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Separation of endosomes by aqueous two-phase partition and free-flow electrophoresis

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Abstract

We have developed two endosome models to evaluate the separation of endosome populations by aqueous two-phase partition. In the first model, bovine kidney endosomes were used. In the second model, HeLa endosomes were identified in homogenates by means of a latent drug-(capsaicin-)inhibited NADH oxidase (NOX). Endosomes were first isolated by aqueous two-phase partition. To separate early and late endosomes, the endosomes were incubated with ATP to acidify the endosome interiors by activating a proton-translocating ATPase. Thus far, we have been able to resolve the early and late endosomes from any source only by preparative free-flow electrophoresis and not by phase-partition. Previous studies have shown that gravitational forces may be important for separation of endosomes by phase partition. Low-speed centrifugation ($\leq$12.5 *g*) during phase resolution altered the activity of the latent NADH oxidase used as a marker for HeLa cell endosomes.

Keywords: Aqueous two-phase systems; Endosomes

1. Introduction

The endosomal–lysosomal system of mammalian cells consists of several organelle ‘compartments’ broadly classified as early, intermediate and late endosomes, plus dense lysosomes [1–3]. These subcellular particles are involved in a variety of functions related to normal intracellular processing of material, as well as in disease and drug response. Endosomes play key roles in the acquisition of immunity (antigen presentation, drug delivery and processing, receptor regulation, signal transduction, transcellular transport, uptake of serum lipoproteins, viral infection, antimicrobial activity, immune surveillance, cancer, and a myriad of normal physiological functions including tissue growth, wound healing and differentiation [3]. Present understanding of the complexity and functioning of endocytic compartments, and the pathway from cell surface to digestive granule is limited primarily by an inability to resolve and fractionate the different compartments from one another. Obtaining even small amounts of better-resolved fractions would aid the investigation of biochemical organelle–function relationships and the design of more efficient purification methods. Development of new fractionation methodologies would facilitate investigation of the exocytic pathway as

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well as the function of specialized plasma membrane regions.

Aqueous two-phase partition in combination with preparative free-flow electrophoresis has been shown to provide the resolving power necessary to subfractionate endosomes [4–7]. However, present subfractionations are still limited in resolution. The extent that resolution is affected by gravity may be considerable. The objective of the present study was to develop experimental systems amenable to an investigation of the effects of gravity on the two-phase partition of endosomes.

2. Experimental

2.1. Reagents and chemicals

Poly(ethylene glycol) (PEG) 3350 and 8000 were purchased from Fisher Scientific (Chicago, II, USA). Dextran T-500 was from Pharmacia (Piscataway, NJ, USA). Other reagents were from Sigma Chemical (St. Louis, MO, USA).

2.2. Preparation of HeLa cells

HeLa S cells were grown by Cellex Biosciences (Minneapolis, MN, USA) collected by centrifugation and shipped frozen in 0.1 *M* sodium acetate, pH 5 in a ratio of 1-ml packed cell volume to 1 ml of acetate. The cells were thawed at room temperature, resuspended and then incubated at 37°C for 1 h. The cells were removed by centrifugation at 35 000 *g* for 60 min (Sorval) and were refrozen and stored at −70°C.

2.3. Isolation of endosomes from fresh or frozen HeLa cells or fresh or frozen kidney

1. The fresh or frozen and thawed cell pellet from 10 l of cultured HeLa S cells was resuspended in ca. 24 ml of 1 m*M* $NaHCO_3$ (bicarbonate) containing 0.2 m*M* EDTA and the resuspended cells were incubated on ice for 10 to 30 min. For kidney, 40 g of tissue were finely chopped and placed in 1 m*M* bicarbonate–EDTA solution in a ratio of 1 g of kidney per 2 ml of medium. The kidney was homogenized immediately without preincubation.
2. Either the incubated HeLa or the kidney were homogenized for 30 to 40 s at 12 000 *g* in 8-ml aliquots for HeLa and 20-ml aliquots for kidney using a Polytron 20 ST low-shear tissue homogenizer.
3. The homogenates were centrifuged 10 min at 1000 *g* (HB-4 rotor, Sorvall).
4. The supernatant was removed carefully with a pasteur pipette and centrifuged at 20 000 *g* for 60 min (SW28 rotor, Beckman L3-50 centrifuge).
5. During the 60-min centrifugation, the 16-g two-phase systems of Table 1 were prepared. Four 16-g systems were prepared for each kidney homogenate and one 16-g system was prepared for each HeLa homogenate. The systems were prepared in 30-ml Corex glass centrifuge tubes. Dextran was added first followed by poly-

Table 1
Composition of the 16-g (6.4%) two-phase systems

Reagent	Amount		
	5.8% system	6.0% system	6.4% system
20% Dextran T500 (Pharmacia)	4.64 g	4.8 g	5.12 g
Add 40% PEG 3350 (Fisher)	6.96	7.2	7.68
Add 0.2 *M* potassium phosphate buffer, pH 7.2	0.32 ml	0.32 ml	0.32 ml
Add water to total	16 g	16 g	16 g

(ethylene glycol) to the total weights indicated. The 0.2 *M* buffer (0.32 ml) was added followed by water to a total of 16 g.

6. The 20 000 *g* pellet was resuspended in 2 ml 0.2 *M* potassium phosphate(with HeLa) or 8 ml 0.2 *M* potassium phosphate buffer (with kidney). Of the resuspended pellets, 2 g was placed on the 6.4% dextran system to a final weight of 16 g. Each tube was covered with parafilm and inverted vigorously 40 times in the cold and centrifuged at 1000 *g* for 5 min at 4°C to resolve the phases. Also the 6.0- and 5.8-g systems (which had no sample) were inverted and centrifuged as well.
7. The top phase of the 6.4% dextran system was removed and diluted and pelleted by centrifugation (20 000 *g* for 30 min).
8. The top phase of the 6.0% dextran system was added to the bottom phase of the 6.4% dextran system. The tube was inverted vigorously 40 times in the cold and centrifuged to resolve the phases (1000 *g* for 5 min at 4°C).
9. The upper phase was removed with a pasteur pipette and centrifuged as above. To the bottom phase was added the top phase of the 5.8% dextran system. The tubes were covered with parafilm, inverted vigorously 40 times in the cold and centrifuged at 1000 *g* for 5 min at 4°C to resolve the phases.
10. The top and bottom phases were separated and the partitioned membranes were collected by centrifugation as above. The final pelleted top phase was the starting material for the preparative free-flow electrophoretic separation.
11. The final upper phase pellet was held on ice prior to resuspension in 2 ml of 50 m*M* Tris–MES, pH 7, and incubation in the absence and presence of ATP (see below) followed by free-flow electrophoresis. If necessary, the procedure can be interrupted at this step and the final upper phase pellet may be frozen or stored overnight on ice.
12. For incubation without ATP and with ATP to induce negative diffusion potentials, the resuspended upper phase from step 11 was then pelleted in a microfuge for 5 min. The supernatant was removed carefully and the soft pellet was divided into 2 parts. The first was resuspended in 1 ml of 2.5 m*M* Tris–MES, 50 m*M* KCI, 3 m*M* $MgCl_2$ and 5 m*M* DTT, pH 7.0 and incubated for 15 min at room temperature (−ATP). The second was incubated as above but with 1 m*M* ATP added (+ATP).

2.4. Preparative free-flow electrophoresis

The conditions for preparative free-flow electrophoresis were: flow-rate, 2.0 ml per fraction per hour, injection rate 2.5 ml/h, the voltage 1150 V and amperage about 175 mA. Just before injection, 1.5 ml of chamber buffer (10 m*M* acetic acid, 10 m*M* triethanolamine, 0.25 *M* sucrose, 0.5 m*M* $MgCl_2$ and 5 m*M* glucose, pH 6.5 was added to the mixture. After electrophoresis, the absorbance of 2-ml fractions was determined at 280 nm. Based on absorbance, various fractions were combined and the endosomes collected by centrifugation (20 000 *g*, 30 min).

2.5. Centrifugation during resolution of phases

To test the response of the two-phase partitioning to imposed centrifugal forces, the two-phase samples were centrifuged in a refrigerated (4°C) swing-out rotor (Sorvall HB-4) or, after transfer to 13×100 mm disposable borsilicate glass tubes, at room temperature (IEC HN-SII table top centrifuge) for 5 min during phase resolution. Uncentrifuged, paired samples were covered with parafilm and rested horizontal at the same temperature as during centrifugation (on ice for 4°C, bench top for room temperature) in place of the centrifugation.

3. Results

3.1. Isolation from kidney endosomes and HeLa and resolution of early and late endosome populations

The utility of preparative free-flow electrophoresis for resolution of early and late endosomes was demonstrated both for endosomes of bovine kidney and endosomes from HeLa cells. For the bovine, the

starting material was crude endosomal preparations obtained by differential centrifugation.

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 2.2. Representative electron micrographs are shown in Fig. 1.

Brush border membranes were concentrated in the upper phase of the initial 6.4% dextran–PEG phase

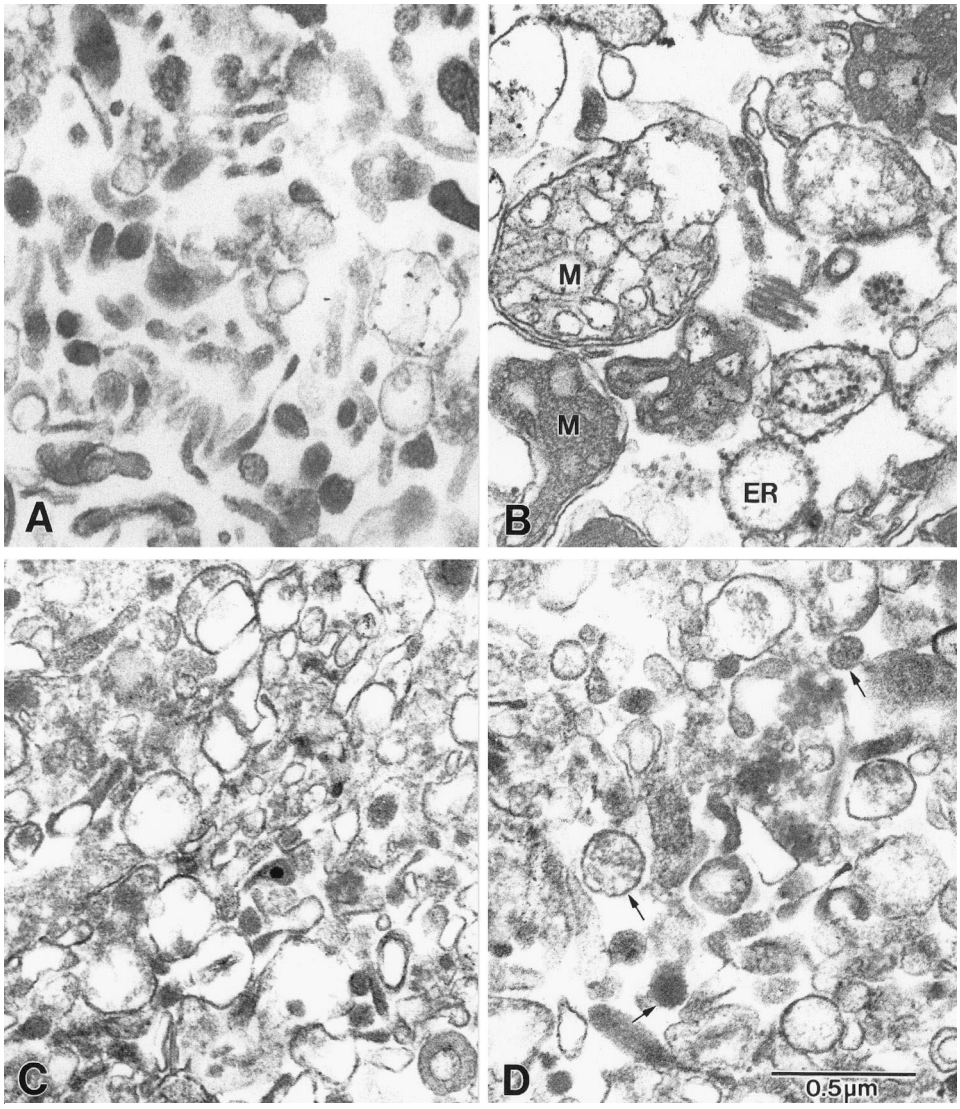

Fig. 1. Electron micrographs of cell fractions from bovine kidney prepared by partitioning in a 5.8% phase system. (A) Upper phase enriched in endosomes. (B) Lower phase containing mitochondria (M) and vesicles with attached ribosomes derived from rough endoplasmic reticulum (ER). (C and D) Electrophoretic fractions from the 5.8% upper phase after incubation for 10 min with 1 m*M* ATP. (C) Fraction enriched in early endosomes with lowest electrophoretic mobility. (D) Fraction enriched in late endosomes with greatest electrophoretic mobility. Small arrows indicate endosomes with electron-dense content. Scale bar=1 μm.

separation [5]. The lower phase still contained basolateral plasma membranes, endosomes, mitochondria and endoplasmic reticulum. An upper phase equilibrated from a 6.0% dextran–PEG phase system extracted the basolateral plasma membranes [5]. An upper phase, equilibrated from a 5.8% dextran–PEG phase system, extracted the endosomes in a third partitioning step (Fig. 1A). The bulk of the mitochondria, endoplasmic reticulum, nuclei and other internal membranes were retained in the lower phase (Fig. 1B).

The endosome fractions prepared by aqueous two-phase partition were subsequently separated into early and late endosomes by preparative free-flow electrophoresis after incubation with 1 m*M* ATP to acidify the interiors of the late-endosome fraction (Fig. 2). In contrast, in the absence of the ATP incubation, the kidney endosome migrated as a single, nearly symmetrical peak. The absolute electrophoretic mobility is not strictly comparable between separations. Normally the total endosomes (−ATP) and early endosomes (+ATP) appear to exhibit similar electrophoretic mobilities [5] (see also Fig. 3). The early endosomes had mostly empty-appearing interiors (Fig. 1C) whereas many of the late endosomes contained electron dense contents (Fig. 1D). When endosomes were prepared from HeLa cells in a manner parallel to those for bovine kidney, similar results were obtained. When separated by fractional aqueous two-phase partition, the vesicles with endosomal properties favored the upper phase in the 5.8% polymer partitioning step (Fig. 4A) whereas mitochondria, endoplasmic reticulum and other contaminating membranes remained in the lower phase (Fig. 4B).

If the preparations of endosomes were then divided and one portion incubated with 1 m*M* ATP for 5 min, two peaks of material based on electrophoretic mobility were obtained (Fig. 3). One peak

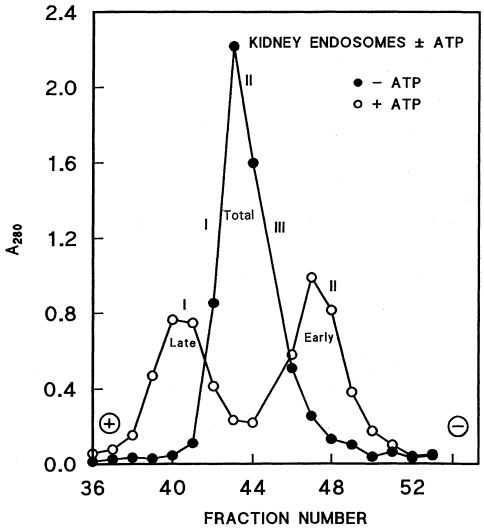

Fig. 2. Free-flow electrophoretic separation of bovine kidney endosomes prepared by aqueous two-phase partition. In the absence of ATP, the distribution of components was symmetrical (Total). When equal portions of the preparation were incubated for 10 min with 1 m*M* ATP, an ATP-induced region of particles with increased electrophoretic mobility indicative of late endosomes with acidified interiors [5] was observed as well as a less electrophoretically mobile fraction containing the early endosomes.

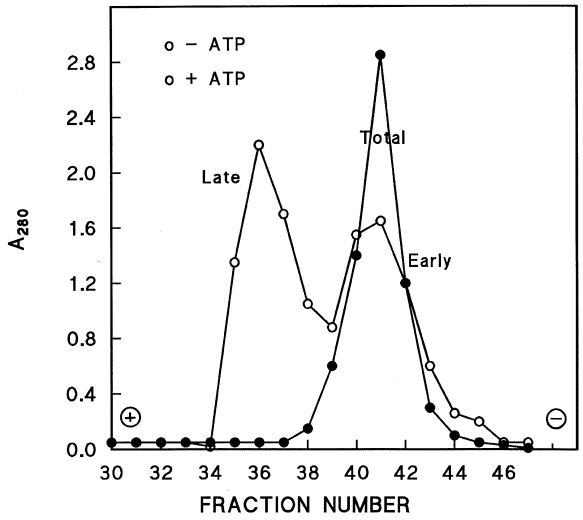

Fig. 3. As in Fig. 2 except a preparation of endosomes from HeLa cells prepared by aqueous two-phase partition. In the absence of ATP a symmetrical distribution of components was observed. After addition of 1 m*M* ATP for 10 min, an ATP-induced population of particles with increased electrophoretic mobility was observed indicative of late endosomes with acidified interiors. Approx. 30% less material was loaded for total endosomes due to a problem with the injector syringe.

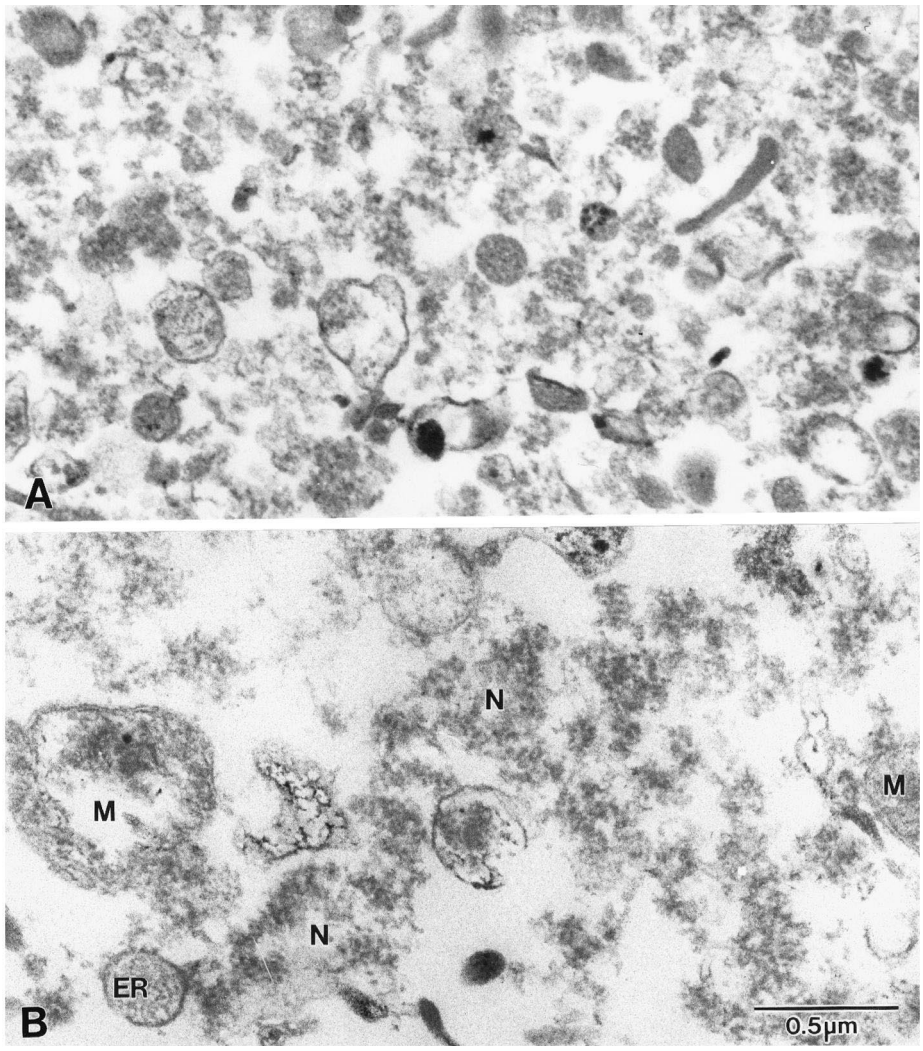

Fig. 4. Electron micrograph of endosome fraction prepared from HeLa homogenates by partitioning with a 5.8% phase system. A. Upper phase enriched in the endosomal marker, latent capsaicin-inhibited plasma membrane NADH oxidase. The preparation was enriched in endosomal vesicles. B. Lower phase depleted in the endosomal marker, latent capsaicin-inhibited plasma membrane NADH oxidase. The preparation contained predominantly swollen mitochondria (M), nuclear fragments (N) and vesicles with attached ribosomes derived from the rough-surfaced endoplasmic reticulum (ER). Scale bar=0.5 μm.

corresponded in electrophoretic mobility to the starting material and is considered to represent early endosomes. The second peak with a greater electrophoretic mobility induced by ATP addition represents the late endosomes capable of acidification of their interiors. The appearance of the two endosome fractions are shown in Fig. 5. Some endosomes with electron dense content were present in the late-endosome fraction (arrows, Fig. 5B).

3.2. Response of HeLa endosome isolation by aqueous two-phase partition to imposed gravitational fields

For the first studies with gravitational fields imposed by centrifugation, endosomes were isolated from HeLa S cells grown in culture. As a marker, a drug-responsive protein disulfide–thiol interchange (DTI) protein with NADH-oxidase activity [8,9] was

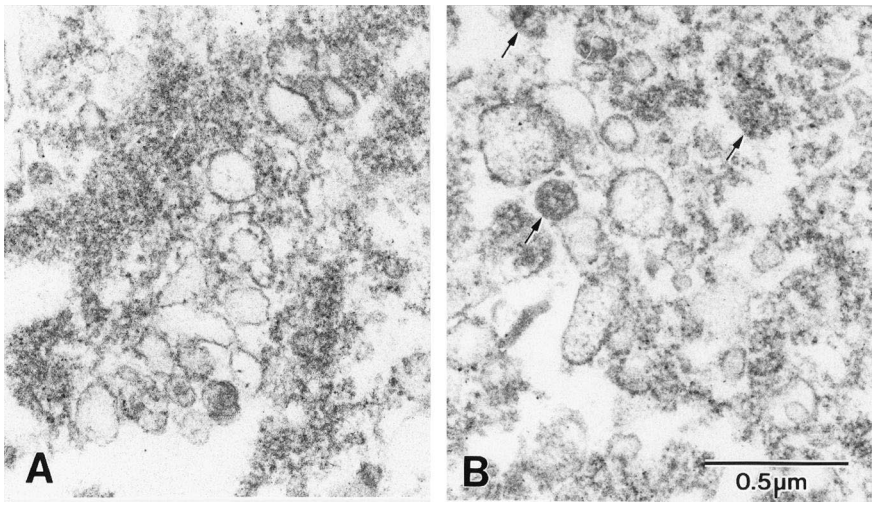

Fig. 5. Electron micrographs of endosome subfractions prepared from HeLa homogenates by preparative free-flow electrophoresis as described in Fig. 4 and incubated for 10 min with 1 m*M* ATP. A. Fraction enriched in early endosomes with lowest electrophoretic mobility. B. Fraction enriched in late endosomes with greatest electrophoretic mobility. Small arrows indicate endosomes with electron-dense content.

used (Table 2). This activity is located at the external surface of HeLa cells and is inhibited by a small group of quinone-site inhibitors including the vanilloid antitumor agent capsaicin (8-methyl-*N*-vanillyl-6-noneamide) (Table 2) [8,9]. With plasma membrane vesicles isolated from HeLa cells, NADH-oxidase activity was located at both the external and internal plasma membrane surfaces. With sealed, right side-out vesicles, only the capsaicin-inhibited activity at the external surface was measured. The total activity was about 1 nmol/min/mg protein. After addition of 1 μ*M* capsaicin, this activity dropped to 0.4 nmol/min/mg protein.

Table 2
NADH-oxidase activity of right side-out plasma membrane vesicles isolated from HeLa cells and response to 0.1% Triton X-100 and 1 μ*M* capsaicin

Triton X-100	Capsaicin	NADH oxidase, nmol/min/mg protein	
		Total	Δ Capsaicin
None	None	1.0±0.15[a]	
	1 μ*M*	0.4±0.1[b]	−0.6
0.1%	None	1.6±0.2[c]	
	1 μ*M*	1.2±0.15[a]	−0.5

Values not followed by the same letter are significantly different ($P<0.05$).

With right side-out plasma membrane vesicles, the capsaicin-inhibited activity was not increased by solubilizing the vesicle membrane with 0.1% Triton X-100 detergent. If the membranes were solubilized with 0.1% Triton X-100, the total activity increased to about 1.6 nmol/min/mg protein but the capsaicin-inhibited component remained the same at approximately 0.6 nmol/min/mg protein.

The NOX protein is bound to the outer leaflet of the plasma membrane but can be released from cells by dropping the pH to 5 [10]. The HeLa cells, once stripped of their plasma membrane-located NOX activity by treatment at pH 5, yielded plasma membranes now lacking the activity (Table 3). However, the activity associated with endosomes remained (Table 4).

The drug-responsive activity present in endosomes is located on the inside of the endosomal vesicles (Table 4). If an impermeant substrate such as NADH is used to assay the activity, endosomes from HeLa cells will not show drug-inhibited NOX activity as long as the endosomal membrane is intact. Once the endosomal membrane is dissolved with Triton X-100, the activity will appear (Table 4).

For the centrifugation experiments, an endosome-enriched fraction was prepared from HeLa cells stripped of their external, drug-responsive NADH

Table 3
Depletion of NADH-oxidase activity inhibited by capsaicin from plasma membranes by incubation of HeLa S cells at 37°C for 2 h in the presence of 0.1 *M* sodium acetate, pH 5.0

Treatment	NADH-oxidase activity, nmol/min per mg plasma membrane protein		
	No capsaicin	1 μ*M* capsaicin	100 μ*M* capsaicin
PBS, pH 7.0 2 h, 37°C	0.9+0.3	0.4±0.2	0.4±0.2
0.1 *M* sodium Acetate, pH 5.0 2 h, 37°C	0.2±0.01	0.2±0.01	0.2±0.05

Frozen HeLa S cells were thawed at room temperature, incubated as above and plasma membranes were then isolated from the incubated cells and analyzed for NADH-oxidase activity inhibited by capsaicin
Average of three determinations+standard deviations.

oxidase and from which most of the plasma membranes had been removed. A two-phase separation to concentrate the endosomes was then carried out (Section 2). During this separation, the centrifugal force was varied from approximately 0 to 400 *g*. The centrifugal force was applied for 5 min after which the two phases were resolved and the content of endosomes in the upper phase was determined from the Triton X-100-dependent, capsaicin-inhibited NOX activity (Fig. 6). The endosome fractions not treated with Triton X-100 lacked the activity (located inside the endosomes). The activity appeared only with Triton X-100 treatment (0.1% Triton X-100) (Table 4). The crude endosome fractions contained a NOX activity resistant to cyanide but the capsaicin-inhibited activity characteristic of the plasma membrane (Table 2) was lost (Table 3). Upon treatment with Triton X-100, the NOX activity was enhanced by 40% (Table 4). This enhanced activity was 60% inhibited by 1 μ*M* capsaicin to a degree nearly identical with that observed for plasma membrane vesicles (Table 2).

These experiments have been repeated several times and the results are reproducible. The Triton-activated and capsaicin-inhibited NOX activity in the

Table 4
Activation of NADH-oxidase activity of an endosome-enriched fraction of HeLa cells by Triton X-100 and inhibition by 1 μ*M* capsaicin

Triton X-100	Capsaicin	NADH oxidase, nmol/min /mg protein	
		Total	Δ Triton
None	None	0.6±0.07[a c]	
	1 μ*M*	0.6±0.02[a]	
0.1%	None	0.85±0.02[b]	0.25±0.02
	1 μ*M*	0.7±0.06[c]	0.1±0.05

Values not followed by the same letters are significantly different ($P<0.05$).

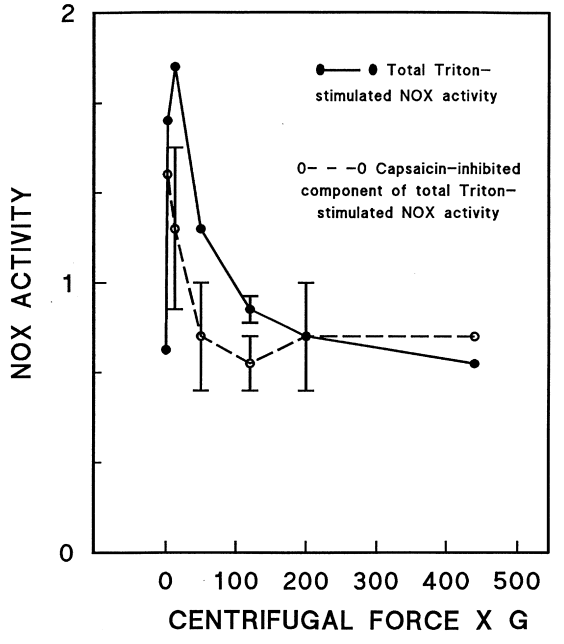

Fig. 6. Response to centrifugation of the Triton X-100-stimulated NADH oxidase (NOX) activity of a HeLa cell endosome fraction and inhibition by 1 μ*M* capsaicin. The preparations were centrifuged in a swing-out rotor for 5 min during the resolution of the two phases. Units of specific activity are nmol/min per mg protein.

upper phase (the phase normally enriched in endosomes) was enhanced by the low-speed centrifugation of 2 and 12.5 *g* but not by higher centrifugal forces (50 *g* and above) (Fig. 6).

4. Discussion

Phase partitioning is based on differentially distributing particles and/or macromolecules between the two immiscible aqueous phases which form when neutral polymer(s) such as poly(ethylene glycol) (PEG) and dextran are mixed at a few percent (w/w concentration in buffered media [11–13]. The systems consist of a less dense, PEG-rich phase floating on top of a denser, dextran-rich phase. Partitioning consists of adding the sample of interest to a phase system, gently mixing to emulsify the phases, and analyzing them following emulsion demixing. The phases mix readily due to low interfacial tensions, e.g., 5 μN/m, and following a coalescence period of a few seconds, rapidly demix due to phase convection. Demixing proceeds rapidly, e.g., 0.5 cm/s, due to phase-density differences, e.g., 0.04 g/ml [14–16]. Particles generally partition between one phase and the phase interface and sampling usually follows the formation of a planar, bulk interface. Results are expressed as the coefficient (K) ratio (or percent) of material (re)partitioning into the less dense phase compared with the rest of the system.

Phase partitioning is influenced by gravity [14–16]. The demixing necessary for its use as a separation technique is driven by gravity. Secondly, as in electrophoresis [17], particle separations are negatively affected by gravity-related sedimentation and phase convection. Sedimentation, from the less dense to the denser phase, or phase interface, reduces the distribution, and limits the time the phases are allowed to demix before sampling to under an hour for a 10-ml system in a 10-mm diameter tube. The rapidity of convective demixing randomizes the distributions obtained in two ways. The orphan particles attached to the interface of small phase droplets in the complementary phase where, due to short demixing times, they are sampled before reaching the bulk interface. Secondly, the drag associated with rapid phase demixing may remove particles from the interface. Brooks et al. [18] calculated that in a typical system the energy necessary to remove a 3.5 μM diameter biological particle from the phase interface is 1 to 5×10^{-6} dyne but the Stokes' viscous drag force related to unit gravity acting on such a particle may reach over 1×10^{-4} dyne [14]. This may be a significant determinant of partition. Smaller particles would require greater gravitational or related forces to achieve a similar effect.

Phase partition is exponentially influenced by surface charge for particle separations on the basis of both surface charge [12,19–21] and affinity ligand interaction [20]. Partitioning has been the subject of microgravity experimentation. Brooks was the first to propose studying phase partitioning in low *g* using weak electric fields to control particle mobility plus the demixing and disposition of the phases [22].

Endosomes are defined as the heterogeneous population of prelysosomal, acidic organelles which play pivotal roles in the sorting and targeting of internalized membranes (and their contents) and which direct their specific transport to appropriate intracellular destinations [2]. A first major compartment is the aggregate of incoming vesicles, some clathrin-coated and some uncoated, derived from the plasma membrane and rapidly modified by fusion of compartments. These are referred to by various authors as primary or secondary pinosomes, early pinosomes or early endosomes. They have a short intracellular half-life and either recycle to the plasma membrane or fuse with other endosomal compartments [23]. Pinosome contents are eventually delivered into endosomes, yet it is not known to what extent endosomes are independent organelles and may share common proteins with the plasma membrane. Resident endosomal membrane proteins, if any, remain to be identified, let alone harnessed for biotechnical control of intercellular functions. A third major player in endocytic membrane traffic is the lysosome. Primary lysosomes deliver newly synthesized digestive enzymes to endosomes (post vesicle fusion) with formation of secondary lysosomes (digestive vacuoles). These can participate in repeated cycles of intracellular digestion which, for example, may lead to the formation of residual (exocytic) bodies whose contents are later discharged from the cell.

Endosomes exhibit both structural and functional heterogeneity. For example, internalized (cell sur-

face) receptor-ligand complexes are first delivered to endosomal vesicles and associated tubules near the internal periphery of the cell (early endosomes). Membrane receptors are recycled, whereas ligands destined for lysosomal digestion are transported, via relatively unknown mechanisms, to the cell's interior where they are sequestered into distinct endosomal vesicles or multivesicular bodies. The two broad populations of 'receptor-positive' and 'receptor-negative' endosomes are morphologically and biochemically distinguishable [24–26] and can be resolved by centrifugal techniques [27–29]. Probes sensitive to pH have recently shown that the interior endosomal environment decreases in pH as endosomes traverse the endocytic pathway. Internal pH is now established as a biochemical indicator of endosomal heterogeneity however present fractionations are of a quality to permit determination if pH heterogeneity reflects the existence of distinct subpopulations.

In order to further understand the complexities of the endocytic–lysosomal system, it is necessary to be able to subfractionate endosomes and related particles. Particle heterogeneity, equivalence of apparent densities, and slight, although significant, differences in bulk surface structure and contents indicate a need for new separation methods. Preparative free-flow electrophoresis has proven to be most promising. Harms et al. [30–32], Henning and Heidrich [33] and others used FFE to purify lysosomes. Early and late endosomes have been resolved using FFE by Marsh et al. [24] and Evans and Flint [34]. With FFE, separations are rapid enough that detailed kinetic analysis of ligand transport through the endosome pathway is possible [35,36].

The electrophoretic mobility of endosomes and lysosomes is greater than that of most other cell membranes, with the possible exception of the plasma membrane [24,34–36]. Despite considerable progress towards the use of FFE to isolate and subfractionate endosomes, the technique has just started to be systematically explored. Lysosomes exhibit greater anodal mobility than other endosomes. The chemical groups and membrane structure responsible for such mobility differences are not known. Acidic sialglycoproteins are present but are associated with the luminal side of the lysosomal membranes [37]. Marsh et al. [24] have reported that trypsinization enhances endosomal mobility differences, which might suggest differences in membrane lipid composition [19]. Affinity methods have not been attempted. Their development may require a better understanding of endosomal subpopulation surface differences. Tulp et al. [38] and Amigorena et al. [39] have used immunoaffinity partition to identify a novel endocytic compartment in β-lymphocytes containing new class II histocompatibility antigens.

Another way to improve FFE separation is via preenrichment usually by centrifugal methods such as Percoll gradient purification [40,41]. However, the buoyant density differences exploited in the technique are not overt. Thus, endosome fractions frequently are contaminated with mitochondria. For this reason, and a desire to avoid trypsinization effects [19], the authors began several years ago to explore phase partitioning to isolate contaminating plasma membrane and mitochondria. This led to studies on the partition behavior of endosome subpopulations. Work supported in concept by the successful fractionation of other cell components via both single-tube [12,42,43] and countercurrent distribution [12,42,44]. Morré et al. have recently reported significant advances in the isolation and purification of endosomes from both rat liver and rat kidney [5].

Crucial to these advances has been the development of a protocol to further purify endosomes from endosome-enriched centrifugation pellets using phase partitioning [4–7]. The original experiments involved subjecting the endosome fraction from Percoll gradients to single-step partition at 4°C in a two-phase system composed of 5.8% (w/w) each of poly(ethylene glycol) (PEG) 3350 (Union Carbide) and dextran T500 (Pharmacia). It was found that following phase demixing the endosomes were retained in the PEG-rich, less dense, phase while mitochondrial contaminants were found in the dextran-rich 'bottom' phase [4]. This lead to a two-phase partition being developed as a primary isolation procedure for endosome preparation. The crude 20 000 *g* pellet was first partitioned in a system similar to that above but containing 6.4% (w/w) of each polymer. Following demixing, endosomes were found in the dextran-rich phase while plasma membranes were found in the PEG-rich phase. The plasma membranes could then be isolated by phase dilution followed by centrifugation. Endosomes were isolated by combining the dextran-rich phase they

were suspended in with fresh PEG-rich phase so as to again obtain a system containing 5.8% (w/w) of each polymer. Following demixing the endosome-containing, PEG-rich phase could be isolated from the mitochondrial-enriched dextran phase [5].

Endosomes purified by this rapid two-step partition technique could be subjected to further fractionation via free-flow electrophoresis. Particles with the fastest anodal electrophoretic mobility were recently identified by a monensin-(monovalent ionophore) shift experiment as a population of late endosomes. Receptor and content markers could then be utilized to identify intermediate and early-endosome fractions [7]. Although it might be possible, using affinity and other techniques, to achieve even better separations by phase partitioning or FFE, the double-technique method described above is currently a method of choice for the preparation and fractionation of endosomes [5].

Endosome electrophoresis is very sensitive to pH and ion concentration. Optimization of pH can improve separations, as can manipulation of cation type and concentration in the running buffer. Late endosomes are normally larger than early endosomes which may favour increasing buffer viscosity. It also aids their quantification via microscopy. The one well-established biochemical characteristic which distinguishes early intermediate and late endosome classes from each other is their ability to pump protons. The ATP-requiring, transmembrane pumps are acquired during endosome maturation and are most evident in late endosomes. The ability of various endosome fractions to acidify their interiors has recently been exploited in their fractionation [5]. The method involves incubation of partition-purified endosome fractions with ATP for about 15 min in a buffer of low monovalent ion concentration (rate limiting for proton release [45]). This treatment increases the electrophoretic mobility of late endosomes [5]. It is interesting to note that Österberg et al. [46] reported similar behavior in PSL microspheres suspended in acidified solutions prior to electrophoresis.

The HeLa cell system developed to test the response of endosome isolation to imposed centrifugal force during aqueous two-phase demixing has several novel features. HeLa endosomes were identified from a capsaicin-inhibited oxidation of NADH associated with the outer surface of the plasma membrane. This activity could be released quantitatively by low pH treatment of frozen HeLa cells [10]. After release of the cell surface capsaicin-inhibited NADH oxidase, the only capsaicin-inhibited NADH oxidase remaining in the cell would be that present at the interiors of endocytic vesicles and accessible only after detergent treatment of the membranes to expose the internal NADH oxidase site. This was confirmed by experiment where a low specific activity NADH-oxidase activity of 0.25 nmol/min/mg protein could be induced in a crude endosome preparation from HeLa cells by treatment with Triton X-100. This activity was 60% inhibited by 1 μM capsaicin whereas in the absence of Triton X-100 treatment, the fractions were devoid of a capsaicin-inhibited NADH oxidase.

The activity of the capsaicin-responsive NADH oxidase in the 5.8% upper phase of the PEG-dextran system was enhanced about 2 fold by low-speed centrifugation of 2 to 12.5 g during phase demixing. Forces of 50 g or greater were without effect. However, for early and late endosomes which are readily resolved by preparative free-flow electrophoresis [5], aqueous two-phase partition, with or without imposed centrifugal force during demixing, has thus far failed to result in a useful separation. However, it is of interest that total endosome partition may be aided somewhat by low level centripetal forces [47], but not to the same extent as shown by the HeLa NADH oxidase endosome marker reported here.

The reason it may be more difficult to separate early- and late-endosome models by aqueous two phase partition compared to preparative free-flow electrophoresis has been addressed previously [5]. We have determined that the common parameter among free-flow electrophoretic separations is not entirely due to charged molecules at the vesicle surface but may relate, as well, to the ability of the vesicles to create an (outside) negative diffusion potential. This may be why there is no direct correlation between membrane composition and electrophoretic mobility. Charged surface molecules, while a primary determinant of electrophoretic mobility, may be augmented by altered local surface charges within the double layer region [48,49].

We suggest that organelle separations that are possible by free-flow electrophoresis and not accomplished readily by other separation techniques, in-

cluding aqueous two-phase partition, may be influenced by the ability of different organelle subclasses to alter their local surface-charge distribution. Thus, we have suggested that electrophoretic mobility (ζ potential) can be modified during particle separation and is related to the diffusion potential of the membrane [5].

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