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Uptake by neuroblastoma cells of glucosylceramide, glucosylceramide glucosidase, its stimulator protein, and phosphatidylserine

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Serum-free cultured neuroblastoma cells (clone NIE-115) have been shown to absorb emulsified glucosylceramide, glucosylceramide glucosidase, an activator protein for the enzyme, and phosphatidylserine from a synthetic medium. Uptake of the enzyme was augmented by phosphatidylserine, and vice versa. Uptake of the enzyme-lipid complex was further augmented by the activator protein. It appears likely that the activator forms a complex only with the enzyme-lipid complex, not with the individual components. Two uptake mechanisms for the enzyme seem to be involved, one of which (the complex with activator proteins and acidic lipid) is sensitive to mannosyl phosphate groups. Hydrolysis of absorbed glucosylceramide was slow unless the medium was supplemented with the acidic phospholipid or glucosidase. The most rapid disappearance of stored glycolipid took place when the ternary mixture was added to the cell medium, enzyme + activator protein + phosphatidylserine. These findings may be relevant to enzyme replacement therapy for Gaucher disease.

Introduction

Some genetic disorders characterized by low-efficiency enzymes can, in theory, be treated by injecting the normal enzyme. One must hope that most of the normal enzyme molecules will reach the required sites of action and survive long enough to eliminate accumulated substrate. The enzyme can be injected intravenously in the free form, as a complex with an antibody, or as an inclusion of a liposome or red cell ghost. Alternatively, it can be modified chemically to resist proteolytic degradation or enable it to enter cells via specific receptors. We describe a new system in which the

enzyme is bound noncovalently to its natural activators and then effectively taken up by cells in vitro.

The model system was developed with cultured neuroblastoma cells of murine origin (clone NIE-115), grown under serum-free conditions. The enzyme studied was glucosylceramide (GlcCer) β -glucosidase (EC 3.2.1.45). This is the enzyme which occurs in defective form in individuals with Gaucher disease. A striking result of the genetic defect is a large accumulation of the substrate, a glucosphingolipid which is a precursor of several nonionic glycolipids and most of the gangliosides. Another striking aspect of the disorder is an accumulation of a family of activator proteins, called cohydrolase here. The activator proteins stimulate not only the glucosidase but also sphingomyelinase and galactosylceramide β -galactosidase [1–3].

Cohydrolase forms a complex with glucosidase,

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apparently in conjunction with an acidic phospholipid such as phosphatidylserine [4,5]. The complex exhibits a lowered K_m for its substrate as well as higher activity. Additional evidence for the formation of a ternary complex has come from density gradient centrifugation [6] and tests with an inactivator of the enzyme [7]. The acidic phospholipid itself also acts as a stimulator of the enzyme. Immobilized cohydrolase, attached to Sepharose 4B, binds the crude enzyme and affords significant purification [4]. This paper describes cellular uptake from the culture medium of the various substances involved.

Materials and Methods

Chemicals

Cohydrolase was isolated from bovine spleen [8] and stored at 4°C in buffer A (5 mM phosphate (pH 7)/40 mM NaCl) containing 0.02% NaN₃. It was separated into two fractions, one that does not adhere to concanavalin A-Sepharose, and one that does bind and is eluted with 0.5 M NaCl and 0.5 M methyl α -mannoside in 5 mM (pH 7) phosphate [9]. The latter form, after dialysis against buffer A, was used in most of these experiments.

Bovine brain phosphatidylserine (PS) was from Avanti Polar Lipids and phosphatidyl[U-¹⁴C]-serine (dioleoyl) was from Amersham. A solution containing the former or a mixture of both (26 cpm/ μ g) was evaporated to dryness, sonicated in a cold bath with culture medium, and sterilized by filtration (0.2 μ m Acrodisc assembly, Gelman Scientific). Sodium taurocholate was from Calbiochem Behring. Mannose 6-phosphate, methyl α -mannoside, calf thymus DNA, glucose 6-phosphate, mannose, and concanavalin A-Sepharose CL-4B were from Sigma. A pentamannoside monophosphate was prepared from *Halo-bacterium holstii* [10].

GlcCer (glucocerebroside), isolated from a Gaucher spleen, was dissolved in chloroform/methanol with half its weight of Myrj 52 (a solid polyoxyethylene stearate with a hydrophile-lipophile balance rating of 16.9 from ICI Americas Inc., Wilmington, DE). The solution was evaporated to dryness and converted to a milky emulsion with 0.9% NaCl or culture medium in an

ultrasonic bath and sterilized by filtration just before addition to the medium. Some of the GlcCer was converted to [6-³H]glucosylceramide [11].

Glucocerebroside was purified and delipidated from fresh human placenta by cholate extraction, ammonium sulfate precipitation, acid precipitation, butanol extraction, and affinity chromatography [12]. The enzyme was dialyzed against culture medium and concentrated under vacuum in the dialyzer just before use.

Cells

Murine neuroblastoma cells (clone NIE-115), passage 10–14, were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented (in amounts per liter) with 5 mg of insulin, 20 nmol progesterone, 0.1 mmol putrescine, 30 nmol selenite, 100 mg of transferrin, 1.4 mg of vitamin B-12, 7 μ g of biotin, and 0.2 mg of thioctic acid [13]. Subculture was achieved by inoculating $4 \cdot 10^5$ cells into 75 cm² plastic tissue culture flasks containing 20 ml of medium supplemented with 10% fetal bovine serum (GIBCO) without antibiotics. After 24 h of culture at 37°C in 10% CO₂/humidified air, the medium was removed and replaced with 20 ml of serum-free medium. The cells were fed on days 4 and 6, and every day thereafter, by adding 10 ml of fresh medium and removing 10 ml of medium.

After 6 days of culture in serum-free medium, the medium was removed and replaced with 20 ml of serum-free medium containing a small volume of test substance(s). Control and experimental flasks were incubated at 37°C in duplicate. At the end of the test incubation, the medium was removed and the cells were incubated for 5 min in Puck's D1 solution without trypsin [14]. They were then removed from the flasks, pooled, isolated by centrifugation, suspended in phosphate-buffered saline (pH 7), sonicated on ice, and assayed.

Assays

Cohydrolase concentrations were measured with an immunoassay [15] with two portions of a cell extract, each purified with a size exclusion column and analyzed in duplicate. The uptake of cohydrolase was calculated from the formula,

$$\% \text{ uptake} = 100 \cdot (C_{\text{experimental}} - C_{\text{control}}) \cdot \text{wt. protein/wt. CH}$$

where C is the concentration of cohydrolase in the control or experimental (cohydrolase-treated) cells, wt. protein is the weight of protein in the cohydrolase-treated cells, and wt. cohydrolase is the weight of cohydrolase added to the flasks. A similar calculation was used for the enzyme, with activity units instead of weights. This formula, which assumes that the endogenous level within the cells is unchanged by the uptake, corrects for the small differences in protein content between the different pooled groups.

Glucosidase activity was determined by a 30-min incubation in acetate pH 5.4 and Triton X-100, 0.5 mg/ml, with either methylumbelliferyl-2- β -D-glucopyranoside or with [^3H]GlcCer as substrate. In the latter case, sodium taurocholate (5 mg/ml) was included [16]. One cerebroside or methylumbelliferyl-2- β -D-glucopyranoside unit of activity is the amount of enzyme which hydrolyzes 1 nmol/h of substrate. Assays were run with two different amounts of sample to ensure the absence of interfering materials; the observed specific activities generally agreed within 4%. The endogenous cohydrolase in the cells was too low to affect the measured glucosidase activities when methylumbelliferyl-2- β -D-glucopyranoside was the substrate. Tests of the enzyme assay system with labeled GlcCer as the substrate showed that the presence of 4 μg PS in the 0.2 ml of incubation medium or 50 pg of cohydrolase with the phospholipid did not significantly change the observed activities.

Radioactivity was determined by scintillation counting of the wet cells or lipid extract in a water-miscible system. Analysis of the cell lipids was carried out by extracting cells with hexane/isopropyl alcohol 3:2 and chromatographing the lipids on silica gel-coated TLC plates (E. Merck, No. 5763-7) with chloroform/methanol/water 24:7:1. The water contained 1 M NH_4OH and KCl, 2.5 mg/ml.

Protein was determined by a colorimetric precipitation-Folin reaction with bovine serum albumin as standard [17] and DNA was determined by the diaminobenzoic acid fluorescence method [18].

Results

Occurrence of cohydrolase and glucosidase in the cells

The NIE-115 cells, cultured in normal (10% fetal bovine serum) and in serum-free media, were found to contain cohydrolase (Table I), at a concentration similar to that found in mouse brain [15]. Its concentration, relative to total cell protein, did not change appreciably with time over the 7-day period studied. In contrast, the specific activity of glucosidase rose greatly. The two culture conditions employed yielded similar values and all later experiments were done in serum-free media with cells that had been grown in serum-free medium for 6 days.

Uptake of cohydrolase by cells

The cells were found to absorb cohydrolase from the medium, the amount increasing with time (Fig. 1). In this study, the activator preparation included the two types of protein – the kind which binds to concanavalin A-Sepharose and the kind which does not. The former type was 56% of the total. Calculating from the individual control flasks for each time point, one sees that the cells took up 9, 13, 14, and 19% of the added factor by the end of 1, 2, 4, and 6 h, respectively. The reproducibility of the system is illustrated by the horizontal nature of the lower curve (control cells) and by the duplicate sample values in the figure.

Comparison of the two kinds of cohydrolase in a 6-h incubation showed that the concanavalin binding form was taken up more efficiently: 36% vs. 18% (Table II). This form was used in all the later experiments. Significantly, we found that cohydrolase was not removed from the cells on washing, although some loosely bound protein was lost. This shows that the absorbed cohydrolase was firmly bound to the surface or internalized. In addition, the data show that endogenous cohydrolase did not leak out of the cells into the medium. The concentration of cohydrolase in the control cells, 1.72 ng/ml protein, was similar to the values found in other experiments (Table I and Fig. 1).

Since cohydrolase is a glycoprotein, we were interested in determining whether the uptake phenomenon involved a mannosyl phosphate receptor

TABLE I

GROWTH PROPERTIES OF NIE-115 CELLS WITH RESPECT TO TOTAL PROTEIN, GLUCOSYLCERAMIDE GLUCOSIDASE, AND COHYDROLASE CONTENT

The protein values are derived from two culture dishes, each containing 20 ml medium, and the other values are based on the total protein values. The glucosidase activities are in methylumbelliferyl-2- β -D-glucopyranoside units. Each measurement shown is a mean of duplicate assays.

Age (days)	Protein (mg)		Glucosidase activity (a) (nmol/h per mg)		Cohydrolase concentration (b) (ng/mg)		Ratio (a)/(b)	
	+ serum	- serum	+ serum	- serum	+ serum	- serum	+ serum	- serum
3	3.8	5.0	10	7	1.7	1.5	6	4
4	5.7	5.9	15	14	1.6	1.6	9	9
6	16	10.9	23	16	1.5	1.5	15	11
7	24	25	25	22	1.8	1.6	14	13
8	22	18	32	29	1.6	1.5	20	19
10	28	24	38	33	1.7	1.5	22	21

on the cell surface. When cells were incubated for 6 h with 2 mM mannose 6-phosphate, almost complete blockage of cohydrolase uptake was obtained (upper part of Table III). Mannose phosphate alone (line 2) had little or no effect on the endogenous cell level of cohydrolase. The uptake of cohydrolase from the normal medium was 49%,

based on the average of the two control groups (lines 1 and 2). This was reduced by 86% when mannose phosphate was included in the incubation.

Another experiment (lower part of Table III) showed that other carbohydrates also interfered with cohydrolase uptake. A pentamannoside

TABLE II

UPTAKE OF TWO COHYDROLASE (CH) FORMS BY INCUBATED CELLS

Conditions as in Table I. Two flasks were incubated under each condition for 6 h and the pooled media were saved for analysis. The cells from each dish were washed for 5 min at 37°C with 5 ml of Puck's D1 solution without trypsin, pooled, then sonicated in 5 mM phosphate (pH 7) containing 1 M NaCl and analyzed. The cells of group CH.ConA- were incubated with 13.5 ng of non-binding cohydrolase in two flasks; group CH.ConA+, with 15 ng of concanavalin A-binding cohydrolase. The degree of variability in the cohydrolase assays is indicated in the duplicate values shown for cohydrolase in the cells.

Cells	Fraction analyzed	Protein content (mg)	Cohydrolase content (ng)	Cohydrolase present (% of added CH)
Control	medium	12.0	0.0	-
	wash	1.15	0.0	-
	cells	7.4	12.9, 12.6	-
CH.ConA +	medium	12.0	7.08	47
	wash	1.45	0.0	-
	cells	7.4	17.9, 18.2	36 *
CH.ConA -	medium	13.5	7.68	57
	wash	1.45	0.0	-
	cells	7.5	15.2, 14.9	18 *

* These values have been corrected for the endogenous level of cohydrolase.

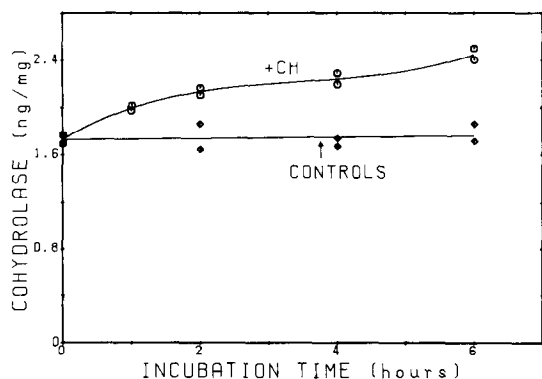


Fig. 1. Uptake of cohydrolase (CH) by neuroblastoma cells as a function of time. Each flask contained 7.5 ng of mixed types of cohydrolase in 20 ml of medium and the cells from two flasks were pooled for assay. The lower curve shows the cohydrolase contents of control cells incubated without added cohydrolase.

monophosphate was quite effective, reducing cohydrolase uptake by 67% at a concentration of only 300 ng/ml. Mannose itself (10 mM) reduced cohydrolase uptake by 63% and 2 mM glucose 6-phosphate also showed some blocking activity: 33%.

Effect of glucosidase on cohydrolase uptake

Uptake of cohydrolase was not appreciably improved by adding glucosidase to the medium (Table IV). In this study, 7.5 ng of cohydrolase were added to the 20 ml of medium in each dish, giving an uptake of 49% in 6 h. Including glucosidase (495 methylumbelliferyl-2- β -D-glucopyranoside units/flask) brought the uptake of cohydrolase to 54%. The enzyme uptake, 24%, was the same with or without cohydrolase. From these findings, it would appear that the two proteins do not interact in the absence of lipids. It may be noted that the amount of enzyme taken up was far greater than the endogenous level. The recovery of added enzyme in cells and medium was 74% with or without added cohydrolase.

In a similar experiment, with PS in the medium of all flasks but two (20 μ g/ml), cohydrolase uptake was 45% without enzyme and 40% with enzyme (Table V). However, the glucosidase uptake was definitely improved by cohydrolase, increasing from 31% without to 48% with cohydrolase. The latter observation can be interpreted to mean that cohydrolase does combine with the enzyme if PS is present, and that this complex is absorbed more efficiently than free enzyme. The

TABLE III

EFFECT OF CARBOHYDRATES ON UPTAKE OF COHYDROLASE (CH)

Conditions as in Methods section. Duplicate flasks containing cells were incubated 6 h with buffer alone, or with cohydrolase (375 pg/ml), or with a carbohydrate, or with both, then assayed for cohydrolase content of the cells. Some of the values in this table are shown as individual results of duplicate analyses. The uptake values for cohydrolase are based on the average cohydrolase content of all the controls. M-6-P, mannose 6-phosphate; (Man)₅P, pentamannoside monophosphate; G-6-P, glucose 6-phosphate.

Group	Total protein (mg)	CH concentration (ng/mg protein)	CH uptake (%)
Control	9.6, 9.9	1.73, 1.79	—
M-6-P alone (2 mM)	9.6, 9.9	1.62, 1.68	—
Cohydrolase alone	10.5, 9.9	2.49, 2.38	49
CH + M-6-P	10.2, 9.6	1.82, 1.82	7
Control	8.85	1.65, 1.69	—
G-6-P (2 mM)	8.70	1.62, 1.59	—
(Man) ₅ P	8.63	1.63, 1.60	—
Mannose (10 mM)	9.38	1.56, 1.59	—
Cohydrolase alone	9.08	2.42, 2.39	48
CH + G-6-P	8.70	2.20, 2.13	32
CH + (Man) ₅ P	8.33	1.89, 1.86	14
CH + mannose	8.33	1.97, 1.89	17

TABLE IV

INTERACTION BETWEEN COHYDROLASE AND GLUCOSIDASE

Data are derived from two pooled dishes for each condition. Cohydrolase (125 μ l, 60 pg/ μ l in buffer A) and glucosidase (275 μ l, 1.8 methylumbelliferyl-2- β -D-glucopyranoside units/ μ l in medium) were added to each flask, as shown below. The medium for control cells was supplemented with the corresponding volume of buffer or medium. The values in parantheses are the percent of added cohydrolase (CH) or enzyme taken up by the cells.

Group	Total protein (mg)	Cohydrolase concentration (ng/mg protein)	Glucosidase activity * (nmol/h)	
			in cells	in medium
Control	10.5	1.73	25	24
+ CH	9.4	2.52 (49%)	24	56
Control	12.3	1.79	23	13
+ enzyme	10.1	1.74	262 (24%)	475
Control	11.0	1.71	20	14
+ CH + enzyme	12.2	2.38 (54%)	258 (24%)	474
Zero time	11.7	1.60	23	11

* Total activity.

failure to observe a corresponding increased uptake of cohydrolase in the presence of both PS and glucosidase can be attributed to the use of a relatively large molar excess of the activator (i.e., the increased uptake of cohydrolase could not be measured accurately enough).

Uptake of cerebroside glucosidase

The above observation, that enzyme uptake was enhanced by cohydrolase in the presence of PS, was studied further. Control cells were incubated

for 6 h without added enzyme, with PS alone (20 μ g/ml), or with cohydrolase alone (375 pg/ml), or with both. This yielded cells having unchanged enzyme specific activity (top four lines, Table VI). Incubation with exogenous glucosidase alone (14 cerebroside units/ml) resulted in 24% of the added enzyme being taken up. As noted before, this represented a large increase above the endogenous level. Inclusion of PS with the enzyme produced a markedly higher uptake (37%). Inclusion of cohydrolase with the enzyme, without phospholi-

TABLE V

INTERACTION BETWEEN COHYDROLASE AND GLUCOSIDASE IN THE PRESENCE OF PHOSPHATIDYLSERINE

Conditions as in Table IV, except that all flasks (except the last set) contained 20 μ g/ml of PS. The values in parentheses are the percent of added cohydrolase (CH) or enzyme taken up by the cells.

Group	Total protein (mg)	Cohydrolase concentration (ng/mg protein)	Glucosidase activity * (nmol/h)	
			in cells	in medium
Control	6.8	1.44	38	15
+ CH	7.7	2.31 (45%)	41	29
Control	8.5	1.45	40	21
+ enzyme	7.4	1.45	349 (31%)	371
Control	7.1	1.51	37	49
+ CH + enzyme	6.8	2.39 (40%)	515 (48%)	264
No PS control	6.6	1.38	34	18

* Total activity.

TABLE VI

UPTAKE OF GLUCOSYLCERAMIDE GLUCOSIDASE FROM CULTURE MEDIA AUGMENTED WITH COHYDROLASE AND PHOSPHATIDYLSERINE

The initial enzyme concentration was 14 cerebroside units per ml medium, the cohydrolase (CH) concentration was 375 $\mu\text{g}/\text{ml}$, and the PS concentration was 20 $\mu\text{g}/\text{ml}$. The uptake percentage was corrected for the endogenous cellular enzyme in the control cells.

Medium	Total protein (mg)	Glucosidase activity * (nmol/h)		Uptake of enzyme (% of added amount)
		in cells	in medium	
Standard medium	10.4	74	45	—
+ PS	10.6	78	45	—
+ CH	10.2	78	39	—
+ CH + PS	11.0	75	41	—
Enzyme medium	10.6	209	287	24
+ PS	10.1	285	180	37
+ CH	10.9	235	278	28
+ CH + PS	10.6	368	146	53

* Total activity.

pid, had little effect on the enzyme uptake (28% uptake), as noted in Table IV. When all three components were added, a further enhancement was seen: 53% of the enzyme was taken up.

An uptake experiment like the above was run with the use of methylumbelliferyl-2- β -D-glucopyranoside in the enzyme assay system. The cells were incubated for 6 h with 17 methylumbelliferyl-2- β -D-glucopyranoside units of glucosidase per ml. Here the fractions of enzyme taken up under the four conditions were 21, 34 (+PS), 25 (+CH), and 53% (+PS + cohydrolase). The specific activity of the enzyme in the last set of cells was 10-times that in the untreated cells. The recovery of added enzyme in cells + medium was 62–72%, with and without activators, so one cannot attribute the increases in enzyme uptake to protection by PS or cohydrolase against enzyme inactivation.

A time comparison of the enzyme uptake in the presence of PS, 3 vs. 6 h of incubation, showed that most of the uptake was complete within 3 h, with or without added cohydrolase: 28–30% uptake without the activator, 45–50% with the activator. Doubling or tripling the cohydrolase concentration in the medium did not increase the enzyme uptake or recovery from the dishes. From this observation, it would appear that we used a large molar equivalent excess of cohydrolase, com-

pared to the enzyme. The same conclusion was drawn before from the data of Table IV.

In a test of carbohydrate interference, the uptake of glucosidase was studied as above, in the presence of 20 $\mu\text{g}/\text{ml}$ of PS. This resulted in absorption of 31% of the added enzyme. The amount of uptake was unaffected by the presence of 1 mM mannose 6-phosphate or the pentamannoside monophosphate (0.3 $\mu\text{g}/\text{ml}$). Evidently the enzyme-PS complex is not absorbed by neuroblastoma cells via a mannosyl phosphate receptor. This is in agreement with the report that this enzyme is absorbed by human fibroblasts by a non-mannosyl phosphate mechanism [19].

When, in the same experiment, glucosidase uptake was enhanced by inclusion of both PS and cohydrolase in the medium, the uptake of enzyme was 54% of the amount added and both mannose derivatives blocked the enhancement, bringing it back to 32 and 30%, respectively. It seems likely from these observations that part of the enzyme (31%) entered the cells by one mechanism and that the other portion (54–31 = 23%) entered the cells by forming a complex with cohydrolase (and PS) which entered by the same mechanism responsible for cohydrolase uptake.

A comparison of different PS concentrations for their ability to enhance glucosidase uptake showed that the enhancement was maximal at PS

concentrations above 50 $\mu\text{g}/\text{ml}$ in the medium (Fig. 2). Recoveries of enzyme in cells and media were 83–87%, so it is evident that the enhancement by PS was not the result of improved recovery.

Uptake of labeled phosphatidylserine

When the medium contained only radioactive PS (10 $\mu\text{g}/\text{ml}$) for 6 h, less than 1% of the lipid was taken up by the cells. When 700 methylumbelliferyl-2- β -D-glucopyranoside units of enzyme were included in the 40 ml of medium, much more phospholipid was absorbed (9%). The uptake was proportional to the concentration of lipid, rising to as high as 35% of the added lipid (lower curve of Fig 3).

When cohydrolase (375 pg/ml) was added to the medium together with glucosidase, the uptake of PS was enhanced even further (upper curve of Fig. 3). The recovery of added lipid in cells and medium was 78–91%, unaffected by the presence or absence of the two proteins.

Uptake of glucosylceramide and its degradation

An important question is whether the glucosidase taken up by the cells reached a subcellular

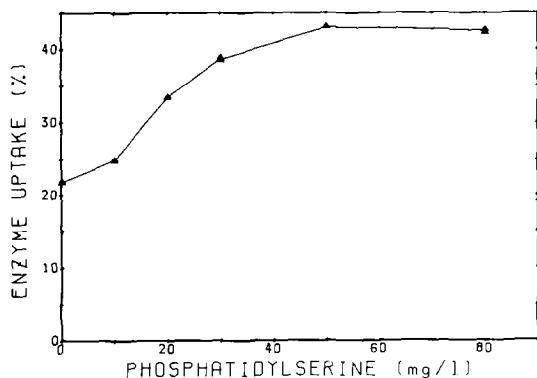


Fig. 2. Dependence of glucocerebrosidase uptake (percent of enzyme added to flasks) on concentration of phosphatidylserine in the medium. Two flasks containing 607 cerebrosidase units were incubated for 6 h with differing concentrations of PS. The enzyme uptake was calculated from the activity in the cells and the activity added. The duplicate values are too close to distinguish except for the 30 and 80 μg points. The total cell protein in the two flasks at each time point was 6.0–6.2 mg.

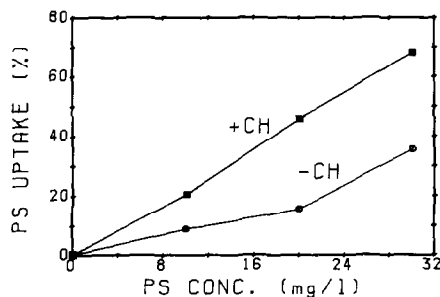


Fig. 3. Dependence of phosphatidylserine uptake (cpm in cells from two flasks) on the concentration of PS in the medium and the presence or absence of cohydrolase. Each flask contained 350 methylumbelliferyl-2- β -D-glucopyranoside units of glucosidase; half the flasks (upper curve) contained 375 pg/ml of cohydrolase. Note that the tick marks on the x axis are 4 units apart.

location that is useful for the accelerated hydrolysis of GlcCer. It was first necessary to produce a model version of Gaucher disease in the cells. This has been achieved readily in intact mice by injection of a GlcCer glucosidase inactivator, conduritol B epoxide [20,21], or an emulsion of GlcCer glucosidase in the nonionic detergent, Myrj 52 [21]. The neuroblastoma cells were first examined with the detergent alone to make sure it was not highly toxic. A concentration of 0.1 mg/ml Myrj showed no visible signs of toxicity after 24 h, but 0.3 mg/ml produced some floating dead cells and all cells were killed by 1 mg/ml Myrj.

An emulsion was prepared with 25 μg GlcCer and 12.5 μg Myrj per ml of medium, and incubated with cells for 24 h. The cells were washed and the lipids were examined by TLC and charring. It was apparent that the cerebrosidase, normally not detectable at the level examined, had become a major lipid component of the cells. Maintenance of washed cells in fresh medium for 2 or 5 days did not result in significant loss of cerebrosidase. A similar failure to hydrolyze exogenous labeled GlcCer by human cultured fibroblasts has been observed [22]. While the GlcCer concentration used in our experiment was higher than the observed 20 $\mu\text{g}/\text{ml}$ limit for nontoxicity in macrophage cultures [23], there was no sign of toxicity in our cells, as judged visually and by constancy of protein and DNA contents per culture (Table VII).

To increase the sensitivity of the cells to exoge-

TABLE VII

EVALUATION OF CELLS FOR PROTEIN AND DNA CONTENT AFTER TAKING UP GLUCOCEREBROSIDE AND PHOSPHATIDYLSERINE

Cells in two flasks were incubated for 24 h with an emulsion of GlcCer (25 $\mu\text{g}/\text{ml}$) and Myrj 52, then washed and incubated further in serum-free medium. An additional set (lower part of table) was incubated with an emulsion of GlcCer/Myrj and PS (10 $\mu\text{g}/\text{ml}$).

Incubation medium	Post-uptake period (days)	Total protein (mg)	Total DNA (μg)	DNA/protein
GlcCer/Myrj	0	17.5	148	8.4
	2	18.0	150	8.3
	5	16.7	153	9.1
GlcCer/Myrj + PS	0	16.6	148	8.9
	2	17.1	152	8.9
	5	16.5	150	9.1
Control cells (no lipid)		15.8	149	9.4

nous materials, we repeated the experiment with lower concentrations of GlcCer (5 $\mu\text{g}/\text{ml}$). The resultant GlcCer spot on the TLC plate was fainter than before, but nevertheless quite distinct. At this level too there was little or no detectable disappearance of the glycolipid over a 5-day period. Phosphatidylserine (5 $\mu\text{g}/\text{ml}$), added with the GlcCer, was also absorbed but the elevation in cellular PS level was found to disappear after 2 days. The PS was clearly effective in speeding the disappearance of GlcCer. Our observations are consistent with the report that an emulsion of GlcCer with PS, injected intravenously into rats, resulted in uptake of the cerebroside by liver and spleen and its subsequent rapid disappearance [24].

The ability of exogenous glucosidase, cohydro-lase, and PS to speed the disappearance of GlcCer from the model Gaucher cells was tested under the same conditions. Cells were loaded with cerebroside by a 24-h incubation with emulsified GlcCer, 5 $\mu\text{g}/\text{ml}$. TLC of the lipids (Fig. 4) showed again that a distinct amount of cerebroside had entered the cells (lane B vs. control cells, lane A) and that most of the cerebroside was still present after 24 h of exposure to stock medium (lane C vs. B). Cells which were exposed to glucosidase (550 cerebroside units) for 24 h showed a distinct decrease in cerebroside content (D vs. C). Exposure to PS alone, 5 $\mu\text{g}/\text{ml}$, produced an even more effective disappearance of the lipid (E vs. D) and the combination of enzyme and PS was more effective than either alone (F vs. D or E). Glucosidase with

cohydro-lase (375 $\mu\text{g}/\text{ml}$) was not more effective than glucosidase alone (G vs. D), an observation to be expected from the previous data. The stron-

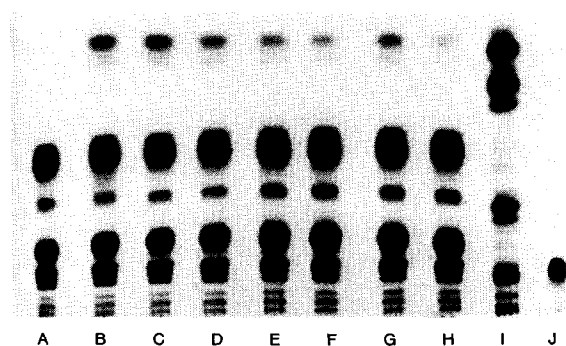


Fig. 4. Thin-layer plate showing neuroblastoma lipids, developed with chloroform/methanol/aqueous KCl in ammonium hydroxide. The highest spot is due to GlcCer. Each lane was derived from 1/30th of the lipids extracted from two flasks of cells. Lane A is from cells treated only with stock medium; lanes B–H are from cells which had been loaded with glucocerebroside for 24 h, then washed with fresh medium and treated for another 24 h with various media. Lane B: cells extracted immediately after washing, showing the accumulated glucolipid. Lane C: cells exposed only to stock medium for 24 h, showing only slight disappearance of glucolipid. Lane D: exposed 24 h to medium augmented with glucosidase. E: exposed 24 h to PS medium. F: exposed to glucosidase + PS. G: glucosidase + cohydro-lase. H: exposed to all three, glucosidase + PS + cohydro-lase. Standards in lane I: three families of galactosylceramide at the top, sulfatide at the center, sphingomyelin near the bottom. Lane J: phosphatidylserine, a little faster moving than sphingomyelin on this plate. The darkest spot in the cell lipids is phosphatidylethanolamine and the next darkest is phosphatidylcholine.

gest effect was produced by a mixture of the three, enzyme + cohydrolase + phospholipid, which produced almost complete disappearance of the stored glucolipid (lane H).

In a similar experiment, the effect of PS plus cohydrolase (without added enzyme) was tested. The presence of labeled PS during the second 24 h incubation period (9500 cpm in each pair of culture dishes) was found to stimulate the disappearance of the glycolipid, as noted before. The presence of cohydrolase alone had little or no effect on the amount of GlcCer remaining in the cells, and cohydrolase did not enhance the effect of PS.

The amounts of radioactivity found in the cells were 879 and 723 cpm (about 8%), with and without added cohydrolase, respectively. Only a very small proportion of the absorbed labeled serine in the phospholipid was found in other cell components after the 24-h period. This makes it unlikely that the effect of PS was due to conversion to other substances.

A repetition of the experiment shown in Fig. 4 was made with labeled GlcCer to place the observations on a more precise basis and to prove that the stimulation of GlcCer disappearance was

due to hydrolysis rather than enhanced addition of sugar moieties. The control cells (Table VIII) took up 93 out of 200 μg of GlcCer added. Cohydrolase produced a small but measurable stimulation of GlcCer disappearance, which had not been detected in the previous visual estimation experiments. Otherwise, the results were much like those observed in Fig. 4. An important finding was that the polar lipids, eluted from the silica gel columns with chloroform/methanol/water 10:10:1, contained only a very small amount of radioactivity (3% or less). Moreover, the ganglioside fraction, obtained by partitioning the total lipids between chloroform, methanol, and water [25], also contained very little activity (3% or less). These observations show that the decreased activity in the cells treated with CH + PS + glucosidase was not the result of enhanced synthetic activity (to form oligosaccharide ceramides).

Discussion

Our observations are consistent with previously published evidence indicating that glucosidase forms a complex with PS [4-7]. We found, by enzyme assay and radioactivity monitoring (Table VI and Figs. 2 and 3), that each of these substances enhanced the uptake of the other. The complex of the two substances, once they were absorbed by the cells, was effective in stimulating the hydrolysis of previously absorbed GlcCer (Table VIII and Fig. 4). Phosphatidylserine alone also stimulated GlcCer hydrolysis, evidently interacting within the cells with the endogenous glucosidase, which was not in a fully activated state. Cultured fibroblasts and lymphocytes from some Gaucher patients have also been shown to respond similarly to PS added in vitro [26,27]. It may be noted that our assay for glucosidase in vitro (Table VI, line 6) did not show the stimulation effect, apparently because the absorbed PS - 0.8 μg in the assay system - dissociated by dilution in the assay procedure.

That cohydrolase and PS do not interact with one another was indicated by the failure of either substance to enhance the uptake of the other. This too agrees with previous work [5,6], which showed that cohydrolase had little stimulatory effect on glucosidase in vitro unless an acidic lipid was

TABLE VIII
DISAPPEARANCE OF STORED GLUCOCEREBROSIDE FROM NEUROBLASTOMA CELLS

Two dishes of cells were preloaded with GlcCer (100 cpm/ μg) for 24 h, washed with fresh medium, then incubated for 24 h with medium containing one or more additions. The lipids in the cells were extracted and the GlcCer was isolated by silica gel chromatography. The decrease in cellular GlcCer was calculated by comparison with the radioactivity of GlcCer in cells that were not incubated for the second 24 h period. CH, cohydrolase.

Addition	GlcCer decrease (% of control)
None	2
Glucosidase	22
Phosphatidylserine	38
Cohydrolase	11
Glucosidase + PS	52
Glucosidase + CH	27
Cohydrolase + PS	24
Glucosidase + PS + CH	74

present. However, cohydrolase did enhance PS uptake by the cells when the latter was bound to glucosidase. From Table VIII, lines 1 and 4, it would appear that the endogenous glucosidase in the neuroblastoma cells was almost fully saturated with cohydrolase.

The mechanism of glucosidase uptake remains obscure and we know only that PS and PS + cohydrolase improve its absorption. Perhaps the enzyme, without added acidic lipid, binds to acidic lipids in the plasma membrane and the complex is then incorporated into a type of lysosome. The complex of enzyme and lipid, presented to the cells exogenously, may contain the enzyme as part of a micellar structure dominated by the lipid. This micelle may enter the cells by permeating the plasma membrane. Cohydrolase, on the other hand, seems to be absorbed by a sugar-sensitive receptor region (Table III). The ternary complex formed between cohydrolase, PS and glucosidase appears to be absorbed by this mechanism, since its uptake could be blocked by mannose phosphate and pentamannosyl phosphate despite the possible presence of micellar PS. The pentasaccharide has been found to strongly inhibit glucuronidase uptake, apparently through its competition for mannosyl phosphate receptors [10].

We found by direct measurement that PS and cohydrolase together improved the ability of cells to take up GlcCer glucosidase and, importantly, that the enzyme taken up in this manner was in a cellular location that allowed it to act on its stored substrate. This observation may be of value in the treatment of Gaucher disease. Administration of the ternary complex would utilize chemically unmodified enzyme and therefore may be safer than the chemical derivatization approach, in which the enzyme is attached chemically to an oligosaccharide or glycoprotein and might thus produce immune reactions.

The injection of Gaucher patients with highly purified glucosidase has not yet proved to be effective enough to reverse the symptoms [28,29]. One could hypothesize that there is already a great surplus of cohydrolase in these patients and that the injected enzyme ought to reach the cohydrolase and become activated. Apparently this does not happen. One possible explanation is that the amount of available acidic phospholipid, which is

needed for forming the active ternary complex, is inadequate. It is possible, therefore, that glucosidase should first be combined with PS before injection.

Experiments with mice, in which the level of liver GlcCer rose as the result of injecting emulsified GlcCer or a glucosidase inactivator, showed that the animals responded with a rapid increase in the level of liver cohydrolase [21]. This response, which mimicked that of Gaucher patients, suggests that the activator protein is elevated by the cells in order to stimulate the endogenous glucosidase and normalize the GlcCer level. In view of the known role of acidic phospholipids in activating the glucosidase, and our uptake findings, it is possible that the cellular level of PS is another important controlling factor.

Phosphatidylserine may also play a specific role in cellular uptake mechanisms. It has been shown that red cells coated with this lipid are taken up by the reticuloendothelial system much more effectively [30]. It also aids the uptake of sterols when mixed with sterol carrier protein [31,32], the uptake of γ -aminobutyric acid by synaptosomes [33], and the uptake of galactosylceramide and lactosylceramide by cultured fibroblasts [34]. In this last study, the acidic lipid was found to accelerate the hydrolysis of the glycolipids, a finding like ours.

Since cells and intact animals [21] readily absorb GlcCer, when emulsified with Myrj 52, it becomes possible to induce a model form of Gaucher disease *in vitro* and *in vivo*. This may ease the problem of testing chemotherapeutic treatments for the disorder. While the cell type used in the present study is derived from brain, the symptoms of Gaucher disease are derived primarily from reticuloendothelial cells, which could be studied by this approach.

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