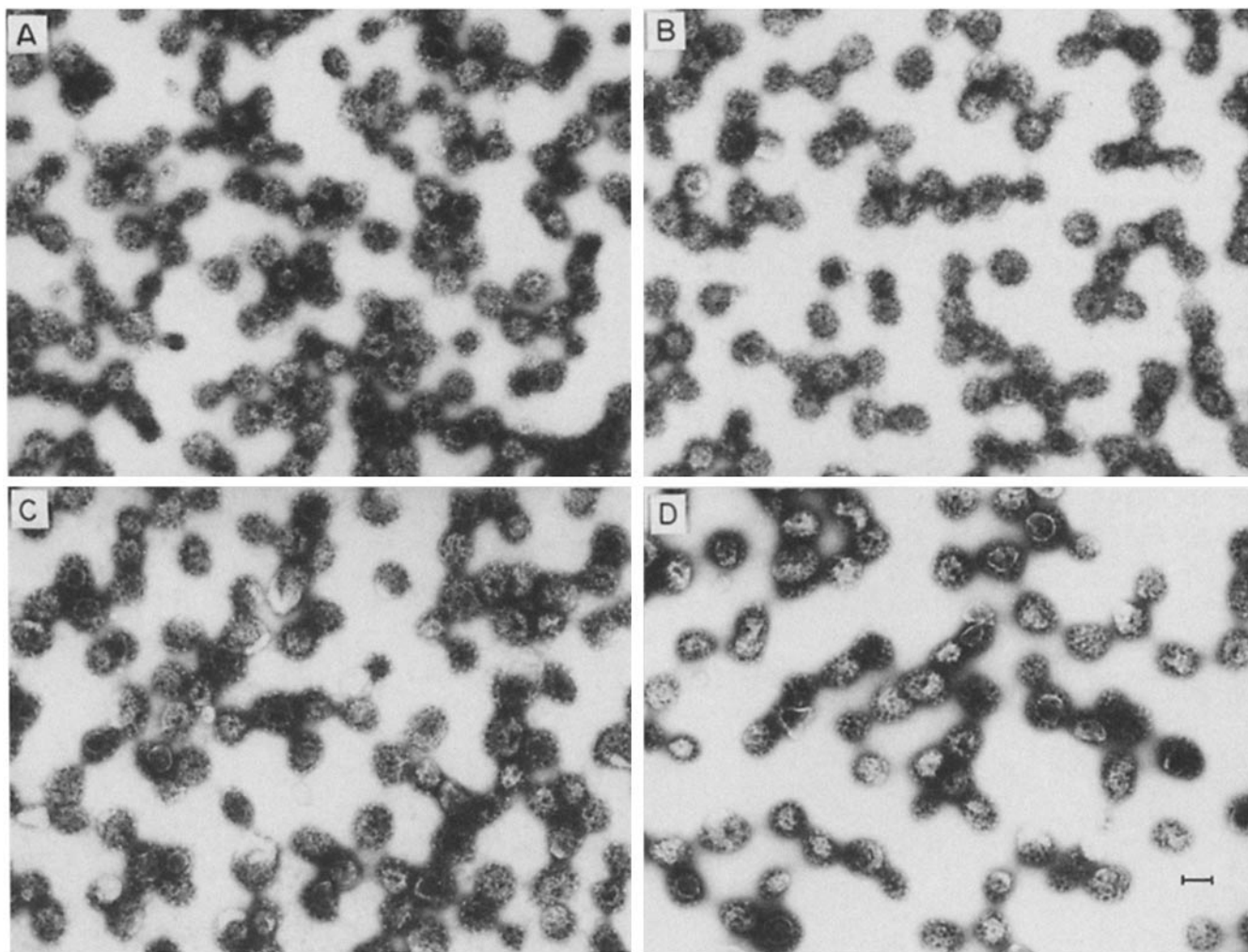


Figure 3. Electron microscopy of fractionated CVs. Agarose gel-fractionated CVs were pooled into eight fractions, concentrated by ultracentrifugation, and resuspended in MES buffer. Aliquots were fixed with glutaraldehyde, negatively stained, and examined on a Hitachi electron microscope. (A) Pool 1; (B) pool 3; (C) pool 5; (D) pool 7. Bar, 100 nm.

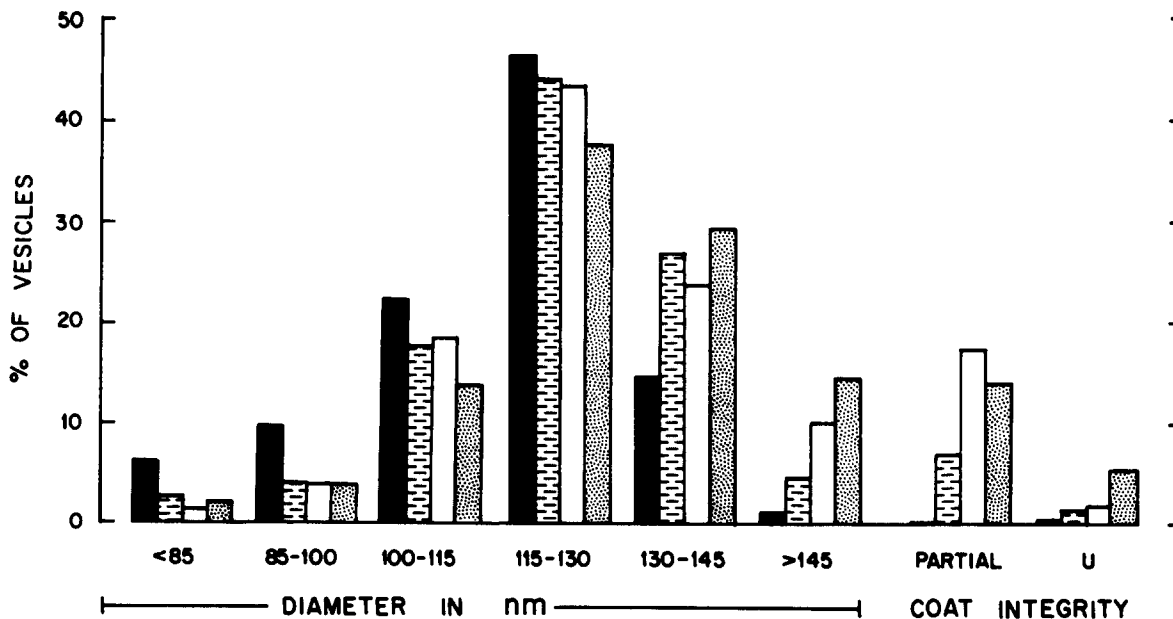


Figure 4. Size distribution of charge-fractionated CVs. Aliquots of agarose gel-fractionated CV pools 1, 3, 5, and 7 were fixed with glutaraldehyde, stained with uranyl acetate, and examined by electron microscopy. Vesicle diameters were measured as described in Materials and Methods. Solid bars, pool 1; dashed bars, pool 3; open bars, pool 5; stippled bars, pool 7. The number of particles sized was 643 from pool 1, 563 from pool 3, 833 from pool 5, and 588 from pool 7. Only intact CVs were sized. The partially uncoated vesicles (*PARTIAL*) and smooth or uncoated CVs (*U*) are indicated as a percentage of the total vesicles examined in each fraction.

agarose gel procedure, there was an enrichment of larger classes of CVs in the slow-migrating populations (Fig. 4).

CV Subpopulations Possess Similar Coat Proteins but Differ in Total Protein Composition

Equal aliquots of fractionated CVs were treated with 2 M urea, 12 mM Tris-HCl, pH 8.2 for 2 h at room temperature. Vesicles were separated from the solubilized coat proteins by ultracentrifugation (100,000 g for 30 min), and both the

pellets and the supernatants were subjected to SDS PAGE (Fig. 5). The coat proteins which were removed by the urea Tris-HCl treatment appear identical among all fractions (Fig. 5, lanes 9-16), suggesting that the electrophoretic differences between the CV subpopulations are not due to differences in coat composition. Examination of the stripped vesicles by SDS PAGE (Fig. 5, lanes 1-8) reveals several polypeptides which segregate specifically with the slow-migrating CV populations (see arrows).

To further establish that differences in the clathrin coats

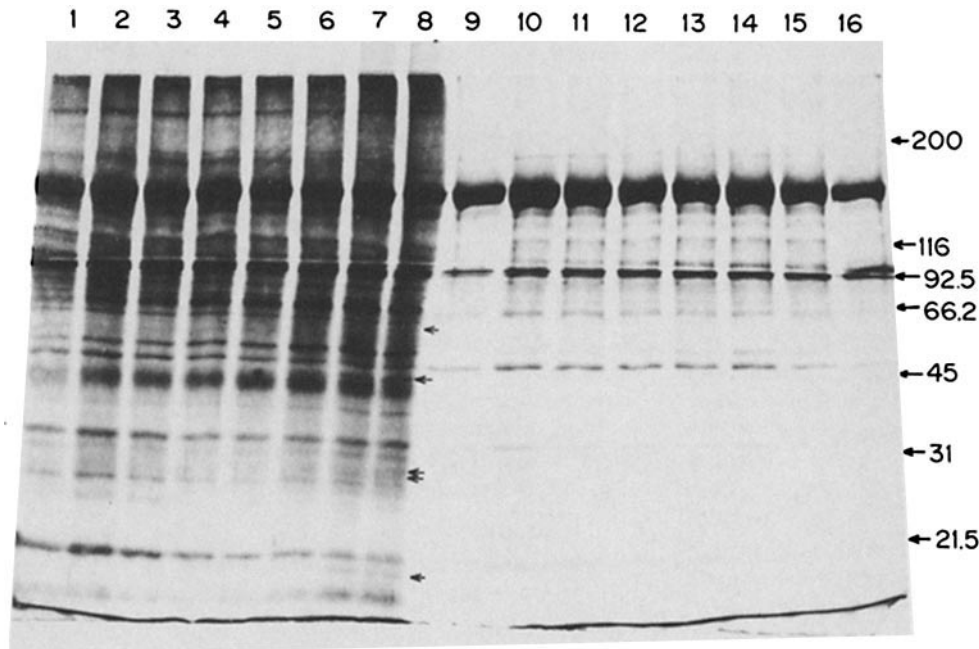


Figure 5. Silver-stained SDS PAGE of stripped CVs and coat proteins. The fractionated CVs were stripped by treatment with urea-Tris as described in the text. Aliquots of stripped vesicles and soluble coat proteins were applied to an 8/12% acrylamide gel as in Fig. 2. (Lanes 1-8) Vesicle pools 1-8, respectively; (lanes 9-16) the urea-Tris-soluble coat proteins from pools 1-8, respectively. Arrows indicate species which appear present in some pools but not others.

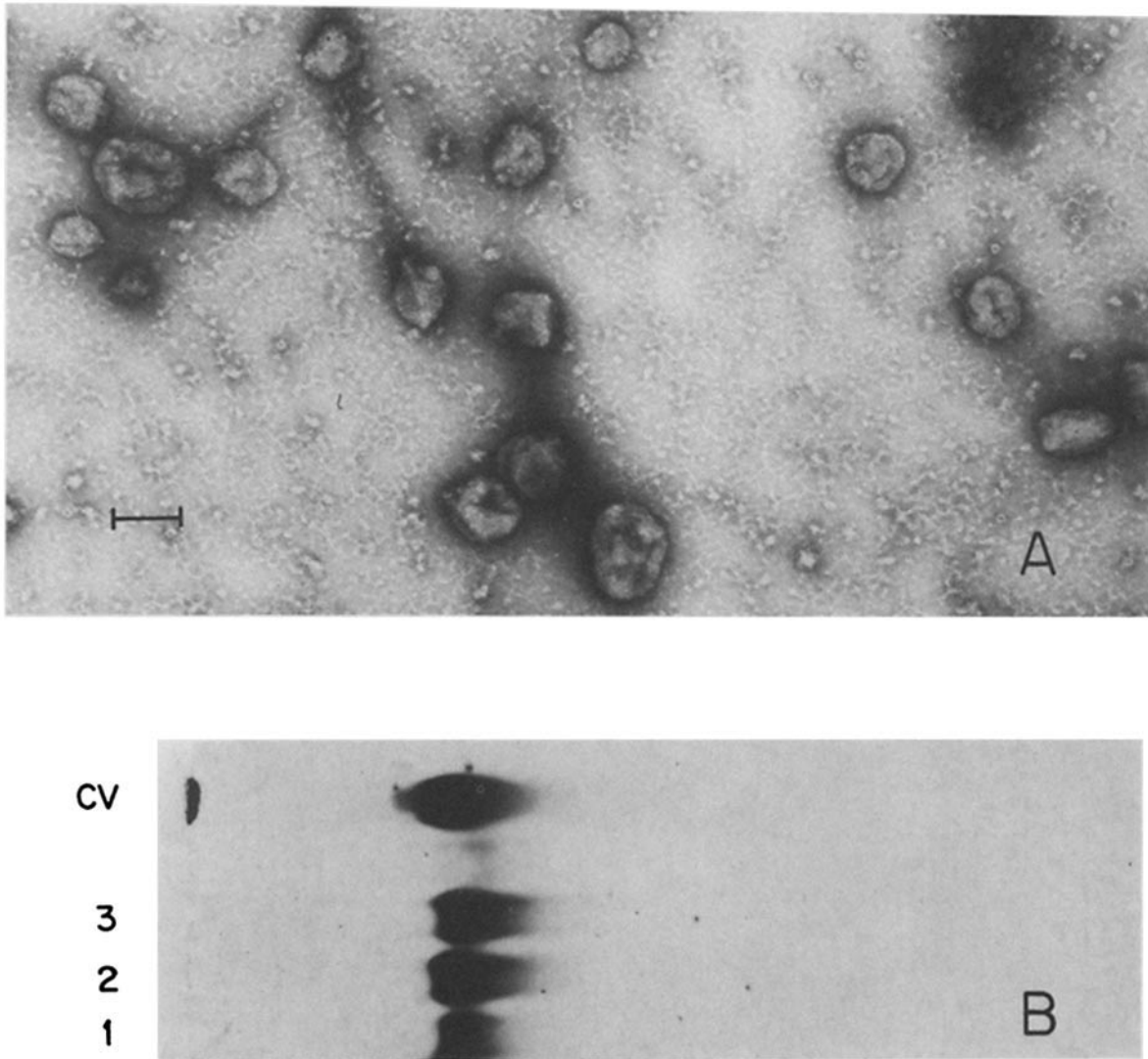


Figure 6. Agarose gel electrophoresis of urea-solubilized CVs. Samples of fractionated CVs were treated with 2 M urea in MES buffer and run in an agarose gel containing 2 M urea in addition to 50 mM MES buffer, pH 6.5. (A) Aliquot of urea-treated vesicles before electrophoresis, showing the stripped vesicles amid a lawn of intact triskelions. (B) Agarose gel which was run for 45 h (approximately twice as long as usual) and then stained with Coomassie Brilliant Blue. (Lane CV) Unfractionated CVs; (lane 1) pool 2 CVs; (lane 2) pool 4 CVs; (lane 3) pool 6 CVs. Compare the relative migration distances to those in Fig. 1, lanes 2–4.

did not cause the separation of the different populations on the agarose gel, CV fractions which were separated on the preparative agarose were treated with 2 M urea for 1 h at room temperature. This concentration of urea has been shown to dissociate clathrin triskelions from CVs at neutral pH (30, 36). The stripped vesicles and intact triskelions (Fig. 6 A) were then applied to an agarose gel that also contained 2 M urea. Under these conditions, the major Coomassie-staining bands (clathrin triskelions), whether derived from fast-, moderate-, or slow-migrating CVs, moved to the same position on the agarose gel (Fig. 6), also suggesting that the mobility differences in intact CVs are not due to differences in clathrin composition but rather due to charge heterogeneity in the underlying vesicles. Attempts to directly measure the mobility of the stripped vesicles themselves were unsuccessful, as they had such reduced mobilities in the agarose gel that differences could not be detected.

Two-dimensional electrophoresis of slow- and fast-migrating CVs revealed numerous differences in polypeptide

composition between the two populations (Fig. 7). Proteins specific to the fast-migrating CVs (Fig. 7 B, in the M_r 68,000–80,000 region) appeared predominately in the basic region of the gel, whereas the majority of proteins specific to the slow-migrating CVs were acidic (Fig. 7 A, M_r 48,000 and several species M_r 50,000–90,000). As clathrin iodinated poorly under the conditions used here (see Materials and Methods), additional differences between the two populations became apparent when agarose-separated CVs were iodinated and subjected to two-dimensional electrophoresis followed by autoradiography (Fig. 7, C and D). Several acidic species and a species of M_r 43,000 (arrow) were present exclusively in the slow-migrating population.

Subpopulations Exhibit Heterogenous Glycoprotein Composition

Equal quantities of fractionated coated vesicles were stripped of their coat proteins as described above. Both the

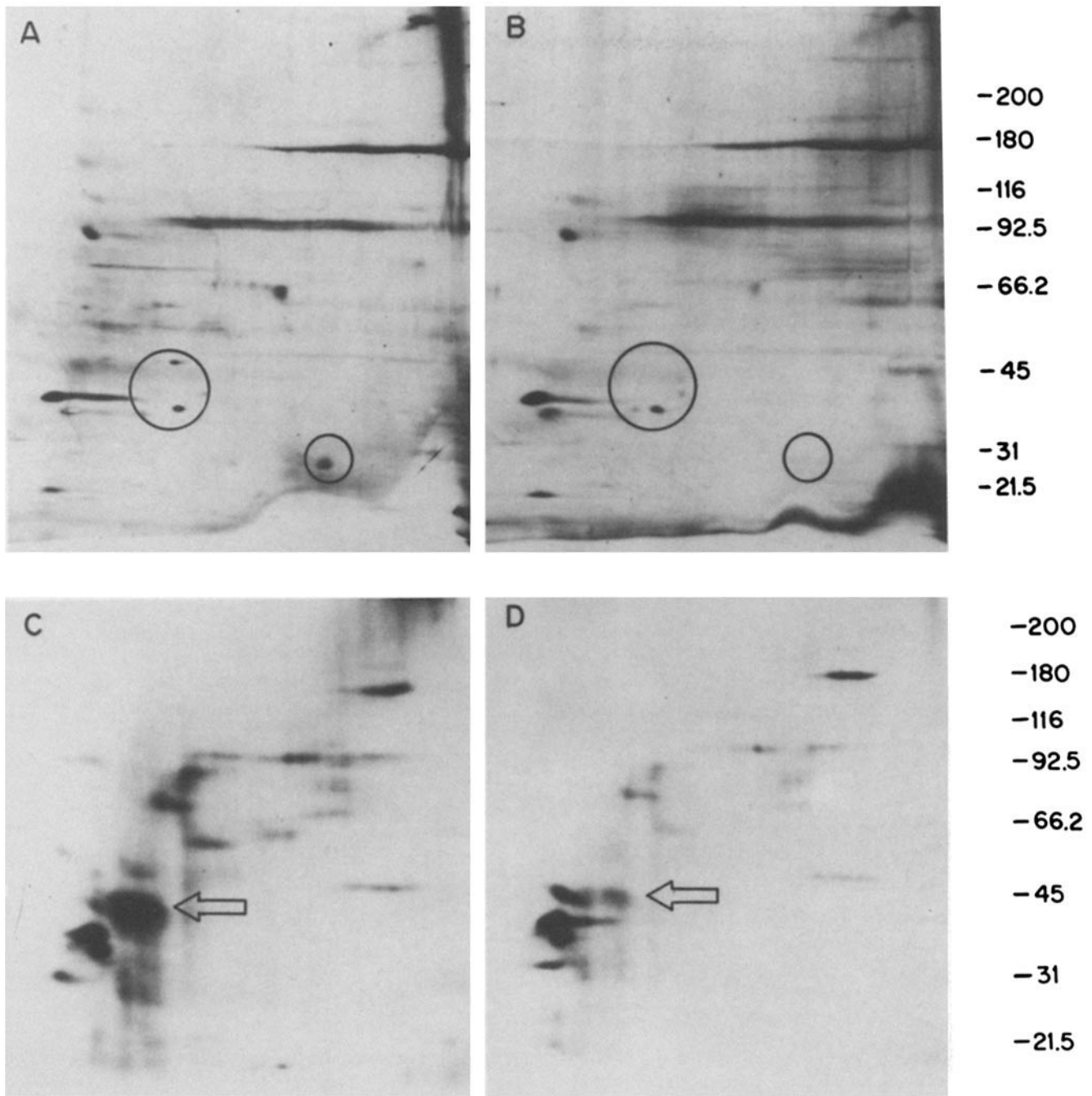


Figure 7. Two-dimensional PAGE of CV proteins. (A) Silver-stained gel showing differences between slow- and fast-migrating CVs (pools 7, 2). (A) Pool 7; (B) pool 2. Circles indicate some regions of different protein content. Equal amounts of protein were loaded on each gel. (B) Autoradiograms of two-dimensional separation of iodinated slow- and fast-migrating CVs. (C) Pool 7; (D) pool 2. Equal amounts of protein were loaded onto each gel. Arrow indicates position of a polypeptide greatly enriched in the slow-migrating CVs. Anode is at the left.

vesicle-associated proteins and the urea/Tris-soluble coat proteins were separated on SDS PAGE, transferred to nitrocellulose sheets by electrophoretic transfer, and examined for lectin-binding proteins as described in Materials and Methods. Several concanavalin A-binding proteins were detected, having mobilities of M_r 82,000, 50,000 and 38,000. These glycoproteins appeared to be dramatically enriched in the slow-migrating population of CVs (Fig. 8, arrows), while no concanavalin A-binding proteins were detected in the stripped coat proteins (Fig. 8, lanes 9-13). Greater diversity

was observed in populations of CVs that were probed with WGA (Fig. 9). Here, a polypeptide of M_r 195,000 was a predominate species in the early CVs, and was most abundant in pooled fractions 3-4 (Fig. 9, top arrow). The majority of other WGA-binding species predominated in the slow migrating CVs. Again, no WGA-binding species were detected among the urea/Tris-solubilized coat proteins. From the results presented in Figs. 8 and 9, it appears that the majority of glycoproteins detectable with the two lectins are found in the slower (late-eluting) fraction of CVs.

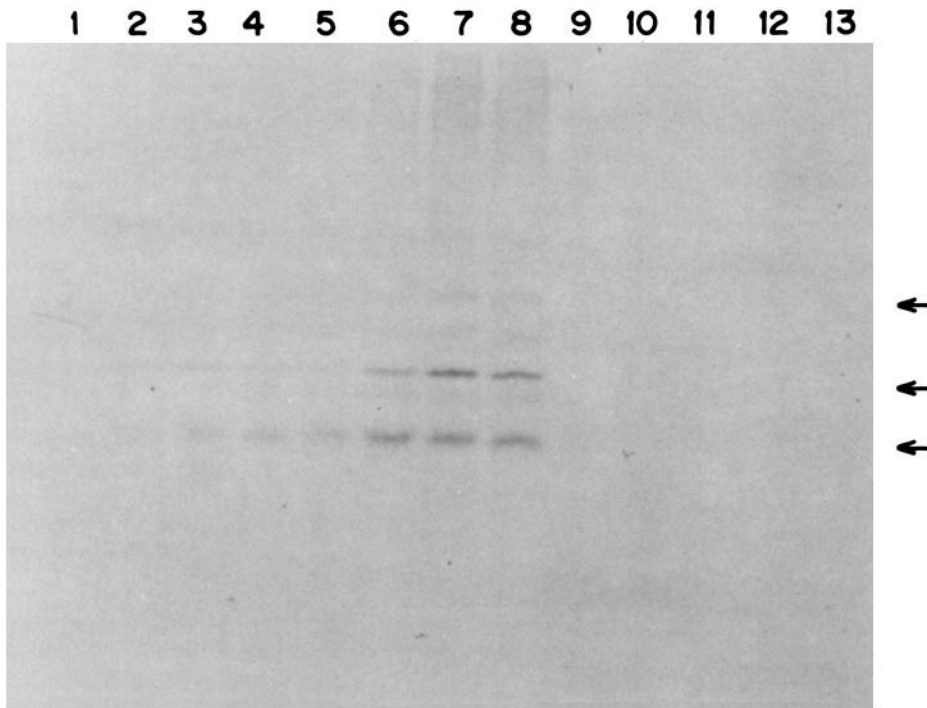


Figure 8. Concanavalin A-binding proteins in fractionated CVs. Stripped CVs and CV coat proteins were prepared as described in the text. Equal amounts of protein from each pool were separated on SDS PAGE, transferred to nitrocellulose, and the concanavalin A-binding proteins visualized. (Lanes 1-8) Vesicle proteins from pools 1-8, respectively. (Lanes 9-13) The stripped coat proteins from pools 1, 2, 3, 5, and 7, respectively.

Subpopulations of CVs Are Heterogeneous with Respect to M6P and ASGP Receptor Distribution

CV populations were subjected to SDS PAGE, transferred to nitrocellulose sheets, and probed with antibodies specific for either the M6P receptor or the ASGP receptor. A heterogeneous distribution was observed (Fig. 10). The bulk of the M6P receptor was found in the fast-migrating fractions 3-4, whereas the ASGP receptor appeared to be most concentrated in the slow-migrating fractions 6-7 (Fig. 10).

Endocytic and Exocytic CV Subpopulations

Rat liver was perfused with ^{125}I -ASOR for 40 min at 18°C, then with EGTA (5 mM) for 10 min at 4°C. The liver was homogenized, CVs were purified and separated on the agarose gel system. The protein composition of the eluted CVs was identical to that shown in Fig. 1. The fractions were assayed for protein, counted in a gamma counter to detect the presence of ^{125}I -ASOR, and assayed for AChEase. This enzyme, which is synthesized in the liver and secreted for ex-

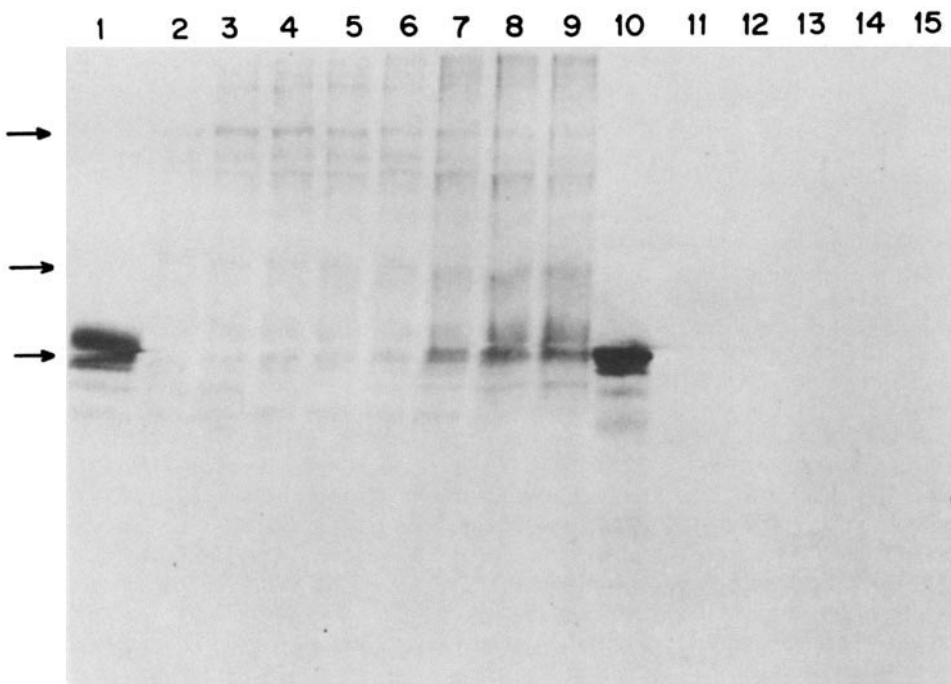


Figure 9. WGA binding proteins in fractionated CVs. Equal amounts of stripped CV proteins and CV coat proteins were treated as in Fig. 7, transferred to nitrocellulose, and reacted with a WGA-horseradish peroxidase conjugate as described in the text. Lanes 1 and 10 contain fetuin. (Lanes 2-9) Vesicle-bound protein from pools 1-8, respectively. (Lanes 11-15) Stripped coat proteins from pools 2, 4, 6 and 8, respectively.

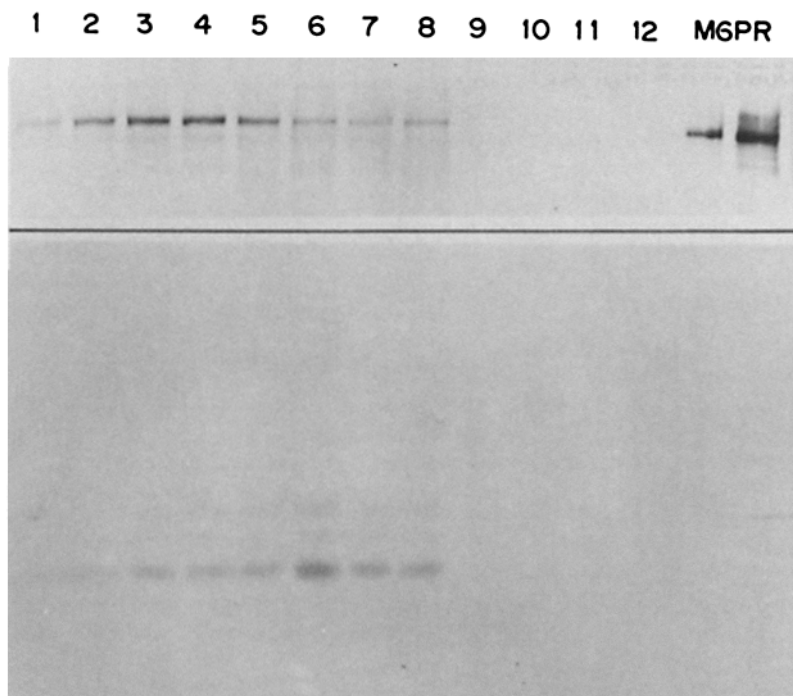


Figure 10. Western blot quantitation of M6P and ASGP receptors in fractionated CVs. Equal amounts of stripped CV proteins and coat proteins were resolved on SDS PAGE, blotted onto nitrocellulose, and reacted with antibodies specific for either the M6P receptor (*upper panel*) or the ASGP receptor (*lower panel*) as described in the text. (Lanes 1-8) Equal amounts of stripped CV proteins from pools 1-8, respectively. (Lanes 9-12) Coat proteins from pools 1, 3, 5 and 7, respectively.

port to other tissues, is a useful marker of exocytic CVs (18). The results, shown in Fig. 11, indicate that the bulk of the AChE activity is found to be coincident with the bulk of the CV protein (fractions 26-29, corresponding to pooled fractions 3-4 of the preparation shown in Figs. 6-8), and also coincident with the M6P receptor peak. The ^{125}I -ASOR was concentrated in CVs which eluted more slowly, coincident with pooled fractions 6-7 of Figs. 5, 8, and 9, and also coincident with the bulk of the ASGP receptor detected by immunoblotting (Fig. 10). The ^{125}I -ASOR was present within the vesicles and not free or adhering to the vesicle surfaces, as free ^{125}I -ASOR mixed with unlabeled crude vesicles was found to migrate extremely slowly on the agarose gel, never associating with vesicles (not shown).

Discussion

Intracellular transport of macromolecules requires that many sorting steps occur as material moves from one membrane-

bound compartment to another. The postulated role of CVs as "molecular filters" (3) suggests that they constitute an important sorting step necessary to initiate various transport routes, by virtue of their ability to selectively include or exclude various membrane proteins from the nascent vesicles. CVs with different origins, contents, and destinations should constitute distinct biochemical entities and as such should be separable by biochemical techniques.

Using a preparative agarose gel system, we fractionated purified CVs and isolated several electrophoretically distinct species. The charge heterogeneity which enables the resolution of these different populations is not directly attributable to differences in electrophoretic mobility of their clathrin coats, as freed triskelions from different populations exhibit identical electrophoretic mobilities in the agarose gel. Furthermore, 2-D gels reveal no detectable charge differences in clathrin and other clathrin-associated coat proteins that could account for the mobility differences of the subpopulations. Finally, as the separation of species on non-sieving aga-

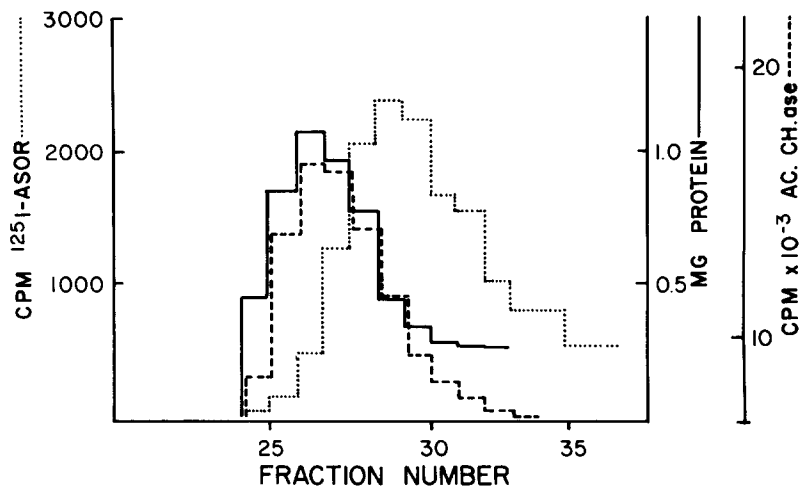


Figure 11. Protein, acetylcholine esterase content, and ligand content of CVs isolated from ^{125}I -ASOR-perfused rat liver. The dotted line indicates ^{125}I cpm, solid line indicates total protein concentration in mg, dashed line indicates AChE activity in cpm (see text for assay conditions).

rose gels is largely determined by surface charge (7, 16, 37), one would predict that larger CVs would migrate faster than smaller CVs, due to the greater amount of clathrin associated with the former and its greater net charge. As the opposite is observed, it appears probable that other factors such as non-clathrin, vesicle-associated polypeptides cause the mobility differences observed here. Antibodies to the cytoplasmic domain of an integral membrane protein have been shown to penetrate the clathrin coat in intact brain CVs (27, 35), demonstrating the accessibility of non-coat, vesicle-associated proteins. This suggests that such proteins are capable of contributing to the surface charge of intact CVs and are therefore able to influence CV mobility in the agarose gel.

Considerable diversity was observed in non-clathrin polypeptides from the CV subpopulations which could account for the observed CV charge heterogeneity. Numerous species resolved by 2-D gels are exclusively present in the slower-migrating CVs, particularly those found in the acidic region of the gel (Fig. 7). As CVs arise from different membranes, the contribution of membrane lipids to the overall surface charge of the vesicle may also contribute to the electrophoretic mobility of intact CVs. It has recently been shown that endocytic and exocytic CVs purified by a density shift technique differ significantly in cholesterol content (18).

The subpopulations were also observed to be heterogeneous in glycoprotein composition, M6P receptor and ASGP receptor content, and AChE and ASOR ligand content. In addition to their clearly demonstrable charge heterogeneity, the CVs thus appear to be biochemically and functionally heterogeneous. One interpretation of the data would be that the endocytic (slow) and exocytic (fast) CVs constitute the two major species separated by this technique, and that the biochemical differences observed reflect the characteristics of these two major classes of CVs. This may oversimplify the case, as there are clearly more than two species that are electrophoretically distinct (Fig. 1); our fractionation procedure defines at least five but in fact a continuum appears to exist.

However, an alternate interpretation of the data would be that the observed compositional heterogeneity (e.g., protein heterogeneity, ligand heterogeneity) is due to contaminating smooth vesicles of discrete charge rather than representing true differences in CV contents. The agarose gel technique we use has been shown to be particularly effective in removing smooth vesicles and uncoated CVs from the intact CVs (18), and the high degree of purity of the CVs obtained in these studies (see Figs. 4 and 5) confirms the value of this technique in this regard. We made repeated efforts to use this technique in the isolation of charge-specific populations of smooth vesicles. Under the buffer and pH conditions used, microsomal smooth vesicles migrate more slowly than the bulk of the CVs and in extremely broad bands which appear to be homogeneous in their protein composition and, in the case of perfused livers, ^{125}I -ASOR content (not shown). We have also attempted to separate (by electrophoresis) smooth vesicles which had been purified using combined sucrose equilibrium and velocity gradients. These vesicles were similar in size and density. As with crude microsomal vesicles broad bands (spanning 20–30 fractions) of apparently homogeneous protein composition were seen. This is not surprising considering that the separation of particles in non-sieving agarose gels is due to their net surface charge (7, 37),

which in the case of smooth vesicles is a function of both membrane charge and vesicle size. Variations in size among vesicles of identical membrane composition therefore create species of different net charge; only species of identical size and charge can possess identical net surface charge, and such homogeneity does not appear to exist among smooth vesicles.

We cannot rule out the possibility that the leading edge of a slow peak of smooth vesicles may contaminate the slower CVs and thus be responsible for the apparent enrichment of glycoproteins in the late-eluting CV fraction. However, it seems unlikely that such a smooth vesicle contamination could account for the relatively narrow bands (8–10 fractions) containing ^{125}I -ASOR (shown in Fig. 11) and the ASGP and M6P receptors (shown in Fig. 10), since smooth vesicles in our hands migrate considerably slower and with a broader distribution. Furthermore, the distribution of the ligands and receptors is not coincident with the degree of possible smooth vesicle contamination observed by electron microscopy (Figs. 3 and 4). The same argument applies to the distribution by the M_r 195,000 glycoprotein seen in Fig. 9. Although we detect increasing numbers of smooth vesicles by electron microscopy in the late-eluting pools (Fig. 4), we feel that these are due to uncoated vesicles produced after electrophoresis during preparation of the samples for electron microscopy (centrifugation and resuspension). This is supported by the greater numbers of partially uncoated CVs observed in these same pools and may reflect an instability inherent in larger CVs. It is also possible that the non-clathrin protein composition of the underlying vesicle may influence CV stability. Recent studies indicate that the presence of M6P receptors stabilizes clathrin coated structures *in vitro*, even in the presence of 1.0% Triton X-100 (25). The fast-migrating CVs are more stable, enriched in M6P receptor and, on the average, smaller in size.

A possible relationship between CV size and function has been previously proposed based upon morphological studies in rat vas deferens (11) and in mouse liver (8). These and other studies suggest that Golgi-derived CVs are generally smaller (60–80 nm) than those which appear to be derived from the cell surface (100–160 nm). Steven et al. (31) have also suggested that size variability may reflect functional differences in CV subpopulations, and that CVs of similar size may differ considerably in the mass of their vesicle contents. Our results are not incompatible with these findings; however, since we find considerable size heterogeneity within each charge-selected population, it seems that size alone is not an adequate criterion for differentiation of endocytic and exocytic CVs in rat liver.

The multiple aspects of CV heterogeneity may reflect the involvement of CVs in many different trafficking routes. In addition to their well-documented role in endocytosis, CVs have also been shown to contain newly synthesized lysosomal enzymes bound to the M6P receptor (5, 6), presumably packaged in Golgi-derived CVs as the first step in the transport of lysosomal enzymes to the lysosome. Studies using immunolocalization techniques at the electron microscopy level have shown that in rat liver the M6P receptor appears to be concentrated in the Golgi apparatus (4, 13), while the ASGP receptor predominates on the plasma membrane (14). Although some CVs derived from the plasma membrane contain both receptors, the ASGP receptor is present in

greater amounts (14). This suggests that the M6P receptor should be primarily located in Golgi-derived CVs, while the ASGP receptor should be primarily found in CVs originating at the plasma membrane. In the agarose subpopulations described here, the electrophoretically fast-migrating population is clearly enriched with the M6P receptor, and is electrophoretically coincident with the CV subpopulation containing exocytic AChE. The ASGP receptor is predominately found in the slow CVs, coincident with those endocytically labeled by ¹²⁵I-ASOR in the perfused rat liver.

The studies presented here demonstrate that considerable heterogeneity exists in CVs purified from rat liver, a relatively homogeneous tissue composed largely of a single cell type. Given the complexity and diversity of the trafficking routes which may be mediated by CVs, the large number of subpopulations observed here is not surprising. The isolation of these functionally distinct species should lead to a better understanding of the role of CVs in intracellular transport.

We would like to thank Dr. Richard Fine for sharing the data in reference 18 in advance of publication. We would like to acknowledge the excellent technical assistance Ms. Hedi Roseboro.

This work was supported by a United States Public Health Service (USPHS) grant (HD-06576) and by grant R0066 from the Cystic Fibrosis Foundation. N. L. Kedersha is the recipient of the USPHS training grant HD-07032. L. H. Rome is the recipient of an American Cancer Society Faculty Research Award.

Received for publication 30 November 1985, and in revised form 19 February 1986.

References

- Bartles, J. R., and A. L. Hubbard. 1984. ¹²⁵I-Wheat germ agglutinin blotting: increased sensitivity with polyvinylpyrrolidone quenching and periodate oxidation/reductive phenylation. *Anal. Biochem.* 140:284-292.
- Blitz, A. L., R. E. Fine, and P. A. Toselli. 1977. Evidence that coated vesicles isolated from brain are calcium-sequestering organelles resembling sarcoplasmic reticulum. *J. Cell Biol.* 75:135-147.
- Bretscher, M. S., J. N. Thomson, and B. M. F. Pearse. 1980. Coated pits as molecular filters. *Proc. Natl. Acad. Sci. USA.* 77:4156-4159.
- Brown, W. J., and M. G. Farquhar. 1984. The mannose-6-phosphate receptor for lysosomal enzymes is concentrated in *cis* Golgi cisternae. *Cell.* 36:295-307.
- Campbell, C. H., R. E. Fine, J. Squicciarini, and L. H. Rome. 1983. Coated vesicles from rat liver and calf brain contain cryptic mannose 6-phosphate receptors. *J. Biol. Chem.* 258:2628-2633.
- Campbell, C. H., and L. H. Rome. 1983. Coated vesicles from rat liver and calf brain contain lysosomal enzymes bound to mannose 6-phosphate receptors. *J. Biol. Chem.* 258:13347-13352.
- Childs, J. D. 1980. Effect of *hoc* protein on the electrophoretic mobility of intact bacteriophage T4D particles. *J. Mol. Biol.* 141:163-173.
- Croze, E. M., D. J. Morre, D. M. Morre, J. Kartenbeck, and W. W. Franke. 1982. Distribution of clathrin and spiny-coated vesicles on membranes within mature Golgi apparatus elements of mouse liver. *Eur. J. Cell Biol.* 28:130-138.
- Dunn, W. A., D. A. Wall, and A. L. Hubbard. 1983. Use of isolated, perfused liver in studies of receptor-mediated endocytosis. *Methods Enzymol.* 98:225-251.
- Farquhar, M. G. 1983. Intracellular membrane traffic: pathways, carriers, and sorting devices. *Methods Enzymol.* 98:1-13.
- Friend, D. S., and M. G. Farquhar. 1967. Functions of coated vesicles during protein absorption in the rat vas deferens. *J. Cell Biol.* 35:357-376.
- Geuze, H. J., J. W. Slot, G. J. A. M. Strous, H. F. Lodish, and A. L. Schwartz. 1983. Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor mediated endocytosis. *Cell.* 32:277-287.
- Geuze, H. J., J. W. Slot, G. J. A. M. Strous, A. Hasilik, and K. von Figura. 1984. Ultrastructural localization of the mannose 6-phosphate receptor in rat liver. *J. Cell Biol.* 98:2047-2054.
- Geuze, H. J., J. W. Slot, G. J. A. M. Strous, J. Peppard, K. von Figura, A. Hasilik, and A. L. Schwartz. 1984. Intracellular receptor sorting during endocytosis: comparative immunoelectron microscopy of multiple receptors in rat liver. *Cell.* 37:195-204.
- Goldstein, J. L., R. G. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature (Lond.)* 279:679-685.
- Gottlieb, M. H., C. J. Steer, A. C. Steven, and A. Chrambach. 1985. Applicability of agarose gel electrophoresis to the physical characterization of clathrin-coated vesicles. *Anal. Biochem.* 147:353-363.
- Hawkes, R. 1982. Identification of concanavalin A-binding proteins after sodium dodecyl sulfate gel electrophoresis and protein blotting. *Anal. Biochem.* 123:143-146.
- Helmy, S., K. Porter-Jordan, E. A. Dawidowicz, P. Pilch, A. L. Schwartz, and R. E. Fine. 1986. Separation of endocytic from exocytic coated vesicles using a novel cholinesterase mediated density shift technique. *Cell.* 44:497-506.
- Johnson, C. D., and R. L. Russell. 1975. A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. *Anal. Biochem.* 64:229-238.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Merril, C. D., D. Goldman, and M. L. Van Keuren. 1983. Silver staining methods for polyacrylamide gel electrophoresis. *Methods Enzymol.* 96:230-239.
- Markwell, M. A. K., and C. F. Fox. 1978. Surface-specific iodination of membrane proteins of viruses and eukaryotic cells using 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril. *Biochemistry.* 17:4807-4817.
- Nandi, P. K., G. Irace, P. P. van Jaarsveld, R. E. Lippoldt, and H. Edelhoch. 1982. Instability of coated vesicles in concentrated sucrose solutions. *Proc. Natl. Acad. Sci. USA.* 79:5881-5885.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 256:4007-4021.
- Pearse, B. M. F. 1985. Assembly of the mannose-6-phosphate receptor into reconstituted clathrin coats. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2457-2460.
- Pearse, B. M. F., and M. S. Bretscher. 1981. Membrane recycling by coated vesicles. *Annu. Rev. Biochem.* 50:85-101.
- Pfeffer, S. R., and R. B. Kelly. 1985. The subpopulation of brain coated vesicles that carries synaptic vesicle proteins contains two unique polypeptides. *Cell.* 40:949-957.
- Rothman, J. E., E. Fries, W. G. Dunphy, and L. J. Urbani. 1982. The Golgi apparatus, coated vesicles, and the sorting problem. *Cold Spring Harbor Symp. Quant. Biol.* 35:797-805.
- Rubenstein, J. L. R., R. E. Fine, B. D. Luskey, and J. E. Rothman. 1981. Purification of coated vesicles by agarose gel electrophoresis. *J. Cell Biol.* 89:357-361.
- Schook, W., and S. Puszkun. 1983. Dissociation and reassociation of clathrin. *Methods Enzymol.* 98:350-358.
- Steven, A. C., J. F. Hainfeld, J. S. Wall, and C. J. Steer. 1983. Mass distributions of coated vesicles isolated from liver and brain: analysis by scanning transmission electron microscopy. *J. Cell Biol.* 97:1714-1723.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Walter, P., I. Ibrahim, and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. I. Signal recognition particle (SRP) binds to the in vitro assembled polysomes synthesizing secretory proteins. *J. Cell Biol.* 91:545-550.
- Weidenmann, B., and W. W. Franke. 1985. Identification and localization of synaptophysin, an integral membrane glycoprotein of *M*, 38,000 characteristic of presynaptic vesicles. *Cell.* 41:1017-1028.
- Weidenmann, B., K. Lawley, C. Grund, and D. Branton. 1985. Solubilization of proteins from bovine brain coated vesicles by protein perturbants and Triton X-100. *J. Cell Biol.* 101:12-18.
- Woodward, M. P., and T. F. Roth. 1978. Coated vesicles: characterization, selective dissociation, and reassembly. *Proc. Natl. Acad. Sci. USA.* 75:4384-4398.
- Yamaguchi, Y., and M. Yanagida. 1980. Head shell protein *hoc* alters the surface charge of bacteriophage T4. *J. Mol. Biol.* 141:175-193.