Acidification and Ion Permeabilities of Highly Purified Rat Liver Endosomes*

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While it is well established that acidic pH in endosomes plays a critical role in mediating the orderly traffic of receptors and ligands during endocytosis, little is known about the bioenergetics or regulation of endosome acidification. Using highly enriched fractions of rat liver endosomes prepared by free flow electrophoresis and sucrose density gradient centrifugation, we have analyzed the mechanism of ATP-dependent acidification and ion permeability properties of the endosomal membrane. This procedure permitted the isolation of endosome fractions which were up to 200-fold enriched as indicated by the increased specific activity of ATP-dependent proton transport. Acidification was monitored using hepatocyte and total liver endosomes selectively labeled with pH-sensitive markers of receptor-mediated endocytosis (fluorescein isothiocyanate asialoorosomucoid) or fluid-phase endocytosis (fluorescein isothiocyanate-dextran). In addition, changes in membrane potential accompanying ATP-dependent acidification were directly measured using the voltage-sensitive fluorescent dye Di-S-C3(5). Our results indicate that ATP-dependent acidification of liver endosomes is electrogenic, with proton transport being accompanied by the generation of an internal-positive membrane potential opposing further acidification. The membrane potential can be dissipated by the influx of permeant external anions or efflux of internal alkali cations. Replacement externally of permeable anions with less permeable anions (e.g., replacing Cl⁻ with gluconate) diminished acidification, as did replacement internally of a more permeant cation K⁺ with less permeant species (such as Na⁺ or tetramethylammonium). ATP-dependent H⁺ transport was not coupled to any specific anion or cation, however. The endosomal membrane was found to be extremely permeable to protons, with protons able to leak out almost as fast as they are pumped in. Thus, the internal pH of endosomes is likely to reflect a dynamic equilibrium of protons regulated by the intrinsic ion permeabilities of the endosomal membrane, in addition to the activity of an ATP-driven proton pump.

Receptor-mediated endocytosis is the major pathway for the cellular uptake of nutrients, hormones, enzymes, immunoglobulins, toxins, and viruses. In most cases, these ligands are internalized via coated pits and coated vesicles and then rapidly delivered to endosomes where ligands typically dissociate from their receptors. Free receptors are then recycled back to the plasma membrane while the discharged ligands are delivered to lysosomes and degraded (1, 2). This molecular sorting of receptors and ligands is facilitated by the acidic pH found in endosomes, an environment which favors the disruption of many receptor-ligand complexes (2, 3). Dissipation of the pH gradient, using ionophores or acidophilic weak bases, generally inhibits receptor-ligand dissociation and rapid receptor recycling (3).

Like lysosomes and many secretory organelles, endosomes lower their internal pH via an NEM⁻-sensitive proton ATPase (3-5). However, the internal pH in these various organelles varies considerably (from pH 4.6 to >6.5) and is even thought to vary between different endosome subpopulations (3, 6). A number of experiments performed using intact cells have shown that internalized macromolecules encounter a progressively lower pH as they traverse the endocytic pathway en route to lysosomes (7-11). Thus, endosomes at different stages of maturation differ in their internal pH, indicating that endosome acidification is likely to be subject to regulation. Since endosomal pH may determine the rate and intracellular site at which receptor-ligand dissociation occurs, the regulation of endosome acidification is likely to play a critical role in controlling the intracellular traffic of internalized receptors and ligands (2, 3).

In spite of considerable interest, few details are known concerning the actual mechanism by which endosomes lower or control their internal pH. This has been largely due to difficulties in preparing sufficient quantities of highly purified, functionally intact organelles to permit the detailed study of the proton transport and ion permeabilities of the endosomal membrane. Recently, we found that free flow electrophoresis (FFE) provides a rapid and effective method for the isolation of active endosome populations from tissue culture cells (12, 13). In this paper, we show that FFE can be used in conjunction with sucrose density gradient centrifugation for the isolation of endosomes from rat liver in sufficient quantity to permit a more complete understanding of how endosomes lower their internal pH. Our results demonstrate that ATP-dependent acidification in endosomes is electrogenic and is controlled not only by the activity of the H⁺-ATPase itself, but also by the ion permeability characteristics of the endosomal membrane.

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1 The abbreviations used are: NEM, N-ethylmaleimide; AMP-PNP, adenylyl-5'-yl-imidodiphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazinenuethanesulfonic acid; FFE, free flow electrophoresis; TEA, triethanolamine; FITC-dextran, fluorescein isothiocyanate-derivatized dextran; ASOR, asialoorosomucoid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; TMA, tetramethylammonium hydroxide.
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MATERIALS AND METHODS

Animals—Male Sprague-Dawley rats, 180–200 g, were purchased from the Charles River Breeding Laboratories (Wilmington, MA) and were fasted 24 h before use.

Biochemical Reagents—Unless noted otherwise, all reagents were obtained by Sigma. FITC-celite as described previously for the preparation of FITC-conjugated transferrin (15, 16). Unconjugated FITC was removed by dialysis against Tris-buffered saline and finally by centrifugation at 100,000 g. FITC-ASOR was stored frozen (−20°C) at a concentration of 20 mg/ml. The potential sensitive fluorescent dye 3,3'-dipropylthiodiacarbocyanine iodide (Di-S-C₆(5)) was the generous gift of Dr. R. H. Moseley (Yale Liver Center).

Selective Labeling of Rat Liver Endosomes—Endosomes were obtained by centrifugation using FITC-celite as described previously for the preparation of endosomes. FITC-dextran (Mr 40,000) and were fasted 24 h before use.

The G1 fraction was concentrated by centrifugation (100,000 g, 1 h) and resuspended in TEA buffer to a final concentration of 1 mg of protein/ml (19). As described in detail for the isolation of endosomes from tissue culture cells (12, 13), the sample was next subjected to gentle trypsin treatment by incubation for 5 min at 37°C with 0.2% chymotrypsin (5 μg/ml) for 10 min yielding pellet (P3) and supernatant (S3) fractions. S3 was diluted with TEA buffer to the initial homogenate volume (40 μg) and centrifuged 1 h at 100,000 g. The resulting membrane potential was then dissipated with 1 mM chloromethyl ketone-trypsin (mg of enzyme; 22, 23).

Selective Labeling of Rat Liver Endosomes—Prior to isolation, endosomes were selectively labeled by in situ perfusion of rat liver using well characterized markers of fluid phase or receptor-mediated endocytosis (4, 14, 22, 23). For a pH-sensitive fluid phase marker, FITC-dextran was perfused at a concentration of 5 mg/ml (in Hank’s B) for 5 min at 37°C. Since FITC-dextran would be expected to label endosomes in both parenchymal and non-parenchymal cells, we also used FITC-conjugated ASOR, which is internalized only by hepatocytes via the asialo-glycoprotein receptor (22). For these experiments, endosomal labeling was carried out by perfusion with 5 μg/ml FITC-ASOR for 10 min at 37°C, and fluorescence was shown to deliver the ligand to endosomes and not to lysosomes (22, 23) (see below). Specific binding and uptake of FITC-ASOR was demonstrated by competition with a 10-fold excess of non-derivatized ASOR which reduced FITC-ASOR uptake to an amount undetectable in liver homogenates by fluorescence spectrophotometry (now shown).
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Labeling with pH-sensitive probes ensured that only endocytic vesicles would contribute to acidification activity observed in vitro (4), detailed analysis of the bioenergetics of ATP-driven proton transport still requires highly purified preparations of organelles. Because endosomes have a buoyant density similar to that of many other smooth membranes, separation of endosomes from Golgi, endoplasmic reticulum (ER), and plasma membranes has proved difficult using conventional techniques. Since FFE provides a rapid and effective method for the purification of endosomes from tissue culture cells (12, 13), optimal separation of rat liver endosomes by FFE required a brief treatment of the sample with low concentrations of trypsin. The endosome-enriched Golgi fraction (G1) was resuspended at 1 mg/ml protein, incubated with 0.2% trypsin/mg protein for 5 min at 37 °C followed immediately by the addition of a 10-fold excess of soybean trypsin inhibitor at 0 °C. The trypsin treatment affected neither acidification nor the protein profiles (as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis), but was absolutely required to achieve anodal deflection of endosomes away from the bulk of membrane and cytosolic proteins (12, 13, 24).

A partially enriched endosome fraction was first prepared by differential and isopycnic centrifugation (18) prior to the FFE step. Using ATP-dependent acidification as a functional enzymatic marker for endosomes to follow purification, both FITC-dextran and FITC-ASOR-labeled endosomes were enriched in several previously described Golgi fractions (18). Briefly, after perfusion a postnuclear supernatant was prepared from the labeled liver homogenate and centrifuged to yield low speed 30,000 x g (P3) and high speed 100,000 x g (P4) pellets. These pellets were then fractionated by flotation in discontinuous sucrose density gradients (18). As shown in Table I, ATP-dependent acidification was most enriched at the 0.25/0.86 M ("G1") and 0.86/1.0 M ("G2") sucrose interfaces. The G1 fraction exhibited the highest specific activity for ATP-driven proton transport, approximately 100-fold enriched relative to protein as compared to the starting material (i.e. the P4 pellet). With the exception of the trans-Golgi marker UDP-galactosyltransferase (40-fold enrichment), only slight enrichment of marker enzymes for organelles other than endosomes was found in the G1 fraction; therefore, G1 was selected for further purification by FFE. Importantly, lysosomes (β-hexosaminidase activity) were actually depleted in this fraction.

As previously found for preparation of endosomes from tissue culture cells (12, 13), optimal separation of rat liver endosomes by FFE required a brief treatment of the sample with low concentrations of trypsin. The endosome-enriched Golgi fraction (G1) was resuspended at 1 mg/ml protein, incubated with 0.2% trypsin/mg protein for 5 min at 37 °C followed immediately by the addition of a 10-fold excess of soybean trypsin inhibitor at 0 °C. The trypsin treatment affected neither acidification nor the protein profiles (as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis), but was absolutely required to achieve anodal deflection of endosomes away from the bulk of membrane and cytosolic proteins (12, 13, 24).

The separation profile of G1 after FFE is shown in Fig. 1. FITC-ASOR-labeled endosomes migrated as a single peak which was well separated from a major unshifted peak containing UDP-galactosyltransferase activity (Golgi) and NADPH-cytochrome c reductase activity (ER). The specific activity of ATP-dependent acidification in the peak fraction (G35) was increased 2-3-fold relative to that measured in the starting G1 fraction (Table I). Marker enzymes for lysosomes (β-hexosaminidase) and mitochondria (not shown) were barely detectable after FFE. While a portion of the plasma membrane marker enzymes 5'-nucleotidase (not shown) and alkaline phosphodiesterase I was associated with the unshifted peak, more than half of the alkaline phosphodiesterase I co-migrated at least in part with the anodally deflected endosomal fractions. However, alkaline phosphodiesterase I specific activity was 7-10-fold lower than for ATP-dependent acidification but represented <0.1% of the initial enzyme.

### Table I

Relative specific activities of marker enzymes in subcellular fractions of rat liver

| Marker enzyme | Fraction |
|---------------|----------|
|               | H P3 P4 L1 L2 G1 G2 FFE (35) |
| ATP-dependent acidification | | | | | | |
| FITC-dextran | 0.40 ± 0.1 | 0.80 ± 0.2 | 42 ± 9 | 14 ± 2 | 100 ± 20 | 52 ± 5 | 265 ± 42 |
| FITC-ASOR | 0.25 ± 0.07 | 0.90 ± 0.1 | 40 ± 3 | 3.2 ± 0.3 | 86 ± 8 | 22 ± 4 | 170 ± 30 |
| INT-reductase | 1.0 | 0.20 ± 0.03 | 0.04 ± 0.008 | 0.04 ± 0.009 | 0.03 ± 0.009 | ND | ND | ND |
| β-Hexosaminidase | 1.0 | 0.3 ± 0.3 | 0.06 ± 0.2 | 3.2 ± 0.2 | 2.1 ± 0.2 | 0.63 ± 0.3 | 0.3 ± 0.8 | 0.7 ± 0.3 |
| NADH-cytochrome c reductase | 1.4 | 0.24 ± 0.3 | 18 ± 2 | 1.5 ± 0.4 | 3.2 ± 0.4 | 2.5 ± 0.3 | 9.2 ± 0.5 | 4.3 ± 1.5 | ND |
| Alkaline phosphodiesterase I | 1.0 | 1.4 ± 0.2 | 1.0 ± 0.1 | 7.8 ± 0.4 | 3.0 ± 0.3 | 5.3 ± 0.5 | 4.4 ± 0.6 | 25 ± 0.6 |
| 5'-Nucleotidase | 1.0 | 1.35 ± 0.2 | 0.91 ± 0.1 | 1.7 ± 0.2 | 2.6 ± 0.3 | 1.9 ± 0.2 | 4.3 ± 0.3 | ND |
| UDP-galactosyltransferase | 1.0 | 0.66 ± 0.1 | 2.50 ± 0.5 | 89 ± 8 | 85 ± 6 | 40 ± 5 | 39 ± 5 | ND |

* Specific activities were determined in each of the indicated fractions as units/mg of protein. With the exception of ATP-dependent acidification (see below), these values were then expressed relative to the specific activity (±S.D.) determined in the initial homogenate (which was assigned an arbitrary value of 1). These values do not account for percent recovery at each step. The derivation of each individual fraction is defined in the text: H (initial homogenate); P3 (30,000 x g pellet); P4 (100,000 x g pellet); L1 (0.25/0.86 M sucrose interface after sucrose gradient centrifugation of P3); L2 (0.86/1.0 M sucrose interface after sucrose gradient centrifugation of P3); G1 (0.25/0.86 M sucrose interface after sucrose gradient centrifugation of P4); G2 (0.86/1.0 M sucrose interface after sucrose gradient centrifugation of P4); FFE (35) (fraction 35 after free flow electrophoresis, containing endosome peak). The data were pooled from six separate experiments.

* Due to the turbidity of the initial homogenate, it was impossible to obtain an initial value for ATP-dependent acidification in this fraction (H). Therefore, ATP-dependent acidification is expressed as percent ATP-dependent FITC fluorescence quenching per mg of protein in each sample. Although arbitrary, comparison of these values provides a direct indication of relative enrichment of ATP-dependent acidification activity and, thus, of endosome purification. For example, the FITC-dextran containing endosomes in the G1 sucrose interface fraction, measured as having a specific activity of acidification of 100, were 100-fold enriched relative to protein compared to the P4 pellet fraction from which it was derived (specific activity ~1.0). The FEE step resulted in an additional 2.7-fold enrichment. 100% of the acidification activity measured in P4 (and P3) could be recovered from the sucrose gradients; >70% of this activity was divided between the G1 (L1) and G2 (L2) fractions, with G1 (L1) having the higher specific activity (and containing 2-3-fold more total activity) as indicated in the Table.

* ND, not detected.

* 5'-Nucleotidase activity was not detected in fraction 35 due to insensitivity of the assay (relative to alkaline phosphodiesterase) and due to the severalfold dilution that occurs during the electrophoresis step.
activity found in the homogenate or P4 fraction. 5'Nucleotidase activity, measured using a less sensitive assay, was not reproducibly detectable in the shifted fractions after FFE. The recovery of all enzyme activities, fluorescence, and protein from the G1 fraction after the FFE step was >90%.

Characterization of Rat Liver Endosomal Fractions—The degree to which the endosomes were enriched in the anodally deflected fractions was estimated by determining the recovery and relative specific activities of various organelle marker enzymes at each stage of purification (Table I). As mentioned above, the starting material, i.e., the G1 fraction from the sucrose flotation gradient, was already 90–100-fold enriched in ATP-dependent acidification (using either FITC-ASOR or FITC-dextran as endocytic markers) relative to the 100,000 × g pellet, significantly more than the enrichment of galactosyltransferase (40-fold), NADH-cytochrome c reductase (8-fold), alkaline phosphodiesterase I (5-fold), and 5' nucleotidase (2-fold) or the lysosomal marker β-hexosaminidase (0.3-fold). After separation by FFE, the endosomes were enriched an additional 2–3-fold in the anodally shifted fractions: final enrichment of 265 ± 42-fold for FITC-ASOR- and 170 ± 30-fold for FITC-dextran-labeled endosomes. The apparent difference in enrichment for the two markers was not statistically significant but may reflect the fact that fluid-phase and receptor-bound endocytic tracers can accumulate in different endosome subpopulations.

More importantly, FFE further depleted the endosomal fraction of markers for ER, Golgi apparatus, plasma membrane (5' nucleotidase), lysosomes, and mitochondria (the latter were undetectable even in the G1 and G2 fractions prior to electrophoresis). Only alkaline phosphodiesterase I was enriched (25-fold) in the shifted endosome fraction, and presumably reflected that fraction of plasma membrane ectoenzymes which exist as intrinsic components of endocytic vesicle membrane in rat liver (25, 26). This suggestion is supported by the fact that the enrichment of alkaline phosphodiesterase I in the G1 fraction by FFE was comparable (4-fold) to that observed for the endosome markers FITC-ASOR and FITC-dextran. It is less likely that the co-migrating alkaline phosphodiesterase I represented contaminating plasma membrane, since we have previously found that 125I-labeled Chinese hamster ovary (CHO) cell surface proteins do not appear in the shifted endosome fraction after FFE, whereas alkaline phosphodiesterase I partially co-localizes with endosomes (12, 13). In addition, the fraction of alkaline phosphodiesterase I in the final endosome peak was minor and represented <0.02% of the initial alkaline phosphodiesterase I activity in the homogenate; this contrasted with the approximately 20% recovery of FITC-ASOR in this fraction. 5'-Nucleotidase, determined using a less sensitive enzyme assay, was not reproducibly detected in the shifted fractions (which were diluted severalfold relative to G1).

Isolated Endosomes Have an Acidic Internal pH—To determine whether the isolated endosomes retained an acidic internal pH in vitro, FFE-purified FITC-ASOR-labeled endosomes were next resuspended in an isosmotic buffer containing 150 mM KCl and FITC fluorescence intensity monitored as a function of time. As shown in Fig. 2, the fluorescence signal increased gradually, reflecting a continual increase in internal pH. Using a pH calibration curve generated using intravascular FITC-ASOR (not shown), the initial pH was estimated to be between 5.0 and 5.5 immediately after isolation. Dissipation of the initial pH gradient required about 40 min and was dependent on the presence of external cations (K+ or Na+), as well as on their concentration (dissipation was significantly slower at concentrations <100 mM; not shown).

While the addition of the H+ ionophore FCCP to freshly isolated endosomes in the presence of KCl only slightly enhanced the rate of proton efflux, the K+ ionophore valinomycin greatly stimulated H+ efflux, even in the absence of FCCP (Fig. 2). These data indicate that the endosomal mem-

![Fig. 1. Separation of an endosome-enriched Golgi fraction by free flow electrophoresis.](image1)

**Fig. 1.** Separation of an endosome-enriched Golgi fraction by free flow electrophoresis. Hepatocyte endosomes were labeled with FITC-ASOR by in situ perfusion for 10 min at 37 °C. Following homogenization, a high speed (100,000 × g) microsomal pellet (P4) was centrifuged in a discontinuous sucrose gradient and an enriched endosome-Golgi fraction (G1) collected. The G1 fraction was then subjected to separation by FFE and the distribution of cell protein (closed squares), FITC-ASOR (closed diamonds), and several marker enzymes were determined. Other marker enzyme activities shown are: Golgi membranes, galactosyltransferase (open diamonds); ER, NADPH-cytochrome c reductase (closed triangles); plasma membrane, alkaline phosphodiesterase I (open triangles); lysosomes, β-hexosaminidase (open squares). Positions of the anode and cathode are indicated. All activities are expressed as arbitrary units/ml (12) where 100 arbitrary units/ml corresponds to: 6 pg of protein; FITC, 1.4 (1-h, assay); β-hexosaminidase, A410 = 1.5 (2-h assay); galactosyltransferase, 1700 cpm of trichloroacetic acid-precipitable [3H]-galactose (1-h assay); NADPH-cytochrome c reductase, A340 = 0.25/min (kinetic assay).

![Fig. 2. Dissipation of the pre-existing pH gradient in isolated endosomes.](image2)

**Fig. 2.** Dissipation of the pre-existing pH gradient in isolated endosomes. Rat liver endosomes labeled with FITC-ASOR were purified by free flow electrophoresis (fraction 35 in Fig. 1) and then added to cuvettes (2 µg of protein each) containing 150 mM KCl buffer, 150 mM KCl + 1 µM FCCP, or 150 mM KCl + 1 µM valinomycin. In each case, the initial FITC fluorescence intensity increased gradually, reflecting a dissipation of the initial acidic internal pH. While addition of the K+ ionophore valinomycin accelerated the decay of the pH gradient, the addition of the H+ ionophore FCCP had little effect. After dissipation of the initial pH gradient, endosomes were re-acidified by addition of 2.5 mM K+ -ATP (in the presence of MgCl2). Presumably due to their increased conductance for H+, the ATP-driven acidification of FCCP-treated endosomes was significantly reduced. A rapid increase in FITC fluorescence was obtained by adding the K+/H+ ionophore nigericin (1 µM) which would be expected to immediately bring the internal endosomal pH to that of the suspending medium (i.e. pH 7.4).
brane has intrinsic permeabilities for H⁺ and K⁺ which allow electrically coupled exchange of internal H⁺ for external cations, thus allowing for the dissipation of the initial pH gradient in KCl buffer. The fact that H⁺ efflux was stimulated by valinomycin, but not by FCCP, indicated further that the K⁺ permeability, and not the H⁺ permeability, was rate-limiting for dissipation of the H⁺ gradient. Thus, the FITC-ASOR-containing endosomes were more permeable to H⁺ than to K⁺. As expected, addition of the K⁺/H⁺ ionophore nigericin led to a rapid release of internal H⁺ (Fig. 2).

Importantly, identical results were obtained (for this and all subsequent experiments involving FITC-labeled probes) when the G1 and G2 fractions were used without further enrichment by FFE. This demonstrated that neither the trypsin treatment needed to prepare samples for FFE nor the electrophoresis itself had any effect on H⁺ or K⁺ permeability of FITC-ASOR or FITC-dextran-labeled endosomes from rat liver.

**Characteristics of the Endosomal Proton Pump—Adding ATP to acidic endosomes resulted in no further decrease in FITC fluorescence, suggesting that there is a maximum pH gradient that can be established. Therefore, all experiments designed to characterize the ATP-dependent acidification of endosomes were performed under conditions of ionic equilibrium, i.e., after incubation of the endosomes for 1–24 h at 4°C in cation-containing buffers to dissipate any pre-existing H⁺ gradients (Fig. 2). For all experiments, equivalent results were obtained as long as the internal pH was permitted to equilibrate with that of the suspending medium, irrespective of the length of preincubation. In fact, FFE-purified endosomes could be incubated at 4°C for up to 80 h without loss of acidification activity. As shown in Fig. 2, addition of ATP to endosomes previously allowed to dissipate their pre-existing pH gradients exhibited a rapid nigericin-reversible decrease in FITC fluorescence. ATP-driven acidification was observed in FCCP-treated endosomes, indicating that even when freely permeable to H⁺, the endosome’s limited K⁺ permeability was sufficient to stabilize a transmembrane pH gradient via an electrochemical potential.

We first examined the nucleotide specificity of the endosomal proton pump. Acidification was found to be specific for ATP, as earlier observed for crude preparations of endosomes from tissue culture cells (10). Addition of other nucleotides such as CTP, GTP, or the nonhydrolyzable ATP analog AMP-PNP, did not support acidification of FFE-purified endosomes (not shown).

Next, we examined the effects of several potential H⁺-ATPase inhibitors on acidification. Total inhibition of ATP-driven proton transport was achieved after 5 min incubation with 10 μM NEM, the best characterized inhibitor of vacuolar H⁺ pumps (3, 5). Inhibitors of either the mitochondrial-type F₁,F₀-ATPase (efrapeptin) or the gastric K⁺/H⁺-ATPase (Na⁺V0₄) had no effect on acidification, as described for endosomes from tissue culture cells (not shown) (4).

The Endosomal Proton Pump Is Electrogenic—NEM-sensitive proton pumps present in clathrin-coated vesicles, estrogen-induced proton transport was achieved after 5 min incubation with 10 μM NEM, the best characterized inhibitor of vacuolar H⁺ pumps (3, 5). Inhibitors of either the mitochondrial-type F₁,F₀-ATPase (efrapeptin) or the gastric K⁺/H⁺-ATPase (Na⁺V0₄) had no effect on acidification, as described for endosomes from tissue culture cells (not shown) (4).

**Fig. 3. Electrogenicity of endosome acidification.** Endosomes labeled with FITC-dextran were purified by FFE and pre-equilibrated (0.8 μg) overnight with 150 mM KCl (panel a) or K⁺-gluconate (panel b). ATP-dependent fluorescence quenching was determined in the same medium in the presence or absence of 1 μM valinomycin (val). Substitution of Cl⁻ (panel a) with the less permeant organic anion gluconate (panel b) inhibited acidification. This inhibitory effect could be overcome by the K⁺ ionophore valinomycin. Direct measurement of potential changes in endosomes with a voltage-sensitive dye Di-S-C₃(5) is shown in panels c and d. In panel c, K⁺-induced diffusion potentials were used to verify the ability of the dye to partition in accordance with predictable differences in transmembrane potential. FFE-purified endosomes (40 μg of protein) were equilibrated with 10 mM K⁺-gluconate overnight and then diluted into buffer containing 1 mM K⁺-gluconate, 10 mM K⁺-gluconate, or 100 mM K⁺-gluconate (as indicated) and 0.25 μM Di-S-C₃(5). 10 μM valinomycin was then added to each cuvette to facilitate the further development of a diffusion potential. As shown, the dye appropriately reports both positive and negative potential changes following dilution into buffers of higher and lower [K⁺], respectively. In panel d, endosomes were equilibrated with 100 mM Na⁺-gluconate and diluted into a cuvette containing the same buffer and 0.25 μM Di-S-C₃(5). After the fluorescence signal reached a stable baseline, 1 mM ATP was added. The ATP-dependent increase in Di-S-C₃(5) fluorescence, indicating the formation of an interior-positive potential, was reversed by 1 μM FCCP, demonstrating that the potential difference was due to H⁺. Addition of 10 μM NEM inhibited the ATP-induced increase in Di-S-C₃(5) fluorescence, suggesting that passive influx of external anions helps to dissipate an interior-positive membrane potential due to H⁺ influx. Consistent with this interpretation, addition of the K⁺ ionophore valinomycin to gluconate-equilibrated endosomes (Fig. 3b), completely restored the acidification observed in the presence of Cl⁻, presumably by facilitating the efflux of internal K⁺ down the electrical gradient. As expected, addition of valinomycin to KCl-equilibrated endosomes had.
that ATP-dependent acidification of endosomes is characterized by electrogenic H+ influx, which can be influenced by the membrane’s permeability to anion or cations.

To provide further evidence that ATP-coupled proton transport was electrogenic, we next used the positively charged potential sensitive dye Di-S-C3(5). This dye partitions between intra- and extravesicular space in response to the membrane potential (30). It is important to note that unlike the pH-sensitive FITC-labeled endocytic probes, this externally added voltage probe is nonselective and will partition across any membrane present. As a result, the use of Di-S-C3(5) is absolutely dependent on the availability of a homogenous, highly purified population of organelles.

We first validated that Di-S-C3(5) could detect changes in membrane potential in FFE-purified endosomes by calibrating the fluorescence signal after creating K+-valinomycin diffusion potentials. This was accomplished by first loading endosomes with 10 mM K-gluconate and diluting them into buffers of higher or lower K+ concentrations. Dilution into a medium containing 1 mM K-gluconate generated an interior-negative membrane potential, due to K+ efflux down the concentration gradient, and was accompanied by a decrease in Di-S-C3(5) fluorescence due to quenching resulting from the dye influx and/or binding to the endosomes (Fig. 3c). On the other hand, dilution from 10 mM K+ into a buffer with a higher K+ concentration (100 mM) generated an interior-positive potential and which led to an increase in fluorescence. As expected, these differences were accentuated by the addition of valinomycin which increased the endosomes’ permeability to K+ (Fig. 3c).

Having demonstrated that Di-S-C3(5) could be used to report changes in membrane potential, we next determined whether ATP-dependent proton pumping is accompanied by membrane potential alterations. After equilibrating endosomes in Na+-gluconate buffer containing 0.25 mM Di-S-C3(5), addition of ATP resulted in a rapid increase in fluorescence, reflecting dye efflux concomitant with acidification (Fig. 3c). This effect could be reversed by the H+ ionophore FCCP, demonstrating that the fluorescence increase was indeed due to H+ uptake. In contrast, CTP and AMP-PNP, two nucleotides which do not support acidification, failed to alter the Di-S-C3(5) fluorescence signal (not shown). Likewise, 10 mM NEM inhibited the ATP-dependent fluorescence increase (Fig. 3d). Thus, the fluorescence signal was not generated as an artifact of nucleotide addition (21). Taken together with the anion replacement experiments (Fig. 3, a and b), these data demonstrate the existence of an electrogenic H+ pump in rat liver endosomes. No attempt was made to quantify changes in membrane potential using Di-S-C3(5), however, due to quantitative inaccuracies characteristic of this method and due to the fact that although highly enriched, FFE-purified endosomes may still contain contaminating membrane vesicles of indeterminate volume capable of generating a signal with Di-S-C3(5).

**Influence of Ion Substitution on ATP-dependent Acidification**—Since the interior-positive membrane potential due to H+ transport would be dissipated by the influx of permeable external anions or efflux of internal alkali cations, the extent of acidification will in part be determined by the ion permeability characteristics of the endosomal membrane. To determine the anion and cation permeabilities of rat liver endosomes, we next tested the effect of anion and cation substitutions on ATP-dependent acidification under conditions of ionic equilibrium.

**Anion Permeability**—Endosome-enriched fractions were preincubated with the Na+ salts of various anions prior to determining the rate and extent of ATP-induced acidification in media of identical compositions. As shown in Fig. 4a, acidification was supported to different extents by different anions. The rate and extent of acidification decreased in the following order: Cl− > gluconate = sulfate = isethionate > NO3− > SCN−. Thus, substitution of less permeant anions such as gluconate, sulfate, or isethionate for a more permeable anion such as Cl− decreased acidification. Interestingly, however, inhibition of acidification occurred in the presence of highly permeable anions such as SCN− and NO3− at concentrations ranging from 12 to 150 mM.

The effect of NO3− appeared to be due to a direct inhibition of acidification activity since NO3− was also found to block H+ transport in the presence of the permeant anion Cl− (not shown). In addition, the addition of NO3− to acidic endosomes after the addition of ATP led to the dissipation of the pH gradient. Indeed, NO3− has previously been found to be an inhibitor of acidification of other organelles in plant and mammalian cells (3, 31).

The inhibitory effect of SCN− on ATP-dependent acidification, however, is difficult to reconcile with its normally high membrane permeability that might be expected to stimulate acidification. Indeed, such stimulation has been observed for ATP-driven H+ transport in renal cortical endocytic vesicles and Golgi vesicles (21, 32, 33).

**Cation Permeability**—We next investigated the endosomal membrane’s cation permeability by determining the effect of cation substitution on ATP-dependent H+ transport. FITC-dextran-containing endosome-enriched Golgi fractions were pre-equilibrated with the Cl− salts of various cations for 24 h on ice prior to the determination of ATP-induced H+ transport. As shown in Fig. 4b, ATP-driven H+ transport was stimulated by Na+ > K+ > choline > TMA+.” These data demonstrate that ATP-dependent endosome acidification has no absolute or specific cation requirement, unlike the gastric K+/H+-ATPase, for example. The differential ability of the various cations to support electrogenic H+ almost certainly

![Fig. 4. Effect of anions and cations on ATP-dependent acidification.](image-url)
reflects the differential membrane permeabilities to these ions since continued H⁺ influx will be compensated by the efflux of internal cations. Therefore, the more permeant the internal cation, the more acidification. Accordingly, the endosomal membrane is more permeant for Na⁺ and K⁺ than for choline and TMA⁺.

It is thus likely that both anion and cation permeabilities play a role in ATP-dependent acidification. Even in medium containing Cl⁻, acidification is decreased in the presence of impermeant cations such as TMA⁺, and vice versa, in the presence of Na⁺ or K⁺-gluconate.

**Ion Permeabilities Can Regulate ATP-driven Proton Transport**—We have demonstrated that the activity of the endosomal proton pump can be regulated by the development of an interior-positive membrane potential which, in turn, can be regulated by the intrinsic ion conductances of the endosomal membrane. Since the endosomal membrane also exhibits a significant conductance for protons (Fig. 2), it is possible that electrically coupled H⁺/K⁺ (or Na⁺/H⁺) exchange may also serve to control internal pH in endosomes. This was evaluated by observing the rate of dissipation of an ATP-generated pH gradient in buffers containing either K⁺ or Na⁺.

FFE-purified endosomes were equilibrated in 25 mM KCl or 25 mM NaCl and H⁺ transport was initiated by adding ATP. When maximum acidification had been obtained, ATP was “removed” by the addition of glucose and hexokinase, which resulted in a rapid dissipation of the pH gradient (Fig. 5a). The half-time of decay of the pH gradient was ~2.5 min in K⁺ and ~4 min in Na⁺.

Similar results were obtained when, instead of removing ATP, the endosomes were rendered freely permeable for H⁺ by the addition of FCCP (Fig. 5b). Under these conditions, where cation permeability was the only rate-limiting factor affecting H⁺ efflux, the decay of the pH gradient was again faster in K⁺ than in a Na⁺ buffer. Taken together, these results indicate that rat liver endosomes are more permeable to K⁺ than to Na⁺. Hence, the reduction of acidification activity in K⁺ buffer even under conditions of ionic equilibrium (Fig. 4b) can be explained by a significantly higher permeability to K⁺ which favors H⁺ efflux by electrically coupled K⁺/H⁺ exchange. Fig. 5 also demonstrates that endosomes are significantly permeable to H⁺, indicating that acidification is likely to be associated with a dynamic flux of H⁺.

To directly demonstrate the effect of ion permeabilities on acidification, experiments were also performed with different ion compositions on both sides of the membrane. Endosomes were equilibrated with NaCl and diluted into a KCl buffer or vice versa. As shown in Fig. 6a, when the more permeable cation (i.e., K⁺) was inside, acidification was favored. Thus, optimizing conditions for the efflux of internal cations facilitates ATP-driven H⁺ influx.

The effect of anion gradients on ATP-dependent acidification was also investigated. Endosomes were loaded with Na⁺-gluconate or NaCl and diluted into NaCl or Na⁺-gluconate. As shown in Fig. 6b, when the more permeable anion was outside, acidification was favored. This result may also reflect a direct stimulation of the endosomal proton pump by external Cl⁻, as demonstrated for the proton pump isolated from bovine adrenal chromaffin granules (34).

Finally, the general potentiating effect of permeable anions and cations on acidification is illustrated in Fig. 6c. No acidification was observed if both anions and cations were

**Fig. 5. Ion permeabilities of the endosomal membrane.** FFE-purified endosomes labeled with FITC-dextran were equilibrated for 1 h at 4°C and then acidified by addition of 2.5 mM ATP in 25 mM KCl or NaCl (containing isosmotic sucrose). Decay of the ATP-induced pH gradient was determined after depleting the ATP concentration with 10 mM glucose and hexokinase (12 units) (panel a). The rate at which the pH gradient dissipated was faster when K⁺ was present as compared to Na⁺. Similarly, rendering the endosomal membrane freely permeable to H⁺ with 1 μM FCCP, resulted in more rapid dissipation in the presence of K⁺ than Na⁺ (panel b).

**Fig. 6. Ion requirements of ATP-dependent proton transport.** Panel a, a possible requirement for internal versus external impermeable cations was determined by equilibrating (1 h) FFE-purified, FITC-dextran-labeled endosomes in 25 mM NaCl or KCl (in) followed by dilution into isosmotic buffer containing 25 mM KCl or NaCl (out), respectively. 2.5 mM ATP was added to initiate acidification. Panel b, the effect of internal versus external permeant and impermeant anions on ATP-driven acidification was determined by preloading endosomes with 25 mM TMA-Cl⁻ or 25 mM TMA-glucuronate (in) and diluting into 25 mM TMA-glucuronate or 25 mM TMA-Cl⁻ buffer (out), respectively. Panel c, endosomes were equilibrated in buffer containing 300 mM sucrose, 25 mM Na⁺-gluconate, or 25 mM K⁺-glucuronate (in) and then diluted into sucrose alone or 25 mM TMA-Cl⁻ (out). Complete absence of internal and external anions and cations completely abolished ATP-dependent acidification. A gradual increase in the rate and extent of ATP-dependent acidification was obtained by substituting a more permeant cation (K⁺) inside as well as by the presence of a permeable anion (Cl⁻) externally.
When endosomes were equilibrated with Na+-gluconate or completely omitted, i.e. using endosomes in isosmotic sucrose. Both cation and anion permeabilities can play important roles in regulating acidification by an electrogenic H$^+$ pump.

**DISCUSSION**

The bioenergetics of endosome acidification was investigated using highly purified rat liver endosomes selectively labeled with pH-sensitive endocytic tracers. We have shown that the rat liver endosomal H$^+$ pump operates by an electrogenic mechanism and thus belongs to the class of NEM-sensitive vacuolar ATPases (3). Due to the high H$^+$ permeability of the endosomal membrane, continuous inward H$^+$ pumping was required to maintain the pH gradient. In addition, we found that the magnitude of the pH gradient established was influenced by the permeability of the endosomal membrane for cations and anions. Together, these findings demonstrate that acidification is associated with an unexpectedly dynamic flux of H$^+$ across the endosomal membrane. Thus, the net accumulation of intravesicular H$^+$ at equilibrium must therefore reflect both the rate of ATP-driven H$^+$ pumping and the membrane's other permeability characteristics. The basic features of rat liver endosome acidification are similar to those described for two other endocytic vacuole populations, estrogen-induced rat liver multivesicular bodies (28, 31) and renal cortical cell endocytic vesicles (21), although the origin, purity, and function of these earlier preparations were less certain.

Our detailed investigation was facilitated by a new approach to endosome isolation, FFE, which has previously been applied only to tissue culture cells (12, 13). We have shown that FFE, in combination with sucrose density gradient centrifugation, can be successfully used for the isolation of a endosomal fraction from rat liver which is essentially devoid of other intracellular organelles known to contain H$^+$ pumps, such as Golgi, lysosomes, and mitochondria. Although the electrophoretic separation is dependent on a brief trypsin treatment, trypsinization had no quantitative effect on the bioenergetics of isolated endosomes of progressively lower pH, ranging from pH > 6.3 to 5 (2, 6–11). Since different receptor-ligand complexes may dissociate at characteristic pH values, and since ligand dissociation can influence the pathway and efficiency of receptor recycling (2, 3), the regulation of pH in endosome subpopulations may play an important role in controlling membrane traffic during endocytosis. Given that all organelles along the endocytic pathway are likely to contain the same H$^+$ pump, other mechanisms must be provided to regulate the activity of this ATPase.

It seems unlikely that pH regulation is accomplished by controlling the number of ATPase molecules present in each compartment. Endosomes contain a small internal volume (approximately 10$^{-15}$ ml for a spherical endosome 0.5-μm in diameter); therefore only limited numbers of H$^+$ are needed to lower the internal pH to <6. In addition, the observed pH differences among endosomes are relatively slight (<1–1.5 pH units). Since endosomes were found to be highly permeable to H$^+$ in vitro and, in fact, leaked protons almost as fast as they were pumped in, continuous H$^+$ pumping was required to maintain the pH gradient. If this situation approximates the permeability properties of endosomes in vivo, a high rate of H$^+$ flux would provide an ideal mechanism for controlling intravesicular pH in intact cells by regulating the net accumulation of internal H$^+$ in dynamic equilibrium with cytosolic H$^+$. The equilibrium would be subject to the permeability characteristics of the endosomal membrane since permeability to other anions and cations can control both the rate of H$^+$ efflux by H$^+$-cation exchange and the activity of the voltage-sensitive, electrogenic H$^+$-ATPase by alterations in membrane potential. It will now be important to determine whether the anion and cation permeabilities of the endosomal membrane are themselves subject to regulation (e.g. by phosphorylation) or whether different endosome subpopulations exhibit different permeability characteristics.

In addition, we recently found that endosomes which occur “early” on the endocytic pathway represent a distinct population involved in receptor recycling which is generally less acidic than kinetically “late” endosomes (13). Since early and late endosomes have markedly different protein compositions, it is indeed possible that these two subpopulations also have distinct ion permeability properties which are responsible for their different pH values. We have already identified at least one potential difference: the presence of the Na$^+$/K$^+$-ATPase is restricted to early endosomes from CHO cells (30). This Na$^+$/K$^+$-ATPase appears to attenuate ATP-dependent acidification of early endosomes by facilitating the development of the interior-positive membrane potential which in turn inhibits the activity of the H$^+$-ATPase. While such a situation may serve to help regulate pH in some cell types, we have not found similar evidence supporting a regulatory role for Na$^+$/K$^+$-...
ATPase in rat liver endosomes.\(^2\)

The endocytic pathway in hepatocytes is complex, involving not only constitutive recycling of receptors at the sinusoidal (or basolateral) plasma membrane, but also the transport of certain receptors and ligands to lysosomes, the Golgi, or to the bile canalicular (apical) plasma membrane. The experiments discussed here have focused only on the "total" endosome population labeled with fluid phase or receptor-bound markers destined primarily for lysosomes. While the basic bioenergetics of acidification of this population have now been defined, it will be important to define the properties of endosome populations labeled with fluid phase or receptor-bound markers and transport of certain receptors and ligands to lysosomes, the Golgi, or to the bile canalicular (apical) plasma membrane. The experiments discussed here have focused only on the "total" endosome population labeled with fluid phase or receptor-bound markers destined primarily for lysosomes. While the basic bioenergetics of acidification of this population have now been defined, it will be important to define the properties of endosomes involved in the other transport pathways found in polarized epithelial cells.

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REFERENCES
1. Steinman, R. M., Mellman, I. S., Muller, W. A., and Cohn, Z. A. (1983) J. Cell Biol. 96, 1–27
2. Helenius, A., Mellman, I., Wall, D., and Hubbard, A. (1983) Trends Biochem. Sci. 8, 245–250
3. Mellman, I., Fuchs, R., and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663–700
4. Galloway, C. J., Dean, G. E., Marsh, M., Rudnick, G., and Mellman, I. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3334–3338
5. Al-Awqati, Q. (1986) Annu. Rev. Cell Biol. 2, 179–199
6. Yamashiro, D. J., Tycko, B., Fluss, S. R., and Maxfield, F. R. (1984) Cell 37, 789–800
7. Kielian, M. C., Marsh, M., and Helenius, A. (1986) EMBO J. 5, 3103–3109
8. Murphy, R. F., Powers, S., and Cantor, C. R. (1984) J. Cell Biol. 98, 1757–1762
9. Tanasugarn, L., McNeil, P., Reynolds, G. T., and Taylor, D. L. (1984) J. Cell Biol. 98, 717–724
10. Meron, M., Schlesinger, P., Brooks, J. M., Meehring, J. M., Meehring, T. J., and Sly, W. S. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5315–5319
11. Yamashiro, D. J., and Maxfield, F. R. (1987) J. Cell Biol. 105, 2713–2721
12. Marsh, M., Schmid, S., Kern, H. T., Harms, E., Male, P., Mellman, I., and Helenius, A. (1987) J. Cell Biol. 104, 875–886
13. Schmid, S., Fuchs, R., Male, P., and Mellman, I. (1988) Cell 52, 73–80
14. Dunn, W. A., La BADie, J. H., and Aronson, N. N., Jr. (1979) J. Biol. Chem. 254, 4191–4196
15. van Renswoude, J., Bridges, K. R., Harford, J. B., and Klausner, R. D. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6186–6190
16. Roff, C., Fuchs, R., Mellman, I., and Robbins, A. R. (1986) J. Cell Biol. 103, 2293–2298
17. Seglen, P. O. (1976) Methods Cell Biol. 13, 29–64
18. Khan, M. N., Savoie, S., Bergeron, J. J. M., and Posner, B. I. (1986) J. Biol. Chem. 261, 8462–8472
19. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
20. Hubbard, A. L., Wall, D. A., and Ma, A. (1983) J. Cell Biol. 96, 217–229
21. Sabolic, I., and Burchardt, G. (1986) Am. J. Physiol. 250, F817–F826
22. Wall, D. A., and Hubbard, A. L. (1985) J. Cell Biol. 101, 2104–2112
23. Musilier, S. C., and Hubbard, A. L. (1986) J. Cell Biol. 102, 932–942
24. Schmid, S. L., and Mellman, I. (1988) in Cell Free Analysis of Membrane Transport (Morré, D. J., Howell, K. E., Cook, G. M. W., and Evans, W. H., eds) pp. 35–49, Alan R. Liss Inc., New York
25. Widnell, C. C., Schneider, J.-J., Pierre, B., Baudhuin, P., and Trouet, A. (1982) Cell 28, 61–70
26. Stanley, K. K., Edwards, R. M., and Luzio, J. P. (1980) Biochem. J. 186, 59–69
27. Forgac, M., Cantley, L., Wiedenmann, B., Alstiel, L., and Branton, D. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1300–1303
28. Van Dyke, R. W., Hornick, C. A., Belcher, J., Scharschmidt, B. F., and Havel, R. J. (1985) J. Biol. Chem. 260, 11021–11026
29. Ohkuma, S., Moriyama, Y., and Takano, T. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2758–2762
30. Harikumar, P., and Reeves, J. P. (1983) J. Biol. Chem. 258, 10403–10410
31. Van Dyke, R. W. (1986) J. Biol. Chem. 261, 15941–15948
32. Glickman, J., and Al-Awqati, Q. (1984) J. Clin. Invest. 73, 1704–1710
33. Glickman, J., Croen, K., Kelly, S., and Al-Awqati, Q. (1983) J. Cell Biol. 97, 1303–1308
34. Fuchs, R., Schmid, S. L., and Mellman, I. (1989) Proc. Natl. Acad. Sci. U. S. A., in press