Purification of Coated Vesicles by Agarose Gel Electrophoresis

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ABSTRACT We have applied agarose gel electrophoresis as a novel step in the purification of clathrin-coated vesicles. Preparations of coated vesicles obtained by sedimentation velocity and isopycnic centrifugation are resolved into two distinct fractions upon electrophoresis. The slower migrating fraction contains smooth vesicles, whereas the faster contains only coated vesicles and empty clathrin coats. The faster mobility of the coated vesicles is primarily caused by the acidic nature of clathrin. Coated vesicles from three different cell types have different mobilities. In each case, however, all of the major polypeptides previously attributed to coated vesicles comigrate with the now homogeneous particles, even though a powerful ATPase activity is completely removed.

Evidence is accumulating that coated vesicles provide a fundamental mechanism for the specific transfer of membranes and membrane-associated molecules among a variety of intracellular compartments in higher cells (1). Coated vesicles account for most if not all of receptor-mediated endocytosis (2) and appear also to function in the transport of proteins to the plasma membrane (3), to secretion granules (4, 5) and to lysosomes (6).

Biochemical studies of coated vesicles were greatly facilitated by the purification of these vesicles from other intracellular organelles by Pearse (7). She discovered that the coat has a strikingly simple composition, its principal constituent on a mass basis being a protein of 180,000 mol wt which she termed clathrin. Clathrin assembles to form a polyhedral lattice that encases a small (~50–100 nm diameter) membrane-bounded vesicle.

Although clathrin can account for more than half of the protein of coated vesicles, other polypeptide species are found in preparations from a wide variety of sources. Especially prominent are families of polypeptides in the regions of 100,000, 55,000, and 33,000 mol wt. It has been difficult to judge whether these are authentic constituents of coated vesicles, for even the best coated vesicle preparations usually are contaminated by other membranes. A more complete purification would also be helpful to determine whether enzyme activities, such as ATPase (8), are truly caused by coated vesicles. In this paper we report a novel step leading to a far more complete purification of coated vesicles, by electrophoresis through an agarose gel.

MATERIALS AND METHODS

Agarose Gel Electrophoresis

Solid agarose, Seakem HGT(P) (Marine Colloids, Rockland, Maine, 04841), was dissolved (usually 0.15% wt/vol, see Fig. 7) in a solution of 50 mM 2-(*N*-morpholino)ethane sulfonic acid (MES) pH 6.5, using microwave radiation to boil the water. Volumes were readjusted after the agarose was totally dissolved. The solution was poured onto a horizontal electrophoresis apparatus (20 × 13.2 cm) identical to that used for electrophoresis of nucleic acids (9). Typically, 200 ml of agarose solution was used. Sample wells were formed in the agarose by placing a plastic comb into the fluid. Electrophoresis was performed at 0.75 V/cm while recirculating the 50 mM MES, pH 6.5 electrophoresis buffer (with a pump) to prevent pH gradients from forming. Larger voltage gradients caused streaking of the sample. Typically, electrophoresis was carried out for 24 h at 5°C.

Visualization of the Sample

The turbidity of as little as 10 μg of vesicles directly revealed their position in the gel. Vesicles could also be stained in the gel with 100 μg/ml of aniline naphthaline sulfonic acid (ANS) for 1 h. After destaining for 30 min, the vesicles were photographed by placing the gel on a horizontal short-wave UV lamp. A yellow filter aided in removing background illumination.

The most sensitive method for localizing the vesicles was by staining the gel with Coomassie Blue. Because of the fragility of the gel, it was best to dry it before staining. To accomplish this, we placed the gel on filter paper, and the filter paper was placed upon paper towels. The paper towels were changed when they became wet. The gel dehydrated to form a thin film of agarose within 30 min. Proteins were stained by immersing the dried gel in 0.025% (wt/vol) Coomassie Blue, 10% (vol/vol) acetic acid, 25% (vol/vol) isopropanol for 10 min. The gel was transferred to 10% acetic acid and 12.5% isopropanol until the background stain was removed. This technique easily revealed 1 μg of protein. The dried gel was also autoradiographed. In addition, proteins were transferred to diazotized paper and antigens were revealed with antibody (10).

To characterize the protein constituents in different regions of the gel, we cut the gel into fractions with a razor blade. The slices were transferred to individual tubes and were heated to 95°C in the presence of 60 mM Tris HCl, pH 6.8, 20% glycerol, 70 mM β-mercaptoethanol, 2% SDS, 0.001% bromphenol blue. This solution was applied to a 10% SDS polyacrylamide gel (11) and electrophoresed. The proteins were detected by Coomassie Blue staining or by autoradiography.

Electron Microscopy of Electrophoresed Particles

Regions of the gel of interest were excised with a razor blade and transferred to a glass Dounce homogenizer (Kontes Co., Vineland, N. J.). The agarose was broken with five strokes and the homogenate was centrifuged at 17,600 *g* for 30

s in an Eppendorf 5412 centrifuge. Typically, 20% of the coated vesicles were in the supernate. A 5-μl aliquot was removed from the supernate and was applied to a Formvar- and carbon-coated copper grid. The excess solution was removed by blotting the grid with filter paper, and the sample was stained with five drops of a 1% solution of uranyl acetate and observed in a Philips EM 300 electron microscope.

Isolation of Coated Vesicles and Vesicular Stomatitis Virus

Coated vesicles were prepared from 1 kg of bovine brains by the method of Pearse (7). Coated vesicles from Chinese hamster ovary (CHO) cells and bovine liver were prepared by variations of Pearse's method.[1] Vesicular stomatitis virus (VSV) was prepared as described by Barenholz et al. (12). Before electrophoresis, samples were centrifuged at 17,600 *g* for 10 s to remove precipitates. If the large aggregates were not removed, they obstructed the origin of the gel and prevented the entry of the smaller particles.

Assay for ATPase in Agarose Gel Fractions

After electrophoretic separation of coated vesicles from vesicle contaminants, the agarose gel was soaked in 50 mM Tris-HCl, pH 7.4, 50 mM KCl, and 0.25 mM EGTA for 1 h at 4°C, changing the solution twice. The gel was sliced in 0.5-cm fractions and assayed directly for ATPase in 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 0.25 mM EGTA, and 2 mM MgATP ([8-^{3}H]ATP, sp act, 30 Ci/mmol, 200 μCi/ml per reaction; Amersham Corp., Arlington Heights, Ill.) in a total volume of 0.50 ml for 3 h at 37°C with vigorous shaking. 1-μl aliquots were removed and applied to prewashed polyethyleneimine-impregnated cellulose strips, for thin-layer chromatography (MN300; Brinkmann Instruments, Inc., Westbury, N. Y.). The 0.6 × 4.5 cm strips had been prespotted with 1 μl of 50 mM ATP, 50 mM ADP as markers visible under UV light. The strips were eluted at room temperature in an ascending solvent containing 1 M formic acid and 0.5 M LiCl. The strips were dried, sectioned into fractions containing the UV-visualized ATP or ADP spots, and counted with 2 ml Aquasol (New England Nuclear, Boston, Mass.).

Assay for Protein and Lipid Phosphorus in Agarose Gel Fractions

After electrophoresis, fractions of interest were cut out of the gel and homogenized in a 1-ml Dounce homogenizer. The volume of the dispersed agarose was measured, and SDS was added to a final concentration of 0.2%. The agarose was melted by heating it to 100°C for 2 min. Measurement of protein was made by directly assaying 25- to 50-μl aliquots of the molten agarose solution in a 500-μl reaction volume by the method of Lowry (13), using bovine serum albumin as a standard. During the assay, the agarose precipitated. This precipitate was removed by a 5-min, 3,000 rpm centrifugation step before measurement of the optical density. Lipid phosphorus was determined by extracting the molten agarose solution with 5 vol of chloroform:methanol (1:2) (14). Rapid mixing by vortexing aided in the extraction. The agarose and proteins formed a precipitate that was removed by centrifugation at 2,000 rpm for 5 min. Then, 1 vol each of chloroform and H_2O were added to the supernate; the resulting mixture was vortexed for 5 min and then centrifuged for 5 min at 2,000 rpm to resolve the two phases. The organic phase was removed, and the aqueous phase was re-extracted with 1 vol of chloroform. The organic phases were combined and the solvent was evaporated by a stream of nitrogen gas. The nonvolatile residue was assayed for phosphorus by the method of McClare (15).

RESULTS

Separation of Coated from Smooth Vesicles

Fig. 1 shows a photograph of an ANS-stained agarose gel of brain coated vesicles, empty baskets, and vesicles derived from coated vesicles. The coated vesicle preparation resolves into two fractions. Reconstituted empty baskets have the same mobility as the faster fraction; clathrin-depleted vesicles have the mobility of the slower fraction. Electron microscopy of the two fractions shows that the faster migrating particles are coated vesicles and empty coats, and that the slower ones are smooth-surfaced vesicles (Fig. 2). When coated vesicles (the fast fraction) were removed from the gel and re-electrophoresed, only a single species, the fast band, was obtained (data not shown). The fact that empty baskets but not vesicles co-migrate with coated vesicles demonstrates that the coat confers the size and charge characteristics that determine the coated vesicle's mobility.

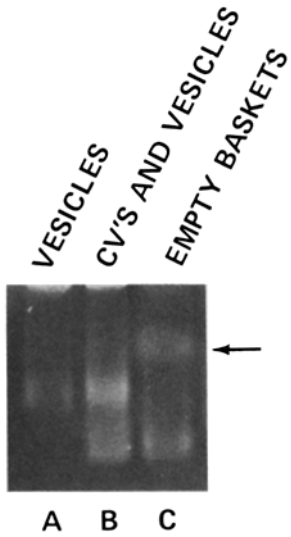

FIGURE 1 Photograph of an ANS-stained gel of bovine brain coated vesicles (*CV's*) (lane *B*), vesicles (lane *A*) and empty baskets (lane *C*) after electrophoresis in 0.15% agarose. All of the samples migrate towards the positive electrode. To obtain the vesicles and empty baskets, we extracted coated vesicles with 2 M urea. The vesicles were separated from the solubilized coated proteins by sedimentation at 130,000 *g* for 15 min in a Beckman airfuge, followed by a similar washing in 2 M urea. The washed vesicles were resuspended in 50 mM MES, pH 6.5. The supernate of the 2 M urea extract, containing the coat proteins, was diluted 10-fold with 50 mM MES, pH 6.5. This allowed the coat proteins to form empty baskets (18). The arrow in lane *C* points to large aggregates of coat proteins that did not properly reform baskets. The origin is at the top of the gel.

Resolution of Coated Vesicles and Coats from Different Sources

Fig. 3 shows that coated vesicles from three sources have different mobilities. The bovine brain coated vesicle band migrates 20% faster than the band of bovine liver and 25% faster than the band of CHO cell coated vesicles. That these bands indeed represent coated vesicles was confirmed by electron microscopy. Empty baskets present in the brain and liver preparations also migrate differently, demonstrating that the coats are different. All of the coated vesicles migrate faster than VSV (Fig. 3) and mouse mammary tumor virus (data not shown).

Fig. 4 shows that electrophoresis can resolve a mixture of coated vesicles from bovine brain and CHO cells. Brain coated vesicles were mixed with a trace of [^{35}S]-methionine-labeled CHO coated vesicles. After electrophoresis of this sample, the agarose gel was cut into fractions, each of which was electrophoresed on an SDS polyacrylamide gel to display the protein composition of each region of the agarose gel. Fig. 4*A* (the

[1] Rubenstein, J. L. R., R. E. Fine, E. J. Patzer, M. D. Snider, and J. E. Rothman. Manuscript submitted for publication.

Coomassie Blue-stained gel) shows that the clathrin of brain coated vesicles is found primarily in fractions 7 and 8. Similarly, the other major groups of brain coated vesicle proteins, 100,000, 55,000, and 33,000, also are found in largest amounts co-electrophoresing with clathrin, confirming that they are authentic constituents. Fig. 4*B* shows the autoradiograph of the same gel seen in Fig. 4*A*. The radioactive proteins, from the CHO coated vesicles (including CHO clathrin), are found predominantly in fractions 6 and 7, displaced one fraction from the brain coated vesicles. Therefore, brain coated vesicles electrophorese faster than CHO coated vesicles, as judged both by fluorescent staining of the agarose gel and by examination of the proteins associated with coated vesicles.

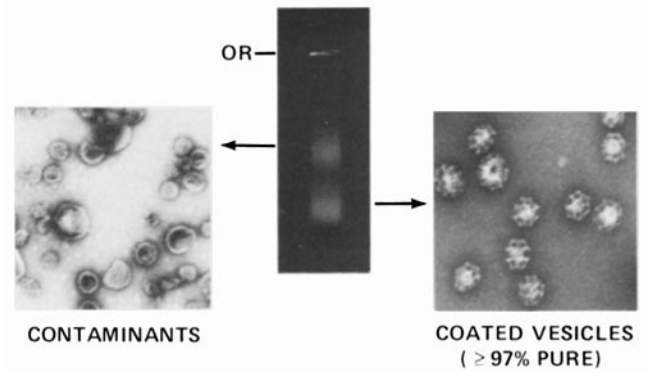

FIGURE 2 Photograph of an ANS-stained gel of bovine brain coated vesicles after electrophoresis in 0.3% agarose. The fast and slow bands were eluted from the gel and examined in the electron microscope. *OR*, origin.

Electrophoretic Preparations are Essentially Homogeneous

Electrophoresis through agarose separates brain coated vesicles from coated vesicles from other tissues, from contaminating smooth vesicles, and from VSV. We quantitated the abso-

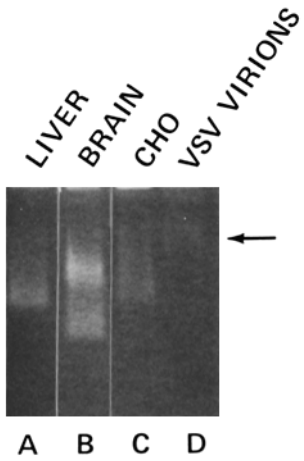

FIGURE 3 Photograph of an ANS-stained gel of coated vesicle preparations from three different sources, after electrophoresis in 0.15% agarose. In each case, the fastest band consisted of coated vesicles. Material electrophoresing more slowly consisted of smooth surface vesicles. Because of the slower mobility of the liver and CHO coated vesicles, longer times of electrophoresis were required to separate these coated vesicles from their contaminants. Lane *D* contains VSV virions. The arrow points to their position in the gel, not easily seen in this reproduction.

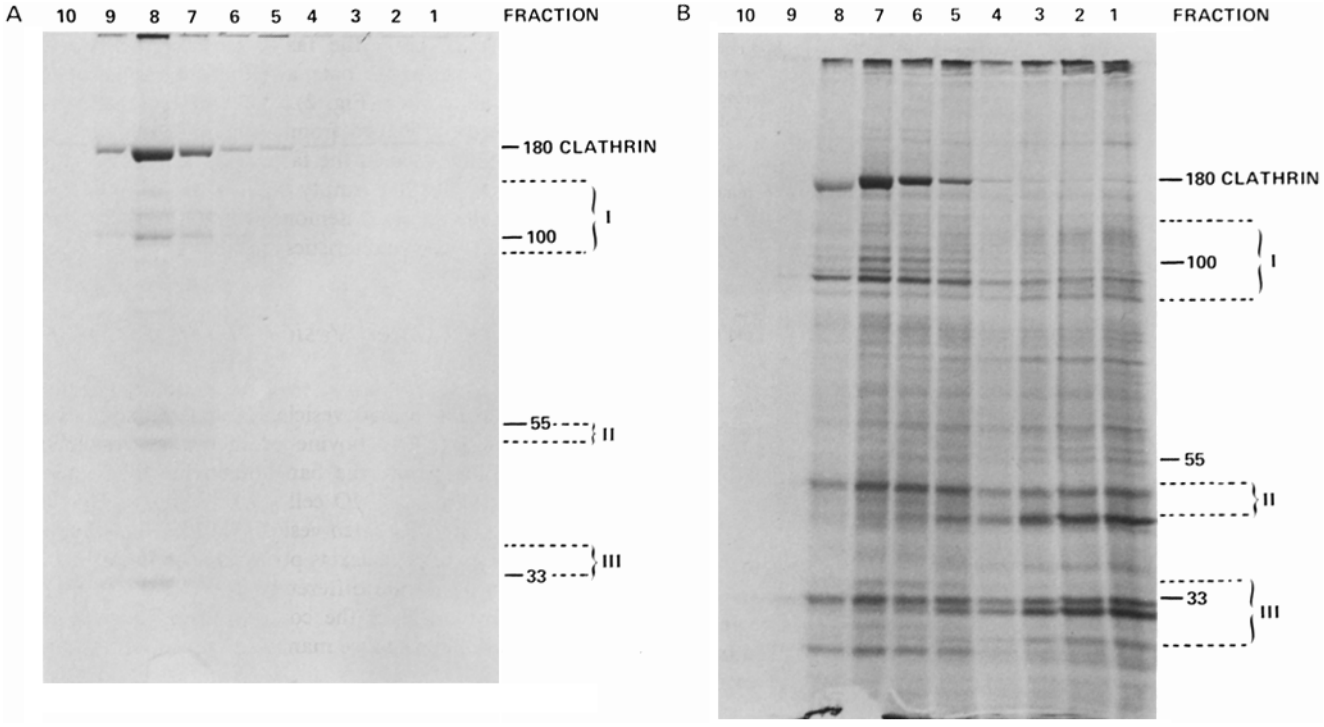

FIGURE 4 Analysis of fractions of an agarose gel by SDS polyacrylamide gel electrophoresis. Coated vesicles from bovine brain and a trace of [^{35}S]methionine-labeled coated vesicles from CHO cells were first co-electrophoresed in a 0.15% agarose gel. Then, slices of the agarose gel were subject to SDS gel electrophoresis. (*A*) Coomassie Blue stained SDS polyacrylamide gel, revealing the distribution in the agarose gel of the polypeptides found in the bovine brain coated vesicle preparation. Fraction 1 includes the origin of the agarose gel. (*B*) Autoradiograph of the same SDS polyacrylamide gel shown in part *A*, revealing the [^{35}S]methionine-labeled proteins derived from the CHO cell coated vesicle preparation.

lute purity of the coated vesicles by electron microscopy and the relative purity by the lipid to protein ratio. The agarose gel-purified bovine brain preparation contained 94% coated vesicles, 4% empty baskets (judged not to contain a vesicle inside the clathrin basket), and 2% vesicles. CHO cell preparations contained 92% coated vesicles, 5% empty basxets, and 3% vesicles. In each case >500 particles were counted. Without the agarose gel, typically only 50–80% of the vesicles were coated. The same results were obtained when the particles were simply adsorbed to grids and when they were quantitatively collected by ultracentrifugation directly on the electron microscope grid (E. J. Patzer and J. E. Rothman, data not shown).

It is also of interest to compare the lipid to protein ratio of the coated vesicles before and after electrophoresis, for this ratio should decrease because of the extensive removal of contaminating smooth vesicles. Before electrophoresis, a brain coated vesicle preparation had 250 nmol lipid phosphorus/mg protein. After electrophoresis, the coated vesicle fraction had a ratio of 150 nmol lipid phosphorus/mg protein, whereas the vesicle fraction had a ratio of 250 nmol lipid phosphorus/mg protein.

ATPase Activity is Caused by Contaminating Smooth Vesicles

The ability to remove this large amount of contaminant smooth vesicles prompted us to ask whether the ATPase associated with cruder brain coated vesicle preparations (8) is still found with the agarose gel-purified coated vesicles. Fig. 5 shows that after electrophoresis the distribution of the ATPase

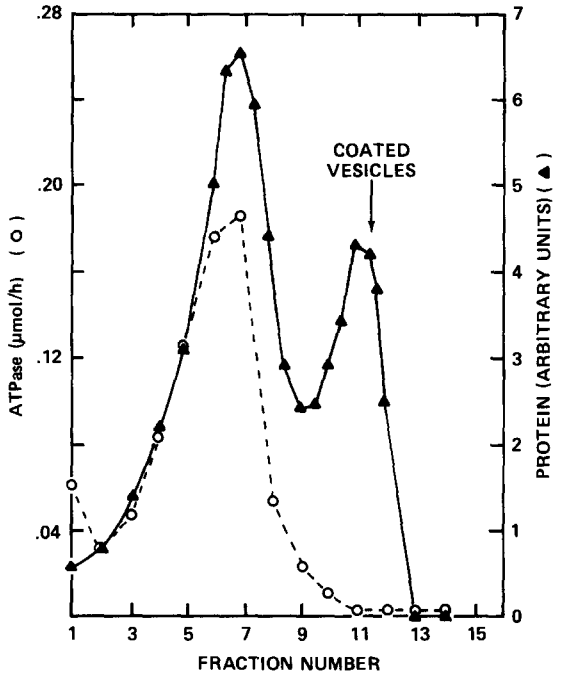

FIGURE 5 The ATPase found in coated vesicle preparations from bovine brain (8) separates from coated vesicles upon electrophoresis in 0.25% agarose. The position of the coated vesicles (arrow) in the agarose gel was determined from the location of clathrin (see Fig. 4). ATPase activity (O) was measured as described in Materials and Methods. The distribution of protein was estimated crudely by a densitometer tracing of the Coomassie Blue-stained agarose gel (▲).

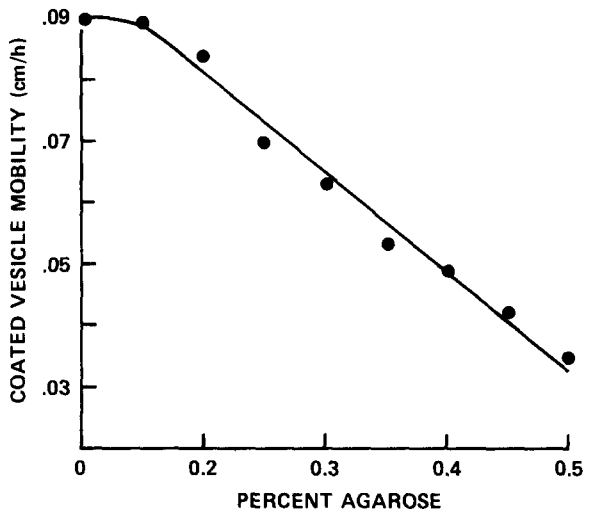

FIGURE 6 Electrophoretic mobility of bovine brain coated vesicles as a function of agarose concentration (wt/vol %). Electrophoresis was performed at 5°C using 0.75 V/cm. The slower migrating vesicle contaminants in the coated vesicle preparation showed a similar decrease in mobility with increasing agarose concentration (data not shown). Finally, the rate of migration was constant with time.

does not overlap with the distribution of brain coated vesicles. This experiment demonstrates that this enzyme is not an integral part of coated vesicles and further underscores the utility of electrophoresis in agarose for identifying the components of pure coated vesicles.

The agarose gel can separate particles on the basis of charge. For example, increasing the concentration of phosphatidylserine from 1 to 30 mol percent in sonicated phosphatidylcholine vesicles increased the mobility in a 0.15% gel 10-fold from 0.4 to 5.0 cm/h at 25°C in an electrical field of 5 V/cm. Fig. 6 shows that decreasing the concentration of agarose from 0.5 to 0.15% increases the mobility of brain coated vesicles threefold. A further decrease to 0.10% does not change the mobility. The decreased mobility as a function of agarose concentration in the 0.15–0.5% range suggests that the rate of migration is determined in part by particle size. Therefore, both the size and charge of particles are important determinants of electrophoretic mobility.

DISCUSSION

We have applied agarose gel electrophoresis to the purification of coated vesicles in which particles are separated according to differences in size and charge. Similar methods have been used in the separation of a wide variety of virions (16, 17). Conventional preparations of coated vesicles contain contaminant smooth vesicles that can be removed by agarose gel electrophoresis (Figs. 1–3). This purification step decreases the lipid to protein ratio from 250 nmol lipid phosphorus/mg protein to 150 nmol lipid phosphorus/mg protein. Both of these values are significantly lower than the value of 410 nmol lipid phosphorus/mg protein, reported by Pearse (7).

It is important to realize that the prevalence of empty coats in coated vesicle preparations can vary considerably (18). It is quite common for empty coats to account for more than half of all coated particles. Therefore, a relatively small fraction of smooth vesicles can account for much if not most of the total membrane material in a preparation. The agarose gel is thus especially useful when coated vesicle membranes are being

studied. For example, this technique revealed that the ATPase activity, assumed to be a part of coated vesicle membranes (8), is actually an impurity (Fig. 5).

Despite the significant purification obtained, the families of 100,000-, 55,000-, and 33,000-dalton proteins are still retained in coated vesicles, providing evidence that these are indeed authentic constituents. Coated vesicles from different tissues of the same animal migrate differently (Fig. 3), and this difference is entirely on account of their coats, because the empty baskets co-electrophorese with complete coated vesicles (Fig. 1), and reconstituted empty baskets from brain and liver coated vesicles migrate differently (data not shown). The different mobilities could be caused by size and/or charge variation among coat types.

The agarose gel procedure should be useful in a variety of circumstances that require the separation of small membranes (<200 nm diameter), virions, and coated vesicles for preparative or analytical purposes. For coated vesicles, the best separations are achieved when agarose concentrations of ~0.15 to 0.3% are employed. The major limitation of the gel as a preparative step is the difficulty in eluting particles from the agarose. For coated vesicles, only ~20% of the protein is eluted from the gel. However, the agarose gel can also be employed as a powerful analytical tool (as in Figs. 1–5) under circumstances not requiring elution of native particles. When the gel is dissolved, extracted, assayed, or autoradiographed, the recovery of material is far more complete. One can anticipate that the gel will prove most useful as an analytical procedure.

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REFERENCES

1. Roth, T. F., J. A. Cutting, and S. B. Atlas. 1976. Protein transport: a selective membrane mechanism. *J. Supramol. Struct.* 4:527–548.
2. Goldstein, J. L., R. G. W. Anderson, and M. Brown. 1979. Coated pits, coated vesicles, and receptor mediated endocytosis. *Nature (Lond.).* 279:679–684.
3. Rothman, J. E., and R. E. Fine. 1980. Coated vesicles transport newly synthesized membrane glycoproteins from the endoplasmic reticulum to the plasma membrane in two successive stages. *Proc. Natl. Acad. Sci. U. S. A.* 77:780–784.
4. Franke, W. W., M. R. Luder, J. Kartenbeck, H. Zorban, and T. W. Kennan. 1976. Involvement of vesicle coat material in casein secretion and surface regeneration. *J. Cell Biol.* 69:173–195.
5. Jamieson, J. D., and G. E. Palade. 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. *J. Cell Biol.* 34:577–596.
6. 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.
7. Pearse, B. M. F. 1975. Coated vesicles from pig brain: purification and biochemical characterization. *J. Mol. Biol.* 97:93–98.
8. 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.
9. McDonell, M. W., M. N. Simon, and F. W. Studier. 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *J. Mol. Biol.* 110:119–146.
10. Renart, J., J. Reiser, and G. R. Stark. 1979. Transfer of proteins from gels to diazo benyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proc. Natl. Acad. Sci. U. S. A.* 76:3316–3120.
11. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
12. Barenholz, Y., N. F. Moore, and R. R. Wagner. 1976. Enveloped viruses as model membrane systems: microviscosity of vesicular stomatitis virus and host cell membranes. *Biochemistry.* 15:3563–3570.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
14. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.
15. McClare, C. W. F. 1971. An accurate and convenient organic phosphorus assay. *Anal. Biochem.* 39:527–530.
16. Pfankuch, E. 1940. Über die Spaltung von Virusproteinen der Tabakmosaik-Gruppe. *Biochem. Z.* 306:125–129.
17. Childs, J. D., and H. C. Birnboim. 1975. Polyacrylamide gel electrophoresis of intact bacteriophage T4D particles. *J. Virol.* 16:652–661.
18. Woodward, M. P., and T. F. Roth. 1978. Coated vesicles: characterization, selective dissociation, and reassembly. *Proc. Natl. Acad. Sci. U. S. A.* 75:4394–4398.