Lysosomal Enzyme Trafficking between Phagosomes, Endosomes, and Lysosomes in J774 Macrophages

ENRICHMENT OF CATHEPSIN H IN EARLY ENDOSONES

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In this study we take advantage of recently developed methods using J774 macrophages to prepare enriched fractions of early endosomes, late endosomes, dense lysosomes, as well as phagosomes of different ages enclosing 1-μm latex beads to investigate the steady state distribution and trafficking of lysosomal enzyme activity between these organelles. At steady state these cells appear to possess four different cellular structures, in addition to phagolysosomes, where acid hydrolases were concentrated. The first site of hydrolase concentration was the early endosomes, which contained the bulk of the cellular cathepsin H. This enzyme was acquired by phagosomes significantly faster than the other hydrolases tested. The second distinct site of lysosomal enzyme concentration was the late endosomes which contain the bulk of cathepsin S. The third and fourth large pools of hydrolases were found in two functionally distinct types of dense lysosomes, one only of which was found to be secreted in the presence of chloroquine or bafilomycin. Among this secreted pool was soluble furin, generally considered only as a membrane-bound trans-Golgi network resident protein. Thus, the organelles usually referred to as “lysosomes” in fact encompass a growing family of highly dynamic but functionally distinct endocytic organelles.

De Duve’s (1) now classic studies of the “lysosome” led to the concept that this membrane enclosed structure, visualized by the electron microscopic studies of Novikoff and others, defined a single type of acidic organelle that encloses all the mature acid hydrolases in the cell. That this simple view has survived so long (see e.g., Refs. 2 and 3) is surprising when one considers the extensive data published since approximately 1980 showing that in many cell types lysosomal enzymes at steady state are enriched in at least two physically distinct organelles, the so-called late endosomes (low density lysosomes or prelysosomes) and the structures now referred to as lysosomes (4, 5). In human fibroblasts these two sets of organelles were shown to divide the total cellular acid hydrolase pool equally between them (6), although in most cell types investigated 80–90% is usually maintained in the dense lysosomes (4). The term lysosome is now operationally defined as the kinetically most distal compartment of the endocytic pathway that is relatively dense in fractionation studies and which is devoid of recycling receptors such as the two mannose-6-phosphate receptors (see Refs. 4 and 7).

We have previously proposed that the late endosomes and the dense lysosomes interact in some fashion such that the Lgp-Lamp family of (lysosomal) membrane proteins are in similar concentrations in the membranes of both organelles (8, 9). That the two sets of structures equilibrate in some way is also argued by cell-cell fusion experiments (11, 12) and by other in vivo data (13). Nevertheless, these two organelles are distinct as seen by the presence of high concentrations of the cation-independent mannose-6-phosphate receptor, as well as Rab7 in the late endosomes, but not in lysosomes of cells such as normal rat kidney or the presence of the regulatory subunit of the cyclic AMP-dependent protein kinase in the late endosomes (as well as other locations) but not in lysosomes of Madin-Darby bovine kidney cells (14). In J774 macrophages, which we used for this study, the two Rab proteins, Rab5 and Rab7, while mostly depleted from lysosomes are enriched in both early and late endosomes (15), a clear difference to all the other cell types investigated (9). For these reasons we emphasize that the organelles operationally referred to here as early endosomes, late endosomes, and lysosomes from J774 cells may not all be strictly equivalent to the organelles we and others have defined more precisely in cells such as normal rat kidney, baby hamster kidney, Madin-Darby canine kidney cells, or hippocampal neurons (9). However, these fractions from J774 cells have been extensively characterized in our previous studies (15).

There is also considerable evidence that, in addition to late endosomes and lysosomes, early endosomes can contain small but functionally significant amounts of some lysosomal enzymes (see Ref. 5 for review). The simplest way to rationalize this early endosome pool is that it represents newly synthesized hydrolases that have been delivered in clathrin-coated vesicles that originate from the trans-Golgi network (16, 17). In the study by Ludwig et al. (16) significant amounts of newly synthesized lysosomal enzymes were detected in the early endosomes by both biochemical and electron microscopic approaches. In the case of rabbit alveolar macrophages, Diment et al. (18) could show that ~35% of the total cathepsin D activity was associated with “light endosomal membranes”; significantly, a fraction of this putative early endosome pool was at least transiently membrane-bound, by a mannose-6-phosphate-independent mechanism, suggesting the possibility of a specific and more residential association with the membranes of (early) endocytic organelles by an unknown mechanism. Indeed, the...
idea of a mannose 6-phosphate-independent mechanism for keeping a number of newly synthesized hydrolases membrane-bound at different stages in their transport from the endoplasmic reticulum to the endocytic pathway is also consistent with many other lines of evidence (e.g. Refs. 18–21).

Biodegradable organisms such as bacteria, yeast, or protozoans as well as non-degradable particles such as carbon, asbestos, or latex beads can be taken up by phagocytes, especially by macrophages and neutrophils. In most cases, after their formation phagosomes can fuse with elements of the endocytic pathway, a process enabling the transfer of hydrolytic enzymes into the phagosome. The available evidence argues that newly formed phagosomes can fuse with early endosomes (22–25) as well as with later endocytic organelles (26–30).

In the present study we made use of a recently developed method using J774 macrophages to prepare enriched fractions of operationally defined early endosomes, late endosomes, as well as lysosomes that had accumulated colloidal gold (15). In addition, we made use of our recent approach to prepare highly purified fractions of phagosomes (phagolysosomes) enclosing 1-μm latex beads (29). Most of the data in this study deal with careful kinetic measurements of the parallel activities of 10 mostly well known acid hydrolases in these different organelles. The results argue that in J774 cells different acid hydrolases are selectively enriched in at least four different types of endocytic organelles at steady state. MATERIALS AND METHODS

Cells—J774 cells were cultured as described previously (29). For experiments using horseradish peroxidase (HRP)2 over 70% confluent cells were incubated 30 min at 37 °C with 2 mg/ml HRP (type II, Sigma) in Dulbecco’s modified Eagle’s medium supplemented with 1 mg/ml mannan (Sigma, Munich), followed by a chase incubation of 30 or 90 min in marker-free Dulbecco’s modified Eagle’s medium. For measurement of the secretion (HRP and cellular enzymes), the cell culture media were replaced, after a brief rinse with PBS, by 10 ml Heps in PBS, with or without 180 μM chloroquine, and the cells were further incubated at 37 °C for the indicated times. Extracellular enzyme activities were determined in the cell culture supernatant after 5 min centrifugation at 1200 rpm. The intracellular activity of HRP was measured after extensive washing of the cells with PBS at 4 °C in the lysate of harvested cells. In these experiments no significant amount of enzyme could be detected in the medium, as was confirmed by washing the cells twice under the same conditions at 4 °C (results not shown). For treatment with inhibitors, cells were incubated in the different preincubation, pulse, and chase media supplemented with 10 ml Heps and either 100 mM bafilomycin A1, 180 μM chloroquine, or 20 μg/ml cycloheximide (all from Sigma, Munich).

Preparation of Endocytic Fraction and Phagosomes—Late endosomes and lysosomes were isolated from one set of macrophages. The procedure was a modification of the method described by Tjelle et al. (15). Briefly, for the preparation of late endosomes and lysosomes, J774 cells were allowed to internalize ovalbumin-conjugated gold particles (10 nm) for 3 h and chased overnight to trace the bulk of gold into the lysosomal compartment. Cells with or without gold were homogenized and centrifuged at 750 × g for 7 min. For early endosomes the homogenate was mixed 1:1 with 50% sucrose, 3 mM imidazole buffer, pH 7.4, 1 mM dithiothreitol followed by a layer of 500 μl of HB. After centrifugation at 56,000 × g for 30 min, the early endosomes were collected from the top of the self-formed Percoll gradient. Late endosomes and lysosomes were isolated from a different set of macrophages using ovalbumin-conjugated gold particles (10 nm) to achieve a density shift between late endosomes and lysosomes. The gold was internalized into J774 for 3 h and chased overnight to trace the bulk of gold into the lysosomal compartment. The cells were homogenized and centrifuged at 750 × g for 7 min. The gold-containing fraction was found in the nuclear pellet, whereas the late endosome fraction stayed in the PNS. This PNS was layered onto 17% Percoll/HB with a 50% sucrose cushion underneath to eliminate gold particles left over in the PNS. After centrifugation at 56,000 × g for 1 h, the late endosomes accumulated as a band in the Percoll gradient close to the sucrose cushion. The nuclear pellet was considered gold-containing lysosomes and was resuspended in 17% Percoll/HB and layered onto a 64% sucrose cushion. A centrifugation at 40,000 × g for 30 min separated the gold-filled lysosomes, which pelleted through the sucrose cushion, from the nuclei remaining on top of the gradient. The characterization of these fractions is described by Tjelle et al. (15) and Jahraus et al.1

It was shown by Tjelle et al. (15) and Jahraus et al.1 that the transferrin receptor is enriched in the early endosomal fraction but is essentially below detection in both the late endosome and lysosome fractions. Moreover, the early endosome specific marker EEA1 (31) is detected only in the early endosome fraction by Western blotting.1 In contrast cathepsin S is highly enriched in the late endosome fraction.1 In addition early and late endosome fractions could be distinguished by kinetic studies with internalized horseradish peroxidase which forms a plateau in the early and late endosomes after a 5-min pulse and a 5-min pulse plus a 25-min chase, respectively. As for the late endosomes/lysosomes the kinetics of HRP entry into the (isolated) endocytic compartments varied slightly from experiment to experiment (15).1 Although in some sets of experiments HRP reached the late endosome (HRP activity increased), it was not significant in others.1 Nevertheless these two sets of organelles can be differentiated both by their structure (15) and by their in vitro fusogenic properties.1

The preparation of phagosomes enclosing 1-μm latex beads was done exactly as described by Desjardins et al. (29). The quantification of phagosome number was done using a 10–100-fold dilution by measurement of A at 600 nm. For the standard curve, latex bead solution with different number of beads was determined by counting them in a hemocytometer. The determination of protein was done by the BCA-Microassay (Pierce).

Preparation of Membranes—Early endosomal vesicles or PNS were lysed by 10-fold dilution of the fraction in 5 mM carbonate plus 150 mM NaCl at pH 11 or in 5 mM phosphate plus 150 mM NaCl at pH 7, supplemented with either 0.5% Triton X-100 or 0.5% saponin, or, according to the method of Ref. 32, by dilution in hypotonic 5 mM phosphate at pH 7 followed by 8 strokes through a 0.4 × 30 needle, followed by incubation in 0.3 or 1 mM NaCl or in 5 mM mannose 6-phosphate (Sigma, Munich). The samples were incubated under the different conditions for 30 min at 4 °C before the membranes were pelleted by centrifugation for 40 min at 100,000 × g in a Ti 70 rotor (Beckman). The enzyme activities were measured in the supernatant before and after the centrifugation as described below.

Enzyme Assays—HRP activities were determined at 37 °C, using as substrate 1.2-phenylenediamine (Sigma, Munich) in a concentration of 1 mg/ml in citric acid/phosphate buffer plus 0.13% hydrogen peroxide, and 1 mM sulfuric acid was used as a stop reagent. Quantification was done spectrophotometrically via measurement at 490 nm. All cellular enzyme activities were measured fluorometrically after pretreatment of the cellular fractions with 0.2% Triton X-100 for 20 min at 4 °C. The assays were performed at 37 °C with a substrate concentration of 0.1–1 mM using specific substrates, buffers, and pH for the different enzymes as listed in Table I with the exception of cathepsin S which was determined as described by Kinoshita and Wiedner (33). In the case of cathepsin H some of the assays were also done in the presence of 1 μM puromycin to exclude the presence of aminopeptidase activity which also cleaves Arg-AMC (34). It should be noted that since the substrate used to detect cathepsin L (Z-Phe-Arg-AMC) may also react with cathepsin S (35), we refer to this activity as cathepsin B + L. In contrast the substrate used to detect cathepsin B (Z-Arg-Arg-AMC) is specific for this enzyme (35).

In the case of the 4-methylumbelliferyl-derived substrates (Sigma, Munich, or Calbiochem, Bad Soden) the quantification of fluorochromes was done using an excitation (Ex) wavelength of 364 nm and emission (Em) at 448 nm in a 10-fold dilution of 0.1 mM acetate at pH 10.6 in the case of the 7-aminomethylcoumarin (AMC)-derived substrates (all from Bachem, Heidelberg) at Ex370 nm/Em460 nm in a 10-fold dilution of 0.1 mM chlороacetic acid, 0.1 mM acetate at pH 4.5. The amounts of enzyme activities were expressed as units, where 1 unit is the amount of enzyme that cleaves 1 nmol of substrate per min.

Immunoelectron Microscopy—J774 macrophages were allowed to internalize 16 nm gold/BSA conjugates for 2 h at 37 °C followed by an overnight chase in medium free of gold. They were subsequently fed 5 nm gold/BSA for 5 min to fill early endosomes. The cells were removed
with proteinase K fixed with 4% formaldehyde and cryosections prepared which were labeled with rabbit anti-rat cathepsin H (36) followed by 10 nm gold-protein A. Profiles of early endosomes (containing at least one 5-nm gold particle) and late endocytic structures (containing at least one 16-nm gold particle) were systematically sampled and photographed. The density of labeling for cathepsin H over early endosomes, late endocytic structures, and the nucleus was counted as described by Griffiths (10) using a systematic sampling procedure.

**Enzyme-linked Immunosorbent Assay—**The vesicular fractions were lysed by 10 cycles of thawing and freezing, followed by 10 strokes through a 0.4 × 30 needle. After centrifugation for 5 min at 13,000 rpm in an Eppendorf centrifuge to remove the gold, i.e. in the lysosomal fraction, different amounts of the supernatants, containing 1–3 μg of protein, were coated on microtiter plates (Greiner, Nüttlingen) by incubation in 0.44% carbonate buffer overnight at 4 °C. As a standard, different amounts (2–120 ng) of human liver cathepsin H (Sigma, Munich) were coated in the same way. Blocking was done by incubation with 1% BSA in PBS, 0.1% Tween 20 for 1.5 h at 37 °C. For detection we used as primary antibody the rabbit anti-rat cathepsin H antibody, described above, in a concentration of 9 μg/ml in PBS, 0.1% BSA. As a second antibody we used a goat anti-rabbit IgG antibody conjugated with HRP (Bio-Rad, Munich) in a working dilution of 1:500 in PBS. The incubation time for each antibody was 1 h at 37 °C. The quantification of bound HRP activity was carried out as described above.

**RESULTS**

**Distribution of Acid Hydrolases in Endocytic Organelles—**By using the method developed by Tjelle et al. (15) we prepared enriched fractions of the three different endocytic compartments in J774 cells, namely early endosomes, late endosomes, and lysosomes. The rationale for this approach is to allow cells to internalize ovalbumin-coated gold particles (10 nm) which were then chased overnight into late endocytic structures. As seen by electron microscopy the bulk of this gold is found in large aggregates in mostly spherical vesicles (lysosomes), whereas low levels of gold are present also in other more heterogeneous structures which we define as late endosomes (see Fig. 3 and Ref. 9). Following homogenization the bulk of the gold-containing organelles pelleted at low speed with the nuclear fraction; from this the lysosomal fraction could be purified using a second centrifugation on a Percoll gradient. The PNS from this preparation was used to enrich the late endosomes, and the PNS from a separate set of cells was used to prepare the early endosome fraction. For details of the characterization of these three different fractions, see “Materials and Methods.”

When the three endocytic fractions were assayed for the activities of 11 different acid hydrolases, 9 behaved as expected with the bulk of activities in the lysosomal fraction. The rest of the activities of these nine enzymes was distributed as expected from earlier studies (4) with more in the late endosome fraction than in the early endosomes (Fig. 1). There were three exceptions to this general pattern. The first was β-hexosaminidase whose activity in the early endosome fraction was significantly higher than that found in the late endosomes. The second and most striking exception to the general pattern was the activity of cathepsin H in that the bulk of the activity (∼70%) of this enzyme (35) is found to be insensitive to 1 μM puromycin (data not shown), so that a contribution of cellular aminopeptidases (34, 37) could be excluded. Since the activity of this enzyme in the early endosome fraction was not significantly affected by treatment of the cells for up to 3 h with 20 μg/ml cycloheximide before isolation of the fractions (results not shown) it appears that, for the most part, cathepsin H in the early endosome pool is probably resident rather than a transient passenger.
enzyme can be retained in a dynamic organelle with a high flux gain insights into the mechanisms by which this "soluble" endosomes, we performed some preliminary experiments to nuclear matrix (background) (Fig. 4). That on late endosomes/lysosomes as well as that over the nuclear matrix (background) (Fig. 4). That on late endosomes/lysosomes as well as that over the nuclear matrix (background) (Fig. 4). That on late endosomes/lysosomes as well as that over the nuclear matrix (background) (Fig. 4). That on late endosomes/lysosomes as well as that over the nuclear matrix (background) (Fig. 4).

After the experimental part of this project was completed, we found in a parallel study that cathepsin S was highly enriched in the late endosome fraction by immunoblot analysis. We therefore analyzed the activity of this protease in the three fractions. As seen in Fig. 1 also the activity of this enzyme is significantly higher in the late endosome fraction, although the magnitude of the increase in the late endosomes over the lysosomes was significantly less than that seen by immunoblot analysis.

Cathepsin H in Early Endosomes—Since the activity for cathepsin H was found to be enriched in early endosomes, we decided to investigate the location of this protein using a rabbit antibody against rat cathepsin H that has previously been extensively characterized (36). This antibody is specific for cathepsin H since it neither recognizes the closely related cathepsins B, L, or D nor the closely related plant protease papaip (34). Since attempts to identify cathepsin H by immunoblotting were not successful, we set up a quantitative enzyme-linked immunosorbent assay to determine the amount of cathepsin H from each endocytic fraction adsorbed to microtiter wells. Purified cathepsin H was used to quantify the reaction, using a HRP-based detection system. As shown in Fig. 2 the relative concentrations of cathepsin H in the early endosome and lysosome fractions by this approach was similar to that seen by enzymatic activity (Fig. 2).

We next looked at the distribution of cathepsin H using immunogold EM. For this, we used thawed cryosections of J774 cells that had internalized 16 nm gold to label late endocytic structures and 5 nm gold (5 min) to label early endosomes. These sections were labeled with the rabbit anti-cathepsin H antibody followed by protein A-gold (Fig. 3). The bulk of the labeling was detected in early endosomes, and only low levels were detected in 16 nm gold-filled organelles (mostly lysosomes). Although the overall extent of the labeling on these thin cryosections was low, a quantitative analysis confirmed that the early endosome labeling was significantly higher than that on late endosomes/lysosomes as well as that over the nuclear matrix (background) (Fig. 4).

Since the above data showed that in J774 macrophages the bulk of cathepsin H protein and activity was localized to early endosomes, we performed some preliminary experiments to gain insights into the mechanisms by which this "soluble" enzyme can be retained in a dynamic organelle with a high flux rate of both fluid and membrane components. For this, we took advantage of the procedure of Authier et al. (32) in which the membrane fraction of interest is hypotonically disrupted, followed by the determination of enzyme activities in the supernatant before and after high speed centrifugation. Following preparation of the early endosomal vesicles under isomolar conditions without homogenization, only ~25% of the cathepsin H activity was recovered in the supernatant (Fig. 5A). This level of "free" cathepsin H was not significantly affected by homogenization under hypotonic conditions followed by incubation in 0.3–1 M NaCl, whereas a small but insignificant increase in the extent of solubilization was seen following incubation with 5 mM mannose 6-phosphate. The supernatant pool approached 50% with 0.5% saponin. The only treatments we found to solubilize the bulk of cathepsin H activity were 0.5% Triton X-100 or a pH 11 treatment (Fig. 5A) (which was also found to inhibit activity by ~70%; results not shown). Collectively, these data suggest that the bulk of cathepsin H is maintained in the early endosome by a mannose 6-phosphate-independent mechanism, perhaps as a peripheral (lumenal) membrane protein.

The Bulk of Furin Activity Is Soluble—We were surprised to see furin activity behaving in these experiments as a typical lysosomal enzyme since furin is normally considered a trans-Golgi network-spanning membrane protein (38). We therefore investigated the fraction of this activity which was soluble and that which was membrane-bound. For this we carried out the same procedure as for cathepsin H. As shown in Fig. 5B under isomolar conditions 25% of furin activity was soluble. Following hypotonic conditions, however, ~80% of the activity of this enzyme became soluble.

Transfer of Acid Hydrolases to Phagosomes—Phagosomes are newly formed particles enclosing vesicles that are initially devoid of acid hydrolases but, following a delay, acquire the full complement of these enzymes following fusion with the organelles traditionally referred to as lysosomes. Since our latex bead phagosomes can be highly purified from cells at any stage after their internalization, we decided to investigate the kinetics of acquisition of acid hydrolases by phagosomes and compare these data with our previous experiments that investigated transfer of internalized acid hydrolases and the Lamp membrane proteins into phagosomes (29). For this, the enzyme activities were estimated in purified phagosomes prepared at different times after latex internalization. As shown in Fig. 6 it took between 4 and 8 h after the addition of the beads to cells in order for the isolated phagosomes to acquire their full complement of most of the hydrolases tested. The most striking exception was again cathepsin H which was most rapidly acquired by latex phagosomes; the activity of this enzyme measured at the earliest time point (20 min) was the same as that seen at the latest time point (1-h pulse followed by a 24-h chase). We saw a consistent drop in the activity of cathepsin H at the 2–4-h time point (Fig. 6) whose significance is unclear. A quantitation of the percentage of total cell activities of the various hydrolases in phagosomes is shown in Table II. The relative amounts of these enzymes analyzed in 4-h phagosomes range from 1.4% (cathepsin H) to 30% (cathepsin B + L) of the total cellular pool of these enzyme activities (Table II).

A closer investigation of Fig. 6 showed other interesting patterns. Although it took only 2 h for phagosomes to obtain their full complement of α-mannosidase, it took 24 h for them to reach their peak concentration of β-glucosidase. Noteworthy, however, the remaining seven hydrolases reached the phagosomes with essentially identical kinetics, reaching a plateau at 4–8 h. These results can be contrasted with the ~1 h for equilibration of the fluid marker HRP between phagosomes and late endocytic structures and compared with the ~6–8 h

![Fig. 2. Quantitation of cathepsin H by enzyme-linked immunosorbent assay in the different endocytic fractions of J774. The estimation of the amount of cathepsin H was done using as a standard human liver cathepsin H. The values shown represent the mean ± S.E. from four experiments.](image-url)
needed for late endosomes and lysosome-derived Lamp proteins to reach their plateau level in phagosomes (29).

3 Effect of Chloroquine and Bafilomycin A1—Many earlier studies in the literature have shown that acidotropic amine reagents such as chloroquine or ammonium chloride can have significant and often complex effects on phagosome and endosome functions. Among these effects are a neutralization of the low pH of the lumen of endocytic organelles (39) as well as various effects on phagosome-endosome or phagosome-lysosome fusion (22, 27). We therefore investigated the effects of chloroquine, as well as the more specific vacuolar proton ATPase inhibitor bafilomycin A1 (40), on the process of lysosomal enzyme acquisition by phagosomes.

When latex bead uptake was allowed to occur in the continued presence of either 180 μM chloroquine or 100 nM bafilomycin A1, there was a complete block of acquisition by phagosomes of the activities of six out of the eight hydrolases tested. In the case of β-hexosaminidase the block in delivery was of the order of 80%. In contrast, the delivery of cathepsin H was enhanced by both drugs (Fig. 7). The simplest interpretation of these unexpected results was that raising the luminal pH of cell vacuolar organelles leads to a block in fusion between phagosomes and late endosomes but an enhanced rate of fusion of phagosomes with the early endosomes, where the bulk of cathepsin H resides.

We next investigated whether chloroquine or bafilomycin A1 led to any depletion of the hydrolases from endocytic compartments that would normally deliver them to the phagosomes. Strikingly, under these conditions, we saw a complete depletion from the cells of three of the eight hydrolases tested (cathepsin B, B₁L, and furin) (Fig. 8A). In addition, the activity of β-glucuronidase was diminished by about 50% (Fig. 8A). At the same time the activities of four other enzymes (cathepsin H, α-galactosidase, DPPII, and β-hexosaminidase) remained essentially unchanged (Fig. 8A). That the activities of cathepsin B, B₁L, and furin were indeed depleted from the cells was shown directly by experiments showing that these activities were released into the medium of living cells by a process that was enhanced by chloroquine (Fig. 8B). Besides this first group of hydrolases which were quantitatively secreted in the presence of acidotropic drugs (and therefore could not arrive in phagosomes), a second group of enzymes existed that showed no significant secretion (Fig. 8B) but, likewise, did not reach the phagosomes (Fig. 7). The most prominent exam-
ples of the latter were DPPII, β-glucuronidase, and β-hexosaminidase. This experiment provides evidence for different pools of lysosomal enzymes that are differentially affected by bafilomycin and chloroquine. Collectively, these results argue that in the presence of chloroquine or bafilomycin the bulk of cathepsins B, B₁L, and furin that would normally be targeted to newly synthesized phagosomes became secreted, whereas the other hydrolases remained in the cell in a pool that was now inaccessible to phagosomes.

We next asked whether chloroquine would have any effect on the retention of hydrolases already acquired by phagosomes. For this, J774 cells were allowed to internalize beads for 1 h at

![FIG.5] Percentage of soluble activity of cathepsin H (A) and furin (B) in subcellular fractions of J774 cells. Cathepsin H activity was determined in early endosomal vesicles and furin activity in the postnuclear supernatant, both before and after high speed centrifugation of the total membranes. Before pelleting the membranes the subcellular fractions were diluted either in isotonic buffer or in a hypotonic buffer followed by homogenization and incubation at the indicated conditions for 30 min at 4 °C. The values shown represent the mean ± S.E. of three experiments. *, the total of cathepsin H activity at pH 11 was only 30% that found at pH 7. **, under these conditions no furin activity could be detected.

![FIG.6] Enzyme activities of phagosomes of J774 cells at different times after formation of the phagosomes. In case of the 20-min time point cells were incubated for 20 min with latex beads, while for the later time points cells were incubated with a pulse of beads for 1 h followed by further incubation in medium free of beads until the indicated time points. Phagosomes were isolated, and the enzyme assays were performed. Values shown represent the mean ± S.E. of five experiments and are expressed relative to a fixed number (10^{10}) of latex beads.

| Enzyme activity | Phagosomal % |
|-----------------|--------------|
| α-Glycosidases  |              |
| α-Galactosidase | 17.9 ± 3.7   |
| α-Mannosidase   | 12.9 ± 3.9   |
| β-Glycosidases  |              |
| β-Glucosidase   | 12.5 ± 3.4   |
| β-Glucuronidase | 23.5 ± 3.5   |
| β-Hexosaminidase| 22.2 ± 3.8   |
| Proteases and peptidases |     |
| Cathepsin B     | 25.5 ± 7.8   |
| Cathepsin B + L | 29.6 ± 9.9   |
| Cathepsin H     | 014 ± 0.8    |
| DPP II          | 24.8 ± 4.9   |
| Furin           | 068 ± 4.4    |

The isolation of phagosomes were done after 1-h pulse plus 3-h chase incubation of cells with latex beads as described under “Material and Methods.” The enzyme activities were determined in the PNS and in the phagosomal fraction, and the phagosomal associated activity was correlated to the total activity in the PNS, which was set as 100%. The values shown are mean ± S.D. of three different experiments. DPP, dipeptidyl peptidase. 37 °C followed by 1 h of chase. They were then treated with chloroquine and incubated for an additional 1 or 2 h at 37 or 4 °C before isolating the phagosomes and analyzing six hydrolases. As shown in Fig. 9, cathepsins B, B₁L, and furin behaved identically in that their enzyme activities were selectively transferred out of phagosomes within 1 h after adding...
the chloroquine. When the temperature was lowered from 37 to 4 °C, this effect disappeared. In contrast, at 37 °C DPPII, β-hexosaminidase and β-glucuronidase were depleted from phagosomes at a significantly lower rate, being equivalent only to the levels seen for the three other more mobile enzymes at the low temperature. This argues that during phagosome fusions different classes of lysosomal enzymes can be sorted, not only into but also out of phagosomes at different rates.

Secretion of a Fluid Phase Marker—Evidently, under different conditions significant amounts of some acid hydrolases can be secreted by J774 macrophages. The fact that this secretion was unaffected by a 4-h treatment of cycloheximide (data not shown) as well as the fact that in the presence of chloroquine essentially all the cell-associated activities of some enzymes could be secreted (see Fig. 8) suggested that the secreted pool must originate from both late endosomes and lysosomes. In that case, it was relevant to ask whether a bulk endocytic marker internalized by J774 cells could also be secreted and whether that secretion could be modulated by chloroquine.

For this, J774 cells were pulsed with HRP (in the presence of yeast mannan to prevent binding to the macrophage mannose receptor) for 30 min at 37 °C and then chased for either 30 or 90 min at the same temperature. Subsequently, the cells were either treated with chloroquine for a further 60 min or left untreated. After this time the amount of HRP found in the medium or remaining cell-associated was estimated. As seen in Fig. 10, after a 30-min pulse and an additional chase period of 90 min there was a secretion of ~25% of the total cell-associated HRP, a value that was not significantly increased in the presence of chloroquine for the last hour chase. An increase in the chase time to 150 min did not affect the amount of HRP secreted in the absence of chloroquine. However, when chloroquine was added for the last 60 min of this latter condition the bulk (58%) of the cellular HRP was now secreted into the medium (Fig. 10B). This shows that, in addition to their unusually high level of secretion of a fluid phase internalized marker from late endocytic compartments, J774 cells appear to possess a late endocytic organelle, presumably a lysosome, that is significantly accessible to HRP only after a 120–180-min chase.
or cells were incubated for a further 1 or 2 h at 37 °C (point (arrows). After addition, phagosomes were isolated immediately before isolation of the phagosomes. The enzyme activities were then determined. Values shown represent the means ± S.E. of three experiments.

internalization period and which appears to have all the hallmarks of a regulated secretory vesicle.

**DISCUSSION**

*Cathepsin H in Early Endosomes*—A novel observation from our study was the finding that cathepsin H is enriched in early endosomes of J774 macrophages, as determined by its enzymatic activity, by protein determination, and by immuno-EM. This cysteine protease thus appears to be the first example of an acid hydrolase that is more concentrated in early endosomes than in all other endocytic compartments. The information that is available about this enzyme (it is predominantly an aminopeptidase as well as an endopeptidase with a pH optimum of ~6.5 (33, 41, 42)) fits well with estimations of the pH in the early endosomes (43, 44). This enzyme is found in a relatively high concentration in a number of tissues such as kidney and spleen and, significantly with respect to the present study, also in macrophages (45). Although J774 macrophages possess detectable levels of almost all the hydrolases analyzed in their endosomes, a finding consistent with many earlier studies (5), cathepsin H is the first example of a vacuolar hydrolase whose activity is the highest in the most proximal endocytic compartment. The precise mechanism by which the cathepsin is retained in the early endosomes must await a more detailed study.

**Fusion of Phagosomes with Endocytic Compartments in Vivo**—It has long been known that phagosomes acquire a full complement of lysosomal acid hydrolases during their interactions with endocytic organelles. It has also been well established that this mixing of contents is a consequence of fusion events (26, 29, 46–48). We have recently shown that latex bead phagosomes fuse in vitro in an age-dependent fashion with enriched fraction of early endosomes, late endosomes, and lysosomes, prepared as in the present study. In that study phagosomes until ~5–7 h of age were fusogenic with early and late endosomes, whereas their fusogenic life with lysosomes was extended to ~13 h. After this time phagosomes lost their capability to fuse in vitro. In vivo EM studies confirmed that fusion of 24-h phagosomes with gold-filled endocytic organelles is significantly reduced, although a low level of content mixing could be detected using an HRP-based assay (49).

It is striking that the length of the high fusion-competent state of phagosomes in the in vitro study coincides very well with the times needed here for 7 of the 10 acid hydrolases we tested to reach their steady states in phagosomes. In other words phagosomes appear to be switched to the “low fusion” state (4–13 h; depending on the target) only after they have received a full complement of these enzymes (4–8 h). By this reasoning it is interesting to note that most of the phagosomal hydrolases probably come from lysosomes since, at least in vitro, the fusion of phagosomes with both early and late endosomes is turned off before the phagosomes have acquired their full capacity of acid hydrolases. Moreover, the lysosome fraction is the richest source of most of the hydrolases of interest. Interestingly, the in vitro active fusogenic life of the latex phagosomes with lysosomes (13 h) exceeds the ~8 h needed to transfer essentially the full complement of acid hydrolases, as well as the Lamp proteins (29), to the newly formed phagosomes in vivo. That the fusion condition after 13 h is "low" rather than "off" (49) is also supported by our finding here that the phagosomes acquire their full complement of β-glucosidase only after 24 h; a low level of fusion must be continuing for this time. Thus, this enzyme is somehow selectively retained in the lumen of late endocytic organelles.

The acquisition of a full complement of cathepsin H activity within 20 min of phagosome internalization supports earlier data showing that phagosomes can fuse avidly with early endosomes in vivo (30, 25) as they do in vitro (23). An effective block in the acquisition by phagosomes of the bulk of the other nine hydrolases tested, mostly acquired from late endosomes and lysosomes, was seen in the presence of chloroquine and...
baflomycin A1. In contrast, the activity of cathepsin H in phagosomes was not decreased but rather seemed to be increased by these drugs. We interpret these results to indicate that a pH below neutrality needs to be maintained in at least one of the partners in the proposed fusion between phagosomes and late endosomes or lysosomes, but it is clearly not essential for the early presumed fusion of phagosomes with early endosomes. Although the molecular details are unclear the idea emerges that by tampering with pH (as well as perhaps other factors), these acidotropic drugs can inhibit some membrane transport processes (late endosomes/lysosomes to phagosomes) while stimulating others (lysosomes containing cathepsins B, B + L, and soluble furin to the plasma membrane). In this respect our results have similarities to the work of D’Arcy Hart and Young (22) who showed that in mouse macrophages ammonium chloride blocks phagosome-lysosome fusion but enhances phagosome-endosome fusion; however, in an earlier study chloroquine was found to greatly enhance phagosome lysosome fusion (46). A recent in vitro study of yeast Saccharomyces cerevisiae by Haas et al. (50) has shown that the homotypic fusion between vacuoles in yeast is inhibited by baflomycin A1 arguing that the presence of a proton gradient seems to be essential for this fusion event.

Selective Secretion of Hydrolytic Enzymes—Our results again highlight the well known ability of macrophages (including macrophage cell lines) as well as other cells of the immune system to secrete significant amounts of enzymatically active vacuolar hydrolases, which has been shown for various hydrolases (51–55) including different cathepsins (19, 56–58). In a number of these earlier studies the secretion of hydrolases was significantly enhanced in the presence of acidotropic drugs, a phenomenon we could confirm here. However, in J774 cells the secretion of hydrolases and an enhancement of this process by baflomycin and chloroquine was only seen in the case of cathepsin B, B + L, and soluble furin, whereas cathepsin H and the other six late endocytic organelle-enriched enzymes were retained by the cell. The secretion of a subset of lysosomal enzymes could be correlated with a basal and relatively high level of secretion of internalized HRP from a late endocytic organelle, probably a lysosome, and again, this secretion-competent pool could be significantly elevated by acidotropic drugs. Collectively, these data argue that cathepsin B, B + L, soluble furin activity, as well as a significant fraction of kinetically late internalized HRP may be localized to a specialized form of secretory lysosome.

Although we have no morphological data on the identity of the hypothetical lysosome/secretory granule, there is an extensive literature describing secretory granules that appear lysosome-like based on their content of marker proteins, as well as lysosomes that look and behave like secretory granules and are capable of regulated exocytosis (see Refs. 9 and 59). This phenomenon has been documented in a wide range of tissues (see Refs. 60–63 and references therein). In most, if not all of these cases, these two kinds of vesicles appear to originate from the late endosome rather than the trans-Golgi network (summarized in Refs. 9 and 59). Moreover, a late endocytic compartment-derived vesicle has been shown to exocytose bulk membrane and lumenal contents in antigen presenting cells, a phenomenon believed to be important for antigen presentation (64). Many earlier studies had in fact provided EM and biochemical evidence for an exocytosis of lysosomes in a number of cells, and significantly, this process was enhanced by chloroquine (65–67). A recent study by Rodriguez et al. (68) has extended these findings by describing a calcium-dependent exocytosis of lysosomes in fibroblasts and epithelial cells. Finally, our data extend the published work of Rozhin et al. (20), Reddy et al. (57), and Ulbricht et al. (58) who have all provided evidence for the regulated secretion of a subset of enzymatically active (mature) acid hydrolases in different cells, including macrophages.

It has recently been shown that individual vesicles of the late endocytic organelles can have different pH values (69), and in conjunction with earlier estimates of lysosomal pH being as low as 4–4.5, it seems that the pH of these vesicles can vary from 4, or even below, up to neutrality. Our data here, as well as the data of others (summarized in Ref. 5), argue that late endosomes and lysosomes are functionally heterogeneous organelles. When one also considers the dynamic and complex behavior exhibited by endosomes and lysosomes as seen in video microscopic observations of living macrophages,4 it becomes evident that the current textbook notion of a lysosome as a functionally monogamous, homogenous class of vesicles seems now to be seriously outdated.

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