Transport and processing of β -hexosaminidase in normal and mucopolipidosis-II cultured fibroblasts

Effect of monensin and nigericin

Georgirene D. VLADUTIU

Children's Hospital of Buffalo and State University of New York at Buffalo, 219 Bryant Street, Buffalo, NY 14222, U.S.A.

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The univalent-cation ionophores monensin ($4.0\ \mu\text{M}$) and nigericin ($0.5\ \mu\text{M}$) inhibited the abnormal excretion of β -hexosaminidase from mucopolipidosis-II cultured fibroblasts by 62 and 76% respectively, with a corresponding intracellular accumulation of the enzyme. As shown by lectin binding, the enzyme which accumulated in monensin-treated cells did not contain galactose residues, whereas the corresponding enzyme from nigericin-treated cells was galactosylated. The results suggest that monensin acts at an early point in the process of hydrolase glycosylation, and nigericin acts later, both presumably within the Golgi region, allowing the accumulation of different glycosylated forms of the enzyme. The intra- and extra-cellular distribution of β -hexosaminidase in ionophore-treated normal cells was essentially unchanged, whereas concanavalin A precipitability of excreted enzyme was increased and its ability to be taken up by deficient fibroblasts was decreased. The bivalent-cation ionophore A23187 ($1\ \mu\text{M}$) reduced β -hexosaminidase excretion from mucopolipidosis-II cells by 82%, and by 96% when used with EGTA ($1\ \text{mM}$). However, there was no intracellular accumulation of enzyme, suggesting that the effect of this ionophore was restricted to the inhibition of synthesis. It therefore appears that the actual transport of β -hexosaminidase in mucopolipidosis-II cells is affected by univalent-cation ionophores in a selective manner. These findings suggest that individual ionophores could be used to identify the sites of hydrolase oligosaccharide processing in the Golgi region by causing intermediate glycosylated forms of the transported hydrolase to accumulate in a specific Golgi compartment preceding the blocking site of the ionophore.

The most commonly occurring oligosaccharide structures in the glycoproteins of higher organisms, including lysosomal enzymes, begin with an *N*-glycosyl or *N*-acetylglucosamine linkage to asparagine residues (Schachter, 1981). The initial step in the synthesis of this linkage occurs in the rough endoplasmic reticulum and involves the assembly of a large, high-mannose oligosaccharide on a dolichol pyrophosphate carrier, with subsequent transfer to an asparagine residue on the polypeptide. The resulting $\text{Glc}_3\text{Man}_9(\text{GlcNAc})_2\text{Asn}$ structure is trimmed of its glycosyl residues by neutral glycosidases in the rough endoplasmic reticulum, then transferred to the Golgi region where mannose residues are phosphorylated and

'capped' with *N*-acetylglucosamine (simple high-mannose oligosaccharides) or further trimmed by a Golgi mannosidase to $\text{Man}_5(\text{GlcNAc})_2\text{Asn}$ (Tabas & Kornfeld, 1979) and eventually to $\text{Man}_3(\text{GlcNAc})_2\text{Asn}$. A variety of glycosyl residues are then added to produce complex oligosaccharide chains (Miller *et al.*, 1981a). Although these modifications occur in the Golgi region, it is not known exactly where each step occurs or where secretory and non-secretory forms of lysosomal enzymes are sorted from each other (Farquhar & Palade, 1981). Certain ionophores have the potential for aiding in the delineation of these processes, on the basis of reported studies of their effects on the processing and transport of many glycoproteins. The univalent-cation ionophores monensin and nigericin appear to perturb the migration

Abbreviation used: ML II, mucopolipidosis II.

of Golgi-derived vesicles in a variety of cells and consequently inhibit the secretion of various glycoproteins, both secretory and membrane-bound (see Tartakoff, 1983, for review). Ultrastructural (Tartakoff & Vassalli, 1977, 1978; Ledger *et al.*, 1980) and autoradiographic (Tartakoff & Vassalli, 1978) studies further suggest that the primary site of action of monensin is in the Golgi region. We have shown that monensin reduces excretion and stimulates the intracellular accumulation of β -*N*-acetyl-D-glucosaminidase (EC 3.2.1.30; β -hexosaminidase) in fibroblasts derived from patients with I-cell disease [mucopolipidosis II (ML II)] while having comparatively little effect on the distribution of β -hexosaminidase in normal fibroblast cultures (Vladutiu & Rattazzi, 1980).

ML-II fibroblasts preferentially excrete most of their newly synthesized lysosomal enzymes in a complex glycosylated form, resulting in an intracellular deficiency of hydrolases (Wiesmann *et al.*, 1971; Vladutiu & Rattazzi, 1975). The primary defect in ML II appears to be a deficiency of a specific phosphotransferase, located in the Golgi region (Hasilik *et al.*, 1980, 1981; Reitman & Kornfeld, 1981; Reitman *et al.*, 1981; Tabas & Kornfeld, 1980), which mediates the transfer of glucosamine phosphate to terminal mannose residues on hydrolases. A second Golgi-region enzyme, α -*N*-acetylglucosamine phosphodiesterase (Varki & Kornfeld, 1980; Waheed *et al.*, 1981a,b), found in both normal and ML-II cells, removes the terminal *N*-acetylglucosamine residue to uncover phosphomannose recognition residues, presumably necessary for the receptor-mediated targeting of lysosomal enzymes to the lysosomes (Kaplan *et al.*, 1977; Sando & Neufeld, 1977; Ullrich *et al.*, 1978).

Other investigators have reported alterations in the composition of oligosaccharide chains of glycoproteins such as transferrin and fibronectin during monensin treatment (Strous & Lodish, 1980; Ledger *et al.*, 1983). Therefore the purpose of the present study was to (1) determine if and to what extent glycosylation of β -hexosaminidase in fibroblasts is affected by selected ionophores, and (2) attempt to assign relative sites in the Golgi region at which these ionophores act on β -hexosaminidase. The ML-II fibroblast cultures provide a particularly good model for these studies, since over 85% of the β -hexosaminidase associated with these cells in a 24 h period is newly synthesized (Vladutiu & Rattazzi, 1979).

Our findings show that univalent-cation ionophores can be used as pharmacological probes to stimulate the intracellular accumulation of different glycosylated forms of a given hydrolase. Preliminary results from the present study have been published in abstract form (Vladutiu, 1981)

Materials and methods

Materials

The ionophores A23187, monensin and nigericin were gifts from Dr. R. Hamill, Eli Lilly and Co. (Indianapolis, IN, U.S.A.). *Ricinus communis* (castor bean) agglutinin-120 (ricin-120) was obtained from Miles Laboratories (Elkhart, IN, U.S.A.). *Clostridium perfringens* neuraminidase type VI and *Escherichia coli* alkaline phosphatase type III R were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ML-II fibroblast cultures from patients A. S. and L. T. were generously given by Dr. Arnold Miller (University of California at San Diego, La Jolla, CA, U.S.A.) and Dr. George Thomas (Johns Hopkins University, Baltimore, MD, U.S.A.) respectively. Normal fibroblast culture (GM-969) and Sandhoff-disease fibroblast culture (GM-294) were obtained from the Genetic Mutant Cell Repository (Camden, NJ, U.S.A.).

Cell culture and enzyme assays

Fibroblast cultures were maintained in 25 cm² flasks or 35 mm-diameter 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 Biological Co., Grand Island, NY, U.S.A.). The collection of fibroblast secretions, cell extraction, and enzyme-assay methods were described previously (Vladutiu & Rattazzi, 1975). The fluorogenic substrate, 4-methylumbelliferyl *N*-acetylglucosaminide (Pierce Chemical Co., Rockford, IL, U.S.A.) was used for the assay of β -hexosaminidase and for the detection of electrophoretic isoenzyme patterns as described previously (Vladutiu & Rattazzi, 1975). 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).

Neuraminidase treatment and protein determination

The treatment of fibroblast-derived β -hexosaminidase preparations with *Clostridium perfringens* neuraminidase for 1 h at 37°C has been described (Vladutiu & Rattazzi, 1975). Protein concentrations were measured by the Coomassie Blue dye-binding method of Bradford (1976), with bovine serum albumin as a standard.

Treatment of cell cultures with ionophores

Ionophores were incubated with fibroblast cultures in serum-free Ham's F-10 medium + 0.25% human serum albumin in 35 mm Petri dishes for 24 h at 37°C. The concentrations of ionophores used were determined by titrations based on previous studies (Vladutiu & Rattazzi, 1980). Cell

monolayers were harvested by trypsin treatment and extracted by sonication as described previously (Vladutiu & Rattazzi, 1975), and culture fluids were assayed for β -hexosaminidase and lactate dehydrogenase directly in experiments involving the distribution of intra- and extra-cellular enzyme. In lectin-precipitation studies, larger batches of cells were used (four 75 cm² flasks). Cell monolayers were harvested in the usual way and kept on ice during the three 15 s bursts of sonication. Culture fluids were concentrated 25–50-fold by ultrafiltration at 4°C (XM-100 filters; Amicon Corp., Lexington, MA, U.S.A.). The duration of the concentration period did not exceed 30 min. Cell extracts and concentrated culture fluids were then dialysed against 1000 vol. of saline (0.85% NaCl) at 4°C overnight before use in lectin-precipitation studies.

Ricin-120 precipitation of β -hexosaminidase

The ricin agglutinin-120 used in these experiments was specific for terminal galactose residues (Debray *et al.*, 1981). The lectin was diluted in 0.05 M-NaH₂PO₄/0.02 M-NaCl, pH 6.8, to a concentration range of 180–870 μ g/ml. Reaction mixtures containing equal volumes of enzyme solution derived from neuraminidase-treated ML-II cell-culture fluid and lectin solution (20 μ l of enzyme containing 12–15 units of activity + 20 μ l of lectin dilution) were incubated in 1 ml plastic conical tubes for 20 h at room temperature for precipitation. The tubes were centrifuged at 40000g for 5 min and the supernatants assayed for unbound β -hexosaminidase activity to determine the optimal concentration of ricin which maximally precipitated the enzyme.

As controls for the inactivation of enzyme due to the time of incubation at room temperature or to the lectin itself or its buffer, equivalent concentrations of β -hexosaminidase were incubated in control buffer (0.5 M-citrate/phosphate buffer, pH 5.5) or in specific lectin buffer with or without lectin for 20 h at room temperature. β -Hexosaminidase activities in all preparations, including enzyme incubated in control buffer, were not decreased below that of the control.

Concanavalin A precipitation of β -hexosaminidase

β -Hexosaminidase derived from normal and ML-II cell cultures was incubated with serial dilutions of concanavalin A by the same procedure as that used for the ricin-120 titration, except that the concentration range of lectin was 25–200 μ g/ml and the dilution buffer was 0.018 M-NaH₂PO₄/Na₂-PO₄/1 M-NaCl/0.1 M-MgCl₂, pH 6.5.

Results

Distribution of β -hexosaminidase in ML-II fibroblast cultures

Table 1 shows that treatment of cultures with monensin (4 μ M) or nigericin (0.5 μ M) during a 24 h period induced an intracellular accumulation of β -hexosaminidase to 157 and 233% respectively of the untreated-control value while decreasing both the excretion and the total activity in ML-II fibroblast cultures. However, the perturbation of Ca²⁺ flux by A23187 and the removal of Ca²⁺ with EGTA caused a pronounced inhibition of excretion and of total culture-associated β -hexosaminidase activity in ML-II cultures without an intracellular accumulation of the enzyme. The total

Table 1. *Effect of cation flux effectors on β -hexosaminidase excretion by ML-II fibroblasts*

Duplicate 4-day cultures of ML-II fibroblasts in 35 mm Petri dishes (patient L.T. or A.S., 15–25 μ g of protein/dish) were used in all experiments. Each culture was preincubated with 1 ml of serum-free medium + 0.25% human serum albumin for 24 h at 37°C. Culture fluids were exchanged for fresh media containing the appropriate additions, incubated for 8 h at 37°C; culture fluids were exchanged with fresh media plus additions and incubated for an additional 16 h. Cells were harvested, extracted in 75 μ l of 0.05 M-phosphate/citric acid buffer, pH 5.5, assayed for total β -hexosaminidase activity and protein. Culture fluids (1 ml/dish) were assayed for β -hexosaminidase and lactate dehydrogenase activity. The latter never exceeded 0.02% of total cell enzyme. Fibroblasts from patient L.T. were used in all experiments, except those with nigericin, in which fibroblasts from patient A.S. were used. Values in parentheses show the results as percentages of corresponding untreated control values.

Additions (37°C, 24h)	β -Hexosaminidase activity		
	Units/ml of culture fluid	Units/cell extract	Total units
None	136.8	47.4	184.2
Nigericin (0.5 μ M)	32.8 (23.9)	74.8 (157)	107.6 (58)
None	312.0	66.6	378.6
Monensin (4.0 μ M)	118.0 (37.8)	155.0 (232.7)	273.0 (72)
None	196.0	38.9	234.9
EGTA (1 mM)	34.3 (17.5)	29.5 (75.8)	63.8 (27)
A23187 (1 μ M) + EGTA (1 mM)	8.7 (4.4)	32.8 (84.3)	41.5 (17.7)

Fig. 1. Ricin precipitation of ML II-cell and culture-fluid β -hexosaminidase

β -Hexosaminidase derived from ML II cells and culture fluids (patient A. S.) and incubated with ionophores for 24 h at 37°C was precipitated with ricin at a concentration of 1 μ g of ricin/unit of enzyme before and after neuraminidase treatment. Data are expressed as percentages of total activity which was precipitated by ricin. Abbreviations used: C, control; Mon, monensin; Nig, nigericin. ■, Untreated; ▨, neuraminidase-treated.

activities in control cultures varied, owing to the use of two different cell lines for these experiments and different cell passages. The distribution of β -hexosaminidase in normal cultures treated with either ionophore was not appreciably altered [for nigericin, results not shown; for monensin, previously reported (Vladutiu & Rattazzi, 1980)].

Ricin-120 binding of β -hexosaminidase

The optimal concentration of ricin-120 that completely precipitated neuraminidase-treated ML-II-cell-excreted β -hexosaminidase was 1 μ g of

ricin/unit of enzyme. This ratio of lectin to enzyme was then used for a batch precipitation of β -hexosaminidase from each ML-II-cell extract and culture fluid which had been collected in the presence of monensin or nigericin in order to determine the percentage of total enzyme which was precipitable with ricin. The results are presented in Fig. 1. The fact that neuraminidase-treated ML-II-cell excreted enzyme was 100% precipitable with ricin-120 indicated that, after the removal of sialic acid residues, endogenous galactosidases did not continue the glycolytic 'trimming' of the complex oligosaccharide chains on the enzyme molecules under the experimental conditions used. Therefore the ricin precipitation assay served as a good indicator of the extent of galactosylation of β -hexosaminidase. The results in Fig. 1 show that the residual β -hexosaminidase excreted from ML II fibroblasts in the presence of monensin and nigericin had lost approx. 90 and 99% respectively of its ricin-binding capacity, suggesting that this enzyme did not acquire complex oligosaccharides before excretion. The enzyme which accumulated inside ionophore-treated cells was derived from newly synthesized enzyme, since ML-II cells usually excrete almost all newly synthesized β -hexosaminidase (Vladutiu & Rattazzi, 1979; Hasilik & Neufeld, 1980). The enzyme that accumulated inside monensin-treated ML-II fibroblasts (0.5–4 μ M monensin) was not more precipitable with ricin than untreated ML-II-cell-extract enzyme. However, the enzyme that accumulated in nigericin-treated cells for a 24 h period increased in ricin precipitability to approx. 38%, in parallel with the increase in enzyme activity in the cells (Table 1), indicating that nigericin allowed both the galactosylation and an intracellular accumulation of β -hexosaminidase. It should be mentioned that fibroblasts from ML-II patient L. T. used for this experiment excrete sialylated β -hexosaminidase that is only precipitable with ricin after neuraminidase treatment. It has been our experience and that of others (Miller *et al.*, 1981b; van Elsen & Leroy, 1979), however, that some ML-II cell lines excrete hydrolases which are galactosylated, but not sialylated, indicating heterogeneity in the biochemical manifestations of ML II.

Intra- and extra-cellular β -hexosaminidase derived from normal fibroblast cultures incubated with serum-free medium alone were only 10% precipitable with ricin. Monensin and nigericin treatment for 24 h did not alter the distribution or the ricin-binding properties of intra- and extra-cellular β -hexosaminidase in normal cultures (results not shown). This is probably due to the minimal synthesis of new enzyme in normal cells compared with ML-II cells during 24 h incubation (Vladutiu & Rattazzi, 1979).

Fig. 2. Concanavalin A titration of 600–750 units of β -hexosaminidase/ml in ML-II (a and b) and normal (c and d) fibroblast extracts and culture fluids from untreated control cultures or cultures treated with 0.5 μ M-monensin, 0.5 μ M-nigericin or 5 mM-glucosamine

Each point represents the mean result for duplicate precipitations. Key to symbols: (a) Monensin-treated: $\blacktriangle$, culture fluid; $\bullet$, cell; controls: $\triangle$, culture fluid; $\circ$, cell. (b) Nigericin-treated: $\blacksquare$, culture fluid; $\blacklozenge$, cell; glucosamine-treated: $\bullet$, culture fluid. (c) Monensin-treated: $\blacktriangle$, culture fluid; $\bullet$, cell; controls: $\triangle$, culture fluid; $\circ$, cell. (d) Nigericin-treated: $\blacksquare$, culture fluid; $\blacklozenge$, cell.

Concanavalin A binding of β -hexosaminidase

The intracellular β -hexosaminidase from ionophore-treated normal and ML-II-fibroblast cultures was precipitable with concanavalin A to the same extent as enzyme derived from the corresponding untreated cultures. Of the ML-II-cell-excreted-enzyme preparations, only the enzyme from monensin-treated cultures had decreased precipitability (Fig. 2). Enzyme excreted from nigeri-

cin- or monensin-treated normal cells was more precipitable with concanavalin A than untreated control enzyme. A concanavalin A precipitation of β -hexosaminidase excreted from glucosamine-treated ML-II cell cultures was included in Fig. 2(b) as a control to demonstrate altered glycosylation. Glucosamine is known to inhibit asparagine-linked glycosylation of proteins (Koch *et al.*, 1979).

Table 2. Uptake of β -hexosaminidase excreted from nigericin- and monensin-treated normal fibroblasts

Duplicate or triplicate cultures of β -hexosaminidase-deficient fibroblasts (4–8 μ g of protein/dish) were preincubated with serum-free medium + 0.25% human albumin + kanamycin (20 μ g/ml), followed by 5 h uptake with fresh serum-free medium containing albumin, kanamycin and 300 units of β -hexosaminidase derived from normal culture fluids collected under the conditions described.

Collection conditions of enzyme* used for uptake	β -Hexosaminidase uptake (units/ μ g of cell protein)
No additions	0.185 (100%)†
Nigericin (0.5 μ M)	0.021 (11.4%)
Monensin (4 μ M)	0.011 (5.9%)

* β -Hexosaminidase used for uptake experiments was collected from the culture fluids of normal fibroblasts incubated for 24 h with serum-free Ham's F-10 medium + 0.25% albumin + kanamycin (20 μ g/ml) and including the designated compounds. The culture fluids were concentrated by ultrafiltration and dialysed against 500 vol. of saline (0.85% NaCl) before use in uptake experiments.

† Values in parentheses show results as percentages of the uptake of enzyme collected with 'No additions'.

Uptake properties of β -hexosaminidase from ionophore-treated normal cultures

The endocytosis of β -hexosaminidase collected from the culture fluids of nigericin- and monensin-treated normal fibroblasts was decreased to 11.4 and 5.9% respectively of the uptake of untreated control enzyme (Table 2), indicating that phosphomannose residues were either not available or not present for recognition and subsequent uptake. Similar low-uptake results were obtained with enzyme collected in the presence of 10 mM- NH_4Cl + ionophores. Treatment with NH_4Cl or other amines alone usually enhances the excretion of high-uptake enzyme from normal fibroblasts (Vladutiu, 1982; Gonzalez-Noriega *et al.*, 1980).

Discussion

In recent years, ionophores have gained importance as pharmacological tools to differentiate enzymically and functionally distinct compartments in various cell types (Tartakoff, 1982). Monensin, in particular, has been found to inhibit the migration of secretory and membrane proteins from (or through) the Golgi region without inhibiting either protein synthesis or the initial transport from the rough endoplasmic reticulum to the Golgi region (Tartakoff, 1982). The present work is a continuation of a previous study in which monensin inhibited the excretion of at least one

lysosomal enzyme, β -hexosaminidase, from ML-II fibroblasts (Vladutiu & Rattazzi, 1980). We report here that a substantial intracellular accumulation of β -hexosaminidase occurred only in ML-II cells treated with the univalent-cation ionophores nigericin and monensin and not with the bivalent-cation ionophore A23187. It is likely that β -hexosaminidase which has accumulated intracellularly is located within swollen Golgi cisternae and is unable to be translocated through the Golgi region, as has been observed immunocytochemically by Ledger *et al.* (1980) in the case of procollagen and fibronectin in monensin-treated fibroblasts. We have observed a similar immunocytochemical localization of β -hexosaminidase in ionophore-treated fibroblasts (G. D. Vladutiu, unpublished work).

The somewhat lowered total activity in monensin- and nigericin-treated cultures is likely due to a modest feedback inhibition of enzyme synthesis. The decreased total activity and absence of accumulation in cells treated with A23187 and/or EGTA strongly suggest that inhibition of protein synthesis was the primary effect of Ca^{2+} depletion in these cells, as observed by others studying the effect of A23187 on different glycoproteins (Tartakoff & Vassalli, 1978). It appears, therefore, that inhibition of hydrolase excretion with a corresponding intracellular build-up is characteristic of univalent- but not bivalent-cation-ionophore treatment.

The concanavalin A-precipitability of intra- and extra-cellular β -hexosaminidase from ML-II cells was relatively unaffected by ionophore treatment, except for a reduced precipitation of excreted enzyme from monensin-treated cells. In normal cells the enhanced precipitation of extracellular enzyme from both monensin- and nigericin-treated cultures perhaps may be explained by the findings of Ledger *et al.* (1983). These investigators found a similar increase in the affinity for concanavalin A of fibronectin excreted from monensin-treated fibroblasts, presumably due to an increase in the proportion of small oligosaccharides in the excreted glycoprotein compared with larger, more complex, oligosaccharides in fibronectin from untreated cultures. Alternatively, ionophores may prevent Golgi mannosidases from completely trimming high-mannose oligosaccharide chains by separating the mannosidases compartmentally from the new glycoproteins trapped in vacuolated areas of the Golgi region (Tartakoff & Vassalli, 1977).

In ML-II cell cultures monensin appeared to act at a site in the Golgi region preceding the site of galactosylation, since accumulated intracellular β -hexosaminidase was not precipitable with ricin. Inactivation of galactosyltransferase may have

Fig. 3. Diagrammatic representation of vesicle-mediated hydrolase transport through the rough endoplasmic reticulum (RER) Golgi region and then to either lysosomes or excretion via fusion with the plasma membrane

Arrows 1 and 2 indicate the postulated sites of monensin and nigericin action, respectively. Arrows a, b, and c indicate the possible pathways of hydrolase excretion.

occurred in the *trans*-Golgi region due to monensin treatment; however, monensin does not affect galactosyltransferase when added directly to an assay system *in vitro* (Wilcox *et al.*, 1982). Furthermore, although monensin decreased the incorporation of galactose in fast-transported protein in neurons from bullfrog dorsal-root ganglia (Hammerschlag *et al.*, 1982), this was probably not due to an inhibition of galactosyltransferase, since many fast-transported proteins which were decreased were not normally galactosylated anyway (Stone & Hammerschlag, 1981).

Further proof of monensin's action at a point before the *trans*-Golgi region has been presented by Griffiths *et al.* (1983). In Semliki-Forest-virus-infected baby-hamster kidney cells, monensin prevented the conversion of high-mannose oligosaccharides bound to viral membrane proteins into complex oligosaccharides.

It is particularly interesting that nigericin treatment of ML-II cells stimulated an intracellular accumulation of galactosylated β -hexosaminidase. This finding suggests that nigericin acted later in the Golgi region than monensin, allowing the conversion of high-mannose oligosaccharides into complex oligosaccharides. Like the action of weak bases, nigericin is also known to raise lysosomal pH in mouse peritoneal macrophages within minutes of its addition to a culture (Ohkuma & Poole, 1978). However, unlike amines, although ionophores presumably raise fibroblast lysosomal pH and also inhibit receptor-mediated endocytosis of hydrolases (Vladutiu & Rattazzi, 1980) and other glycoproteins (for review, see Tartakoff, 1983), they do not stimulate significant enzyme release. Furthermore, the enzyme that is released is not in the high-uptake form. Monensin only stimulated a 1.6-fold increase in β -hexosaminidase excretion during 24 h incubation, while actually promoting an intracellular build-up (Vladutiu & Rattazzi, 1980). Nigericin had no effect (G. D. Vladutiu, unpublished work), and chloroquine, an amine, stimulated a 4.3-fold increase during 24 h incubation (Vladutiu, 1982).

As a possible mechanism for the amine-induced

secretion of hydrolases, Gonzalez-Noriega *et al.* (1980) postulated that the elevation of lysosomal pH by amines prevents enzyme-receptor-complex dissociation and recycling of receptors to the endoplasmic reticulum and Golgi region. Consequently, new high-uptake enzyme en route to lysosomes with available phosphomannose recognition residues would not find free receptors to bind and would, therefore, be excreted.

The fact that ionophores do not stimulate a similar release of high-uptake enzyme is probably due to the physical trapping of newly synthesized enzymes in the swollen cisternae of the Golgi region and not related to receptor recognition and transport.

In preliminary studies we have observed a 90% decrease in the $^{32}\text{P}_i$ labelling of immunoprecipitated intracellular β -hexosaminidase from monensin- and nigericin-treated normal fibroblasts compared with untreated controls and a normal to increased amount of label in the excreted enzyme, which was alkaline phosphatase-insensitive (G. D. Vladutiu, unpublished work). Enzyme may therefore be excreted before uncapping of the recognition residue by phosphodiesterase.

Rothman (1982) has postulated that the Golgi region exists essentially as two organelles in tandem with the sequential passage of proteins from the early to the late cisternae. Phosphorylation of the mannose recognition residue on β -hexosaminidase normally occurs in the early *cis* region (Pohlmann *et al.*, 1982), and uncapping occurs in the late *cis* region, whereas complex-oligosaccharide-chain formation occurs in the *trans* region (Rothman, 1982). On the basis of our findings we have schematically represented the sites of monensin and nigericin action and the alternative routes of secretion in Fig. 3.

In conclusion, alterations in the complex glycosylation and transport of β -hexosaminidase in ML-II cell cultures occurred in the presence of monensin and nigericin, and phosphorylation and/or uncapping of phosphomannose residues was altered in normal cells. These ionophores appear to be active at topologically distinct regions,

at which they affect β -hexosaminidase glycosylation. Future studies should include the use of these ionophores in localizing different glycosylated forms of hydrolases in fibroblasts using immunocytochemical methodology.

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