ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 166, 382 389 (1975)

Synthetic Inhibitors of Glucocerebroside β-Glucosidase

JUNG C. HYUN, RADHEY S. MISRA, DAVID GREENBLATT, AND NORMAN S. RADIN

Mental Health Research Institute and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48104

Received June 28, 1974

The glucocerebrosidase of human placenta was studied with various potential inhibitors. Several compounds that resemble the lipoidal product of enzyme action, ceramide, proved to be excellent inhibitors, acting by mixed modes (primarily noncompetitively). These were *N*-decyl-DL-erythro-3-phenyl-2-amino-1,3-propanediol and several *p*-substituted derivatives. These compounds were also highly effective in rat spleen toward glucocerebroside and *p*-nitrophenyl β-glucoside as substrates. The compounds were inactive toward the analogous enzyme, galactocerebrosidase of rat brain, and were slightly stimulatory toward the rat brain enzyme which makes galactocerebroside. Longer and shorter *N*-alkyl groups proved to be less effective. Decanoic acid amides of phenylaminopropanediol and related compounds proved to be relatively inert, although some were stimulatory. Deoxycorticosterone β-glucoside was a moderately effective noncompetitive inhibitor and is apparently hydrolyzed by a different glucosidase. *p*-Nitrophenyl β-glucoside was also a moderately effective inhibitor, acting by mixed modes. *p*-Chloromercuribenzenesulfonate was a good inhibitor, presumably acting on a sensitive cysteine residue. It is concluded that cerebrosidase contains two sensitive sites, one catalytic and the other allosteric, each containing an important anionic group and able to bind glucosides and ceramide-like compounds.

The development of inhibitors for specific enzymes has in the past yielded many important findings, so it seems only appropriate to attempt this for glucosyl ceramide β-glucosidase (glucocerebrosidase). A particular reason for attempting this in the case of this enzyme is that the activity of the enzyme is greatly reduced in the human genetic disorder, Gaucher's disease, and it would be very helpful if one could induce a model form of the disorder in a laboratory animal by means of an inhibitor. We have previously described a very effective inhibitor, *N*-hexyl-*O*-glucosyl sphingosine (1), which was found to produce model forms of Gaucher's disease in cultured brain and skin cells (2, 3). However, attempts to utilize the cerebroside analog in rats led to little accumulation of glucocerebroside, apparently due to excessively rapid degradation and excretion of inhibitor.[1] The search for a similar inhibitor, with possibly better characteristics *in vivo*, was, therefore, continued and the initial results are reported here.

MATERIALS AND METHODS

Preparation and assay of glucocerebrosidase. Most of the work reported here was done with a partially purified preparation of the enzyme from human placenta. The enzyme was extracted with sodium taurocholate as described in a separate paper (4), but with an extraction period of only 1 h. It was then precipitated with acetone and enriched about 20-fold by means of an affinity column containing a substrate-like group (details to be reported separately). The final preparation had a specific activity of 8400 nmoles/mg protein/h under the assay conditions described below. An aliquot containing 30 such units was used in each incubation tube.

Each compound tested as a potential inhibitor was

[1] Unpublished work, Kenneth R. Warren and Norman S. Radin.

dissolved in methanol or chloroform–methanol 2:1, and an aliquot containing 75 μmoles was evaporated to dryness in the incubation tube. To this was added an emulsion of glucocerebroside, buffer (11.1 μmoles citric acid + 28.9 μmoles dipotassium phosphate, pH 5.4), and enzyme solution in a total volume of 0.25 ml. The substrate emulsion was made from 2 mg of [^{14}C]stearoyl glucosyl sphingosine (880,000 cpm), Tween 20 (10 mg), and Myrj 59 (5 mg), dispersed in 5 ml of 3% sodium taurocholate (A grade, 98% pure, Calbiochem). Each incubation tube contained 0.1 ml (55 nmoles) of cerebroside emulsion. The tubes were incubated 30 min at 37°C and the radioactive ceramide produced by the enzyme was isolated and assayed by liquid scintillation counting. Details of the procedure are in a separate paper (4).

Control tubes without inhibitor usually yielded about 15 nmoles of ceramide. The rate of hydrolysis was constant over a period of at least 2 h.

Preparation of synthetic amines. Amino alcohols were obtained as gifts (5) or purchased. D-Threo-3-(*p*-nitrophenyl)-2-amino-1,3-propanediol was obtained from Aldrich Chemical Co. and L-threo-3-phenyl-2-amino-1,3-propanediol and 1,2-epoxydecane came from Research Organic/Inorganic Chemical Corp., Sun Valley, CA. The amine group was alkylated with 1-bromoalkanes by a modification of a previously described procedure (1). In a typical preparation, 4 nmoles each of amine and alkyl bromide were heated in 1 ml of *n*-butanol at 100°C in a small screw-capped test tube. The components were partitioned as before between NaOH, water, methanol, and chloroform and purified with a silica gel column with a mixture of chloroform and methanol, sometimes supplemented with 2 *N* ammonium hydroxide. Depending on the chain length of the alkyl group and number of hydroxyl groups, the ratio of the three solvents varied from 92:8 to 85:15:1.5 to 60:40:4 (the chloroform + methanol volumes totalled 100 and the ammonia generally constituted 1/10 of the methanol).

Thin-layer chromatography on binder-free silica gel with chloroform–methanol–concd. ammonium hydroxide 70:10:1 generally showed that the reaction mixture, prior to column purification, contained some of the original amine, the *N*-alkyl derivative (faster migrating), and a spot just above the desired compound. The latter is presumed to be the disubstituted (tertiary) derivative. Identities were established by the pink color due to ninhydrin spray, which reacts with primary and secondary amines, and the blue or whitish color produced by alkaline bromothymol blue, which gives this reaction with primary, secondary, and tertiary amines. A charring spray (6) detected these compounds as well as some faster-moving materials, possibly unreacted alkyl bromide and alkyl butyl ether, from the solvent. The column separation sometimes yielded a small amount of contaminating tertiary amine in the desired secondary amine. Yields were generally 40–50%.

In the case of nitro compounds, the nitro group was reduced at atmospheric pressure with hydrogen and PtO_2 in EtOH. The aromatic amino group was then acylated with decanoyl chloride as described before (5). Secondary amines bearing a 2-hydroxyl group on the alkyl chain were prepared from 8 mmoles of 1,2-epoxydecane and 4 mmoles of primary amine, heated with stirring at 76–80°C in 2 ml methanol and 1 ml water. Additional portions of epoxydecane (0.5 ml) were added every 2 h, for a total of 8 h of heating. The hydroxy amine derivative was purified as above and the final product was converted to the hydrochloride to form a solid derivative.

Other compounds. Amide analogs of the alkyl amines were prepared for a previous study (7) by acylation of the amino alcohols with acyl chlorides in sodium acetate–water–tetrahydrofuran. Methyl and phenyl glucosides were obtained from Pfanstiehl Laboratories, Waukegan, IL. *o*-Nitrophenyl glycosides were obtained from Schwarz/Mann and Research Products International Corp., Elk Grove Village, IL. *p*-Chloromercuribenzenesulfonate and deoxycorticosterone β-glucoside were obtained from Sigma Chemical Co. The drugs and antibiotics tested were commercial drugs, obtained as gifts. Phlorizin was from Chemicals Procurement Laboratories, College Point, NY. Amygdalin (glucosyl glucosyl mandelonitrile) and Nordefrin [DL-erythro-3-(3′,4′-dihydroxyphenyl)-2-amino-3-propanol] were from Schwarz/Mann, Orangeburg, NY. Helicin (β-glucosyl salicylaldehyde) was from Aldrich Chemical Co., Milwaukee.

Assay for aryl glucosidase activity. Rat spleen was homogenized in water and portions containing 4 mg of tissue were incubated in a volume of 1 ml containing 2.5 μmoles of *p*-nitrophenyl β-D-glucoside and 100 μmoles of Na-acetate, pH 5.0 (8). After 60 min at 37°C the reaction was stopped with 0.02 ml of 100% TCA, 0.9 ml of the supernatant liquid was transferred to 1.6 ml of 1 M sodium carbonate, and the absorbance was measured at 420 nm.

RESULTS

N-Alkylated amines as inhibitors. A series of sphingosine analogs was alkylated with 1-bromodecane and found to be quite effective inhibitors (Table I). 3-Phenyl-2-amino-1,3-propanediol is an aromatic analog of sphingosine, in which the unsaturated aliphatic fatty chain is replaced by a phenyl group. In sphingolipids, the 2-position has the D-configuration and the 3-position also has the D-configuration, ordinarily referred to as erythro. The data in

TABLE I

INHIBITION OF PLACENTAL GLUCOCEREBROSIDASE BY DERIVATIVES OF 3-PHENYL-2-DECYLAMINO-1,3-PROPANEDIOL (0.3 mM)

Aromatic substituent	Configuration of alkyl chain	Inhibition (% of control)
—	DL-erythro	98
—	L-threo	59
—	D-threo	39
p-Nitro	DL-erythro	98
p-Nitro	L-threo	52
p-Nitro	D-threo	60
p-Amino	DL-erythro	98
p-Decanoylamido	DL-erythro	99

Table I (lines 1-3) show that the threo configuration lowered the effectiveness of the inhibitor. Lines 4 to 6, for the *p*-nitro derivatives, show the same thing. Substituents of various sorts in the *p*-position seemed to have no effect at the concentration used here (0.3 mM). A test in the same series with DPAPD[2] lacking an OH group in the 1-position led to a distinct loss of effectiveness (34% inhibition). When an hydroxyl group was inserted in the 2-position of the *n*-decyl group of the secondary amine, the effectiveness of L-threo-DPAPD rose to 78% but the effectiveness of the *p*-nitro-D-threo compound dropped to 49%.

Very similar results were obtained when these inhibitors were tested with whole placental homogenate, incubated for 2 h with substrate and Na citrate, pH 5.5.

In order to determine the relative inhibitory effects of the highly active amines more clearly, we tested them at lower concentrations (Table II). It now became apparent that a *p*-substituent is useful, although the exact type seems relatively unimportant. These derivatives of DPAPD were quite effective even at 6 μM, but not as effective as the glucosyl amine previously described, hexyl glucosyl sphingosine. The galactosyl analog (last line) was fairly inhibitory at 0.12 mM but essentially inactive at 6 μM. Thus, we see that the addition of a β-glucosyl group to the alkyl amine (a ceramide analog) distinctly enhances the binding to the glucosidase.

Similar relationships were seen when the inhibitors were tested with rat spleen homogenate, assayed over a 2-h period, but the effectiveness at any given concentration was not as great as noted with the human placental enzyme. For example, DPAPD produced 24% inhibition at the 6 μM level, while hexyl glucosyl sphingosine produced 79% inhibition at this level.

To compare the specificity of these amines, Dr. Yuh-Nan Lin kindly assayed them at the 6 μM level with a partially purified preparation of rat brain galactocerebrosidase (7). Very little inhibitory activity was observed. Dr. Kenneth Warren also tested the amines at the 0.3 mM level with the rat brain system which forms galactocerebroside from ceramide and UDPGal (9). All the aromatic amines were slightly stimulatory (about 15%).

In the case of *N*-hexyl glucosyl sphingosine, tested with rat spleen glucocerebrosidase, we had found that the amine acts as a competitive inhibitor (1). This is to be expected from the close structural similarity between the amine and the substrate. The aromatic, nonglucosylated analogs might act differently. This was investigated with *p*-amino-DPAPD, with varying concentrations of substrate (taurocholate concentration being kept constant). A Hof-

TABLE II

INHIBITION BY SECONDARY AMINES OF PLACENTAL GLUCOCEREBROSIDASE AND PIG BRAIN GALACTOCEREBROSIDASE

Amine tested	Percentage inhibition				
	Glucosidase				Galactosidase
	120 μM	30 μM	6 μM	1.2 μM	6 μM
DPAPD	95	82	28	2	7
p-Nitro-DPAPD	100	92	66	19	5
p-Amino-DPAPD	95	96	50	21	2
p-Decanoylamido-DPAPD	97	91	68	23	5
N-Hexyl-*O*-glucosyl-sphingosine	100	99	92	70	5
N-Hexyl-*O*-galactosyl sphingosine	51	21	5	1	3

[2] DPAPD is *N*-decyl-DL-erythro-3-phenyl-2-amino-1,3-propanediol. Ceramide in *N*-acyl sphingosine. Glucosyl ceramide is glucocerebroside.

stee plot was prepared (Fig. 1), as recommended by Dowd and Riggs (10), although simple visual examination cannot always indicate the type of inhibition. The calculated data for Fig. 1 show that the uninhibited enzyme had a K_m of 13 μM and a V of 21 μmoles/h/mg, while the inhibited enzyme had a K_m of 9.3 μM and a V of 11 μmoles/h mg. Thus, the amine acts as a mixed-type inhibitor, partially at the catalytic site but primarily at a noncompetitive site. This is evidence for the existence of a noncatalytic binding site that acts on amines resembling a portion of the substrate structure.

The influence of the chain length of the *N*-alkyl group was determined at the 6 μM level with homologs of DPAPD and *p*-nitro-DPAPD. In this comparison, DPAPD produced 39% inhibition, while the C_{14} and C_6 homologs produced 32% and 0% inhibition, respectively. With nitro-DPAPD, the inhibition was 59% and the C_{14} and C_6 homologs produced 40% and 3% inhibition, respectively. The chain length effect is quite striking, particularly in the shorter direction. In contrast, we had found that the hexyl derivative of glucosyl sphingosine was the most effective homolog (1). Presumably the chain length difference between glucosylated and nonglucosylated analogs is due to slight differences in the conformation of the two sites which bind the two different groups of inhibitors.

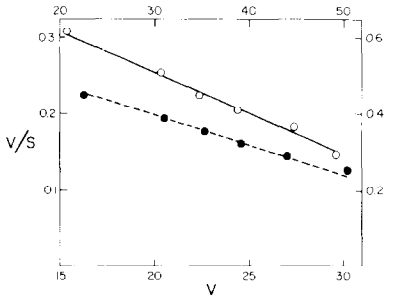


FIG. 1. Hofstee plot relating hydrolysis of glucocerebroside to substrate concentration in the presence and absence of 6 μM *p*-amino-DPAPD. V = nmoles/h and S = μM. Conditions as in text but with varying concentrations of cerebroside, Tween, and Myrj (taurocholate constant at 12 mg/ml). The scales on the left and bottom refer to the uninhibited enzyme, plotted as the broken line.

Amides as inhibitors. 3-Phenyl-2-amino-1,3-propanediol amides of decanoic acid and related compounds were tested at the 0.3 mM level (Table III). Inhibitory effects were generally weak, the strongest coming from *N*-octanoyl-L-phenylalaninol (line 3 from bottom of table). Unexpectedly, we found appreciable stimulation of glucocerebrosidase by *N*-decanoyl-L-threo phenylpropanediol (line 2). When this amide was tested at a higher concentration, 1.2 mM, the stimulation was 47%. It would be of interest to test homologs and analogs of this compound to see if even higher stimulation could be obtained, since such a stimulator might have therapeutic value to patients with Gaucher's disease.

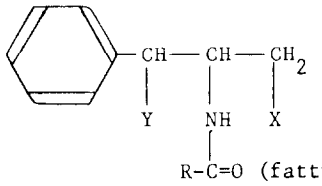
This stimulation is reminiscent of the stimulation we had observed earlier with the decanoic acid amide of 2-amino-2-methyl-1-propanol, a somewhat truncated analog of ceramide. This compound had given as high as a 60% stimulation of rat brain galactocerebrosidase (11) but tests with glucocerebrosidase showed it to be inactive.

Since one of our goals is to develop inhibitors that are specific to the different sphingolipid enzymes, it is encouraging to note that some of the amides in Table III are very good inhibitors of galactocerebrosidase from rat brain (7), yet are relatively inert to the analogous glucosidase.

Glycosides as inhibitors. An assortment of commercially available glycosides and their derivatives was found to give rather small effects (Table IV). Methyl β-glucoside and *o*-aminophenyl β-glucoside produced some stimulation, while deoxycorticosterone β-glucoside produced modest inhibition. The latter is an aliphatic glucoside which may occur in nature and which is rapidly hydrolyzed by a partially purified glucosidase from rabbit liver (12). Although the structure of the steroidal glucoside does not resemble that of glucocerebroside, both are lipoidal glucosides and might be attacked by the same enzyme. As a test of this possibility, we made a kinetic study of the inhibition by the steroidal glucoside. The parallelism of the two lines in the Hofstee plot, with and without inhibitor, showed that the inhibi-

TABLE III

EFFECT OF SYNTHETIC AMIDES ON HUMAN PLACENTAL GLUCOCEREBROSIDASE



Aromatic substituent	X	Y	Configuration	Fatty acid	Stimulation (%)	Inhibition (%)
—	OH	OH	DL-erythro	10:0	6	
—	OH	OH	L-threo	10:0	14	
—	OH	OH	D-threo	10:0	4	
p-Nitro	OH	OH	DL-erythro	10:0	4	
—	H	OH	DL-erythro	8:0		1
—	H	OH	DL-erythro	10:0		13
3,4-Dihydroxy	H	OH	DL-erythro	10:0		18
—	H	OH	DL-erythro	h10:0[a]		3
—	H	OH	DL-erythro	12:0	2	
—	H	OH	DL-erythro	16:0	1	
—	OH	H	L	8:0		33
—	OH	H	L	10:0		23
—	OH	H	L	16:0		17

[a] The h in h10:0 refers to the presence of a 2-hydroxy group in the decanoic acid moiety.

TABLE IV

EFFECT OF D-GLYCOSIDES ON GLUCOCEREBROSIDE HYDROLYSIS BY HUMAN PLACENTAL ENZYME

Glycoside (0.3 mM)	% Inhibition	% Stimulation
Methyl α-glucoside	4	
Methyl β-glucoside		18
Phenyl β-glucoside	5	
o-Aminophenyl β-glucoside		11
o-Nitrophenyl β-glucoside	0	0
p-Nitrophenyl β-glucoside	31	
o-Decanoylamidophenyl β-glucoside	8	
Helicin	1	
Phlorizin	4	
Deoxycorticosterone β-glucoside	30	
Amygdalin		1
Glucose		5
N-Palmitoyl-*O*-lactosyl-DL-ceramide	2	

tion was noncompetitive. Thus, it would appear that human placental glucocerebrosidase possesses a second, noncatalytic site that can bind other glucosides without affecting the K_m for the substrate.

The degree of inhibition (%) was constant with time over a 3-h period, indicating that the steroidal glucoside acted in a simple manner.

o-Aminophenyl glucoside was tested because of its structural resemblance to glucosyl sphingosine, which is a good inhibitor of glucocerebrosidase (1). However, it proved to be slight stimulator (Table IV). *o*-Nitrophenyl glucoside was inert as an inhibitor, but the *p*-nitro analog was moderately inhibitory. The inertness of lactosyl ceramide shows that it is not an effective substrate for the enzyme, i.e., glucocerebrosidase does not have endoglucosidase activity toward this glycosphingolipid.

A test with several β-galactosides (of methanol, *o*-aminophenol, *o*-nitrophenol, and *o*-decanoylamidophenol) showed them to be without effect on the enzyme. Free glucose (0.05–0.5 mM) had a slight stimulatory effect while ceramide (0.5 mM) had a slight inhibitory effect.

Glucosidases are often evaluated with *p*-nitrophenyl β-glucoside as substrate. The highly purified preparation of placental glucocerebrosidase made by Pentchev *et*

al. (13) showed some activity toward the aromatic glucoside, so the question arises: does the enzyme act on both the natural and unnatural glucosides? A kinetic analysis of our preparation with 0.6 mM nitrophenyl glucoside (Fig. 2) showed that this glucoside acts as a mixed type inhibitor. This probably means that it acts at two sites, the catalytic site (as a poor substrate), and the secondary, allosteric binding site. Presumably the latter is the point which also binds deoxycorticosterone glucoside and our series of aromatic *N*-decylamines.

A similar observation was made with cerebroside sulfate sulfatase, which was inhibited noncompetitively by 0.51 mM nitrocatechol sulfate (14). This unnatural substrate, typically used to measure the enzyme acting on the lipid sulfate, presumably binds to a moderating site on the enzyme as well as to the catalytic site. Another unnatural aryl glycoside used as a substrate to assay for a lipid α-galactosidase, methylumbelliferyl galactoside, also was found to act at two sites in the case of trihexosidyl ceramide galactosidase (15).

The inertness of phlorizin with our enzyme (Table IV) is of interest because this glucoside has been reported to inhibit kidney aryl glucosidase very strongly (24). Phlorizin is itself also a substrate for the glycosidase of intestine which acts on glucocerebroside and other glycosides (25).

Miscellaneous inhibitors. While the glucocerebrosidase from bovine spleen is not inhibited by Cu^{2+} or *N*-ethylmaleimide (16), the enzyme in human skin fibroblasts is inhibited rather effectively by *p*-chloromercuribenzenesulfonate (17). We too found the mercury compound to be a strong inhibitor, yielding 55% inhibition at 5 μM. It seems likely that the human enzyme does contain a reactive, vital cysteine residue.

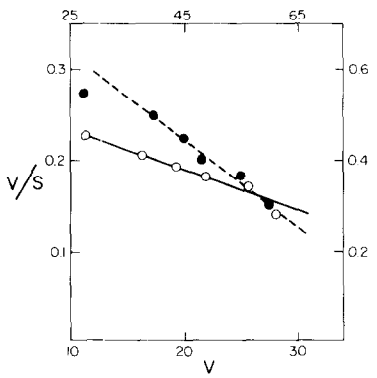


FIG. 2. Hofstee plot as in Fig. 1 except that the inhibitor was 0.6 mM *p*-nitrophenyl glucoside.

We also tested some amines of pharmaceutical interest, although their structures were rather unlike that of cerebroside. Streptomycin B and neomycin B, amino sugar glycosides, proved inert at the 0.3 mM level. Neamine, a glycoside of a diamino analog of glucose, produced 8% inhibition, while neobiosamine B (a related compound) yielded 26% inhibition. 2-Amino-2-deoxyglucose resulted in only 6% inhibition, as did the *N*-acetyl derivative. Chloroquine and chlorpromazine, lipoidal amines, showed 4 and 28% inhibition, respectively. Chlorpromazine concentrates in lysosomes (the presumed site of glucocerebrosidase) (18) and prolonged administration of the psychoactive drug might produce some accumulation of glucocerebroside. However the typical symptoms of chlorpromazine toxicity do not resemble those of Gaucher's disease.

Specificity of inhibitor action. We have shown that our best inhibitor, *n*-hexyl glucosyl sphingosine, is a very efficient inhibitor of both glucocerebrosidase and aryl glucosidase (1–3). This means that the glucoside blocks at least two different glucosidases. It seemed likely that DPAPD, which lacks the glucose moiety but retains the ceramide-like moiety, might block only glucocerebrosidase. Accordingly, we tested 6 μM DPAPD derivatives with an homogenate of rat spleen and *p*-nitrophenyl glucoside as substrate. (Our partially purified glucocerebrosidase had too little aryl glucosidase activity to be useful in this comparison.) *N*-Hexyl glucosyl sphingosine again proved very effective (98% inhibition), while *p*-amino-DPAPD produced 78% inhibition and its *N*-decanoyl derivative inhibited by 83%. The same compounds, tested with the same homogenate but with glucocerebroside as substrate (incubated 20 min under the conditions used with placental enzyme) resulted in 90, 53, and 96% inhibi-

tion, respectively. Thus, we have the unexpected result that compounds resembling ceramide in structure can be strong inhibitors of at least two β-glucosidases. However, the enzymes differ in their sensitivity to the different inhibitors.

DISCUSSION

This investigation has disclosed by means of kinetic studies of glucocerebrosidase that the enzyme possesses a second binding site which, on combining with any one of several types of compounds, acts to slow the enzyme's activity very effectively. Nitrophenyl glucoside and the secondary amines resembling ceramide both acted at the secondary site, as well as the catalytic site, while a steroid glucoside acted only at the secondary site. The glucocerebrosidase of rat intestine, which may well be a different enzyme from the one studied here, was reported to be weakly inhibited by glucosyl glucose (cellobiose); the action was of the mixed type, also implying the presence of two binding sites (19).

Our finding that a substance resembling the substrate could act at an inhibitory site distinct from the catalytic site is like our similar finding with galactocerebrosidase (7). At that time we offered an evolutionary hypothesis to explain the phenomenon and we believe that the explanation could well hold for glucocerebrosidase. The hypothesis holds that glucocerebrosidase was a smaller molecule many eons ago, but that an error in gene duplication produced a partially or wholly doubled enzyme molecule bearing two identical active sites. With the passing of time, mutations caused loss of catalytic activity at one of the sites, which nevertheless retained some ability to recognize substances resembling glucocerebroside in structure.

Human placental glucocerebrosidase was also inhibited weakly by *p*-aminophenyl-β-thioglucoside, the K_i being 6.25 mM (13). The thio compound acted as a competitive inhibitor. Our previous study of the rat spleen enzyme (1) had shown that glucosyl sphingosine was quite effective, better than gluconolactone, and that D-sphingosine itself was quite good (59% at 0.3 mM). Curiously, DL-dihydrosphingosine was a weak inhibitor (16% at 0.3 mM); the L-form might be inert. Gatt had previously shown that sphingosine and gluconolactone were good inhibitors of the brain cerebrosidase (20).

Studies with aryl glucosides as substrates have produced findings of potential applicability to glucocerebrosidase. Two amino derivatives of D-glucose have been found to be very effective inhibitors: 5-amino-5-deoxyglucose (Nojirimycin, an antibiotic) (21) and 1-aminoglucose (a 1-amino-glycoside) (22). The K_i values for Nojirimycin and various plant β-glucosidases were 0.6–7 μM and the K_i for yeast β-glucosidase and glucosylamine was 2.3 μM. It appears likely that the amine groups of these sugars combine electrostatically with the similar anionic group at the active site in cerebrosidase that binds our amino inhibitors. Even though the amine group in Nojirimycin seems far away from the active site, it no doubt exists as part of a ring structure, the nitrogen being in contact with the C-1 carbon of the glucose. These observations constitute further evidence for the presence of an important anionic group close to the active site of a variety of glucosidases. The location of this group is highly specified, since we found 2-amino-glucose to be rather inert.

The synthesis of a reactive inhibitor that could react covalently with glucosidase has been reported (23). An analog of inositol containing an epoxy group, conduritol B epoxide, was found to inactivate several aryl glucosidases. Incubation of a 1 mM solution for 2 h at 25°C led to 92% loss of activity. The enzymes could be protected against this inactivation by gluconolactone so it seems likely that the epoxy compound was bound to the active site of the enzymes, possibly reacting to form an ester with the same carboxyl group in the catalytic site that seems to be binding our synthetic amines. Evidence for ester formation was produced by the use of labeled inhibitor (26) and it was concluded that the pK of the binding group was about 6.1. Similar observations were made with the 6-bromo derivative of the above epoxide (27).

ACKNOWLEDGMENTS

This study was supported by U.S. Public Health Service Grant NS-03192 and National Science Foundation Grant GB-36735. We are indebted to Inez Mason, Carol Seidl, and Jeff Gerber for laboratory assistance. David Greenblatt carried out some of these experiments while an undergraduate student in the Honors Program. Dr. Marvin McMaster kindly carried out the catalytic reductions.

REFERENCES

1. Erickson, J. S., and Radin, N. S. (1973) *J. Lipid Res.* **14,** 133–137.
2. Dawson, G., Stoolmiller, A. C., and Radin, N. S. (1974) *J. Biol. Chem.* **249,** 4638–4646.
3. Warren, K. R., Schafer, I. A., Sullivan, J. S., Petrelli, M., and Radin, N. S., submitted for publication.
4. Hyun, J. C., and Radin, N. S., submitted for publication.
5. Arora, R. C., and Radin, N. S. (1972) *J. Lipid Res.* **13,** 86–91.
6. Fewster, M. E., Burns, B. J., and Mead, J. F. (1969) *J. Chromatogr.* **43,** 120–126.
7. Arora, R. C., Lin, Y.-N., and Radin, N. S. (1973) *Arch. Biochem. Biophys.* **156,** 77–83.
8. Gatt, S., and Rapport, M. M. (1966) *Biochim. Biophys. Acta* **113,** 567–576.
9. Brenkert, A., and Radin, N. S. (1972) *Brain Res.* **36,** 183–193.
10. Dowd, J. E., and Riggs, D. S. (1965) *J. Biol. Chem.* **240,** 863–869.
11. Arora, R. C., and Radin, N. S. (1972) *Lipids* **7,** 56–69.
12. Mellor, J. D., and Layne, D. S. (1971) *J. Biol. Chem.* **246,** 4377–4380.
13. Pentchev, P. G., Brady, R. O., Hibbert, S. R., Gal, A. E., and Shapiro, D. (1973) *J. Biol. Chem.* **248,** 5256–5261.
14. Porter, M. T., Fluharty, A. L., De La Flor, S. D., and Kihara, H. (1972) *Biochim. Biophys. Acta* **258,** 769–778.
15. Ho, M. W. (1973) *Biochem. J.* **133,** 1–10.
16. Weinreb, N. J., and Brady, R. O. (1972) *in* Methods in Enzymology (Ginsburg, V., ed.), Vol. 28, pp. 830–834, Academic Press, New York.
17. Kanfer, J. N., Stein, M., and Spielvogel, C. (1972) *in* Sphingolipids, Sphingolipidoses, and Allied Disorders (Volk, B. W., and Aronson, S. M., eds.), pp. 225–236, Plenum Press, New York.
18. Brosnan, C. F., Bunge, M. B., and Murray, M. R. (1970) *J. Neuropathol. Exp. Neurol.* **29,** 337–353.
19. Brady, R. O., Gal, A. E., Kanfer, J. N., and Bradley, R. M. (1965) *J. Biol. Chem.* **240,** 3766–3770.
20. Gatt, S. (1966) *Biochem. J.* **101,** 687–691.
21. Reese, E. T., Parrish, F. W., and Ettlinger, M. (1971) *Carbohyd. Res.* **18,** 381–388.
22. Lai, H.-Y. L., and Axelrod, B. (1973) *Biochem. Biophys. Res. Commun.* **54,** 463–468.
23. Legler, G. (1966) *Z. Physiol. Chem.* **345,** 197–214.
24. Abrahams, H. E., and Robinson, D. (1969) *Biochem. J.* **111,** 749–755.
25. Leese, H. J., and Semenza, G. (1973) *J. Biol. Chem.* **248,** 8170–8173.
26. Legler, G. (1968) *Z. Physiol. Chem.* **349,** 767–774.
27. Legler, G. (1970) *Z. Physiol. Chem.* **351,** 25–31.