Regulation of Phagosomal Acidification

DIFFERENTIAL TARGETING OF Na\(^+/\)H\(^+/\) EXCHANGERS, Na\(^+/K^+\)-ATPases, AND VACUOLAR-TYPE H\(^+\)-ATPases

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David J. Hackam‡§‖, Ori D. Rotstein§, Wei-Jian Zhang‡§, Nicolas Demurex‡, Michael Woodside‡, Olivia Tsai§, and Sergio Grinstein‡‖

From the ‡Division of Cell Biology, The Hospital for Sick Children, and the †Department of Surgery, Toronto Hospital, University of Toronto, Toronto M5G 1X8, Canada

Vacuolar-type (V) ATPases are thought to be the main determinant of phagosomal acidification. In phagosomes containing mycobacteria, which ostensibly impair the delivery of V-ATPases to the phagosomal membrane, the pH would be expected to be near neutral. This prediction was tested by microfluorescence ratio imaging using macrophages from mice susceptible to mycobacterial infection. Although less acidic than their counterparts containing dead bacteria, phagosomes containing live Mycobacteria bovis were nearly 1 pH unit more acidic than the cytosol, suggesting the existence of alternate H\(^+\) transport mechanisms. We therefore investigated whether Na\(^+/\)H\(^+/\) exchange (NHE) contributes to phagosomal acidification. Immunoblotting, reverse transcriptase-polymerase chain reaction, and pharmacological studies indicated that NHE1 is the predominant isoform of the exchanger in macrophages. Fractionation revealed that NHE1 is incorporated into the phagosomal membrane, and measurements of pH indicated that it is functional in this location. Nevertheless, acidification of the lumen of phagosomes containing either latex beads or live M. bovis was insensitive to (3-methylsulfonyl-4-piperidinobenzoyl)-guanidine methanesulfonate, a potent inhibitor of NHE1. This has been due to the absence of an appropriate lumen to cytosol Na\(^+\) gradient, because the phagosomal membrane was found to be devoid of Na\(^+/K^+\) pumps. Unexpectedly, the acidification of M. bovis phagosomes was fully reversed by specific inhibitors of the vacuolar H\(^+\)-ATPase, suggesting that ATPases are present only transiently or in reduced quantities in the phagosomal membrane. Alternatively, acid equivalents accumulated in endosomes by V-ATPases may be delivered to the mycobacterial phagosome by carrier vesicles devoid of ATPases.

Leukocytes internalize invading pathogens into a membrane-bound organelle called the phagosome. The microbes are initially encircled by extensions of the plasmalemma, and the resulting nascent phagosome subsequently matures upon fusion with endosomes, lysosomes, and possibly other vesicular compartments (1–3). Acidification of the phagosomal interior is a critical component of the microbialicidal response; not only is the low pH directly toxic to many microorganisms but, in addition, lytic enzymes secreted into the phagosomal lumen function optimally at acidic pH. Phagosomal acidification has been attributed principally to the activity of vacuolar type H\(^+\)-ATPases (V-ATPases), which have been shown to accumulate in the phagosomal membrane as it matures within the cell (1, 4). The importance of V-ATPase-mediated acidification to microbial elimination is highlighted by the failure of macrophages to kill organisms such as Mycobacterium tuberculosis. This and other species of mycobacteria avoid acid-mediated degradation by preventing insertion of H\(^+\) pumps into the phagosomal membrane (5, 6). Interestingly, the growth of mycobacteria ceases at pH <6.2 (7), suggesting that their ability to modulate phagosomal pH is an important determinant of intracellular survival.

Despite the ostensible absence of V-ATPases, phagosomes containing live mycobacteria were noted to be significantly more acidic than the extracellular or cytosolic milieu (i.e. pH 6.2 versus 7.0–7.4) (5). This observation suggests the existence of additional mechanisms of phagosomal acidification. Because fusion of late endosomes and lysosomes with the phagosome is inhibited in cells infected with mycobacteria (6, 8, 9), it is conceivable that plasmalemmal transporters, internalized during phagosome formation, may contribute to the V-ATPase-independent acidification. A possible candidate for early phagosomal acidification is the Na\(^+/\)H\(^+/\) exchanger, or NHE. This antiporter is present in the plasma membrane of virtually all mammalian cells, where it catalyzes the electroneutral exchange of one Na\(^+\) for one H\(^+\) (10, 11). The exchange reaction, which is characteristically susceptible to inhibition by amiloride and its analogs, is driven by the concentration gradients of these ions and does not require direct expenditure of metabolic energy (12). Under physiological conditions, the inward Na\(^+\) gradient generated by the Na\(^+/K^+\)-ATPase drives H\(^+\) out of the cell. Because it is constitutively present in the surface membrane, it is likely that the NHE is incorporated into the phagosomal membrane during microbial internalization. At the phagosomal membrane, its forward activity would exchange

1 The abbreviations used are: V-ATPases, vacuolar type H\(^+\)-ATPases; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; NCS-CF, N-hydroxysuccinimidy1 5-(and 6)-carboxyfluorescein; HOE 694, (3-methylsulfonyl-4-piperidinobenzoyl)-guanidine methanesulfonate; BCECF, Na\(^+/\)H\(^+/\) exchanger; PBS, phosphate-buffered saline; pH\(_i\), extracellular pH; pH\(_o\), intracellular pH; pHb, phagosomal pH; BCG, bacillus Calmette-Guerin strain of M. bovis; PCR, polymerase chain reaction; bp, base pair(s).

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† Recipient of a postdoctoral fellowship from the Medical Research Council of Canada and an Ethicon Society of University Surgeons research award.

‡ International Scholar of the Howard Hughes Medical Institute and cross-appointed to the Dept. of Biochemistry at the University of Toronto. To whom correspondence should be addressed: Division of Cell Biology, Hospital for Sick Children, 555 University Ave., Toronto, M5G 1X8, Canada. Tel.: 416-813-5727; Fax: 416-813-5028; E-mail: sga@sickkids.on.ca.
Na\(^+\), taken up from the extracellular space during phagocytosis, for cytosolic H\(^+\), resulting in phagosomal acidification. On thermodynamic grounds, the NHE could support an acidification of up to 2 pH units, provided that intraphagosomal Na\(^+\) was maintained at extracellular levels. This could in turn be accomplished by the continued operation of the Na\(^+\)/K\(^+\)-ATPase, which might be similarly internalized upon phagosome formation. In this regard, the Na\(^+\)/K\(^+\)-ATPase is known to be incorporated into endocytic compartments where it can remain functional, as judged by its effects on endosomal pH (13, 14). The latter observation, however, is not universal, and discrepant results have been reported (15).

The present experiments were performed to define the mechanisms contributing to the acidification of phagosomes containing live mycobacteria. Specifically, we assessed if the NHE is in fact internalized into phagosomes and whether it contributes to the early stages of phagosomal acidification. We also examined the presence and activity of the Na\(^+\)/K\(^+\)-ATPase, to define if a suitable ionic gradient would be available for sustained NHE function in the phagosome. Finally, we considered whether V-ATPases may play a direct or indirect role in the acidification of phagosomes containing live mycobacteria.

**EXPERIMENTAL PROCEDURES**

**Materials, Solutions, and Antibodies**

Nigericin, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), acetoxyethyl ester, fluorescein isothiocyanate, and N-hydroxysuccinimydyl 5- (and 6)-carboxyfluorescein (NHS-CF) were from Molecular Probes Inc. (Eugene, OR). Pepstatin A, phenylmethylsulfonyl fluoride, imidyl 5-(and 6)-carboxyfluorescein (NHS-CF) were from Molecular Probes Inc. (Eugene, OR). Fabulins were from Sigma. Compound HOE 694 (3-methylsulfonyl-4-piperidinobenzoyl)-guanidine methanesulfonate) was a generous gift from Hoechst (Frankfurt, Germany).

Polynuclear antibodies to the NHE1 isofrom of the Na\(^+\)/H\(^+\) exchanger were raised by injecting rabbits with a fusion protein containing the last 157 residues (658–815) of the human homologue as described (16). Polyclonal antibodies to the 39-kDa subunit of the V-ATPase were raised by injecting rabbits with a fusion protein containing the last 157 residues (658–815) of the human homologue as described (16). The Na\(^+\)/H\(^+\)-ATPase is known to be incorporated into endocytic compartments where it can remain functional, as judged by its effects on endosomal pH (13, 14). The latter observation, however, is not universal, and discrepant results have been reported (15).

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**Cell and Bacterial Cultures**

The murine cell line J774 was obtained from ATCC (Rockville, MD) and was maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 5% penicillin/streptomycin (Life Technologies Inc.) at 37 °C under 5% CO\(_2\). Resident peritoneal macrophages were obtained from the *Mycobacterium* susceptible mice strain C57BL/6 (Taconic Farma Inc., Germantown, NY) as described (18). Briefly, the peritoneal cavities of 6–8-week-old mice were lavaged with 10 ml of ice-cold PBS. The cells, comprising approximately 30% macrophages as determined by Wright staining, were washed three times in ice-cold PBS and then resuspended in Hapes-buffered medium RPMI 1640 with 10% fetal calf serum. Cells (1 × 10\(^6\)/ml) were then incubated with bacteria as described below and plated on glass coverslips. Non-adherent cells (predominantly lymphocytes) were removed by washing with fresh medium.

*M. bovis* strain BCG, sub-strain Montreal (BCG), was obtained from the Armand Frappier Institute (Laval, Quebec, Canada) and main-

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Calibration of the fluorescence ratio versus pH was performed for each experiment by equilibrating the cells in isotonc K\(^+\)-rich medium (6.0 or 7.4) and adding the fluorescence ratio imaged on the Leiden microscope (20). Caliibrant was used to perfuse the cells with K\(^+\)-rich medium containing 1 mM ouabain for 1 h at 23 °C. The cells were then washed with K\(^+\)-free medium and to the corresponding fluorescence ratio (21).

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**Measurement of Cytosolic pH (pH\(_i\))**—For fluorescence determinations, cells were plated to 60–70% confluence on glass coverslips. To measure pH\(_i\), they were incubated with 2 μM BCECF acetoxyethyl ester for 15 min at 37 °C. Fluorescence of BCECF was measured essentially as in Ref. 20. Briefly, the coverslip was placed in a thermostatted Leitz-Microscope on the stage of a Zeiss IM-35 microscope with a 63 × 1.4 N.A. oil-immersion objective. A Sutter filter wheel was used to alternate position the two excitation filters (500 ± 10 and 440 ± 10 nm) in front of a xenon lamp. To minimize dye bleaching and photodamage, neutral density filters were used to reduce the intensity of the light reaching the cells. The excitation light was directed to the cells via a 510-nm dichroic mirror, and fluorescence emission was collected by a 535 ± 25 nm bandpass filter. Data were recorded every 30 s by irradiating the cells for 50 ms at each of the excitation wavelengths. Images were captured with a cooled CCD camera (Princeton Instruments Inc., NJ). Image acquisition was controlled by the Metafluor software (Universal Imaging Corp.), operating on a Pentium Dell computer (Dell Inc., Canada). The resulting ratio images were displayed on-line, and regions of interest encompassing individual cells were defined.

**Measurement of Phagosomal pH (pH\(_{ph}\))**—Measurements of pH\(_{ph}\) were obtained through the combined application of video microscopy and fluorescence measurements. Cells were imaged for 2 min, to allow for adherence to occur. For pH measurement, the coverslips were mounted on the Leiden chamber as above, and excitation was alternated at 490 and 440 nm for 250 ms, capturing images at 1-min intervals. The sample was continuously illuminated at 620 nm by placing a red filter in front of the transmitted incandescent source. By placing an additional dichroic mirror in the light path, the red light was directed to a video camera, allowing continuous visualization of cell morphology and of the course of phagocytosis by digital interference contrast microscopy, while the fluorescent light was directed onto a 535 ± 25 nm emission filter placed in front of the cooled CCD camera used for fluorescence detection (see Ref. 22 for details). Calibration of fluorescence ratio versus pH was obtained with BCECF acetoxyethyl ester as above.

A representative experiment designed to determine the pH of phagocytosed particles is illustrated in Fig. 1, which proceeds sequentially from top to bottom. The colorimetric pH scale to the right of the figure provides an assessment of the particle pH as displayed in the pseudo-color ratio image. Because not all the particles that associate with cells become internalized, it was essential to identify those beads or bacteria...
The new pH medium pH from line beads. Therefore, sensitivity to added NH$_4$Cl or to extracellular acidification was induced by addition of defined concentrations of NH$_4$Cl, and following stabilization of cytosolic or phagosomal pH, rapid alkalinization that had phagocytosed fluoresceinated particles, as detailed above. Addition of nigericin altered pH but had no acute effect on intraphagosomal particles (Fig. 1). Finally, addition of bafilomycin, an inhibitor of the V-ATPase (Fig. 1), or NH$_4$Cl as NH$_3$ and becomes protonated in the phagosomal lumen. Neither bafilomycin nor NH$_4$Cl are expected to affect extracellular beads. SEC as NH$_3$ and becomes protonated in the phagosomal lumen. Neither bafilomycin nor NH$_4$Cl are expected to affect extracellular beads. Samples were analyzed in a model 443 flame photometer (Instrumentation Laboratories, Lexington, MA) and compared with Na$^+$ standards.

**Microsomal and Plasma Membrane Preparations**

To obtain a microsomal fraction, 1.5 $\times$ 10$^7$ cells were sedimented (250 $\times$ g for 10 min) and resuspended in 2 ml of homogenization buffer (250 mM sucrose, 20 mM Hepes, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM pepstatin A, 1 mM EDTA, pH 7.4). Homogenization was performed by 50 strokes of a Dounce homogenizer, at which time approximately 90% of cells were broken as monitored by microscopy. The resulting homogenate was subjected to centrifugation at 1800 $\times$ g for 10 min to remove nuclei and unbroken cells. The resulting supernatant was sedimented at 100,000 $\times$ g for 1 h yielding the "microsomal fraction." Protein concentration was determined with the bicinchoninic acid protein assay reagent (BCA assay, Pierce) using bovine serum albumin as a standard.

A plasma membrane preparation was obtained by a slight modification of the method of Lutz et al. (24). J774 cells plated on 10-cm Petri dishes were bled by Gentle shearing the plasma membrane from 1 ml Tris-Cl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM pepstatin A, 1 mM EDTA, pH 7) through a 10-ml syringe using an 18-gauge needle oriented at a 45° angle to the surface of the dish, until no intact cells were detected by light microscopy. Cell membranes still adherent to the dish were then scraped into 100 ml of 1/5 x Laemmli's sample buffer (25) and concentrated 5-fold by lyophilization. Typically, 15 Petri dishes were used per preparation. Protein concentration was then determined by the method of Lowry et al. (26) after precipitation of proteins using 10% trichloroacetic acid.

**Phagosome Isolation**

A phagosome fraction was obtained as described (2). Briefly, cells were plated on 10-cm Petri dishes until they reached approximately 80% confluence. Latex beads (0.8 $\mu$m diameter blue dyed, Sigma) were added to the cells, and the mixture was incubated for 2 h at 37 °C. The cells were then washed three times in ice-cold PBS plus protease inhibitors (10 min each, with continuous shaking) and disrupted in a Dounce homogenizer until approximately 90% of cells were broken as assessed by light microscopy. The homogenate was then subjected to centrifugation at 350 $\times$ g for 5 min. The resulting supernatant was mixed with 80% sucrose and 3 ml imidazole, pH 7.4, and applied to the bottom of a discontinuous sucrose gradient composed of the following steps: 10, 25, 35, and 40% sucrose-imidazole, 2 ml each. The gradient was subjected to centrifugation at 100,000 $\times$ g for 60 min, and the phagosome fraction was collected from the 10–25% interphase. This was added to 15 ml of PBS and sedimented at 40,000 $\times$ g for 15 min. The phagosomal pellet was solubilized for immunoblotting following SDS-polyacrylamide gel electrophoresis. Purity was evaluated by transmission electron microscopy, as described (18).

**Polyacrylamide Gel Electrophoresis and Immunoblotting**

Samples were solubilized in Laemmli's sample buffer (25), resolved by SDS-polyacrylamide gel electrophoresis using the Protean II minigel system (Bio-Rad), and transferred onto nitrocellulose membranes. Membranes were then immersed in blocking buffer (0.25% gelatin, 1% ethanolamine, 1 x Tris, pH 9.0) for 2 h. Blots were incubated with primary antibody solution overnight at 4 °C (antibodies to V-ATPase, NHE1, and the a$_1$, a$_2$, and b$_1$ subunits of the Na$^+$-K$^+$-ATPase were used at a 1:1000 titer, whereas LAMP2 antibodies were used at 1:4). The blots were then washed three times for 10 min each in antibody buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 0.04% Nonidet P-40, pH 7.5). Blots were next incubated with anti-rabbit IgG for V-ATPase, NHE1, and the b$_1$ subunits of the Na$^+$-K$^+$-ATPase (titer, 1:10000), anti-mouse IgG for the a$_1$ and a$_2$ subunits of the Na$^+$-K$^+$-ATPase, or anti-rat IgG (titer, 1:10,000) for anti-LAMP2. Membranes were washed and developed using the enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham Corp.).
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NHE4, 5

PCR product, 680 bp; NHE3, 5

as a template but did not yield discernible products when any of

incubation with the antigenic purified fusion protein for

t room followed by a 1/1000 dilution of Cy3-labeled donkey anti-rabbit IgG. The

the polyclonal antibody raised to the 39-kDa subunit of the V-ATPase,

ice-cold PBS, and blocked with 5% donkey serum in PBS for

t room temperature, confirming its specificity.

Where indicated, 5 µM nigericin was added in KCl medium at pH 7.4. Representative of seven separate experiments.

Isolation of RNA, Reverse Transcription and Polymerase Chain Reaction

Total RNA was isolated from α106 cells by guanidinium thiocyanate/phenol/chloroform extraction, based on the method of Chomczynski and Sacchi (27). Poly(A)+ RNA was purified by affinity chromatography with an oligo(dT)-cellulose column (Pharmacia Biotech Inc.). J774 mRNA was then reverse-transcribed, and the complementary DNA was amplified by the polymerase chain reaction, using the GeneAmp RNA PCR kit (Perkin-Elmer) and a Perkin-Elmer DNA thermal cycler model 480. After completion of the PCR reaction (35 cycles), a 10-µl sample of the PCR tube was analyzed by electrophoresis on a 0.8% agarose gel pre-stained with 0.5 mg/ml ethidium bromide, and the gel was photographed under UV illumination. For illustration, the photograph was scanned and labeled using Adobe Photoshop software (Adobe Systems, Inc, Mountain View, CA). Four isoform-specific sets of primers were used, which hybridized to unique regions of the rat NHE1, NHE2, NHE3, and NHE4. Primers were as follows: NHE1, 5′ primer, CCT ACG TGG AGG CCA AC; 3′ primer, CAG CCA ACA GGT CTA CC; size of the PCR product, 429 base pairs (bp); NHE2, 5′ primer, GCT GTC TCT GCA GGT GG; 3′ primer, CGT TGA GCA GAG ACT CG; size of PCR product, 680 bp; NHE3, 5′ primer, CTT CTG CTA CCT GCT GC; 3′ primer, CAA GGA CAG CAT CTC GC; size of PCR product, 574 bp; NHE4, 5′ primer, CTG AGC TCT GTG GCT TC; size of PCR product, 381 bp. All four sets of primers yielded the expected PCR products when linearized pcMV plasmids containing the full-length clone of the corresponding isoform were used as a template but did not yield discernible products when any of the other isoforms was used as template.

Immunofluorescence Microscopy

Immunofluorescence studies were performed on peritoneal macrophages from C57BL/6 mice that had phagocytosed live M. bovis essentially as described (28). Briefly, macrophages were allowed to internalize mycobacteria that had been labeled with NHS-CF (0.01 mg/ml) as above. The concentration of NHS-CF was one-tenth that used for fluorescence ratio imaging studies, to prevent any green fluorescence from crossing over into the rhodamine filter set. Cells were fixed for 3 h with 4% paraformaldehyde in PBS at room temperature and then washed in PBS containing 100 mM glycine for 10 min. The cells were then permeabilized in 0.1% Triton X-100 in PBS for 20 min at 22 °C, washed in ice-cold PBS, and blocked with 5% donkey serum in PBS for 1 h at room temperature. Coverslips were then incubated with a 1:1000 dilution of the polyclonal antibody raised to the 39-kDa subunit of the V-ATPase, followed by a 1/1000 dilution of Cy3-labeled donkey anti-rabbit IgG. The immunoreactivity of the anti-39-kDa antibody was eliminated by pre-incubation with the antigenic purified fusion protein for 1 h at room temperature, confirming its specificity.

After staining, cells were mounted using Slow Fade (Molecular Probes, Eugene, OR) and sealed with nail polish. Fluorescence was analyzed using a Leica Model TCS4D (Heidelberg, Germany) laser confocal microscope with a 63 × objective. Compositions of confocal images were assembled and labeled using Photoshop and Illustrator software (Adobe, Mountain View, CA). All experiments were performed at least four times. Representative confocal images are displayed where appropriate.

Miscellaneous Methods and Data Presentation

To assess the affinity and specificity of the NHE1 antibody, positive controls consisted of human platelets and NHE1-transfected Chinese hamster fibroblasts lacking endogenous exchanger, which were obtained as described (29). Negative controls for the NHE1 antibody consisted of non-transfected, antiport-deficient fibroblasts and fibroblasts transfected with NHE2. Preparations of rodent brain used as positive controls for the Na+−K+ ATPase antibody were prepared as described (30). Immunoblots were scanned using the Hewlett-Packard Desk ScanII imaging system (Falo Alto, CA) and labeled with graphics software by Adobe (Mountain View, CA). Immunoblot quantification was performed using a PDI model DNA35 scanner (Protein Data bases, Inc.) and the Discovery Series one-dimensional gel analysis software (Version 1.3). All immunoblot experiments were performed at least three times. Unless otherwise indicated, data are expressed as means ± S.E. of the specified number of experiments (n).

RESULTS

The pH of Phagosomes Containing M. bovis (BCG) in Peritoneal Macrophages—Perusal of earlier publications revealed that, although ostensibly devoid of V-ATPases, phagosomes containing live mycobacteria are more acidic than the surrounding cytosol (5, 7). To validate this important observation, we compared the pH of phagosomes induced by live and heat-killed BCG in resident peritoneal macrophages obtained from C57BL/6 mice. This strain of mice was selected due to its susceptibility to infection by mycobacterial species, including M. bovis (BCG), Mycobacterium avium, and Mycobacterium intracellulare, which remain viable and replicate within phagosomes of the host macrophages (31–33). Phagosomal pH was determined by fluorescence ratio imaging of single cells, using bacteria labeled with a pH-sensing fluoroprobe (see “Experimen
tal Procedures”). As summarized in Fig. 2A, the phagosomal pH of live BCG was significantly more alkaline than that of dead BCG (6.5 ± 0.05 versus 5.6 ± 0.06, respectively; p < 0.05). These values are consistent with earlier studies of the pH of internalized Mycobacteria in macrophages from susceptible mice (5, 7). The phagosomal pH of both live and dead BCG could be similarly dissipated by nigericin, confirming the intracellular location of the bacteria (Fig. 2B). Moreover, the calibration of dead and live bacteria was indistinguishable (not shown), indicating comparable responsiveness of the covalently attached pH probe. Importantly, the phagosomal pH of live BCG was nearly 1 pH lower than the cytoplasmic pH (Fig. 2B), suggesting active acidification of the phagosomal lumen. The differential pH of phagosomes containing dead versus live bacteria could not be explained by differences in cytoplasmic pH, which was identical in the two cases (Fig. 2B).

Biochemical and Functional Characterization of NHE in J774 Cells—We next sought to determine the mechanism re-
Fig. 3. NHE1 is the predominant isoform of the Na⁺/H⁺ exchanger in J774 cells. A, immunoblotting with polyclonal anti-NHE1 antibodies. Samples of human platelets (PLT), or microsomes prepared from J774 cells, PS127 cells (hamster fibroblasts transfected with the human NHE1), PS120 cells (antiport-deficient hamster fibroblasts), or AP-1/NHE2 cells (hamster fibroblasts transfected with human NHE2) were subjected to electrophoresis and blotting and were probed with a polyclonal rabbit antibody raised against the C-terminal 157 amino acids of NHE1. B, reverse transcription and PCR. Messenger RNA was extracted from J774 cells and used as a template for reverse transcription-polymerase chain reaction with isoform-specific primers. M, molecular weight markers. P, template was linearized pCMV plasmid containing the full sequence of rat NHE1 (lane 1), NHE2 (lane 4), NHE3 (lane 7), or NHE4 (lane 10), hybridized with the corresponding primers. J774, template was J774 mRNA, hybridized with primers specific for NHE1 (lane 2), NHE2 (lane 5), NHE3 (lane 8), or NHE4 (lane 11). (minus), controls used similar template and primers as J774, but reverse transcriptase was omitted. Representative of three separate experiments. C, sensitivity of Na⁺/H⁺ exchange to HOE 694. Cytosolic pH was measured in BCECF-loaded J774 cells by ratio fluorimetry. The cells were acid-loaded by an ammonium pre-pulse, and recovery was initiated by perfusion in Na⁺-containing medium. Where indicated, 1 μM HOE 694 was present. Data are means ± S.E. of four determinations. D, concentration dependence of the inhibitory effect of HOE 694, derived from experiments like that in C.

sponsible for the acidification of phagosomes containing live BCG. Because previous reports have indicated that phagosomes induced by live *Mycobacteria* do not contain vacuolartype ATPases (5, 34), we investigated whether other ion transporters, namely the NHE, can contribute to phagosomal acidification. Although functional studies can be readily performed using resident murine macrophages (see below), the biochemical assessment of the presence of NHE in phagosomes requires large amounts of material that are impossible to obtain from mice. We therefore complemented the functional analyses with molecular studies in J774 macrophages. This cell line, which was isolated from a susceptible strain of mice, behaves similarly to macrophages from C57BL/6 mice that are internalized mycobacteria survive and thwart phagosome-lysosome fusion (7, 35).

To define the presence and activity of the Na⁺/H⁺ exchanger in phagosomes, it was essential to define initially the isoform(s) of the antiporter expressed by J774 cells. To date, five isoforms of the Na⁺/H⁺ exchanger have been identified (NHE1 to NHE5) (36–42). NHE1, the “housekeeping” isoform, is present in nearly all mammalian cells examined. The other isoforms have a more restricted tissue distribution. NHE2, NHE3, and NHE4 are abundant in epithelial cells of kidney, intestine, and stomach, whereas NHE5 resides primarily in brain, spleen, and testis. Because murine peritoneal macrophages were shown earlier to express NHE1 (43), the presence of this isoform in J774 cells was assessed first. Microsomal fractions were subjected to electrophoresis and probed with affinity purified antibodies to NHE1. Platelets, known to be rich in NHE1 (44), and NHE1-transfected fibroblasts (PS 127 cells) were used to verify the effectiveness of the antibodies (Fig. 3A). As anticipated, a polypeptide of ≈110 kDa, corresponding to the known size of NHE1 (36), was recognized by the antibody. A band of identical size was also detected in membranes isolated from J774 cells. The specificity of the immunoreactivity was confirmed by comparison with microsomes obtained from antiport-deficient fibroblasts (PS120) or from antiport-deficient cells transfected with NHE2, which failed to react with the antibody (Fig. 3A).

These findings indicate that NHE1 is expressed in J774 cells but do not rule out the expression of other isoforms. Because specific antibodies to all other isoforms are not currently available, we utilized reverse transcription-polymerase chain reaction to assess the presence of NHE1 through NHE4 in the cultured macrophages. Isoform-specific primers that hybridize to unique regions of NHE1, NHE2, NHE3, and NHE4 were used. All four sets of primers yielded the expected PCR products (Fig. 3B, lanes 1, 4, 7, and 10) when linearized pCMV plasmids containing the full-length cDNA clone of the corresponding isoform were used as template. When cDNA obtained by reverse transcription of J774 mRNA was used as a template, the NHE1 primers yielded a product of ≈500 bp (Fig. 1B, lane 2), in good agreement with the immunoblotting data of Fig. 3A. In contrast, all the other sets of primers did not yield discernible products (Fig. 3B, lanes 5, 8, and 11). Omission of reverse transcriptase prevented appearance of the 500-bp product, ruling out contamination with genomic DNA. Thus, the predominant isoform expressed in J774 cells is NHE1.

The presence of NHE5, an incompletely characterized isoform (45), was not assessed biochemically. To exclude the possibility that this or other heretofore unidentified isoforms contribute to Na⁺/H⁺ exchange in J774 cells, a pharmacological
approach was undertaken. We used compound HOE 694, an amiloride analogue, known to inhibit the individual NHE isoforms at differential concentrations. As shown in Fig. 3C, the antiport activity of J774 cells, assessed as the \( \text{Na}^{+} \)-dependent recovery from a cytosolic acid load, could be effectively inhibited by compound HOE 694 (1 \( \mu \text{M} \)). The concentration of HOE694 required for half-maximal inhibition was 64 \( \mu \text{M} \) (Fig. 3D), similar to that reported to inhibit NHE1 (46) and much lower than that needed to inhibit other isoforms (e.g. \( K_c \) of 5 and 650 \( \mu \text{M} \) for NHE2 and NHE3, respectively). These data are consistent with the notion that NHE1 is the main, and possibly the only, isoform of the antiport operating in J774 cells.

**Presence of NHE in the Phagosomal Membrane**—Having established the presence of NHE1 in J774 cells, we investigated whether this antiporter is internalized during phagosome formation. To this end, we compared the density of NHE1 in plasma membranes and phagosomal membranes. Surface (plasma) membranes were prepared by an adherence and shearing procedure (see “Experimental Procedures”). The relative purity of this preparation was indicated by a ≥10-fold enrichment in \( \text{Na}^{+}/\text{K}^{+} \)-ATPase, compared with whole cell lysates (data not shown). Moreover, the plasma membrane preparation was largely devoid of LAMP-2, a late endosome-lysosome marker, and of V-ATPase, which is present in various endomembrane compartments (Fig. 4). As expected, immunoblotting experiments revealed that NHE1 is abundant in the plasma membrane (Fig. 4).

Phagosomes were purified from J774 cells that had been allowed to ingest latex beads. The purity of the resulting population was assessed by transmission electron microscopy (see Ref. 18 for representative micrograph). These mature phagosomes were rich in LAMP-2 and V-ATPases, indicating enrichment in Na-H exchanger 1 (NHE1).

**Contribution of the V-ATPase and of NHE to Phagosomal Acidification**—Having established that both V-ATPases and NHE are present in the phagosomal membrane, we next investigated their relative contribution to phagosomal acidification. This was accomplished by application of selective inhibitors of the pumps (bafilomycin and concanamycin) or antiporters (HOE 694). The presence of these inhibitors did not affect the number of cells undergoing phagocytosis. As illustrated in Fig. 5, internalization of latex beads in otherwise untreated cells was followed by a rapid acidification of the phagosomal lumen, which equilibrated at \( \text{pH}_{\text{i,phag}} = 6.0 \pm 0.1 \) (\( n = 7 \)). By contrast, in cells exposed to a combination of bafilomycin and concanamycin (100 \( \text{nM} \) each), \( \text{pH}_{\text{i,phag}} \) acidified marginally, if at all, remaining at a level indistinguishable from the cytosolic pH (\( \text{pH}_{\text{i,plasm}} = 7.3 \pm 0.1 \); \( n = 7 \)). HOE 694 was used to evaluate the contribution of NHE1. Because the binding site for this and related inhibitors is believed to be on the extracellular (luminal) aspect of the

![Phagosomal Acidification in Macrophages](https://example.com/figure5.png)
residual acidification recorded in phagosomes with live BCG is not dependent on NHE activity.

Assessment of Phagosomal \( \text{Na}^+ / \text{H}^+ \) Exchange Activity—The data summarized above indicate that, despite being present on the phagosomal membrane, NHE1 does not contribute measurably to phagosomal acidification. It was therefore of interest to determine whether the phagosomal antiporters are functionally active. Given the very acidic interior of the phagosome, transport by NHE in the forward mode (luminal \( \text{Na}^+ \) for cytoplasmic \( \text{H}^+ \)) is unlikely to be detectable. Instead, we attempted to measure exchange activity in the reverse direction. That NHE1 can operate in reverse in J774 cells was first documented by measuring the activity of the plasmalemmal antiport. Cells were initially loaded with \( \text{Na}^+ \) by incubation for 30 or 60 min in \( \text{K}^- \)-free medium with ouabain. Under these conditions, the cytosolic \( \text{Na}^+ \) content increases from 16.5 \( \pm \) 1.5 to 71.5 \( \pm \) 3.5 and 114 \( \pm \) 6 \( \mu \)M, respectively (Fig. 6A). Upon resuspension in a \( \text{Na}^- \)-free medium, the outward gradient for \( \text{Na}^+ \) drove \( \text{H}^+ \) inward, inducing a pronounced cytosolic acidification (Fig. 6B). Three lines of evidence confirmed that such an acidification is mediated by reverse NHE. First, the pH change is greatly inhibited by HOE 694 (Fig. 6, B and C). Second, the acidification rate was greater when the extracellular concentration of \( \text{H}^+ \) was higher (cf. results at \( \text{pH} \) 6.0 and 7.0 in Fig. 6C). Finally, the acidification was minimal when extracellular \( \text{Na}^+ \) was present, minimizing the driving force for \( \text{H}^+ \) uptake (Fig. 6C).

By having demonstrated the ability of NHE1 to operate in reverse in J774 cells, we investigated whether the phagosomal antiporters are functional. For this purpose, cells were \( \text{Na}^+ \)-loaded as above, and \( \text{pH}_p \) was measured. Under these conditions, reverse \( \text{Na}^+ / \text{H}^+ \) activity is predicted to dissipate the phagosomal pH gradient. This prediction was borne out when using an exogenous \( \text{Na}^+ / \text{H}^+ \) exchanging ionophore, namely monensin. As illustrated in Fig. 7A, addition of this ionophore to control cells caused phagosomal alkalization. Importantly, the rate of alkalization was much greater in \( \text{Na}^+ \)-loaded cells than in controls, implying that pH dissipation occurs as a result of exchange of cytosolic \( \text{Na}^+ \) for luminal \( \text{H}^+ \).

The basal phagosomal pH was not affected when J774 cells were loaded with \( \text{Na}^+ \) using ouabain (see Fig. 7B). This finding could be interpreted to mean that NHE1 is not functional in the phagosomal membrane. However, it is conceivable that, although exchange of cytosolic \( \text{Na}^+ \) for \( \text{H}^+ \) may indeed be occurring, it is readily offset by a more vigorous V-ATPase, capable of maintaining \( \text{pH}_p \) at an acidic level. This possibility was analyzed by comparing the rate of dissipation of the acidic \( \text{pH}_p \) upon addition of concanamycin and bafilomycin (Fig. 7B). As expected, the V-ATPase inhibitors induced a gradual alkalization of \( \text{pH}_p \) in cells containing normal \( [\text{Na}^+]_o \). Importantly, the rate of dissipation was consistently greater in \( \text{Na}^+ \)-loaded cells, suggesting the occurrence of reverse \( \text{Na}^+ / \text{H}^+ \) exchange (Fig. 7, B and C). Accordingly, addition of HOE 694 markedly reduced the rate of alkalization. It is noteworthy that the NHE inhibitor, which was added during the final phases of \( \text{Na}^+ \) loading and was present during phagocytosis, did not affect that ability of the cells to accumulate \( \text{Na}^+ \) (Fig. 6A).

Exclusion of the \( \text{Na}^+ / \text{K}^- \) ATPase from the Phagosome—Although activity of NHE1 was demonstrable in the reverse direction, the antiporter was seemingly unable to contribute to phagosomal acidification by operating in its forward mode. As shown in Fig. 5A, phagosomal acidification was insignificant in the presence of V-ATPase inhibitors. We therefore considered the possibility that, while the antiporter was present and functional, the prevailing ionic gradients may be inappropriate for induction and maintenance of an acidic \( \text{pH}_p \) by the NHE. Suspected transfer of \( \text{H}^+ \) into the phagosomal lumen would require the presence of a steady lumen-to-cytosol \( \text{Na}^+ \) gradient, which could in principle be maintained by the \( \text{Na}^+ / \text{K}^- \)-ATPase. We therefore proceeded to determine whether this enzyme is present and active in the phagosomal membrane. Purified phagosomal and plasma membrane fractions were subjected to electrophoresis and immunoblotted using antibodies to the individual subunits of the \( \text{Na}^+ / \text{K}^- \)-ATPase. As shown in Fig. 8, both the \( \alpha_1 \) and \( \beta_1 \) subunits of the ATPase were present in the plasma membrane, as expected. By contrast, these \( \text{Na}^+ / \text{K}^- \)-ATPase subunits were not detectable in the phagosome. Similar results

FIG. 6. Reverse \( \text{Na}^+ / \text{H}^+ \) exchange in \( \text{Na}^+ \)-loaded J774 cells. A, \( \text{Na}^+ \) concentration of J774 cells before (0 min) and after 30 or 60 min of incubation with 1 mM ouabain in \( \text{K}^- \)-free solution. Where indicated 1 \( \mu \text{M} \) HOE 694 was added during the last 15 min of the incubation. Na\(^+\) content was measured by flame photometry, and cell volume was determined electronically using the Coulter-Channelizer. Data are means \( \pm \) S.E. of four determinations. B, cytosolic pH determinations in \( \text{Na}^+ \)-loaded cells. Cells were loaded with \( \text{Na}^+ \) for 60 min as in A and stained with BCECF during the last 10 min of incubation. The traces start upon resuspension of the cells in \( \text{K}^- \)-rich (Na\(^-\)-free) medium, pH 7.0, in the presence and absence of 1 \( \mu \text{M} \) HOE 694. Data are means \( \pm \) S.E. of five determinations. C, effect of extracellular cation composition and \( \text{pH} \) on the rate of intracellular acidification. Cells were acid-loaded, and cytosolic pH was measured as in B. The extracellular medium was either \( \text{Na}^+ \)- or \( \text{K}^- \)-rich, at the indicated pH. HOE 694 (1 \( \mu \text{M} \)) was present where specified. Ordinate, maximal rate of acidification, measured within 1.5 min of resuspension. Data are means \( \pm \) S.E. of five determinations.
for the α1 subunit were obtained 30 min after phagosome formation (Fig. 8), reducing the likelihood of proteolysis accounted for our failure to detect the Na⁺/K⁺-ATPase. To exclude the possibility that another isoform of the Na⁺/K⁺-ATPase accumulated in the phagosome, the presence of the α2 subunit was probed. This isoform, which is present in brain cells (left–most lane in Fig. 8), was not detectable in either the plasma membrane or the phagosomes of J774 cells. Jointly, these results indicate that Na⁺/K⁺-ATPases are either not incorporated into forming phagosomes or are rapidly removed from the sealed phagosomes during the early stages of phagosomal maturation.

Role of V-ATPases in the Acidification of Phagosomes Containing Mycobacteria—Despite its presence in the phagosomal membrane, NHE does not appear to contribute to luminal acidification. Thus, the partial, yet significant acidification recorded in phagosomes containing live mycobacteria remains unexplained. Although V-ATPases are reportedly absent from such phagosomes (5, 34), we considered the possibility that H⁺ pumping may nevertheless play a role in phagosomal acidification. This can be envisaged to occur in two ways as follows: V-ATPases may be transiently present in the phagosomal membrane, causing luminal acidification, which may be followed by removal of the pumps from the membrane by vesicular budding. Alternatively, pump-mediated accumulation of acid may occur in the endosomal lumen, possibly followed by delivery of acidic fluid to the phagosome by means of carrier vesicles, themselves devoid of V-ATPases. In both instances, bafilomycin or concanamycin-sensitive acidification of phagosomes would ensue, in the absence of detectable V-ATPases in the phagosome.

To assess these possibilities, macrophages from C57BL/6 mice were allowed to internalize either live or dead BCG and, after establishing the base-line pH, concanamycin was added. As shown in Fig. 9A, the pH of internalized BCG was rapidly dissipated after addition of concanamycin. Importantly, after inhibition of the V-ATPase, the phagosomal pH approached the level of the cytosolic pH (cf. Figs. 2 and 9), and a comparable extent of dissipation was noted for live and dead BCG phagosomes (Fig. 9B). Together these data suggest that the V-ATPase is the principal and perhaps the sole mechanism of acidification of internalized mycobacteria.

Incorporation of Proton Pumps onto Mycobacterial Phagosomes—At first glance, the results of Fig. 9 appear to be incompatible with earlier reports that V-ATPases are not detectable in mycobacterial phagosomes (5). This apparent discrepancy may be due to the use of different species of mycobacteria, i.e. avium (5) versus bovis (this study). We therefore examined the possible presence of V-ATPases in BCG-containing phagosomes. Macrophages from C57BL/6 mice were allowed to internalize live, fluoreseinated BCG, and the presence of the V-ATPase on the phagosomal membrane was determined by indirect immunofluorescence, using the antibody to the 39-kDa subunit of the Na⁺/K⁺-ATPase (Fig. 10, B and G). In some instances, staining with the V-ATPase antibody was noted to outline the surface of the internalized mycobacteria (Fig. 10F), implying that V-ATPases were present on the phagosomal surface. In the remainder of the cells (85–90% of the total) BCG did not colocalize with the V-ATPase (e.g. Fig. 10, B–E, G, and H).

This suggests that in the majority of cases the V-ATPase is not present on the mycobacterial phagosome or associates with it.
only transiently. Alternatively, V-ATPases may be present in most phagosomes, but their abundance is below the level of immunodetection.

**DISCUSSION**

The purpose of the present study was to determine the mechanisms contributing to phagosomal acidification in general, with special interest in phagosomes containing mycobacteria. Phagosomal acidification is reduced but not completely eliminated by live mycobacteria; indeed an acidification of approximately 1 pH unit was recorded after internalization of live BCG (Fig. 2), in agreement with previous observations (5, 7). Because V-ATPases were reportedly excluded from the mycobacterial phagosome, we first investigated whether NHE molecules internalized from the surface membrane during phagocytosis contribute to acidification. Using a combined biochemical, molecular, and pharmacological approach, we determined that the housekeeping NHE isoform, NHE1, was expressed by J774 cells. More importantly, we found that NHE1 is incorporated into phagosomal membranes. At this site, it is in principle poised to exchange intraphagosomal Na$^+$ ions for cytoplasmic H$^+$ ions, thereby potentially contributing to phagosomal acidification. The extent to which NHE can theoretically contribute to phagosomal acidification can be estimated on thermodynamic grounds (Equation 1), based on the knowledge that the exchange reaction is electroneutral, with a 1:1 stoichiometry. Because at equilibrium

$$\frac{[\text{Na}^+]_{\text{phag}}}{[\text{Na}^+]_{\text{cytosol}}} = \frac{[\text{H}^+]_{\text{cytosol}}}{[\text{H}^+]_{\text{phag}}}$$  
(Eq. 1)

the ratio of cytosolic to phagosomal (luminal) Na$^+$ will dictate to what extent the phagosomal pH ($pH_p$) can deviate from the cytosolic pH. Assuming that the phagosomal [Na$^+$] is maintained at extracellular levels ($\approx 140$ mM) and that cytoplasmic [Na$^+$] approximates 10–15 mM (23), pH$_p$ could in principle become up to two pH units more acidic than the cytosolic pH, approaching the values reported in many studies (e.g. Refs. 48–50). Thus, NHE could conceivably contribute importantly to phagosomal acidification.

The preceding calculations assume that the concentration of phagosomal Na$^+$ would be maintained throughout the exchange process by independent means and/or that the buffering capacity of the phagosomal interior is negligible. The latter is certainly not the case: direct determination of the buffering power of latex phagosomes by pulsing with weak electrolytes
Several mechanisms may account for the depletion of Na\(^+\)\,-ATPases from the phagosome. A segregation mechanism may exclude the pumps from the areas of the membrane that may exclude the pumps from the areas of the membrane that are being internalized. This could conceivably occur through interactions with cytoskeletal components. The Na\(^+\)/K\(^+\)-ATPase is known to interact with ankyrin, which in turn attaches to the actin cytoskeleton (52, 53). This may provide a means of excluding the ATPase from the forming phagosome. Alternatively, the pumps may be internalized and then rapidly removed during phagosomal maturation (1). Regardless of the underlying mechanism, depletion of Na\(^+\)/K\(^+\)-ATPases from the phagosome can be envisaged to be advantageous to the cells in at least two ways. It would serve to maintain a normal complement of pumps on the surface, preventing their wasteful degradation in the phagolysosomal compartment. In addition, elimination of Na\(^+\)/K\(^+\)-ATPases from the phagosome would preclude the build-up of an electrogenic potential across the phagosomes. Such an internally positive potential would antagonize the V-ATPase, which is similarly electrogenic (54). In this regard, the plasmalemmal Na\(^+\)/K\(^+\)-ATPase is known to be incorporated into early endosomes, where it seemingly remains active (13, 14), although its effects on endosomal pH are not universally observed (15). By generating a potential across the endosomal membrane, the Na\(^+\)/K\(^+\)-ATPase is believed to limit the acidification of early endosomes.

It is conceivable that mature phagosomes attain a much more acidic pH than endosomes due, at least in part, to the effective removal of Na\(^+\)/K\(^+\)-ATPases.

The results of experiments using HOE 694 (Fig. 5) rule out NHE as a likely source of the partial, yet significant acidification observed in phagocytes infected with mycobacteria. On the other hand, our experiments indicate that the acidification of phagosomes containing live BCG can be entirely suppressed by addition of concanamycin (or bafilomycin; not shown), specific antagonists of the V-ATPase (55). These findings are, at first glance, incompatible with the results of Sturgill-Koszycki et al. (5), who demonstrated that mycobacterial phagosomes lack V-ATPases. We therefore undertook studies of V-ATPase localization by immunofluorescence in cells infected with BCG. Like Sturgill-Koszycki et al. (5), we were unable to detect V-ATPases in the majority of the phagosomes. However, 10–15% of the phagosomes were lined by ATPases. These phagosomes may contain dead or otherwise inactive mycobacteria. On the other hand, it is conceivable that association of V-ATPases with the phagosome is transient. Acidification of mycobacterial phagosomes may have occurred through the internalization of plasmalemmal V-ATPases, known to be present on the surface of macrophages (51, 56) or by delivery of endosomal ATPases, followed by their subsequent removal through selective budding off the phagosomal membrane. This model, however, is not tenable, since in the steady-state concanamycin rapidly dissipated the acidification of all phagosomes tested, implying that an endogenous leak of H\(^+\) was being continuously offset by ongoing pumping. Hence, V-ATPases must have been present in the phagosome at the time of analysis. A second possibility is that a reduced number of phagosomal V-ATPases escaped detection by immunoblotting and immunofluorescence.

A third, more attractive hypothesis is presented schematically in Fig. 11. Under normal circumstances, fusion of lyosomes with phagosomes results in the transfer of V-ATPases to the phagosomal membrane, contributing to phagosomal acidification (Fig. 11A). This step appears to be impaired by mycobacteria, accounting for the paucity of pumps in most phagosomes (Fig. 11B). It is nevertheless possible that acid equivalents are delivered to the phagosomal lumen by carrier vesicles (ACV in Fig. 11B), which are derived from endosomes,
yet are devoid of V-ATPases. Although unable to interact with lysosomes, the mycobacterial phagosome can fuse with endo-

somes and/or vesicles derived thereof (57). Continued vesicular 

traffic to the phagosome would explain the sustained, concana-

cycin-sensitive acidification in a compartment that lacks AT-

Pases. The observed leakage of protons could be due, at least in 

part, to the removal of fluid from the lumen during the course 

of vesiculation of the phagosomal membrane, which would be 

necessary to maintain a steady phagosomal size.

In summary, we found that NHE, but not Na⁺/K⁺ ATPases, are present in phagosomes. Although present in the phagoso-

mal membrane in a functional state, NHE1 does not contribute noticeably to the acidification of phagosomes. Even in those instances where V-ATPases are scarce or absent, as in the case of mycobacterial phagosomes, the acidification was found to be obliterated by V-ATPase inhibitors. This observation points to the existence of a mechanism whereby delivery of acid (equiva-

lents) to phagosomes is not necessarily accompanied by de-

tectable levels of V-ATPases on its membrane.

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