

Hydrolase Secretion Is a Consequence of Membrane Recycling

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ABSTRACT *Acanthamoeba* releases lysosomal hydrolases continuously into the culture medium. This release is specific for lysosomal hydrolases, but not other cellular proteins, and is energy dependent. The secreted hydrolases can be separated into two groups on the basis of their secretion kinetics: one is secreted at ~15% of the cellular activity per hour and the other at ~5%. Intracellularly the lysosomal hydrolases are restricted almost exclusively to secondary lysosomes where the hydrolases demonstrate a differential pH-dependent binding to membrane. Hydrolase secretion is not the result of secondary lysosomes' fusing with the plasma membrane since soluble and particulate lysosomal contents are not released at the same rate. Together the data suggest that the secreted hydrolases are trapped in shuttle vesicles that cycle membrane from secondary lysosomes to the cell surface. The inner membrane and content of these vesicles undergo a marked pH shift when, following fragmentation from lysosomes, these vesicles fuse with plasma membrane. This rapid pH shift and the differential pH-dependent membrane binding of hydrolases appear to account for the heterogeneous hydrolase secretion kinetics.

A variety of unicellular organisms and mammalian cells in culture are known to secrete lysosomal hydrolases. While this secretion by some cells appears to be due to a modification of the enzyme structure, leading to an error in its cellular processing and compartmentalization (11), hydrolase secretion by other cells appears to be concomitant with endocytosis and intracellular digestion (6, 14, 27). For most cell types the subcellular source of the secreted hydrolases and the significance of the process are unknown. Differences in the secretion kinetics of several lysosomal hydrolases from *Dictyostelium* have prompted Dimond et al. (7) to propose that the secreted hydrolases are released from functionally and structurally different lysosomal subpopulations. Vick and Blum (26) similarly interpreted their results in a study of hydrolase secretion by *Tetrahymena*. We report here that in *Acanthamoeba*, as in *Dictyostelium* and *Tetrahymena*, secreted hydrolases can be separated into several groups on the basis of their secretion kinetics. This heterogeneity in the kinetics of hydrolase secretion by *Acanthamoeba*, however, does not result from differences in hydrolase compartmentalization but from differences in hydrolase binding to membranes. This binding is reversible and is pH dependent. We also report evidence suggesting that the secreted hydrolases are those that are trapped in the vesicular shuttle that returns membrane from the digestive vacuole to the plasma membrane.

MATERIALS AND METHODS

Cells: *Acanthamoeba castellanii* (Neff strain) were harvested from axenic cultures that had grown to a cell density between 3 and 8×10^5 cells/ml. These cells were washed twice in filter-sterilized ($0.45 \mu\text{M}$ filter; Nalge, Rochester, NY) growth medium (15) and were then resuspended in fresh growth medium prior to experimentation.

Secretion of Lysosomal Enzymes: Secretion of acid hydrolases was measured in amoeba cultures with a density of 2×10^6 cells/ml, incubated at 30°C in a reciprocal shaking water bath (100 oscillations/min). At the appropriate sampling time, aliquots of amoebae in media were centrifuged (500 g, 2 min). The supernatant fraction was passed through a $0.22\text{-}\mu\text{m}$ filter (Millipore Corp., Bedford, MA) and rapidly frozen at -40°C . The pellet was washed twice in cold (4°C), 0.1 M phosphate buffer, pH 6.8, resuspended to the original volume in the same buffer with 0.1% Triton X-100, disrupted in a tissue homogenizer, and rapidly frozen. Additional aliquots of amoebae in media were treated with an equal volume of 3% glutaraldehyde in 0.1 M phosphate buffer, pH 6.8. These samples were used to monitor the cell density with a cell counter (Particle Data, Inc., Elmhurst, IL).

Enzyme Assays: Acid phosphatase and *N*-acetyl- β -hexosaminidase activities were assayed using *p*-nitrophenyl-derivatives, whereas β -glucosidase, β -galactosidase, α -galactosidase, β -glucuronidase, and β -xylosidase activities were assayed using 4-methylumbelliferyl derivatives (cf. reference 1). Fluorimetric assays of esterase (10) were modified and employed carboxyfluorescein diacetate as substrate (Molecular Probes, Junction City, OR). Unless specified, all of the reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Acid phosphatase activity was assayed in 0.25 M acetate buffer, pH 4.5, esterase in 0.2 M PIPES buffer, pH 7.5, and β -glucosidase in 0.25 M citrate buffer, pH 3.5. All of the other glycosidases were assayed in 0.2 M citrate buffer, pH 4.6. To increase the solubility of the esterase, α -glucosidase, β -galactosidase, and β -

xylosidase substrates, these reagents were first dissolved in dimethyl sulfoxide and subsequently diluted with buffer. The final concentration of dimethyl sulfoxide in the reaction mixture was 2%. Concentrations of dimethyl sulfoxide >10% did not interfere with the assays. To prevent the loss of hydrolase activity from samples with low protein concentration, β -lactoglobulin was routinely added to the reaction mixture to give a final concentration of 0.2 mg/ml.

After the appropriate incubation period, which generally ranged from 10 to 30 min, the assays employing the *p*-nitrophenyl and 4-methylumbelliferyl derivatives were terminated by the addition of a stopping reagent containing 0.133 M glycine, 0.083 M sodium carbonate, and 0.067 M sodium chloride, pH 10.7. Released *p*-nitrophenol was measured spectrophotometrically at 410 nm on a Gilford modified Beckman DU spectrophotometer (Gilford Instruments, Inc., Oberlin, OH), and released 4-methylumbelliferone was measured fluorometrically on an SLM DME-2 spectrofluorometer (SLM, Urbana, IL) with excitation at 360 nm and emission at 448 nm. Carboxyfluorescein released in the esterase assay was monitored continuously for 8 min in the spectrofluorometer with excitation at 490 nm and emission at 520 nm. All of the hydrolase assays were performed in the presence of 0.1% Triton X-100 and under conditions in which the production of fluorogen or chromagen was linear with the length of the assay and the amount of sample used.

Assays for malate dehydrogenase activity employed oxalacetate in 0.1 M phosphate buffer, pH 7.5 as substrate. The oxidation of NADH in these assays was monitored continually for 5 min at 340 nm with a Beckman Acta III spectrophotometer (Beckman Instruments, Inc.). All of the enzyme assays were performed at 30°C, except for the esterase and malate dehydrogenase assays which were performed at 25°C. Protein concentration was determined using the Peterson (20) modification of the Lowry procedure (16).

Isolation of Phagocytic Vesicles: Phagocytic vesicles were isolated from amoebae that had ingested polyvinyltoluene, 2.02- μ m beads (Dow Diagnostic Products, Indianapolis, IN), or *Saccharomyces cerevisiae*. Prior to their use, the latex beads were dialyzed against water, and the yeast were lipid-extracted (4), heat-treated at 100°C for 5 min in 0.1 M phosphate buffer, pH 6.8, and washed twice in growth medium. These particles were added to amoeba cultures (2×10^6 cells/ml) to give a final concentration of 0.8 mg of beads per 10^6 amoebae or 3.0 mg of yeast per 10^6 amoebae. After either 25 or 120 min in a reciprocal shaking water-bath at 30°C, amoebae were washed free of uningested particles by centrifugation at 500 g in growth media and then washed twice in 10 mM TES buffer pH 6.8 (*N*-tris-[hydroxymethyl]methyl-2-aminoethanesulfonic acid). Amoebae that had ingested yeast were resuspended at 10^7 amoebae/ml in 12% sucrose (wt/vol) and were disrupted in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ). The homogenate was centrifuged at 300 g for 2 min, and the pellet containing the yeast phagosomes was washed three times in 12% sucrose. Phagosomes were isolated from amoebae that had ingested latex beads by a modification of the procedure described by Wetzel and Korn (29). Amoebae washed twice with 10 mM TES, pH 6.8 were resuspended to 10^7 cells/ml in 30% sucrose and disrupted in a Dounce homogenizer. 5 ml of the homogenate placed at the bottom of a centrifuge tube were mixed with 5 ml of 30% sucrose and overlaid with 6 ml of 20%, 10 ml of 12%, and 12 ml of 5% sucrose (wt/vol), and then centrifuged (No. 27 rotor; Beckman Instruments, Inc.) for 90 min at 100,000 g. The bead phagosomes accumulated between the 5 and 12% sucrose layers.

Density Gradient Fractionation: Control amoebae or amoebae incubated for 30 min in growth media containing 100 μ g/ml of the fluorescent dye, Lucifer yellow, were washed twice in growth medium and twice in 10 mM TES buffer, pH 6.8. These cells were resuspended at 10^7 cells/ml in 0.25 M sucrose and disrupted in a Dounce homogenizer. The homogenate was centrifuged at 500 g for 5 min, and 3 ml of the supernatant fraction was carefully overlaid on top of a step gradient consisting of 34 ml of 10% Percoll (Pharmacia, Inc., Piscataway, NJ), in 12% sucrose overlaid with 27 ml of 12% sucrose. These 64-ml gradients were centrifuged in a No. 21 rotor (Beckman Instruments, Inc.) at 110,000 g for 60 min and then separated into 2-ml fractions collected from the top of the gradient with the aid of a Buchler Auto Densi-Flow (Buchler Instruments, Fort Lee, NJ). The fractions were assayed fluorometrically for Lucifer yellow with excitation at 430 nm and emission at 540 nm (23) or assayed for hydrolase activity and protein. Fractions recovered from a gradient centrifuged without amoeba homogenate were used to correct for Percoll interference with the protein assay. Percoll did not interfere with the hydrolase assays or measurements of Lucifer yellow.

Processing of Carboxyfluorescein Cross-linked to Plastic Beads: Carboxyfluorescein diacetate (5 mg) was dissolved in 200 μ l of dimethyl formamide and added dropwise with continual stirring to 1.3 ml of 50 mM pyridine buffer, pH 5.0, containing 100 μ M 1-ethyl-3-(dimethylaminopropyl) carbodiimide and 0.5 ml of 1.75 μ M polystyrene primary amino beads (2.5% solid) (Polysciences, Warrington, PA). After a 5-min incubation at room temperature, 10 ml of pyridine buffer was added and the beads were centrifuged at 10,000 g for 5 min. The beads were repeatedly washed in distilled

water, and aliquots of the supernatant were monitored for the presence of carboxyfluorescein. Four washes were usually sufficient to reduce the amount of noncross-linked carboxyfluorescein in the supernatant fraction below detectable levels. These particles were added to amoeba cultures (2×10^6 cells/ml) to give a final concentration of 0.5 mg of beads per 10^6 amoebae. After 30 min in a reciprocal shaking water-bath at 30°C, amoebae were washed free of uningested beads and resuspended in fresh media. At selected times, aliquots were removed and the cells and growth medium were separated and processed as described above. Additional aliquots were removed and diluted with an equal volume of 3% glutaraldehyde in 0.1 M phosphate buffer, pH 6.8. Aliquots of cell homogenates and growth medium diluted with a 20-fold excess of 0.1 M glycine buffer, pH 10.7 were assayed for carboxyfluorescein as described above. Aliquots of cells in growth medium fixed in glutaraldehyde were washed free of excess beads and carboxyfluorescein in 0.1 M glycine buffer, pH 10.7. The ingested beads were extracted with dioxane and quantified spectrophotometrically (28).

Fluorescence Microscopy: Endocytosed Lucifer yellow and carboxyfluorescein as well as carboxyfluorescein released from phagocytosed plastic beads was visualized with fluorescence microscopy using a Zeiss microscope equipped with a halogen light source, a 450- to 490-nm band pass filter and a 520-nm barrier filter (Carl Zeiss, Inc., Thornwood, NY).

Membrane-binding of Hydrolases and Carboxyfluorescein: Yeast phagosomes, prepared as described above and resuspended in distilled water, were rapidly frozen in liquid nitrogen and thawed at 30°C, three times. This preparation was then centrifuged at 300 g for 2 min to remove the yeast. The supernatant fraction was divided into two aliquots which were diluted with an equal volume of 50 mM acetate buffer with a pH of either 4.5 or 6.0. After a 15-min incubation at 0°C, aliquots of these preparations were centrifuged (No. 40 Beckman rotor) for 60 min at 100,000 g. For some experiments the recovered pellets were resuspended in distilled water to the original volume, and the supernatant and pellet fractions, as well as aliquots not centrifuged, were assayed for hydrolase activities. For experiments measuring the reversibility of hydrolase binding, aliquots of the soluble hydrolases recovered from the pH 6.0 supernatant were dialyzed against a 1,000-fold greater volume of 50 mM acetate buffer, pH 4.5. An aliquot of the phagosomal membranes recovered from this same preparation was resuspended in the dialyzed supernatant. This sample, as well as phagosomal membranes recovered from the pH 4.5 pellet and resuspended to the original volume in 50 mM acetate buffer, pH 6.0, were centrifuged again for 60 min at 100,000 g. The recovered supernatant fractions and pellet fractions were resuspended in distilled water and assayed for hydrolase activity.

To measure the membrane binding of carboxyfluorescein, phagosomal membranes in distilled water were incubated for 15 min at 0°C with an equal volume of 50 mM acetate buffer, pH 4.5 or 6.0, with 1 μ M carboxyfluorescein. These preparations were centrifuged at 100,000 g for 60 min and the recovered pellet and supernatant fractions were assayed for carboxyfluorescein. For experiments measuring the reversibility of carboxyfluorescein binding, aliquots of the 100,000-g pellets resuspended in distilled water were incubated with an equal volume of acetate buffer containing carboxyfluorescein. Pellets recovered from preparations with pH 4.5 were incubated with buffer at pH 6.0, and pellets recovered from preparations with pH 6.0 were incubated at pH 4.5. After a 15-min incubation at 0°C these preparations were centrifuged at 100,000 g for 60 min and the recovered pellet and supernatant fractions were assayed for carboxyfluorescein. Similarly treated preparations in which the phagosomal membranes were omitted served as controls.

RESULTS

Secretion of Lysosomal Enzymes

Acanthamoeba releases lysosomal hydrolases into its growth medium at rates that are linear with time (Fig. 1). The cellular specific activity and rate of release of nine hydrolases are summarized in Table I. Hydrolase release is specific, i.e., all of the hydrolases are secreted at rates much greater than cell lysis (Table I) that, as estimated by the appearance of malate dehydrogenase (25) in the growth medium, is <0.25% of the cells per hour. Although the release of all of the hydrolase activities is energy-dependent, as demonstrated by the observation that the incorporation of 1 mM NaN_3 into the growth medium blocked hydrolase release (Fig. 1), all of the hydrolases are not released at the same rate. Five of the hydrolases, acid phosphatase, β -glucosidase, α -glucosidase, α -galactosid-

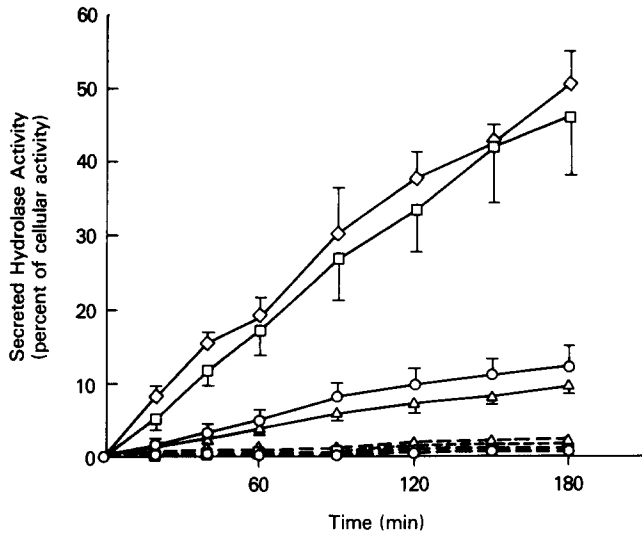


FIGURE 1 Secretion of lysosomal hydrolase activities. The secreted activities, measured in the presence (dashed lines) or absence of 1 mM NaN_3 , are expressed as percents of the cellular activity present at the initiation of the experiment. Vertical lines through experimental points represent standard deviations. \diamond , *N*-acetyl- β -hexosaminidase; \square , β -glucuronidase; \circ , β -glucosidase; Δ , acid phosphatase.

TABLE I
Hydrolase Specific Activity and Rate of Secretion

Hydrolase	Cellular specific activity* nmol/min·mg protein	Rate of secretion* %/h
Acid phosphatase	264.2 (26.1)	3.53 (0.25)
β -Glucosidase	6.37 (1.60)	4.51 (1.69)
α -Glucosidase	2.23 (0.38)	6.79 (0.54)
α -Galactosidase	2.67 (0.41)	2.10 (0.29)
β -Xylosidase	0.28 (0.07)	3.60 (0.30)
<i>N</i> -acetyl- β -hexosaminidase	92.3 (24.8)	17.77 (3.00)
β -Glucuronidase	0.75 (0.16)	19.33 (1.80)
β -Galactosidase	0.95 (0.18)	12.96 (1.33)
Esterase	0.14 (0.01)	19.86 (2.11)

* Values in parentheses are standard deviations calculated from the means of 10 separate experiments.

† Values expressed as percent cellular activity released per hour.

ase, and β -xylosidase, are released at rates between 2 and 7% of the cellular activity per hour, whereas four other hydrolases, β -galactosidase, *N*-acetyl- β -hexosaminidase, β -glucuronidase, and esterase, are released more rapidly at rates between 13 and 20% per hour (Table I). The experiments reported below investigate possible explanations for the variation in secretion rates.

Hydrolase Enrichment of Phagolysosomes

To test the hypothesis that specific hydrolases may be differentially enriched in *Acanthamoeba* lysosomes and thereby be differentially secreted, we isolated secondary lysosomes loaded with yeast or polyvinyltoluene beads from homogenates of amoebae that were fed these particles and we measured the enrichment of eight hydrolase activities as compared with whole homogenates. The results, shown in Table II, demonstrate that all of the hydrolase activities are enriched to the same extent in both yeast and bead phagolysosomes. Hydrolase enrichments calculated from phagolysosome prep-

arations isolated 25 or 120 min after the addition of particles to amoeba cultures were not significantly different (data not shown).

Density Gradient Centrifugation

Although cytochemical studies of *Acanthamoeba* (2, 22) have identified the digestive vacuole as the largest cellular compartment of hydrolase activities, there may be other populations of lysosomes in *Acanthamoeba*. We attempted to separate lysosomal subpopulations by density gradient centrifugation. Fig. 2, *A* and *B* show the distributions of protein and three lysosomal hydrolase activities on a density gradient formed during high speed centrifugation. These three hydrolase activities, and five others not shown, had an almost identical bimodal distribution with similar amounts of activity found in each peak. The first peak formed at a density of 1.04 g/ml and the second at 1.06.

The fluorescent dye Lucifer yellow was readily pinocytosed by *Acanthamoeba* and was sequestered into large vacuoles. These large vacuoles, 2-5 μM in diameter, as observed with fluorescence microscopy, are presumed to be equivalent to amoeba digestive vacuoles that are similar in size and abundance. This and previous studies (see Table II and text; 3) have demonstrated that recently interiorized material is rapidly incorporated into secondary lysosomes (digestive vacuoles).

To determine whether the hydrolase peaks separated on the density gradient are derived from digestive vacuoles, amoebae allowed to pinocytose Lucifer yellow or to phagocytose yeast were homogenized and fractionated on density gradients. Fluorescence microscopy revealed that the fluorescence distribution remained punctate throughout the experiment. On density gradients the fluorescent dye had a bimodal distribution identical to that of the acid hydrolases (Fig. 2*C*). When homogenates of amoebae that had ingested yeast were centrifuged at 500 *g* for 5 min, 85-90% of the total cellular activity of each hydrolase was recovered in the pellet. When an aliquot of the supernatant fraction was layered onto a density gradient, the two hydrolase peaks were greatly reduced in size (Fig. 2*D*) and no other hydrolase peaks were apparent. The low speed pellet recovered from control amoebae that had not ingested yeast, contained only 10-15% of the cellular activity of each hydrolase. When amoebae that had ingested Lucifer yellow were fed yeast and processed as described

TABLE II
Hydrolase Enrichment in Phagolysosomes

Hydrolase	Hydrolase enrichment*	
	yeast phago- somes†	bead phago- somes
Acid phosphatase	8.4 (1.3)	13.6 (1.3)
β -Glucosidase	9.3 (1.9)	14.8 (2.9)
α -Glucosidase	8.0 (1.0)	14.1 (2.8)
α -Galactosidase	9.4 (1.4)	15.6 (3.1)
β -Xylosidase	9.3 (1.9)	13.9 (3.1)
<i>N</i> -acetyl- β -hexosaminidase	8.9 (1.5)	11.7 (1.4)
β -Glucuronidase	9.0 (2.6)	13.9 (2.1)
β -Galactosidase	8.8 (1.3)	14.2 (3.5)

* Specific activity of phagosomes

† Specific activity of whole homogenate.

‡ Values in parentheses are standard deviations calculated from the means of four experiments.

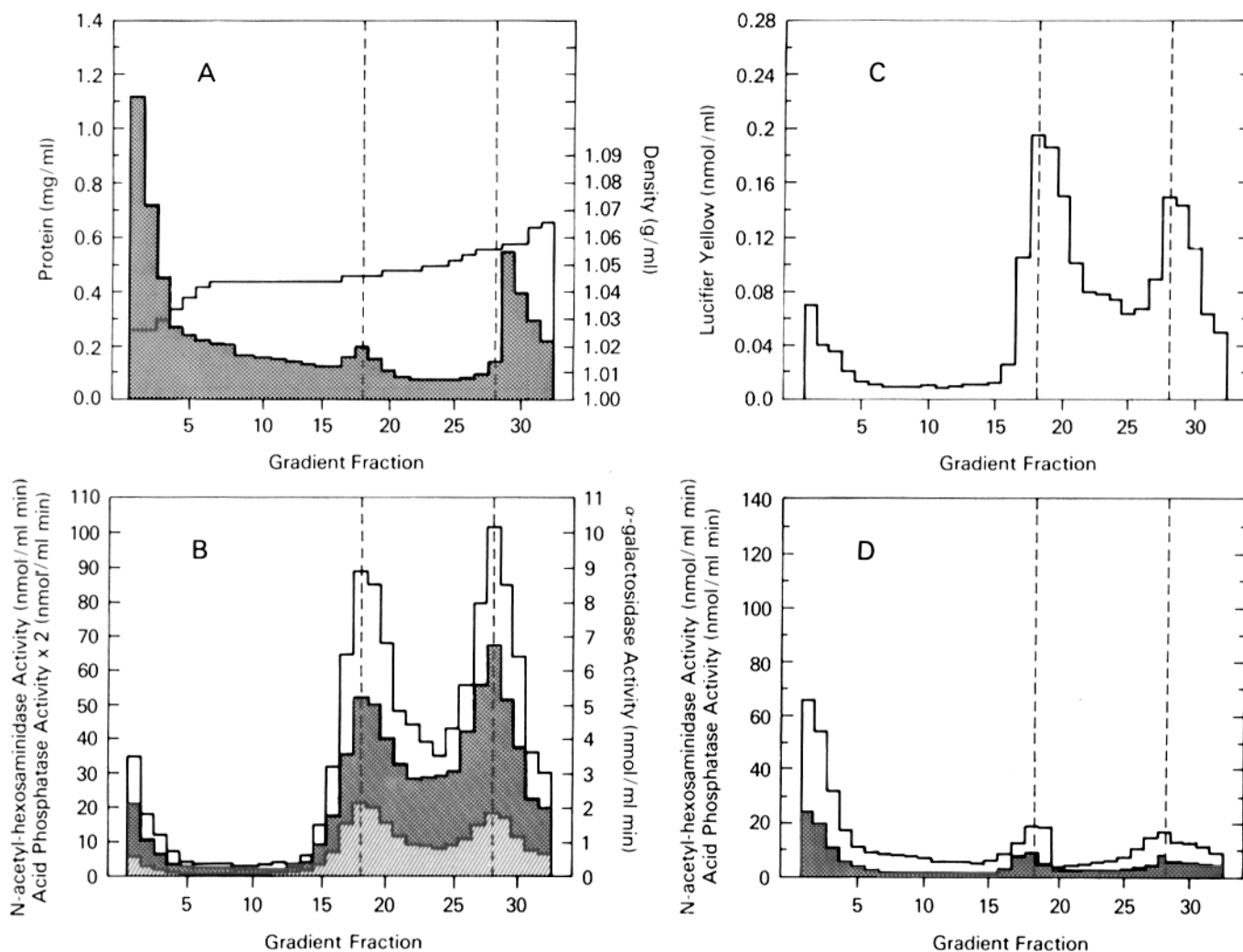


FIGURE 2 Fractionation of postnuclear supernatants on self-forming density gradients. (A) Density (open bars); protein (stippled bars). (B) Acid phosphatase, open bars; *N*-acetyl- β -hexosaminidase, stippled bars; β -galactosidase, hatched bars. Between 85 and 90% of the total cellular activity of the hydrolases was recovered in the postnuclear supernatant. (C) Fractionation of Lucifer Yellow in a postnuclear supernatant prepared from amoebae allowed to ingest the dye for 30 min. 90% of the total cellular amount of the dye was recovered in the postnuclear supernatant. (D) Acid phosphatase (open bars) and *N*-acetyl- β -hexosaminidase (stippled bars) activities in fractions of postnuclear supernatants prepared from amoebae that had ingested yeast. Only 10% of the total cellular acid phosphatase and 15% of the *N*-acetyl- β -hexosaminidase activities are recovered in the postnuclear supernatants.

above, 90% of the ingested Lucifer yellow was recovered in the low-speed pellet and both dye peaks on the density gradient were greatly reduced in size. These results suggest that the acid hydrolases are almost exclusively restricted to digestive vacuoles and that other compartments, if they exist, are quantitatively insignificant.

Exocytosis of Carboxyfluorescein and Plastic Beads

Carboxyfluorescein diacetate, a substrate for lysosomal esterase activity, can be covalently cross-linked to amino-derivatized plastic beads. Following endocytosis of these particles, enzymatic hydrolysis released highly fluorescent carboxyfluorescein that did not diffuse into the cell cytoplasm but remained sequestered within the amoeba vacuolar compartment. These properties make carboxyfluorescein diacetate coupled to plastic beads a suitable tool for determining whether the soluble content of digestive vacuoles is secreted without loss of the particulate content. To test whether this

occurs, we allowed amoebae to ingest plastic beads that had carboxyfluorescein diacetate covalently cross-linked to their surface. After a brief incubation period uningested beads were removed, cells were resuspended in fresh media, and the amoebae and growth media were monitored for plastic beads and carboxyfluorescein. The results of a representative experiment, presented in Fig. 3, show that while the plastic beads were retained in amoebae, carboxyfluorescein was hydrolyzed off the beads and was secreted linearly with time. Greater than 90% of the ingested beads present 1 h after initiation of the experiment remained inside the amoebae 3 h later, while >50% of the carboxyfluorescein label was excreted during this same time. The excretion of carboxyfluorescein was blocked when 1 mM NaN_3 was incorporated into the growth medium.

pH-Dependent Binding of Hydrolases and Carboxyfluorescein to Lysosomal Membranes

A morphometric study of the *Acanthamoeba* vacuolar compartment (5) suggested that small vesicles shuttle membrane

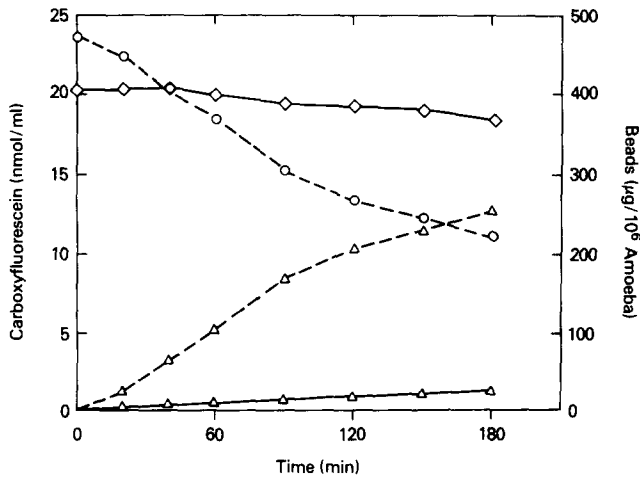


FIGURE 3 Secretion of carboxyfluorescein and beads. Amoebae were allowed to ingest beads covalently coupled with carboxyfluorescein diacetate for 30 min and were then washed free of uningested beads. Cells resuspended in fresh media were monitored for the retention of beads (\diamond), retention of carboxyfluorescein (\circ), and secretion of carboxyfluorescein (\triangle). It is also shown that secretion of carboxyfluorescein by amoebae is inhibited by media with 1 mM NaN_3 (\triangle).

from secondary lysosomes to the plasma membrane. During this cycling, vesicle membrane undergo marked changes in pH. The luminal surface of the phagolysosomal membranes is exposed to an acidic pH, and, as the membrane cycles back to the plasma membrane, this same membrane surface is exposed to growth medium at a more neutral pH. To test whether hydrolases and carboxyfluorescein are differentially bound to lysosomal membranes in a pH-dependent manner, we prepared a crude membrane fraction from isolated phagosomes, resuspended them in acetate buffer at pH 6.0 or 4.5 with or without carboxyfluorescein, and measured the percent of carboxyfluorescein and eight different hydrolases that sedimented with the membranes during high-speed centrifugation. The data in Table III demonstrate that while all of the hydrolases sedimented with the membrane pellet to a greater extent at pH 4.5 than at 6.0, the difference in hydrolase binding between these pH values was very different for some of the hydrolases. The difference in binding for *N*-acetyl- β -hexosaminidase, β -glucuronidase, and β -galactosidase, three of the hydrolases that are rapidly excreted by *Acanthamoeba*, was much greater than that for acid phosphatase, α -glucosidase, β -glucosidase, α -galactosidase, and β -xylosidase, hydrolases which are slowly excreted. The percent of each of the hydrolase activities that was recovered in the membrane pellet from preparations at pH 6.0 was very similar to that observed from preparations at pH 6.8 (data not shown).

The binding of hydrolases to membranes was reversible, as shown by the data in Table IV. Similar percents of hydrolase activity sedimented with the membrane pellet when the phagolysosome preparation was treated with acetate buffer, pH 4.5, and when the pellet and supernatant fraction from preparations treated at pH 6.0 were recombined subsequent to changing the supernatant fraction pH to 4.5 by dialysis. Additionally, the activity that sediments with the membrane pellet from phagolysosomal preparations in acetate buffer at pH 4.5 can be released if these membranes are resuspended in acetate buffer at pH 6.0. Carboxyfluorescein also demon-

TABLE III
pH-Dependent Hydrolase Binding to Membranes

Hydrolase	Activity bound*		Difference in binding [†]
	pH 4.5	pH 6.0	
Acid phosphatase	4.8 (2.2)	0.0 (0.0)	4.8
β -Glucosidase	11.9 (3.0)	5.1 (1.4)	6.8
α -Glucosidase	12.5 (2.7)	7.9 (2.0)	4.6
α -Galactosidase	23.1 (2.6)	16.9 (2.5)	6.2
β -Xylosidase	11.5 (3.6)	3.2 (1.2)	8.3
<i>N</i> -acetyl- β -hexosaminidase	21.0 (2.5)	2.7 (1.0)	18.3
β -Glucuronidase	31.7 (4.8)	10.0 (2.0)	21.7
β -Galactosidase	55.0 (7.2)	30.1 (1.6)	24.9

* Values in parentheses are standard deviations calculated from the means of six experiments.

[†] Binding at pH 4.5 minus binding at pH 6.0.

TABLE IV
Hydrolase Binding to Membranes Is Reversible

Hydrolase	Recombination			
	pH 4.5	pH 6.0	pH 6.0 then pH 4.5*	pH 4.5 then pH 6.0 [†]
	%	%	%	%
β -Glucosidase	12.9	3.2	7.9	57.6
α -Glucosidase	11.8	6.3	14.6	46.8
α -Galactosidase	20.7	14.2	24.4	76.6
<i>N</i> -acetyl- β -hexosaminidase	18.3	3.8	12.8	35.6
β -Glucuronidase	35.2	14.0	32.0	41.1
β -Galactosidase	53.8	31.4	46.1	58.0
Carboxyfluorescein	11.7	1.5	9.2	1.5

Values represent the percent activity bound.

* Membranes at pH 6.0 reincubated with dialyzed supernatant at pH 4.5.

[†] Membranes at pH 4.5 reincubated with buffer at pH 6.0.

strated a pH-dependent binding to phagosomal membranes which, like that of the hydrolase, is reversible (Table IV).

DISCUSSION

Primary lysosomes, secondary lysosomes, and fragmentation vesicles formed from secondary lysosomes (5) are the potential sources of the secreted hydrolases in *Acanthamoeba*. Of these, primary lysosomes undoubtedly play a quantitatively minor role since, as shown here, acid hydrolases in *Acanthamoeba* are almost exclusively restricted to secondary lysosomes. A similar partition of acid hydrolases is apparent in cytochemical studies of *Acanthamoeba*, where primary lysosomes are rarely observed (22; T. Hohman, unpublished observations). Since the particle and soluble content within the same secondary lysosomes are not excreted at the same rate, it appears that hydrolase secretion does not result from the fusion of secondary lysosomes with the plasma membrane. Morphometric studies of *Acanthamoeba* (5) have suggested that the amoebae are able to maintain their characteristic high endocytic rates by recirculating membrane between the cytoplasmic vacuolar system and the plasma membrane. Membrane appears to be returned to the cell surface via small vesicles that fragment from secondary lysosomes. Evidence reported here indicates that this vesicle shuttle is the source of the secreted hydrolases.

If, as suggested above, all the secreted hydrolases are re-

leased from a single source, why are they secreted at different rates? There are a number of mechanisms that can be proposed to account for this observed heterogeneity. Among these are the possibilities that (a) individual hydrolases are enriched to different extents in secondary lysosomes and that the rate of hydrolase secretion is directly proportional to enrichment, (b) there are subclasses of lysosomes that differ in their hydrolase content and in the rate at which they fuse with the plasma membrane, (c) individual hydrolases are differentially bound to lysosomal membranes and are not equally available for secretion, and (d) secreted lysosomal enzymes are specific products generated during cellular processing of hydrolases and have specific activities that are greatly different from their precursors; those that have specific activities higher than their precursors appearing to be secreted at a high rate and, conversely, those that have specific activities lower than their precursors appearing to be secreted at a lower rate.

The first of these mechanisms does not appear to be functioning in *Acanthamoeba* since hydrolases that were secreted at very different rates are similarly enriched in preparation of secondary lysosomes. Whether the second mechanism is functioning is uncertain. Centrifugation of steep density gradients resolved the hydrolases into a single activity peak. With shallower gradients the hydrolase activities were separated into two peaks, reflecting two subpopulations of lysosomes. These two populations of lysosomes cannot account for the heterogeneity in hydrolase secretion since all of the hydrolases are equally distributed between them. However, we cannot rule out the possibility that there are subpopulations of lysosomes that differ in their hydrolase content, but that cannot be separated on the basis of differences in density. Subpopulations of lysosomes differing in density are not unique to *Acanthamoeba* but have also been observed in fibroblasts (21).

Although we have no evidence in support of the first two proposed mechanisms, we did observe differential binding of acid hydrolases to phagosomal membranes, which may account for the heterogeneity in hydrolase secretion. Whereas the percent of each hydrolase that is membrane-bound at an acidic pH (4.5) was larger than that bound at a more neutral pH (6.0), the difference between the percent bound at these two pH values varied among the hydrolases. These differences in pH-dependent binding, which may reflect hydrolase availability for secretion, clearly separate the hydrolases into the same two groups as does the enzyme secretion kinetics of the hydrolases.

These data suggest that the secreted lysosomal enzymes are those that are trapped in shuttle vesicles that cycle membrane from secondary lysosomes to the cell surface. During membrane recycling the shuttle vesicles begin as a portion of a secondary lysosome where the luminal membrane surface is exposed to the acidic lysosomal pH. Estimates of lysosomal pH using an assortment of techniques and various cell types range from 3.5 to 5.5 (8, 9, 12, 13, 18, 19). When the shuttle vesicle fragments from the secondary lysosome, the hydrolases bound to the membrane become trapped, along with small volumes of the lysosomal contents. Within the shuttle vesicles, which have a high surface-to-volume ratio, the membrane-bound hydrolases form a large fraction of the trapped enzymes. Following fusion with the cell membrane, the luminal surface of the shuttle vesicle membrane is exposed to the growth medium where the pH is 6.8. This pronounced pH shift and the differential pH-dependent membrane binding of

hydrolases results in heterogeneous hydrolase secretion kinetics.

Carboxyfluorescein, which enters the cells as carboxyfluorescein diacetate covalently coupled to plastic beads, was released into the growth medium at a rate similar to that of the rapidly secreted hydrolases. This release was blocked by the incorporation of sodium azide into the growth medium. The probable route of secretion is identical to that of the hydrolases since, unlike the situation in mammalian cells (24), neither carboxyfluorescein nor carboxyfluorescein diacetate permeates *Acanthamoeba* plasma membrane. Following interiorization of these fluorescent labels either free in solution or bound to plastic beads, the fluorescence remained punctate.

Carboxyfluorescein demonstrated a pH-dependent binding to phagolysosomal membrane similar to that of the rapidly secreted hydrolases. Since the volume transported to the cell surface via the shuttle vesicles must be very small (5), we expect soluble phagolysosomal contents to be secreted at rates similar to or less than those of the slowly secreted hydrolases. The pH-dependent membrane binding of carboxyfluorescein probably accounts for its rapid secretion.

Recent observations (18) suggest that cells may be surrounded by a boundary layer that has a pH more acidic than that of the growth medium. For *Amoeba proteus* the pH of the boundary layer was reported to be 0.9 u below that of the defined medium. The presence of a similar boundary layer surrounding *Acanthamoeba* is compatible with our model of hydrolase secretion since all of the hydrolases were similarly bound at pH 6.0 and 6.8.

Although differences in the pH-dependent binding of hydrolases to lysosomal membrane correlate well with the observed differences in hydrolase secretion kinetics, we cannot eliminate the possibility that there are other mechanisms functioning as well. In our studies of hydrolase binding to membranes we observed similar results with both acetate and HEPES buffer (data not shown). When these buffers were replaced with citrate buffer, none of the hydrolases bound to membrane at either pH. Clearly, pH is not the only factor regulating hydrolase binding, although it is a major one.

The physiological significance, if any, of hydrolase secretion in *Acanthamoeba* is unknown. McIntyre and Jenkin (17) have reported that chemotaxis in a closely related amoeba depends on factors released from the food source as a result of interaction with substances (speculated to be enzymes) secreted by the amoebae. It is difficult to imagine what other utility secretion of hydrolases might have for an amoeba with a simple, nonsexual life cycle. For the moment, the most reasonable guess is that the hydrolase secretion is concomitant with membrane recycling and as such may provide important clues to the details of that process.

Received for publication 29 June 1983, and in revised form 23 September 1983.

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