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STUDIES OF HUMAN ACID BETA-GLUCOSIDASE AND TYPE 1 GAUCHER DISEASE

City University of New York PH.D. 1981

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STUDIES OF HUMAN ACID β-GLUCOSIDASE AND TYPE 1 GAUCHER DISEASE

by

BRIDGET SHAFIT-ZAGARDO

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1981

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STUDIES OF HUMAN ACID β-GLUCOSIDASE AND

TYPE 1 GAUCHER DISEASE

by: Bridget Shafit-Zagardo

Advisor: Professor R.J. Desnick

ABSTRACT

Human placental acid β-glucosidase (EC 3.2.1.45) was purified 3900-fold to a specific activity of 270,000 nmoles/h/mg protein and was found to be free of 12 lysosomal hydrolases. Included among the standard purification steps and hydrophobic chromatography were chromatography on dextran sulfate-Sepharose and sucrose gradient ultracentrifugation. Partially purified enzyme bound to dextran sulfate-Sepharose was eluted with crude taurocholate, resulting in a high yield and a 10-fold purification. Sucrose gradient ultracentrifugation was useful at the later stages of purification, separating β-glucosidase from the two major contaminating hydrolases, β-glucuronidase and β-hexosaminidase B.

A fluorescent natural substrate assay was developed using the compound 12-[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]-amino dodecanoic acid-glucosyl ceramide (NBD-glucer). This substrate was specific for acid β-glucosidase, was sensitive in the picomole range and was easily synthesized. The ratio of the amount of natural:artifical (4-methylumbelliferyl-β-D-glucopyranoside, 4MUG) substrate hydrolyzed was ∿10:1. The apparent K_m's of acid β-glucosidase for the natural and artifical substrates were 0.285 mM and 2.65 mM, respectively. The K_m observed with the

natural substrate was in agreement with published values for the enzyme utilizing the radiolabeled natural substrate.

An electrophoretic system using cellulose acetate has been developed for the resolution of acid and neutral β-glucosidase in human tissue homogenates. Electrophoresis of homogenates from normal and Type 1 Gaucher disease tissues revealed two fluorescent bands of β-glucosidase activity which correspond to the acid and neutral enzymes separated by concanavalin A-Sepharose chromatography. The acid enzyme had only β-glucosidase activity, hydrolyzing both the natural (NBD-glucer) and the artifical (4MUG) substrates.

Neutral β-glucosidase (EC 3.2.1.21) also exhibited α-L-arabinosidase (EC 3.2.1.55), β-D-galactosidase (EC 3.2.1.23) and β-D-xylosidase (EC 3.2.1.37) activities, and did not hydrolyze the natural substrate, NBD-glucer.

In homogenates of cultured skin fibroblasts, only acid β-glucosidase was observed which co-electrophoresed with the acidic activity in other tissue homogenates. The acidic activity in tissue and fibroblast homogenates from Type 1 Gaucher disease appeared to co-electrophorese with the acid β-glucosidase in normal tissues, but had markedly reduced activity.

Acid β-glucosidase effector isolated from normal and Type 1 Gaucher fibroblast and spleen homogenates was found to retard purified acid β-glucosidase migration during electrophoresis on cellulose acetate. Following a 55% ammonium sulfate cut, the effector was separated from acid β-glucosidase. Sucrose gradient ultracentrifugation demonstrated that the effector, isolated from normal and Type 1 Gaucher spleen homogenates, bound to the purified enzyme and increased its specific activity.

Finally, the structural gene for human acid β-glucosidase has been

assigned to chromosome 1 using somatic cell hybridization techniques for gene mapping. The human enzyme was detected in mouse RAG cell-human fibroblast cell hybrids by a sensitive double antibody immunoprecipitation assay using a mouse anti-human acid β-glucosidase antibody. No cross-reactivity between mouse β-glucosidase and human acid or neutral β-glucosidase was observed. Fifty-two primary, secondary, and tertiary man-mouse hybrid lines, derived from three separate fusion experiments, were analyzed for human acid β-glucosidase and enzyme markers for the human chromosomes. Without exception, the presence of human acid β-glucosidase in these hybrid clones was correlated with the presence of human chromosome 1 or its enzymatic markers, phosphoglucumutase-1 (PGM1) and fumarate hydratase (FH). All other human chromosomes were eliminated by the independent segregation of acid β-glucosidase and their respective enzyme markers and/or chromosomes. Using a RAG x human fibroblast line with mouse-human rearrangement of human chromosome 1, the locus for acid β-glucosidase was limited to the region 1p11 to 1qter.

FORWARD

Portions of this thesis have been presented in the following publications:

Shafit-Zagardo, B., Devine, E.A., and Desnick, R.J., 1980, Electrophoretic separation of neutral and acid β-glucosidase isozymes in human tissues. Biochim. Biophys. Acta 614:459-465.

Shafit-Zagardo, B., Devine, E.A., Smith, M., Arredondo-Vega, F., and Desnick, R.J., Assignment of the gene for acid β-glucosidase (GBA) to human chromosome 1. Am. J. Hum. Genet., in press.

Gatt, S., Dinur, T., Barenholz, Y., Ben-Gerson, Z.L., Rosenthal, J., Devine, E.A., Shafit-Zagardo, B., and Desnick, R.J., Assay of glucosyl ceramide using fluorescent derivatives of glucocerebroside. In: Methods of Enzymology, in press.

Shafit-Zagardo, B., and Turner, B.M., Human β-glucosidase inhibition by sulfates and purification by affinity chromatography on dextran sulfate-sepharose. Biochim. Biophys. Acta, in press.

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Aconitase, mitochonrdrial	ACON2
Aconitase, soluble	ACON1
Adenosine deaminase	ADA
Adenylate kinase, mitochondrial	AK2
Adenylate kinase, soluble	AK1
Approximately	~
N-N'-bismethylene acrylamide	bis
Bovine serum albumin	BSA
Centigrade (degrees)	°C
Centimeter	cm
Concanavalin-A	con A
Distilled water	H_2O
Enolase	ENOL
Esterase D	ESD
α-Fucosidase	FUCA
Fumarate hydratase	FH
Galactokinase	GALK
β-Galactosidase 1	GLB1
Glucose-6-phosphate dehydrogenase	G6PD
Glucosephosphate isomerase	GPI
α-Glucosidase	GLUA
β-Glucosidase (acid)	GBA
β-Glucosidase (neutral)	GBN
β-Glucuronidase	GUSB

Glutamate oxaloacetate transamindase, soluble	GOT1
Glutathione reductase	GSR
Gram	g
Hexosaminidase A	HEXA
Hexosaminidase B	HEXB
Hour	h
Hypoxanthine, aminopterin, thymidine	HAT
Hypoxanthine-guanine phosphoribosyl-transferase	HGPRT
Kilograms	kg
Lactate dehydrogenase A	LDHA
Lactate dehydrogenase B	LDHB
Liter	l
Malate dehydrogenase, soluble	MDH1
Malic enzyme, soluble	ME1
Mannosephosphate isomerase	MPI
2-Mercaptoethanol	2-ME
4-Methylumbelliferyl	4MU
4-Methylumbelliferyl-β-D-glucopyranoside	4MUG
Microgram	ug
Microliter	ul
Milliamps	ma
Milligrams	mg
Millililiter	ml
Millimeter	mm
Millimolar	mM
Minute	min
Molecular weight	MW

Nanometer	nm
Nanomole	nmol
12-[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]	NBD
NBD-glucosyl ceramide	NBD-glucer
Nucleoside phosphorylase	NP
Optical density	OD
Peptidase A	PEPA
Peptidase B	PEPB
Phosphoglucomutase-1	PGM1
Phosphoglucomutase-2	PGM2
Phosphoglycolate phosphatase	PGP
Primary somatic cell hybrids	P
Retention factor	R_f
Second	sec
Secondary somatic cell hybrids	S
Sodium dodecyl sulfate	SDS
Disodiumethylenediaminetetraacetate	EDTA
Superoxide dismutase, mitochrondrial	SOD2
Superoxide dismutase, soluble	SOD1
Tetramethylethylenediamine	TEMED
Thymidine kinase	TK
Trichloroacetic acid	TCA
Ultraviolet	UV
Units	U

BACKGROUND AND RATIONALE

I. Gaucher Disease:

A. Subtypes and Clinical Aspects: Gaucher disease is a family of disorders resulting from the deficient activity of the lysosomal hydrolase, acid β-glucosidase or glucocerebroside:β-glucosidase (EC 3.21.45), and the subsequent accumulation of its substrate, glucosyl ceramide (Figure 1). There are 3 clinically distinct subtypes of Gaucher disease. The features shared by patients with each of these forms include an autosomal recessive mode of inheritance, hepatosplenomegaly and Gaucher cells in the bone marrow (1,2).

The Gaucher cell is an enlarged lipid-laden histiocyte that is found dispersed throughout the reticuloendothelial system, especially in the red pulp of the spleen, the sinusoids and medullary portions of lymph nodes, sinusoids of the liver and the bone marrow.

Under phase microscopy, the characteristic Gaucher cell ranges from 20 to 100 um in diameter with an eccentric nucleus. The cytoplasm contains fibrils with a "crinkled paper" appearance. Rod shape inclusion bodies are discernible by Normarski interference microscopy. The Gaucher cells stain positively for acid phosphatase when phenylphosphate is used as substrate. The cells will also stain for carbohydrates when the periodic acid Schiff reagent is present (2).

The most common form of Gaucher disease is Type 1 or the adult form. This subtype was first described by Phillipe Gaucher in 1822, and his original description was recognized by the use of his name as the eponym for the disease. Although Type 1 Gaucher disease is about 30 times more prevalent in Ashkenazi Jews than in all other groups, it has a panethnic distribution. The incidence in individuals of Ashkenazi ancestry is 1 in

every 2500 births and the heterozygote frequency is about 1 in 25. The age of onset varies from the first week of life to the eighth decade, and the clinical expression is highly variable, ranging from mild to severe. There is no neurological involvement in patients with Type 1 disease. The progressive accumulation of the glycosphingolipid substrate, glucosyl ceramide, in Gaucher cells in the bone marrow and the lysosomes of reticuloendothelial cells causes the pathologic alterations which lead to the clinical manifestations of the disease. Accumulation of Gaucher cells in the bone marrow results typically in chronic bone pain and pathologic fractures. Substrate accumulation in the spleen causes splenomegaly, and the hematologic consequences of thrombocytopenia and anemia. Other organs whose function is impaired by progressive substrate deposition include the lungs, lymph nodes, intestines and liver. Severe involvement can lead to debilitating and incapacitating disease.

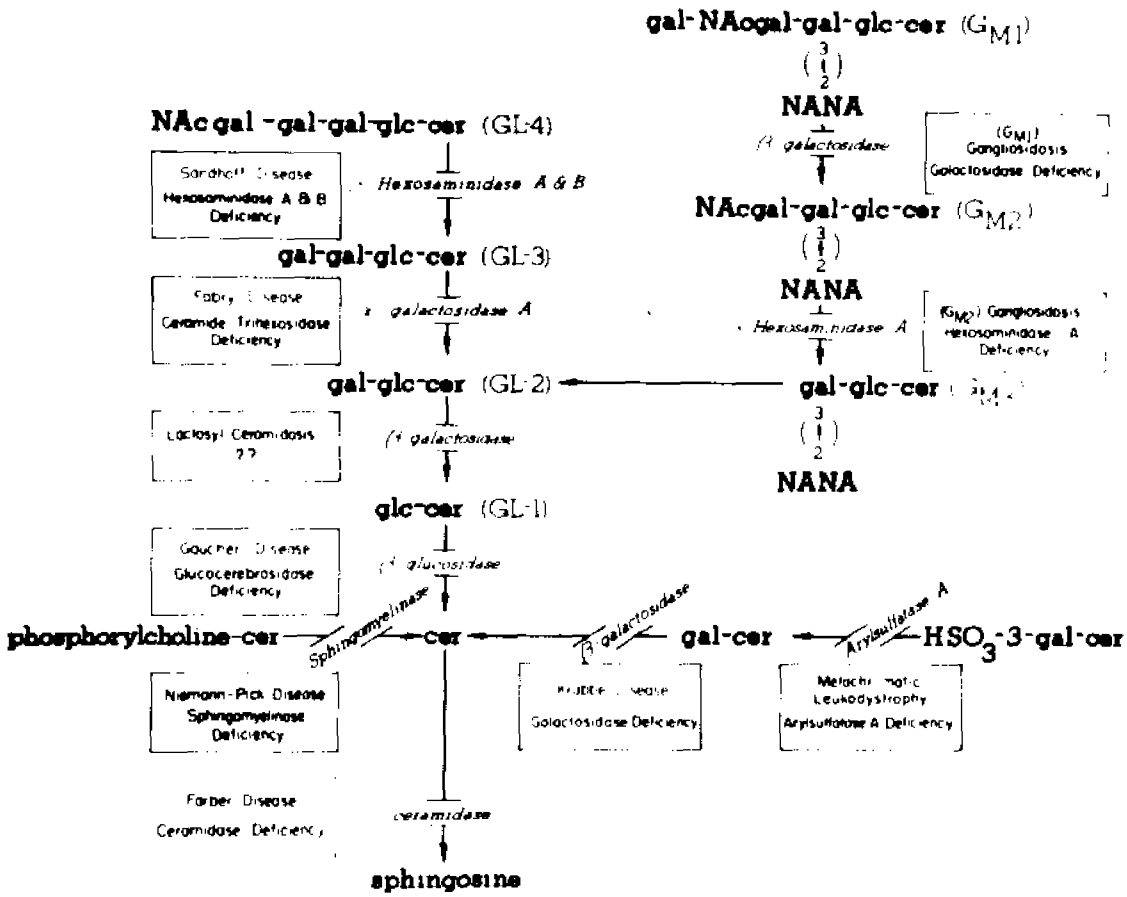
Type 2 or infantile Gaucher disease occurs in all ethnic groups and symptoms are usually apparent by 6 months of life. Severe neurological involvement leads to death in early childhood. The neurologic signs include strabismus, muscular hypertonicity and retroflexion of the head; some patients have seizures. In addition, these infants have severe involvement of the reticuloendothelial system with remarkable hepatosplenomegaly.

The third clinical subtype, Type 3 or juvenile Gaucher disease, is characterized by neurologic involvement occurring in late childhood or adolescence. Convulsive episodes, abnormal electroencephalograms, hypertonicity, strabismus and coordination difficulties are seen in patients with this subtype. These patients also have marked reticuloendothelial involvement. Type 3 Gaucher disease occurs primarily in a genetic isolate

Figure 1: Catabolic Pathway of the Sphingolipids

The position of each genetically determined metabolic block and the resulting sphingolipidosis is indicated. Gaucher disease is shown as a deficiency of acid β-glucosidase. Normally, the enzyme hydrolyzes glucosyl ceramide (GL-1) to ceramide (cer).

GL-4: globoside; GL-3: globotriglycosyl ceramide; GL-2: lactosyl ceramide; GM1: II^3-α-N-acetylneuraminosyl-gangliotetraglycosylceramide; GM2: II^3-α-N-acetylneuraminosyl-gangliotriglycosylceramide; GM3: II^3-α-N-acetylneuraminosyl-lactosylceramide; NANA: n-acetylneuraminic acid; SO_3H-galcer: sulfatide; gal-cer: galactosylceramide.


gal-NAcgal-gal-glc-cer (G_{M1})
NANA
β galactosidase
(G_{M1}) Gangliosidosis
Galactosidase Deficiency
NAcgal-gal-glc-cer (G_{M2})
NANA
β Hexosaminidase A
(G_{M2}) Gangliosidosis
Hexosaminidase A
Deficiency
NAc gal -gal-gal-glc-cer (GL-4)
Sandhoff Disease
Hexosaminidase A & B
Deficiency
β Hexosaminidase A & B
gal-gal-glc-cer (GL-3)
Fabry Disease
Ceramide Trihexosidase
Deficiency
α galactosidase A
gal-glc-cer (GL-2)
gal-glc-cer
NANA
Lactosyl Ceramidosis
??
β galactosidase
glc-cer (GL-1)
Gaucher Disease
Glucocerebrosidase
Deficiency
β glucosidase
phosphorylcholine-cer
Sphingomyelinase
cer
β galactosidase
gal-cer
Arylsulfatase A
HSO_3-3-gal-cer
Niemann-Pick Disease
Sphingomyelinase
Deficiency
Krabbe Disease
Galactosidase Deficiency
Metachromatic
Leukodystrophy
Arylsulfatase A Deficiency
Farber Disease
Ceramidase Deficiency
ceramidase
sphingosine

among individuals of a highly consanguineous community in Norrbotten, Sweden (2,3).

B. Enzymatic Defect: Gaucher disease is characterized by the deficient activity of the lysosomal hydrolase, acid β-glucosidase (1,2). Normally this membrane-bound enzyme catalyzes the cleavage of glucosyl ceramide to glucose and ceramide (Figure 2). Glucosyl ceramide is comprised of a long chain fatty acid linked to the nitrogen atom on carbon-2 of sphingosine forming a complex sphingolipid called ceramide. A molecule of glucose is linked by a β-glycosidic bond to carbon 1 of the sphingosine moiety of ceramide (Figure 3). The primary source of accumulating glucosyl ceramide is senescent granulocytes, since lactosyl ceramide, its metabolic precursor, is the major glycosphingolipid in polymorphonuclear leukocytes (1,2; Figure 1). The clinical symptoms observed in Gaucher disease are due to excessive intralysosomal accumulation of the glycosphingolipid, glucosyl ceramide, in reticuloendothelial cells (4-7).

C. Acid β-Glucosidase:

Assay Systems: The deficiency of acid β-glucosidase in Gaucher disease was first demonstrated by Brady et al. (1) using chemically synthesized glucosyl ceramide which was radiolabeled in the carbon 1 of the glucose moiety. Reduced levels of acid β-glucosidase activity were found in the spleen of patients with Gaucher disease (Table 1). Using nonlabeled glucocerebroside and an artificial substrate, p-nitrophenyl-β-D-glucopyranoside, Patrick confirmed these findings (18).

The fluorogenic substrate, 4-methylumbelliferyl-β-D-glucopyranoside (4MUG) is the most commonly used artificial substrate. In the presence of β-glucosidase, 4MUG is cleaved to glucose and fluorescent 4-methylumbelliferone (Figure 4) which is sensitively quantitated using a fluorometer.

Figure 2: Degredation of glucosyl ceramide to ceramide and glucose by membrane-bound acid β-glucosidase.

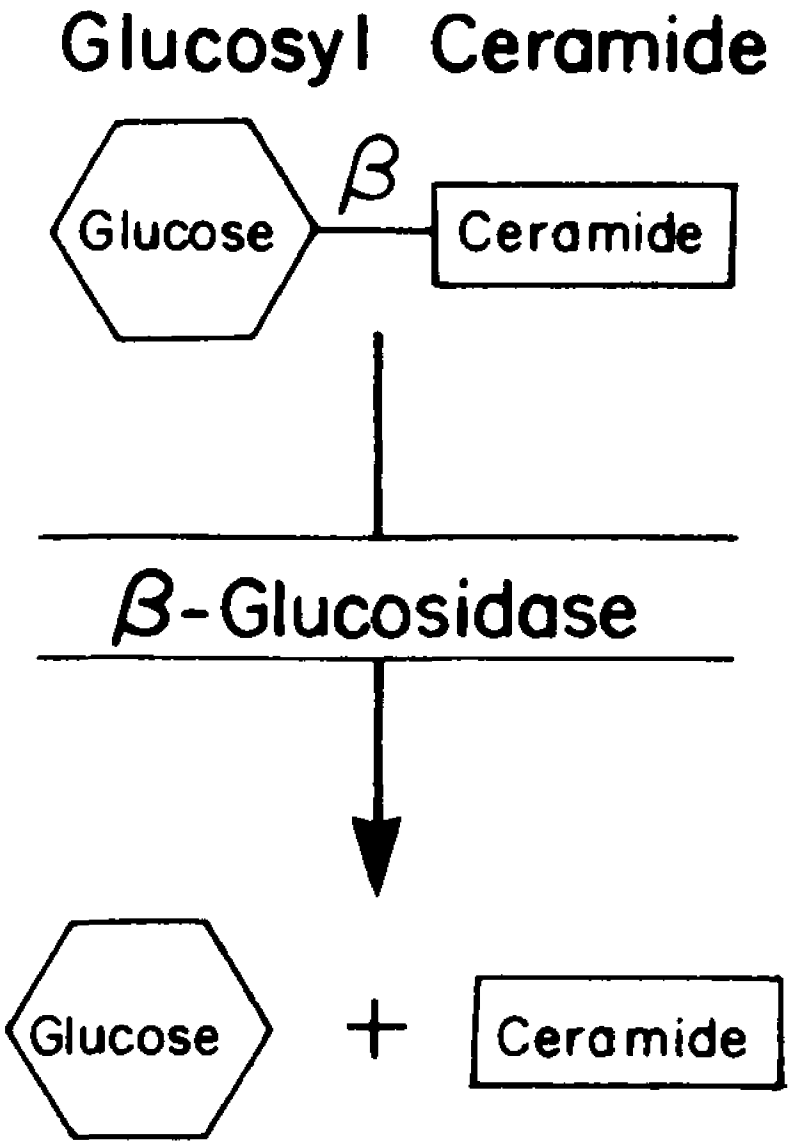

Glucosyl Ceramide
Glucose
β
Ceramide
β-Glucosidase
Glucose
+
Ceramide

Figure 3: The structure of glucosyl ceramide, the natural substrate of acid β-glucosidase.

Ceramide consists of the long chain amino alcohol, sphingosine, to which a long chain fatty acid is joined by an amide bond at the nitrogen atom on carbon 2 of sphingosine. A glucose molecule is linked to carbon 1 of the sphingosine moiety of ceramide by a β-anomeric bond (2).

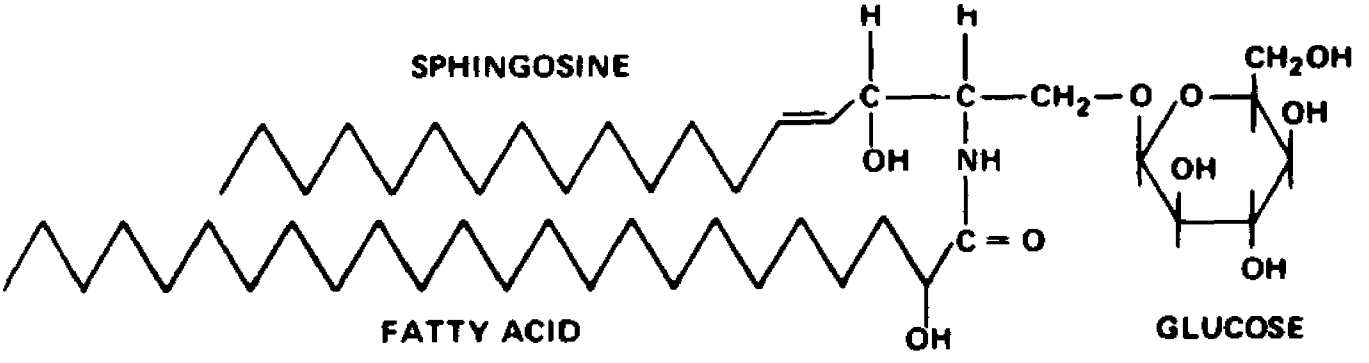

SPHINGOSINE
H
H
C
C
CH2
O
O
CH2OH
OH
OH
NH
OH
C = O
OH
OH
FATTY ACID
GLUCOSE

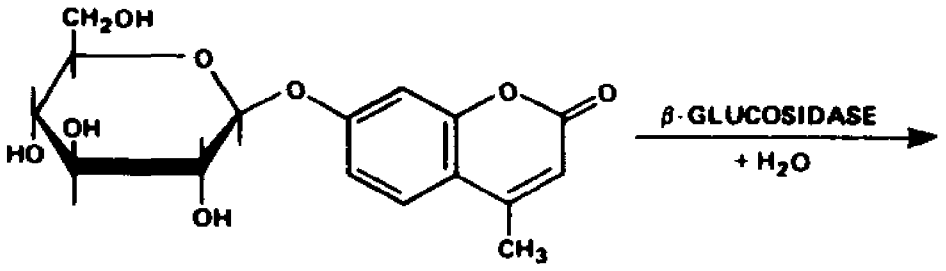
Table 1

Acid β-Glucosidase Specific Activity Observed in Control and Type 1 Gaucher (adult form) Spleens.*

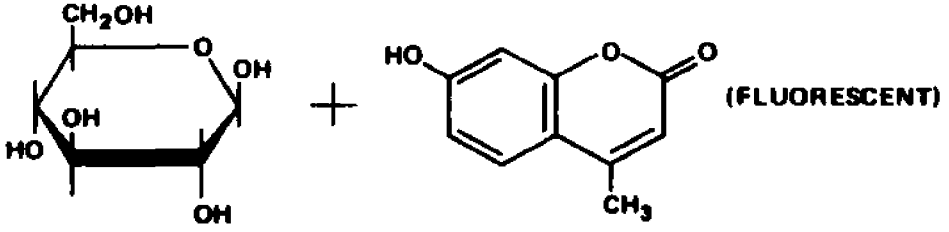
Condition	Patient	Age	Sex	Enzymatic Activity
		(years)		mumoles cleared/mg protein/h
Sickle cell anemia	MS	12	F	0.11
Congenital hemolytic anemia	WR	76	F	0.13
Hemolytic anemia	DS	15	F	0.10
Aplastic anemia	FB	68	F	0.13
Tetralogy of Fallot	CD	11	F	0.08
Mitral stenosis	JD	40	M	0.09
Congenital spherocytosis	FA	4	M	0.15
Idiopathic thrombocytopenia purpura	SM	37	F	0.11
Congenital hemolytic anemia	CF	14	F	0.10
Chronic nephritis	RP	21	M	0.12
			Mean	0.11 ± 0.001 SE
Gaucher's disease, adult form	GM	33	F	0.008
Gaucher's disease, adult form	AK	3	F	0.006
Gaucher's disease, infantile form	SZ	1	M	0.001
Gaucher's disease, adult form	SG	39	M	0.022
Gaucher's disease, adult form	SL	3	M	0.010
Gaucher's disease, adult form	PA	4	M	0.020
Gaucher's disease, adult form	JR	13	M	0.005
Gaucher's disease, adult form	TM	5	M	0.015
Gaucher's disease, adult form	MW	12	F	0.019
Gaucher's disease, adult form	GK	14	F	0.021
Gaucher's disease, adult form	SK	15	F	0.032
			Mean	0.015 ± 0.002 SE

*The Substrate Utilized was D-[1-^{14}C]-Glucosyl Ceramide (1).

Figure 4: The structure of 4-methylumbelliferyl-β-D-glucopyranoside (4MUG), the artificial substrate for β-glucosidase. In the presence of β-glucosidase, the substrate is hydrolyzed to glucose and fluorescent 4-methylumbelliferone, which can be quantitated in a fluorometer (2).



4-METHYLUMBELLIFERYL-β-D-GLUCOPYRANOSIDE



Using 4MUG, reduced β-glucosidase activity is found in liver, spleen (9, 24), leukocytes (11-15) and fibroblasts (16-20) from patients with Types 1, 2 and 3 Gaucher disease. In addition, a chromogenic substrate, 2-hexadocanoyl-amino-4-nitro-β-glucopyranoside recently has been used for the detection of individuals affected with Gaucher disease (21,22); however, this substrate offers no advantages over the 4MUG substrate.

D. Differences in Acid β-Glucosidase Activity Observed in Subtypes of Gaucher Disease: Acid β-glucosidase from cultured skin fibroblasts can be solubilized in an active form by treatment of cell homogenate preparations with Triton X-100 and taurocholate. This solubilization of fibroblast β-glucosidase caused a shift in the pH optimum from pH 4.5 (membrane-bound enzyme) to pH 6.0 (solubilized enzyme) (17). When compared with the enzyme from control fibroblasts, the acid β-glucosidase activity from Type 1 Gaucher patients of Ashkenazi origin was rapidly inactivated at 50°C (17). As shown in Figure 5, the enzymatic activity from control fibroblasts underwent a rapid loss of activity with an average half-life of 18.1 minutes. The enzymatic activity from all Jewish Type 1 Gaucher patients tested was inactivated more rapidly.

Fibroblasts from Ashkenazi patients with Type 1 disease were also found to have an altered pH curve. As shown in Figure 6, the pH curve for acid β-glucosidase in Type 1 solubilized fibroblasts showed a more alkaline pH optimum than that found in normal fibroblasts (17). In contrast, the β-glucosidase from patients with Types 2 and 3 disease was qualitatively indistinguishable from the normal enzyme in terms of pH optima and thermal stability. No difference in K_m was observed among the Gaucher subtypes (17).

When compared with cell homogenates from normal individuals the

Figure 5: Thermostability of detergent-solubilized fibroblast β-glucosidase at 50°C.

Activity is expressed as a percentage of the activity of the unheated sample. Each point represents an average obtained from several different experiments. Vertical bars represent ± 1 standard deviation (17). Chronic Form = Type 1 Gaucher homozygote of Ashkenazi ancestry.

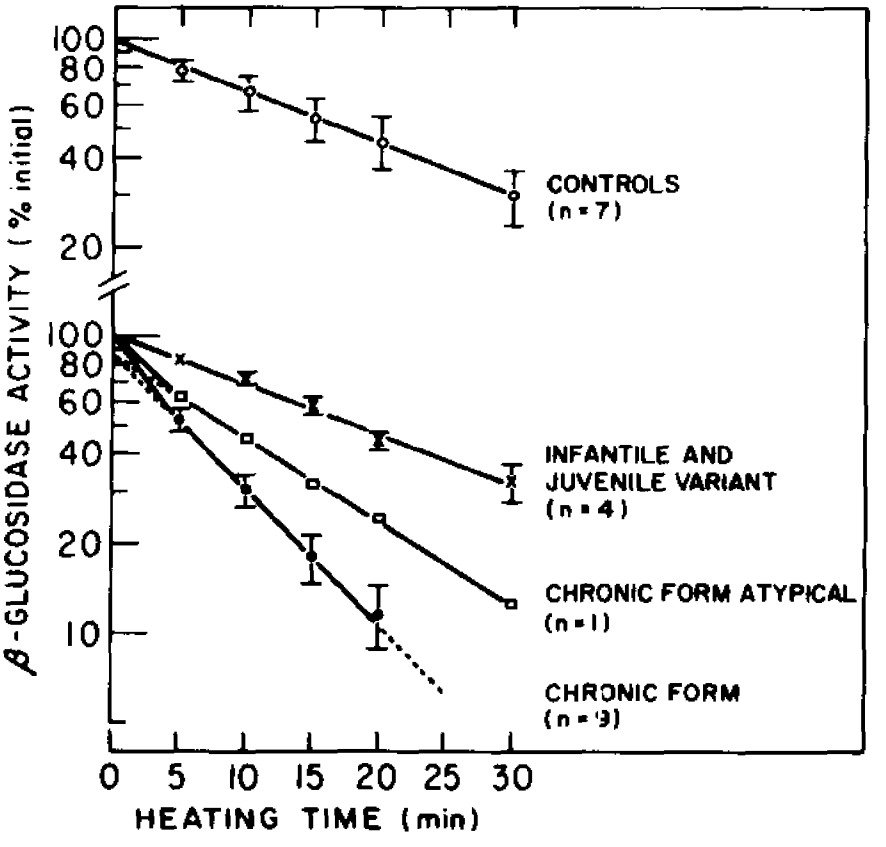
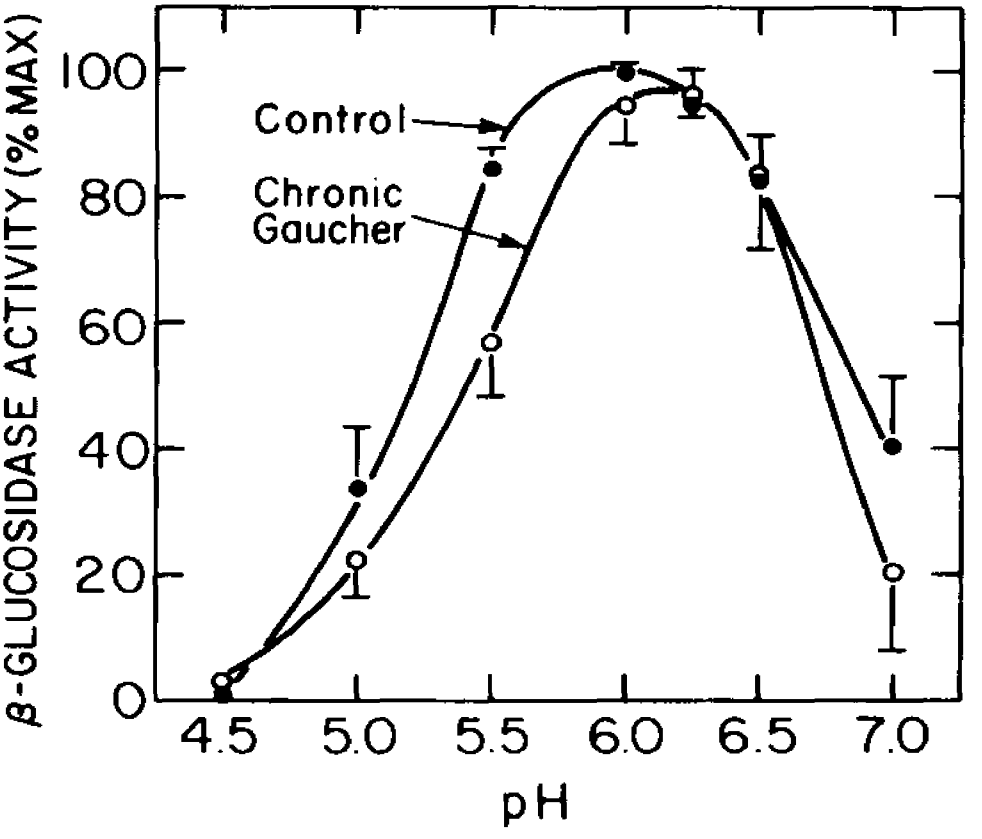

β-GLUCOSIDASE ACTIVITY (% initial)
100
80
60
40
20
100
80
60
40
20
10
CONTROLS
(n = 7)
INFANTILE AND
JUVENILE VARIANT
(n = 4)
CHRONIC FORM ATYPICAL
(n = 1)
CHRONIC FORM
(n = 3)
0 5 10 15 20 25 30
HEATING TIME (min)

Figure 6: Effect of pH on activity of solubilized β-glucosidase from control (n=7) and Type 1 Gaucher (chronic) fibroblasts (n=8). For each sample, the β-glucosidase activity was calculated as a percentage of the activity at the pH optimum. Vertical bars represent + 1 standard deviation (17).


β-GLUCOSIDASE ACTIVITY (% MAX)
100
80
60
40
20
0
Control
Chronic
Gaucher
4.5
5.0
5.5
6.0
6.5
7.0
pH

residual β-glucosidase activities observed in Types 1, 2 and 3 Gaucher disease are 20-30%, 10% and 10-15%, respectively.

E. Heterozygote Detection for Gaucher Disease: Heterozygote detection can be performed by assaying acid β-glucosidase activity in leukocytes (15,18) and cultured skin fibroblasts (17). Although some overlap does occur with the values observed for normal individuals, the majority of heterozygotes tested for Gaucher disease demonstrated activity intermediate between that of normal and affected individuals. These enzyme assays were optimized for the greatest difference in specific activity between normal and obligate heterozygotes (15,17-18).

II. β-Glucosidase Isozymes:

A. Tissue Isozymes: β-Glucosidase isozymes have been identified in normal human tissues by various colorimetric and fluorogenic artificial substrates (8,9,11,16, 21,22).

Two isozymes of membrane-bound β-glucosidase with activity towards 4MUG have been reported in cultured long term lymphoid lines (12), peripheral blood leukocytes (12,13) and spleen (23). One of these isozymes has a pH optimum of 5.0, a K_m of 0.4 mM and is rapidly inactivated at pH 4.0 (12). The function of this isozyme is unknown. The second isozyme has a pH optimum of 4.5, a K_m of 0.8 mM, is stable at acidic pH (12) and is known as glucocerebroside: β-glucosidase (EC 3.2.1.45) or acid β-glucosidase. Only this isozyme is expressed in cultured skin fibroblasts (16,17,22). Acid β-glucosidase is present in lysosomes, is activated in the presence of anionic detergents and acid phoshpholipids (17,24), and has a strong affinity for concanavalin A (25,26) indicating that the enzyme is a glycoprotein. Acid β-glucosidase cleaves both the artificial

substrates (8,16,17,21,22) and its natural substrate, glucosyl ceramide (1,8).

Neutral β-glucosidase (EC 3.2.1.22) is a soluble enzyme which is not expressed in fibroblasts but is found in kidney, spleen, liver and brain extracts (9,27,28). It has a broad pH optimum from 5.5 to 6.5, is inhibited by sodium taurocholate (24) and it hydrolyzes steroid glucosides. The function of the soluble β-glucosidase is unknown although it is found to have a broad substrate specificity and in addition to β-glucosides (9,27, 28) will hydrolyze β-galactosides, β-xylosides, β-fucosides and α-arabinosides.

B. Electrophoretic Separation of β-Glucosidase Isozymes: Three electrophoretic systems have been described to separate and visualize the isozymes of β-glucosidase (29-31). The neutral isozyme has been easily detected on starch gel and cellulose acetate gel electrophoresis. Electrophoretic analysis of the acid isozyme has been hindered by the inability of the native enzyme to migrate in conventional electrophoretic systems. Recent modifications have allowed migration and visualization of the acid isozyme in polyacrylamide (29), cellulose acetate gel electrophoretic (30) and isoelectric focusing gel (31) systems. However, none of these methods simultaneously resolved the acid and neutral isozymes.

The presence of two β-glucosidase isozymes in leukocytes was confirmed by cellulose acetate electrophoresis (30), although the same investigators were unable to resolve the isozymes in other tissue extracts. The polyacrylamide gel system (29), and the isoelectric focusing system (31) were able to visualize normal and Type 1 Gaucher activities in fibroblast extracts. No differences in the migration of normal or Type 1 Gaucher fibroblast activities was observed in the polyacrylamide gel

electrophoresis system. Isoelectric focusing of Triton X-100 solubilized activity from normal fibroblasts, revealed a major isozyme with an isoelectric point at pH 4.80 and two very minor fractions with isoionic points at 4.67 and 4.55. The minor pH 4.55 enzyme appeared in Types 1 and 2 Gaucher fibroblast extracts, however, the major band was absent in these extracts (31).

III. Purification and Characterization of Acid β-Glucosidase:

A. Normal, Human Acid β-Glucosidase: Several investigators have partially purified acid β-glucosidase (32-39). Attempts to purify and characterize human acid β-glucosidase have been hampered by its lipophilic nature and strong association with the lysosomal membrane. By conventional and hydrophobic chromatography, Pentchev et al. (32) were able to purify human placental acid β-glucosidase 4,000-fold with a yield of 5%. Glycerol and dithiothreitol were required to stabilize the purified enzyme. Ho (40) reported an affinity technique employing an immobilized glycoprotein effector, purified from the spleen of a patient with Gaucher disease (see Section IV). This immobilized effector specifically bound acid β-glucosidase in the presence of phospholipid and a 190-fold purification was achieved. Although successful, the applicability of this method to large-scale purification was limited by the availability of the effector glycoprotein and its lack of stability over prolonged storage.

Recently, higher yields have been achieved by affinity chromatography on concanavalin A-Sepharose (34,41) and on hydrophobic gels such as octyl- and decyl-agarose (36) and phosphatidyl serine-agarose (34). These affinity methods are relatively non-specific but can be used in sequence to obtain good purification. Braidman and Gregoriadis (41) combined concana-

valin A-Sepharose chromatography and an ethanol/chloroform precipitation to purify human acid β-glucosidase 1500-fold with a final yield of 1.4%. Dale and Beutler (34) also exploited the hydrophobic and glycoprotein nature of the enzyme in their purification procedure. The enzyme was chromatographed on the hydrophobic support, phosphatidyl serine-Sepharose 4B, as well as on the glyco-affinity support, concanavalin A-Sepharose. They obtained a 6,000-fold purification with a 60% yield. They noted (34) that the 60% yield represented a "modest exaggeration" since the pH shifted from pH 5.0 to 6.0 during the purification while all the enzymatic assays were performed at pH 6.0. Furthermore, con-A contamination of the acid β-glucosidase preparation proved to be highly toxic to cells and, therefore, not suitable for administration in enzyme replacement therapy trials (36). Furbish et al. (36) utilized butanol extraction and hydrophobic chromatography to purify placental acid β-glucosidase. A 30% yield was obtained with a 3,000-fold purification. Table 2 summarizes the specific activities obtained using the above purification procedures. The degree of purity of these preparations could not be determined on native polyacrylamide gel electrophoresis since the enzyme would not enter the gel. On SDS gel electrophoresis, Dale and Beutler (34) found multiple bands and Furbish et al. (36) observed two major bands each with an apparent molecular weight of approximately 67,000. Pentchev et al. (32) noted that the denatured enzyme migrated as a single band with a molecular weight of 60,000. Following Sephadex G-200 chromatography, the molecular weight of acid β-glucosidase varied from 60,000 - 300,000 depending on the method of extraction (32,36). Furbish et al. estimated the molecular weight to be 87,000 - 92,000 (36). The apparent K_m of β-glucosidase with the natural substrate was reported to be 65 uM (32) and 87 uM (36) and the

Table 2

Specific Activity Obtained Using
The Published Purification Protocols

Specific Activity*				
4MUG	D-[1-^{14}C]-Glucosyl Ceramide	Purification (fold)	Yield (%)	Reference
0.078	1.0	4,000	5.0	32
--	0.03	190	60.0	40
0.15	--	6,000	60.0	34
--	0.165	1,500	1.4	41
--	1.1	3,000	30.0	36

*10^6 nmoles/h/mg protein

pH range extended from pH 4.5 to 7.5 (36).

B. Comparison of Acid β-Glucosidase from Normal and Gaucher Spleens: Using butanol extraction and hydrophobic chromatography, acid β-glucosidase was purified from normal and Gaucher patients' spleen with specific activities of 8.5×10^5 and 5.4×10^4 nmoles/h/mg protein, respectively (38). Although the acid β-glucosidase activities from the two sources co-purified, the specific activity of the Gaucher spleen enzyme was 6% of the specific activity of the normal β-glucosidase. Table 3 shows that the enzyme preparations were similar with respect to pH optimum and substrate specificity. Anti-β-glucosidase antibodies from the normal and Gaucher enzyme demonstrated equivalent amounts of cross-reacting immunological material in Type 1 Gaucher spleen. This data suggests the molecular defect in Type 1 Gaucher disease involves a structural alteration of acid β-glucosidase rendering the enzyme catalytically deficient (40).

IV. Acid β-Glucosidase Effector Molecule:

A. Purification and Characterization: A low molecular weight (10,000 to 20,000), heat stable glycoprotein, isolated from normal and Gaucher patients' tissues, has been shown to stimulate crude as well as purified acid β-glucosidase activity (40,42-45), using both the artificial substrate, 4MUG (42-44) and the natural substrate, glucosyl ceramide (40,43,45-47). Using 4MUG, Ho et al. (42) found that the effector stimulated acid β-glucosidase activity over a wide pH range as well as shifting the pH optimum from pH 6.0-6.5 to approximately pH 4.5. This activation was also observed with the natural substrate (45). The effector was resistant to organic solvent extraction, heat (100°C, 10 min), TCA precipitation and pronase digestion (42).

Table 3

Kinetic Properties of Purified Human Spleen Acid β-Glucosidase (38)

Parameter	Enzyme	
	Normal	Gaucher
K_m, M X 10^{-5}		
Glucocerebroside*	10.9 ± 0.9	12.6 ± 0.6
4-Methumbelliferyl-β-D-glucopyranoside	89 ± 6	178 ± 45
Maximum velocity, nmol/mg protein/h		
Glucocerebroside	8.5 X 10^5	5.4 X 10^4
4-Methylumbelliferyl-β-D-glucopyranoside	4.2 X 10^5	3.1 X 10^4
pH optimum with glucocerebroside	5.0 - 6.0	5.0 - 6.0

*Glucocerebroside = Glucosyl ceramide

The effector stimulated acid β-glucosidase activity in the presence of acid phospholipids such as phosphatidic acid, phosphatidylserine and phosphatidylinositol, and Triton X-100, but not in the presence of sodium taurocholate (42,45,48,49). However, Peters et al. (44) observed no additional stimulation of acid β-glucosidase activity by the effector in the presence of phospholipids.

The effector appears to stimulate acid β-glucosidase activity by binding directly to the enzyme (40). Purified effector coupled to cyanogen bromide-activated Sepharose selectively bound acid β-glucosidase in the presence of phosphatidylinositol (Figure 7). The enzyme was eluted by altering the pH (4.5→ 7.0). The acid β-glucosidase did not bind to Sepharose 4B alone. The effector column did not bind β-galactosidase or β-N-acetyl-glucosaminidase; furthermore, β-galactosidase activity was inhibited 90% in the presence of effector (0.1 mg/ml) (40).

Using conventional ion exchange chromatography, the heat stable effector was purified from normal and Gaucher spleen (44). The two effectors differed in molecular weight (8,803 and 11,044 respectively), amino acid and carbohydrate composition and in their ability to activate human liver acid β-glucosidase. The effector from normal spleen was only 6% as active (on a protein basis) as that from Gaucher spleen. Amino acid analysis determined that Gaucher effector contained approximately 2 times more leucine, methionine, and half-cystine residues than the normal effector. Conversely, the normal effector contained higher amounts of alanine, arginine, glycine, glutamic acid and proline, indicating structural differences between the two effector molecules. The effector substance from control spleen contained negligible amounts of carbohydrate whereas the Gaucher effector contained substantial quantities of glucosamine, mannose,

Figure 7: Chromatography of acid glycosidases (40).

(a) acid β-glucosidase effector coupled to Sepharose.

(b) Sepharose column.

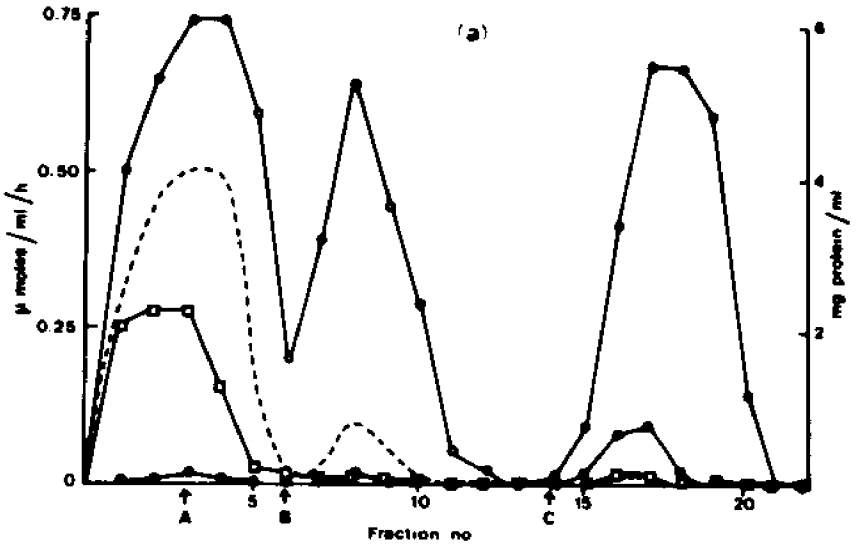
o-o = hexosaminidase

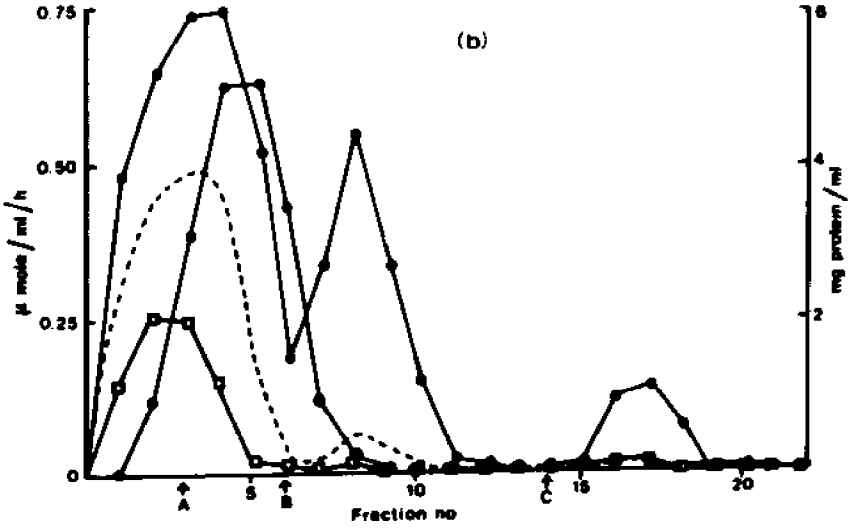
□-□ = β-galactosidase

●-● = acid β-glucosidase activity assayed with [^{14}C]-stearoylglucosylsphingosine

↑ indicates different buffers

— — — protein


(a)
0.75
0.50
0.25
0
μ moles / ml / h
mg protein / ml
6
4
2
A
5
B
10
C
15
20
Fraction no


(b)
0.75
0.50
0.25
0
μ moles / ml / h
mg protein / ml
6
4
2
A
5
B
10
C
15
20
Fraction no

fucose, galactose and sialic acid (44). To date, these results have not been substantiated by other investigators.

B. Physiological Role of the Effector Molecule: It is generally accepted that Gaucher spleen contains an effector which is able to interact with acid β-glucosidase from normal tissues and stimulate enzymatic activity *in vitro*. However, the physiological role of the effector in the degradation of glycosyl ceramide has not been determined. Activating factors have been described for cerebroside sulfatase (51) and β-hexosaminidase (52-54); the physiological significance of these factors also remains unknown. In contrast to the effector-substrate complexes observed for G_{M1} and G_{M2} ganglioside and sulfatide (51-54), the acid β-glucosidase-effector complex is the first example of an enzyme-effector interaction known for a lysosomal enzyme.

Ho suggested that the effector, along with an acidic phospholipid, was an important component in the cleavage of glucosyl ceramide by acid β-glucosidase (48). Others have argued that sodium taurocholate or acid phospholipids alone stimulate acid β-glucosidase activity more than the Gaucher effector and that the factor does not play a significant role in the metabolism of glucosyl ceramide (44,50). The findings of Peters *et al.* (44) that the effectors from normal and Gaucher spleen differ does not support a physiologic role, especially with the reduced stimulatory effect of the normal effector.

Possibly, the function of the effector in Gaucher spleen is to serve as a natural detergent in order to increase catalysis of glucosyl ceramide by the altered residual acid β-glucosidase.

V. Somatic Cell Hybridization:

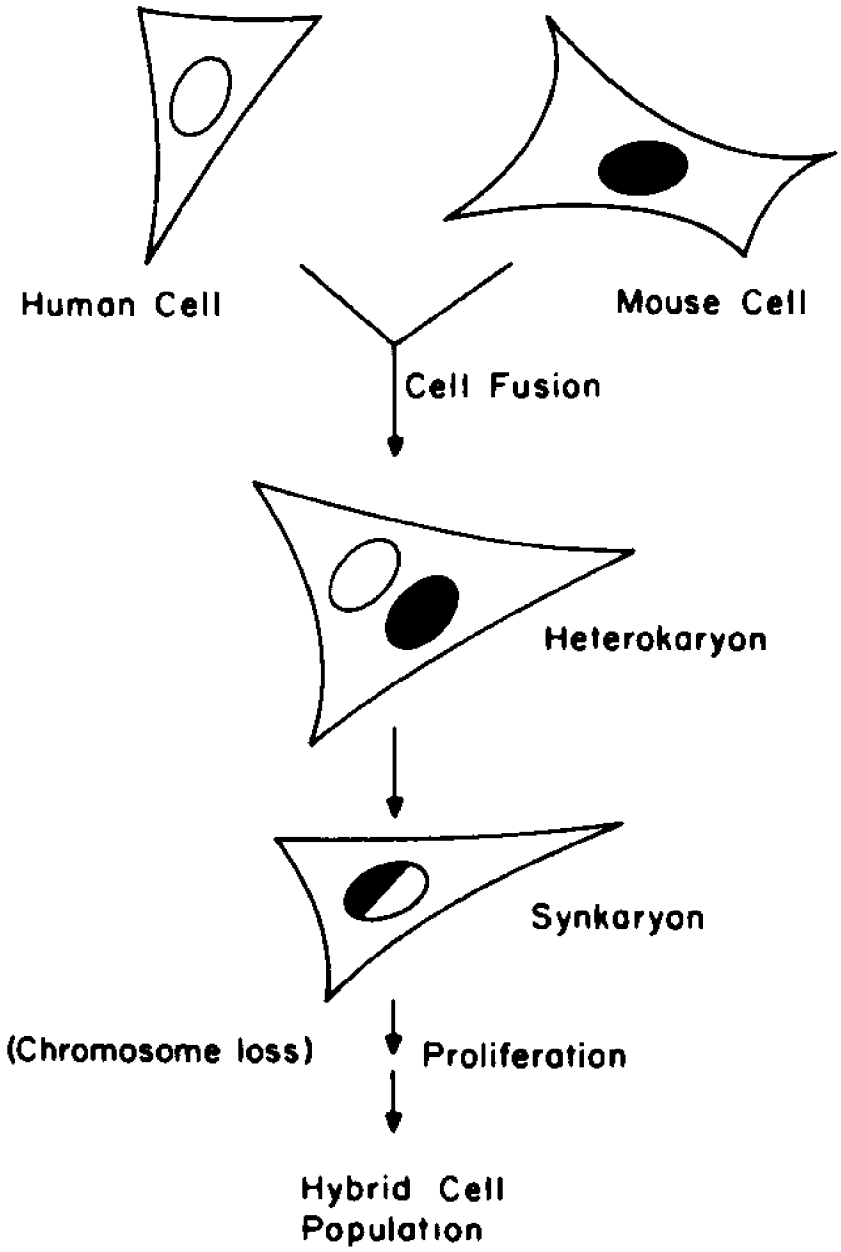
A. Chromosomal Localization of Human Genes: Somatic cell hybridization provides a powerful approach to human chromosome mapping (55,56). The first step in the process is cell fusion of human somatic cells with cells from another species (mouse, rat, Chinese hamster) (57,58) facilitated by inactivated viruses such as Sendai virus (59) or chemical agents such as polyethylene glycol (60). As shown in Figure 8, the nuclei of the hybrid cells contain the chromosomes of both parent cell types. Since hybrid cells constitute only a small proportion of the cells in mixed cultures, a selection system for the isolation of hybrid cells free from both parental cell types is used. In the HAT (hypoxyanthine, aminopterin, and thymidine) system (61), one parental cell line is deficient in thymidine kinase (TK), an enzyme of the pyrimidine salvage pathway, while the other parental cell line is deficient in hypoxanthine-guanine phosphoribosyl-transferase (HGPRT), an enzyme of the purine salvage pathway. The addition of aminopterin to the medium blocks both purine and pyrimidine de novo synthesis in these cells, and exogenous preformed purine (hypoxanthine) and pyrimidine (thymidine) is needed.

Thus, in the presence of HAT medium, only complemented hybrid cells survive. Once free of parental cells, the hybrid clones can be isolated and used for gene mapping. In further cell divisions, there is preferential loss of human chromosomes from man-rodent hybrid cells. The identification of human and rodent chromosomes is possible by specific banding techniques including Giemsa 11 staining (62) and quinicrine (63).

For actual mapping of the gene to the human chromosome, interspecies gene products must be identifiable by some cellular variation, i.e., electrophoretic variation in enzyme mobility, variation in isoelectric

Figure 8: The formation of hybrid cells by cell fusion.

Using either chemical agents or viruses, heterokaryons are formed, each cell retaining its nucleus. Following mitosis, the chromosomes combine within a single nucleus to form a synkaryon. By preferential loss of human chromosomes, populations of hybrid cells are formed (56).


Human Cell
Mouse Cell
Cell Fusion
Heterokaryon
Synkaryon
(Chromosome loss)
Proliferation
Hybrid Cell
Population

point, or detection of a species specific antibody and the subsequent design of an assay to determine precipitable product (Ouchterlony, immunoprecipitation). Ruddle and Creagan (56) and others have developed clone panels for gene mapping. Clone panels assist in the assignment of a locus to a chromosome. The location of a specific gene product to a particular human chromosome can be demonstrated by synteny testing, i.e., the concordance between the presence or absence of a specific chromosome and the specific human gene product in each hybrid clone.

For regional mapping, i.e., mapping of a human gene locus to a specific segment of a chromosome, a human cell with a chromosomal rearrangement is used. This rearrangement can involve a segment of a human chromosome translocated onto a rodent chromosome. Deletions or duplications of a specific segment of the chromosome, i.e., the long or the short arm, are also useful.

B. The Use of Antibody for Gene Product Isolation: Recently, the use of antiserum specific for a human protein, in conjunction with an Ouchterlony double diffusion or a double antibody immunoprecipitation assay has become a powerful tool for gene mapping (64). Purified human proteins injected subcutaneously or intraperitoneally, into either rabbits or more preferably, a rodent species are capable of making specific human antiserum (64,65). A human enzyme-antibody complex can be visualized by staining for protein (Coomassie Blue) following Ouchterlony double diffusion (64,65). The observed precipitin band showing a line of complete identity confirms the presence of the specific human protein in the human control and the hybrid clones. No precipitin band should be observed in either the rodent control or hybrids lacking the specific human protein and human chromosome. In the double immunoprecipitation assay, a second

antibody is used to form large aggregates that will precipitate enzyme-antibody complexes which can then be assayed for enzymatic activity. Thus, the selective precipitation of the human protein and not the rodent protein in human-rodent somatic cell hybrids, can determine the location of the human gene on its chromosome.

OBJECTIVES

The overall objective of this research was to investigate the biochemical and genetic characteristics of the human lysosomal hydrolase, acid β-glucosidase or glucocerebroside:β-glucosidase (EC 3.2.1.45). It was reasoned that characterization of the normal enzyme would provide insight into the nature of the enzymatic defect(s) observed in each of the subtypes of Gaucher disease. Biochemical, genetic and immunologic techniques were developed in order to accomplish this research. The specific research objectives were:

1) to purify acid β-glucosidase from human placenta,
2) to develop a sensitive and specific natural substrate assay for acid β-glucosidase,
3) to develop an electrophoretic system to detect electrophoretic differences in the residual activities in type 1 Gaucher disease and normal tissues,
4) to purify the acid β-glucosidase effector molecule from Gaucher and normal tissues and to determine its effect on acid β-glucosidase,
5) to produce antibodies to the purified human acid β-glucosidase by immunization of Balb/C mice, and
6) to determine the chromosomal localization of the human gene for acid β-glucosidase.

MATERIALS AND METHODS

I. β-Glucosidase Activity Using 4-Methylumbelliferyl-β-D-Glucopyranoside (4MUG) As Substrate:

A. β-Glucosidase Assay: β-Glucosidase activity was assayed with the fluorogenic substrate, 4MUG (RPI Corp., Elk Grove Village, IL). The reaction mixture contained the substrate solution (5.0 mM 4MUG, 60 ul; 0.2 M sodium phosphate-citric acid buffer, pH 6.0, 30 ul; 0.12% Triton X-100 and 1% crude sodium taurocholate, 10 ul) and column fractions or solubilized cell extract (20 ul). The reaction mixture was incubated for 15 min at 37°C and then was terminated with 4.0 ml of 0.085 M glycine-carbonate buffer, pH 10.0. The liberated 4-methylumbelliferone was quantitated in a Turner Model 111 fluorometer (G.K. Turner Assoc., Palo Alto, CA). One unit (U) of enzymatic activity represented 1 nmol of 4MUG hydrolyzed per h at 37°C.

B. pH Determination: For pH versus activity determinations, the pH of the sodium phosphate-citric acid buffer was varied from 4.5 to 7.0 by mixing 0.2 M sodium phosphate and 0.1 M citric acid to the required pH.

C. K_m Determination: For K_m determination of the purified acid β-glucosidase, the substrate concentration was varied from 0.25 mM to 5.0 mM. The results were graphed as a 1/v vs. 1/[S] plot.

II. Purification of Human Placental Acid β-Glucosidase:

A. Tissue Preparation: Placental tissue was homogenized in two volumes of distilled water and centrifuged for 30 min at 10,000 x g. All steps were carried out at 4°C. The pellet was washed in distilled water, resuspended in a volume of the extraction buffer (0.05 M phosphate-citric acid, pH 6.0, containing 0.06% Triton X-100 and 0.1% crude sodium tauro-

cholate) equal to three times the original weight of tissue, and was centrifuged as above. The solubilized extract was first precipitated with 33% ammonium sulfate and then the supernatant was precipitated with 55% ammonium sulfate (18). The pellet was resuspended in 0.06% Triton X-100 and then dialyzed overnight against 5.0 mM sodium phosphate-citric acid buffer, pH 4.0, containing 5.0 mM 2-mercaptoethanol (2-ME) and 5.0 mM ethylenediaminetetracetic acid (EDTA). The dialyzed solution was extracted with n-butanol by the gradual addition of 20% (v/v) n-butanol with rapid stirring at 2°C. After mixing 30 min, the extract was centrifuged (10,000 x g, 30 min) and the lower aqueous layer was removed and dialyzed against Binding buffer (0.05 M phosphate/citric acid, pH 6.5, 0.02% Triton X-100).

B. Dextran Sulfate Chromatography:

1. Preparation of Dextran Sulfate-Sepharose: Dextran sulfate (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) was bound to Sepharose 4B via a lysine spacer using a method based on that described by Eichmann and Greenblatt (66). In a typical preparation, 10 g of cyanogen-bromide-activated Sepharose 4B (Pharmacia) was swollen in 1 mM HCl and washed sequentially with 1 L 1 mM HCL and 100 ml 0.5 M $NaHCO_3$, pH 8.5. The activated Sepharose was suspended in 40 ml 0.5 M $NaHCO_3$, pH 8.5, containing 20 mg/ml lysine. The pH was adjusted to 8.5 and the mixture stirred gently overnight at room temperature. The gel was then washed with 500 ml aliquots of 0.2 M $NaHCO_3$, pH 8.5; 0.1 M sodium acetate, pH 4.5; distilled water and 0.5 M $NaHCO_3$, pH 8.5. The washed gel was resuspended in 40 ml of 0.5 M $NaHCO_3$, pH 8.5. Activation of dextran sulfate was carried out by mixing 20 ml of cyanogen bromide (Pierce Chemical Co., Rockford, IL), 100 mg/ml in water, with 20 ml of dextran sulfate, also at 100 mg/ml in water. The

mixture was stirred for 15 min at room temperature. The pH was then raised to 11.0-11.5 by addition of 1 N NaOH and maintained at this pH. When the pH had stabilized (5-10 min), the mixture was added to the lysine-Sepharose suspension and stirred gently at room temperature for 8-16 h. Unreacted sites were then blocked by addition of 2 volumes of 1 M glycine and the gel was put through the washing cycle described above. The gel was washed extensively with binding buffer prior to use.

2. Chromatography of β-Glucosidase: The enzyme was applied to a 100 ml affinity column of dextran sulfate. The flow rate was 80-100 ml/h. The column was washed with binding buffer until absorbance at 280 nm fell to baseline. The enzyme was then eluted with a crude sodium taurocholate gradient, 0-5 mg/ml in binding buffer. Fractions containing β-glucosidase activity were pooled, diluted 1:1 with distilled water and adjusted to pH 4.5 with 0.5 M citric acid.

C. Butanol Treatment: n-Butanol extraction was performed as above. The sample was dialyzed against 0.1 M sodium citrate buffer, pH 5.0, containing 2% (v/v) n-butanol, 5.0 mM EDTA and 5.0 mM 2-ME.

D. Octyl-Sepharose Chromatography: The dialyzed preparation was applied to an octyl-Sepharose column (Pharmacia) (36), prewashed with 0.1 N NaOH and octyl-Sepharose buffer (0.1 M sodium citrate, pH 5.0, containing 5.0 mM EDTA and 5.0 mM 2-ME). The column was washed with this buffer until the absorbance at 280 nm reached baseline. Bound enzyme was eluted with 80% (v/v) ethylene glycol in octyl-Sepharose buffer. Fractions containing β-glucosidase activity were concentrated (Amicon YM10) and dialyzed in sucrose ultracentrifugation buffer (0.15 M phosphate-citric acid buffer, pH 6.0, containing 0.02% Triton X-100, 5.0 mM EDTA, 1.0 mM 2-ME).

E. Sucrose Density Centrifugation: The enzyme was layered on linear sucrose gradients (5 to 20%, w/w). Tubes were centrifuged in a Beckman Model L5-75 ultracentrifuge at 82,000 x g in a SW-27.1 rotor for 48 h at 4°C to a final w^2t of 1.1 x 10^{12} rad^2/ sec. Fractions (0.5 ml) containing β-glucosidase activity were concentrated (Amicon YM10), dialyzed overnight against the ultracentrifugation buffer containing 25% glycerol and then stored at -20°C.

F. Protein Determinations:

1. Lowry Method (67):

Reagents:

Solution 1: 1.0 ml 1% $CuSO_4 \cdot 5H_2O$; 1.0 ml 2% Na,K Tartrate; 98.0 ml 2% Na_2CO_3 in 0.1 N NaOH.

Phenol Reagent Solution: (2N) (Fisher Scientific Products, Pittsburg, PA). (Folin-Ciocalteau Reagent) 1:1 dilution with distilled water.

Assay:

In a 12 X 75 mm test tube: 200 ul appropriately diluted enzyme or protein standard was mixed with 2.0 ml of Solution 1. After 15 min, 0.2 ml of phenol reagent was added while vigorously vortexing the tube. After 30 min, the sample was read in a Gilford spectrophotometer at 750 nm. The protein concentration was determined from a standard curve (mg/ml) using bovine serum albumin as the protein standard.

2. Fluorescamine Method (68):

Equipment: Filters for Turner fluorometer: primary - Corning 7-51, 390 nm peak; secondary - Wratten #4, 465 nm cutoff; Wratten 10% or 1% neutral density filter.

Reagents:

Borate Buffer: 0.2 M sodium borate buffer, pH 9.0 (12.4 gm boric acid, MW = 61.84 dissolved in 840 ml of distilled water, pH to 9.0 with about 6.5 ml 10 N NaOH, dilute to 1 liter).

Fluorescamine Reagent: 300 ml of acetonitrile (Burdick and Jackson Laboratories, Inc., Muskegon, MI) was swirled in a dry 500 ml Erlenmeyer flask containing about 100 gm white, non-indicating drierite. (The blue indicating drierite is unsuitable as the blue dye is soluble in acetonitrile.) After about 5 min, the acetonitrile was filtered through a cone of drierite in Whatman #1 paper. Dry acetonitrile (200 ml) is then added to 60 mg of fluram (Pierce) and stored in a dry reagent bottle.

Assay:

In a 10 X 75 mm test tube mix: 0.1 ml of appropriately diluted enzyme or protein standard, and 0.9 ml of borate buffer. While vortexing, 0.35 ml of fluorescamine reagent was added to each tube. After 5 to 15 min, fluorescence was determined on the fluorometer and the protein calculated [(dilution) (turner units) (constants) = ug protein].

III. Assay of Acid β-Glucosidase Using 12-[N-Methyl-N-(7-Nitrobenz 2-Oxa-1,3-Diazol-4-YL)]-Amino Dodecanoic Acid (NBD)-Glucosyl Ceramide:

A. Synthesis of NBD-Glucosyl Ceramide: The fluorescent natural substrate, NBD-glucosyl ceramide (NBD-glucer), has been synthesized in collaboration with Dr. S. Gatt, Professor of Neurochemistry, Hebrew University, Jerusalem.

The natural fatty acid of glucosyl ceramide was removed by controlled acid hydrolysis to yield glucosyl phychosine (69). By condensing the fluorescent fatty acid, NBD-dodecanoic acid (Molecular Probes, Plano, TX) with glucosyl phychosine, NBD-glucosyl ceramide was synthesized. The condensation was done according to the procedure of Hammastron (70) using the carbodiimide linkage, N-ethyl-N'-(3-dia-ethylaminopropyl)-carbodiimide hydrochloride. The NBD-glucosyl ceramide was purified by preparative thin-layer chromatography on 0.5 mm thick plates of silica gel HR (Analabs, Newark, DE) in a solvent system consisting of chloroform:methanol: H_2O (85:15:1.5). When chromatographed, the NBD-glucosyl ceramide migrated with the same R_f as glucosyl ceramide isolated from the spleen of a Gaucher patient.

B. NBD-Glucosyl Ceramide Assay:

1. Reagents: NBD-glucosyl ceramide:0.025 mM in chloroform/methanol, 2:1, v/v; glucosyl ceramide:0.225 mM in chloroform/methanol, 2:1, v/v; cutscum (Fisher Scientific), 5% (w/v) in chloroform/methanol, 2:1, v/v; 0.2 M sodium phosphate/ citric acid buffer, pH 5.3, containing 5.0 mg/ml sodium taurocholoate; heptane, isopropyl alcohol (Fisher) - all reagents were redistilled.

2. Assay: 0.1 ml of NBD-glucosyl ceramide, 0.1 ml glucosyl ceramide and 10 ul cutscum were pipetted into 13 X 100 mm test tubes. The mixture was evaporated under nitrogen gas and resuspended in 100 ul of phosphate/citric acid buffer containing 5.0 mg/ml taurocholate which was pre-incubated at 37°C. Enzyme and water were added to a final volume of 0.2 ml and the tubes were incubated for 1 h at 37°C. Heptane (2.0 ml) was added followed by 0.6 ml of isopropyl alcohol and 0.4 ml of water. After each addition, the tube was vigorously vortexed. The tubes were then

centrifuged at room temperature in a bench-top centrifuge at 8,000 x g. A 2.0 ml upper, heptane phase, containing cleaved ceramide, was removed and the fluorescence was determined using a Perkin-Elmer fluorometer, Model 204-A (Perkin-Elmer Corp., Elmwood, NJ) with an excitation wavelength of 460 nm and an emission wavelength of 515 nm. Uncleaved fluorescent glucosyl ceramide remained in the lower phase.

C. Calculation of Picomoles of Fluorescent Ceramide Produced: A mixture of heptane:isopropyl alcohol:water (2.0 ml:0.6 ml:0.6 ml) was centrifuged and the upper phase was removed as described above. Varying concentrations of NBD-ceramide (6.5×10^3 picomoles/ml → 4.0 picomoles/ml) were dissolved in the upper phase and the fluorescence measured in the Perkin-Elmer fluorometer. NBD-ceramide was generously supplied by Dr. S. Gatt. A standard curve of picomoles NBD-ceramide vs. fluorescence was constructed for each scale of the fluorometer. For each scale of the standard curves, the fluorescent value for 100 picomoles of NBD-ceramide was noted. Thus,

$$\text{picomoles ceramide/ml} = \frac{\text{fluorescence obtained from assay (2 ml volume) (10) (100)}}{\text{fluorescence of 100 picomoles NBD-ceramide}}$$

The 10 represents the 10-fold dilution of the NBD-glucosyl ceramide by the natural glucosyl ceramide in the actual assay.

D. pH Determination: The pH of the phoshpate/citric acid buffer was varied from pH 3.5 to 7.0 with 0.5 increments over the total range and 0.1 increments from pH 5.0 to 6.0 as described in Section IIIB.

E. Sodium Taurocholate Determination: The assay was performed as

described in Section IIIB. The taurocholate concentration was varied from 2.5 to 40 mg/ml.

F. Discrimination Assay Between NBD-Glucosyl Ceramide and Non-Fluorogenic Glucoysl Ceramide: The assay was performed as described in Section IIIB with the following modification: the molarity of the glucosyl ceramide was varied from 0.250 mM to 0.125 mM while the NBD-glucosyl ceramide varied from 0.125 mM to 0.0 mM. The results were plotted as fluorescent substrate hydrolyzed vs. the % fluorescent NBD-glucosyl ceramide present in the assay.

G. K_m: The assay was performed as described above however, the total glucosyl ceramide concentration was varied from 0.0125 mM to 0.250 mM. The results were plotted according to Lineweaver-Burke graphs as 1/v vs. 1/[S].

IV. Acid β-Glucosidase Characterization:

A. Isoelectric focusing column: Isoelectric focusing columns were run according to the method of Behnke (71).

Gel Plug: A mixture of 0.23% tetramethylene-diamine (TEMED, Bio-Rad, Richmond, CA) in H_2O, 1 ml; 8% acrylamide solution (38.8 gm acrylamide, 1.2 gm BIS-acrylamide [Bio-Rad] to 100 ml H_2O), 1.2 ml; and 1.8 ml of H_2O was gently stirred and degassed with N_2 for 30 sec; ammonium persulfate, 1.5 mg/ml; 4ml, was added. A gel plug was poured into a 0.8 cm X 15 cm glass tube, whose bottom was covered with parafilm and layered with water. Upon polymerization, the parafilm was removed and the gel was soaked in 3% sulfuric acid overnight. A 5.2 ml gradient of 0-75% ethylene glycol in H_2O containing 5 mM EDTA, 5 mM 2-ME, 0.05% Triton X-100, 1.85% of pH 3.5-10 ampholines (Pharmacia), 0.15% of pH 4-6 and pH 3.5-5 ampholines,

and 0.3% of pH 9-11 ampholines were added to the tube and 200 ul of acid β-glucosidase was then layered on top. The column was inserted into a reservoir holder and unused holes were plugged with rubber stoppers. The upper portion of the reservoir (cathode) was filled with 3% ethylenediamine solution (250 ml) and the lower reservoir (a glass beaker) was filled with 250 ml 3% H_2SO_4 (anode). The voltage was constant at 200 volts. The solutions were focused for 5 hr at 4°C. After focusing, the ethylenediamine solution was removed, the column was attached to a ring stand and sample was removed from the bottom by puncturing the gel with a 22 gauge needle. Approximately 22 fractions of 0.2 ml each were collected and assayed for acid β-glucosidase, β-hexosaminidase B and β-glucuronidase; the latter two activities were assayed as markers. The pH was determined for each tube using a microelectrode (Fisher Scientific Company).

B. SDS-gel electrophoresis: SDS-gel electrophoresis was performed according to the procedure of Laemmli (72).

1. Reagents:

30% acrylamide (stock): 29.2% acrylamide (Bio-Rad) and 0.8% N,N'-bis-acrylamide (Bio-Rad).

Lower gel buffer: 1.5 M Tris-HCl, pH 8.8, containing 0.4% sodium dodecyl-sulfate (SDS, Bio-Rad).

Upper gel buffer: 0.5 M Tris-HCl, pH 6.8, containing 0.4% SDS.

Coomassie Brilliant Blue stain: Coomassie Brilliant Blue (Becton Dickinson and Co.; Orangeburg, NY) 1.25 grams, was dissolved in 227 ml methanol, 46 ml glacial acetic acid and brought to 500 ml volume with distilled water. The stain was filtered through #1 Whatman filter paper.

Destain: Methanol, 50 ml; glacial acetic acid, 75 ml, brought to 1 l volume with distilled water.

2. Procedure: A 10% gel, consisting of stock acrylamide, 4 ml; lower gel buffer, 3 ml; H_2O, 5 ml, was de-gassed and 10% ammonium persulfate in H_2O, 40 ul and TEMED, 6 ul were added. A thin vertical gel (11 cm X 15 cm X 0.5 mm) was poured and H_2O was gently layered on top. The gel was polymerized at room temperature. Upon polymerization, the H_2O was removed and stacking gel, consisting of stock acrylamide, 1.5 ml; upper gel buffer, 2.5 ml; TEMED, 10 ul; 10% ammonium sulfate, 30 ul; and H_2O, 6 ml, was layered on top of the gel. Water was again layered on top of the gel. Each sample contained 5 ug protein in a final concentration of 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-ME and 0.001% bromophenol blue. Protein markers were from the molecular weight calibration kit (Pharmacia). The proteins were dissociated by boiling for 2 min. Electrophoresis was performed with a constant current of 15 ma until the bromophenol blue marker reached the bottom of the gel ($\sim$ 2 h). The gel was emersed in Coomassie Blue stain for 1 h and de-stained overnight. Results were plotted as log molecular weight vs. the amount of cm the protein samples migrated from the top of the gel.

V. Other Glycosidases Assayed:

A. Fluorometric Assays: Enzymes were all assayed with 4-methylumbelliferyl-(4MU)-glucosides (RPI). Substrate and buffer concentrations were as follows (73,74):

α-L-arabinosidase (EC 3.2.1.55): 0.4 mM 4MU-α-L-arabinopyranoside in 0.2 M sodium citrate buffer, pH 5.0, 100 ul; 20 ul enzyme.

β-D-xylosidase (EC 3.2.1.37): 0.4 mM 4MU-β-D-xylopyranoside in 0.2 M

sodium citrate, pH 5.0, 100 ul; 20 ul enzyme.

β-D-galactosidase (EC 3.2.1.23): 0.8 mM 4MU-β-D-galactopyranoside in 0.2 M sodium acetate buffer, pH 4.5 and pH 7.0, 100 ul; 20 ul enzyme.

α-D-mannosidase (EC 3.2.1.24): 6 mM 4MU-α-D-mannopyranoside in 0.3 M sodium phosphate-citric acid buffer, pH 4.2 and 6.0, 100 ul; 20 ul enzyme.

β-D-hexosaminidase (EC 3.2.1.30): 3 mM 4MU-β-D-n-acetyl-β-D-glucosaminide in 0.04 M sodium phosphate-citric acid, pH 9.4, 100 ul; 20 ul enzyme.

β-D-glucuronidase (EC 3.2.1.31): 10 mM 4MU-β-D-glucuronide in 0.1 M acetate buffer, pH 4.8, 100 ul; 20 ul enzyme.

α-D-galactosidase (EC 3.2.1.22): 10 mM 4MU-α-D-galactopyranoside in 0.15 M sodium phosphate-citric acid, pH 4.6, 100 ul; 20 ul enzyme.

α-L-fucosidase (EC 3.2.1.51): 1.2 mM 4MU-α-L-fucopyranoside in 0.1 M sodium phosphate-citric acid buffer, pH 4.8, 100 ul; 20 ul enzyme.

neutral β-D-glucosidase (EC 3.2.1.21): 5 mM 4MUG in water, 60 ul; 0.2 M sodium phosphate-citric acid buffer, pH 6.0, 30 ul; water, 10 ul; 20 ul enzyme.

All assays were incubated at 37°C for 15-30 min and then terminated with 4 ml 0.085 M glycine/sodium carbonate buffer, pH 10.0. Samples were read in the Turner fluorometer as described above.

B. Spectrophotometric Assays: All spectrophotometric assays were read in the Gilford spectrophotometer.

arylsulfatases (EC 3.1.6.1): arylsulfatase A and B (ASA and ASB) (75):

ASA: 10 mM p-nitrocatechol sulfate in 0.5 M sodium acetate buffer, pH 5.2 containing 0.05 M sodium pyranophosphate and 1.7 M sodium chloride, 200 ul; 200 ul enzyme.

ASB: 50 mM p-nitrocatechol sulfate in 0.5 M sodium acetate buffer, pH 6.0, containing 10 mM barium acetate, 200 ul; 200 ul enzyme.

ASA samples were incubated at 37°C for 30 min. ASB samples were incubated for 30 min and 90 min and the two readings subtracted to correct for hydrolysis of substrate by ASA. The reactions were stopped with 2 N NaOH, 600 ul. Samples were read at an absorbance of 515 nm.

α-glucosidase (EC 3.2.1.20)(76): Acid and neutral α-glucosidase activities were measured at pH 4.0 and 6.3, respectively. The reaction mixture for the assay with maltose as substrate contained 5-20 ug of cellular protein; 20 mM maltose in 0.05 M phosphate-citric acid buffer, pH 4.0, 40 ul; in a total volume of 50 ul.

After incubation for 60 min at 37°C, the reaction was stopped with 0.1 M Tris-HCl buffer, pH 7.0. Released glucose was measured with a modification of the glucose colorimetric enzymatic method, procedure no. 510 (Sigma Chemical Co., St. Louis, MO). Glucose oxidase (10 U/ml), horseradish peroxidase (4 U/ml) and o-dianisidine-diHCl (1.25 mg/ml) were added to the Tris-HCl buffer used to terminate the α-glucosidase reaction. After 30 min incubation, the reaction was stopped with 4 N HCl, 30 ul; and the absorbance was read at 420 nm.

VI. Tissue Culture Conditions:

A. Growth Conditions: All fibroblast lines (human and mouse) were grown in RPMI 1640 medium containing 10% fetal calf serum (GIBCO, Grand Island, NY), 1% L-glutamine (GIBCO) and 1% pencillin-streptomycin (GIBCO) using standard tissue culture techniques (12).

B. Maintenance of Cell Cultures: Confluent cells from one 75 cm^2 flask were exposed to 0.25% trypsin (GIBCO) for 10 min, transferred to a

sterile 15 ml conical tube and centrifuged at room temperature for 10 min at 1000 x g. The pellet was resuspended in 4 ml of culture medium, and 1 ml was transferred to each of four 75 cm^2 flasks. 10 ml of culture medium (10 ml) was added and the cells were gassed for 10 sec with 5% CO_2 (12).

C. Harvesting of Cell Cultures: Early confluent cells were harvested by sequential exposure to 0.25% trypsin (GIBCO) and 0.2% EDTA in 0.9% NaCl approximately 24 h after a change of medium. The cells were transferred to a 15 ml conical tube and centrifuged at room temperature for 10 min at 1000 x g. The pellet was washed twice in 0.9% NaCl and the dry pellet stored at -4°C (12).

VII. Solubilization of Acid β-Glucosidase In Fibroblast Extracts:

The cell pellet from one 75 cm^2 flask was suspended in 0.3 ml of distilled water and freeze/thawed five cycles in an acetone-dry ice slurry. A 0.2 ml aliquot was mixed with 0.7 ml of 0.05 M sodium phosphate-citric acid buffer, pH 6.0, containing 0.1 ml of 0.6% Triton X-100 and 1% crude sodium taurocholate (Lot No. 6420410, Gallard and Schlesinger Chemical Mfg. Corp., Carle Place, NY). The suspension was incubated for 15 min at 25°C, centrifuged for 30 min at 30,000 x g at 4°C, and then the supernatant was removed for assay.

VIII. Tissue Sample Preparation:

Tissues from normal individuals and homozygotes with Type 1 Gaucher disease were collected at surgery or autopsy (within 2 h of death) and were processed immediately or frozen at -70°C prior to use. Optimal results were obtained with fresh tissues, since freezing reduced the acid β-glucosidase activity. Skin fibroblasts were obtained, cultured, and

harvested as described above.

A. For Chromatography on Concanavalin A-Sepharose (Con A-Sepharose): Tissues were minced and homogenized in distilled water (3:1, w/v) containing 0.05% Triton X-100 and centrifuged at 15,000 x g for 30 min at 4°C. The supernatants were diluted in 3 vols 0.05 M sodium citrate, pH 6.0 with 1 M NaCl, 5 mM each of $MgCl_2$, $MnCl_2$, and $CaCl_2$ and 0.02% NaN_3 (standard chromatographic buffer).

B. Cellulose Acetate Electrophoresis: Tissues were homogenized in 40 mM sodium phosphate containing 20% ethylene glycol and 0.05% Triton X-100; the buffer was adjusted to pH 7.0 with 0.5 M citric acid (standard electrophoretic buffer). After homogenization, 1 vol of ethanol/chloroform (2:1, v/v) was added dropwise to 2 vols of the homogenate, gently mixed for 5 min at 25°C, and then centrifuged at 4,000 x g for 10 min at 4°C; the supernatants were electrophoresed immediately. Fibroblasts were freeze/thawed five times in the standard electrophoresis buffer, ethanol/chloroform treated and the whole cell homogenate was immediately used for electrophoresis. Optimal separation of the acid and neutral isozymes were attempted by systematically varying electrophoretic conditions including ionic strength (20 mM-50 mM phosphate), pH (6.0-8.0), the concentrations of Triton X-100 (0-0.15%) and ethylene glycol (0-50%). In addition, the presence of phosphatidic acid (0.1 mg/ml) or sodium taurocholate (0.02%) had no effect on isozyme migration.

IX. Chromatographic Separation of Acid and Neutral β-Glucosidase:

Supernatants from about 5 g of tissue were diluted in the standard chromatographic buffer, mixed with 2 ml of con A-Sepharose (Pharmacia) at 25°C for 2 h and then poured into a disposable polypropylene column. The

column was washed with this buffer (about 20 ml) until no β-glucosidase activity was detected in the eluant. Bound β-glucosidase activity was then eluted with 20 ml of buffer containing 1 M of 1-O-methyl-α-D-glucopyranoside (Sigma); over 90% of the bound activity was recovered in the first 5 ml fraction. The buffer wash and 1-O-methyl-α-D-glucopyranoside fractions were pooled separately and dialyzed overnight against 2 l of standard electrophoretic buffer. Pooled fractions were concentrated by ultrafiltration, using an Amicon PM10 filter.

X. Electrophoretic Separation of The Acid and Neutral β-Glucosidases:

A. Electrophoretic Conditions: A cellulose acetate slab gel (17 X 16 X 0.35 cm, Cellogel) was immersed in the standard electrophoretic buffer for a minimum of 15 min, blotted with Whatman No. 1 filter paper and immediately placed on the electrophoresis apparatus. Cellogel and the horizontal electrophoresis tank were purchased from Kalex Scientific Co., Manhasset, NY. Samples with at least 6 U of β-glucosidase activity were applied to the gel. A constant voltage of 240 V and an initial current of 25 mA were applied for 2.5-4.0 h at 4°C.

B. Enzyme Activity Stains for Cellulose Acetate Gel Electrophoresis: The gels were stained for β-glucosidase activity with a filter paper overlay containing 3 mM 4MUG in 0.06 M phosphate citrate buffer, pH 4.0 or 6.0, containing 0.12% Triton X-100 and 0.2% sodium taurocholate; for β-galactosidase with 0.8 mM 4-methylumbelliferyl-β-D-galactopyranoside in 0.2 M sodium acetate, pH 4.5; for β-xylosidase with 0.4 mM 4-methylumbelliferyl-β-D-xylopyranoside in 0.2 M sodium citrate, pH 5.0; or for α-L-arabinosidase with 0.4 mM 4-methylumbelliferyl-α-L-arabinopyranoside in 0.2 M sodium citrate, pH 5.0. The gel was placed in a sealed, moist

chamber and incubated at 37°C for 1-2 h. After incubation, the gel was placed in an NH_4OH chamber for 3 min at 25°C. Activity bands were visualized with long wave ultraviolet light and photographs were taken with Polaroid Type 51 film using a Wratten No. 4 filter.

IX. Effector Studies:

A. Assay for the Effector: One unit of effector is defined as that amount which would stimulate acid β-glucosidase activity two-fold. The effector was assayed as follows: 5 mM 4MUG in 0.2 M sodium acetate buffer, pH 5.5, 100 ul; acid β-glucosidase, 20 ul and effector 10-30 ul. The assay was performed as described in Methods Section IIA.

B. Isolation of Crude Effector from Normal and Gaucher Fibroblasts: Cell pellets from one 75 cm^2 flask were suspended in 0.2 ml of distilled water and freeze/ thawed five cycles in an acetone-dry ice slurry. The homogenate was then heated at 100°C for 10 min and subsequently digested for 4 hrs at 50°C with 1 mg/ml of pronase (grade B, Calbiochem, LaJolla, CA) (42).

C. Isolation of Effector from Gaucher and Normal Spleen: This protocol was essentially that of Peters et al. (44): One hundred grams of tissue were minced 1:1 (w/v) in cold distilled water, homogenized in a Waring blender at high speed for 2 min, and centrifuged in a Sorvall RC-2B at 40,000 x g for 60 min. The supernatant was boiled at 100°C for 4 min, filtered through gauze and centrifuged at 4°C for 10 min at 10,000 X g. The pH was brought to 4.4 with 1.0 M acetic acid, allowed to stir at room temperature for 15 min and centrifuged as above. The supernatant was concentrated 5-fold (Amicon UM-2), ethanol precipitated (1:9 v/v) and centrifuged at 20,000 X g for 20 min. The precipitate was resuspended in dis-

tilled water, made 2% with 12% TCA, centrifuged (20,000 X g, 20 min) and the supernatant dialyzed against distilled water using benzoylated dialysis tubing (Sigma). All further steps were performed at 4°C. The effector was adjusted to pH 7.0 using solid dibasic sodium phosphate and applied to a DEAE-cellulose column (2.5 X 8 cm) pre-equilibrated with 10 mM sodium phosphate buffer, pH 7.0. The effector was eluted using a 500 ml linear gradient of sodium chloride (0-0.5 M) in the above buffer. Fractions containing the effector were determined by stimulation of acid β-glucosidase activity during the standard assay. The above fractions were pooled, dialyzed against 10 mM sodium phosphate, pH 7.0, and applied to a 0.8 X 8 cm DEAE-cellulose column. The effector was eluted with a 300 ml sodium chloride gradient (0.1 to 0.25 M) and active fractions were pooled and dialyzed as above.

D. Stimulation of Acid β-Glucosidase Using Control and Gaucher Fibroblast Effector: Effector from fibroblast cell extracts were assayed for protein using the fluram method (Methods, Section IC). Effector was added to purified enzyme from 0.0-0.6 mg protein/sample. As controls, equivalent amounts of BSA in water were added to one set of samples and distilled H_2O was added to a second. The assay was performed as described above.

F. Lipase Treatment of Effector: Effector was treated with 4 mg/ml triacylglycerol lipase (EC 3.1.1.3) (Type VI, Sigma) in 0.2 M sodium phosphate-citric acid, pH 7.7, for 1 h at 37°C and dialyzed overnight in 0.2 M sodium acetate buffer, pH 5.5. The effector was added to purified acid β-glucosidase, assayed, and electrophoresed as described below.

F. Electrophoresis of Acid β-Glucosidase in the Presence of Effector: Conditions for electrophoresis of acid β-glucosidase homogen-

ates from fibroblasts and purified placenta were identical to Section X. One U of effector was added to the enzyme prior to application of the sample to the cellogel.

G. Effect of Phospholipids vs. Effector on Acid β-Glucosidase Activity: The standard effector assay was done in the presence and absence of 1% crude sodium taurocholate, 0.015% Triton X-100.

H. Sucrose Ultracentrifugation of Effector and Acid β-Glucosidase: Acid β-glucosidase with and without effector were layered on linear sucrose gradients (5% to 20%, w/w) in 0.2 M sodium acetate buffer, pH 5.5 containing 0.05% Triton X-100, 1 mM 2ME and 5 mM EDTA. Tubes were centrifuged in a Beckman Model L5-75 ultracentrifuge at 120,000 X g in a SW-56 rotor for 18 hr at 4°C to a final w^2t of 0.843 X 10^{12} rad^2/sec. 0.5 ml fractions were collected and the acid β-glucosidase activity plus and minus effector were compared.

I. Heat Stability Studies: To determine whether the effector stabilizes acid β-glucosidase activity, heat stability curves were performed in the presence and absence of effector. The buffer solution for heating experiments was made up according to the procedure of Turner and Hirschhorn (17) as follows: 0.2 M sodium phosphate-citric acid, pH 6.0, 5 vols; distilled water, 4 vols; 0.6% Triton X-100, 1 vol; BSA was added to a final concentration of 2 mg/ml. Equal volumes of this buffer solution and purified placental acid β-glucosidase were mixed and 100 ul aliquots dispensed into glass tubes. 1 U of effector or equivalent protein was also added, sealed and heated in a 50°C water bath for up to 30 min. Heating was terminated by placing the tubes on ice. Acid β-glucosidase activity was assayed as described in Methods, Section IIA.

XII. Antiserum Against Human Acid β-Glucosidase:

A. Preparation of Antiserum Against Human Acid β-Glucosidase: Balb/C mice (Jackson Laboratories, Bar Harbor, ME) were inoculated with four bi-weekly intraperitoneal injections of 5 ug of purified enzyme in Freund's complete adjuvant. When 2 U of acid β-glucosidase activity from human fibroblast extracts were titrated against increasing amounts of the antiserum, optimal precipitation of acid β-glucosidase was obtained with 2 ul of antiserum and 20 ul of rabbit anti-mouse Ig. The precipitated enzyme-antibody complex retained catalytic activity and was quantitated by the standard enzymatic assay for β-glucosidase as described in Section II. The antiserum selectively precipitated human acid β-glucosidase; no β-glucosidase activity was precipitated from mouse, rat, or Chinese hamster fibroblasts.

B. Antibody Specificity for Human Acid β-Glucosidase: To determine the specificity of the immunoprecipitation assay for acid β-glucosidase, partially purified neutral β-glucosidase isolated from human liver as described in Section VIII, was used in place of fibroblast cell extracts. No neutral β-glucosidase activity was detected in the immunoprecipitate. Assays of the fibroblast enzyme-antibody complex for the presence of other enzymes, including β-glucuronidase, β-hexosaminidase, α-glucosidase at pH 4.0 and 6.0, β-galactosidase at pH 4.5 and 7.0, α-L-arabinosidase, and β-D-xylosidase as described in Section V, proved negative, thus further demonstrating the specificity of the antibody for human acid β-glucosidase.

XIII. Standard Immunoprecipitation Assay For Human Acid β-Glucosidase:

Cell extracts from parental lines of individual hybrid clones were

assayed for acid β-glucosidase activity; 2 U were added to each assay tube. The volume was brought to 200 ul with the immunoprecipitation buffer [0.2 M sodium phosphate-citric acid buffer, pH 6.0, containing 0.05% Triton X-100, 10.0 mM 2-ME, 1.0 mM EDTA and 1.0 mg/ml bovine serum albumin (BSA)]. Anti-acid β-glucosidase antibody (2 ul) was added and the mixture incubated for 30 min at 37°C. Rabbit anti-mouse immunoglobulin was added and the mixture incubated for 1-2 h at 37°C. Blanks contained 2 ul of normal mouse serum instead of the anti-GBA antiserum. Samples were centrifuged in 1.0 ml conical polypropylene tubes at 27,000 x g for 20 min in a Sorvall RC-2B centrifuge. The supernatant was retained and assayed for β-glucosidase activity. The pellet was washed with 0.5 ml of immunoprecipitation buffer and centrifuged as above. The pellet was assayed for β-glucosidase activity with the following modifications: 100 ul of substrate solution (see above) was added directly to the pellet and incubated for 1 h at 37°C. The reaction mixture was stopped with 1.0 ml of 0.085 M glycine-carbonate buffer, pH 10.0, the mixture was transferred to a 10 X 75 mm glass test tube and fluorescence was determined. For each hybrid clone, the background fluorescence in the blank was subtracted and the activity in the immunoprecipitate was expressed as the percent of total activity recovered in supernatant and pellet.

XIV. Cell Fusion:

A. Human and Mouse Parental Cells: The parental lines used for hybridization were the mouse RAG cell line (77) and three human fibroblast lines (fetal lung, fetal liver and fetal kidney) from different sources. Parental cells were grown in RPMI 1640 medium as described above.

B. Somatic Cell Hybrids: The hybrids were generously provided by

Dr. Moyra Smith. The preparation of these hybrids were as follows: Parental cells were fused by centrifugation through a 7-50% polyethylene glycol gradient (78). Heterokaryons were cultured in the hypoxanthine/aminopterin/thymidine selective medium (79). Hybrid cells were then cloned using the technique of Ham and Puck (80). After 10-20 passages, selected primary hybrid clones were recloned, giving rise to secondary clones, and for some hybrids, tertiary clones were recloned from subclones.

C. Determination of The Human Chromosomal Constitution of Hybrid Clones: Marker enzymes for specific human chromosomes were determined by established electrophoretic methods (81-88). Metaphase spreads were prepared (89) and the chromosomes were banded to distinguish mouse from human chromosomes with the Giemsa 11 technique (90) and subsequently destained and banded with quinacrine hydrochloride fluorescence (91). Human chromosomal constitution of individual hybrid clones was based on enzyme marker data and/or cytogenetic analysis in which at least 20% of the metaphase spreads contained a specific chromosome. Human acid β-glucosidase immunoprecipitation assays, marker enzyme electrophoreses and cytogenetic analyses were performed on cell hybrids harvested from the same passage.

RESULTS

I. Purification of Human Acid β-Glucosidase:

A. Purification Procedure: The results of a typical purification are shown in Table 4. The procedure incorporates two novel steps. These are chromatography on dextran sulfate-Sepharose and sucrose density gradient ultracentrifugation.

Human acid β-glucosidase was strongly inhibited by dextran sulfate and this inhibition was found to be alleviated by crude sodium taurocholate. A dextran sulfate affinity column was synthesized and efficiently bound human acid β-glucosidase. As shown in Figure 9, β-glucosidase activity was eluted by a sodium taurocholate gradient (0-5 mg/ml) with a sharp peak of activity at 2 mg/ml taurocholate. Most of the UV-absorbing material was attributable to the taurocholate and a discrete peak coinciding with enzyme activity was not detected. Subsequent elution with 5 M NaCl gave a sharp peak of UV-absorbing material but did not release additional enzyme.

Dextran sulfate chromatography provided a 10-fold purification step with a 60-70% recovery. More significantly, the procedure separated β-glucosidase from several other hydrolases including α-arabinosidase, β-xylosidase, α-mannosidase, α-galactosidase, β-galactosidase, α-fucosidase, arylsulfatase A and B and α-glucosidase. Recovery of β-glucuronidase and β-hexosaminidase B (two major contaminants) was less than 20% of that applied, respectively. It was notable that efficient binding of acid β-glucosidase to the column could be achieved only after butanol extraction of the sample.

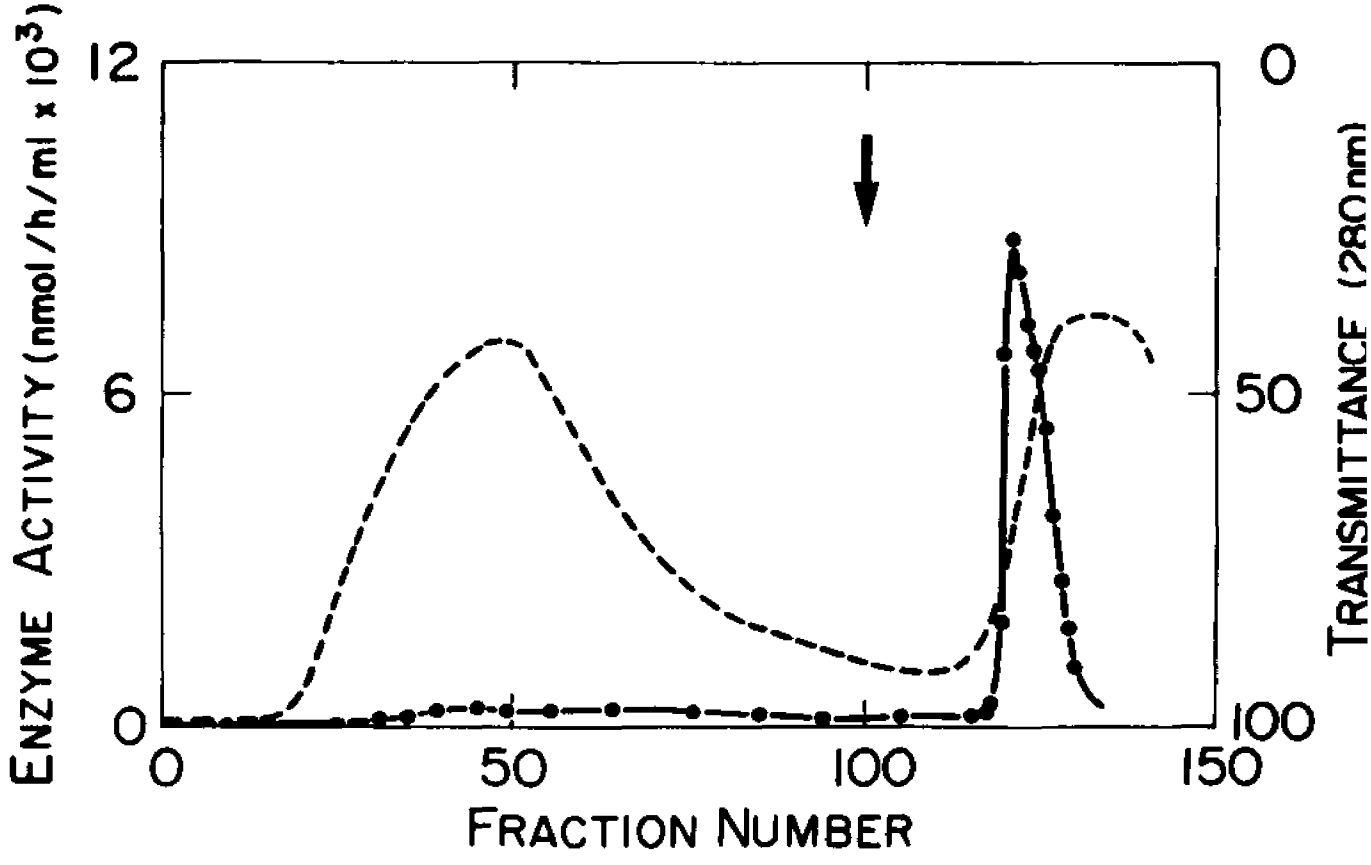
Chromatography on octyl-Sepharose resulted in a 40-fold purification of the enzyme eluted from the dextran sulfate column, however, the sample

Table 4

Purification of Acid β-Glucosidase from Human Placenta (1.3 kg)

Fraction	Vol (ml)	Activity nmoles/ h/ml	Protein mg/ml	Specific Activity nmoles/h/mg protein	Total Units nmoles/h	%- Yield	Fold- Purification
Extract Homogenized in (2X distilled H_2O	2,850	287	4.16	69	817,000	100	-
33% Ammonium Sulfate Supernatant	3,100	225	1.68	134	697,000	85	2
55% Ammonium Sulfate Precipitate, (resuspended) Post-Acid Dialysis	112	5,364	8.7	617	601,000	73	9
20% (v/v) Butanol Extraction	110	5,460	9.2	593	601,000	74	9
Dextran Sulfate Chromatography	88	3,520	0.529	6,660	310,000	38	97
20% (v/v) Butanol Extraction	93	2,110	0.397	5,300	196,000	24	77
Octyl- Sepharose Chromatography	5.2	18,400	0.085	217,000	95,800	12	3,140
Sucrose Gradient Ultracentrifuga- tion	2.7	19,100	0.070	270,000	51,600	6.3	3,920

Figure 9: Elution of human placental β-glucosidase from dextran-sulfate-Sepharose (30 ml bed volume) by crude sodium taurocholate (0-5 mg/ml). The sample previously was butanol extracted and dialyzed overnight against binding buffer. Sample volume was 100 ml; 9.2 mg protein/ml. The acid β-glucosidase activity was 5460 nmol/h/ml. Fraction volume was 5.5 ml. The column was run at 4°C.


ENZYME ACTIVITY (nmol/h/ml x 10³)
12
6
0
0
50
100
150
FRACTION NUMBER
0
50
100
TRANSMITTANCE (280nm)

remained contaminated with β-hexosaminidase B and β-glucuronidase activity. The final purification step, sucrose density ultracentrifugation, gave only a slight increase in specific activity, but more importantly, removed all the β-hexosaminidase B and β-glucuronidase activity.

The procedure outlined in Table 4 gave a 3900-fold purification of acid β-glucosidase with a yield of 6%. Considerable loss of activity occurred during the final stages of purification. However, the purified enzyme retained activity for several months when stored at -20°C in 25% glycerol.

B. Inhibition of Enzyme Activity by Sulfates: Dextran sulfate was found to be an effective inhibitor of purified acid β-glucosidase. Inhibition was independent of substrate concentration, but was found to be sensitive to the concentration of either phospholipid or crude sodium taurocholate. Typical results are shown in Table 5. Similar results were obtained with chondroitin sulphate, a sulphated mucopolysaccharide, whereas sodium sulphate had little effect.

C. NBD-Glucosyl Ceramide Assay vs. 4MUG Assay: Figure 10 shows the scheme for the synthesis of fluorescent NBD-glucer. The fatty acid was cleaved by acid hydrolysis and subsequently extracted from the glucoysl phychosine with hexane. NBD-dodecanoic acid was joined to the glucosyl phychosine via hydrolysis and a carbodiimide linkage. NBD-glucer was purified by chromatography on silica gel HR plates. The assay was optimized for lipid (taurocholate, 2.5 to 40 mg/ ml), detergent (cutscum, 0 to 10%) and pH (3.5 to 7.0). The pH optimum for acid β-glucosidase was 5.3 and the final cutscum and sodium taurocholate concentrations were 0.25% and 2.5 mg/ml, respectively. The assay was linear with respect to both time (0-30 min) and protein.

Table 5

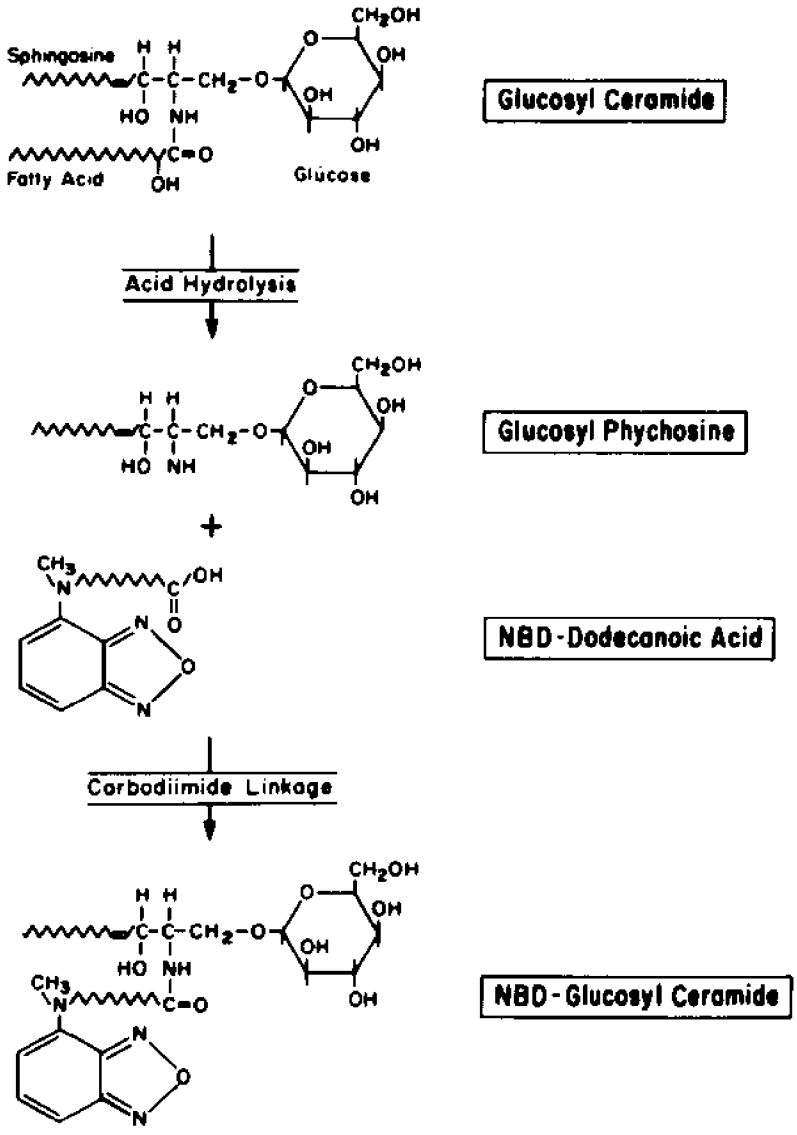
Effect of Sulfates and Phosphatidylinositol on Purified Acid β-Glucosidase Activity

Substrate (5 mM)	Phosphatidyl-Inositol (mg/ml)	Inhibitors (4 mg/ml) % of Control Activity			
		None	Dextran Sulfate	Chondroitin Sulfate	Sodium Sulfate
4MUG	0.1	100	60	83	99
	0.05	100	24	73	83
	0.02	100	12	37	76

Figure 10: Synthesis of NBD-glucosyl ceramide.

Glucosyl ceramide undergoes acid hydrolysis to cleave the fatty acid. Glucosyl phychosine is then linked to the fluorescent fatty acid, NBD-dodecanoic acid, via a carbodiimide linkage. The final product, NBD-glucosyl ceramide, is further purified by silica gel thin layer chromatography.

Synthesis of NBD-Glucosyl Ceramide



NBD = 12-[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]-amino-

To conserve the fluorescent NBD-glucer, natural substrate assays were performed by diluting the NBD-glucer 10-fold with natural glucosyl ceramide. Figure 11 demonstrates the results of a mixture of fluorescent NBD-glucer with natural glucosyl ceramide. A linear relationship was observed between the degree of NBD-glucer and fluorescence. Acid β-glucosidase hydrolyzed the NBD-glucer and the natural substrate with equal efficiency. This confirmed that NBD-glucer was a suitable substrate for acid β-glucosidase.

Table 6 shows a small scale (0.7 Kg) purification of acid β-glucosidase assayed with both the natural substrate, NBD-glucer, and the artificial substrate, 4MUG. Both substrates demonstrated purification of acid β-glucosidase. The acid β-glucosidase had a 10-fold higher specific activity when assayed with NBD-glucer than with 4MUG. Apparent K_m values of 0.285 mM and 2.65 mM were observed for acid β-glucosidase using NBD-glucer and 4MUG, respectively. The difference in K_m reflects the stronger avidity of the enzyme for the natural substrate compared to the artificial substrate.

II. Characterization of Human Acid β-Glucosidase:

A. Effect of Phospholipid on Acid β-Glucosidase Activity: Either phospholipid or crude sodium taurocholate stimulates purified acid β-glucosidase activity with the artificial substrate as well as the natural substrate. Using 4MUG as substrate, phosphatidic acid was the most effective of the phospholipids tested, in terms of both maximum activity and activity at low concentrations of phospholipid. Maximum activities achieved with phosphatidylserine, phosphatidylinositol (both at 0.2 mg/ml)

Figure 11: Indiscriminant hydrolysis of NBD-glucosyl ceramide and glucosyl ceramide by acid β-glucosidase.

The molarity of glucosyl ceramide varied from 0.250 mM to 0.125 mM; the NBD-glucosyl ceramide varied from 0.125 mM to 0.0 mM. Final substrate concentration in the assay was 0.250 mM.

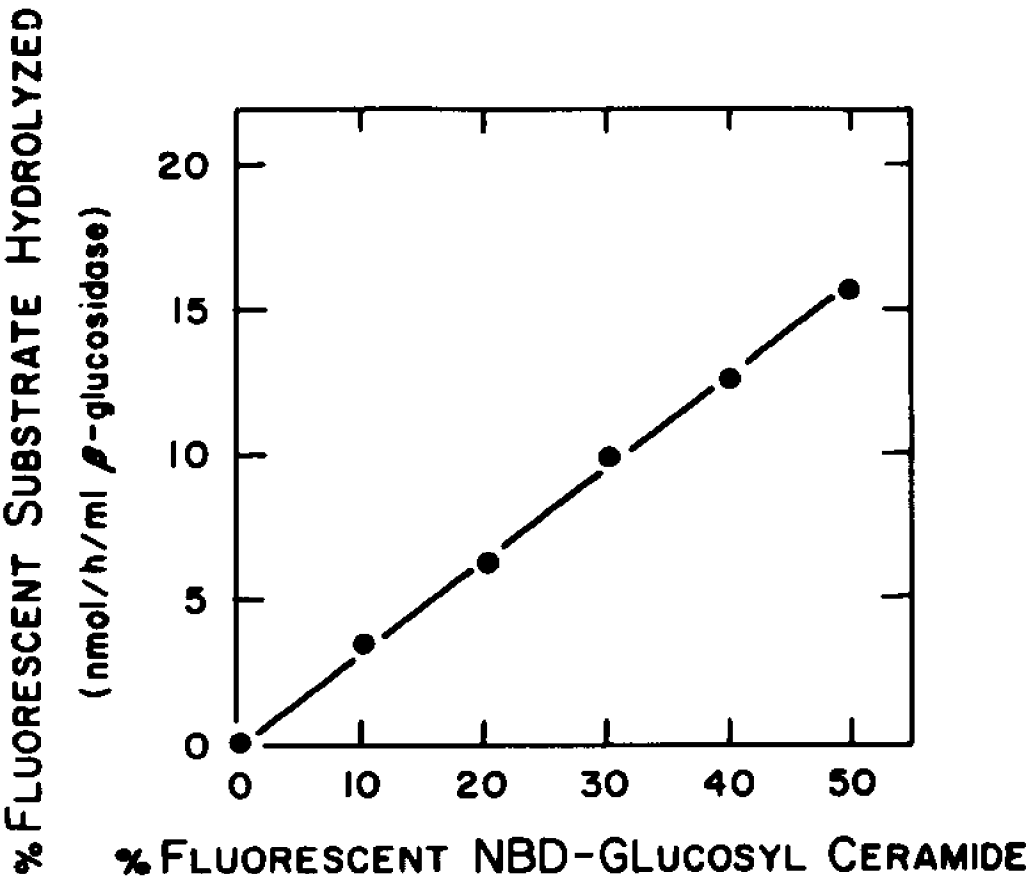

% Fluorescent Substrate Hydrolyzed
(nmol/h/ml β-glucosidase)
20
15
10
5
0
0
10
20
30
40
50
% Fluorescent NBD-Glucosyl Ceramide

Table 6

Small Scale Purification of Placental Acid β-Glucosidase (0.7 kg) with both the Natural Substrate, NBD-glucer, and the Artificial Substrate, 4MUG

Step	Specific Activity (nmol/h/mg protein)	
	4MUG	NBD-glucer
Extract 1	15	184
33% Ammonium Sulfate	34	422
55% Ammonium Sulfate Supernatant Post-Acid Dialysis	209	2,220
20% (v/v) Butanol Extraction	305	3,040
Dextran Sulfate Chromatography	3,400	25,100
Octyl-Sepharose Chromatography	36,200	370,000
Sucrose Gradient Ultra-centrifugation	37,100	416,000

and with crude taurocholate (1 mg/ml) were 80%, 65% and 70%, respectively, of that obtained with phosphatidic acid (0.2 mg/ml). Addition of both phospholipids and taurocholate together to the reaction caused no further increase in activity. All assays in which the concentration of phospholipid was varied contained 0.02% Triton X-100.

The effect of pre-incubation with phospholipids and taurocholate on the sedimentation of purified acid β-glucosidase through sucrose gradients was studied. Gradients were run in the presence and absence of Triton X-100. As shown in Figure 12A, when Triton X-100 was present in the gradient, pre-incubation with either phospholipid or taurocholate had no effect on either recovery of activity or sedimentation rate. However, in the absence of Triton X-100 (Figure 12B), recovery of enzyme activity was extremely low except in the sample pre-incubated with phospholipid; the sample also sedimented more rapidly than the enzyme run in the presence of only Triton X-100. It was clear that taurocholate effects neither the stability of the enzyme nor its sedimentation rate to the same extent as phospholipids. Thus, pre-incubation in the presence of phospholipid both stabilized the enzyme and caused an increase in sedimentation rate. The latter effect was reversed by Triton X-100. Thus, while phospholipid and taurocholate had similar effects on enzymatic activity, they differed in their effects on stability and sedimentation properties.

B. Isoelectric Focusing: Figure 13 shows the separation of acid β-glucosidase from β-hexosaminidase B and β-glucuronidase by isoelectric focusing. The gradient was linear from pH 3.5 to 7.7, and 95% of the acid β-glucosidase activity was recovered. The pI of acid β-glucosidase was approximately 4.35. β-Glucuronidase and β-hexosaminidase B have pI's of approximately 7.3 and 7.5, respectively. Variation from one isoelec-

Figure 12: Preparative sucrose gradient ultracentrifugation of acid β-glucosidase.

A. Purified placental acid β-glucosidase was pre-incubated with 0.02% Triton X-100 (●—●) and Triton X-100 containing 1.0 mg/ml crude sodium taurocholate (▼—▼), or 0.2 mg/ml phosphatidyl serine (X-X).

B. Acid β-glucosidase was pre-incubated with sucrose gradient buffer (●—●), crude sodium taurocholate (▼—▼) or phosphatidyl serine (X-X). Note that in the absence of Triton X-100 there is reduced enzymatic activity as well as a shift in the migration of the gradient containing phosphatidyl serine.

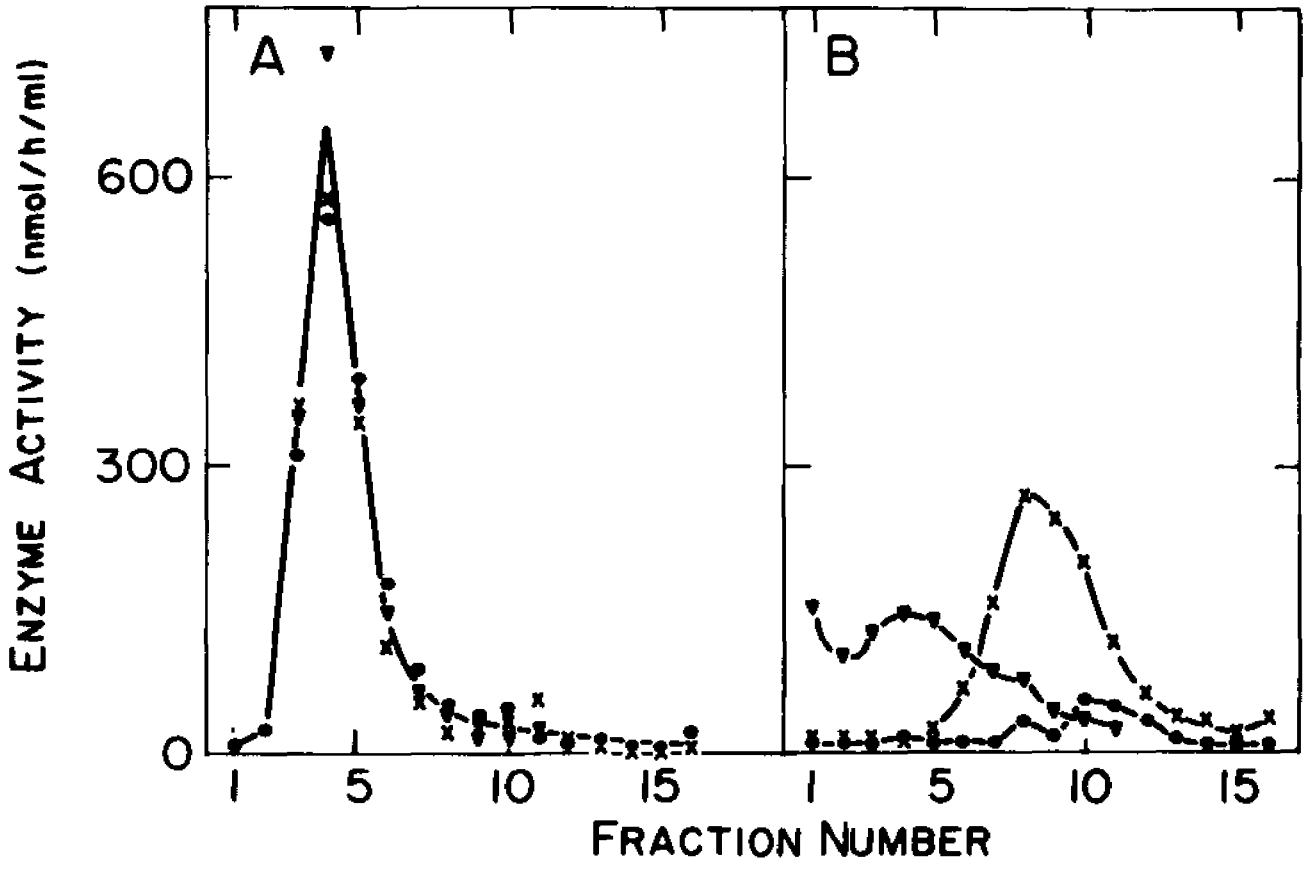
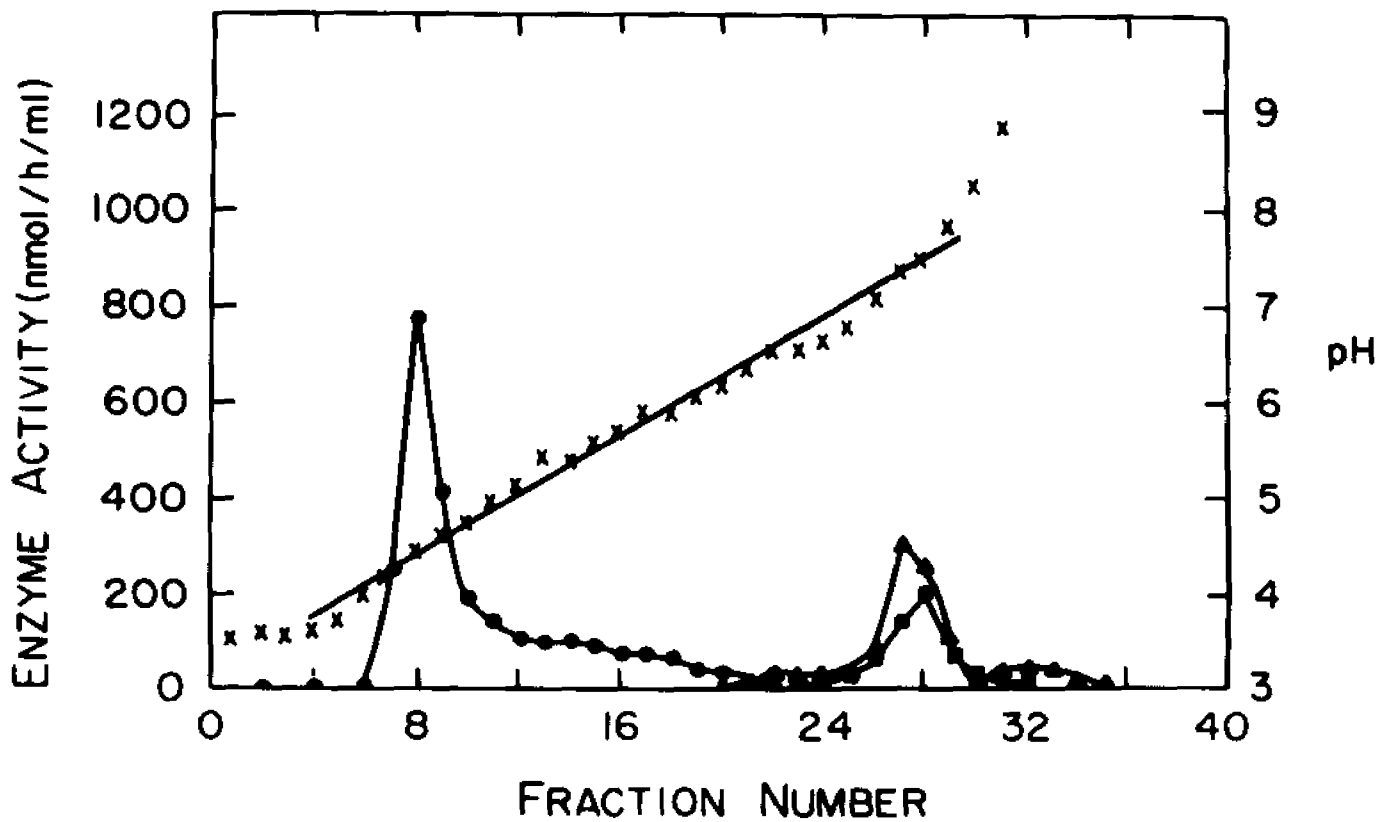

A
B
600
300
0
1
5
10
15
1
5
10
15
ENZYME ACTIVITY (nmol/h/ml)
FRACTION NUMBER

Figure 13: Isoelectric focusing of partially purified acid β-glucosidase from human placenta.

An isoelectric focusing column was run with a linear gradient from pH 3.5 to 7.5. Acid β-glucosidase activity (●—●); β-glucuronidase activity (▲—▲); β-hexosaminidase B activity (●—■); pH values (X-X).


ENZYME ACTIVITY (nmol/h/ml)
1200
1000
800
600
400
200
0
9
8
7
6
5
4
3
pH
0
8
16
24
32
40
FRACTION NUMBER

tric focusing run to another varied in pI by approximately 0.05. The values obtained for β-glucuronidase and β-hexosaminidase B coincided with previously published values (92,93). Srivastava et al. (93) observed a pI of 7.9 for β-hexosaminidase B and Glaser et al. (92) found the pI of β-glucuronidase to be 7.0-7.5. This column was useful not only in determining the pI of acid β-glucosidase but also in totally separating the enzyme from β-glucuronidase and β-hexosaminidase B.

C. SDS gel electrophoresis: Multiple bands were observed for the purified acid β-glucosidase upon SDS-polyacrylamide gel electrophoresis. Two major bands were present with molecular weights of about 67,600 and 79,400. Three minor bands with molecular weights of 61,700, 39,000 and 26,000, respectively were also present. Ovalbumin, aldolase, ribonuclease A, and chymotrypsin were used as protein markers as shown in Table 7. The purified enzyme preparation was assayed for 12 different lysosomal hydrolases and found to be free of any of these enzymatic activities.

III. Cellulose Acetate Electrophoresis and Chromatographic Separation of Acid and Neutral β-Glucosidases:

Figure 14 shows the cellulose acetate electrophoresis pattern of β-glucosidase activity from crude homogenates of normal liver, kidney, brain, and cultured skin fibroblasts. Two fluorescent bands of β-glucosidase activity were visualized in liver, kidney, spleen, and brain homogenates with 4MUG. In these tissues, the maior band was the more anodal (fast) band. In cultured skin fibroblasts only one band was observed which corresponded to the less anodal (slow) activity.

Two peaks of β-glucosidase activity from splenic and hepatic homogenates were separated by chromatography on con A-Sepharose and further

Table 7

SDS Polyacrylamide Gel Electrophoresis of
Purified Placental Acid β-Glucosidase

Protein Markers and Acid β-Glucosidase	mm Migrated during Electrophoresis	Log MW	Subunit MW*
Ovalbumin	31	4.63	45,000
Aldolase	36	4.60	40,000
Ribonuclease A	54	4.14	13,700
Chymotrypsin	61	4.04	11,000
	52	4.11	13,000
Acid β-Glucosidase			
major bands	24	4.90	79,433
	26	4.83	67,608
minor bands	42	4.42	26,303
	37	4.59	38,905
	29	4.79	61,650

*Subunit molecular weight for acid β-glucosidase was extrapolated from a plot of the log of the protein marker's molecular weight vs. the mm the subunits migrated during electrophoresis.

Figure 14: Fluorescent bands of β-glucosidase activity in normal human tissue homogenates after electrophoresis on a cellulose acetate gel and staining with 4MUG.

Lane 1, brain; lane 2, liver; lane 3, kidney; lane 4, cultured skin fibroblasts, Arrow = point of application; A = acidic β-glucosidase; N = neutral β-glucosidase.

characterized. As shown in Figure 15, the activity eluted in the buffer wash electrophoresed as a single activity band which co-migrated with the fast band of β-glucosidase activity in the crude homogenates. The β-glucosidase activity which bound to the lectin column was eluted with 1.0 M of 1-O-methyl-α-D-glucopyranoside and electrophoresed as a single band which co-migrated with the slow activity band observed in the crude tissue homogenates.

The β-glucosidase activity eluted in the buffer wash did not have activity for the natural substrate, NBD-glucer, although it demonstrated activity for 4MUG. Alternatively, the β-glucosidase activity eluted with 1-O-methyl-α-D-glucopyranoside had activity with both NBD-glucer and 4MUG substrate.

Figure 16 shows the pH profiles for β-glucosidase activity from the crude homogenate, the buffer wash and the 1-O-methyl-α-D-glucopyranoside eluant from con A-Sepharose chromatography of normal liver and spleen homogenates. In each tissue, the β-glucosidase activity which bound to the lectin column had an acid pH optimum ($\sim$ 5.0) and co-electrophoresed with the slow activity band observed in crude homogenates. The hepatic and splenic activities which eluted in the buffer wash had a broader, more neutral pH optima ($\sim$6.3) and co-electrophoresed with the fast activity band of tissue homogenates. Based on these results, the faster migrating activity band was identified as neutral β-glucosidase and the slower migrating activity band as acid β-glucosidase.

The electrophoretic profiles of the splenic and fibroblast homogenates from homozygotes with Type 1 Gaucher disease and normal individuals are shown in Figure 17. When equal amounts of protein were electrophoresed, the acid β-glucosidase in the Gaucher tissue homogenates appeared

Figure 15: Electrophoresis of normal hepatic β-glucosidases before and after separation by chromatography on con A-Sepharose.

Lane 1, total liver homogenate; lanes 2 and 3, buffer wash; lane 4, 1-O-methyl-α-D-glucopyranoside eluant.

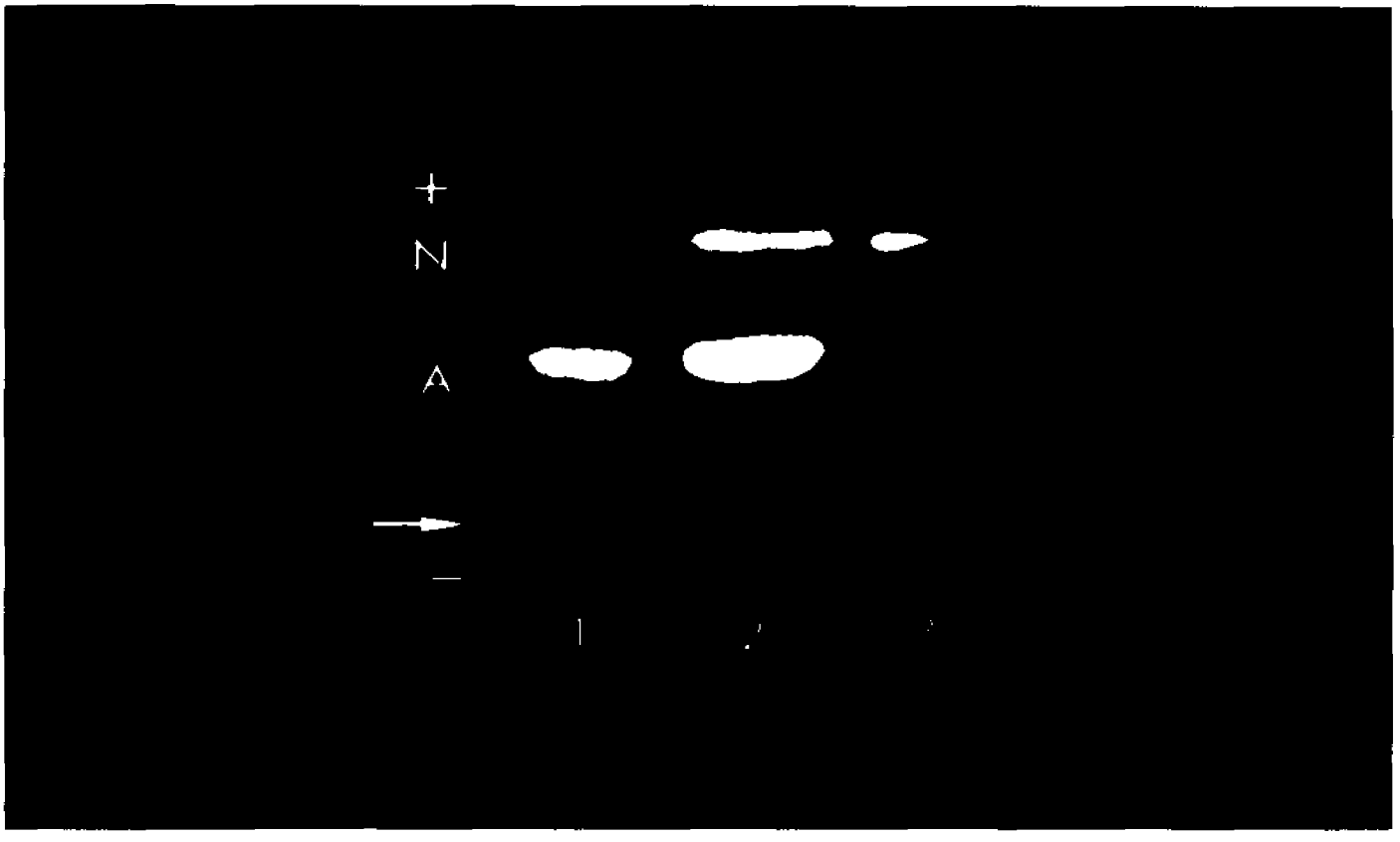

+
N
A
—

Figure 16: pH profiles of β-glucosidase activity from normal human hepatic (a-c) and splenic (d-f) homogenates before and after chromatography on Con A-Sepharose. Total tissue homogenates (a and d); buffer wash (b and e); 1-O-methyl-α-D-glucopyranoside eluant (c and f).

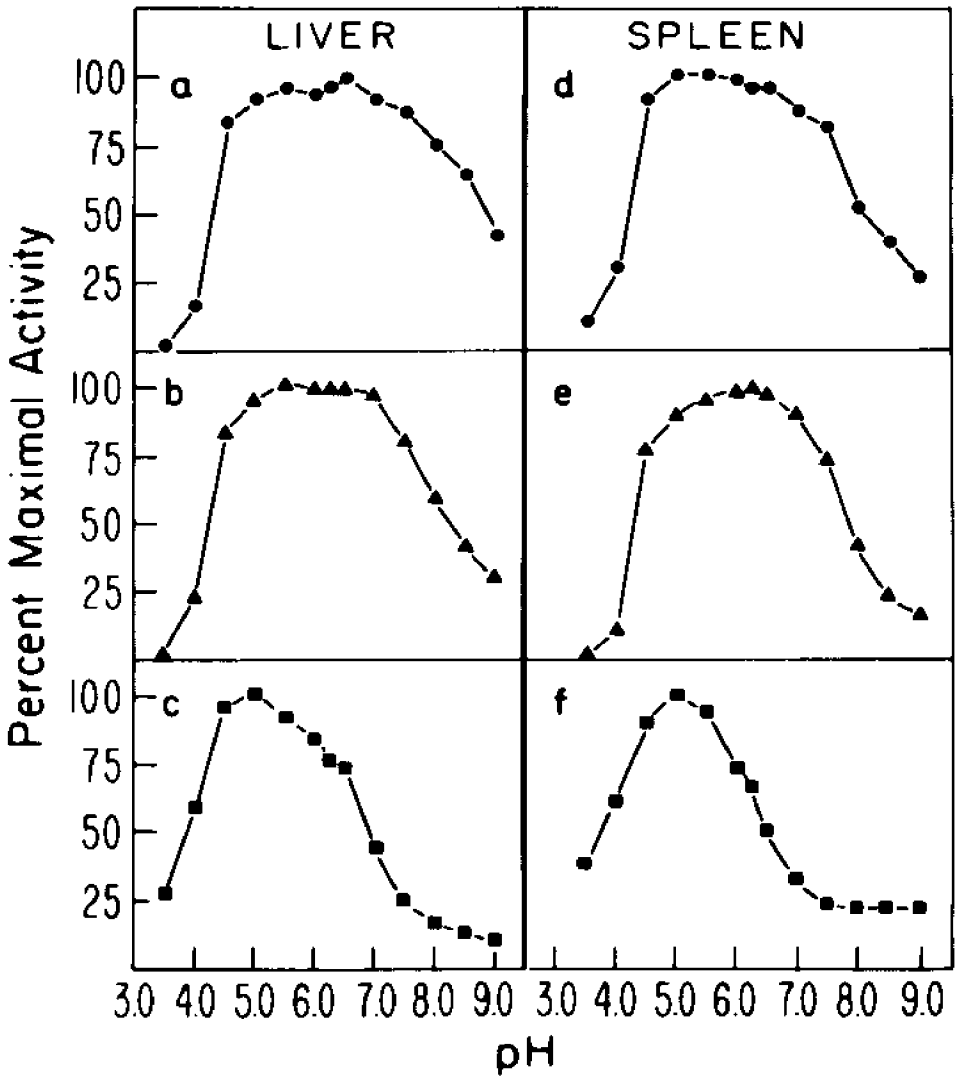
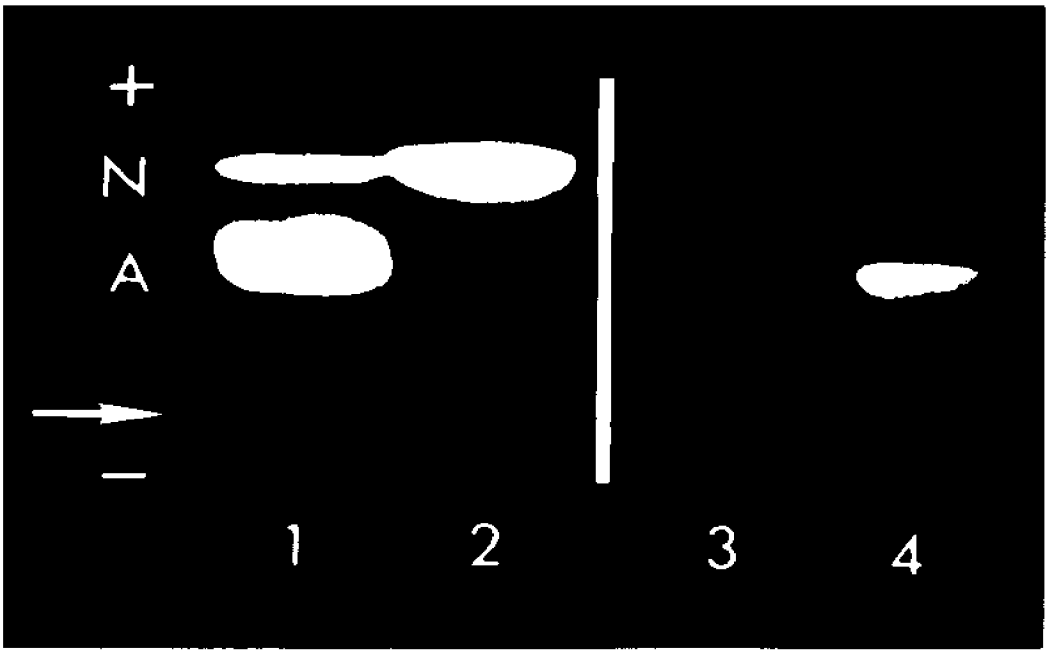

LIVER
SPLEEN
a
b
c
d
e
f
100
75
50
25
Percent Maximal Activity
3.0 4.0 5.0 6.0 7.0 8.0 9.0
pH

Figure 17: Fluorescent bands of β-glucosidase activity after cellulose acetate electrophoresis of homogenates from normal and Type 1 Gaucher disease tissues.

Lanes 1 and 2, normal and Type 1 Gaucher spleen, respectively; lanes 3 and 4, Type 1 Gaucher and normal skin fibroblasts, respectively. Equal amounts of total β-glucosidase activity from normal and Gaucher tissue homogenates were applied.


+
N
A
1
2
3
4

to migrate with, but had markedly diminished activity compared to the acidic activity in normal tissue homogenates. Prolonged electrophoresis (> 4 h) resulted in inactivation of the acid enzyme from Type 1 Gaucher tissue homogenates. In contrast, the neutral activity in hepatic and splenic homogenates from Gaucher and normal tissues co-migrated and appeared to have similar activities when the same amounts of homogenate protein were applied. The neutral β-glucosidase activity co-electrophoresed with α-L-arabinosidase, β-D-xylosidase, and the more anodal β-D-galactosidase activities when visualized with the appropriate substrate. Following electrophoresis, normal fibroblast homogenates revealed only acid β-glucosidase activity when stained with 4MUG; when incubated with its substrate, β-galactosidase activity band did not co-migrate with the acid β-glucosidase activity band. Furthermore, within the sensitivity of the method, no bands of activity were visualized when incubated with substrates for β-D-xylosidase or α-L-arabinosidase (data not shown).

IV. Studies of the Effector Molecule for Acid β-Glucosidase:

In homogenates of cultured skin fibroblasts, only acid β-glucosidase is expressed. As noted in Section III Results, the acid β-glucosidase activity in tissue and fibroblasts from Type 1 Gaucher disease appeared to co-migrate with the acid isozyme in normal tissues but had markedly reduced activity.

As shown in Figure 18, the acid β-glucosidase activity band from Type 1 Gaucher fibroblast homogenates (lane 1) occasionally migrated slightly less anodally than the homogenates from normal individuals (lanes 2). When mixing experiments were performed, the samples consistently migrated to the activity band of the Gaucher individuals. Purified acid β-glucosi-

dase from placenta migrated more anodally than the fibroblast samples (lane 3). When the placental enzyme was mixed with the fibroblast homogenates of normal (lane 5) and Type 1 Gaucher patients (lane 4), the activity band remained at the positions noted for normal and Type 1 fibroblast homogenates, respectively (lanes 2, 1).

As demonstrated in Figure 19, electrophoresis of fractions obtained from the purification of acid β-glucosidase were found to migrate more anodally following a 55% ammonium sulfate fractionation (lane 2). The supernatant from the 55% ammonium sulfate cut was concentrated, TCA-treated and mixed with the resuspended pellet containing acid β-glucosidase activity; following electrophoresis, the enzyme once again migrated less anodally (data not shown). The alteration in electrophoretic migration of purified acid β-glucosidase was also observed in the presence of effector isolated from the spleens of a Type 1 Gaucher homozygote and a normal individual (lanes 6 and 7, respectively). The purified effector retarded the electrophoretic migration of acid β-glucosidase regardless of whether or not the effector was boiled, lipase- or pronase-treated.

Figure 20 shows that effector isolated from normal and Type 1 Gaucher homozygote fibroblasts increased the specific activity of the purified acid β-glucosidase. As more effector was added, enzymatic activity also increased. Equal amounts of bovine serum albumin (BSA) added to the purified enzyme had no effect on its activity. Purified acid β-glucosidase mixed with effector, isolated from normal and Type 1 Gaucher homozygote spleen homogenates, demonstrated increased specific activity. However, the effector from Gaucher spleen homogenates was more effective than the effector from normal spleen homogenates in stimulating enzymatic activity (data not shown). Sucrose gradient ultracentrifugation demonstrated that

Figure 18: Cellulose acetate electrophoresis of normal and Type 1 Gaucher fibroblast homogenates and purified placental acid β-glucosidase.

Lane 1, Type 1 Gaucher fibroblast homogenate; lane 2, normal fibroblast homogenate; lane 3, purified placental acid β-glucosidase; lane 4, Type 1 Gaucher fibroblast homogenate and purified acid β-glucosidase; lane 5, normal fibroblast homogenate and purified acid β-glucosidase. Equal amounts of total β-glucosidase activity were applied. Samples were equalized for protein using BSA.

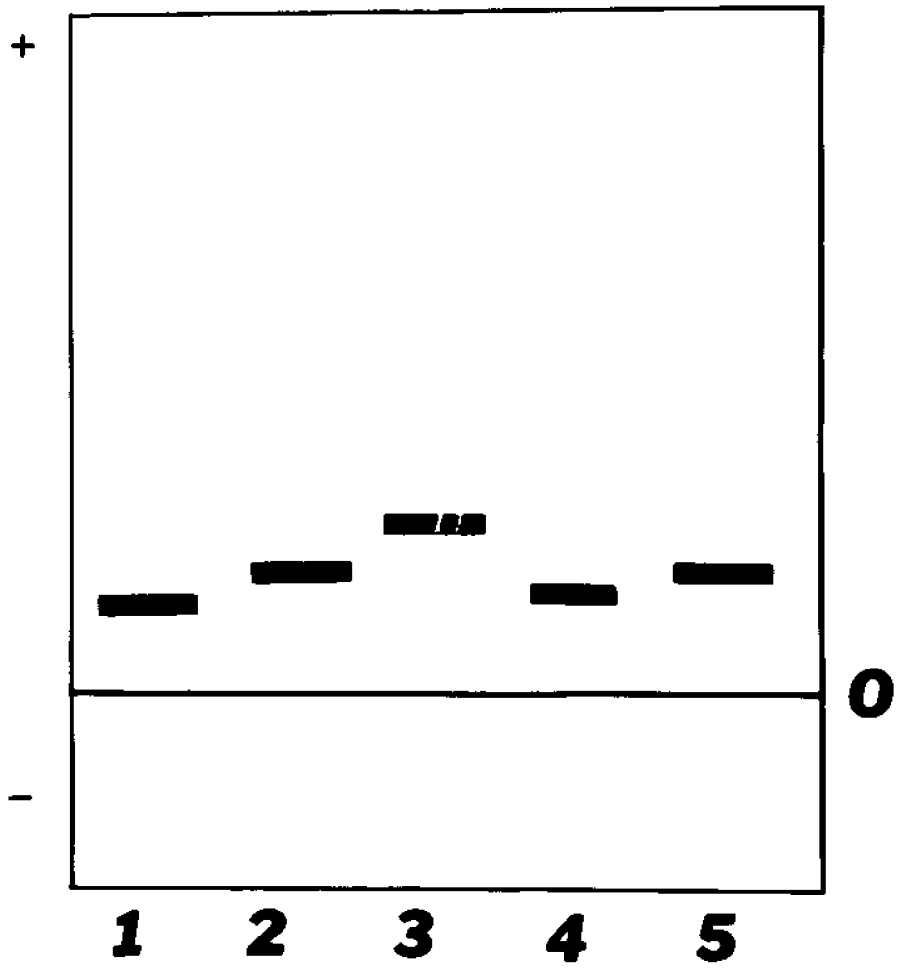

+
0
–
1
2
3
4
5

Figure 19: Cellulose acetate electrophoresis of placental acid β-glucosidase fractions and purified enzyme in the presence and absence of effector.

Lane 1, placental acid β-glucosidase in crude extract; lane 2, 33% ammonium sulfate fractionation; lane 3, 55% ammonium sulfate fractionation (resuspended pellet); lane 4, 20% (v/v) butanol extraction; lane 5, purified enzyme post-sucrose gradient ultracentrifugation; lane 6, purified enzyme with effector isolated from Type 1 Gaucher spleen homogenate; lane 7, purified enzyme with effector isolated from normal spleen homogenate. One unit of effector was utilized. One unit of effector is defined as that amount which would stimulate acid β-glucosidase activity two-fold.

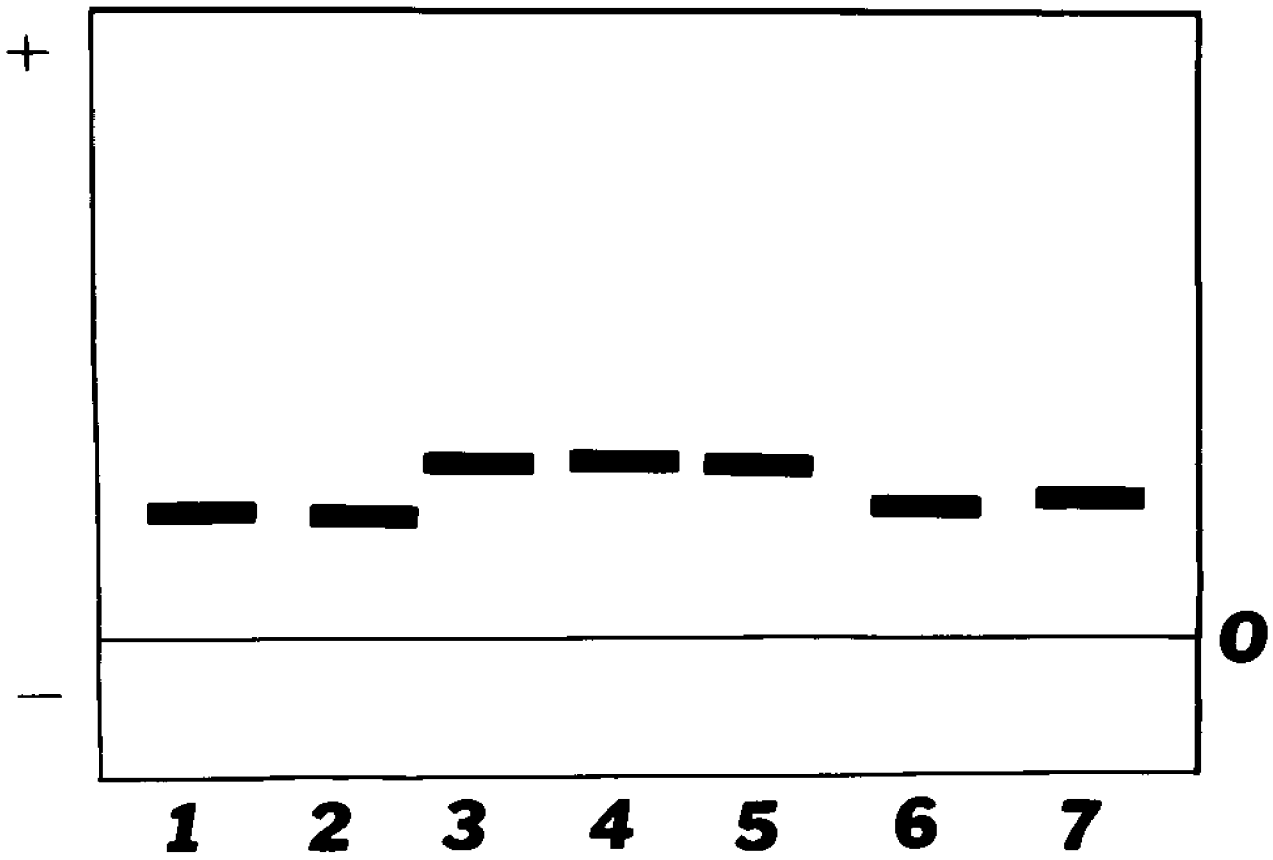

+
−
0
1
2
3
4
5
6
7

Figure 20: Human placental acid β-glucosidase activity observed in the presence and absence of effector.

Effector from normal (■–■) and Type 1 Gaucher (▲–▲) fibroblast homogenates. BSA (●–●) was added to the purified enzyme as a control. The amount of effector protein was varied from 0.0 to 0.5 mg/sample.

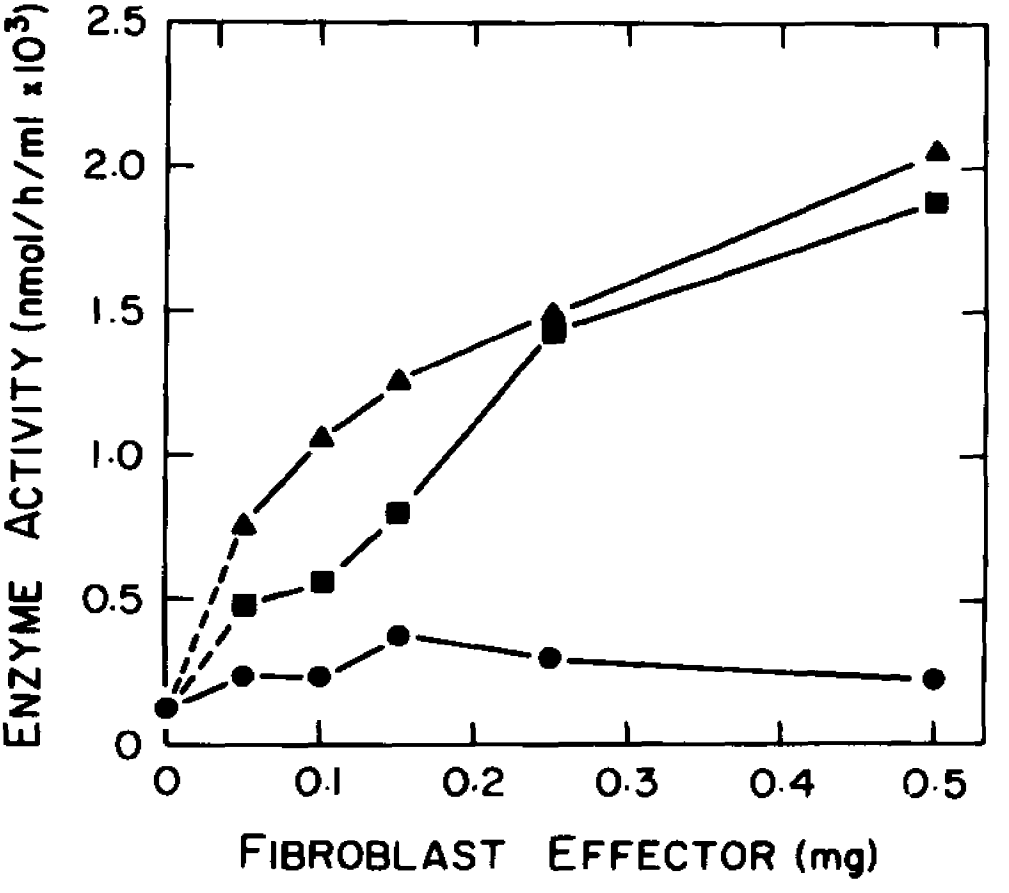
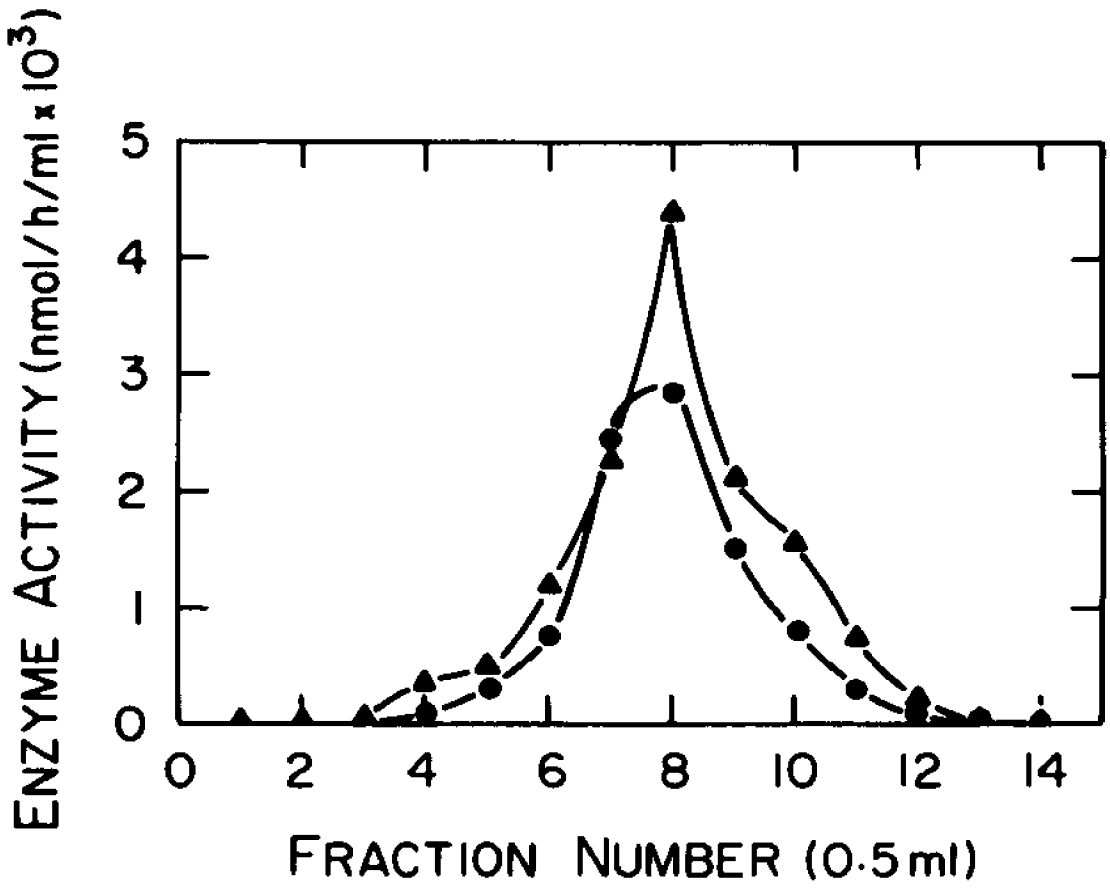

ENZYME ACTIVITY (nmol/h/ml x10³)
2.5
2.0
1.5
1.0
0.5
0
0
0.1
0.2
0.3
0.4
0.5
FIBROBLAST EFFECTOR (mg)

Figure 21: Sucrose density ultracentrifugation of purified acid β-glucosidase from human placenta in the presence and absence of 1 U of effector.

Fractions (0.5 ml) were assayed for acid β-glucosidase activity using the artificial substrate, 4MUG. Purified acid β-glucosidase minus effector (●–●). Purified acid β-glucosidase pre-incubated with effector (▲–▲).


ENZYME ACTIVITY (nmol/h/ml x 10³)
0
1
2
3
4
5
0
2
4
6
8
10
12
14
FRACTION NUMBER (0.5 ml)

the effector bound to purified acid β-glucosidase. The enzyme was run in the presence and absence of effector (1 U) and as shown in figure 21, enzyme activity increased two-fold in the presence of the effector. The effector did not alter the sedimentation rate of the enzyme.

Heat stability studies performed with purified acid β-glucosidase minus its effector molecule demonstrated a $t_{1/2}$ of 4.5 minutes. Addition of effector to either normal or Type 1 Gaucher fibroblasts had no effect on the enzymatic stability at 37°C or 50°C. Furthermore, pre-incubation with the effector for 5 min at 37°C for 30 min at room temperature or 30 min on ice had no effect on either enzyme stability or on activity.

V. Gene Mapping:

A. Specificity of Anti-Human Acid β-Glucosidase In Hybrid Clones:

A double antibody immunoprecipitation assay for human acid β-glucosidase was developed (Table 8). In this system, purified human placental acid β-glucosidase activity was depleted from the supernantant and 45% of the total recovered activity was present in the immunoprecipitate. Acid β-glucosidase activity in solubilized human fibroblast extracts was also precipitated by the antiserum; typically 33-45% of the total recovered activity was precipitated. In the absence of the anti-acid β-glucosidase antibody and/or the rabbit anti-mouse Ig, no acid β-glucosidase activity was detected in the immunoprecipitate. Partially purified neutral β-glucosidase from human liver was not immunoprecipitated nor was any β-glucosidase activity precipitated from mouse (RAG), rat, or Chinese hamster fibroblasts. In addition, the immunoprecipitate showed no enzymatic activity when assayed for the presence of other glycosidases including, α-L-arabinosidase, β-galactosidase at pH 4.5 and 7.0, α-glucosidase at pH

Table 8

Specificity of the Immunoprecipitation Assay

For Human Acid β-Glucosidase (GBA)

Enzyme Source	Anti-GBA Antibody	Rabbit Anti-Mouse Ig	% Total Activity β-Glucosidase Recovered	
			Supernatant	Pellet
Purified Human Placenta GBA	+	+	65	35
Human Fibroblast Extract	+	+	72	28
"	+	-	100	ND ‡
"	-	+	100	"
"	NMS †	+	100	"
Partially Purified Human Liver GBN*	+	+	100	"
RAG Fibroblast Extract	+	+	100	"
RAT Fibroblast Extract	+	+	100	"
Chinese Hamster Fibroblast Extract	+	+	100	"

*GBN (neutral β-glucosidase) was partially purified from human liver as described in Methods and used as the β-glucosidase source in this assay.

† NMS = normal mouse serum

‡ ND = not detectable

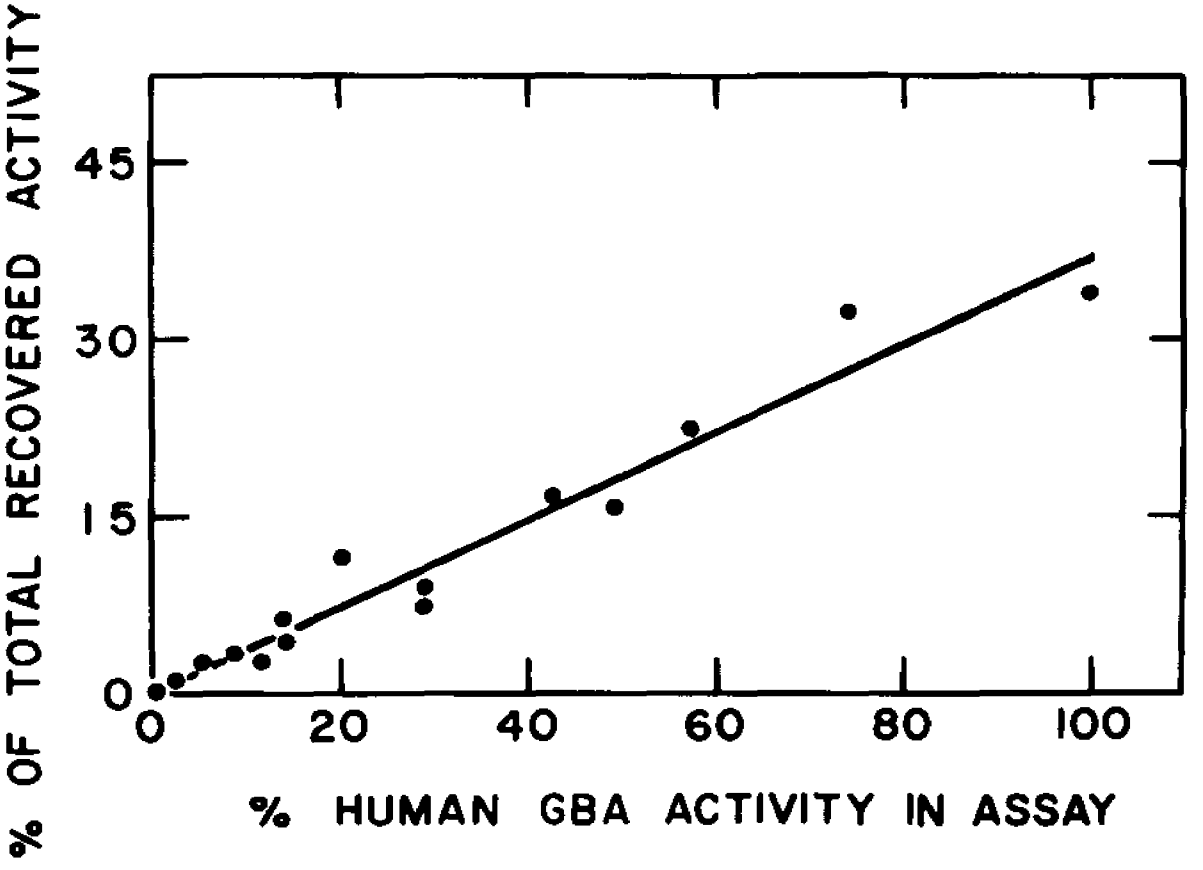
4.0 and 6.0, β-glucuronidase, β-hexosaminidase B and β-D-xylosidase. Thus, the antiserum was specific only for the human acid β-glucosidase isozyme.

For detection of human acid β-glucosidase in hybrid clones, the sensitivity of the competitive immunoprecipitate assay for human acid β-glucosidase was determined in the presence of mouse β-glucosidase activity. Figure 22 shows that the anti-human acid β-glucosidase antibody permitted the sensitive and specific precipitation of human acid β-glucosidase in mixtures containing varying percentages of human and mouse fibroblast extracts. The assay was linear over the entire range of 0 to 2 U of the human enzyme and allowed the sensitive detection of human acid β-glucosidase in man-mouse hybrid clones.

For each set of immunoprecipitation assays, diploid human fibroblast extracts were used as controls. A mean value of 37.8% (range 30.6-44.9%, n=12) of total recovered β-glucosidase activity was found in the human control immunoprecipitates. On the basis of gene dosage, it was estimated that homogeneous hybrid lines carrying a single human chromosome coding for acid β-glucosidase would have about 1/3 of the total recovered β-glucosidase activity in the immunoprecipitate compared to control diploid human fibroblasts. Clones which were not homogeneous for the chromosome containing the acid β-glucosidase structural gene would contain fewer molecules of human acid β-glucosidase. Only clones containing a human chromosome 1 in at least 30% of the metaphases studied were considered positive for that chromosome. Therefore, the maximal expected percent immunoprecipitated activity in a heterogeneous clone with 30% of the metaphase spreads containing human chromosome 1 would be 30% times the maximal % immunoprecipitated activity in diploid human fibroblasts (37.8)

Figure 22. Immunoprecipitation of human acid β-glucosidase in mixtures of parental mouse and human fibroblast extracts.

The total amount of acid β-glucosidase activity (2 U) in the assay was the same in all cases; however, the mixtures contained the indicated percentage of human fibroblast extract.


% OF TOTAL RECOVERED ACTIVITY
45
30
15
0
0
20
40
60
80
100
% HUMAN GBA ACTIVITY IN ASSAY

times 1/3 to adjust for gene dosage in the hybrid cell, i.e., 0.3 X 37.8% X 1/3 = 3.78% of total recovered activity. Thus, hybrids were scored positive for human acid β-glucosidase when the % of immunoprecipitated acid β-glucosidase was greater than 4% of total recovered activity [or greater than 10% of the human diploid fibroblast control activity precipitated (see Table 10)].

B. Segregation Analysis of Human Acid β-Glucosidase in Cell Hybrids: Segregation of human acid β-glucosidase and the enzyme markers for the human chromosomes in primary and secondary hybrid clones is shown in Table 9. Table 10 shows the human chromosome complements and the immunoprecipitation data in representative secondary or tertiary clones selected for the presence or absence of human acid β-glucosidase. Based on these analyses, all human chromosomes except chromosome 1 were excluded from gene assignment for human acid β-glucosidase. The segregation of human acid β-glucosidase activity in the hybrids demonstrated 100% concordant expression of the enzyme with phosphoglucomutase 1 (PGM1), fumarate hydratase (FH) and the intact chromosome 1. All the other chromosomes had a discordant frequency for acid β-glucosidase ranging from 0.26 to 0.75 and therefore, could be eliminated.

C. Regional Localization of Acid β-Glucosidase on Chromosome 1: A hybrid cell line ($R/KidA_{10}$) with a human-mouse rearrangement involving human chromosome 1 was used to further localize the gene for acid β-glucosidase. As shown in Figures 23 and 24, the chromosomal rearrangement was cytogenetically defined as human 1pter → p11 to a mouse chromosome by Giemsa 11 and Q-banding. Consistent with the cytogenetic analysis, the hybrid line was positive for human chromosome 1 short arm markers, enolase 1 (ENO1), PGM1 and α-fucosidase (FUCA), and was negative for the long arm

Table 9

Segregation of Human Chromosome Markers and Human Acid α-Glucosidase In Primary and Secondary Somatic Cell Hybrids

Enzymes*	Chromosome	Concordant +/+ P*	Concordant +/+ S†	Concordant -/- P	Concordant -/- S	Discordant +/- P	Discordant +/- S	Discordant -/+ P	Discordant -/+ S	Frequency Discordant
PGM1, FH	1	14	19	8	11	0	0	0	0	0.00
MDH1	2	1	0	3	0	7	1	0	1	0.69
GLB1	3	6	7	0	0	2	0	4	4	0.43
PGM2	4	3	4	1	0	3	3	3	1	0.56
HEXB	5	3	8	1	4	3	2	4	3	0.43
SOD2, ME1	6	3	4	4	0	7	0	4	1	0.52
GUSB	7	1	2	0	0	5	0	2	0	0.70
GSR	8	0	4	5	1	2	3	2	2	0.47
AK1, AK3, ACON1	9	14	4	2	4	0	4	7	0	0.31
GOT1	10	3	0	1	1	4	1	4	0	0.64
LDHA	11	1	1	5	4	7	5	2	2	0.59
LDHB, PEPB	12	7	5	5	3	4	0	2	1	0.26
ESD	13	4	3	0	1	2	2	4	0	0.50
NP	14	3	5	1	5	4	1	3	0	0.36
HEXA, MPI	15	5	7	1	0	5	2	5	6	0.58
PGP	16	2	4	3	3	2	2	3	0	0.37
GLUA, GALK	17	0	-	4	-	2	-	1	-	0.75
PEPA	18	7	4	2	1	1	0	5	1	0.33
GPI	19	10	8	5	9	1	5	2	3	0.26
ADA	20	5	1	4	2	4	2	1	1	0.40
SOD1	21	9	3	1	1	0	0	6	3	0.39
ACON2	22	4	0	2	3	2	3	4	0	0.50
G6PD	X	2	-	1	-	2	-	3	-	0.63

*Enzyme markers were performed by starch or cellulose acetate gel electrophoresis as previously described (81-88).

* P = Primary; † S = Secondary

Table 10

Segregation of Human GBA and Human Chromosomes in RAG X Human Fibroblast Secondary and Tertiary Hybrid Clones

Cell Line	Human GBA			Chromosomes																				
	% Immunoprecipitate*	% Human Control†	Pos/Neg‡	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Human Fibroblast	37.8	100.0																							
RAG	0.0	0.0																							
RAG X F_2 Liver:																									
R/F_2Li 1B	14.7	38.8	+	+	-	+	+	-	+	-	-	+	+	-	-	-	+	-	-	+	+	-	+	+	+
R/F_2Li 1C	17.0	44.9	+	+	-	+	-	+	+	-	-	+	-	+	+	+	-	-	+	-	-	+	-	+	-
R/F_2Li 1E	8.7	23.0	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	-
R/F_2Li 1F	5.0	13.2	+	+	-	+	+	+	+	-	-	+	+	-	-	-	-	+	+	-	+	-	-	+	+
R/F_2Li 1G	13.7	36.2	+	+	-	+	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	+	+	-
R/F_2Li F_3	2.4	6.3	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
RAG X F_3 Lung:																									
R/F_3Lu $E_{21}G$	7.3	19.3	+	+	-	+	-	+	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
R/F_3Lu E_2H	0.9	2.3	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-
R/F_3Lu E_{14}	2.9	7.6	-	-	-	-	+	-	+	-	-	-	-	+	+	-	+	-	+	-	-	-	-	+	+
RAG X F_3 Kidney:																									
R/F_3Kid A_1	7.3	19.3	+	+	-	-	-	+	-	-	-	+	-	-	-	+	-	+	-	-	-	+	-	+	+
R/F_3Kid A_4	7.5	19.8	+	+	-	+	-	+	-	-	+	+	-	-	-	-	-	+	+	-	-	+	-	+	-
R/F_3Kid A_{12}	5.6	14.3	+	+	-	-	-	-	-	-	+	-	+	-	+	-	-	+	+	+	+	+	-	+	+
R/F_3Kid Q_1	13.7	36.2	+	+	-	+	-	+	-	-	-	-	-	-	+	+	+	-	-	+	+	+	-	+	-
R/F_3Kid Q_3	7.4	19.5	+	+	-	+	-	+	-	-	+	-	-	-	+	-	-	+	+	-	-	-	-	+	+
R/F_3Kid A_3	0.0	0.0	-	-	-	+	-	+	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-
R/F_3Kid U_3	0.5	1.3	-	-	-	+	+	+	-	-	+	+	-	-	-	-	-	+	+	+	+	+	-	+	+

* Percentage of total recovered activity in immunoprecipitate.

† GBA activity in immunoprecipitate of hybrids expressed as a percentage of activity immunoprecipitated from human fibroblast controls.

‡ Hybrids scored as positive had ≥10% control human parental fibroblast GBA activity in immunoprecipitates.

Figure 23. Metaphase chromosome spreads showing the human 1(p11 → pter)/ mouse chromosome translocation carried in the mouse-human hybrid line R/KidA$_{10}$.

A) Giemsa 11 banding and B) Quinacrine fluorescent banding. The translocation chromosome is indicated by the arrow.

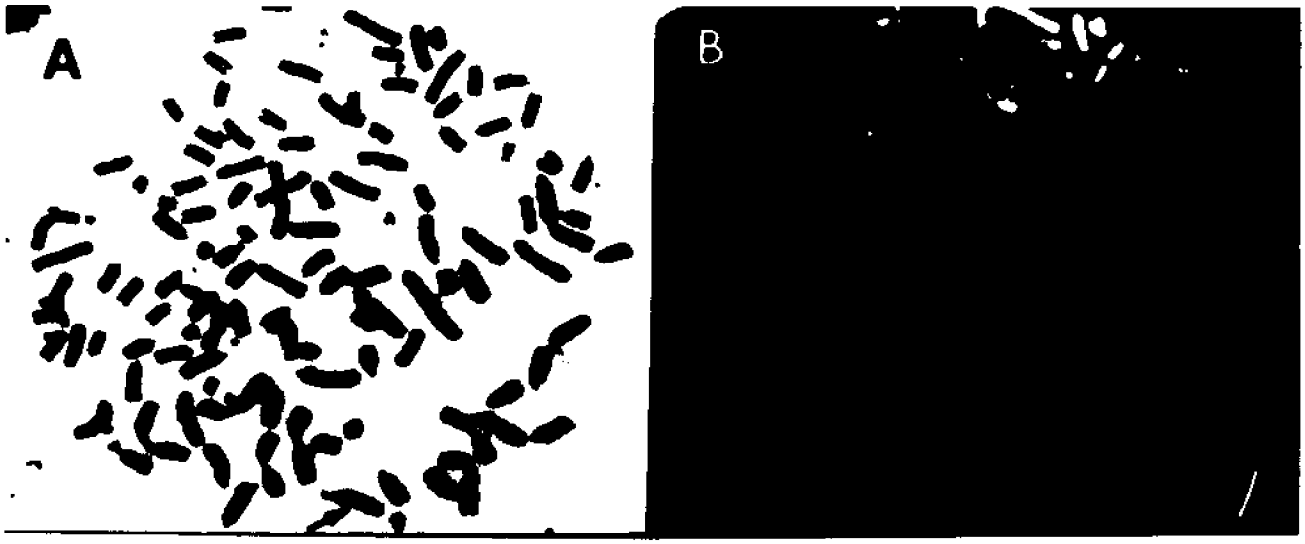

A
B

Figure 24. Chromosome 1 from metaphase spreads of normal human cells and the R/KidA$_{10}$ hybrid clone carrying the mouse-human chromosome 1 (p11→ pter) translocation.

A) Normal human chromosome 1 banded by Giemsa 11 and quinicrine, respectively; B and C) The translocated chromosome banded with Giemsa 11 and quinicrine, respectively. The darkly staining mouse chromosomal material is detected by the Giemsa 11 stain while the quinicrine banding demonstrated the translocation to involve the p region of human chromosome 1.

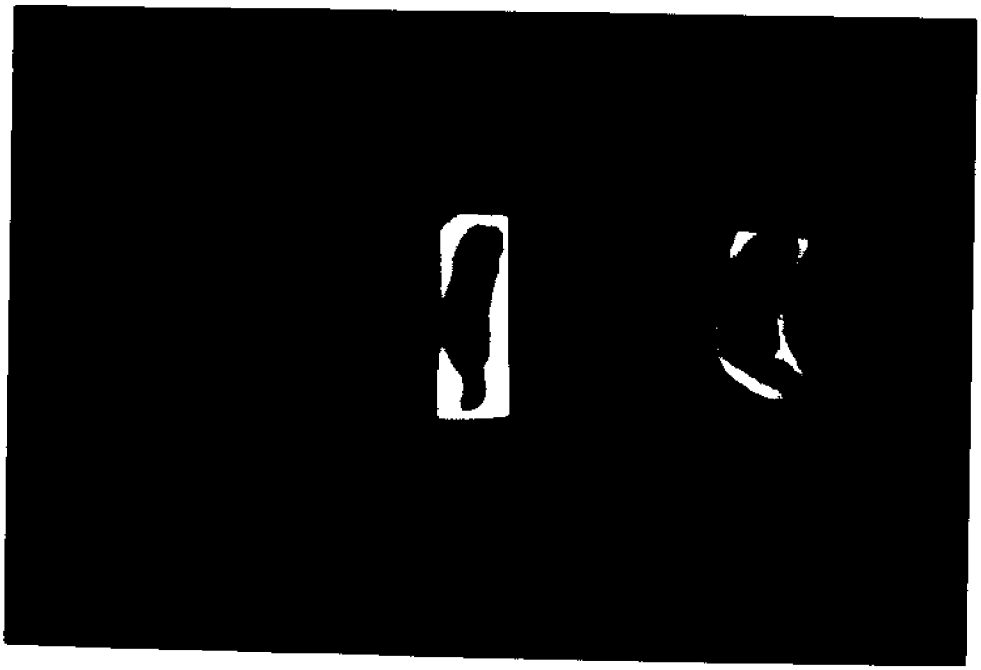
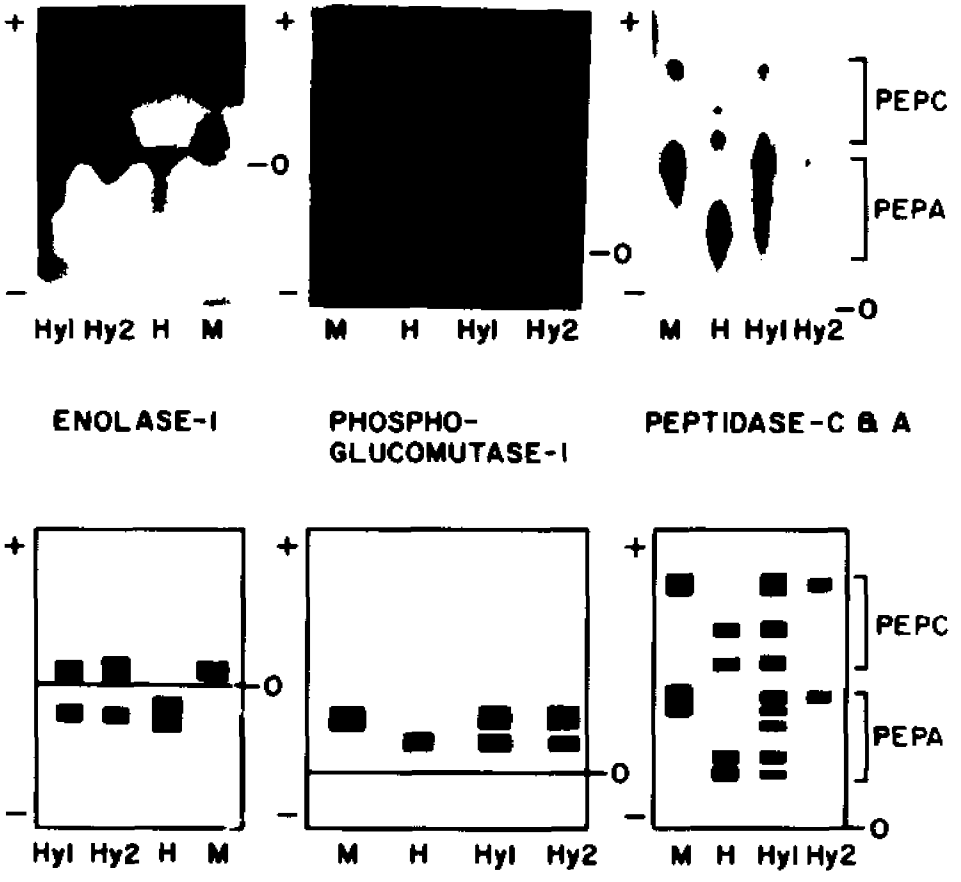


Figure 25. Starch gel electrophoresis of enzymatic markers for chromosome 1.

Fibroblast extracts of mouse (M), human (H), R/Lung E_{21} (Hy1) and R/KidA_{10} (Hy2) were electrophoresed and stained for enzymatic activity of various chromosome 1 enzyme markers. The mouse-human hybrid line, Hy1, which carries an intact human chromosome, demonstrated both mouse and human isozymes for all the chromosome 1 markers tested. The Hy2 hybrid line, which carries the chromosome 1 (p11 → pter)/mouse translocation, was positive for the short arm markers, enolase 1 (ENO1) and phosphoglucomutase 1 (PGM1) and negative for the long arm marker, peptidase C (PEPC). These results confirmed the cytogenetic data. The isozyme pattern of each gel is shown diagramatically below.


+
−
O
Hyl Hy2 H M
M H Hyl Hy2
PEPC
PEPA
ENOLASE-I
PHOSPHO-
GLUCOMUTASE-I
PEPTIDASE-C & A

markers, peptidase C (PEPC) and FH. Representative gels for ENO1, PGM1 and PEPC are shown in Figure 25. The absence of detectable acid β-glucosidase immunoprecipitable activity in this hybrid eliminated assignment of the acid β-glucosidase structural gene to the region 1pter → p11.

DISCUSSION

I. Purification of Human Placental Acid β-Glucosidase:

Acid β-glucosidase was enriched 3900-fold over the initial placental extract, and the final enzyme preparation was free from 12 other hydrolases. Affinity chromatography on dextran sulfate-Sepharose proved to be a useful step in the purification of acid β-glucosidase. Butanol extraction of the partially purified β-glucosidase preparation enhanced enzyme binding to the dextran sulfate column. This enhanced binding may be explained by the exposure of some hydrophobic sites which were previously shielded by lipids or other membrane components. The column would be expected to bind cationic proteins and, despite attempts to minimize this type of interaction, considerable ionic binding did occur. The enzyme itself could be eluted with salt gradients, but such elution gave only a 2-fold purification and low yield. In contrast, a crude sodium taurocholate gradient (0-5 mg/ml) gave a 10-fold purification. Thus, the ability of the column to give good purification was dependent upon the specificity of the eluant. Following the addition of crude sodium taurocholate, a major peak of UV-absorbing material was eluted with 5M NaCl, but no acid β-glucosidase activity was detected.

The inhibition of β-glucosidase activity by sulfated macromolecules might be attributed to the interaction of these compounds with either the enzyme or activator(s) which enhance the activity of the solubilized enzyme. However, the binding of the enzyme to immobilized dextran sulfate and its specific elution with crude sodium taurocholate, suggests a direct interaction between the inhibitor and the enzyme itself.

The sucrose density gradient ultracentrifugation was useful in later

stages of the purification, separating the acid β-glucosidase from both major lysosomal contaminants, β-hexosaminidase B and β-glucuronidase. β-Glucuronidase was easily separated from the acid β-glucosidase using a hydroxyapetite column. However, β-hexosaminidase B remained, and only a two-fold increase in specific activity was observed. Con-A Sepharose, wheat germ lectin and phenyl-Sepharose also were unsuccessful in separating acid β-glucosidase from β-hexosaminidase B. Rechromatography on octyl-Sepharose did not increase the specific activity of the enzyme and ion exchange and Sephadex chromatography were not useful as acid β-glucosidase activity was rapidly lost.

Although the yield of the purified enzyme was low (6%), the addition of glycerol and 2-ME stabilized the purified acid β-glucosidase and stability was retained for months when stored at -20°C. The final preparation had a specific activity of 0.27 X 10^6 nmoles/h/mg protein as compared to specific activities of 0.078 X 10^6 nmoles/ h/mg protein (32) and 0.15 X 10^6 nmoles/h/mg protein (34) reported for the enzyme using the artificial substrate, 4MUG. The purity of the enzyme preparation was difficult to establish since acid β-glucosidase does not migrate into native polyacrylamide gels and when taurocholate was added to allow migration, this compound interfered with protein staining (29). Upon SDS-polyacrylamide electrophoresis, multiple bands were observed, indicating that the enzyme was not homogeneous. The two major bands observed had molecular weights of 67,600 and 79,400, similar to the doublet observed by Furbish et al. (36). It is not known whether the three minor bands represent breakdown products or other contaminants.

II. Evaluation of NBD-Glucosyl Ceramide Activity:

A fluorescent natural substrate for β-glucosidase has been recently synthesized. The procedure is straightforward, and the fluorescent derivative, NBD-glucer, is far easier to prepare than the radioactive substrate (94). The NBD-glucer assay was rapid, very sensitive and much less enzyme was needed since the fluorescent product was detectable in picomole quantities. This sensitivity was achieved with a 10-fold dilution of the NBD-glucer with the natural glucosyl ceramide permitting conservation of the fluorescent substrate. The linear relationship observed between initial enzyme rates and the ratio of NBD-glucer:glucosyl ceramide demonstrated the inability of the acid β-glucosidase to discriminate between the two natural substrates (Figure 11). It was shown (Table 6) that acid β-glucosidase readily hydrolyzed NBD-glucer. Furthermore, the ratio of NBD-glucer:4MUG activities (∿10:1) stayed fairly constant throughout the purification demonstrating that the same enzyme was being purified and assayed with both substrates. Similarly, Pentchev et al. (32) found a 13-fold difference in specific activity between the natural substrate D-[1-^{14}C]-glucosyl ceramide and 4MUG. The 3-4 fold higher apparent K_m observed using the NBD-glucer (0.287 mM) vs. the radiolabeled natural substrate (0.065-0.87 mM) could be explained by the different detergent conditions (32,36) used in the respective assays. It has been well documented that protein, enzyme and detergent concentrations can each affect the K_m when the substrate is a lipid (95,96). The NBD-glucer assay was linear with time (up to 30 min) and with increasing enzyme concentration at the levels used in these experiments. The enzyme demonstrated a Km of 0.287 mM when assayed from 0.0125 mM to 0.250 mM total glucosyl ceramide. Substrate was not soluble at greater than 0.250 mM using the described

buffer and taurocholate mixture. Therefore, the assays were performed with a substrate concentration below the K_m. To obtain an accurate specific activity for each enzyme sample, dilutions of each sample were assayed for 10 min. For this short incubation period, only a small portion (∿20%) of the substrate was hydrolyzed, assuring that first-order kinetics were maintained.

III. Studies of the Effects of Phospholipids on Purified Acid β-Glucosidase:

Various purified phospholipids have been shown to be effective activators of the purified acid β-glucosidase and to give a maximum specific activity comparable to that obtained with taurocholate preparations. Phosphatidic acid was found to be slightly more effective than the substituted phospholipids, phosphatidyl serine and phosphatidyl inositol. Phospholipids were also effective in alleviating inhibition of the enzyme by dextran sulfate suggesting that the inhibitory effect of sulfated macromolecules on purified acid β-glucosidase may occur through binding to a site involved in binding the negatively charged phosphate moiety of the phospholipid. This conclusion was consistent with the observation that the neutral phospholipids, phosphatidyl ethanolamine and phosphatidyl choline, were ineffective activators of acid β-glucosidase (45,97).

It would be expected that hydrophobic sites would also play a role in stabilizing the enzyme-phospholipid interaction. Evidence for this can be deduced from the effects of the non-ionic detergent Triton X-100 on acid β-glucosidase. The influence of this detergent on the sedimentation rate of the putative enzyme-phospholipid complex (Figure 12) and the inhibition of enzyme activity by relatively low detergent concentrations may both be

attributable to disruption of the enzyme-phospholipid interaction. These studies indicate that the stability and activity of human acid β-glucosidase are dependent on a complex array of ionic and non-ionic interactions.

IV. Separation of Acid and Neutral β-Glucosidases:

Electrophoresis on cellulose acetate slab gels permitted the resolution of both the acid and neutral β-glucosidases in normal tissue homogenates. This was important in determining whether residual neutral or acid β-glucosidase activity was present in purified preparations since the artificial substrate, 4MUG cannot distinguish between the two enzymes. The identity of the acid and neutral enzymes was based on the following experimental findings: (1) Chromatography on con A-Sepharose resulted in the separation of the two β-glucosidase activities and permitted characterization of their respective pH profiles. The acidic activity bound to this lectin indicating its glycoprotein nature, similar to other acidic lysosomal hydrolases (25). The more neutral activity did not bind suggesting a different carbohydrate structure or the lack of post-translational glycosylation. Following electrophoresis, the acidic and neutral activities resolved by con A-Sepharose each co-migrated with only one of the activity bands present in hepatic or splenic homogenates. (2) Only the acidic activity band was observed in normal fibroblasts, consistent with the previous demonstration that only the acidic activity is expressed in these cultured cells (12,17). In addition, (3) the markedly reduced activity of the slower migrating band in tissue homogenates from Type 1 Gaucher homozygotes supports the identification of this electrophoretic band as acid β-glucosidase responsible for residual glucocerebrosidase

activity. (4) The neutral β-glucosidase activity band exhibited activity with other artificial glycoside substrates as previously reported (28), whereas the acidic activity band only had activity toward 4MUG. Finally, (5) only acid β-glucosidase activity, which bound to con A-Sepharose had activity towards the natural substrate, NBD-glucer. The neutral enzyme had no activity toward the natural substrate.

The acidic activity band in Type 1 Gaucher tissue homogenates was visualized and appeared to co-migrate with the acidic activity in normal tissue homogenates. The most likely explanation for the loss of activity observed on electrophoresis is a point mutation which altered the stability as well as the activity of the residual acid β-glucosidase in Type 1 Gaucher disease. Support for this hypothesis was described by Turner and Hirschhorn (17). They found that fibroblast homogenates from Type 1 Gaucher patients were more thermolabile than homogenates from normal controls.

This electrophoretic method differs markedly from a recently described system for β-glucosidase (30) in which (1) only one activity band was visualized in normal hepatic and splenic homogenates, (2) acid and neutral β-glucosidases were not identified, and (3) no activity was observed in Gaucher fibroblast homogenates. In part, this might be explained by the use (in these studies) of ethylene glycol in the electrophoretic buffer and the ethanol-chloroform treatment which enhanced the entrance and migration into the gel of the acidic activity from normal and especially from Type 1 Gaucher tissue homogenates. This finding may be related to the known interaction of the membrane-bound acidic activity with phospholipids (24,49), and suggests that the defective acid β-glucosidase in Type 1 Gaucher disease may also have an altered binding to

crucial lipids, substrate, effector molecules or other hydrophobic moieties in the membrane. A more recent report (29), described a method for the polyacrylamide gel electrophoresis of only the acid enzyme. Unfortunately, this system requires large amounts of enzyme activity and taurocholate which selectively inhibits neutral β-glucosidase (24).

The enzymes, identified by the electrophoretic and lectin-binding studies, correspond to the acid membrane-bound activity which is deficient in Type 1 Gaucher disease and the more neutral, soluble activity which may be involved in the post-translational processing of glycoproteins. The electrophoretic separation of these enzymes should provide the means to investigate the molecular nature and interrelationships of the β-glucosidase deficiencies in Gaucher disease subtypes.

V. Evaluation of the Role of the Acid β-Glucosidase Effector:

Mixtures of effector isolated from either normal or Type 1 Gaucher homogenates (fibroblasts or spleen) bound to purified placental acid β-glucosidase and altered the enzyme's electrophoretic migration. When purified acid β-glucosidase was mixed with effector the enzyme consistently migrated less anodally. When mixtures of enzyme and effector were subjected to sucrose gradient ultracentrifugation (Figure 21), enzyme-effector complexes were distinguishable by a two-fold increase in acid β-glucosidase activity observed in the presence of effector as opposed to enzyme alone. This effect was not caused by additional protein in the preparation since equal amounts of BSA did not stimulate enzyme activity. When enzyme fractions from the purification of acid β-glucosidase activity were monitored electrophoretically, the enzyme appeared to lose its effector molecule after a 55% ammonium sulfate fractionation step. The resus-

pended pellet from the ammonium sulfate cut contained acid β-glucosidase activity while the supernatant, which had no enzyme activity, contained the effector molecule. The effector was partially purified from the supernatant and the presumed effector-enzyme complex was reconstituted. Upon electrophoresis, the enzyme-effector complex migrated less anodally than the enzyme alone. Furthermore, this complex migrated to the same position as the acid β-glucosidase activity prior to the 55% ammonium sulfate fractionation.

The alteration in migration of the enzyme caused by addition of the effector molecule may be caused by (1) a change in the net charge of the enzyme-effector complex as opposed to enzyme alone or (2) a conformational change in the enzyme caused by binding of the effector molecule. During the acid β-glucosidase purification, phospholipids or lipids may be stripped off and the enzyme may have more negatively charged sites exposed on its surface. These sites may be masked when the enzyme-effector complex is formed, or a conformational change occured making the enzyme-effector complex less electronegative, thus causing it to migrate less anodally.

Effector from normal and Type 1 Gaucher tissue was found to increase the enzyme activity of purified acid β-glucosidase (Figure 20). As the amount of effector increased, enzyme activity also increased. On a protein to protein basis, the effector from normal and Gaucher fibroblasts stimulated enzyme activity to the same extent. When effector was isolated from the spleens of a control and a Type 1 Gaucher individual, more effector activity was observed in the Gaucher spleen than the normal spleen. Peters et al. (44) observed that the Gaucher effector isolated from Type 1 Gaucher spleen homogenate gave a higher specific activity than that of the effector from normal spleen. The normal effector was only 6% as

effective as the Gaucher spleen effector in stimulating acid β-glucosidase activity of human liver homogenates (44). It is possible that different concentrations of effector are present in different organs. Since the spleen is one of the major target organs affected in Gaucher disease, a higher concentration of effector might be present. The effector could bind to the enzyme and by direct interaction the enzyme:effector complex might solubilize and hydrolyze the substrate. However, when effector from the spleens of five patients with Type 1, 2 and 3 Gaucher disease were isolated and studied (98), no correlation between the amount of effector present and the severity of the disease or age of onset was found.

Although acidic phospholipids and taurocholate stimulate acid β-glucosidase more effectively, the effector may still play a role in ameliorating the physiological pathology of Gaucher disease.

VI. Chromosomal Localization of the Human Acid β-Glucosidase Gene:

The structural gene for human acid β-glucosidase has been localized to chromosome 1 using somatic cell hybridization techniques and a double antibody immunoprecipitation assay specific for the human acid β-glucosidase isozyme. This is the first time a chromosomal assignment has been designated for this enzyme. In all 52 hybrid clones examined, 100% concordant expression of human acid β-glucosidase and the enzymatic markers for chromosome 1, PGM1 and FH, was observed (Table 9). Cytogenetic analysis of these hybrid lines also demonstrated segregation of acid β-glucosidase with an intact chromosome 1 (Table 10). All other human chromosomes showed discordancy for acid β-glucosidase as determined by either enzyme marker or cytogenetic analyses.

The assignment of acid β-glucosidase to chromosome 1 was strengthened

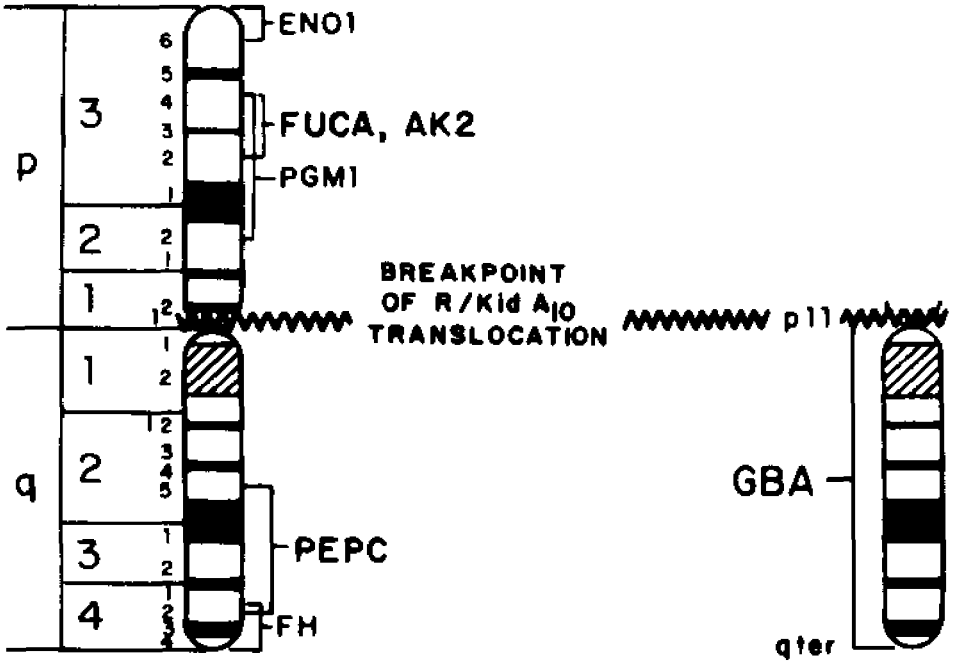
by the following factors. First, the somatic cell hybrids used were derived from the fusion of a mouse RAG cell line with human fibroblasts from three different individuals. This excludes a potential source of error arising from a single fusion containing a large number of hybrid subclones (99). Second, only the acid β-glucosidase isozyme was expressed in human fibroblast cell lines (17), thereby eliminating the possibility of precipitating the human neutral β-glucosidase isozyme. In support, partially purified hepatic neutral β-glucosidase was not precipitated by the antiserum. Third, the antibody to human acid β-glucosidase was prepared in Balb/C mice which decreased the probability of any cross-reactivity between the human and mouse acid β-glucosidase isozymes. In fact, no cross-reactivity between the mouse, rat, or Chinese hamster fibroblast β-glucosidase and the human enzyme was observed. Furthermore, the immunoprecipitation assay proved to be a sensitive and reliable method to detect human acid β-glucosidase activity in the presence of the mouse isozyme.

In addition to assigning the structural gene for human acid β-glucosidase to chromosome 1, the gene locus has been further localized using a hybrid line with a human chromosome 1 (pter→ p11)/mouse chromosome translocation. Enzyme marker analyses substantiated the cytogenetic data; as ENO1, FUCA and PGM1, which are all within the translocated region 1pter → p11, were present in this hybrid line, while PEPC and FH (localized at 1q25 → 1q42 and at q42→ qter, respectively) were absent. No acid β-glucosidase activity was detected in this hybrid indicating that the translocated segment of chromosome 1 did not have the locus for acid β-glucosidase. Thus, the region on human chromosome 1 to which the structural locus for acid β-glucosidase has been localized, 1p11 → qter, is illustrated in Figure 26.

Figure 26. Regional assignment of human acid β-glucosidase to chromosome 1.

Human chromosome 1 and its enzymatic markers are shown diagramatically on the left. The location of the breakpoint of the translocation carried in the hybrid line $R/KidA_{10}$ is designated by the broken line. The structural gene locus for human acid β-glucosidase has been mapped to the region 1p11 → qter and is depicted diagramatically on the right.

REGIONAL ASSIGNMENT OF HUMAN ACID β-GLUCOSIDASE ON CHROMOSOME 1



CONCLUDING REMARKS

I. Accomplishments:

These studies have provided a useful foundation for further studies on acid β-glucosidase. Several biochemical tools have been developed and utilized. (1) A dextran sulfate affinity column which binds β-glucosidase. (2) The synthesis and the development of a sensitive assay for acid β-glucosidase activity using NBD-glucer. (3) The development of a cellulose acetate electrophoretic system to identify acid and neutral β-glucosidases in various human tissue homogenates. (4) The isolation and characterization of the normal and Type 1 Gaucher effector molecule using cellulose acetate electrophoresis and sucrose density ultracentrifugation. (5) The production of highly specific human acid β-glucosidase raised in Balb/C mice. Finally, (6) the development of a double antibody immunoprecipitation assay to selectively precipitate human acid β-glucosidase in man-mouse hybrid cell lines.

II. Future Studies:

The purified acid β-glucosidase will be useful for many further structural and biochemical studies of the normal enzyme and the mutant form(s) of the enzyme found in the Gaucher subtypes. The dextran sulfate-Sepharose column which binds the normal acid β-glucosidase may not bind the mutant enzyme and thus give some insight to the basic mutation. In this manner, the dextran-sulfate binding site may also be identified. Purified enzyme can be used to make additional antibody both from the normal enzyme and enzyme from the Gaucher subtypes.

The natural substrate, NBD-glucer, may be useful for detection of

carriers and homozygotes for Gaucher disease. The neutral enzymatic activity, present in leukocytes, will not interfere with heterozygote detection in this assay as it does with the 4MUG assay.

Cellulose acetate electrophoresis may be helpful for studies of the other subtypes of Gaucher disease. The electrophoretic migration of tissue homogenates from Type 2 and 3 Gaucher disease is yet to be studied. Furthermore, the isolation and electrophoretic characterization of their respective effector molecules will be of interest.

Highly specific antibodies to acid β-glucosidase raised in mice against purified enzyme, may enable the finer mapping of the gene for acid β-glucosidase to the long arm of chromosome 1. This can be accomplished using cell lines containing translocations and deletions of segments of the long arm of chromosome 1.

Anti-β-glucosidase antibodies may also be used for immunochemical studies of acid β-glucosidase from normal and Gaucher subtypes. Spleen cells from Balb/C mice making anti-β-glucosidase antibody have already been fused with an NS1 Balb/C mouse myeloma cell line and hybridomas have been established. These hybridomas each producing monoclonal antibodies, i.e., antibody to the active site, phospholipid site, etc., may lend some insight to the mutations of the Gaucher subtypes. Using rocket immuno-electrophoresis, the presence of cross-reacting immunologic material (CRIM) may be determined.

In addition, cells grown in tunicamycin, which inhibits glycosylation, would document whether any of the antibodies are specific for the post- translational modifications of the acid β-glucosidase.

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