CHLOROQUINE INHIBITS LYSOSOMAL ENZYME PINOCYTOSIS AND ENHANCES LYSOSOMAL ENZYME SECRETION BY IMPAIRING RECEPTOR RECYCLING

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ABSTRACT

Adsorptive pinocytosis of acid hydrolases by fibroblasts depends on phosphomannosyl recognition markers on the enzymes and high-affinity pinocytosis receptors on the cell surface. In this study, β-glucuronidase binding to the cell surface of attached fibroblasts was found to be saturable and inhibitable by mannose-6-phosphate (Man-6-P). Dissociation of cell-bound β-glucuronidase occurred very slowly at neutral pH, but was greatly accelerated by lowering the pH below 6.0, or by exposure to Man-6-P. Comparison of the maximal cell surface binding and the observed rate of enzyme pinocytosis suggests that the pinocytosis receptors are replaced or reused about every 5 min. Enzyme pinocytosis was not affected by inhibition of new protein synthesis for several hours, suggesting a large pool of internal receptors and/or reuse of internalized receptors.

Chloroquine treatment of normal human fibroblasts had three effects: (*a*) greatly enhanced secretion of newly synthesized acid hydrolases bearing the recognition marker for uptake, (*b*) depletion of enzyme-binding sites from the cell surface, and (*c*) inhibition of pinocytosis of exogenous enzyme. Only the third effect was seen in I-cell disease fibroblasts, which were also less sensitive than control cells to this effect.

These observations are consistent with a model for transport of acid hydrolases that proposes that delivery of newly synthesized acid hydrolases to lysosomes requires the phosphomannosyl recognition marker on the enzymes, and intracellular receptors that segregate receptor-bound enzymes into vesicles for transport to lysosomes. This model explains how chloroquine, which raises intralysosomal pH, can disrupt both the intracellular pathway for newly synthesized acid hydrolases, and the one for uptake of exogenous enzyme by cell surface pinocytosis receptors.

Adsorptive pinocytosis of lysosomal hydrolases was initially recognized as the uptake of "corrective factors" by enzyme-deficient fibroblasts (for review, see reference 14). These corrective factors proved to be acid hydrolases that were secreted into the culture medium by fibroblasts and were also present in body fluids and tissue extracts. The selectivity and saturability of the uptake system

suggested a receptor-mediated uptake process (9, 10, 16, 19, 30). I-cell disease fibroblasts, which were characterized by a deficiency for multiple lysosomal enzymes, were found to secrete enzymes into the extracellular fluid that were not susceptible to pinocytosis by normal fibroblasts. Yet I-cell fibroblasts took up acid hydrolases secreted by normal fibroblasts and retained them normally. These observations led to two hypotheses: (*a*) that there is a common recognition marker for uptake that is shared by many acid hydrolases and is missing from I-cell disease hydrolases (8) and (*b*) that secretion of enzymes into the medium and receptor-mediated uptake are essential steps in transport of acid hydrolases to lysosomes in normal fibroblasts, a suggestion that has come to be called the "secretion-recapture hypothesis" (8, 15).

The common recognition marker hypothesis received strong support from the findings implicating mannose-6-phosphate (Man-6-P) recognition in adsorptive pinocytosis of many acid hydrolases (10, 11, 19, 27). Natowicz et al. (13) recently provided direct evidence for Man-6-P in the recognition marker for human β-glucuronidase, confirming the predictions based on the original indirect evidence. They also presented evidence that the Man-6-P was present on oligosaccharides released by endoglycosidase H. Related evidence has also been presented for human α-*N*-acetyl hexosaminidase (29) and for bovine β-galactosidase (1, 21).

The second hypothesis, the secretion-recapture hypothesis of enzyme localization, has required modification in the light of subsequent data. The discovery that Man-6-P inhibited pinocytosis of acid hydrolases made it possible to test this hypothesis. If enzymes must first be secreted into the medium and then taken up from the medium to reach lysosomes, growth of cells in Man-6-P concentrations that inhibit enzyme uptake should lead to accumulation of enzyme in the medium and to reduction of intracellular lysosomal enzyme levels. However, when this experiment was done in several laboratories (7, 25, 28, 32), Man-6-P failed to depress intracellular enzyme levels significantly and produced only modest increases in extracellular enzyme levels. To explain this result, alternate hypotheses have been presented. Von Figura et al. (31, 32) demonstrated several acid hydrolases on the cell surface by immunologic techniques, and proposed that most of the newly synthesized enzymes are first delivered to the plasma membrane already bound to receptors and then delivered to lysosomes by endocytosis without ever dissociating from the receptors. I-cell enzyme would arrive at the surface unbound and be released into the medium.

Chloroquine and other lysosomotropic amines inhibit the uptake of acid hydrolases. Wiesmann et al. (33) reported that chloroquine had two effects on lysosomal enzymes in fibroblasts: first, inhibiting enzyme pinocytosis by impairing binding at the cell surface, and second, causing loss of endogenous enzyme to the medium. Sando et al. (20) found no evidence that previously internalized α-iduronidase was lost on exposure to chloroquine, and inferred from the kinetics of inhibition of enzyme pinocytosis that amines do not block cell surface binding. However, they confirmed the inhibition of enzyme pinocytosis and suggested that some subsequent step in endocytosis was inhibited by these agents.

The purpose of this report is to present new studies that show different effects of chloroquine on normal and I-cell fibroblasts and to present a model for receptor-mediated transport of acid hydrolases (24, 25) that is consistent with the results presented. Chloroquine treatment of normal fibroblasts had three effects: (*a*) greatly enhanced secretion of newly synthesized acid hydrolases bearing the recognition marker for uptake, (*b*) depletion of enzyme-binding sites from the cell surface, and (*c*) inhibition of pinocytosis of exogenous enzyme. Only the third effect was seen in I-cell fibroblasts. These studies are consistent with a model for enzyme transport (24, 25) that proposes two pathways for delivery of enzyme to lysosomes, an intracellular pathway from the endoplasmic reticulum to lysosomes, and a quantitatively less important pathway involving enzyme pinocytosis by cell surface receptors. We suggest that both pathways depend on the phosphomannosyl enzyme receptor, and that chloroquine disrupts both pathways by impairing receptor recycling.

MATERIALS AND METHODS

Most of the reagents were purchased from Sigma Chemical Co., St. Louis, Mo., or from Fisher Scientific Co., Pittsburgh, Pa. Fluorometric substrates were obtained from Research Products International Corp., Elk Grove Village, Ill. and leucine, L-4,5-^{3}H(N) 40 Ci/mmol, from New England Nuclear, Boston, Mass. Spleens were obtained from the Department of Pathology, Washington University School of Medicine, St. Louis, Mo.

Cell Cultures

Diploid human fibroblasts were obtained from several sources. Tay-Sachs disease fibroblasts (GM-502) were received

from the Human Mutant Cell Repository, Camden, N. J. β-Glucuronidase-deficient cells were established from a skin biopsy obtained from patient J. E. (also available as cell strain GM-151 from Camden). β-Hexosaminidase-deficient fibroblasts from Sandhoff disease patient E. W. were obtained from the Montreal Tissue Repository, Department of Pediatrics, McGill University, Montreal, Canada. I-cell disease fibroblasts from patient L. T. were supplied by Dr. Thaddeus Kelly, Medical College of Virginia, Richmond, Va.

Cultures were maintained at 37°C in 5% CO_2 in minimal essential medium (MEM-Earle's medium, Grand Island Biological Co., Grand Island, N. Y.), supplemented with 15% heat-inactivated fetal calf serum (KC Biological Inc., Lenexa, Kans.), 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate.

Enzyme Preparation

A fraction enriched for "high-uptake" human spleen β-glucuronidase (4) was obtained from enzyme purified as previously described (13).

β-Hexosaminidase B was collected from Tay-Sachs fibroblast secretions. Cells were grown for 10 d to confluence in 490-cm^2 roller bottles (Corning Glass Works, Science Products Div., Corning, N. Y.), washed with saline, and maintained overnight in serum-free Waymouth medium (KC Biological Inc.) containing 1 mg/ml β-hexosaminidase-free human serum albumin. Thereafter, cells were fed every 24 h with 50 ml of Waymouth medium. Collected medium was concentrated to 2 ml by ultrafiltration, using an XM-50 membrane filter (Amicon Corp., Scientific Systems Div., Lexington, Mass.), and dialyzed extensively against 002 M Tris-HCl, 0.01 M Na_2HPO_4, 0.15 M NaCl, and 0.01% NaN_3, pH 7.5.

Assays

Enzyme activities were determined fluorometrically (5). Synthetic substrates were 4-methylumbelliferyl-β-D-glucuronide, 10 mM in 0.1 M sodium acetate, pH 4.8, for β-glucuronidase, and 4-methylumbelliferyl-2-acetamide-2-deoxy-β-D-glucopyranoside, 5 mM in 0.02 M sodium phosphate-citrate, pH 4.4, for β-hexosaminidase. Assays were carried out by incubating at 37°C after adding 25 μl of enzyme to 100 μl of substrate. Reactions were stopped by the addition of 1.8 ml of glycine-carbonate buffer, pH 10.5. One unit of activity is the amount of enzyme that catalyzes the release of 1 nmol of 4-methylumbelliferone per hour. Protein was measured according to Lowry et al. (12).

Binding and Internalization Experiments

Binding of β-glucuronidase was measured in duplicate 35-mm Falcon dishes containing intact cell monolayers. Experiments at 4°C were carried out as follows: We chilled petri dishes for 15 min in a cold room (4°C). The medium was aspirated and cells were washed twice with 5 ml of cold Dulbecco's phosphate-buffered saline (PBS), pH 7.3, containing 1 mg/ml enzyme-free human serum albumin (HSA). We then added 1 ml of PBS-HSA containing β-glucuronidase to the dishes. After a 2-h incubation, the dishes were washed six times with 5 ml of cold PBS and drained, and cell lysates were prepared by the addition of 0.5 ml of 1% sodium deoxycholate per dish.

Reversibility of β-glucuronidase binding to fibroblasts at 4°C was studied as follows: dishes containing the cell-bound enzyme were rinsed as described above, incubated with 1 ml of PBS-HSA in the presence or absence of 10 mM Man-6-P, rinsed twice with 5 ml PBS, and then lysed. Under these conditions, 98% of the β-glucuronidase remained cell-associated after 90 min in buffered saline, but only 5% of the enzyme remained cell-associated after 90 min in buffered saline containing 10 mM Man-6-P.

Binding experiments at 37°C were carried out in cells previously incubated with inhibitors of energy metabolism. For this preincubation, cells were rinsed with PBS-HSA, incubated initially for 30 min at 37°C with fresh PBS-HSA to deplete energy stores, and then incubated for 1 h with PBS-HSA containing 10 mM sodium azide, 10 mM sodium fluoride, and 1 mM sodium cyanide (26). Enzyme binding was measured in preincubated cells, to which enzyme was added in 1 ml PBS-HSA containing the inhibitors of energy metabolism. Under these conditions, little or no β-glucuronidase was internalized, as ~95% of the cell-bound enzyme was released when cells were incubated subsequently for 30 min at 37°C in the presence of 10 mM Man-6-P.

β-Glucuronidase binding was found to be dependent on cell density and, at subsaturating enzyme levels, on the fraction of high-uptake enzyme in a given spleen β-glucuronidase preparation (13). Binding conditions were standardized by using cells grown for 7–14 d after trypsinization and by using enzyme concentrations between one and two times the half-saturating ($K_{binding}$) level for that enzyme preparation.

Internalization experiments were carried out as previously described (10). I-cell and β-glucuronidase- or β-hexosaminidase-deficient fibroblasts were exposed to the indicated concentrations of β-glucuronidase or β-hexosaminidase, respectively. After incubation at 37°C, dishes were chilled on ice and rinsed six times with 3-ml portions of ice-cold PBS. Cell-associated β-glucuronidase was measured in cells disrupted with 0.5 ml of 1% deoxycholate. β-Hexosaminidase was measured in cells disrupted by the addition of 1 ml of distilled water followed by freezing for 20 min at −20°C and then thawing the dishes. The rate of enzyme internalization or uptake is expressed as the amount of enzymatic activity that became cell-associated per unit of time (unit/milligram of cell protein per hour).

Effect of Cycloheximide on Protein Synthesis

Incorporation of added [^{3}H]leucine into acid-precipitable material was measured in those experiments in which the effect of cycloheximide on β-glucuronidase uptake was studied. Incorporation was inhibited ~98% when cells were incubated for 1 h or longer at 37°C in the presence of 0.1 mM cycloheximide in 1 ml of medium containing 2 μCi/ml [^{3}H]leucine.

RESULTS

Effect of Preincubation with Chloroquine on Chloroquine Inhibition of β-Glucuronidase Uptake by I-cell and β-Glucuronidase-deficient Fibroblasts

Fig. 1 presents experiments showing the inhibition of enzyme pinocytosis by chloroquine in I-cell and β-glucuronidase-deficient fibroblasts when the drug was present with the enzyme during a 1-h incubation, and also when the drug was present for varying periods of time before the addition of enzyme. As has been reported previously (20, 33), enzyme pinocytosis was inhibited

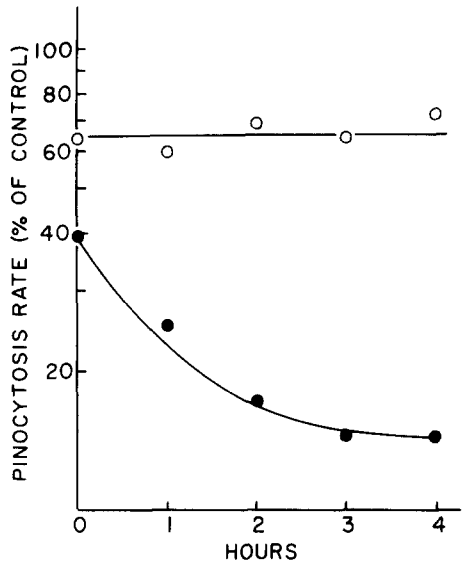

FIGURE 1 Effect of preincubation with chloroquine on the inhibition of pinocytosis by chloroquine in I-cell and β-glucuronidase-deficient fibroblasts. I-cell (○) and β-glucuronidase-deficient (●) fibroblasts were preincubated for 0–4 h at 37°C in Waymouth medium containing 25 μM chloroquine before 1-h measurement of the pinocytosis rate in the presence of chloroquine. Pinocytosis was measured as cell-associated enzyme after a 1-h incubation with 1,000 U/ml of β-glucuronidase. All pinocytosis rates are expressed as the percent of control, i.e., the initial rate of pinocytosis by each cell type in the absence of chloroquine, which was 100 U/mg per h for the I-cell fibroblasts and 116 U/mg per h for the β-glucuronidase-deficient fibroblasts.

by chloroquine. However, the effects of chloroquine were different on enzyme pinocytosis by I-cell and β-glucuronidase-deficient fibroblasts. Firstly, in I-cell fibroblasts, chloroquine inhibited enzyme pinocytosis 35% when the drug and the enzyme were added together at the start of a 1-h incubation. However, preincubation of cells with chloroquine for up to 4 h before addition of enzyme did not enhance the inhibition of enzyme pinocytosis by chloroquine. Secondly, β-glucuronidase-deficient fibroblasts were more sensitive (60% inhibition) than I-cell fibroblasts to the inhibitory effect of chloroquine during a 1-h incubation with enzyme, and, in addition, pinocytosis by these fibroblasts was increasingly inhibited with increasing time of exposure to chloroquine before addition of the enzyme for the 1-h pinocytosis measurement.

Fig. 2 shows an experiment designed to test the reversibility of the time-dependent inhibition by chloroquine of enzyme pinocytosis by β-glucuronidase-deficient fibroblasts. Note again the effect of preincubation with chloroquine on subsequently measured enzyme pinocytosis in the presence of chloroquine. Enzyme pinocytosis fell to 15% of the uinhibited level after 3 h of exposure to the drug. Fig. 2 also shows the rapid reversibility of the inhibition of enzyme pinocytosis. Enzyme pinocytosis recovery was nearly complete in the first hour after removal of the drug. Addition of cycloheximide at the time of removal of chloroquine (Fig. 2) or 1 h before the removal of chloroquine (not shown) did not alter the rate or extent of recovery of enzyme pinocytosis. The cycloheximide results suggest that recovery of the capacity for enzyme pinocytosis on removal of chloroquine does not require new protein synthesis.

Effect of Chloroquine on Previously Endocytosed Enzyme

The reduction of enzyme accumulation in the presence of chloroquine might be a result either of failure of enzyme pinocytosis or of failure to retain

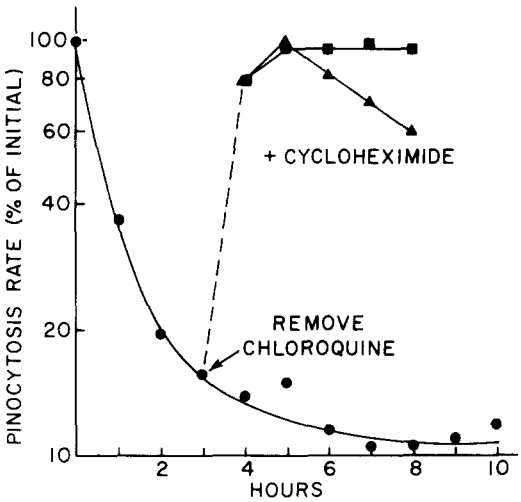

FIGURE 2 Reversibility of the chloroquine effect on β-glucuronidase internalization. Internalization of β-glucuronidase was measured at 37°C over 1 h in β-glucuronidase-deficient cells exposed to 25 μM chloroquine for varying periods before the uptake study. After 3 h, two-thirds of the dishes were washed to remove chloroquine, and then incubated in Waymouth medium alone (■), or in Waymouth's plus 0.1 mM cycloheximide (▲). Chloroquine was present during the uptake period except in those dishes from which chloroquine was removed at 3 h (▲, ■).

internalized enzyme after pinocytosis. Internalized enzyme can be distinguished from enzyme bound to the cell surface because the latter can be displaced from the cell surface by Man-6-P, as was reported by Rome et al. (18). Table I *A* shows that Man-6-P displaced 89% of bound but not internalized β-glucuronidase, i.e., enzyme that became cell-associated during a 2-h binding incubation at 4°C. In contrast, Table I *B* shows that most of the enzyme that became cell-associated during a 3-h incubation at 37°C remained cell-associated in subsequent incubations at either 4° or 37°C with Man-6-P, chloroquine, or both. In addition, Table I *C* shows a similar result from an experiment in which the enzyme pinocytosis during the initial incubation took place in the presence of chloroquine, even though enzyme pinocytosis during the initial incubation was reduced to only 34% of the level seen in the absence of chloroquine. Thus, although chloroquine inhibited pinocytosis, it did not lead to a loss of internalized enzyme.

Effects of Lysosomotropic Amines on Enzyme Secretion

Both chloroquine and NH_4Cl were found to stimulate secretion of acid hydrolases by human fibroblasts. Similar observations on chloroquine were recently reported by Wilcox and Rattray (34). In our studies, the amines enhanced secretion of β-hexosaminidase by every normal fibroblast line examined (three) and by every fibroblast line examined from patients with single-enzyme-deficiency storage diseases (five fibroblast lines, each having a different single-enzyme deficiency). In contrast, amines failed to enhance the already high level of enzyme secreted by I-cell fibroblasts or that secreted by fibroblasts from a patient with mucolipidosis III (GM 2559). Table II shows the effects of these two amines and of Man-6-P on the secretion of hexosaminidase B by Tay-Sachs disease fibroblasts. Tay-Sachs disease fibroblasts were used because they are hexosaminidase A-deficient and secrete mainly hexosaminidase B, which is stable in the medium during the collection periods. Man-6-P, a competitive inhibitor of enzyme pinocytosis (10), had only a small effect on the amount of enzyme secreted by confluent fibroblasts, as had been observed before (7, 25, 28, 32). One can see that chloroquine (25 μM) stimulated hexosaminidase secretion nearly fourfold and NH_4Cl (10 mM) stimulated secretion nearly eightfold. When the fibroblast-secreted hexosaminidase was tested for susceptibility to pinocytosis by fibroblasts after concentration and dialysis, it was clear that the secreted enzyme was rich in high-uptake enzyme (4), i.e., enzyme bearing the phosphomannosyl recognition marker (11). The initial rate of enzyme pinocytosis was nearly four times

TABLE I

Effect of Chloroquine and Man-6-P on Retention of Previously Internalized Enzyme

	Conditions of second incubation (1 h)		
		Cell-associated enzyme	
Conditions of initial incubation with enzyme (4,000 U/ml)	Additives to the media	37°C	4°C
		U/mg	
A. Binding (2 h at 4°C)	None	—	24.5
	+ Man-6-P	—	2.8
B. Internalization (3 h at 37°C)	None	131	162
	+ Man-6-P	132	157
	+ Chloroquine	152	164
	+ Man-6-P + chloroquine	132	152
C. Internalization (3 h at 37°C) with 25 μM chloroquine	None	45	56
	+ Man-6-P	35	53
	+ Chloroquine	49	59
	+ Man-6-P + chloroquine	32	51

Cultures of β-glucuronidase$^-$ fibroblasts were exposed to β-glucuronidase in an initial incubation under the conditions shown, after which the dishes were rinsed and incubated during a second incubation at 37° or 4°C in the presence or absence of 25 μM chloroquine and/or 10 mM Man-6-P as indicated. The cells were rinsed and assayed for cell-associated enzyme.

TABLE II

Effects of Chloroquine and NH_4Cl on Amount and Uptake Properties of β-Hexosaminidase B Secreted by Tay-Sachs Disease Fibroblasts

Growth conditions	Secretion enzyme	Susceptibility to pinocytosis	Added units internalized in 24 h
	U/ml per 24 h	*U/mg per h*	*%*
Medium alone	235	17	5.5
+ Man-6-P	264	38	12
+ Chloroquine	974	62	27
+ Chloroquine + Man-6-P	998	67	31
+ NH_4Cl	1,802	59	24
+ NH_4Cl + Man-6-P	1,658	55	28

Roller bottles of confluent Tay-Sachs fibroblasts were rinsed and incubated for 24 h at 37°C with 50 ml of Waymouth medium, followed by a second 24-h period with 50 ml of fresh Waymouth medium in the presence or absence of 25 μM chloroquine, 10 mM NH_4Cl and/or 10 mM Man-6-P. Secreted β-hexosaminidase was measured in collected medium, which was then concentrated by ultrafiltration in an Amicon filtration unit with an XM 50 filter and dialyzed. Susceptibility of the secreted β-hexosaminidase to pinocytosis by fibroblasts was measured in subconfluent β-hexosaminidase-deficient fibroblasts in 35-mm dishes. 450 U of β-hexosaminidase from each dialyzed concentrate was added to 35-mm dishes in 1 ml of medium, and cell-associated enzyme was measured in the cells after incubation for 2 h at 37°C (to measure the initial rate of pinocytosis) or for 24 h at 37°C (to obtain a minimum estimate of the percent of high-uptake β-hexosaminidase in the enzyme secreted under each condition).

higher for β-hexosaminidase produced by amine-treated cells than for enzyme secreted by untreated cells. A minimum estimate of the percent of high-uptake enzyme in the secreted β-hexosaminidase was provided by the fraction internalized by β-hexosaminidase-deficient fibroblasts during a 24-h exposure. This fraction was five to six times higher for enzyme produced by amine-treated cells than for enzyme produced by untreated cells. Evidence that this uptake depends on the phosphomannosyl recognition marker was provided by two observations: pinocytosis of the secreted enzyme produced under every condition shown in Table II was inhibited by Man-6-P (10) and reduced >90% by treatment of the enzymes with endoglycosidase H (13) (data not shown). As mentioned above, the amines had similar effects on enzyme secretion by normal human fibroblasts to those shown for Tay-Sachs disease fibroblasts. Thus, lysosomotropic amines led to greatly enhanced secretion of hexosaminidase by non-I-cell fibroblasts, and the secreted enzymes were enriched for high-uptake forms bearing the phosphomannosyl recognition marker.

Comparison of the Effects of Ammonium Chloride on Secretion of Newly Synthesized and Previously Endocytosed Lysosomal Enzymes

Failure of chloroquine to induce secretion of pinocytosed enzyme (Table I), coupled with the dramatic enhancement of enzyme secretion, suggested that the amines preferentially affected transport of newly synthesized enzymes and diverted them to the outside. Table III presents an experiment designed to test this hypothesis. β-Glucuronidase-deficient fibroblasts were allowed to pinocytose β-glucuronidase for 24 h and then to equilibrate in enzyme-free medium for the next 24 h, after which NH_4Cl was added to some of the cells. The cells and the medium were studied 48 h later to determine the effect of NH_4Cl on previously endocytosed enzyme (β-glucuronidase) and on β-hexosaminidase. The increase in total β-hexosaminidase over the 48-h exposure (total enzyme minus initial enzyme) was considered "newly synthesized" enzyme. Table III shows that NH_4Cl

TABLE III

Effect of NH_4Cl on β-Hexosaminidase B and on Previously Endocytosed β-Glucuronidase

	Initial enzyme	Final enzyme	
		Control	+NH_4Cl
	U/plate	*U/plate*	*U/plate*
β-Hexosaminidase			
Cellular enzyme	1,130	1,700	1,180
Medium enzyme	—	131	571
Total enzyme	1,130	1,831	1,751
β-Glucuronidase			
Cellular enzyme	362	384	369
Medium enzyme	—	14	29
Total enzyme	362	398	398

Cultures of β-glucuronidase-deficient fibroblasts were incubated with 8,000 U/ml of β-glucuronidase in Waymouth medium for 24 h. The cultures were rinsed and incubated for 24 h with medium alone. Then, cells were incubated for an additional 48-h period with fresh Waymouth medium with or without 10 μmol/ml NH_4Cl before cell extracts, and media were assayed for β-glucuronidase and β-hexosaminidase.

had a very small effect on the distribution of β-glucuronidase that had been previously taken up and presumably localized in lysosomes. By contrast, the distribution of β-hexosaminidase was markedly altered in the NH_4Cl-treated cells. Whereas only 19% of the increase in hexosaminidase was found in the medium from the control cells, 92% of the increase in β-hexosaminidase was present in the medium from the NH_4Cl-treated cells. To be certain that this effect of NH_4Cl on β-hexosaminidase distribution, which differed from its effect on β-glucuronidase distribution, was really attributable to the latter being previously endocytosed enzyme rather than to a basic difference in transport between these two enzymes, a similar experiment was carried out on β-hexosaminidase-deficient cells that had been fed β-hexosaminidase B. Here a similar exposure to NH_4Cl after pinocytosis of β-hexosaminidase B did not stimulate secretion of the previously endocytosed β-hexosaminidase. We inferred from these experiments and from those in Table I that lysosomotropic amines have very little effect on enzymes already in secondary lysosomes, but mainly affect newly synthesized lysosomal enzymes diverted to the extracellular medium at some point en route to secondary lysosomes.

Time and Enzyme-concentration Dependence of Chloroquine Inhibition of Enzyme Pinocytosis by I-Cell Fibroblasts

I-cell fibroblasts provide an interesting model for analysis of the mechanism of enzyme pinocytosis inhibition by chloroquine. These cells have normal pinocytosis receptors and take up normal lysosomal enzymes (8). They produce lysosomal enzymes, but the enzymes do not have the recognition marker for uptake and thus do not act as ligands for the pinocytosis receptors. Kinetic analysis of the inhibition of enzyme pinocytosis by chloroquine in these cells should be less complicated than the analysis in normal fibroblasts in which the drug enhances secretion of high-uptake enzyme that can compete for pinocytosis receptors with the exogenous enzyme whose pinocytosis is being measured. For this reason, we studied the effects of time and enzyme concentration on the effects of chloroquine on enzyme pinocytosis by I-cell fibroblasts. Fig. 3 shows the time-course of enzyme pinocytosis by I-cell fibroblasts and the effects of cycloheximide and chloroquine addition at time 0. In the absence of drugs, the rate of pinocytosis was linear for at least 4 h. Cycloheximide had no significant effect on the rate of enzyme pinocytosis for the first 3 h, which suggests either that there is an internal pool of pinocytosis receptors that can replace those internalized during enzyme pinocytosis, or that cell-surface receptors can be reused after internalization. In contrast to cycloheximide, chloroquine inhibited pinocytosis by I-cell fibroblasts in the first hour (Fig. 3), and the degree of inhibition appeared to increase with time of exposure to chloroquine. The increased inhibition of enzyme pinocytosis with time appeared to require the combined presence of exogenous enzyme and chloroquine, because up to 4 h preincubation of I-cell fibroblasts with chloroquine without added enzyme (Fig. 1) did not enhance the inhibition of pinocytosis by chloroquine when enzyme was added later. These observations led us to predict that the inhibition of enzyme pinocytosis by chloroquine in I-cell fibroblasts would be greater with increasing enzyme concentration. This proved to be the case. As seen in Fig. 4 in the double reciprocal plot of β-glucuronidase concentration and rate of enzyme pinocytosis in the presence and absence of chloroquine, the degree of inhibition by 25 μM chlo-

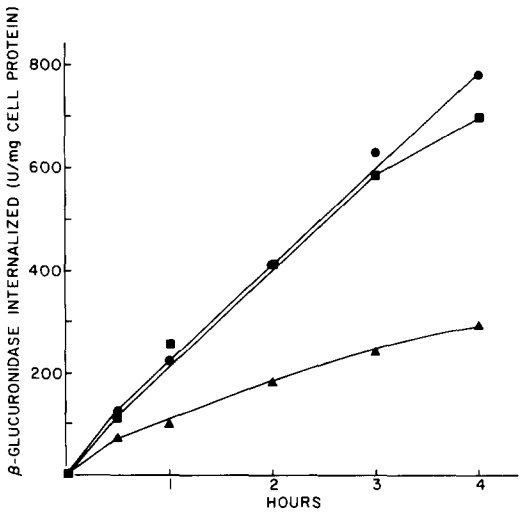

FIGURE 3 Time-course of β-glucuronidase internalization and the effects of cycloheximide and chloroquine. I-cell fibroblasts were incubated at 37°C with 6,000 U of β-glucuronidase in 1 ml of Waymouth medium in the absence of added drug (●), or in the presence of either 25 μM chloroquine (▲) or 0.1 mM cycloheximide (■). After the indicated times, dishes were chilled, rinsed, lysed, and measured for cell-associated enzyme.

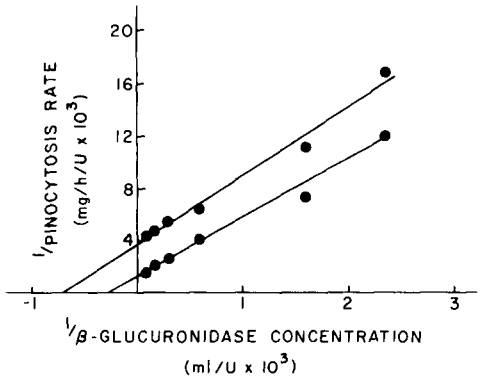

FIGURE 4 Double reciprocal plot of the inhibition by chloroquine of β-glucuronidase pinocytosis in I-cell fibroblasts. Cells were incubated for 1 h at 37°C with increasing concentrations of β-glucuronidase in the presence or absence of 25 μM chloroquine. Cell-associated enzyme was measured after dishes were rinsed with cold PBS. Upper line: with chloroquine; lower line: control.

roquine was 29% for 400 U/ml and 63% for 4,000 U/ml. Similar enzyme-concentration-dependent inhibition of enzyme pinocytosis was seen with NH_4Cl (10 mM) (not shown).

Thus, chloroquine led to progressive depletion of the capacity for enzyme pinocytosis by I-cell fibroblasts only when exogenous enzyme (the ligand for the pinocytosis receptor) was administered with the drug (Fig. 3). This observation, together with data in Fig. 4, suggested that the drug acted after the formation of enzyme receptor complexes to prevent further pinocytosis. Depletion of the capacity for enzyme pinocytosis after initial use of pinocytosis receptors suggested that chloroquine might act to prevent receptor reuse, a process thought to be required for a number of adsorptive endocytosis systems (6).

Kinetics of Cell Surface Binding and Internalization of β-Glucuronidase by I-Cell Fibroblasts in the Absence of Chloroquine

Binding of β-glucuronidase was studied in attached fibroblasts under conditions in which internalization of bound enzyme was prevented by low temperature (4°C) or by incubation of cells, before binding studies at 37°C, with agents that block energy metabolism. Binding at 37°C in energy-poisoned cells was similar to that seen at 4°C in untreated cells, but displacement of prebound enzyme by 10 mM Man-6-P was more efficient at 37°C (96% in 30 min) than at 4°C (78% in 30 min). Fig. 5 *A* and *B* shows the kinetics of binding and internalization of β-glucuronidase by I-cell fibroblasts at 37°C. The two experiments were done simultaneously on the same batch of cells to permit comparisons of the estimated number of receptors at the cell surface and the maximum velocity of internalization. Specific binding was calculated by subtracting the amount of enzyme binding not inhibited by 10 mM Man-6-P (nonspecific binding) from total binding. The "internalized enzyme" represents total cell-associated enzyme, which includes both the internalized enzyme and the enzyme bound to the cell surface. The $K_{binding}$ (1.44×10^{-9} M) was lower than the K_{uptake} (11.6×10^{-9} M) (Fig. 5 *B* and *C*), as reported in the studies of Rome et al. (18). From the double reciprocal plot for binding, the number of apparent specific binding sites at the cell surface was calculated to be 36,800/cell. From the enzyme bound at saturation, $V_{binding}$ (73 U/mg), and from the maximum enzyme uptake observed, V_{uptake} (806 U/mg per hour), one can calculate that cell surface enzyme receptors must be replaced or reused approximately every 5 min. The linear uptake for 3 h in the absence of new protein synthesis (Fig. 3) suggests the existence of a large pool of internal receptors that can replace those internalized through adsorptive pinocytosis and/or reuse of the internalized receptors. Fischer et al. (2) recently reported that there is an internal pool of phosphomannosyl-enzyme receptors that is at least four times as large the number present on the cell surface. However, even if this entire pool were in equilibrium with the cell surface receptors, it would be insufficient to explain the observed rate of enzyme pinocytosis for 3 h in the presence of cycloheximide without invoking recycling of cell surface receptors.

The binding studies on I-cell fibroblasts reported here agree generally with those of Rome et al. (18) who studied direct binding of α-iduronidase to Hurler-syndrome fibroblasts detached by trypsinization and suspended in growth medium to allow partial recovery before study. Under those conditions, roughly 50% of the pretrypsinization levels of internalization were observed, and α-iduronidase binding sites in α-iduronidase-deficient fibroblasts were estimated to be 14,000/cell. Their studies suggested that iduronidase receptors were also replaced or reused approximately every 5 min during enzyme internalization at 37°C.

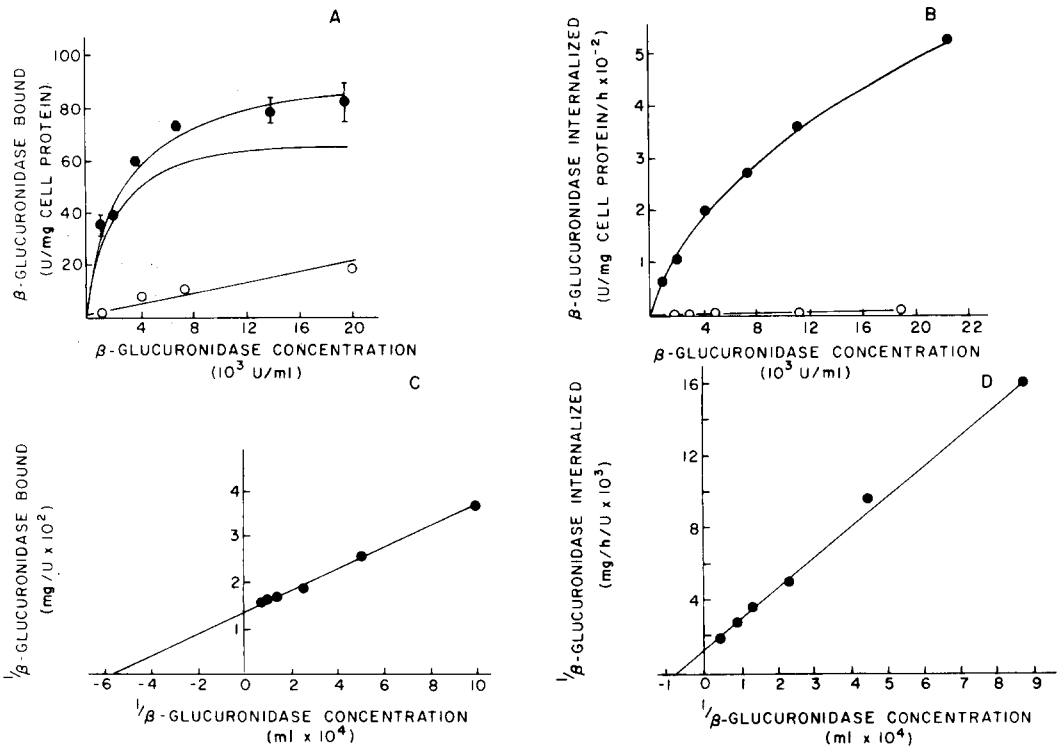

FIGURE 5 Saturation kinetics of binding and internalization of β-glucuronidase at 37°C in I-cell fibroblasts. β-Glucuronidase binding (*A*) at 37°C was carried out in I-cell fibroblasts treated with inhibitors of energy metabolism as described in Materials and Methods. Cultures were incubated for 2 h at 37°C with the indicated concentrations of β-glucuronidase in PBS-HSA containing the inhibitors of energy metabolism with (○) or without (●) 10 mM Man-6-P. Specific binding was calculated by subtracting the nonspecific binding (○) from the total binding (●). For β-glucuronidase uptake studies at 37°C (*B*), cultures were incubated for 1 h with the indicated concentrations of β-glucuronidase in the presence (○) or absence (●) of 10 mM Man-6-P in PBS-HSA. *C* and *D* depict reciprocal plots of specific binding or internalization, respectively.

Effects of Chloroquine on Cell Surface Binding of Enzyme by β-Glucuronidase-deficient Fibroblasts

The inhibition of pinocytosis of exogenous enzyme by chloroquine in I-cell fibroblasts was attributed to impairment of receptor reuse. It is possible to explain the even greater inhibition of enzyme pinocytosis in β-glucuronidase-deficient fibroblasts by a similar mechanism resulting partly from the other effect of chloroquine in these cells of stimulating secretion of high-uptake enzyme forms. If these high-uptake enzyme forms bound to cell surface receptors that were internalized, and if chloroquine blocked reuse of these receptors, a depletion of cell surface receptors could explain the time-dependent inhibition of enzyme pinocytosis by chloroquine in β-glucuronidase-deficient fibroblasts.

Fig. 6 presents an experiment in which enzyme binding at 4°C was measured in β-glucuronidase-deficient cells, some of which had been pretreated with chloroquine. Specific binding was calculated by subtracting the nonspecific binding from the total binding as it was in Fig. 5. From the data in the double reciprocal binding curves (Fig. 6, *inset*) we estimated 38,600 specific binding sites per cell for β-glucuronidase on the control cells and 23,500 sites per cell on the chloroquine-treated cells. The extent of the decrease in binding varied somewhat in different experiments with the enzyme. The decrease in binding sites in Fig. 6 was only 40%,

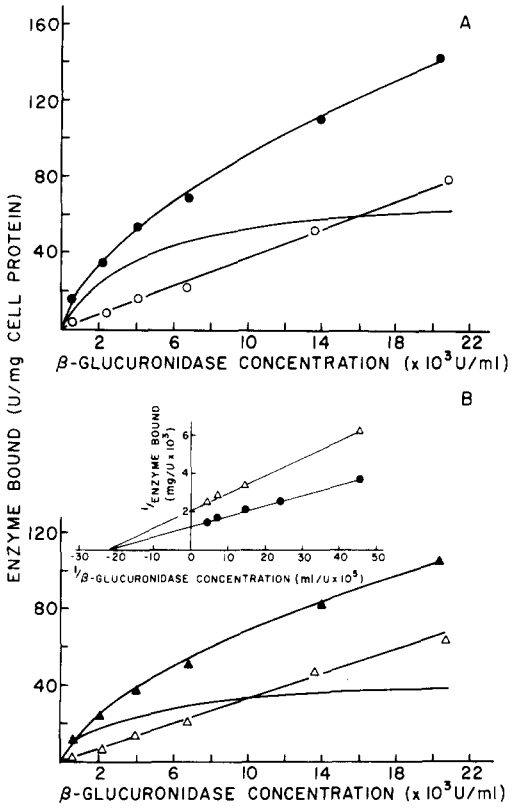

FIGURE 6 Effect of chloroquine on the number of β-glucuronidase binding sites in β-glucuronidase-deficient cells. Cultures were preincubated in the absence (*A*) or presence (*B*) of 25 μM chloroquine in Waymouth medium. After a 3-h preincubation at 37°C, cells were chilled and β-glucuronidase binding was measured by incubating dishes for 2 h at 4°C with increasing concentrations of the enzyme in the presence (○, △) or absence (●, ▲) of 10 mM Man-6-P in PBS-HSA. Cell-associated enzyme was measured after extensive rinsing with cold PBS. Specific binding was calculated by subtracting the nonspecific binding (○, △) from the total binding (●, ▲), respectively. The *inset* depicts reciprocal plots of the specific binding in control (●) and chloroquine-pretreated (△) cells.

but in six other experiments prior exposure to chloroquine for 3–4 h led to a 60–80% decrease in enzyme binding by β-glucuronidase on deficient fibroblasts. The $K_{binding}$ calculated from the data in Fig. 6 is about 3.4×10^{-9} M for both control and chloroquine-treated cells, indicating that the chloroquine treatment of non-I-cell fibroblasts led to a reduction in the number of binding sites, as predicted, without altering the apparent affinity of the binding sites.

Table IV shows additional experiments on the effects of preincubation with amines on subsequently measured cell surface binding of β-glucuronidase at 4°C. The upper portion of Table IV shows the effects of several agents on binding in cells preincubated in the absence of these agents. Note that neither chloroquine nor NH_4Cl interfered with 4°C enzyme binding in cells preincubated without these agents. By contrast, Man-6-P, a known competitive inhibitor of enzyme binding (18) and uptake (10, 19, 27) effectively blocked binding. In addition, preincubation in the presence of Man-6-P and removal of the Man-6-P before binding consistently led to enhancement of cell surface binding, possibly by displacing endogenously produced lysosomal enzyme bound to the cell surface (31, 32). Preincubation at 37°C in the presence of chloroquine (25 μM) or NH_4Cl (10 mM) led to depletion of enzyme binding activity. When Man-6-P was added during the preincubation with chloroquine and ammonium chloride, it completely (chloroquine) or partially (NH_4Cl) prevented the depletion of cell surface binding activity produced by these amines. These observations suggested that the reduction of enzyme binding

TABLE IV

Effect of Preincubation with Amines on β-Glucuronidase Binding at 4°C in a Second Incubation

Conditions			
Preincubation (3 h at 37°C)	Additions to 2nd incubation with enzyme (4,000 U/ml) (2 h at 4°C)	Enzyme bound	
		U/mg	*% of Control*
Medium	None	22.9	—
Medium	+ Man-6-P	4.4	19.2
Medium	+ Chloroquine	22.4	91.8
Medium	+ NH_4Cl	22.0	96.1
+ Man-6-P	None	32.9	143.7
+ Chloroquine	None	13.8	60.3
+ Man-6-P + Chloroquine	None	33.8	147.6
+ NH_4Cl	None	8.3	36.2
+ Man-6-P + NH_4Cl	None	17.2	75.1

Cultures of β-glucuronidase-deficient fibroblasts were preincubated in the presence or absence of 25 μM chloroquine, 10 mM NH_4Cl, and/or 10 mM Man-6-P in Waymouth medium. After a 3-h preincubation at 37°C, cells were chilled, exposed to 4,000 U/ml of β-glucuronidase in phosphate-buffered saline for 2 h at 4°C, washed, and measured for cell-associated enzyme.

sites in non-I-cell fibroblasts was secondary to the chloroquine-induced secretion of high-uptake enzymes that bound to cell-surface receptors and were internalized, and, as suggested above, subsequently measured enzyme pinocytosis was decreased because of impaired reuse of these internalized receptors that were not available for continued enzyme uptake. Presence of Man-6-P in the medium of chloroquine or NH_4Cl-treated cells had little effect on the large stimulation of enzyme secretion produced by the amines (Table II) but inhibited the depletion of enzyme binding activity from the cell surface (Table IV).

Effect of pH on Dissociation of Receptor-bound Enzyme

The lysosome is the destination of many products internalized by adsorptive endocytosis. Chloroquine is a lysosomotropic agent that has been shown to increase the intralysosomal pH (22). We reasoned that the pH elevation caused by lysosomotropic amines might impair enzyme-receptor dissociation in lysosomes, which might, in turn, impair receptor reuse (recycling). Fig. 7 shows the effects of pH on the dissociation of prebound enzyme in the absence of added Man-6-P. Cell-bound enzyme showed minimal dissociation at 37°C between pH 6.5 and 7.5. However, enzyme release was dramatically increased below pH 6.0. Thus, high-uptake enzymes bound cell-surface receptors with little reversibility at neutral pH, but readily dissociated at the pH expected for lysosomes. These observations were consistent with the hypothesis that the normally low intralysosomal pH is important for receptor-bound enzyme to be released in lysosomes and for receptors to be reused. Chloroquine and NH_4Cl have been reported to produce precisely the pH changes in lysosomes (22) that might impair receptor-enzyme dissociation.

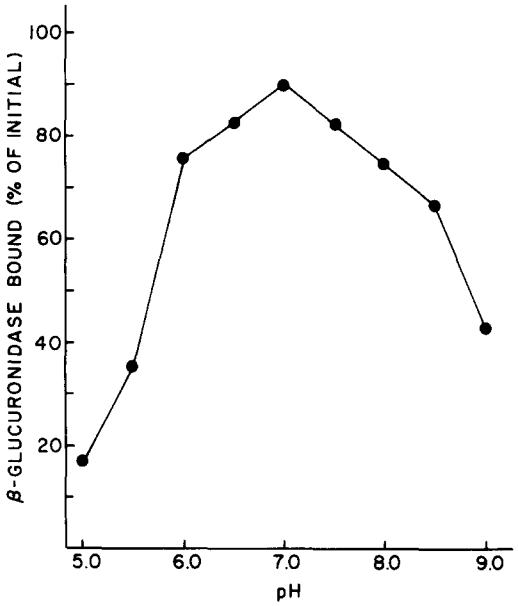

FIGURE 7 Effect of pH on the dissociation of cell surface bound β-glucuronidase. Binding was carried out in the presence of inhibitors of energy metabolism in β-glucuronidase-deficient fibroblasts, as in Fig. 1. After cells were incubated for 1 h at 37°C in the presence of 4,000 U/ml of β-glucuronidase, they were rinsed with PBS-HSA; some were assayed for specifically bound β-glucuronidase, and others were incubated further with 0.015 M maleate-phosphate buffer at differing pH in the presence of 1 mg/ml HSA and the inhibitors of energy metabolism. After incubation for 30 min at 37°C, cells were rinsed with cold PBS and assayed for remaining cell-associated β-glucuronidase.

DISCUSSION

The results presented here allow one to explain the previously reported inhibition of enzyme pinocytosis by chloroquine (20, 33) and other amines (20), and also suggest an attractive model for the normal transport of acid hydrolases. We infer from these results that there are two pathways for enzyme transport that depend on the phosphomannosyl recognition marker on acid hydrolases and its receptor. One mediates transport from the cell surface to lysosomes (via adsorptive endocytosis) and another, from the endoplasmic reticulum to lysosomes. The latter is responsible for delivery of most of the acid hydrolases that reach lysosomes. Amines impair traffic through both pathways. Evidence was presented that amines inhibit adsorptive pinocytosis by impairing receptor reuse. A simple mechanism was suggested to explain the effects of amines on both pathways by the well-known effects of amines on intralysosomal pH (22) and by the observed pH dependence of enzyme dissociation from pinocytosis receptors (Fig. 7).

I-cell fibroblasts provided an opportunity to study the isolated effect of chloroquine on enzyme pinocytosis. These cells produce no endogenous ligands for the pinocytosis receptors, but they make normal receptors and internalize non-I-cell lysosomal enzymes normally (8). Amines had no

significant effect on I-cell enzyme secretion or on I-cell pinocytosis receptors until exogenous ligand was added. When ligand (enzyme) was present, however, chloroquine led to a time-dependent and enzyme-concentration-dependent decay in the capacity for enzyme pinocytosis. We interpret this inhibition to result not from impaired internalization of enzyme-receptor complexes, but from a subsequent impairment of receptor reuse.

The progressive loss of the capacity for enzyme pinocytosis by β-glucuronidase-deficient fibroblasts was associated with a depletion of enzyme binding activity from the cell surface. However, depletion of cell surface binding activity in β-glucuronidase-deficient fibroblasts was blocked by the addition of Man-6-P to the medium. This result is attributed to the ability of Man-6-P to prevent the secreted high-uptake enzymes from binding to cell surface receptors and from being internalized in ligand-receptor complexes. However, experiments not presented here showed that Man-6-P treatment of β-glucuronidase-deficient fibroblasts at either 37° or 4°C after depletion of the cell surface enzyme-binding activity by chloroquine treatment, did not restore the "lost receptors." These observations suggested that the reduction in cell surface binding activity reflects a reduction in the number of receptors on the cell surface, and not an apparent reduction resulting from failure to internalize occupied receptors. Impaired receptor reuse could result in several ways from the amine-induced elevation in intralysosomal pH. The pH change might impair some vesicle-vesicle fusion process, such as pinosome-lysosome fusion, which delivers receptor-bound enzyme to lysosomes. The pH change might also act beyond this step by preventing return of receptors to the cell surface. The observation that elevated pH impairs enzyme-receptor dissociation (Fig. 7) suggested a mechanism whereby pinocytosis receptors may be "trapped" by ligands after pinocytosis. Receptors that fail to release their enzyme in lysosomes might be unable to recycle to the cell surface.

The evidence suggests also that there is an intracellular pathway for transport of acid hydrolases that is also disrupted by amines. The observations that growth of fibroblasts in the presence of Man-6-P at concentrations inhibiting enzyme uptake (*a*) failed to depress intracellular enzyme levels (7, 25, 28, 32) and (*b*) had only marginal effects on extracellular enzyme levels (7, 25, 28, 32, and Table II) suggested that most of the lysosomal enzymes reach lysosomes without being secreted into the extracellular medium. Thus, they must reach lysosomes through an intracellular pathway. In addition, the experiments presented here showed that most newly synthesized enzymes were diverted from this pathway to the extracellular medium by lysosomotropic amines, which had very little effect on lysosomal enzymes that had already reached secondary lysosomes. Finally, the newly synthesized enzyme diverted to the extracellular medium by amines was greatly enriched in high-uptake enzyme forms. We interpret these observations to mean that the major pathway for delivery of lysosomal enzymes from their site of synthesis in the endoplasmic reticulum to their destination in secondary lysosomes is an intracellular pathway, and that this pathway depends upon the phosphomannosyl recognition marker on the enzymes and upon enzyme receptors on intracellular membranes.

The explanation suggested for the effect of amines on enzyme secretion in non-I-cell fibroblasts implies that amines affect both the intracellular pathway and the pinocytic pathway by a similar mechanism. Amines disrupt both pathways by depleting the free receptors involved in enzyme transport and produce the equivalent of a receptor-negative phenotype in non-I-cell fibroblasts. When the free intracellular receptors have been depleted, newly synthesized enzymes remain unbound in the intracisternal space and are secreted, not because they lack the phosphomannosyl recognition marker, as is the case in I-cell disease (7), but because enzymes with normal recognition markers fail to find free receptors.

These observations on the effects of amines on I-cell and non-I-cell fibroblasts led us to suggest a model for the normal transport of enzymes to lysosomes that has the following features: (*a*) Most newly synthesized acid hydrolases are not secreted and recaptured, but normally reach lysosomes without ever leaving the cell. This explains why inclusion of Man-6-P in the growth medium had little effect on the distribution of enzyme between the cells and the medium (7, 25, 28, 32). (*b*) Delivery of enzymes to lysosomes depends on intracellular receptors to which bind newly synthesized enzymes bearing the phosphomannosyl recognition marker. (*c*) Receptor-bound hydrolases collect into specialized vesicles that bud off the endoplasmic reticulum or Golgi complex and deliver the hydrolases to lysosomes. These specialized vesicles may be thought of as primary

lysosomes. (*d*) Continued function of this intracellular pathway depends on the dissociation of enzymes from receptors in lysosomes and on recycling of free receptors to the endoplasmic reticulum or Golgi complex. Amines at least partly disrupt this transport process because they raise the intralysosomal pH (22) above the pH that favors dissociation of enzyme from receptors. The cisternal membranes become depleted of free receptors and subsequently synthesized acid hydrolases have no free receptors to bind. Consequently, acid hydrolases fail to be segregated from products of the endoplasmic reticulum that are destined for export, and pass through the Golgi apparatus and are secreted.

The model proposed for lysosomal enzyme transport differs significantly from previous models (15, 28, 29, 32) in that it proposes that intracellular enzyme receptors mediate transport of newly synthesized acid hydrolases to lysosomes and that the phosphomannosyl recognition marker acts mainly as an intracellular traffic signal to segregate acid hydrolases from secretory products. The extracellular enzyme in this model is not an obligatory intermediate in transport to lysosomes, as in the secretion-recapture model (15), but is the consequence of failure of normal intracellular segregation. Von Figura and Weber (32) demonstrated immunologically several lysosomal enzymes on the cell surface and proposed that most enzymes are first delivered to the plasma membrane tightly bound to receptors, and then internalized without ever leaving the receptors. In this model, segregation of enzymes from secretory products takes place at the plasma membrane. This hypothesis is consistent with the experimental data presented here, if one further postulates that the plasma membrane intermediate is either too tightly bound, or too transient, to be displaced by Man-6-P in the growth medium.

Novikoff (17) has argued that lysosomes arise from a smooth-membrane tubular network that he named GERL, which is closely associated with the endoplasmic reticulum and the concave (trans) face of the Golgi apparatus. Friend and Farquhar (3) and Novikoff (17) identified coated vesicles that stained for acid phosphatase and that appeared to arise from some component of the Golgi complex or from GERL. These were proposed as transport vesicles for acid hydrolases (primary lysosomes). If that interpretation is correct, these would be the structures that we suggest are segregating receptor-bound enzymes for delivery to lysosomes and are also active in the intracellular pathway that amines disrupt by impairing receptor reuse.

The phosphomannosyl "traffic signal" is not related to the signal peptide on secretory proteins. Whether acid hydrolases contain a signal peptide that is important for their secretion into the cisternal space has not been shown. Presumably they contain such a sequence. What has been found recently is that many acid hydrolases are synthesized as precursors that have molecular weights much higher than the average molecular weights of these hydrolases isolated from lysosomes (7, 23). To date, there is no evidence to suggest that the excess polypeptide sequence, although large, has any transport function. There is, however, considerable evidence to implicate the phosphomannosyl recognition marker on acid hydrolases in enzyme transport. We do not suggest that the recognition marker is an alternative to the signal sequence for entry into the cisternal space, but rather that it is a second signal that allows the cells to sort out this class of proteins for delivery to lysosomes from other proteins in the cisternal space having other intracellular or extracellular destinations.

The authors wish to thank Marvin R. Natowicz and Drs. I. Boime and A. W. Strauss for their helpful comments in the preparation of this manuscript and Mrs. Sabra Lovejoy for her assistance in typing the manuscript.

This work was supported by U. S. Public Health Service grant GM 20196 and the Ranken Jordan Trust for the Crippling Diseases of Children. Dr. González-Noriega is the recipient of scholarship grants from Programa de Superación del Personal Académico, Universidad Nacional Autónoma, México D. F., México.

Received for publication 21 December 1979, and in revised form 20 February 1980.

Note Added in Proof: Since submission of this paper, Tietze, Schlesinger, and Stahl (1980. *Biochem. Biophys. Res. Commun.* **93**:1–8) reported that chloroquine inhibited pinocytosis of mannose-terminal glycoconjugates by rat alveolar macrophages. The mannose-glycoprotein receptor on macrophages is a completely different receptor from the phosphomannosyl-enzyme receptor, which is the subject of the studies reported here. In contrast to our results, Tietze et al. found that chloroquine (in larger doses than used here) reduced the number of mannose-glycoprotein receptors on the cell surface of macrophages, whether or not ligand was present. The authors concluded that this receptor was internalized and recycled in the absence of ligand, and that chloroquine impaired the return of this receptor to the cell surface after its internalization.

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