Rapid Analytical and Preparative Isolation of Functional Endosomes by Free Flow Electrophoresis

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Abstract. Endosomes are prelysosomal organelles that serve as an intracellular site for the sorting, distribution, and processing of receptors, ligands, fluid phase components, and membrane proteins internalized by endocytosis. Whereas the overall functions of endosomes are increasingly understood, little is known about endosome structure, composition, or biogenesis. In this paper, we describe a rapid procedure that permits analytical and preparative isolation of endosomes from a variety of tissue culture cells. The procedure relies on a combination of density gradient centrifugation and free flow electrophoresis. It yields a fraction of highly purified, functionally intact organelles. As markers for endosomes in Chinese hamster ovary cells, we used endocytosed horseradish peroxidase, FITC–conjugated dextran, and [^{35}S]methionine-labeled Semliki Forest virus. Total postnuclear supernatants, crude microsomal pellets, or partially purified Golgi fractions were subjected to free flow electrophoresis. Endosomes and lysosomes migrated together as a single anodally deflected peak separated from most other organelles (plasma membrane, mitochondria, endoplasmic reticulum, and Golgi). The endosomes and lysosomes were then resolved by centrifugation in Percoll density gradients. Endosomes prepared in this way were enriched up to 70-fold relative to the initial homogenate and were still capable of ATP-dependent acidification. By electron microscopy, the isolated organelles were found to consist of electron lucent vacuoles and tubules, many of which could be shown to contain an endocytic tracer (e.g., horseradish peroxidase). SDS PAGE analysis of integral and peripheral membrane proteins (separated from each other by condensation in Triton X-114) revealed a unique and restricted subset of proteins when compared with lysosomes, the unshifted free flow electrophoresis peak, and total cell protein. Altogether, the purification procedure takes 5–6 h and yields amounts of endosomes (150–200 μg protein) sufficient for biochemical, immunological, and functional analysis.

ENDOSOMES comprise a heterogeneous population of vacuoles and tubules to which receptors, receptor-bound ligands, and extracellular solutes are delivered after endocytosis (see Helenius et al., 1983; Hopkins, 1983; Steinman et al., 1983; Brown et al., 1983; Pastan and Willingham, 1983; Marsh et al., 1986). Endosomes are responsible for many of the critical events that regulate the traffic, sorting, and processing of internalized macromolecules. The acidic internal pH of endosomes is, for instance, needed for the dissociation of incoming ligand-receptor complexes, allowing the receptors to return to the cell surface and the ligands to be transported to lysosomes for degradation (for review, see Mellman et al., 1986). By selectively rerouting certain receptor-ligand complexes to lysosomes, endosomes serve to regulate receptor expression (Mellman and Plutner, 1984; Beuginot et al., 1984; Ukkonen et al., 1986). In epithelial cells they play a central role in the maintenance of cell polarity (Abrahamson and Rodewald, 1981; Geuze et al., 1984; Pesonen et al., 1984). Endosomes also provide a site of entry into the cytosol for many animal viruses and bacterial toxins taken up by endocytosis (Marsh et al., 1983; Draper and Simon, 1980).

While the functional importance of endosomes is now well established, little is known about their structure, composition, and biogenesis. Apart from the demonstration that endosomes contain an ATP-driven proton pump (Galloway et al., 1983; Merion et al., 1983; Robbins et al., 1983) and the demonstration of a serine protease activity (Diment and Stahl, 1985), few of their biochemical characteristics have been defined. Endosomes are difficult to isolate by standard techniques of cell fractionation as their general properties are similar to many other organelles (e.g., Golgi, endoplasmic reticulum, and plasma membrane). Thus, most current procedures for isolation have relied on endogenous or exogenous perturbants to selectively modify the density of endosomal membranes (Courtoy et al., 1984; Hornick et al.,

1985) and immunoadsorption (Mueller and Hubbard, 1986), or on a lengthy series of physical fractionation techniques (Dickson et al., 1983; Wall and Hubbard, 1985). It is not yet clear whether these approaches provide endosomal fractions of uniformly high yield, purity, and functional competence.

In this paper, we present a new method for the isolation of highly purified endosomes from cultured cells that combines density gradient centrifugation with free flow electrophoresis. The method is rapid and can be used either analytically or preparatively. Most importantly, the isolated organelles are still functionally active and can be used, for example, for the detailed analysis of the bioenergetics of endosome acidification (Fuchs, R., P. Male, and I. Mellman, manuscript in preparation).

Materials and Methods

Cells and Cell Culture

Chinese hamster ovary cells (CHO) were maintained in either suspension or monolayer culture in alpha-MEM supplemented with 5% FCS (J. R. Scientific, Woodland, CA) and penicillin/streptomycin. Fractionation experiments typically used cells harvested from 1 to 3 liters of mid-log phase spinner cultures (5–10 × 10^5 cells/ml). For experiments using CHO monolayer cultures, suspension cells were plated on 100- or 150-mm plastic tissue culture dishes (Falcon Labware, Oxnard, CA) 24–48 h before use. Either confluent or subconfluent cultures were used. Baby hamster kidney (BHK-21)[1] cells were grown in monolayer culture, in either 100-mm plates or 850-cm^2 plastic roller bottles (Falcon Labware) as described previously (Helenius et al., 1980).

Radiolabeling

Total CHO cell proteins were labeled by overnight incubation of subconfluent monolayers (in 100-mm dishes) or log phase suspension cells with [^{35}S]methionine (>600 Ci/mmol, Amersham/Searle, Arlington Heights, IL) in methionine-free alpha-MEM containing 5% dialyzed FCS (1–2 mCi [^{35}S]methionine/5 ml medium; labeling medium). Cell surface proteins were radioiodinated at 0°C by the lactoperoxidase-glucose oxidase method (Hubbard and Cohn, 1975) as previously described (Mellman et al., 1980). Briefly, CHO cells were harvested from spinner cultures and washed extensively with cold PBS. 1 × 10^7 washed cells were suspended in 1 ml PBS containing 1 mM glucose and radioiodinated using 0.75–1.5 mCi carrier-free Na ^{125}I (Amersham/Searle). Labeling was terminated and unincorporated ^{125}I removed by dilution and washing in cold, serum-free alpha-MEM.

Endosome Labeling

Endosomes were labeled by incubation of CHO cells or BHK-21 cells with markers of fluid phase or receptor-mediated endocytosis under conditions that minimized the transfer of internalized tracers to lysosomes (Galloway et al., 1983; Marsh et al., 1983). Fluid phase markers included FITC-conjugated dextran (FITC-dextran; 70,000 mol wt; Sigma Chemical Co., St. Louis, MO) and horseradish peroxidase (HRP; Type IV, Sigma Chemical Co.). FITC-dextran was dialyzed overnight against 250 vol of PBS and used at final concentrations of 6–12 mg/ml in alpha-MEM; HRP was used at 10 mg/ml in alpha-MEM. To label CHO endosomes, the cells were incubated for 10–15 min at 37°C either in suspension or monolayer culture. BHK-21 cell endosomes were labeled by rotating roller bottles of cells at 5 rpm for 15 min at 37°C. At the end of the labeling period, the cells were immediately cooled and washed extensively with ice-cold PBS.

CHO cell endosomes were labeled with Semliki Forest virus (SFV) as a marker of receptor-mediated endocytosis (Marsh and Helenius, 1980; Marsh et al., 1983). [^{35}S]Methionine-labeled SFV was first bound to monolayer cultures in alpha-MEM (pH 6.8) containing 0.2% BSA for 1 h at 0°C. After the removal of unbound virus by washing several times with cold alpha-MEM, the cells were refed with warm medium and incubated for 15 min in a 37°C water bath (Marsh et al., 1983). Greater than 90% of the bound virus was internalized under these conditions, based on the resistance of cell-associated ^{35}S-SFV to removal by subtilisin (1 mg/ml in PBS containing 5 mM EDTA and 5 mM dithiothreitol for 1 h at 0°C; Sigma Chemical Co.). In cells not warmed to 37°C, >90% of the bound virus was susceptible to removal by subtilisin. By centrifugation in Percoll density gradients (see below), ^{35}S-SFV internalized after the 15-min incubation was localized only in low density fractions corresponding to endosomes (Marsh et al., 1983).

Virus Infection

CHO cell monolayers on 100-mm plates were infected with SFV at 50 pfu per cell. To metabolically radiolabel viral spike proteins in the rough endoplasmic reticulum, 4-h infected cells were pulse labeled for 5 min at 37°C with 0.5 mCi [^{35}S]methionine (in methionine-free alpha-MEM), and then immediately washed and harvested in cold PBS (Green et al., 1981). The proteins labeled under these conditions were shown by SDS PAGE to be predominantly the SFV spike glycoproteins.

Cell Homogenization

All manipulations and solutions were at 0–4°C unless otherwise indicated. Cell monolayers were rinsed at least four times with cold PBS and harvested using a rubber scraper. Cells were then collected by centrifugation at 500 *g* (5 min). Suspension cells were washed by repeated centrifugation and resuspension in cold PBS. For experiments involving both monolayer and suspension cultures, the cells were combined at this stage. The washed cell pellet was then resuspended in 50 ml of TEA buffer (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, and 1 mM EDTA, pH 7.4) (Harms et al., 1980) and collected by centrifugation at 750 *g* for 10 min at 4°C.

For CHO cells, washed cell pellets were suspended in cold TEA buffer at a ratio of 5 vol of buffer to 1 vol of packed cells. The cells were then disrupted by two to four passes through a ball bearing homogenizer with a 0.0002-in. clearance (Balch and Rothman, 1985), conditions that resulted in at least 80% cell breakage while leaving >90% of the nuclei intact. Homogenization was routinely monitored by phase-contrast microscopy. The homogenate was first centrifuged at 750 *g* for 10 min and the resulting supernatant centrifuged for 10 min at 950 *g* to derive the postnuclear supernatant.

For BHK-21 cells, the cell pellet was resuspended in TEA buffer (~0.5 ml per roller bottle) and placed on ice for 5 min. The cell suspension was pipetted 10 times up and down through a 10-ml glass pipette (1.5-mm aperture) to disrupt the cells. The postnuclear supernatant was obtained as described above.

Preparation of Microsomes

Microsomes were prepared from postnuclear supernatants by centrifugation at 100,000 *g* for 35 min in an SW55 rotor (Beckman Homogenizer, Beckman Instruments, Inc., Palo Alto, CA). The microsomes were pelleted onto a 0.5-ml cushion of 1 M sucrose in TEA buffer and were resuspended, together with the cushion, with 1.5 ml cold TEA buffer without sucrose to give a final concentration of 0.25 M sucrose. The pellet was dispersed by five passes in a small volume, loose fitting glass Dounce (Kontes Co., Vineland, NJ) and centrifuged at 750 *g* for 5 min to remove any aggregated material. The volume of the resuspended membranes was adjusted with TEA-sucrose to yield a protein concentration of ~1 mg/ml.

Preparation of Crude Golgi Fractions

CHO cell postnuclear supernatants were centrifuged at 100,000 *g* for 30 min in an SW40 rotor (Beckman Instruments, Inc.). Microsomes were collected on a 0.5-ml cushion of 2 M sucrose in TEA buffer and resuspended, together with the cushion, with sucrose-free TEA buffer to a final concentration of 1.2 M sucrose. The microsomes were transferred to an SW 40 tube and overlaid with 4 ml 1 M sucrose and 3 ml 0.6 M sucrose (both in TEA buffer) and centrifuged at 40,000 rpm for 1.5 h. The endosome/Golgi peak was collected from the 1 M/0.6 M sucrose interface. The sucrose concentration was estimated using a refractometer and adjusted to 0.25 M with sucrose-free TEA buffer.

Trypsinization

For most experiments, membranes were treated with N-tosyl-L phenylalanine chloromethylketone (TPCK)-trypsin (Worthington Biochemical Corp.,

1. *Abbreviations used in this paper*: BHK-21, baby hamster kidney cells; DAB, 0.2% diaminobenzidine; FITC-dextran, FITC-conjugated dextran; HRP, horseradish peroxidase; SFV, Semliki Forest virus; TEA buffer, 0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, and 1 mM EDTA, pH 7.4.

Freehold, NJ) before free flow electrophoresis. A range of trypsin concentrations was used (see Results), added at known ratios of trypsin to cell protein (~1 mg/ml, determined by the method of Bradford [1976]). For postnuclear supernatants and microsome fractions, optimal trypsin concentrations were between 1 and 4% by weight (usually 10 μg/ml) and for endosome/Golgi fractions 0.25% trypsin (by weight relative to protein) was used (usually 1–2 μg/ml). Samples were incubated at 37°C for 5 min and the treatment was stopped by returning the samples to 0°C and adding a five-fold excess of soybean trypsin inhibitor (Sigma Chemical Co.).

Free Flow Electrophoresis

Free flow electrophoresis was performed using either the Bender & Hobein ElphorVap 11 (EV11) or ElphorVap 21 (EV21) instruments (Protein Technologies, Inc., Tucson, AZ). Just before each run, the separation chamber was coated with 1% BSA in TEA buffer for 30 min and washed with BSA-free TEA buffer. Similar separation conditions were used for both machines (Harms et al., 1980). TEA buffer (4°C) was used in the separating chamber and run at ~4 ml/fraction per h with a field of 1,400 V at 220 mA (EV11) or 3 ml/fraction per h using 1750 V at 130 mA (EV21). The temperature of the separation chamber was maintained at 5°C. Samples (adjusted to ~1 mg/ml protein) were perfused into the separating chamber at 3–4 ml/h (EV 11) or 1–2 ml/h (EV 21) and collected through a 90-channel peristaltic pump at 4°C. The approximate transit time in the separating chamber was 2 min and individual runs took 30–90 min to complete, depending on the volume of the initial sample.

Percoll Gradient Centrifugation

Centrifugation using self-forming Percoll density gradients was performed as previously described (Galloway et al., 1983). Briefly, samples in TEA buffer were mixed with stock isotonic Percoll (final concentration 27%) and layered onto a 3-ml cushion of 60% sucrose in Quick-Seal tubes (Beckman Instruments). Gradients were centrifuged using a Ti70 or Ti50 rotor (Beckman Instruments, Inc.; 18,000 rpm, 2 h), or a VTi50 rotor (Beckman Instruments, Inc.; 16,000 rpm, 1 h) (Ukkonen et al., 1986). Approximately 24 fractions were collected per gradient.

Enzyme and Protein Assays

Protein was assayed using either the Coomassie Blue binding assay (Bradford, 1976) or by a fluorescamine assay in the presence of 0.1% SDS (Sims and Carnegie, 1975). Since Percoll interferes with this assay, Percoll was removed from density gradient fractions by adjusting the samples to pH 12 with 2 M NaOH and clearing the aggregated Percoll by centrifugation in an Eppendorf microcentrifuge (Brinkman Instruments, Inc., NJ). In experiments involving [^{35}S]methionine-labeled cells, radioactivity was used to follow the distribution of protein.

Marker enzymes for various organelles were measured as follows: *p*-iodinitrotetrozolium violet (INT)-succinate reductase (mitochondria), β-hexosaminidase and β-glucuronidase (lysosomes), NADH-cytochrome *c* reductase and glucose-6-phosphatase (endoplasmic reticulum), and alkaline phosphodiesterase were determined spectrophotometrically (Harms et al., 1980; Pool et al., 1983); galactosyl transferase (Golgi) was assayed using ^{3}H-UDP-galactose (New England Nuclear, Boston, MA) and ovalbumin as an acceptor (Brew et al., 1975). All enzyme assays were carried out in the presence of 0.1% Triton X-100. The association of individual markers with membrane fractions (latency) was estimated by centrifuging samples at 100,000 *g* for 35 min (4°C) and assaying the resultant supernatants and pellets for enzyme activity.

HRP was determined by a modification of previously described procedures (Steinman et al., 1976). *O*-Dianisidine (Sigma Chemical Co.) was dissolved at 83 μg/ml in 50 mM phosphate buffer (pH 5) containing 0.1% Triton X-100. 0.1-ml aliquots of sample were mixed with 0.9 ml of substrate and the reaction started by adding 10 μl of 1% H_2O_2. After incubation for 2–4 min at room temperature, the reaction was stopped by adding 40 μl of 4% NaN_3 and the HRP activity estimated from the absorbance at 460 nm. Kinetic assays demonstrated that HRP activity was linear with respect to time under these conditions.

Acidification Assay

ATP-dependent acidification was determined as previously described using endosomes isolated from cells that had been selectively labeled by endocytosis of FITC-dextran (70,000 mol/wt; Sigma Chemical Co.) (Galloway et al., 1983; Robbins et al., 1983). CHO cells were labeled either in suspension or monolayer culture by incubation for 10–15 min at 37°C in medium containing 5–10 mg/ml FITC-dextran added from stock solutions (50 mg/ml) that had been extensively dialyzed against PBS before use. Organelles were diluted into isotonic buffer containing 145 mM KCl, 5 mM NaCl, 10 mM Hepes–tetramethylammonium hydroxide (TMA) (pH 7.4), and 5 mM $MgCl_2$. Proton transport was initiated by addition of 5 mM ATP (Na^+ or Tris salt, from a 0.5 M stock solution adjusted to pH 7.4), and the resulting pH gradients dissipated using 0.2 μM nigericin (Calbiochem-Behring Corp., La Jolla, CA) or 18 μM carbonylcyanide *m*-chlorophenylhydrazone (Sigma Chemical Co.). Acidification was estimated from the characteristic quenching of FITC fluorescence emission that occurs as a function of decreasing pH. All fluorescence measurements were made at ambient temperature using a spectrofluorometer (model LS-5; Perkin-Elmer Corp., Data Systems Group, Pomona, CA) with excitation and emission wavelengths set at 485 and 515 nm, respectively.

SDS PAGE

SDS PAGE was carried out under reducing conditions using either 4–11% acrylamide gradient gels (Neville and Glassman, 1974) or 10% acrylamide gels (Laemmli, 1970). The samples were electrophoresed either complete or as membrane protein fractions after Triton X-114 phase separation (Bordier, 1981). All samples were precipitated with 10% TCA before being dissolved in sample buffer. Gels were stained in Coomassie Blue and prepared for fluorography using sodium salicylate (Chamberlain, 1979).

Electron Microscopy

Free flow electrophoresis fractions were prepared for electron microscopy by being washed with fresh TEA buffer and centrifuged at 100,000 *g* for 35 min in an SW55 rotor (Beckman Instruments, Inc.). For samples derived from Percoll gradients, the centrifugation-resuspension was repeated several times by collecting the organelles as a loose layer on top of the compact Percoll pellet, resuspending, and recentrifuging until a membrane pellet largely devoid of Percoll was obtained. Membrane pellets were fixed in 2.5% glutaraldehyde in 100 mM cacodylate for 30 min at room temperature, subsequently in 2% osmium tetroxide for 1–2 h, and block stained overnight in 2% aqueous uranyl acetate. In HRP-labeled samples, the glutaraldehyde-fixed pellets were washed with fresh cacodylate buffer and incubated with 0.2% diaminobenzidine (DAB) in cacodylate buffer containing 0.002% H_2O_2 for 2 min. The samples were washed free of DAB, postfixed in reduced osmium, and stained with uranyl acetate. The samples were then dehydrated, embedded in Epon, sectioned, and viewed using a JEOL 100-CX electron microscope.

Results

Free Flow Electrophoresis of Endosomes and Lysosomes

Since intrinsic biochemical markers are not yet known, endosomes can only be identified after labeling with endocytic tracers. For the current experiments, CHO cell endosomes were labeled by 10–15-min exposures at 37°C to HRP, FITC-dextran, or [^{35}S]methionine-labeled SFV. Under these conditions, virtually all of the internalized tracers were present only in the low density fractions after Percoll gradient centrifugation (see below and Robbins et al., 1983). Similarly, no degradation of the labeled virus was detected (as TCA-soluble ^{35}S) during these incubations. By electron microscopy, the general appearance of the HRP-containing organelles was similar to that described for other cultured cells, e.g., BHK-21 cells (Marsh et al., 1986). The labeled vesicles comprised a morphologically heterogeneous population of organelles ranging from large vacuoles (200–500 nm in diameter) to smaller 50–60-nm vesicle profiles that are frequently sections through tubules radiating from the larger vesicles (Marsh et al., 1986).

Since the buoyant density of endosomes is similar to that of other smooth membrane organelles, density gradient centrifugation alone has been generally ineffective at separating endosomes from Golgi, endoplasmic reticulum, or plasma membrane (e.g., Merion and Sly, 1982; Wall and Hubbard,

1985). Thus, we sought other physical parameters that might distinguish endosomes from other cellular organelles and that could be exploited for endosome isolation. Free flow electrophoresis has previously been used to isolate highly purified populations of lysosomes from fibroblasts, lymphoblasts, and rat liver (Harms et al., 1980, 1981; Marsh et al., 1982). Separation is based on differences in surface charge of the particles subjected to electrophoresis, with lysosomes being more negatively charged than most other intracellular membranes (Hannig and Heidrich, 1977). As endosomes and lysosomes are functionally related in the endocytic pathway, it seemed reasonable that free flow electrophoresis could also be used for isolation of endosomes.

The standard conditions for the isolation of lysosomes did not prove effective for endosome separation. However, brief treatment of CHO homogenates with low concentrations of trypsin was found to facilitate subsequent coelectrophoresis and separation of endosomes and lysosomes from other intracellular organelles. CHO cells were labeled in suspension with FITC-dextran for 15 min, washed, and homogenized using a stainless steel ball bearing cell disrupter (Balch and Rothman, 1985). A 100,000 *g* microsomal pellet was prepared from the postnuclear supernatant and resuspended in TEA buffer to 1 mg protein/ml. The microsomal membranes were then injected at the cathode side of the free flow electrophoresis separation chamber with or without prior treatment with various concentrations of trypsin. As shown in Fig. 1, little separation was obtained using untreated membranes: both endosomes (FITC-dextran) and lysosomes (β-hexosaminidase) migrated in a single stream near the cathode. Brief treatment of the membranes with trypsin, however, resulted in a dose-dependent shift of both endosomal and lysosomal markers toward the anode and away from the major protein peak found to contain markers for almost all other organelles (see below and Harms et al., 1980). Trypsin was added by weight (relative to protein in the microsomal fraction) and the membranes incubated for 5 min at 37°C; digestion was terminated by the addition of excess soybean trypsin inhibitor. Lysosomes shifted at lower trypsin concentrations (0.1–0.2% trypsin by weight relative to cell protein) than endosomes (1–2% wt/wt) (Fig. 1). Optimal separation conditions were obtained at trypsin concentrations of 1–2% (corresponding to 10–20 μg/ml trypsin), where both FITC-dextran and β-hexosaminidase markers co-migrated in a single peak 10–12 fractions removed from the unshifted protein peak.

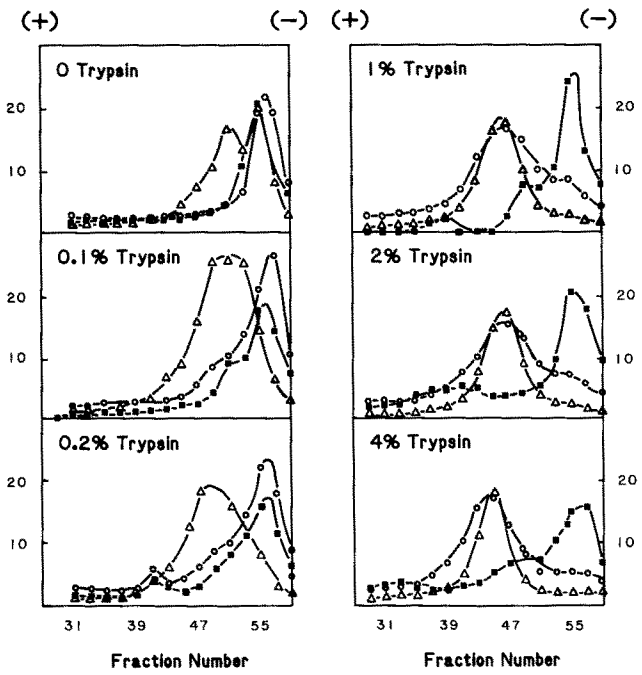

Figure 1. Effect of trypsin on the migration of CHO endosomes and lysosomes during free flow electrophoresis. Crude microsomes were prepared from CHO cells whose endosomes had been labeled in suspension by incubation with HRP for 15 min at 37°C. The protein concentration was determined and the membranes resuspended in TEA buffer at 1 mg/ml protein. TPCK-trypsin (Worthington Biochemical Corp., Freehold, NJ) was then added at the indicated concentrations (wt/wt; 1–40 μg/ml) for 5 min at 37°C before the addition of excess soybean trypsin inhibitor. 0.5-ml samples were then injected into a free flow electrophoresis separation chamber, fractions collected, and assayed for FITC-dextran (*open circles*), β-hexosaminidase activity (lysosomes) (*open triangles*), and total protein (*solid squares*). The positions of the anode (+) and cathode (−) are shown. Activities are given as arbitrary units, but the recovery of protein and both enzymes after electrophoresis was >80% for each sample.

Two-step Analytical Isolation of Endosomes

To determine the extent to which the free flow electrophoresis step resolved endosomes and lysosomes from other organelles, we determined the distribution of several marker enzymes across the sample field. Markers for mitochondria (INT-succinate reductase, Fig. 2 *A*), plasma membrane (cell surface proteins radioiodinated in the cold using lactoperoxidase, Fig. 2 *B*; Na^+,K^+-ATPase, Fig. 2 *C*), and endoplasmic reticulum (SFV spike glycoproteins pulse labeled with [^{35}S]methionine, Fig. 2 *C*) were found to co-migrate with the major peak of protein (Fig. 2 *A*). Golgi membranes (galactosyl transferase activity, Fig. 2 *C*) were resolved into two major peaks, one of which was unshifted and the other deflected slightly toward the anode, as found previously (Morré et al., 1984). Marker enzymes for soluble cytosolic proteins (e.g., glucose-6-phosphate dehydrogenase; data not shown) also migrated with the unshifted peak. Thus, the combined endosome–lysosome pool was largely depleted of marker enzymes for other organelles. Separation of the endosomes from lysosomes in the pooled shifted fraction was accomplished by Percoll density gradient centrifugation, a procedure that effectively resolves low density endosomes from the much higher density lysosomes (Fig. 2 *D*).

This two-step analytical procedure resulted in an enrichment of the endosomal marker HRP of at least 43-fold, with an overall yield of 11% (see Table I). Both of these figures are probably underestimates of the degree of enrichment obtained because much of HRP was likely to have been lost during the purification steps as a result of organelle disruption. Indeed, the use of a receptor-bound ligand (SFV) as an endosome marker resulted in higher yields and purification factors (see below). Nevertheless, even the 43-fold enrichment of the fluid phase endosomal marker HRP was far greater than that observed for lysosomes (β-hexosaminidase; six-fold), plasma membrane (0.03-fold), and mitochondria (0.09-fold).

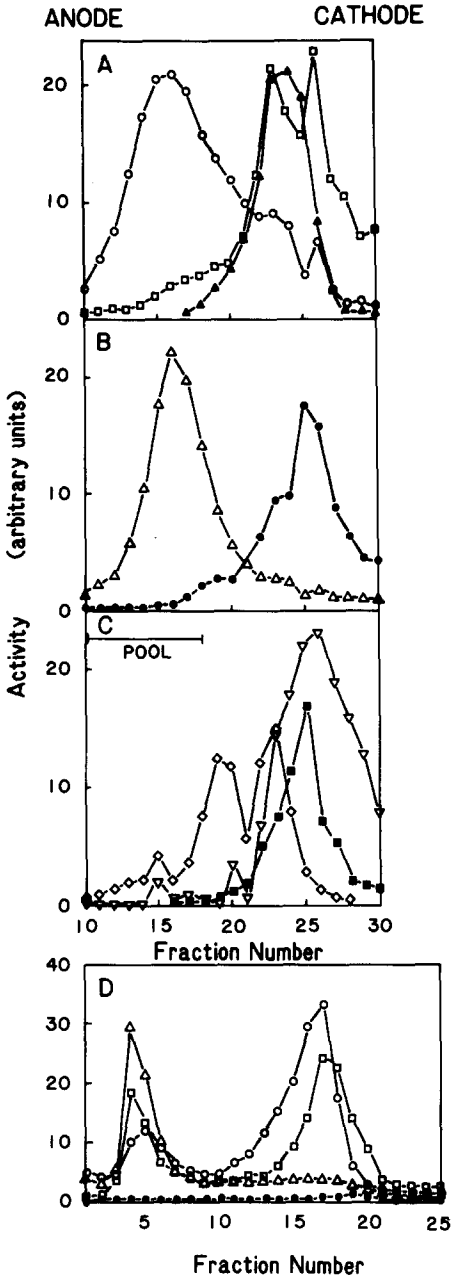

Figure 2. Two-step isolation of endosomes and lysosomes from CHO cells. CHO cells were labeled with 10 mg/ml HRP for 10 min at 37°C, washed with cold PBS, and combined with the cells from one confluent 100-mm plate that had been infected with 50 pfu/cell SFV for 4 h and subsequently pulse-labeled for 5 min with 0.2 μCi/ml [^{35}S]methionine (to provide a marker for the rough endoplasmic reticulum, see Materials and Methods). In a separate experiment, a confluent plate of CHO cells was iodinated at 0°C to provide a marker for the cell surface. Labeled cells were pooled, homogenized, and a microsome fraction was separated by free flow electrophoresis. The fractions containing the endosomal (HRP) and lysosomal (β-hexosaminidase) markers were pooled as indicated, made 27% with isotonic Percoll, and centrifuged at 16,000 rpm for 1 h in a rotor (model VTi50; Beckman Instruments, Inc.). The markers are expressed in arbitrary units (AU). (*A*) Endosomes, HRP (*open circles*) 10 AU ≈ 100 A_{460}; total protein (*open squares*) 10 AU = 20 μg/ml; mitochondria, INT-succinate reductase (*solid triangles*) 10 AU = 100 A_{490}; (*B*) lysosomes, β-hexosaminidase (*open triangles*) 10 AU = 0.4 R_f (at 365 nm excitation/450 nm emission); plasma membrane, ^{125}I (*solid circles*) 10 AU = 2 × 10^4 cpm; (*C*) Golgi membranes, galactosyl transferase (*open diamonds*) 10 AU = 250 cpm; plasma membrane, NA^+,K^+-ATPase (*open triangles*) 10 AU = 1 × 10^3 cpm; rough ER, [^{35}S]methionine (*solid squares*) 10 AU = 4 × 10^4 cpm; (*D*) Percoll density gradient sedimentation of the pooled endosome and lysosome fractions. The heavy density region of the gradient is on the left, and the marker enzymes indicated are HRP (*open circles*), protein (*open squares*), β-hexosaminidase (*open triangles*), and ^{125}I-plasma membrane (*solid circles*). Na^+,K^+-ATPase activity was determined as K^+-stimulated, ouabain-inhibitable ^{32}P-ATP hydrolysis, as previously described (Forbush, 1983).

Isolation of endosomal and lysosomal fractions from CHO cells was both rapid (the entire procedure required 5–6 h) and reproducible, so long as care was taken to ensure the correct trypsin-to-microsomal protein ratio at the digestion step. Thus, microsomal protein was routinely determined (Bradford, 1976) before the addition of trypsin. As few as 5 × 10^7 cells could be processed per run. The procedure was also found to be applicable to cell types other than CHO cells. Results equivalent to those shown in Fig. 2 were obtained using crude homogenates or microsomal membranes prepared from BHK-21 cells. In addition, free flow electrophoresis also permitted the preparation of an enriched endosome fraction from rat liver (Fuchs, R., P. Male, and I. Mellman, manuscript in preparation).

Preparative Isolation and Analysis of Endosomes

To study the biochemical and functional properties of endosomes, it was necessary to develop an isolation procedure for larger quantities of cells (>10^9 per experiment). The procedure described above was limited to analytical scale by the fact that the protein concentration of the injected sample could not exceed 1 mg/ml. Samples at higher concentrations tended to aggregate, limiting the effectiveness of the electrophoresis step. We therefore modified the procedure to preenrich the endosomes before electrophoresis.

Enrichment was accomplished by centrifugation in discontinuous sucrose density gradients, adapting a procedure originally designed for the enrichment of Golgi membranes from CHO cell homogenates (Balch et al., 1984). After endosome labeling, CHO cells were homogenized and microsomes prepared as described above. The microsomal pellet was resuspended in 1.2 M sucrose and layered under a gradient consisting of 1 and 0.6 M sucrose in TEA buffer. After centrifugation, the endosome/Golgi fraction was collected at the 1 M/0.6 M interface and found to be significantly enriched in the endosomal markers, particularly ^{35}S-SFV. As summarized in Table II, significant losses in the recovery of the endocytic tracers accompanied the production of the microsomal and sucrose gradient fractions. Recovery of FITC-dextran in the endosome/Golgi fraction (only 6.6%) was far below that obtained for ^{35}S-SFV (32%), presumably because FITC-dextran (like HRP) is a fluid phase marker that would be lost if any endosomal vesicles were disrupted at any step during the procedure. Nevertheless, the enrichment (relative to protein) of both tracers was enriched in the endosome/Golgi fraction (^{35}S-SFV, 22-fold; FITC-dextran, 4.5-fold) when compared with the enrichments of mitochondrial, endoplasmic reticulum, or lysosomal markers (Table

Table I. Two-step Isolation of Endosomes from CHO Cells

	Activity in initial homogenate				
	Endosomes (HRP)	Lysosomes (β-hexosaminidase)	Plasma membrane (^{125}I)	Mitochondria (1NT-succinate reductase)	Protein ([^{35}S]methionine)
	%	%	%	%	%
Postnuclear supernatant	60 (1)	59 (1)	54 (1)	26 (1)	70
Nuclear pellet	39 (1.5)	40 (1.6)	29 (1.3)	64 (5.8)	30
Microsomes	21 (1.9)	29 (2.7)	20 (2.0)	6.0 (1.3)	13
Free flow electrophoresis endosome/ lysosome pool	11 (16)	11 (16)	0.1 (0.16)	0.03 (0.10)	0.8
Percoll gradient endosome pool	11 (43)	2 (7.9)	0.07 (0.03)	0.01 (0.09)	0.3

Numbers in parentheses indicate the fold-enrichment (relative to [^{34}S]methionine). All values have been normalized to the "specific activity" in the postnuclear supernatant. Data are averaged from three independent experiments.

Table II. Isolation of Endosomes from CHO Cells by Gradient Centrifugation and Free Flow Electrophoresis

	Activity in initial homogenate						
	Endosomes						
	(FITC-dextran)	[^{35}S-SFV]	Golgi (galactosyl transferase)	Lysosomes (β-hexo-saminidase)	Endoplasmic reticulum (glucose-6 phosphatase)	Mitochondria (INT-succinate reductase)	Protein
	%	%	%	%	%	%	*mg*
Homogenate	100	100 (1)	100 (1)	100 (1)	100 (1)	100 (1)	106
Postnuclear supernatant	73 (1)	100 (1.6)	73 (1)	60 (0.9)	72 (1.1)	43 (0.6)	85
Microsomes	37 (2.6)	68 (4.9)	46 (2.8)	43 (2.7)	32 (2.0)	16 (1.0)	14.8
Endosome/Golgi fraction	6.6 (4.5)	32 (22)	21 (4.7)	9.1 (2.0)	9.6 (2.1)	0.7 (0.1)	1.5
Endosome pool	1.7 (10)	12.4 (68)	3.2 (30.9)	1.1 (10.5)	ND	ND	0.17

Numbers in parentheses indicate the enrichment factor, relative to protein (Sims and Carnegie, 1975). All values have been normalized to the "specific activity" in the initial homogenate. Data are averaged from three independent experiments.

II). Only the Golgi marker, galactosyl transferase, was enriched (4.7-fold) to an extent comparable with FITC-dextran but still much less than ^{35}S-SFV.

When injected into the free flow electrophoresis separation chamber, the endosome/Golgi fraction migrated as a single turbid stream. As with crude microsomes, gentle trypsinization was required for the anodal shift of a second stream containing the endosomes. To minimize the severity of digestion, we titrated the amount of trypsin needed to effect separation against the ability of FITC-dextran loaded endosomes to carry out ATP-dependent acidification (Galloway et al., 1983). In contrast to the crude microsomal membranes, optimal separation of endosomes from the endosome/Golgi fraction required treatment with only 0.25% trypsin (corresponding to 1–2 μg trypsin/mg protein). Importantly, incubation with trypsin at this concentration (and up to 1%) for 5 min at 37°C did not affect either the rate or the extent of endosome acidification observed after the addition of ATP (Fig. 3). Thus, in all subsequent experiments, we used trypsin concentrations (0.25%) that were well below the threshold for inactivation of this crucial endogenous endosomal function.

Fig. 4 illustrates the migration of endosomes (labeled with FITC-dextran or ^{35}S-SFV) and other organelles after free flow electrophoresis of the endosome/Golgi fraction. Activities are plotted as percentages of the initial homogenate (see Table II). Endosomes migrated as a broad peak well separated from the major unshifted protein peak (Fig. 4 *A*). FITC-dextran–labeled endosomes generally shifted slightly more toward the anode than ^{35}S-SFV-labeled endosomes, in a position very similar to that of β-hexosaminidase activity (Fig. 4 *B*). Free ^{35}S-SFV added to unlabeled cell homogenates did not shift towards the anode, indicating that the electrophoretic migration of this marker was due to its presence within (and not adsorbed to) endosomes. Glucose-6-phosphatase activity, a marker for endoplasmic reticulum membranes, co-migrated with the unshifted protein peak while galactosyl transferase–containing membranes (Golgi) were again resolved into two peaks, the larger of which shifted to a posi-

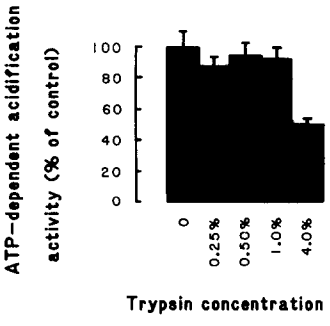

Figure 3. Effect of trypsin on ATP-dependent acidification of the endosome/Golgi fraction. An enriched endosome/Golgi fraction was prepared by sucrose density gradient centrifugation from CHO cells whose endosomes had been labeled with the pH sensitive fluorochrome FITC-dextran. ATP-dependent acidification of this fraction was then determined (Galloway et al., 1983) with or without prior treatment with TPCK-trypsin (0.25–4.0%, wt/wt with respect to protein concentration in the fraction). Trypsin concentrations as high as 1% had no effect on acidification activity whether acidification was expressed as the initial rate or the final extent (after 10 min) of FITC fluorescence quenching due to the addition of ATP.

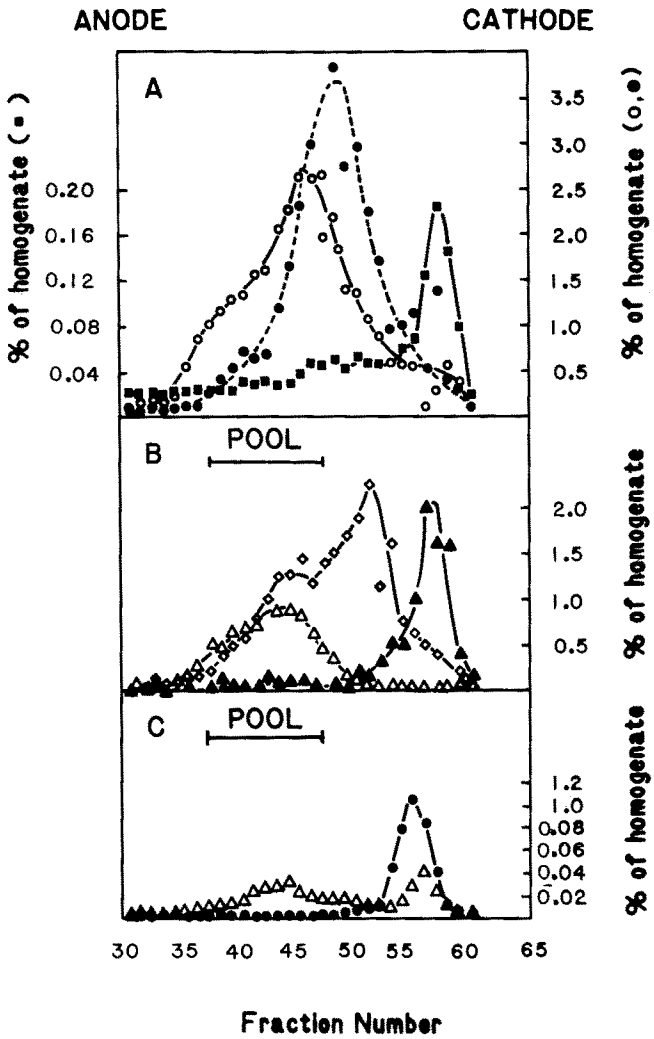

Figure 4. Separation of an enriched endosome/Golgi fraction by free flow electrophoresis. An enriched endosome/Golgi fraction was prepared from CHO cells combined after endosome labeling with FITC-dextran and ^{35}S-SFV and cell surface labeling with ^{125}I. After treatment with 0.25% trypsin (see Materials and Methods), the sample was injected into the free flow electrophoresis separation chamber, fractions collected, and marker enzymes determined. All enzyme and marker activities were normalized and expressed as percentages of that found in the initial homogenate. (*A*) ^{35}S-SFV (*solid circles*), FITC-dextran (*open circles*), total protein (*solid squares*); (*B*) galactosyl transferase (*open diamonds*), β-hexosaminidase (*open triangles*), glucose-6 phosphatase (ER) (*solid triangles*); (*C*) alkaline phosphodiesterase (plasma membrane) (*open triangles*), ^{125}I-plasma membrane (*solid circles*). Fractions pooled for Percoll density gradient analysis are indicated.

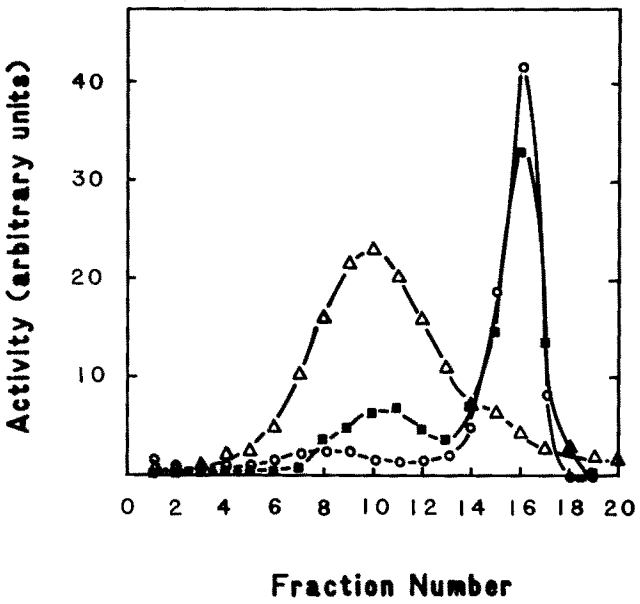

Figure 5. Percoll density gradient centrifugation of endosome/lysosome fractions obtained after free flow electrophoresis. Fractions indicated in Fig. 4 were pooled and centrifuged in 27% Percoll. Shown are markers for endosomes (^{35}S-SFV) (*open circles*), lysosomes (β-hexosaminidase) (*open triangles*), and protein (*solid squares*). Activities were normalized to reflect percentages of the initial homogenate (as in Fig. 4). Galactosyl transferase and alkaline phosphodiesterase activities could not be distinguished from background in the Percoll gradient fractions and are therefore not shown.

tion intermediate between the endosomal and unshifted peaks (Fig. 4 *B*).

The position of plasma membrane markers is shown in Fig. 4 *C*. ^{125}I-Labeled CHO cell surface proteins, labeled with lactoperoxidase-glucose oxidase at 0°C, were found only in the unshifted fractions. In contrast, the small amount of alkaline phosphodiesterase present in the endosome/Golgi fraction was resolved into two distinct peaks, one comigrating with the major unshifted protein peak and the other with the anodally shifted endosomal peak. The latter peak may thus reflect the presence of alkaline phosphodiesterase activity in endosomal membranes as opposed to contaminating plasma membrane vesicles.

In spite of the overlap with a subset of the galactosyl transferase–containing membranes, the free flow step resulted in a considerable additional enrichment of endosomal markers in the pooled fractions indicated on Fig. 4. As shown in Table II, the final degree of purification of ^{35}S-SFV was ~68-fold. While galactosyl transferase activity in this pool was enriched 31-fold, it is not yet clear whether this activity reflects contamination by Golgi cisternae or by the endosome-like elements of the *trans* Golgi network (Griffiths et al., 1985; Geuze et al., 1985; Roth et al., 1986; Marsh et al., 1986) (see below).

The combined endosome pool was further processed by Percoll gradient centrifugation to resolve endosomes from any remaining lysosomes. As shown in Fig. 5, most (>80%) of the protein present in the endosomal pool was associated with low density membranes as opposed to heavy density lysosomes. Thus, the activity of the lysosomal marker enzyme β-hexosaminidase present in the endosomal pool disproportionately reflects the presence of lysosomes due to the sensitivity of the fluorometric assay used to measure hydrolase activity. All of the endosomal marker (FITC-dextran in Fig. 5) was found in the low density region of the gradient.

Electron Microscopy of the Endosomal Fraction

To further characterize the isolated endosomes, electron microscopy was performed on membrane pellets made from the endosome/Golgi fraction and the resultant endosomal pool

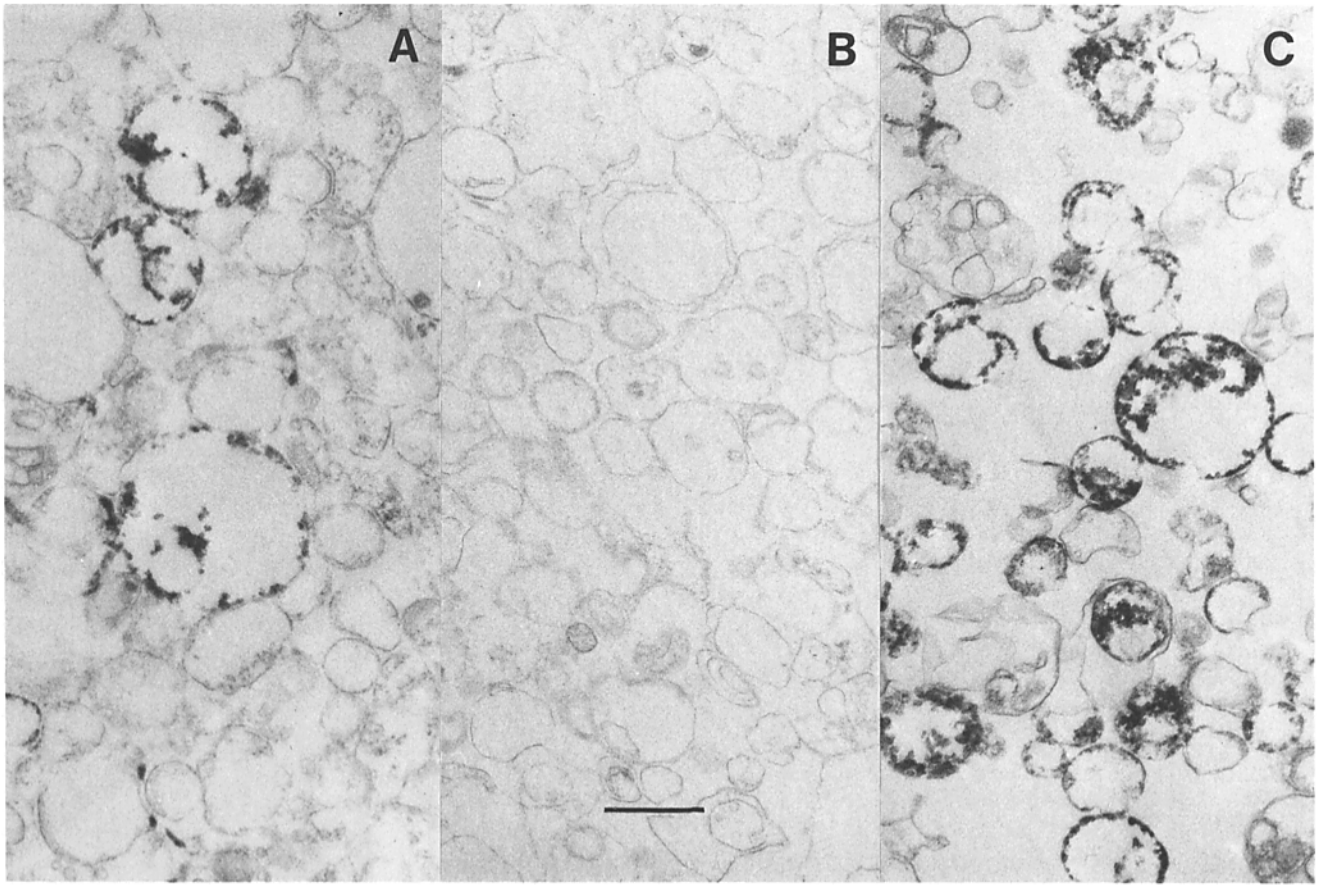

Figure 6. Thin section electron micrograph of isolated endosomes. CHO cells were labeled for 10 min with HRP and an endosome/Golgi fraction prepared and fractionated by free flow electrophoresis as in Fig. 4. Samples from the endosome/Golgi peak and the endosome peak obtained after the electrophoresis step were centrifuged at 100,000 *g* for 35 min and the pellet fixed in 2.5% glutaraldehyde in cacodylate buffer. The fixed pellets were incubated with 0.2% DAB for 5 min, washed by centrifugation with cacodylate, postfixed with osmium tetroxide, and processed for electron microscopy. *A* shows the endosome/Golgi fraction before electrophoresis; the fraction contains many membranous organelles, some of which are recognizable as Golgi cisternae. The electron dense HRP/DAB reaction product can be seen in some of the organelle profiles. *B* and *C* show isolated endosomes with (*C*) and without (*B*) DAB staining. The endosomes appear as mostly empty vacuolar profiles with occasional tubular projections of membrane. The electron dense DAB reaction product is seen in most (~75%) of the vesicular profiles. Bar, 0.5 μM.

isolated from CHO cells labeled with HRP, visualized by reaction with DAB-H_2O_2. As shown in Fig. 6, the initial endosome/Golgi fraction contained a low frequency of HRP-positive vesicles and tubules (Fig. 6 *A*). In contrast, a large proportion (~75%) of the vesicles in the anodally shifted fractions exhibited the HRP reaction product (Fig. 6 *C*). Fig. 6 *B* shows vesicles from the same fraction that were not reacted with DAB, demonstrating the specificity of the reaction product. Positive vesicles ranged in diameter from 0.2 to >0.7 μm, and occasionally had tubular extensions. Similar fields were observed with sections obtained from both the top and the bottom of the organelle pellet. It is not clear whether the empty vesicle profiles in Fig. 6 *C* represent contaminating nonendosomal vesicles, or endosomes that had lost internal HRP due to disruption at some point during the isolation. As discussed above, fluid phase markers such as HRP appear to be less efficiently recovered than do receptor-bound markers (e.g., ^{35}S-SFV).

ATP-dependent Acidification of Isolated Endosomes

As described above, gentle trypsinization of crude microsomes or the endosome/Golgi fraction before electrophoresis did not adversely affect ATP-dependent endosome acidification. To determine whether the final endosome fractions still retained acidification activity, FITC-dextran–labeled endosomes were separated by free flow electrophoresis. Aliquots containing equivalent amounts of FITC fluorescence were taken from the initial homogenate and from the pooled endosome fractions and suspended in spectrofluorometer cuvettes. When ATP and Mg^{++} were added, both samples displayed comparable amounts of fluorescence quenching which was reversed by the addition of the carboxylic ionophore nigericin (Fig. 7). Acidification activity was also retained after Percoll gradient centrifugation. These results indicated that endosomes prepared by free flow electrophoresis were capable of ATP-dependent acidification. The enrichment of ATP-dependent acidification activity relative to protein [^{35}S]methionine) in the endosome fractions was >100-fold over the homogenate, confirming the estimates of purification obtained using ^{35}S-SFV (Table II). Perhaps due to their relative purity, the FITC-dextran–labeled endosomes were exceedingly stable and could be kept up to 24 h at 4°C or 6 h at room temperature without loss of activity. This sta-

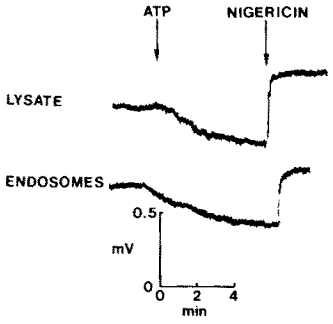

Figure 7. Acidification in isolated CHO cell endosomes. CHO cells were labeled for 10 min with FITC-dextran, homogenized, and fractionated by sucrose gradient centrifugation and free flow electrophoresis as described in Fig. 4. The isolated endosomes and a sample of the initial postnuclear supernatant (lysate) were resuspended into 10 mM Hepes buffer containing 125 mM KCl and ATP-dependent acidification assayed by the decrease in FITC fluorescence emission at 515 nm (Galloway et al., 1983).

bility facilitated their equilibration with various ionic media, thus permitting the detailed investigation of the mechanisms of endosome acidification (Fuchs, R., P. Male, and I. Mellman, manuscript in preparation).

Polypeptide Composition of Endosomes

The polypeptide composition of endosomes was studied by SDS PAGE using fractions obtained from [^{35}S]methionine-labeled CHO cells. The protein patterns were compared with membrane proteins found in lysosomes, with the proteins of the total sample applied to free flow electrophoresis separation, and with the plasma membrane proteins radioiodinated using the lactoperoxidase–glucose oxidase technique. To differentiate between integral membrane proteins and adsorbed soluble protein components, the samples were partitioned in the nonionic detergent Triton X-114, which allows separation of detergent-binding amphiphilic proteins from hydrophilic soluble proteins and peripheral membrane proteins (Bordier, 1981).

The protein patterns obtained for the various fractions are illustrated in Figs. 8 and 9. Fig. 8 contains fractions obtained by the preparative endosome isolation procedure and shows the total protein pattern (*left*) as well as the proteins that partition into the Triton X-114 detergent (*center*) and aqueous (*right*) phases. Lane *1* in each panel shows the pattern of cell surface proteins accessible to iodination by lactoperoxidase. While all fractions displayed complex polypeptide patterns, the proteins recovered in the detergent phase generally showed less well-defined bands than those in the aqueous phase, suggesting that they were glycoproteins. Significant similarities and differences between the various patterns were observed. These allowed the following general conclusions: (*a*) The endosomal protein composition (lane *2* in each panel) was clearly distinct from overall protein in the unshifted protein peak (lane *3*) and in the initial endosome/Golgi fraction (lanes *4* and *5*). Several major proteins appeared to be enriched in the endosomal fraction and ranged in molecular mass from 35 to 150 kD. A 45-kD band (probably actin) that was a major component of the initial sample was virtually absent in the endosomal fraction, further indicating that the electrophoresis step effectively separates the endosomes and lysosomes from most soluble cytosolic proteins. (*b*) Trypsin treatment before electrophoresis had negligible effects on the pattern of labeled proteins in the endosome/Golgi fraction (lanes *4* and *5* were plus and minus trypsin, respectively). Thus, the trypsin-sensitive components responsible for the alteration in electrophoretic mobility did not constitute major proteins in the fraction. (*c*) Although some overlap was apparent, the endosomal protein profile was different from that observed for surface iodinated

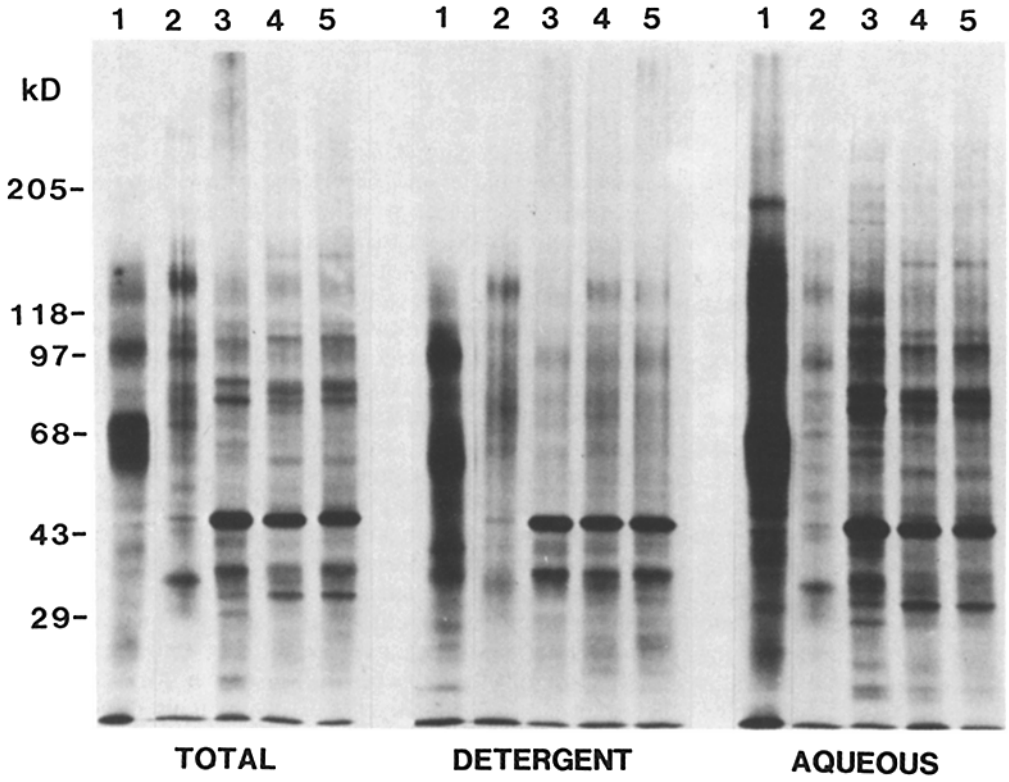

Figure 8. SDS PAGE of CHO cell endosomes. A confluent plate of CHO cells were labeled with 1 mCi [^{35}S]methionine overnight, combined with unlabeled carrier cells, and fractionated by sucrose density gradient centrifugation and free flow electrophoresis. Aliquots of the initial endosome/Golgi fraction (before and after trypsin treatment) and the electrophoretically isolated endosome fraction were lysed in Triton X-114, precipitated with 10% TCA, and subjected to SDS PAGE (5–10% acrylamide gradients) (Laemmli, 1970). (Lane *1*) CHO cell surface proteins labeled with ^{125}I; (lane *2*) endosome/lysosome fraction after free flow electrophoresis; (lane *3*) unshifted free flow fractions corresponding to the protein peak; (lane *4*) initial endosome/Golgi fraction after trypsinization; (lane *5*) endosome/Golgi fraction without trypsin treatment. (*A*) Total protein; (*B*) Triton X-114 detergent phase (membrane proteins); (*C*) aqueous phases (nonmembrane proteins).

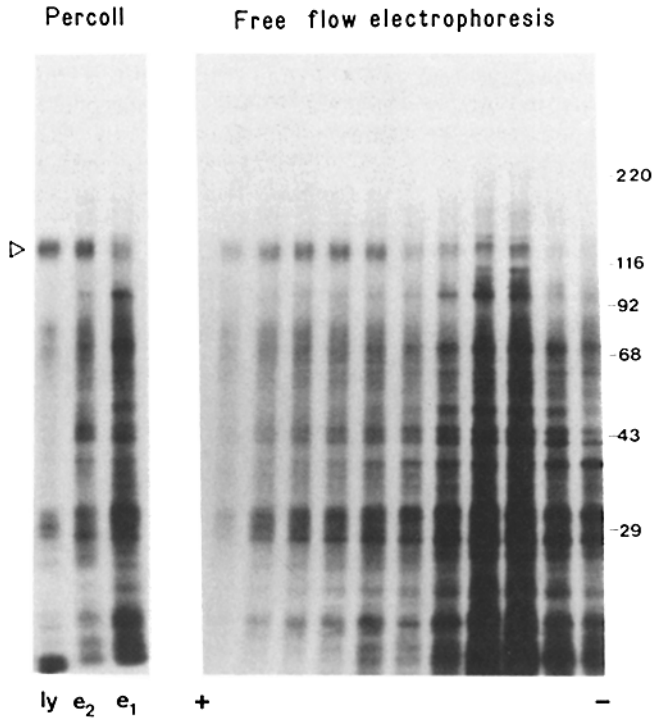

Figure 9. SDS PAGE of fractions obtained after free flow electrophoresis and Percoll density gradient centrifugation. Crude microsomes were prepared from CHO cells labeled with [^{35}S]-methionine and separated by free flow electrophoresis. (*Right*) Samples were taken from approximately every third fraction (beginning at fraction 30, as in Fig. 2) and lysed in Triton X-114. Membrane proteins were isolated by detergent condensation (Bordier, 1981) and subjected to SDS PAGE (4–11% gels) (Neville and Glassman, 1974). The five left-most lanes correspond to the shifted combined endosome–lysosome peak (see Fig. 2). Note the enrichment for labeled proteins at 100–120 kD in these lanes. (*Left*) After centrifugation of the combined endosome–lysosome peak in Percoll density gradients, aliquots were again removed, lysed, and phase-separated using Triton X-114 and analyzed by SDS PAGE. (*ly*) The heavy density lysosome fraction, corresponding to fraction 15 in Fig. 2 *D*. (e_1 and e_2) Samples taken from the low density endosomal fractions, corresponding to fractions 17 and 18 (e_1) and fractions 14 and 15 (e_2) in Fig. 2 *D*.

plasma membrane proteins. This may suggest that endosomal membranes differ in protein composition from that of the plasma membrane, but the result could also be explained by the different labeling methods used. Studies are in progress to compare the two membrane protein patterns more rigorously.

Endosomal and lysosomal membrane proteins are compared in Fig. 9. In this case, the analytical isolation procedure was used because it allowed purification of lysosomes and endosomes from the same original homogenate. The right panel illustrates the separation of Triton X-114–binding membrane proteins in individual fractions taken directly after the free flow electrophoresis step. It is apparent that the five lanes to the left, which represent the combined endosome/lysosome peak, have protein composition different from that of the main protein peak in the lanes to the right. The enrichment of 110–120-kD bands (*arrow*) is observed corresponding to the major lysosomal membrane glycoproteins lgp110 and lgp120, identified previously (Lewis et al., 1985; Granger et al., 1985).

The left panel in Fig. 9 shows the membrane protein patterns after Percoll gradient separation of the endosome/lysosome peak. The lysosomes (*ly*) exhibited a relatively simple protein profile that was greatly enriched in the 110–120-kD band. The endosomal peak was divided into two fractions, corresponding to lighter (e_1) and denser halves (e_2) of the low density endosome peak (see Fig. 2). The proteins in the e_1 pool displayed a variety of lower molecular mass membrane proteins and were relatively depleted of the 110–120-kD band. The pattern of proteins associated with the e_2 pool were intermediate between the low density endosomes and the lysosomes. The results suggested that the endosomal and lysosomal membranes have distinct, but related, polypeptide compositions.

Discussion

Endosomes do not constitute a single, easily identified class of organelles, nor do they behave as a unique and homogeneous fraction when subjected to standard cell fractionation techniques. Even within single cells, endosomes differ widely in structure, cellular location, internal composition, morphology, acidity, density, and size (Helenius et al., 1983; Harford et al., 1983; Murphy et al., 1984; Wall and Hubbard, 1985; Mellman et al., 1986; Marsh et al., 1986). Whether this heterogeneity reflects an underlying process of maturation and organelle movement or the existence of a complex set of independent, permanent organelles is not clear (Helenius et al., 1983), but it certainly complicates any attempts to isolate enriched and representative fractions. These problems are compounded by the endosomes' similarity in physical properties with other smooth membranes, by their relative fragility, and by the lack of known intrinsic markers. The elusive nature of endosomes helps to explain why their existence as important functional components of the endocytic pathway was only recently recognized (Helenius et al., 1983; Hopkins, 1983; Pastan and Willingham, 1983).

The choice of endocytic tracer to monitor endosome purification is important since it alone defines target population. In this study, our purpose was to purify endosomes representative of the entire population of prelysosomal vacuoles, from the early peripheral endosomes to the more mature, multivesicular, perinuclear endosomes (Helenius et al., 1983). Thus, we used well characterized markers that were efficiently internalized and delivered to lysosomes via the standard pathway. HRP and SFV can be visualized by electron microscopy, and both have been shown to occupy the same set of endosomal organelles (Marsh et al., 1986). FITC-dextran offered the advantage that it can be used to monitor endosome acidification in vitro (Galloway et al., 1983). As fluid phase markers, HRP and presumably FITC-dextran are present in both the tubular and vesicular endosomal elements, whereas the virus particles, due to their larger size, tend to be located in the vacuolar portions of the endosomes (Marsh et al., 1986; Helenius et al., 1980).

The membrane association of the virus particles most likely explained the higher recovery of ^{35}S-SFV compared with fluid phase markers. Alternatively, the procedure may have enriched for endosomal elements derived from the

more spherical vacuolar portions of endosomes. The profiles observed after electron microscopy were usually rounded and exhibited few remaining tubules. This could be due to the selective loss of tubules during homogenization and isolation, or to deformation of the endosomes due to osmotic and other stresses during the isolation or fixation processes. Some disruption of endosomes occurred, particularly during the homogenization step where up to 35% of the HRP was rendered nonlatent. Clearly, such unavoidable losses during lysis and subsequent steps would tend to decrease the specific activity of a sequestered marker. Thus, it is likely that our final values for purity and recovery of endosomes are underestimated.

While free flow electrophoresis has previously been used in cell and organelle fractionation (Hannig and Heidrich, 1977; Harms et al., 1980, 1981; Morré et al., 1984; Pesonen et al., 1984), this is the first time that it has been successfully used for preparative and analytical purification of endosomes. An indication of its potential usefulness was first obtained by Debanne et al. (1982) who reported that asialotransferrin-containing organelles migrated slightly more anodally than bulk protein peak during free flow electrophoresis of rat liver homogenates. We have found that in CHO cells, BHK-21 cells, as well as in rat liver cells (Fuchs, R., P. Male, and I. Mellman, manuscript in preparation), this separation could be dramatically improved by the mild trypsin treatment before electrophoresis. Since the trypsinization step was arrived at empirically, we can, at present, only speculate as to why it increases the electrophoretic mobility of the endosomes. Conceivably, the trypsin may remove basic proteins adsorbed to the cytoplasmic aspects of the endosomal membrane, e.g., elements of the cytoskeleton (Beardmore, J., and C. Hopkins, personal communication). Alternatively, the trypsin might facilitate the disaggregation of organelles in the homogenate. While it would have been preferable to avoid a protease step, we found that the trypsin did not drastically affect the intrinsic properties of endosomes. SDS PAGE analysis of homogenates before and after trypsin digestion did not reveal massive degradation of protein nor loss of major protein bands. The digestion did not affect the latency of endocytic markers, the buoyant density of the organelles, or the capacity of the endosomes to exhibit ATP-dependent acidification in vitro. To what extent other endosome-specific functions are affected will become clear as additional assays are developed. We are testing other procedures to substitute for the trypsin step but have thus far been unable to obtain satisfactory results.

Golgi-derived, galactosyltransferase-containing membranes remain the major contaminants of the endosomal fractions. Studies from other laboratories have conversely indicated that endosomes constitute a major contaminant in Golgi fractions (Posner et al., 1980; Bergeron et al., 1985). In fact, for the preparative isolation of endosomes, we have relied on Golgi fractions as the starting material. The close intracellular proximity of endosomes and *trans* elements of the Golgi complex have been noted in many cell types and it has been suggested that endocytosed material, in fact, passes through the *trans* Golgi elements en route to lysosomes or the plasma membrane (Pastan and Willingham, 1983; Yamashiro et al., 1984; Snider and Rogers, 1985). However, recent studies by Griffiths and Simons (1986) indicate that the HRP-containing endosomes in the *trans* Golgi region are distinct from *trans* Golgi elements containing newly synthesized G protein in vesicular stomatitis virus–infected BHK-21 cells. This suggests that exocytic and endocytic compartments in this region of the Golgi are separate. The free flow electrophoresis profile of galactosyltransferase-containing membranes shows two distinct peaks, with only the more anodally migrating one displaying partial overlap with the endosomal peak. Whether there is actually a co-distribution of the galactosyltransferase and endocytic markers in the same vacuoles, or merely a similarity in the physical properties of two or more unrelated membrane populations, remains to be determined.

The isolation procedures described here provide a starting point for the biochemical and functional analysis of endosomes in vitro. Among the major advantages over previous methods is the relative rapidity of electrophoretic isolation. The analytical procedure requires <3 h, allowing up to 10 samples to be processed per day. The procedure also yields endosomes of high purity that appear to be functionally intact, at least with respect to ATP-driven acidification. Purification does not depend on endogenous or externally added density perturbants, thus allowing its adaptation to a variety of cell and tissue types. The main disadvantages are the cost of the equipment needed to conduct the electrophoretic separation and the necessity for trypsin digestion.

The isolated endosomes have already proved useful for the detailed analysis of the bioenergetics of endosome acidification (Fuchs, R., P. Male, and I. Mellman, manuscript in preparation). We are currently pursuing the biochemical analysis of the endosomal fractions to identify structural and functional components that may be unique to the endosomal membrane or to endosome subpopulations.

We wish to thank Helen Plutner, Jürgen Kartenbeck, Brigitte Nade, Eva Bolzau, and David Robertson for advice and assistance, and Margaret Moench for artwork.

This work was supported by grants from the National Institutes of Health (to I. Mellman and to A. Helenius), the Deutsche Forschungsgemeinschaft (to E. Harms), the Cancer Research Campaign/Medical Research Council (to M. Marsh), the Helen Hay Whitney Foundation (to S. Schmid), and by a NATO Travel Award.

Received for publication 2 December 1986, and in revised form 22 December 1986.

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