The effect of chloroquine on the distribution of newly synthesized and old β -hexosaminidase in fibroblasts

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Most of the newly synthesized β -N-acetyl-D-glucosaminidase (EC 3.2.1.30; β -hexosaminidase) in normal fibroblast cultures is excreted during 24 h incubation with serum-free medium. In this study, this new enzyme only comprises about one-half of the excreted pool as determined by a near total inhibition of [14 C]leucine incorporation into the excreted enzyme in the presence of cycloheximide, with only a 46% reduction in enzyme activity. These data indicate that nearly equal fractions of new and old enzyme are normally excreted by fibroblasts. Incubation of normal fibroblast cultures with chloroquine (25 μ M) for 24 h doubled the amount of extracellular β -hexosaminidase activity from 15% to 37% of total culture activity while reducing the incorporation of [14 C]leucine into intra- and extracellular enzyme by 66 and 29% of control, respectively. Therefore, it appears that chloroquine inhibited enzyme synthesis while enhancing the excretion of old as well as newly synthesized enzyme. Chloroquine and cycloheximide together reduced the [14 C]leucine incorporation into intracellular enzyme by more than either agent alone, indicating a combined effect on enzyme synthesis and/or degradation. β -Hexosaminidase-deficient fibroblasts that contained endocytosed enzyme spontaneously excreted 10% of their enzyme during 24 h incubation with serum-free medium and 18% in the presence of mannose 6-phosphate (2 mM). These results indicated that about one-half of the excreted enzyme still possessed its phosphomannosyl recognition residues and actually re-entered the cells. Chloroquine stimulated the excretion of an additional 15% of the total endocytosed enzyme at 48 and 72 h after endocytosis. These data suggest that new, old and endocytosed β -hexosaminidase are all excreted by fibroblasts, that this excretion is enhanced by chloroquine, and that a fraction of the excreted enzyme retains its phosphomannosylated residues needed for re-uptake and transport inside the cells.

Human fibroblasts endocytose exogenous lysosomal hydrolases through a receptor on their plasma membrane which specifically recognizes phosphomannosyl (mannose 6-phosphate) residues on the enzymes (Kaplan *et al.*, 1977; Sando & Neufeld, 1977; Ullrich *et al.*, 1978). The significance of this cell surface system in fibroblasts is still unknown and may be minimal considering that only 12–15% of the total cell-associated β -hexosaminidase regularly utilizes this external route (von Figura & Weber, 1978; Vladutiu & Rattazzi, 1979). A potentially more important role for the phosphomannosyl recognition system may be in the intracellular transport of hydrolases from their site of synthesis in the endoplasmic reticulum to the lysosomes (Gonzalez-Noriega *et al.*, 1980).

A hypothesis proposed by Sly & Stahl (1978)

suggests that newly synthesized hydrolases segregate into secretory and non-secretory products, depending on whether they possess terminal mannose 6-phosphate residues. Those molecules with the marker are bound to receptors inside either common transport vesicles that carry secretory and non-secretory products or in specialized vesicles. In either case, if vesicles fuse with the plasma membrane, only the unbound enzyme is released. In the autosomal recessive disorder known as I-cell disease, in which almost all of the newly synthesized acid hydrolases are constantly excreted (Wiesmann *et al.*, 1971), the phosphomannosyl marker is missing from the hydrolases (Bach *et al.*, 1979; Hasilik & Neufeld, 1980b) and the enzymes are rapidly excreted.

In subcellular fractionation studies, Fischer *et al.*

(1980) found more than 90% of the mannose 6-phosphate-specific binding of β -hexosaminidase B in the Golgi-endoplasmic reticulum-lysosomal (GERL) system of rat liver cells. These data were interpreted to support the hypothesis that the receptors for mannose 6-phosphate play a role in the intracellular transport of newly synthesized hydrolases.

Several studies have used amines to characterize the transport of endogenous and exogenous enzymes in fibroblasts (Wiesmann *et al.*, 1975; Sando *et al.*, 1979; Willcox & Rattray, 1979; Gonzalez-Noriega *et al.*, 1980). One of these groups suggested that only newly-synthesized hydrolases utilize the internal mannose 6-phosphate receptor system during transport, since chloroquine enhances the excretion of newly synthesized hydrolases bearing phosphomannosyl recognition markers. It was further postulated that, by the time hydrolases reach the lysosomes, mannose 6-phosphate is no longer necessary for retention by cells and is removed (Gonzalez-Noriega *et al.*, 1980). Earlier studies also suggested that, although exogenous hydrolases need the recognition marker for specific uptake by cells, the marker is removed after internalization (Glaser *et al.*, 1975). Most studies have given little attention to the possible effect of chloroquine on the excretion of pre-existing enzyme or the lysosomal targeting of endocytosed enzyme.

The purpose of the present study was: (1) to examine in more detail the excretion of newly synthesized and old, presumably lysosomal, β -hexosaminidase in the presence or absence of chloroquine and the protein synthesis inhibitor cycloheximide, in order to determine whether amines selectively enhance the excretion of only newly-synthesized enzyme bearing mannose 6-phosphate; (2) to determine whether internalized enzyme continues to utilize the mannose 6-phosphate recognition system; and (3) to assess whether endocytosed enzyme is susceptible to chloroquine-induced excretion. This study should contribute to understanding the involvement of the mannose 6-phosphate recognition system in different phases of lysosomal enzyme transport and to understanding the effect of chloroquine in stimulating the excretion of lysosomal enzymes by fibroblasts.

Materials and methods

Chemicals

[14 C]Leucine was obtained from New England Nuclear, Cambridge, MA, U.S.A. Chloroquine, cycloheximide and mannose 6-phosphate were obtained from Sigma.

Cell culture

Cultures of normal skin fibroblasts were obtained

from the Genetic Mutant Cell Repository, Camden, NJ, U.S.A. (GM-969) and from our own stock (line 389). Cultures of β -hexosaminidase-deficient fibroblasts from patients with Sandhoff disease were obtained from Dr. U. Wiesmann, University of Berne, Switzerland (line 605) and from our own stock (line 421). Cells were cultured in 25 cm² plastic flasks and 35 mm Petri dishes (Corning Glass Co., Corning, NY, U.S.A.) with Ham's F-10 medium + 15% (v/v) foetal calf serum + kanamycin (20 μ g/ml; Grand Island Biologicals Inc., Grand Island, NY, U.S.A.). Cells used for the preparation of excreted β -hexosaminidase were cultured in 670 cm² glass roller bottles (Bellco Glass Inc., Vineland, NJ, U.S.A.). Methods for the preparation of cell extracts and collection of fibroblast secretions have been described (Vladutiu & Rattazzi, 1975). Cell wash procedures were carried out in Hanks balanced salt solution (Grand Island Biologicals). Cultured cells used in [14 C]leucine incorporation studies were harvested with 0.25% trypsin + leucine (1 mg/ml) then washed and resuspended in Hanks solution containing leucine. The suspensions were frozen and thawed five times and centrifuged at 40 000g for 10 min. Culture fluids were precipitated with (NH₄)₂SO₄ and dialysed against 1000 vol. of 10 mM-phosphate/0.9% NaCl (pH 6.8) for 3 h at 4°C.

Preparation of high-uptake β -hexosaminidase

A preparation enriched with high-uptake enzyme was obtained from roller bottle cultures of normal fibroblasts incubated for 24 h at 37°C with 50 ml each of serum-free medium containing chloroquine (25 μ M) and mannose 6-phosphate (2 mM). The culture fluids were collected, concentrated to 2–5 ml by ultrafiltration and dialysed for 24 h at 4°C against two 500 ml changes of serum-free medium.

Enzyme assays and protein determination

β -Hexosaminidase activity was measured by using the fluorogenic substrate 4-methylumbelliferyl-*N*-acetylglucosaminide (Pierce Chemical Co., Rockford, IL, U.S.A.). One enzyme unit was defined as the activity that liberated 1 nmol of 4-methylumbelliferone/h at 37°C. Lactate dehydrogenase activity in cell culture fluids was determined by the method of Wroblewski & LaDue (1955) and used to assess cytotoxicity under a given set of experimental conditions. The amount of extracellular lactate dehydrogenase never exceeded 0.02% of the total enzyme in any experiment presented in this study. Protein concentrations in the cell extracts were determined by the method of Bradford (1976).

Incubation of cultures with chloroquine and cycloheximide

All cell cultures for individual experiments were maintained in 35 mm Petri dishes and pre-incubated

with serum-free medium for 24 h at 37°C before any combination of mannose 6-phosphate, chloroquine and cycloheximide was added. The highest concentration of the protein synthesis inhibitor cycloheximide (4 μ g/ml) was used which was not cytotoxic while maximally inhibiting the synthesis of β -hexosaminidase by cultured fibroblasts (Vladutiu & Rattazzi, 1981). The 'old' enzyme in this study, therefore, is defined as that enzyme which remains in cells and culture fluids after 24 h incubation with cycloheximide. The extent of protein synthesis was determined by [14 C]leucine incorporation into β -hexosaminidase. 'New' enzyme is defined as the activity remaining in cells and culture fluids after the subtraction of the old enzyme activity. These designations do not differentiate between enzyme that may be in intermediate vesicles en route to lysosomes, and old enzyme, already packaged in lysosomes.

Preparation of antisera

High-titre rabbit antiserum was prepared against purified human placental β -hexosaminidase B according to previously described methodology (Therrien *et al.*, 1971). The placental enzyme used for immunization was purified essentially by the method of Geiger & Arnon (1978) and was a gift from Drs. Alan Appel and Mario Rattazzi (Children's Hospital of Buffalo, NY, U.S.A.). The antiserum was absorbed with insolubilized normal human serum and whole β -hexosaminidase-deficient Sandhoff disease fibroblasts using previously reported methodology (Vladutiu & Rose, 1973). The antiserum was tested for monospecificity by double diffusion in 1% agarose against the placental β -hexosaminidase used for immunization. β -Hexosaminidase activity was visualized on the immunodiffusion plate with the fluorogenic substrate. A counter-stain for protein with Coomassie Blue B indicated a single immune precipitation arc superimposed on the arc with enzyme activity, indicating monospecificity of the antiserum for β -hexosaminidase (Fig. 1).

Immune precipitation of [14 C]leucine-labelled β -hexosaminidase

The β -hexosaminidase in cell and culture fluid extracts from [14 C]leucine incorporation studies was immune precipitated with rabbit anti-(β -hexosaminidase) exactly as described by Hasilik & Neufeld (1980a,b). Soluble immune complexes were further precipitated by incubation with goat anti-(rabbit immunoglobulin G) serum for 1 h at 4°C. Immune precipitates were washed in 0.05 M-Tris/HCl containing 1.2 M-KCl and 1.2% (v/v) Triton X-100, pH 7.5, resuspended in 10 ml of scintillation fluid (Aquasol, New England Nuclear) and counted in a Searle Mark III beta counter.

Fig. 1. Double immunodiffusion of human placental β -hexosaminidase B (1) versus rabbit anti-(human β -hexosaminidase B) serum (2, 3)

(a) β -Hexosaminidase activity was visualized with fluorogenic substrate; (b) counterstained for protein with Coomassie Blue B. A single immune precipitation arc superimposed on the arc with enzyme activity, indicating monospecificity of the antiserum for β -hexosaminidase.

Results and discussion

Time-dependence of chloroquine-induced release of endogenous enzyme

Table 1 shows the time-dependent effect of chloroquine (25 μ M) on β -hexosaminidase excretion by normal fibroblasts. A net increase of 16% in the excretion of total cell-associated enzyme compared with untreated controls was seen only after 24 h exposure to the drug. Longer exposures or concentrations higher than 25 μ M led to cytotoxicity as indicated by finding >0.02% of total lactate dehydrogenase activity in the culture fluid.

Effect of chloroquine and cycloheximide on β -hexosaminidase distribution

It is known that chloroquine prevents re-assimilation of excreted hydrolases (Gonzalez-Noriega *et al.*, 1980). Therefore, mannose 6-phosphate was added

Table 1. *Chloroquine-induced release of β -hexosaminidase from normal fibroblasts*

Cultures of normal fibroblasts (GM-969; approx. 50 μ g of cell protein/dish) were pre-incubated with serum-free Ham's F-10 medium for 24 h at 37°C. Culture fluids were then exchanged for fresh medium with or without 25 μ M-chloroquine. Cells and culture fluids from triplicate cultures were then harvested at the designated time intervals and assayed for β -hexosaminidase and lactate dehydrogenase activities. Values in parentheses indicate % of total enzyme activity. Lactate dehydrogenase activity was absent from the culture fluids.

Time (h)	β -Hexosaminidase distribution					
	No additions			Chloroquine (25 μ M)		
	Units/cell extract	Units/culture fluid	Total units	Units/cell extract	Units/culture fluid	Total units
2	970 $\pm$ 4.9	7.8 $\pm$ 0.8 (0.79)	977 $\pm$ 4.3	804 $\pm$ 71.0	11.4 $\pm$ 1.4 (1.4)	815.6 $\pm$ 72
4	916 $\pm$ 39.0	14.3 $\pm$ 5.0 (1.5)	930 $\pm$ 35.4	1040 $\pm$ 117.6	32.3 $\pm$ 1.1 (3.0)	1072 $\pm$ 117
6	1001 $\pm$ 54.0	17.2 $\pm$ 1.1 (1.7)	1019 $\pm$ 54.6	691 $\pm$ 151	41.3 $\pm$ 6.6 (5.6)	732 $\pm$ 157
8	1009 $\pm$ 29.0	18.2 $\pm$ 2.4 (1.8)	1027 $\pm$ 27.9	784 $\pm$ 43	53.0 $\pm$ 4.1 (6.3)	836.6 $\pm$ 46.9
24	1012 $\pm$ 120.0	52.4 $\pm$ 4.8 (4.9)	1064.7 $\pm$ 123.7	727 $\pm$ 22	194 $\pm$ 18.5 (21)	920 $\pm$ 40

to a set of control cultures during the 24 h incubation described in Table 2 to prevent re-uptake of spontaneously excreted enzyme, thus providing a more valid comparison between spontaneous and chloroquine-induced excretion.

During a 24 h incubation, cycloheximide reduced the total amount of extracellular β -hexosaminidase activity by 46% and had a minimal effect on intracellular enzyme activity, reducing the total culture activity by 14% (Table 2, C and D). Chloroquine doubled the amount of enzyme activity in the culture fluid and reduced the intracellular activity by 35% with an overall 12% reduction in the total culture activity (Table 2, C and E). Together, chloroquine and cycloheximide reduced the intra- and extracellular activity by 44 and 23%, respectively, while reducing the total culture activity by 60%. The total intracellular loss cannot be explained by an enhanced excretion, suggesting that an enhanced degradation or inactivation (Willcox & Rattray, 1979) of intracellular enzyme had occurred. Nevertheless, an enhanced excretion of old enzyme (Table 2, F) did occur compared with excretion in the presence of mannose 6-phosphate and cycloheximide alone (Table 2, D). These data indicate that chloroquine with cycloheximide either enhanced the degradation or inactivated the overall activity. The addition of mannose 6-phosphate to cycloheximide-treated cultures (Table 2, D) had a negligible effect on the intra- and extracellular distribution of enzyme compared with cultures treated with cycloheximide alone (Table 2, B).

Effect of chloroquine and cycloheximide on [14 C]-leucine incorporation into β -hexosaminidase

Approx. 95% of the 14 C-labelled β -hexosaminidase in normal fibroblast cultures was excreted during 24 h incubation (Table 3, A). This represented about 44% of the total excreted enzyme activity since, in

the presence of cycloheximide, the cells still excreted 56% of the enzyme excreted by the control culture (Table 2, A and B). Therefore, it appeared that normally, the excreted enzyme pool consisted of nearly equal fractions of new and old enzyme.

Chloroquine reduced the incorporation of [14 C]-leucine into intra- and extracellular enzyme by 66 and 29%, respectively (Table 3, C), yet the excretion of enzyme quadrupled (Table 1), and the total enzyme activity was reduced by only 9%. Therefore, a large portion of the enzyme excreted in the presence of chloroquine was not newly synthesized. In combination, chloroquine and cycloheximide reduced the labelling of intracellular enzyme by 94%, which is a greater effect than found with either agent alone (Table 3). These results support the view that the combined substances had an additive effect in inhibiting enzyme synthesis.

Effect of chloroquine on endocytosed enzyme

Sandhoff fibroblasts (line 605), which endocytosed exogenous β -hexosaminidase during a 5 h exposure, excreted a net 6% of this enzyme in the presence of chloroquine during the first 24 h after uptake (Table 4). The total culture activity also was decreased by 21%, making it difficult to interpret the 24 h effect of chloroquine on enzyme distribution in the culture. However, when the excretion was induced with chloroquine at 48 and 72 h after uptake, the excretion due to chloroquine increased to 15 and 13%, respectively, while the spontaneous excretion constantly decreased by approx. 50% every 24 h. It thus appears that the intracellular transport system for endocytosed enzyme is susceptible to chloroquine-mediated release of hydrolases at least for 72 h post-uptake while the enzyme becomes less susceptible to spontaneous release.

Approx. 10% of the enzyme freshly endocytosed (5 h uptake) by Sandhoff cell line 421 was found in

Table 2. *Comparative release of endogenous enzyme from normal fibroblasts*

Cultures of normal fibroblasts (GM-969) were pre-incubated for 24 h under the conditions described in Table 1. Cultures were then incubated under conditions described. Cells and culture fluids from triplicate cultures were then harvested and assayed as described in Table 1. Values in parentheses indicate % of total enzyme activity. Similar results were obtained with cell line 389. Values in brackets indicate % of corresponding control in A or C.

Incubation conditions (24 h at 37°C)	Distribution of β -hexosaminidase		
	Units/cell extract	Units/culture fluid	Total units
A No additions	787.1 $\pm$ 63.3 (91)	77.3 $\pm$ 1.4 (9)	864.4 [100]
B Cycloheximide (4 μ g/ml)	734.0 $\pm$ 63.5 (94)	43.4 $\pm$ 5.9 (6)	777.4 [90]
C Mannose 6-phosphate (2 mM)	806.7 $\pm$ 42.3 (85)	144.1 $\pm$ 19.1 (15)	950.9 [100]
D Mannose 6-phosphate + cycloheximide	754.3 $\pm$ 29.9 (92)	62.7 $\pm$ 4.1 (8)	817.0 [86]
E Mannose 6-phosphate + chloroquine (25 μ M)	521.7 $\pm$ 14.2 (62)	311.8 $\pm$ 7.2 (37)	833.5 [88]
F Mannose 6-phosphate + chloroquine + cycloheximide	457.9 $\pm$ 33.8 (81)	110.3 $\pm$ 4.6 (19)	568.2 [40]

Table 3. *Incorporation of [14 C]leucine into β -hexosaminidase in the presence of cycloheximide and chloroquine*

Each flask (25 cm²) of normal fibroblasts (GM-969) was washed twice with 5 ml of Hanks solution, then incubated for 3 h at 37°C with 2.5 ml of serum-free Ham's F-10 medium containing no additions, chloroquine, or chloroquine + cycloheximide. Culture fluids were then replaced with fresh medium containing the same additions plus 1 μ Ci/flask of [14 C]leucine, then incubated as described. Cells and culture fluids were harvested as described in the Materials and methods section. β -Hexosaminidase was immune precipitated as described. The immune precipitates of control cell and media extracts contained 294 and 31 units of β -hexosaminidase, respectively.

Incubation conditions (24 h at 37°C)	Extracts		Media		Total culture	
	Counts/min	% of	Counts/min	% of	Counts/min	% of
		'No additions'		'No additions'		'No additions'
A No additions	59.4	100.0	1120.7	100.0	1180.1	100.0
B Cycloheximide (4 μ g/ml)	9.7	16.3	68.2	6.0	77.9	6.6
C Chloroquine (25 μ M)	20.2	34.0	792.2	70.7	812.4	68.8
D Chloroquine + cycloheximide	3.6	6.0	107.7	9.6	111.3	9.4

Table 4. *Chloroquine-induced release of internalized β -hexosaminidase from Sandhoff fibroblasts*

Cultures of β -hexosaminidase-deficient Sandhoff fibroblasts (line 605) were pre-incubated as described in Table 1, then incubated for 5 h at 37°C with 450 units/dish of high-uptake β -hexosaminidase. Cultures were washed in Hanks solution + 2 mM-mannose 6-phosphate four times, then incubated under the conditions described. The 24 h incubations with chloroquine were initiated at three time intervals: (1) immediately after the 5 h uptake (0 time), (2) 24 h after uptake, and (3) 48 h after uptake. Cultures were then harvested after 24 h incubation with chloroquine and cell extracts and culture fluids were assayed for β -hexosaminidase and lactate dehydrogenase activities. Values in parentheses indicate % of total enzyme activity. The β -hexosaminidase assimilated during the 5 h incubation ranged from 10.1 to 13.6 units/cell extract.

Time of initiation of
chloroquine treatment
following 5 h uptake

(h)	Incubation conditions (24 h at 37°C)	Units/cell extract	Units/culture fluid	Total units
0	No additions	13.1 $\pm$ 0.6	1.4 $\pm$ 0.2 (9.9)	14.5
	Chloroquine (25 μ M)	9.6 $\pm$ 0.4	1.8 $\pm$ 0.2 (15.8)	11.4
24	No additions	10.1 $\pm$ 0.5	0.7 $\pm$ 0.2 (6.1)	10.8
	Chloroquine (25 μ M)	8.0 $\pm$ 0.8	2.1 $\pm$ 0.3 (20.8)	10.1
48	No additions	9.7 $\pm$ 0.5	0.3 $\pm$ 0.04 (3.3)	10.0
	Chloroquine (25 μ M)	7.7 $\pm$ 0.3	1.5 $\pm$ 0.2 (16.3)	9.2

the culture fluid during a 24 h post-uptake incubation (Table 5, A). This amount nearly doubled in the presence of 2 mM-mannose 6-phosphate, indi-

cating that about one-half of the excreted enzyme was still in a high-uptake form and was capable of recycling through the mannose 6-phosphate recep-

Table 5. Release of internalized β -hexosaminidase in the presence or absence of mannose 6-phosphate

Duplicate cultures of β -hexosaminidase-deficient fibroblasts (line 421) were incubated for 5 h at 37°C with 450 units/dish of high-uptake β -hexosaminidase, washed and incubated with 2 mM-mannose 6-phosphate for 1 h. Cultures were then incubated under the conditions described. Cell extracts and culture fluids were assayed for β -hexosaminidase and lactate dehydrogenase activities. The initial mean enzyme uptake in 5 h was 8.5 units/cell extract. Values in parentheses indicate % of total enzyme activity.

Incubation conditions (24 h at 37°C)	Units/cell extract	Units/culture fluid	Total units
A No additions	6.56	0.73 (10)	7.29
B Mannose 6-phosphate (2 mM)	5.18	1.15 (18)	6.33
C Chloroquine (25 μ M)	0	3.03	3.03
D Mannose 6-phosphate + chloroquine	0	3.20	3.20

tor system. This enzyme was not surface-bound, since surface-bound enzyme was eluted with mannose 6-phosphate during the first 1 h following uptake. These data appear to be in disagreement with the findings of Glaser *et al.* (1975). However, these investigators studied the re-uptake of assimilated enzyme only at the end of the 24 h post-uptake period, whereas our experiments measured excretion and re-uptake during the entire 24 h period following uptake. By the end of 24 h, all of the endocytosed enzyme in Glaser's study may have been converted to a low-uptake form.

Following the addition of chloroquine, our cultures excreted more than twice the amount of enzyme found in cultures containing mannose 6-phosphate alone (Table 5, C and D). The presence of mannose 6-phosphate with chloroquine did not significantly enhance the accumulation of extracellular enzyme. However, only the 3–3.2 units found in the culture fluid of the chloroquine-treated Sandhoff cells could be accounted for in the cell cultures, although the mean uptake of β -hexosaminidase in 5 h was 8.5 units/cell extract (Table 5). Chloroquine may have temporarily inactivated the remaining intracellular enzyme (Willcox & Rattray, 1979) as it became concentrated in the lysosomes. No cytotoxicity was observed by the quantification of lactate dehydrogenase activity in the culture fluid.

The percentages of endogenous and endocytosed enzyme which were excreted from fibroblasts due to chloroquine treatment (13–22% of total) are relatively small, indicating that the majority of enzyme was not susceptible to chloroquine-mediated release. A fraction of the total enzyme in each case may have entered the same chloroquine-sensitive vesicle at some point during processing. In fact, there is evidence that endocytosed lysosomal enzymes in fibroblasts migrate to the same subcellular lysosomal location as do endogenous enzymes after 48 h incubation (Bach & Liebmman-Eisenberg, 1979). Furthermore, Tietze *et al.* (1981) have shown that two distinct compartments exist for receptor–ligand complexes in rat macrophage, which differ in their susceptibility to chloroquine.

Since primarily high-uptake enzyme was released in the presence of chloroquine, and chloroquine has been shown to inhibit the dissociation of receptor-bound enzyme (Gonzalez-Noriega *et al.*, 1980), the chloroquine-susceptible vesicles may contain a small enzyme pool which, while in equilibrium with its receptors, remains in the dissociated state as a result of one or more of chloroquine's many effects, e.g., on protein synthesis, electron transport, macromolecules and membrane binding, etc. (Andersson *et al.*, 1980). Dephosphorylation of receptor-dissociated enzyme may be prevented so that the enzyme retains its mannose 6-phosphate residues, yet is inhibited from reassociating with its receptor. Alternatively, the amine may facilitate the dissociation of the enzyme from its receptors (Wiesmann *et al.*, 1975).

The extent of chloroquine-induced excretion of endogenous β -hexosaminidase found in our study agrees with data presented by other investigators using various amines and enzymes (summarized in Table 6). The amount of exogenous β -hexosaminidase excreted in the presence of chloroquine in our study falls between the amounts of two different hydrolases excreted in similar studies (Sando *et al.*, 1979; Wiesmann *et al.*, 1975). Sando *et al.* (1979) may have found a greater release of enzyme if the incubation with amine had been longer. Gonzalez-Noriega *et al.* (1980) also only incubated their cultures with chloroquine for a short period after a 3 h enzyme uptake (Table 6). In later experiments, these investigators allowed a lengthy 24 h uptake, followed by a 24 h chase with medium alone before finally adding chloroquine for the induction of excretion. By this time, much of the endocytosed enzyme was probably no longer susceptible to chloroquine.

It may be postulated that in fibroblasts, the recognition and subsequent binding of hydrolases possessing mannose 6-phosphate residues occur in several lysosomal-type vesicles including vesicles carrying newly-synthesized enzyme (primary lysosomes?), those carrying old enzyme (intermediate vesicles or secondary lysosomes?), and endocytic

Table 6. *Effect of amines on excretion of endogenous and exogenous acid hydrolases from fibroblasts*

Experimental culture conditions	Amine	Hydrolase studied	Results of amine-induced enzyme excretion*	Conclusions
A Wiesmann <i>et al.</i> , 1975				
1 8 h with amine, then 24 h with fresh medium	Chloroquine (5 μ M)	β -Glucuronidase	350% of control release (End)	Amine may displace bound enzyme from lysosomal membrane or interfere with new enzyme placement into lysosomes
2 24 h enzyme uptake, then 13 h with amine	Chloroquine (5 μ M)	Arylsulphatase A	28% net release over control (Exo)	
B Sando <i>et al.</i> , 1979				
1 2.5 h enzyme uptake, then 2.5 h with amine	Chloroquine (25 μ M)	α -L-Iduronidase	12% net release over control (Exo)	Insignificant loss of endocytosed enzyme
C Willcox & Rattray, 1979				
1 24 h with amine	Chloroquine (50 μ M)	β -Hexosaminidase	300% of control release (End)	Amines stimulate excretion rather than inhibit re-uptake of enzyme
D Gonzalez-Noriega <i>et al.</i> , 1980				
1 3 h enzyme uptake, then 1 h with amine + mannose 6-phosphate	Chloroquine (25 μ M)	β -Glucuronidase	No enzyme release (Exo)	Delivery of new enzyme to lysosomes requires mannose 6-phosphate recognition system. Amines enhance secretion of new enzyme bearing mannose 6-phosphate and minimally affect enzymes already in lysosomes
2 24 h medium alone, then 24 h with amine + mannose 6-phosphate	Chloroquine (25 μ M)	β -Hexosaminidase B	425% of control release (End)	
3 24 h enzyme uptake, then 24 h with fresh medium, then 48 h with amine	NH ₄ Cl (10 mM)	β -Glucuronidase	3.7% net release (Exo)	
E Present paper				
1 24 h medium alone, 24 h with amine	Chloroquine (25 μ M)	β -Hexosaminidase	420% of control release (End)	Excreted enzyme pool contains equal fractions of new and old enzyme; chloroquine enhances excretion of both
2 24 h medium alone, 24 h with amine, synthesis inhibitor or both	Chloroquine (25 μ M); cycloheximide (4 μ g/ml)	β -Hexosaminidase	Equal fractions new + old excreted; amine doubled excretion of both (End)	
3 5 h enzyme uptake, 24 h with amine	Chloroquine (25 μ M)	β -Hexosaminidase	13–15% net release (Exo)	

* End, endogenous; Exo, exogenous.

vesicles carrying exogenously endocytosed enzyme. Alternatively, during transport, the enzyme from each category may pass through a common vesicular subgroup which is susceptible to chloroquine-mediated release. Since chloroquine enhances the excretion of only a fraction of the enzyme in each case, and the resulting enzyme is predominantly high-uptake, it may be that: (1) the small fraction of enzyme which is susceptible to chloroquine-mediated release is in equilibrium with its receptors, and chloroquine inhibits enzyme-receptor reassociation regardless of whether the enzyme is new, old or endocytosed; or (2) unbound enzyme (new, old or endocytosed) is released at the cell surface when the vesicles fuse with the plasma membrane, as originally proposed for new enzyme by Sly & Stahl (1978).

The hypothesis suggesting that mannose 6-phosphate residues are required for the migration and targeting of newly-synthesized hydrolases to lysosomes, and that mannose 6-phosphate-deficient hydrolases are excreted (Sly & Stahl, 1978; Gonzalez-Noriega *et al.*, 1980) is attractive. However, our data indicate that mannose 6-phosphate-mediated transport is not confined to newly synthesized enzyme. The inhibition of specific mannosylation and/or phosphorylation of hydrolases in normal fibroblasts leading to the hyperexcretion of newly synthesized hydrolases, i.e. an I-cell-disease phenocopy, would be a positive test of the Sly & Stahl hypothesis. A UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminylphosphotransferase, which mediates the transfer of phospho-*N*-acetylglucosamine to terminal mannosyl residues (Tabas & Kornfeld, 1980; Hasilik *et al.*, 1980), is deficient in I-cell disease fibroblast cultures (Reitman *et al.*, 1981). The deficiency of this enzyme appears to account for the hyperexcretion of newly-synthesized enzyme by I-cell fibroblasts.

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