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 lysosomes (6).

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.

**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 x 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 naphthalene 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.

**Rapid Communications**

**Electron Microscopy of Electrophoresed Particles**

Regions of the gel of interest were excised 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.
s in an Eppendorf 5412 centrifuge. Typically, 20\% of the coated vesicles were in the supernate. A 5-\mu l 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. Vesicular stomatitis virus (VSV) was prepared as described by Barenholz et al. (12). Before electrophoresis, samples were centrifuged at 17,000 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 ([\(\beta\)-\(\gamma\)]-ATP, sp act, 30 Ci/mmol, 200 \(\mu\)Ci/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-\mu l aliquots were removed and applied to prewashed polyethyleneimine-imregnated cellulose strips, for thin-layer chromatography (MN300; Brinkmann Instruments, Inc., Westbury, N. Y.). The 0.6 x 4.5 cm strips had been prespotted with 1 \(\mu\)l of 50 mM ATP, 50 mM ADP as markers visible under UV light. The strips were eluted by a 5-min, 3,000 rpm centrifugation step before measurement of the optical density. Lipid phosphorus was determined by extracting the molten agarose by a stream of nitrogen gas. The nonvolatile residue was assayed for phosphorus and the optical density at 600 nm was subtracted as a blank. 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.

Aspar 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-mi Dounce homogenizer. The volume of the dispersed agarose was measured, and SDS was added to a final concentration of 0.2\%. The agarose gel was melted by heating it to 100\°C for 2 min. Measurement of protein was made by directly assaying 25- to 50-\mu l aliquots of the molten agarose solution in a 500-\mu l 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 centrifugation 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 (16). 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).

Resolution of Coated Vesicles and Coats from Different Sources

Fig. 4 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).

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.

\[\text{Rubenstein, J. L. R., R. E. Fine, E. J. Patzer, M. D. Snider, and J. E. Rothman. Manuscript submitted for publication.}\]
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. 4B shows the autoradiograph of the same gel seen in Fig. 4A. 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.

**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 [35S]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 [35S]methionine-labeled proteins derived from the CHO cell coated vesicle preparation.

**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-
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 prepa-
rations contained 92% coated vesicles, 5% empty baskets, 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 micro-
scope 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 asso-
ciated 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
does not overlap with the distribution of brain coated vesicles.
This experiment demonstrates that this enzyme is not an inte-
gral 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 phosphatidylser-
ine 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
does not overlap with the distribution of brain coated vesicles.
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). Conven-
tional preparations of coated vesicles contain contaminant
smooth vesicles that can be removed by agarose gel electro-
phoresis (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 phos-
phorus/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

**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). Conven-
tional preparations of coated vesicles contain contaminant
smooth vesicles that can be removed by agarose gel electro-
phoresis (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 phos-
phorus/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

---

**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
(A).

**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.
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.

We thank Eric Patzer and Alan Matsumoto for their suggestions and aid in the preparation of this paper, and Debra Young for the typing of the manuscript.

This work was supported by National Institutes of Health grants GM 25662-03 to J. E. Rothman, NS10582-8 to R. E. Fine, and GM-07365 to J. L. R. Rubenstein.

Dr. Fine was on sabbatical leave from the Department of Biochemistry, Boston University School of Medicine (Boston, Mass.).

Received for publication 1 December 1980, and in revised form 19 January 1981.

REFERENCES
1. Roth, T. F., J. A. Cutting, and S. B. Atlas. 1976. Protein transport: a selective membrane mechanism. J. Supramol. Struct. 4:327–548.
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. Lader, J. Kartenbeck, H. Zorban, and T. W. Kennan. 1976. Involvement of vesicle coat material in casein secretion and surface regeneration. J. Cell Biol. 79:133–195.
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. Nearey, B. M. F. 1975. Coated vesicles from pig brain: purification and biochemical characterization. J. Cell Biol. 79:95–98.
8. Blitz, A. L., R. E. Fine, and P. A. Tossell. 1977. Evidence that coated vesicles isolated from brain are calcium-sequestering organelles resembling sarcoplasmic reticulum. J. Cell Biol. 75:132-147.
9. McDonnell, 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. 110:141–146.
10. Renart, J., J. Reiser, and G. R. Stark. 1979. Transfer of proteins from gels to diazo benzoxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. Proc. Natl. Acad. Sci. U. S. A. 76:3126–3130.
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. 39:227–330.
16. Pfankuch, E. 1940. Uber 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. Biol. Chem. 250:459–460.
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.