Journal of Cell Science 108, 2213-2219 (1995)
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Co-localization of hydrolytic enzymes with widely disparate pH optima: implications for the regulation of lysosomal pH

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SUMMARY

Lysosomes are traditionally defined by their acidic interior, their content of degradative 'acid hydrolases', and the presence of distinctive membrane proteins. Terminal degradation of the N-linked oligosaccharides of glycoproteins takes place in lysosomes, and involves several hydrolases, many of which are known to have acidic pH optima. However, a sialic acid-specific 9-*O*-acetyl-esterase and a glycosyl-*N*-asparaginase, which degrade the outer- and inner-most linkages of N-linked oligosaccharides, respectively, both have pH optima in the neutral to alkaline range. By immunoelectron microscopy, these enzymes co-localize in lysosomes with several conventional acid hydrolases and with lysosomal membrane glycoproteins. Factors modifying the pH/activity profiles of these enzymes could not be found in lysosomal extracts. Thus, the function of the enzymes with neutral pH optima must depend either upon their minimal residual activity at acidic pH, or upon the possibility that lysosomes are not always strongly acidic. Indeed, when lysosomes are marked in living cells by uptake of fluorescently labeled mannose 6-phosphorylated proteins, the labeled organelles do not all rapidly accumulate Acridine Orange, a vital stain that is specific for acidic compartments. One plausible explanation is that lysosomal pH fluctuates, allowing hydrolytic enzymes with a wide range of pH optima to efficiently degrade macromolecules.

Key words: lysosome, immunoelectron microsocopy, sialate:*O*-acetylesterase, pH optima

INTRODUCTION

Lysosomes are intracellular digestive organelles (Bainton, 1981; Kornfeld and Mellman, 1989) that are generally assumed to have an internal pH of 4.0-5.5 (Ohkuma and Poole, 1978; Yamashiro and Maxfield, 1987a,b; Anderson and Orci, 1988) generated by proton pumps (Ohkuma et al., 1982; Yamashiro et al., 1983). This is supported by the observation that many lysosomal enzymes have acidic pH optima for activity (Bond and Butler, 1987; Aronson and Kuranda, 1989). However, some lysosomal hydrolases have substantial 'residual activity' in the less acidic pH range (Kirschke and Barrett, 1980; Dawson and Tsay, 1977). Furthermore, prior studies have suggested that a sialic acid-specific *O*-acetylesterase (Higa et al., 1989; Butor et al., 1993a,b) and a glycosylasparaginase (Tollersrud and Aronson, 1992; Maury, 1982; Mononen et al., 1993), which have neutral pH optima, might be found in lysosomes. These apparently paradoxical observations are explored in this study.

Glycoprotein degradation takes place mainly in lysosomes. Unlike lysosomal proteases, which are endo-enzymes (Kirschke and Barrett, 1980; Bond and Butler, 1987; Glaumann and Ballard, 1987), lysosomal glycosidases are generally exo-enzymes, degrading oligosaccharides sequentially from the outer (non-reducing) end (Aronson and Kuranda, 1989). The outermost possible structures on typical N-linked oligosaccharides (NLOs) are *O*-acetyl esters on sialic acids (Diaz et al., 1989). Two immunochemically and structurally distinct sialic acid-specific *O*-acetylesterases are known: one (CSE) is found in the cytosol, and the other (LSE) is found within the lumen of membrane-limited compartments (Higa et al., 1989; Butor et al., 1993a,b). The LSE is a water-soluble glycoprotein which acquires sialylated NLOs as it traverses the Golgi apparatus during biosynthesis (Higa et al., 1989; Butor et al., 1993a,b). It is distinct from previously described 'microsomal' esterases of the endoplasmic reticulum (Mentlein et al., 1987; Takagi et al., 1988; Robbi et al., 1990). Because of its specificity and post-Golgi location, it should be the first enzyme with the opportunity to degrade glycoconjugates carrying *O*-acetylated sialic acids.

Homogeneous LSE from rat liver has a neutral to alkaline pH optimum (Higa et al., 1989). However, subcellular fractionation and density-shift experiments showed co-enrichment of this activity with traditional lysosomal markers, but not with Golgi, ER, or plasma membrane markers (Butor et al., 1993a,b). The apparent lysosomal localization of the LSE was surprising, given its almost complete lack of activity in the acidic pH range (Higa et al., 1989). A glycosyl-*N*-asparaginase that degrades NLOs at the opposite (reducing) end (Tollersrud and Aronson, 1989, 1992; Maury, 1982; Mononen et al., 1993) is also reported to have

similar puzzling properties, i.e. a neutral pH optimum, but a suggested localization to lysosomes by cell fractionation methods (Mahadevan and Tappel, 1967). However, these localization studies were primarily based upon measurements of activity, assuming that no other enzyme with similar specificity existed in membrane-bound compartments. Also, these approaches can be misleading if two organelles of similar density happen to overlap in a gradient. Here, we directly confirm the localization of both these enzymes to lysosomes by immunoelectron microscopy, and pursue the implications of this finding.

MATERIALS AND METHODS

Materials

Most of the materials used in this study were from the Sigma Chemical Company (St Louis), or are listed by Butor et al. (1993a,b).

Enzyme assays

Glycosidases were assayed by following cleavage of paranitrophenol glycosides. The glycosylasparaginase (Tollersrud and Aronson, 1989) and LSE (Butor et al., 1993b) were assayed as previously described. All assays were conducted under conditions where product formation was linear with time, and added enzyme source.

Antibodies

Polyclonal monospecific rabbit antibodies directed against the LSE were obtained as previously described (Butor et al., 1993b). The particular antibody used in this study was shown to react on western blots of rat liver extracts with a single diffuse band of ~60 kDa. After reduction of proteins in the extract, the antibodies reacted only with the two expected LSE subunit bands of ~30 kDa and ~36 kDa (data not shown). No cross-reactivity was noted with any other proteins. The characterization of monospecific antibodies against rat glycosyl-*N*-asparaginase (Tollersrud and Aronson, 1992), α-fucosidase (Fisher and Aronson, 1989; Hancock et al., 1993), cathepsin B (Glaumann and Ballard, 1987), cathepsin D (Ludwig et al., 1991), and lgp-120 (Lewis et al., 1985) have been previously described.

Immunoelectron microscopy

Ultrathin frozen sections of rat hepatocytes were labeled with polyclonal monospecific rabbit antibodies. The antibodies directed against homogeneous LSE, or the 20 kDa subunit of the glycosylasparaginase were coupled to 9 nm large gold particles, while the other monospecific antibodies were coupled to 6 nm gold particles. General details of the procedures followed have been reported elsewhere (Griffiths et al., 1993).

Preparation of crude lysosomal extracts and assay of lysosomal enzymes

Rat livers were homogenized in 0.25 M sucrose 0.2 M KCl, pH 7.0. After a low speed nuclear spin, the homogenate was centrifuged at 10,000 ***g*** for 30 minutes to pellet lysosomes and mitochondria. The pellet was resuspended in three volumes of buffer and lysosomes ruptured by repeated freeze-thaw. Membranes and mitochondria were then repelleted, and the supernatant was examined as representing total lysosomal contents. All enzyme assays were done in 50 mM sodium citrate/50 mM sodium phosphate/50 mM sodium borate, adjusted to the appropriate pH (a multi-buffer system covering the entire range of pH studied, without a change in concentration of anions).

Preparation and uptake of fluorescently labeled lysosomal enzymes

The secretions of the $P388D_1$ macrophage cell line are known to contain a mixture of lysosomal enzymes carrying the phosphomannosyl recognition marker (Varki and Kornfeld, 1983; Hoflack and Kornfeld, 1985). These 'high-uptake' enzymes were collected into serum-free medium, dialyzed, concentrated and labeled with the fluorophores FITC (flourescein isothiocynate), AMCA (7-amino-4-methylcoumarin-3-acetic acid) or Cascade Blue (for the latter, the ethylene diamine form from Molecular Probes was first converted to the isothiocyanate form by thiophosgene treatment). The labeled enzymes were dialyzed, concentrated, and provided to living cells for uptake. Cells were grown on glass coverslips and incubated with the fluorescent enzymes in complete medium for 1 to 4 hours. In some experiments, this was followed by a 16-hour chase to ensure delivery to lysosomes. A control incubation in 5 mM M6P showed decreased uptake (not shown). After initial visualization, Acridine Orange (AO, 5 μM in Hanks' balanced salt solution) was added to the coverslips for 30 seconds. The coverslips were then washed in Hanks' balanced salt solution, and the fluorescent organelles were photographed in a channel that allows simultaneous visualization of both fluorophores.

RESULTS AND DISCUSSION

Colocalization of LSE and glycosyl-*N*-asparaginase with lysosomal markers

To directly explore the subcellular localization of both these enzymes, we used monospecific antibodies to carry out immunoelectron microscopy. Using gold beads of different sizes, we compared the localization of these enzymes with that of three traditional 'acid hydrolases' (α-fucosidase, cathepsin B, and cathepsin D) and a lysosomal membrane glycoprotein, lgp-120 (Mane et al., 1989; Lewis et al., 1985). Antibodies against the latter four proteins should strongly mark only lysosomal and prelysosomal compartments (Griffiths et al., 1988). Indeed, in a series of studies involving different combinations of different antibodies, all four co-localized with one another in typical lysosomal organelles, but also with the LSE and the glycosylasparaginase (complete data not shown, see Fig. 1 for a few examples). Selective enrichment of any one protein was not found in a subset of lysosomes or prelysosomes, nor was any significant signal seen in early endosomes, mitochondria or peroxisomes (Fig. 1). The lysosomal localization of the esterase and the glycosylasparginase is thus confirmed. Also, the co-localization with each of the acid hydrolases is complete, and there is no evidence for a specialized subfraction of lysosomes that are enriched in the two enzymes with higher pH optima.

No evidence for factors modifying pH optima in lysosomes

What explains the discrepancy between this localization and the neutral pH optima of these two enzymes? One possibility is that their low residual activity at acidic pH might be sufficient for their functions. However, the preservation of such an inefficient system seems somewhat unlikely during the evolution of lysosomal function. Another explanation is that their activity at neutral pH is spurious, and that they possess other activities at acidic pH. However, the LSE shows exquisite specificity for sialic acids (Higa et al., 1989), and is the only such enzyme found in lysosomes of rat liver, a tissue known to have high levels of sialic acid *O*-acetylation (Butor et al., 1993a). In the case of the glycosyl-asparaginase, human genetic deficiency of the enzyme causes accumulation of the appropriate substrate, GlcNAc-asparagine (Maury, 1982), indi-

cating that this is indeed its primary substrate. Yet another explanation is that cofactors in lysosomes might lower the pH optima of these enzymes. We therefore studied both activities within a concentrated preparation of total lysosomal contents, in comparison with conventional acid hydrolases (Fig. 2). Both the LSE and glycosyl-asparaginase maintained a pH optima of ~8.0 in this extract, with only ~25% activity at pH 5.0, and almost none at pH 4.5. Thus, within the limits of the assay (it was necessary to disrupt the lysosomal membranes by freeze-thaw to give access to the substrates), there is no evidence for modifying co-factors. As expected, β-galactosidase activity in the same extract had a pH optimum of 3.5, with only 50% activity at pH 5.5. In comparison, α-fucosidase maintained at least 75% of its activity from pH 4.0 to pH 6.8. Thus, four different lysosomal enzymes have pH/activity curves spanning a range from 3.0 to 10.0 (Fig. 2).

Are lysosomes always strongly acidic?

It is difficult to reconcile the above data with the traditional model of a lysosome that always has a strongly acidic interior.

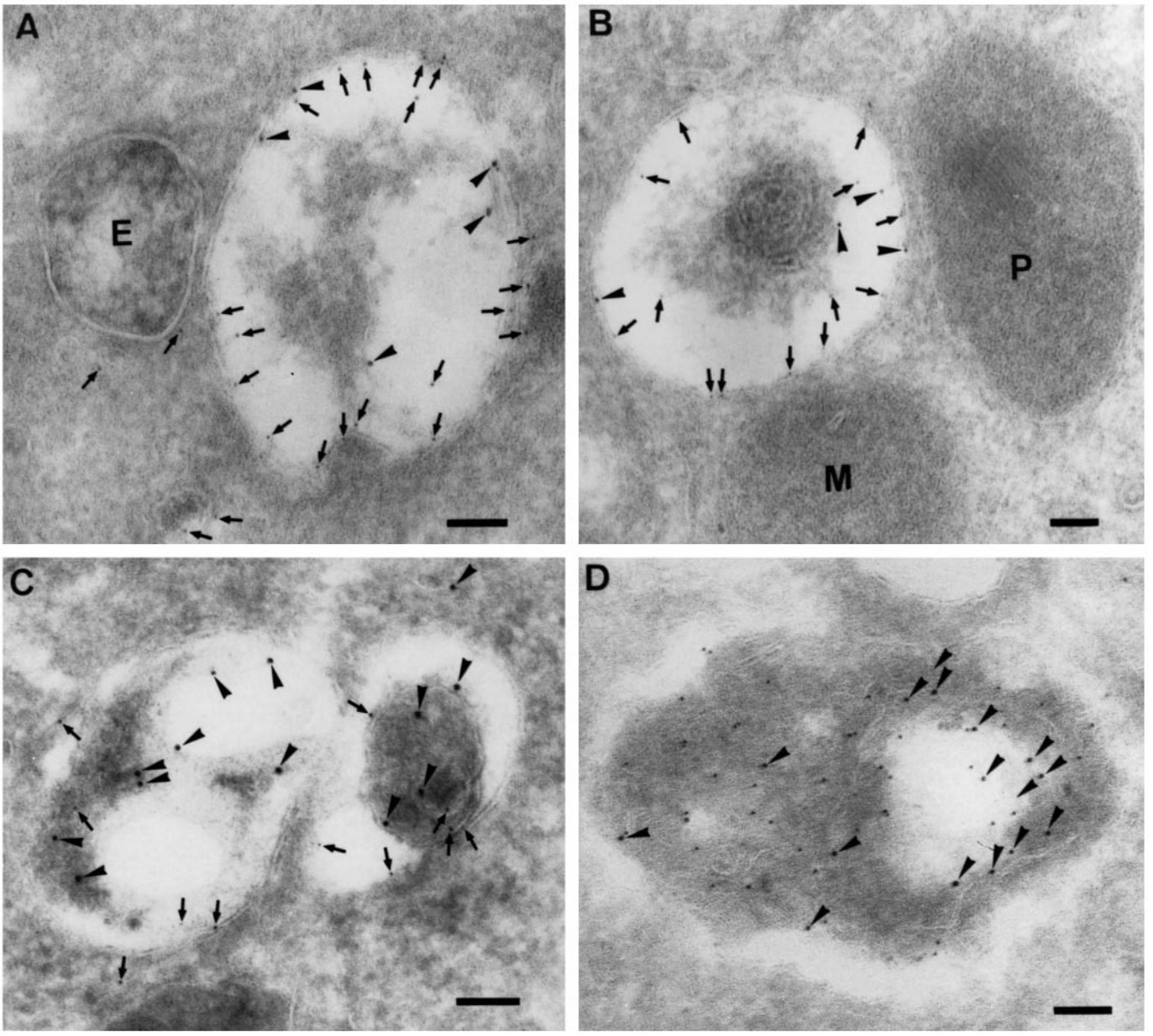

Fig. 1. Glycosyl-*N*-asparaginase and 9-*O*-sialic-acid-esterase (LSE) co-localize with lysosomal enzymes and membrane glycoproteins. Ultrathin frozen sections of rat hepatocytes were incubated with gold-labeled polyclonal monospecific rabbit antibodies as described in Materials and Methods. Antibodies against homogeneous LSE (A,B and D), or against the 20 kDa subunit of the glycosylasparaginase (C) were coupled to 9 nm large gold particles (large arrowheads). The sections were then double-labeled with monospecific antibodies (carrying 6 nm gold particles) against the lysosomal membrane glycoprotein lgp-120, or one of the traditional acid hydrolases cathepsin B, α-fucosidase or cathepsin-D. The examples shown are for lgp-120 (A,B,C, small arrows) and cathepsin B (D, unmarked beads). All other appropriate combinations of the antibodies were examined, and showed similar co-localization (data not shown). Examples of negative organelles are marked as E, early endosome; P, peroxisome; and M, mitochondria.

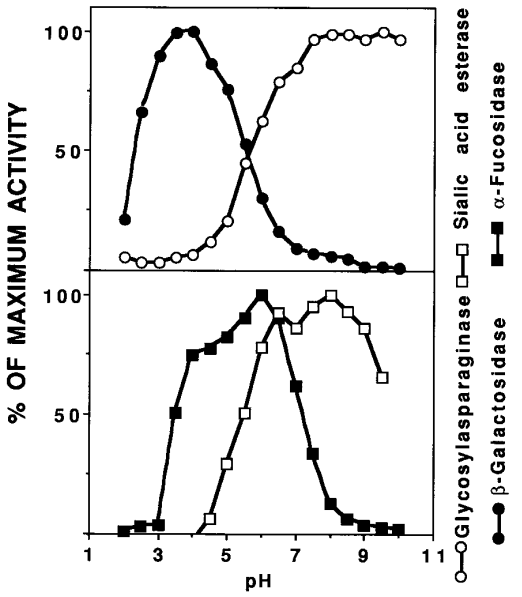

Fig. 2. pH/activity profiles of four lysosomal enzymes in a total lysosomal extract. Rat liver lysosomes were prepared and ruptured by repeated freeze-thaw as described in Materials and Methods. Various enzymes were assayed in the concentrated extract using a multi-buffer system that covers the entire range of pH studied, without a change in concentration of anions. The 100% activities shown are as follows: (●) β-galactosidase, 68.3 nmoles paranitrophenol released per h per μl crude lysosomal contents; (■) α-fucosidase, 14.5 nmoles paranitrophenol released per h per μl; (○) glycosyl-*N*-asparaginase, 1.3 nmoles *N*-acetylglucosamine released per h per μl; (□) 9-*O*-acetyl-sialic-acid esterase, 1.19% [^{3}H]acetate released per h per μl.

An attractive though somewhat heretical explanation is that lysosomal pH might not always be acidic. Rather, the pH might sometimes fluctuate away from acidic values towards neutrality, giving each lysosomal enzyme the opportunity to act under its most optimal conditions. Such a fluctuation might occur by variations in the proton-pumping ability of a given discrete lysosome. Alternatively, transient alkalinization could be due to fusion with less acidic endosomes, phagocytic vesicles, or pinocytotic vesicles.

Since most methods used to identify lysosomes take advantage of their acidic interior (Ohkuma and Poole, 1978; Yamashiro and Maxfield, 1987a,b; Anderson and Orci, 1988), a subset of lysosomes whose pH was neutral at the time of observation may have been previously missed. To detect lysosomes in living cells without regard to internal pH, we used fluorescently labeled phosphorylated lysosomal enzymes, which should bind to cell surface mannose 6-phosphate receptors, and be delivered to lysosomes by endocytosis (Kornfeld and Mellman, 1989; Griffiths et al., 1988; Nolan and Sly, 1987; Kornfeld, 1992; Varki, 1992). Using lysosomal enzymes labeled with any one of three fluorophores (Cascade Blue, AMCA or fluorescein) uptake was carried out for 1 to 4 hours, with or without a chase period (16 hours). The chase was included in some experiments to ensure that the enzymes had cleared the endosomal compartments that they must pass through enroute to the lysosomes. Similar results were obtained using the enzymes labeled with all three fluorophores: a granular and punctate fluorescence pattern typical of endosomes/lysosomes was seen. This is distinctly different from the pattern expected for ER or Golgi, and the dots showed no co-localization with rhodamine 123 (a vital stain for mitochondria, data not shown). The results obtained with FAO rat hepatoma cells and human foreskin fibroblasts (TC718) were similar. Examples of results with fibroblasts using Cascade Blue-labeled enzymes are presented here visually because they gave a stronger signal that was easier to photograph. Following uptake, we co-stained the cells with Acridine Orange (AO), a vital dye that reversibly accumulates in intracellular organelles with an acidic pH (Allison and Young, 1975; Lake et al., 1987; Moriyama et al., 1982). The fluorescence of AO shifts to longer wavelength when it stacks in high local concentration. Thus, red or orange vesicles are much more acidic than yellow ones. Most of the lysosomes observed contained both Cascade Blue and red or orange-yellow AO fluorescence (Fig. 3). However, a significant fraction of the Cascade Blue-stained organelles did not co-stain with AO at all. The number and distribution of these less acidic (? neutral) lysosomes varied between cells, being either scattered throughout the cytoplasm and/or in extended processes, or being confined to well-delimited areas. Granules stained either exclusively with AO or with each fluorophore were observed side-by-side in many different fields examined in each experiment (see Fig. 3 for one example). This makes an artifact caused by lack of penetration of AO into particular areas highly unlikely.

These data suggest that at steady state not all lysosomes have strongly acidic interiors. Short incubations with a range of AO concentrations gave similar results (very high concentrations lead to extreme sensitivity of the lysosomes to light, with bursting upon illumination). During longer incubations, AO cannot be used to determine steady state lysosomal pH precisely. Once it has entered organelles with acidic interiors, AO can undergo covalent or strong non-covalent interactions with various molecules such as glycosaminoglycans (Lüllmann-Rauch and Ziegenhagen, 1991), i.e. wash-out experiments do not work well. Thus, prolonged accumulation of AO should result in irreversible staining of all compartments that were acidic at any time during the period of incubation. In fact, upon prolonged exposure to AO, all of the organelles eventually turn red (e.g. half hour, data not shown), indicating that they all go through a phase when they become strongly acidic. Thus, the proposed fluctuation in pH might take place over a relatively short time span.

A potential artifact to consider is that the fluorescently labeled lysosomal enzymes were internalized for a long time: thus, a fraction of the dye might be released after degradation and could diffuse through the lysosomal membrane, sticking to neutral organelles. This is very unlikely for several reasons. First, the proteins taken up via the mannose 6-phosphate receptor-mediated route are expected to be mainly lysosomal enzymes, which are known to have rather long half-lives in the lysosome. Furthermore, similar results were obtained using three different fluorophores attached to the lysosomal enzymes (Cascade Blue, fluorescein and AMCA), under several different kinetics (1 to 4 hours, with or without a 16 hour chase) in two different cell types (hepatocytes and fibroblasts). Additionally, if such diffusion did occur, one might expect it to result in diffuse staining of all membranes, rather than giving a punctate granular staining pattern typical of lysosomes.

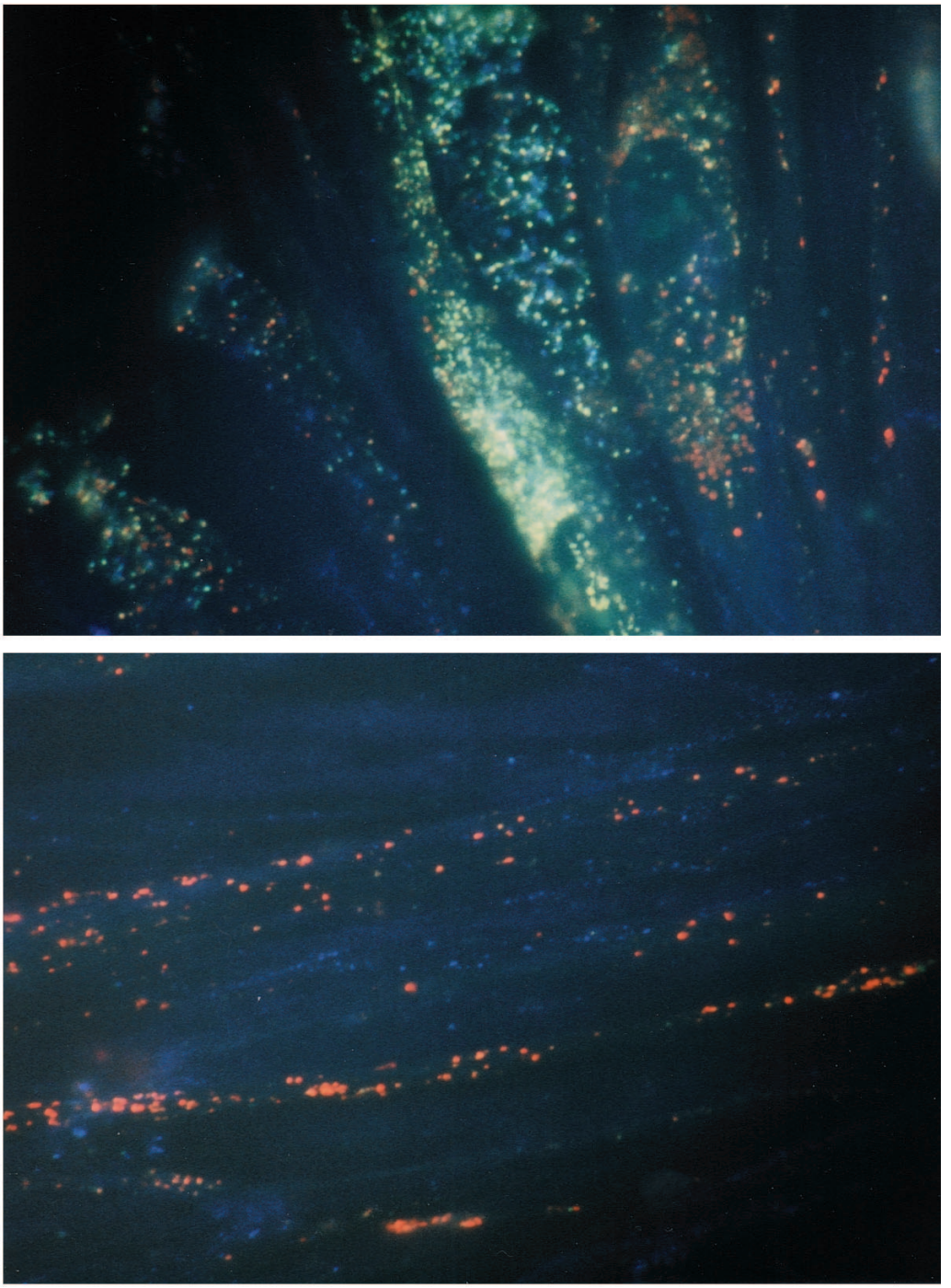

Fig. 3. Fluorescent lysosomal enzymes endocytosed to lysosomes are not all present in acidic compartments. 'High-uptake' lysosomal enzymes from the secretions of the $P388D_1$ macrophage cell line (Varki and Kornfeld, 1983; Hoflack and Kornfeld, 1985) were labeled with fluorophores, dialyzed, concentrated, and added to cultured cells for uptake as described in Materials and Methods. Following incubation for 4 hours and a 16 hour chase, acridine orange was added and fluorescent organelles photographed as described in Materials and Methods. Two representative fields are shown, using Cascade Blue-labeled enzymes and human fibroblasts.

Finally, the fact that all the the granules eventually turn red upon prolonged exposure to AO (see above) makes it likely that they are all lysosomes.

If lysosomal pH indeed fluctuates towards neutrality, how could this have been missed in previous studies? Most prior studies of lysosomal pH used weak bases that change properties only upon entering acidic compartments. Other studies which monitored the quenching of fluoresceinated dextrans at acidic pH generally presented averaged values for whole cells, without a distribution range. However, in one study where measurements of individual lysosomes were actually recorded at single time points (Yamashiro and Maxfield, 1987b), ~80% had a value of <5.50, ~15% were in the range of 5.5-6.0, and a small proportion were actually indicated as having a pH between 6 and 7.5 (see Fig. 3 of Yamashiro and Maxfield, 1987b). Also, most such studies were done in subconfluent cells, where individual cells are spread out and easy to observe. We have noted that AO accumulation appears higher in such sub-confluent cells than in confluent ones, where the AO-negative organelles were more frequently seen.

In the above-mentioned studies of others, the accumulation of fluoresceinated dextrans is assumed to be exclusively in the

lysosomes. We used a different and perhaps more specific marker: the uptake and incorporation of M6P-containing enzymes. Thus, the definition of lysosomes in this discussion (uptake of enzymes carrying M6P, and co-localization of lgp120 with several acid hydrolases) includes organelles traditionally defined as late endosomes or prelysosomes (Griffiths et al., 1988). We suggest that the pH of the late endosomal and lysosomal compartments might be more dynamic than previously thought, fluctuating over a wide range, with a preference for a more acidic state. Direct proof of this hypothesis will require time-lapse analysis of lysosomal pH in living cells, a task made very difficult by the constant saltatory motion of lysosomes.

There are other scattered facts in the literature that support this hypothesis. An alkaline protease has been found associated with purified buffalo kidney lysosomes (Harikumar et al., 1989). Some traditional 'acid' hydrolases have substantial residual activity in the near-neutral range (e.g. α-fucosidase; see Dawson and Tsay, 1977; and Fig. 2). This particular enzyme also shows a further shift in pH optimum towards the neutral range under certain incubation conditions (Dawson and Tsay, 1977). Likewise, the lysosomal protease cathepsin B has a varying pH optimum for different proteins, ranging from 3.5 to 6.0 and a less acidic pH optimum for low molecular mass substrates (Kirschke and Barrett, 1980).

Taken individually, none of the current or prior data prove that lysosomal pH fluctuates. However, taken together, they do indicate the likelihood of a pH cycle in lysosomes. In fact, this would make sense for a digestive organelle with enzymes having pH-dependent activity spanning such a wide range. Such fluctuation would allow different degradative processes to occur in sequence, with acidic and neutral hydrolases alternating in their action. Also, such pH changes might themselves serve a digestive function, by promoting denaturation of macromolecules.

We thank Sandra Diaz, Adriana Manzi, Karin Norgard-Sumnicht, Leland Powell and Eric Sjoberg for useful discussions, and for help with some experiments. This work was supported by a Merit Award from the VA Medical Research Service and grant GM 32373.

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(Received 18 January 1995 - Accepted 6 March 1995)