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Experimental basis for separation of membrane vesicles by preparative free-flow electrophoresis

D. James Morré*[,a], James Lawrence[a], Keri Safranski[a], Timothy Hammond[b], Dorothy M. Morré[c]

[a]*Department of Medicinal Chemistry, Purdue University, West Lafayette, IN 47907, USA*
[b]*Department of Medicine, University of Wisconsin Medical School, Madison, WI 53792, USA*
[c]*Department of Foods and Nutrition, Purdue University, West Lafayette, IN 47907, USA*

Abstract

In practice it has been possible to separate membrane particles of different origins but of similar chemical composition by preparative free-flow electrophoresis. Examples include the vacuolar (tonoplast) and plasma membranes of plants and membranes derived from the *cis* and *trans* regions of the rat liver Golgi apparatus. Yet, when analyzed for intrinsic molecules that might contribute to significant differences in surface charge, the separated membranes were surprisingly similar. As more information was generated, it became apparent that the membranes with greatest electrophoretic mobility (*i.e.* lysosomes, rightside-out tonoplast vesicles and membranes from the trans region of the Golgi apparatus), where those membranes with an inherent ability to acidify their interiors. By so doing, the vesicles generate a membrane potential, negative outside, which might serve as a basis for enhanced electrophoretic mobility. To test the hypothesis, tonoplast membranes were incubated with ATP to drive proton import or with monensin to dissipate the ATP-supported proton gradient. With ATP, mobility was enhanced. Also, when ATP-treated vesicles were analyzed in the presence of monensin, the ATP effect on mobility was reversed. Similarly with Golgi apparatus, mobility of the most electrophoretically mobile portions of the separation was enhanced by ATP and the ATP effect was reversed with monensin. A *trans* origin of the vesicles was verified by assay of the *trans* Golgi apparatus marker, thiamine pyrophosphatase. Finally, incubation with ATP (and reversal by monensin) was employed as an aid to the free-flow electrophoretic separation of kidney endosomes from complex mixtures. These lysosomal derivatives also are capable of acidification of their interiors in an ATP-dependent process and of generating, at the same time, a negative (outside) membrane potential. The findings provide both an experimental basis to enhance membrane separations by preparative free-flow electrophoresis and, at the same time, a theoretical basis to help explain why certain membranes of very similar overall chemical composition may be separated by electrophoretic methods.

1. Introduction

The techniques of preparative free-flow electrophoresis have been employed to separate a variety of cell types, membrane vesicles and macromolecules based on differential electrophoretic mobility in buffered solutions [1,2]. In this technique, the material to be separated is injected as a fine stream into a solution which is flowing perpendicular to the lines of force of an electric field (Fig. 1). Electrically charged particles are deflected from the direction of flow at an angle determined by a combination of flow

* Corresponding author.

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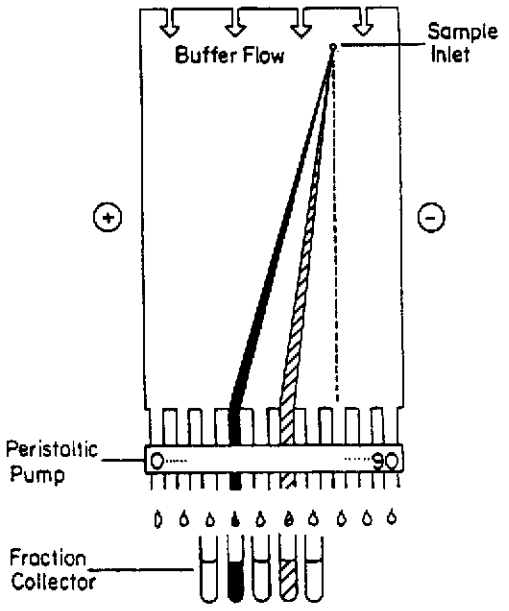

Fig. 1. Principles of preparative free-flow electrophoresis. From Morré *et al.* [16].

velocity and the electrophoretic mobility of the particles. Components with different electrophoretic mobilities move to different extents and are collected separately in a fraction collector after leaving the separation chamber.

Aqueous two-phase partition also has been successfully employed to fractionate membranes [3,4]. However, for the most part, these applications have been primarily to isolate highly purified plasma membranes [5–8], to subfractionate purified plasma membrane into rightside-out and inside-out vesicles [9], or to remove plasma membrane contaminants during the purification of internal membranes [10, 11]. However, with important exceptions using countercurrent distribution [12,13], there has been little progress in the separation of internal membranes by aqueous two-phase partition.

In contrast, preparative free-flow electrophoresis does provide for additional separations. Especially successful have been applications to the separation of vacuole (tonoplast) and plasma membranes of plants [14,15], Golgi apparatus subfractionations [16,17], isolation of endosomes [18,19] and isolation of transport vesicles induced with ATP to bud from transitional endoplasmic reticulum of rat liver [20]. Despite much success in effecting membrane separations, the molecular basis of free-flow electrophoretic separations of the type described above remain unclear.

Normally, intrinsic electrophoretic mobility of cells or membrane vesicles is a function of charged molecules exposed at the cell or vesicle surface. However, it has not been possible to relate electrophoretic separations of internal membranes to the content of charged surface molecules. Lysosomes, which are the most electrophoretically mobile of all internal membranes [21], are much less richly endowed with charged surface molecules than rightside-out plasma membrane vesicles regarded to be extensively coated with sialic acid-rich, anionic glycoprotein chains. Also the Golgi apparatus, which is a polarized structure consisting of *cis* and *trans* elements, can be unstacked and the component cisternae resolved into *cis*-derived fractions of low electrophoretic mobility, *trans*-derived fractions of greatest electrophoretic mobility and a medial fraction of intermediate electrophoretic mobility. However, a chemical gradient of externally exposed charged molecules to explain the differences in electrophoretic mobility has not been detected. There is a *cis*-to-*trans* gradient of sialic acid [16] but this sialic acid should be present at the inside surface of the sealed membrane vesicles and unavailable to influence electrophoretic mobility.

Even more difficult to understand is the basis for the tonoplast-plasma membrane separation of higher plant homogenates. Neither membrane appears to have an abundance of uniquely charged macromolecules at its surface. Sialic acid is absent and even their phospholipid compositions are similar [22]. Yet tonoplast membranes and plasma membranes from plants are readily separated by preparative free-flow electrophoresis.

The relationships described above for free-flow electrophoretic separation of membrane

vesicles are summarized in Fig. 2. What became evident was a correlation between electrophoretic mobility and the ability to accumulate protons. Those electrophoretic components endowed with the greatest electrophoretic mobility were membrane vesicles characterized by ATP-driven proton pumps capable of acidification of their interiors. Such vesicles may develop internal pH values approaching 5.0. These include lysosomes [23], tonoplast vesicles [24–26] and *trans* Golgi apparatus cisternae [27–29]. In parallel to acidification, the vesicles will develop an outside negative diffusion potential. In this report, we provide evidence for a pH-gradient-induced diffusion potential as a major contributing factor to membrane separation by preparative free-flow electrophoresis not based on intrinsic ionizable groups at the cell surface.

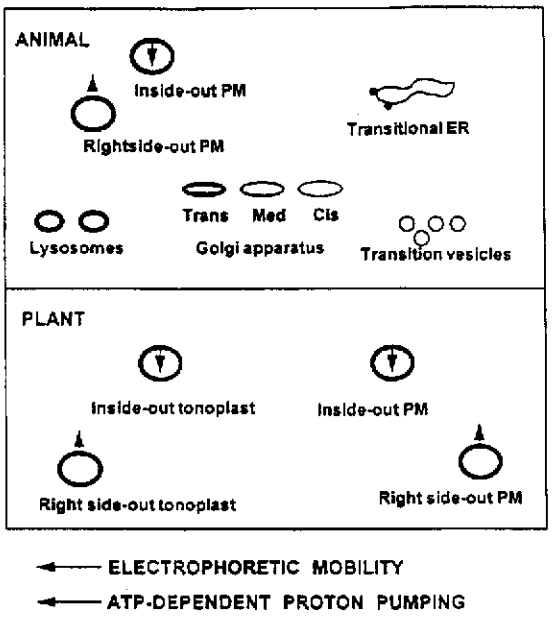

Fig. 2. Summary of particles separated by preparative free-flow electrophoresis showing relative mobility on the ordinate. The correlation is between electrophoretic mobility and the capacity for ATP-dependent inward proton pumping (acidification of vesicle interiors) with a concomitant generation of a membrane potential, negative outside, is indicated.

2. Materials and methods

2.1. Isolation of plant membranes

Seeds of soybean (*Glycine max* L. Merr. var. Williams) were soaked in tap water 4 to 6 h, planted in moist vermiculite, and grown 4 to 5 days in darkness. Segments of 2 cm long, cut from the hypocotyl 5 mm below the cotyledons, were harvested under normal laboratory conditions and used for isolation of membranes. The hypocotyl segments (25 g) were homogenized in 100 ml of a medium containing 25 m*M* Tris–2-(N-morpholino)ethanesulphonic acid (MES) (pH 7.5), 300 m*M* sucrose, 10 m*M* KCl and 1 m*M* $MgCl_2$ using a blender. After filtration through one layer of Miracloth (Chicopee Mills, NY), the filtrate was centrifuged for 10 min at 6000 g_{max} (6000 rpm, Sorvall, HB-4 rotor), and the pellet was discarded. The resulting supernatant was centrifuged for 30 min at 40 000 *g* (Beckman, SW-28 rotor), and the supernatant was discarded. The 40 000-*g* pellets were resuspended in electrophoresis chamber buffer (see below) and centrifuged for 30 min at 40 000 *g*. The final pellets again were resuspended in electrophoresis chamber buffer using about 1 ml per 10 g starting fresh mass of hypocotyl segments. For plant membranes, 10 μM $CaCl_2$ was added to the electrophoresis medium and the pH was 7.5.

2.2. Preparative free-flow electrophoresis

Preparative free-flow electrophoresis was performed on a VAP-22 free-flow electrophoresis device (Bender & Hobein, Munich, Germany). The electrophoresis medium contained 10 m*M* triethanolamine, 10 m*M* acetic acid, 5 m*M* glucose, 0.25 *M* sucrose and 0.5 m*M* $MgCl_2$, final pH 6.5 (1 *M* sodium hydroxide), osmolality 270 mosm, conductivity $5.9 \cdot 10^2$ μS. The conditions for the electrophoresis were: 167 mA, 131 ± 10% V/cm, buffer flow 2.75 ml/h per fraction, sample injection 3.5 ml/h, temperature 6°C. Absorption at 280 nm was determined on collected fractions. Each fraction was pelleted

for 20 min at 20 000 g, and resuspended in 30 m*M* mannitol, 12 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), pH 7.4 (1 *M* Tris). Aliquots were kept for flow cytometry analysis and electron microscopy.

2.3. Assay of thiamine pyrophosphatase

The assays contained 50 m*M* Tris–HCl (pH 8.0), 15 m*M* $CaCl_2$, 3 m*M* thiamine pyrophosphate and 50 to 100 μg protein, in a total volume of 1 ml [30]. Incubations were at 37°C for 30 min and the reaction was terminated with cold 10% trichloroacetic acid. Inorganic phosphate was determined by the method of Harris [31].

2.4. Flow cytometry analysis

Flow cytometry analysis was performed on a Becton Dickinson FACStar Plus flow cytometer interfaced to a VAX 40 computer [32]. Flow cytometry conditions optimized to single molecule detection [33] were utilized as described for small particle resolutions [34,35]. All flow cytometry was in the presence of antifluorescein antibodies to quench fluorescence from extravesicular fluorescein.

2.5. Preparation of renal cortical endosomes

Renal cortical endosomes were prepared by differential Percoll gradient centrifugation [32,36,37]. Prior to harvesting kidneys the endosomal pathway was labeled by administering 100 mg of either non-fluorescent or fluorescein-conjugated dextran (10 000S; Sigma, St. Louis, MO, USA) intravenously. The dextran was small enough to be rapidly filtered through the renal glomeruli and taken up by endocytosis in the renal proximal tubule [38].

2.6. Enrichment of endosomes prior to free-flow electrophoresis by aqueous two-phase partition

A single 5.8% (w/w) aqueous two-phase partition of the endosomal fraction was performed [7,32]. The basic procedure was as described below for incremental aqueous two-phase partition.

2.7. Incremental aqueous two-phase partition fractionation of rat kidney endosomes

For incremental aqueous two-phase partitioning, stock solutions of 20% (w/w) dextran T-500 (Pharmacia) and 40% (w/w) poly(ethylene glycol) PEG 3350 (Union Carbide) were prepared in double-distilled water. For example, the 6.0% (w/w) two-phase system contained 4.80 g of 20% dextran and 2.40 g of 40% PEG in 30-ml Pyrex tubes on ice, after which were added 0.32 ml of 0.2 *M* potassium phosphate at pH 7.2. The total mass of the contents then was adjusted to 14 g with distilled water. Other mass ratios were prepared by varying the amounts of dextran and PEG added to the system, as described [7]. Membranes to be separated were resuspended in buffer, (total volume < 2 ml), added to the two-phase system, and the final mass of the contents was adjusted to 16 g with buffer. The Pyrex tube was covered with Parafilm, and then inverted vigorously 40 times in the cold (9°C). The resultant mixture was centrifuged at 1000 *g* for 5 min at 4°C to separate the phases. The upper phase was removed using a Pasteur pipette and diluted 10-fold with buffer. Membranes were collected from the upper phase by centrifugation (20 000 *g* for 30 min).

2.8. Incubations with ATP and ionophore

To induce negative diffusion potentials, preparations were incubated for 15 min at room temperature in the presence of 1 m*M* ATP contained in 2.5 m*M* Tris–MES (pH 7.0), 50 m*M* chloride (KCl or NaCl), 3 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol (DTT), and 75 to 100 μg protein in a total volume of 1.5 ml, combined with preparative free-flow electrophoresis chamber buffer [10 m*M* triethanolamine–acetic acid (pH 6.5) and 0.25 *M* sucrose]. For their dissipation, 1 μM monensin (final concentration) was added to the chamber buffer during free-flow electrophoresis.

2.9. Electron microscopy

Samples were fixed for electron microscopy with 2% glutaraldehyde in phosphate buffered saline. The samples were then transferred to 1% osmium tetroxide in 0.05 *M* sodium phosphate (pH 7.2) for several hours, the samples were dehydrated in an acetone series followed by embedding in Epon [6,7]. Lead-stained thin sections were examined and photographed using a Philips EM/200 electron microscope.

3. Results

When plant homogenates were incubated with ATP, the mobility of the tonoplast fractions was greatly enhanced (Fig. 3). Plant tonoplast vesicles isolated by preparative free-flow electrophoresis were active in acidification of their interiors and this acidification was ATP-dependent [39]. A shift also was noted in the plasma membrane portion of the separation (fractions 45–50).

Purified tonoplast fractions were analyzed under conditions similar to those of Fig. 3 where the vesicles carried a negative diffusion potential outward. Two distinct populations of vesicles were observed (Fig. 4). The tonoplast fraction having the greatest electrophoretic mobility (T_A) was identified as cytoplasmic side-out (rightside-out) based on the orientation of the stalked ATPase particles at the vesicle surface. To determine ATPase orientation, the freshly-isolated membrane vesicles were contrasted directly on electron microscope grids with phosphotungstic acid (negative staining) [39]. With T_A fractions, the stalked ATPase particles were on the external surface of the vesicles. The vesicles of the tonoplast fractions having the lower electrophoretic mobilities (T_B) also contained stalked ATPase particles on their surfaces but with the

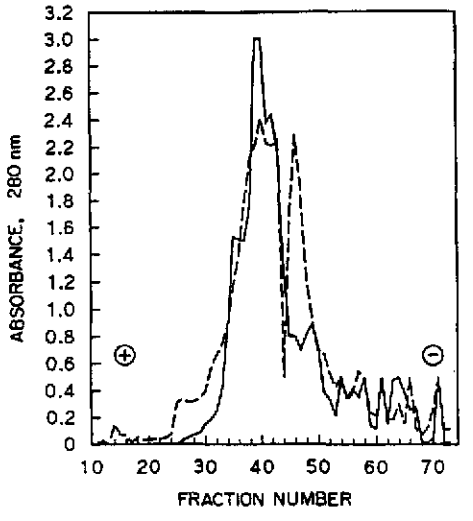

Fig. 3. Free flow electrophoresis separation of a homogenate of plant stems (dark-grown soybean) incubated (---) or not incubated (——) in the presence of ATP. After incubation with ATP, the mobility of the anodel fractions (fractions 22–32) representing tonoplast is increased. Also altered in mobility are fractions 45–50, probably representing plasma membranes. Addition of 1 μM monensin to the electrophoresis chamber buffer after ATP incubation resulted in a loss of the increased mobility of the anodal fractions (not shown).

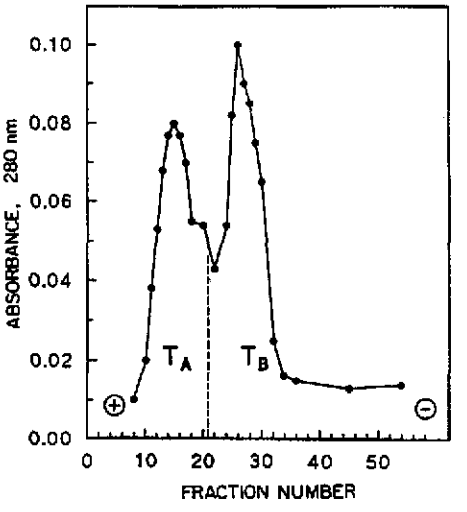

Fig. 4. Electrophoretic separation of purified tonoplast fractions as in Fig. 3 except that the tonoplast was first separated from other membranes of the homogenate as described [39]. The identity of the fraction as tonoplast was verified from analysis of marker enzymes. Two populations of vesicles were resolved. Tonoplast fraction A (T_A) was characterized by stalked ATPase particles on the outside of the vesicle and therefore rightside- (cytoplasmic side-)out [39]. Tonoplast fraction B (T_B) also contained vesicles with stalked ATPase particles but the particles were now on the inside of the vesicle [39]. This population of tonoplast vesicles therefore was inside-out or cytoplasmic side-in.

T_B vesicles the stalked ATPase particles were on the vesicle interiors [39]. This arrangement of ATPase particles identified the tonoplast vesicles of the T_B fraction as being inside-out or cytoplasmic side-in.

A response to ATP was seen as well with Golgi apparatus subfractions. The Golgi apparatus normally distributed over about 10 electrophoretic fractions (Fig. 5). Also correlated with the *cis*-to-*trans* gradient of rat liver Golgi apparatus membranes was the ability to acidify the vesicle interiors [29] in an ATP-dependent manner.

If rat liver Golgi apparatus fractions were incubated with ATP prior to and during electrophoresis (Fig. 6), the separation based on A_{280} was increased from 10 to about 15 fractions. This increase was due to an increased electrophoretic mobility of *trans*-derived Golgi apparatus as shown by measurements of the *trans* Golgi apparatus marker, thiamine pyrophosphatase (Fig. 7). With both A_{280} measurements (Fig. 6) and measurement of thiamine pyrophosphatase (Fig. 8), the mobility shift induced by ATP was reversed by treatment with the ionophore monensin.

The utility of the ATP-induced shift in electro-

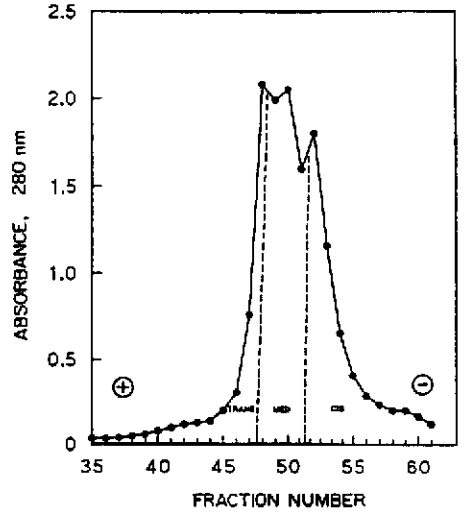

Fig. 5. Preparative free-flow electrophoretic profile depicting a typical Golgi apparatus separation. Values are based on $A_{280\,nm}$. The separations are normally divided for analysis into three regions representing membranes of derivation from the *cis*, medial (MED) and *trans* regions of the Golgi apparatus as indicated.

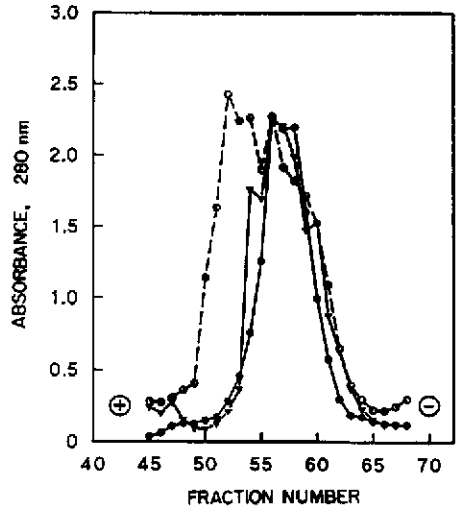

Fig. 6. Preparative free-flow electrophoresis separation of rat liver Golgi apparatus without (●) and with (○) ATP. Total material was correlated with A_{280} as verified by determination of total protein for each fraction (not shown). The effects on material in the electrophoretic fractions of high mobility induced by ATP were reversed by the addition of monensin (▼).

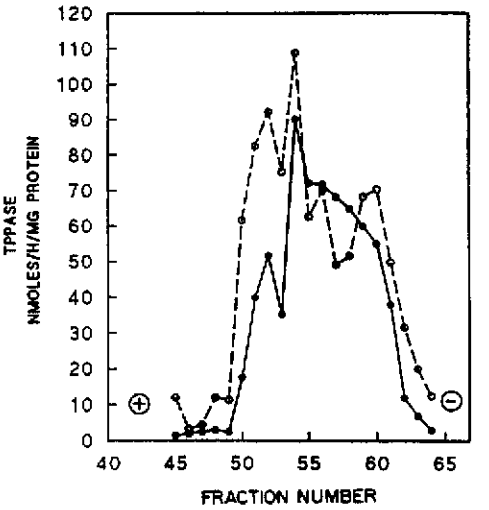

Fig. 7. As in Fig. 6 except a comparison of the specific activity of the trans Golgi apparatus marker thiamine pyrophosphate without (●) and with (○) ATP.

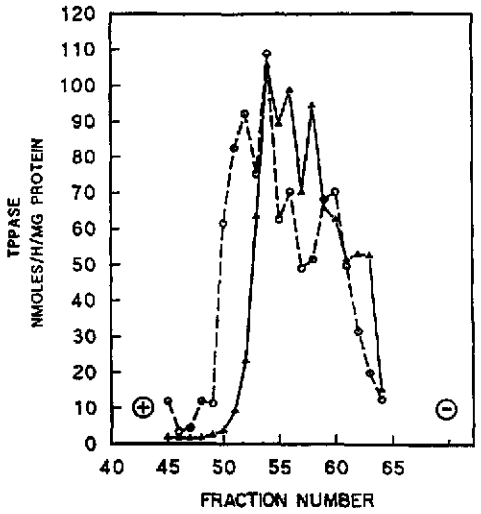

Fig. 8. As in Fig. 7 except a comparison of the electrophoretic mobility with ATP (○) and after the addition of 1 μM monensin during electrophoresis (▲).

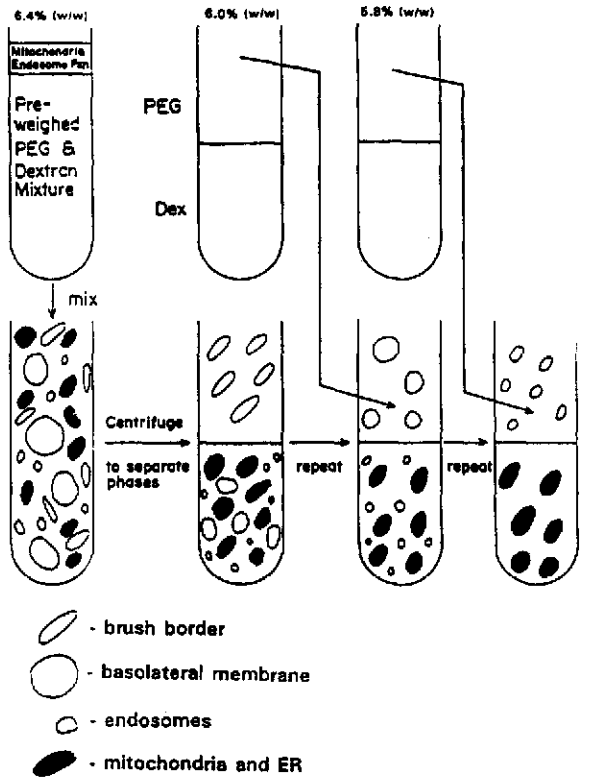

Fig. 9. Results of the incremental two-phase partition for the resolution of different plasma membrane domains and a partially purified endosome fraction from a rat kidney particulate fraction. The initial partition used a 6.4% PEG–dextran phase system. Brush border membranes partitioned into the upper phase. A 6.0% phase system was equilibrated in parallel and the upper phase of this phase system replaced the brush border-containing upper phase of the 6.4% system. After mixing and resolution of the two new phases, the upper phase now contained the remainder of the plasma membrane including basolateral-derived vesicles. A 5.8% phase system also was equilibrated in parallel and the upper phase replaced the plasma membrane-containing upper phase of the second partition. After mixing and resolution into two phases, a third upper phase enriched in endosomes and depleted in plasma membrane was obtained. The fraction, however, was still contaminated by mitochondrial fragments (Fig. 10C). The bulk of the mitochondria, endoplasmic reticulum and nuclei remained in the lower phase after the last partitioning step. Fxn = Fraction.

phoretic mobility in organelle isolation was illustrated by an additional example involving rat kidney endosomes. For these studies, the starting material was a crude endosome preparation obtained by percoll gradient centrifugation as described [34,36,37]. When analyzed by preparative free-flow electrophoresis, these fractions were shown to be mixtures only partially resolved by the electrophoretic method (not shown). The relative distribution of membranes in these studies was determined by electron microscope morphometry.

To remove brush border membranes, the percoll gradient-purified endosomes were first partitioned using a 6.4% dextran–PEG phase system followed by incremental aqueous two-phase partition as described in section 2. The results are summarized diagramatically in Fig. 9 and representative electron micrographs are shown in Fig. 10.

Brush border membranes were concentrated in the upper phase of the initial 6.4% dextran–PEG phase separation (Fig. 10A). The lower phase still contained basolateral plasma membranes, endosomes, mitochondria and endoplasmic reticulum (ER) (Fig. 9). An upper phase equilibrated from a 6.0% dextran–PEG phase system extracted the basolateral plasma membranes (Fig. 9, Fig. 10B). An upper phase equilibrated from a 5.8% dextran–PEG phase system extracted the endosomes in a third partitioning step (Fig. 9, Fig. 10C). The bulk of the

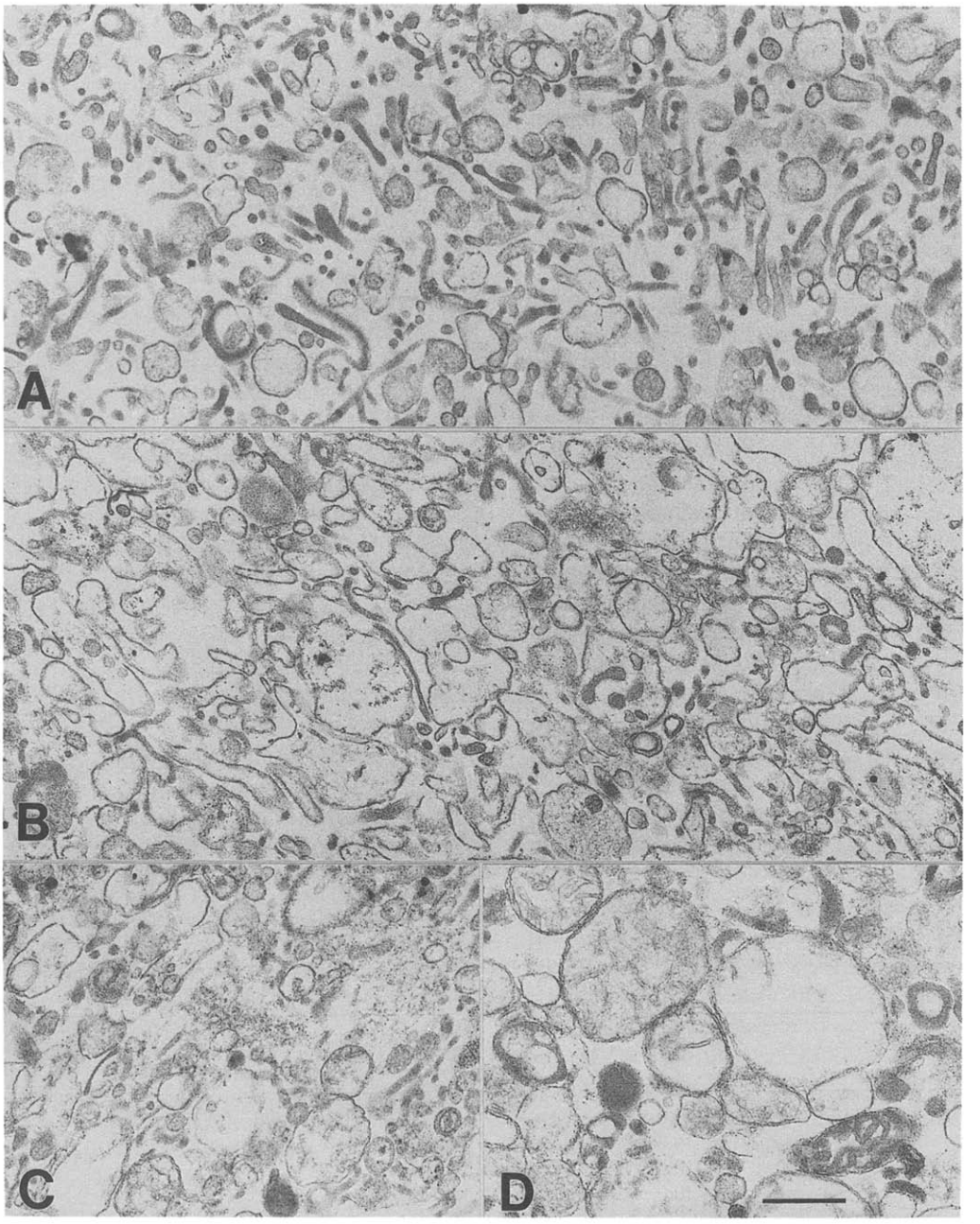

Fig. 10. Electron micrographs of rat kidney membrane fractions obtained by incremental aqueous two-phase separation diagrammed in Fig. 9. (A) Brush border-enriched fraction. (B) Basolateral plasma membrane-enriched fraction. (C) Endosome-enriched fraction. (D) Final lower phase containing mitochondria, nuclear envelope, endoplasmic reticulum and other membrane fragments. Scale bar = 0.5 μm.

mitochondria, endoplasmic reticulum, nuclei and other internal membranes were retained in the lower phase (Fig. 9, Fig. 10D).

The endosome fractions prepared by aqueous two-phase partition, while enriched in endosomes, were still heavily contaminated by mitochondria and/or mitochondrial fragments (Fig. 10C). To further purify endosomes, the pH shift technique was employed (Fig. 11). As shown in Fig. 12A, the particles concentrated in the region of highest electrophoretic mobility induced by ATP treatment, were primarily a population of endosomes or endosome-like vesicles. This region of high electrophoretic mobility was collapsed by the ionophore monensin (Fig. 11) to confirm that the ATP-induced mobility shift was related to proton uptake and concomitant endosome acidification.

The trailing portion of the separation unaffected by ATP or monensin consisted primarily of mitochondria and mitochondrial fragments (Fig. 12B). Also present were other vesicles, some of which might represent early endosomes still incapable of generating a significant proton gradient in response to ATP.

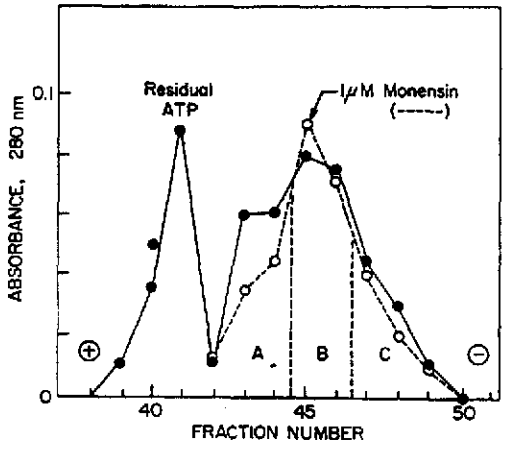

Fig. 11. Free-flow electrophoretic purification of endosomes prepared by aqueous two-phase partition using the ATP shift technique. In the absence of ATP, the distribution of components was symmetrical and mitochondria and endosomes were incompletely resolved (not shown). When equal portions of the preparations were incubated with ATP in the absence or presence of 1 μM monensin an ATP-induced region of particles with increased electrophoretic mobility was observed.

To demonstrate that the pH shift was exhibited by authentic endosomes in the preparations rather than endosome-like vesicles of some other origin, kidney endosomes loaded with FITC (fluorescein isothiocyanate)–dextran were purified by flow cytometry based solely on the criterion of having participated in an endocytic event [40]. When subjected to electrophoretic analysis, these highly purified endosomes also exhibited an enhanced electrophoretic mobility when incubated with ATP (Fig. 13).

4. Discussion

In order to improve free-flow electrophoretic separations of membranes and to provide some basis for the rational design of buffering systems, it would be helpful to know the theoretical basis for particle migration in solution in response to a particular electric field strength.

From our experience and that of others [2], the electrophoretic mobility is largely independent of size and shape of membrane particles within the range of sedimentable particles found in cell or tissue homogenates. Nor does the electrophoretic mobility of a particle in buffered solution correlate exactly with the content of negatively charged surface molecules [14]. A case in point is the widely different electrophoretic mobilities of vesicles derived from vacuoles (tonoplast vesicles) and from the plasma membrane of plants. The two types of membranes are compositionally very similar [22] and have similar surface charges [42]. Yet, they are readily separated by preparative free-flow electrophoresis with tonoplast vesicles having the greater electrophoretic mobility. A difference in mobility is given by vesicles having both inside-out and rightside-out orientations.

In ongoing studies, we have determined that the common parameter among free-flow electrophoretic separations may not be due entirely to charged molecules at the vesicle surface but to the ability of the vesicles to create an (outside) negative diffusion potential as well. This is probably why we are able to separate such markedly similar membranes such as tonoplast

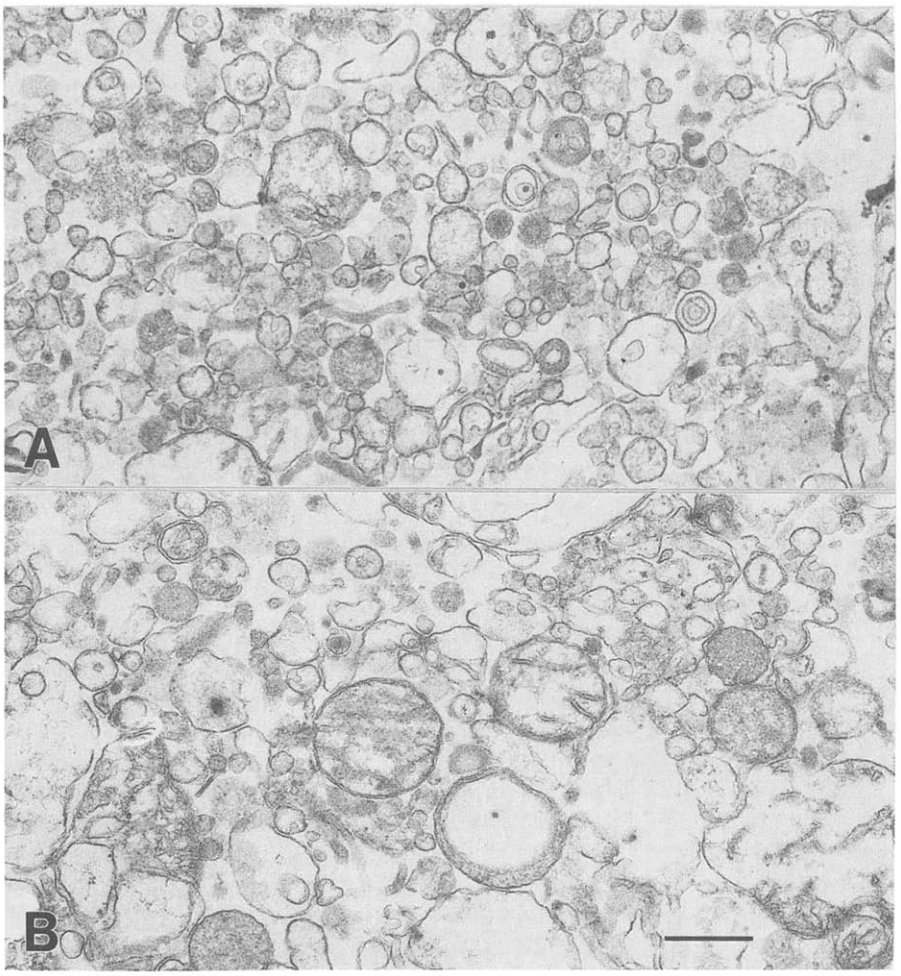

Fig. 12. Electron micrographs of endosomes purified by the ATP-shift technique shown in Fig. 11. (A) Fractions 43 and 44, responsive to ATP and monensin were highly enriched in endosomes. Renal tubules were labeled for 6 h with FITC–dextran to label the endosomes. Only a few mitochondrial fragments remained. (B) Fractions 45–48, unresponsive to ATP and monensin, contained the bulk of the mitochondria and proportionally fewer and smaller endosomes. Scale bar = 0.5 μm.

and plasma membrane of plants and why inside-out plant plasma membrane vesicles migrate more rapidly than rightside-out plasma membrane vesicles under certain conditions but not others. It will also explain why we are able to subfractionate Golgi apparatus membranes so effectively by preparative free-flow electrophoresis and why the monensin treatment is able to shift the electrophoretic mobility of endosomes. Also explained is the lack of direct correlation between membrane composition and electrophoretic mobility. Charged surface molecules, while a primary determinant of electrophoretic mobility may be augmented by diffusion potential.

However, a diffusion potential, negative outside, will not of itself alter the electrophoretic mobility of a particle in buffered solution. Electrophoretic mobility is universally considered to be determined by a mobile ion cloud around the particle, the so-called Debye–Hückel layer [1,43] (Fig. 14). The Debye–Hückel layer is the outer region of an electric double-layer of ions surrounding bioparticles in an electric field. Its distribution around the particle is determined by

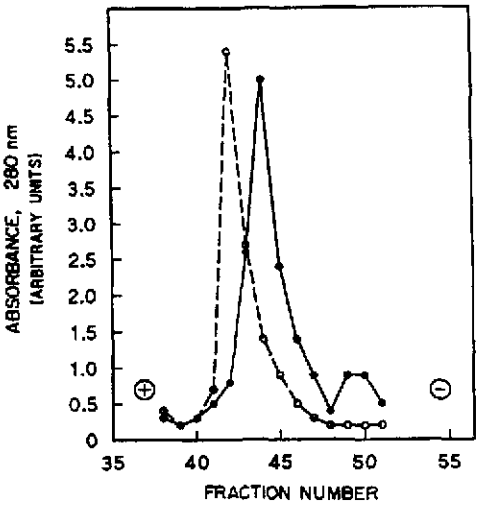

Fig. 13. Endosomes purified by flow cytometry (enrichment of particles containing FITC–dextran) showed the shift in electrophoretic mobility induced by incubation with ATP. From T.G. Hammond and D.J. Morré [40]. ○ = +ATP; ● = no ATP.

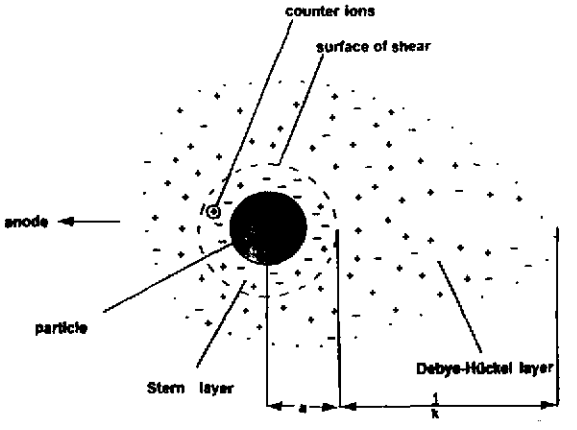

Fig. 14. Theoretical basis for free-flow electrophoretic separations based on diffusion potential. The figure illustrates how the electric double layer of a particle in a solution of electrolytes is thought to be influenced by an electric field [1]. The inner region includes absorbed ions and a diffuse region in which ions redistribute according to the electrical force exerted on the particle. The ζ potential, the so-called actual electrical charge arises from a "surface of shear" at the border with the inner hydrate = Stern layer to then influence electrophoretic mobility. It is the electrical charge at this surface of sheer that we suggest is modified by diffusion potential. Modified from Hannig and Heidrich [1].

both electrical forces and random thermal motion. When subjected to an electric field, the layer is deformed such that the outer ion cloud is shifted in a direction opposite to that of particle movement to expose underlying charged groups and allow their contribution to electrophoretic mobility.

The inner region of the double-layer includes absorbed ions. The ζ potential of the particle, its actual electrical charge, is determined at the surface of shear between the two layers during electrophoresis (Fig. 14). The hydrate or Stern layer at the particle surface includes many absorbed cations which tend to reduce the overall negative charge of the surface. What is missing from our information is a practical theoretical basis for how the composition and arrangement of charged groups underlying the Stern layer may be translated into a given ζ potential of that portion of the ion cloud that influences electrophoretic mobility. That separations by free-flow electrophoresis should be governed by a ζ potential of a region determined of itself by the conditions of electrophoresis (Fig. 14) is the principle theoretical argument advanced here. We argue that, as a result, organelle separations are possible by free-flow electrophoresis that are not accomplished readily by other separation techniques currently employed, including aqueous two-phase partition. The tonoplast and plasma membrane vesicle separation provides one example. It is difficult to completely resolve inside-out plasma membrane vesicles from right-side-out tonoplast vesicles by aqueous two-phase partition. Yet these two types of vesicles are readily resolved by free-flow electrophoresis.

How specific deformations of the Stern layer may relate to ζ potential and how such deformations may be induced and experimentally confirmed is a subject of continuing investigation. We can now alter electrophoretic mobility of tonoplast vesicles, *trans* Golgi apparatus cisternae and certain classes of endosomes by modifying diffusion potential through addition of ATP to induce proton entry or addition of ionophores such as monensin to induce proton release. This provides the primary experimental basis for

suggesting that electrophoretic mobility (ζ potential) can be modified during particle separation and related to a known experimentally determined property (diffusion potential) of the membrane (Fig. 14).

An initial important application of an ATP-induced diffusion potential is in the isolation and subfractionation of endosomes. Endosomes are defined as a heterogeneous population of prelysosomal, acidic organelles which play pivotal roles in the sorting and targeting of internalized membrane and content and that direct their specific transport to appropriate intracellular destinations [44]. A first major compartment is the aggregate of incoming vesicles, some clathrin coated, some without coats, derived by budding from the plasma membrane and rapidly modified by fusion among compartments. These are referred to variously as primary or secondary pinosomes or simply as early pinosomes or as early endosomes. These components have a very short life time and either recycle back to the plasma membrane or fuse with other endosomal compartments [45]. Eventually, pinosome contents are delivered into endosomes. The third major player in endocytic membrane traffic is the lysosome. Primary lysosomes deliver newly synthesized digestive enzymes to the endosomes with the formation, after fusion, of a secondary lysosome or digestive vacuole. These can participate in repeated rounds of digestion, for example, with the resultant formation of residual bodies, the content of which can be discharged from the cell.

Because of the anticipated heterogeneity of endosomal populations and the rather small differences in membrane and content properties that may distinguish different endosomal classes, new separation methods clearly are needed. Of those thus far applied to this difficult study area, preparative free-flow electrophoresis has been among the most effective. Marsh et al. [18] reported that functionally and structurally distinct subpopulations of endosomes could be resolved by preparative free-flow electrophoresis. Since cell fractionation by free-flow electrophoresis was rapid, large amounts of highly enriched early and late endosomes and endosomal subpopulations could, in principle, be prepared for biochemical and functional analyses and/or for antibody production.

Marsh *et al.* [18] reported that the anodal migration of endosomes and lysosomes during free-flow electrophoresis was dependent upon mild trypsinization of the membranes. The reasons for the trypsin requirement was unclear but there was apparently very little or no separations of endosomes or lysosomes from the bulk of the input membranes in the absence of the trypsinization step [18,19,46]. Control experiments [47], suggested little or no detectable alteration in the protein composition or in the functional integrity of either endosomes or lysosomes as a result of trypsinization. Nevertheless, a procedure in which the trypsin treatment could be eliminated would be advantageous for many applications.

It is clear that the electrophoretic mobility of endosomes and lysosomes was greater than that of most of the other membranes with the possible exception of plasma membrane vesicles. This was in contrast, for example, to their buoyant density which may be very similar to that of other membrane systems. The fact that endosomes, together with lysosomes, were separated from most other organelles by free-flow electrophoresis, indicates that differences in the net charge can be exploited for fractionation. Ionizable groups responsible for conferring these charge differences are not known. Acidic sialoglycoproteins are present but associated with the luminal aspects of the lysosomal membrane [48] and are unlikely to contribute. These considerations, taken together with the mobility shift experiments, suggest potentially important roles for diffusion potential as an exploitable parameter to effect endosome and other types of membrane separations involving vesicles with an inherent capacity to develop diffusion potentials, negative outside.

5. References

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