Biochem. J. (1984) **224**, 515–524
Printed in Great Britain

Characterization of the phospholipid requirement of a rat liver β-glucosidase

Alakananda BASU and Robert H. GLEW*
Department of Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, U.S.A.

(*Received 1 August 1984/Accepted 15 August 1984*)

The lipid requirement of membrane-bound rat liver β-glucosidase was investigated using 4-methylumbelliferyl-β-D-glucopyranoside as the substrate. The enzyme was solubilized and delipidated by sequential extraction of a crude lysosomal fraction from rat liver lysosomes with sodium cholate and ice-cold butan-1-ol. Neither saturated nor unsaturated phosphatidylcholine activated this enzyme. In contrast, acidic phospholipids like phosphatidylglycerol (PtdGro) and phosphatidylserine (PtdSer) were effective activators. For the PtdGro series, fatty acid composition was important, with the shorter chain or unsaturated fatty acid-containing PtdGro species being the best activators. Heat-stable factor (HSF) from Gaucher spleen by itself (1–2 μg) had no effect on enzyme activity. However, the same amount of HSF when combined with 10 μg of PtdSer markedly stimulated β-glucosidase activity. In the presence of HSF, di-9-*cis*-octadecenoyl-PtdGro (1 μg) or -PtdSer (5 μg) provided maximum protection of β-glucosidase against heat (60°C) inactivation. In the absence of phospholipids, HSF had no effect on the rate of inactivation of the enzyme by the suicide inhibitor conduritol B epoxide ($t_{0.5}$, 12 ± 0.5 min); the maximum rate of inactivation was achieved in the presence of a mixture of PtdGro (2.5–5 μg) and HSF ($t_{0.5}$, 2.8 min). The combination of PtdSer (10 μg) and HSF (1.3 μg) lowered the K_m for 4-methylumbelliferyl-β-D-glucopyranoside from 24 to 2.7 mM. Inhibition of the enzyme by the glucocerebrosidase substrate analogues *N*-hexyl-*O*-glucosylsphingosine and glucosylsphingosine was influenced by the activator substances. The inclusion of PtdSer and HSF in the β-glucosidase assay medium lowered the K_i of *N*-hexyl-*O*-glucosylsphingosine 20-fold. The same combination of activators decreased the $I_{0.5}$ of the enzyme for glucosylsphingosine from 89.4 to 7.6 μM. A study of log ($V_{max.}/K_m$) versus pH indicated that the PtdSer–HSF pair creates the active site of β-glucosidase, making apparent three ionizable groups on the enzyme with pK values in the range 4.5–5.1.

Glucocerebrosidase (E.C. 3.2.1.45) is a lysosomal β-glucosidase that catalyses the hydrolysis of glucocerebroside to glucose and ceramide. A deficiency of glucocerebrosidase is the cause of Gaucher's disease (Patrick, 1965; Brady *et al.*, 1965*a*).

Most of our knowledge about glucocerebrosidase has been derived from studies where human tissues have served as the source of enzyme; these include: liver (Hultberg & Öckerman, 1970), spleen (Brady *et al.*, 1965*b*; Ho *et al.*, 1973; Pentchev *et al.*, 1978), brain (Daniels *et al.*, 1982), leucocytes (Matoth *et al.*, 1974; Raghavan *et al.*, 1980; Svennerholm *et al.*, 1980), and fibroblasts (Beutler *et al.*, 1971; Mueller & Rosenberg, 1979; Ginns *et al.*, 1982). The enzyme is firmly bound to the lysosomal membrane, and detergents such as Triton X-100 and sodium cholate have been used to extract it in a soluble form (Ho, 1973; Pentchev *et*

Abbreviations used: PtdGro, phosphatidylglycerol; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine; 10:0, didecanoyl; 12:0, didodecanoyl, 14:0, ditetradecanoyl; 15:0, dipentadecanoyl; 16:0, dihexadecanoyl; 18:0, dioctadecanoyl; 18:1, di-9-*cis*-octadecenoyl; 18:2, di-9-*cis*-12-*cis*-octadecadienoyl; 18:3, *di*-9-*cis*-12-*cis*-15-*cis*-octadecatrienoyl; HSF, heat-stable factor; MUGlc, 4-methylumbelliferyl-β-D-glucopyranoside; CBE, conduritol B epoxide; HGS, *N*-hexyl-*O*-glucosylsphingosine; GS, glucosylsphingosine.

* To whom all reprint requests should be addressed.

al., 1973). The human enzyme: (1) is composed of 65–70kDa subunits and is active in a 120kDa form (Wenger & Olson, 1981) that tends to aggregate (Pentchev *et al.*, 1973), (2) is active with non-physiological β-D-glucoside substrates (e.g. 4-methylumbelliferyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside) (Wenger & Roth, 1982), (3) is inhibited by CBE (Kanfer *et al.*, 1975; Hara & Radin, 1979; Daniels *et al.*, 1981), (4) has a strong dependency on lipid activators, especially bile salts and acidic phospholipids (e.g. PtdSer, PtdGro) (Ho & Light, 1973; Blonder *et al.*, 1976; Peters *et al.*, 1976; Dale *et al.*, 1976; Glew *et al.*, 1982; Basu *et al.*, 1984), and (5) is activated markedly by micromolar amounts of a heat-stable factor from bovine (Berent & Radin, 1981*a*) and human (Ho & O'Brien, 1971; Peters *et al.*, 1977*a*; Basu *et al.*, 1984) spleen. The pH optimum of the enzyme is shifted from 5.5–6.0 to 4.6–5.2 by the lipid activators and heat-stable factors (Ho, 1973; Pentchev *et al.*, 1973; Peters *et al.*, 1977*a*).

Whereas there are numerous studies describing the properties of the solubilized enzyme, few reports aimed at characterizing glucocerebrosidase *in situ* using purified lysosomal preparations or membranes have appeared. Because of the difficulty of routinely obtaining fresh lysosomal preparations from human liver to support such studies, we elected to use rat liver as a source of tissue from which to isolate lysosomes. However, because our knowledge of glucocerebrosidase from this source is so limited, in an effort to obtain basic information about the properties of the enzyme that would guide and support our studies on lysosomal particles, we set out to characterize some of the properties of the solubilized and partially purified enzyme from rat liver.

We elected to utilize the fluorometric compound, MUGlc, rather than glucocerebroside as the glucocerebrosidase substrate in the present study because the former is water-soluble, thereby allowing us to exclude from the assay medium agents required to disperse or dissolve glucocerebroside; such agents (e.g. bile salts) render glucocerebrosidase activity insensitive to exogenous phospholipids and HSF (Basu *et al.*, 1984).

The present report documents the marked lipid requirement of the solubilized rat liver enzyme and characterizes the effects of various stimulatory and inhibitory substances on enzyme activity.

Materials and methods

Materials

Rats were purchased from Zivic-Miller, Pittsburgh, PA, U.S.A. Pure sodium taurodeoxycholate and MUGlc were purchased from Sigma Chemical Co. Bovine brain PtdSer and 18:1 PtdCho were obtained from Supelco. Saturated PtdCho species were obtained from Calbiochem-Behring Corp., and unsaturated PtdCho and PtdGro species from Avanti Polar Lipids, Inc. Conduritol B epoxide and HGS were generously provided by Dr. Norman S. Radin of the Department of Biological Chemistry, University of Michigan, Ann Arbor, MI, U.S.A. GS was prepared as described elsewhere (Peters *et al.*, 1977*b*).

Preparation of rat liver β-glucosidase

Male Sprague–Dawley rats weighing 150–200g were fasted overnight and killed by CO_2 asphyxiation. The livers were homogenized in 4vol. of ice-cold buffer containing 0.25M-sucrose, 2mM-EDTA, 5mM-Tris/HCl, pH7.4 (designated SET buffer) and 1mM-phenylmethanesulphonyl fluoride in a Potter–Elvehjem homogenizer. All procedures were performed at 1–4°C. The homogenate was centrifuged in a Sorvall centrifuge at 8000*g* for 10min in an SS-34 rotor. The supernatant was removed and the pellet was rehomogenized in 3vol. of SET buffer and centrifuged as described above. The pooled supernatants were centrifuged in the same rotor at 17000*g* for 15min to provide a crude lysosomal pellet fraction rich in glucocerebrosidase.

Glucocerebrosidase was partially purified and delipidated by extraction of the lysosomal pellet with sodium cholate and butan-1-ol as described by Furbish *et al.* (1977) with some modifications (Basu *et al.*, 1984). The specific activity of the final preparation, determined by using the standard β-glucosidase assay supplemented with 0.2% (w/v) sodium taurodeoxycholate, was in the range 350–500 units/mg of protein.

Although this procedure has been used by several investigators to isolate glucocerebrosidase, in the present report we refer to the enzyme activity measured using MUGlc as β-glucosidase. Maret *et al.* (1981) described the properties of another membrane-bound β-glucosidase in human spleen. Even though our enzyme preparation acts on glucocerebroside and is completely inhibited by the glucocerebrosidase-specific active site reagent CBE, we cannot rule out the presence of some other membrane-bound, nonspecific β-glucosidase, distinct from glucocerebrosidase, in our enzyme preparation.

Preparation of heat-stable factor

The heat-stable factor was purified from the spleen of a patient with type 1 Gaucher's disease exactly as described by Peters *et al.* (1977*a*).

Enzyme assays

β-Glucosidase activity was measured as previously described (Basu *et al.*, 1984). One unit of

enzyme activity is defined as that amount of enzyme which cleaves 1 nmol of glucose from the substrate/h at 37°C.

Protein determination

Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Results

Reconstitution of β-glucosidase activity by various phospholipids

Extraction of cholate-solubilized proteins from a crude preparation of rat liver lysosomes twice with butan-1-ol rendered enzyme activity markedly responsive to the inclusion of phospholipid in the standard β-glucosidase assay. We first compared the effects of a series of PtdGro species containing different fatty acids on β-glucosidase activity (Fig. 1). For saturated PtdGro the most effective activator lipid was 12:0 PtdGro. As the fatty acid chain length was increased, the other members of the saturated PtdGro series became progressively less effective activators, to the extent that 18:0 PtdGro was essentially incapable of raising β-glucosidase activity above the basal level. Of the two 18-carbon-atom unsaturated fatty acid-containing PtdGro species tested, 18:2 PtdGro was the better activator of β-glucosidase.

We similarly tested a variety of PtdCho species; all were totally incapable of activating the β-glucosidase preparation even when the assay was performed in the presence of HSF (1.3 μg/0.1 ml) (results not shown). In the absence of HSF, bovine brain PtdSer was a poor activator. However, the highest activity observed in the present study was achieved when β-glucosidase was assayed in the presence of a mixture of HSF and PtdSer (246 units/mg). Butanol-delipidated rat liver β-glucosidase, in the absence of exogenous phospholipid, was not responsive to HSF (14 versus 15 units/mg).

The PtdGro behaved differently from bovine brain PtdSer in two respects. First, in the absence of HSF several of the PtdGro species are more effective than PtdSer as β-glucosidase activators. Second, the sensitivity of β-glucosidase to HSF is greater with PtdSer than with any of the lipids in the PtdGro series.

The effect of HSF on the stimulation of β-glucosidase activity was determined by using different concentrations of four phospholipids: bovine brain PtdSer, 18:1 PtdCho, 18:1 PtdGro, and 12:0 PtdGro (Fig. 2). In the range 2–10 μg of 18:1 PtdCho/assay, HSF had no significant effect on β-glucosidase activity (Fig. 2*a*). Half-maximum stimulation of β-glucosidase activity in the presence of HSF and one of the other three acidic lipids

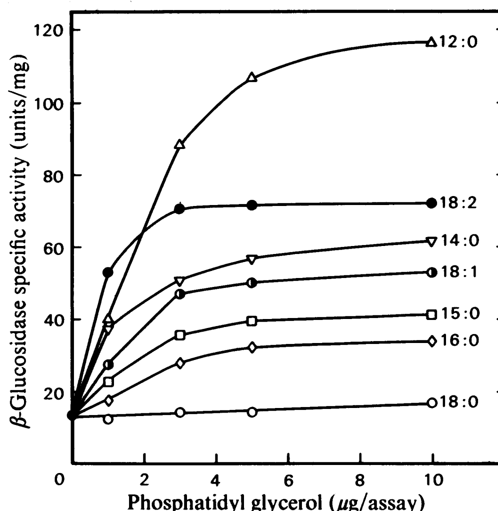

Fig. 1. *Effect of fatty acid chain length and degree of unsaturation of phosphatidylglycerol on the activation of β-glucosidase*

β-Glucosidase activity was determined using the standard assay as described under 'Materials and methods' in the presence of increasing amounts of various phosphatidylglycerols.

(Figs. 2*b*–2*d*) required less than 1 μg of phospholipid/assay.

Effect of activator substances on the rate of inactivation of β-glucosidase at 60°C

In an effort to determine if the HSF and activator lipids might be interacting with β-glucosidase we compared the rates of inactivation of the enzyme at 60°C in the presence of the various lipids (Fig. 3). Dramatic protection was afforded by the mixture of PtdSer and HSF, where the $t_{0.5}$ was increased from 2 min to nearly 10 min. The modifying effect of 18:1 PtdGro on the inactivation of β-glucosidase by heat differs from that seen with PtdSer; a pronounced biphasic result was obtained in the presence of HSF (Fig. 3*c*). The fact that there appears to be a correlation between the ability of phospholipid/HSF mixtures to activate β-glucosidase and their capacity to protect the enzyme from heat inactivation (Figs. 2 and 3) lends support to the idea that the activators interact with β-glucosidase.

Effect of lipid activators and HSF on the inactivation of β-glucosidase by conduritol B epoxide

CBE is a suicide inhibitor of certain β-glucosidases and it acts presumably by forming a covalent bond with a carboxyl group at the enzyme's active site (Legler, 1970; Stephens *et al.*, 1978; Radin, 1982). In an effort to learn more about the effect of

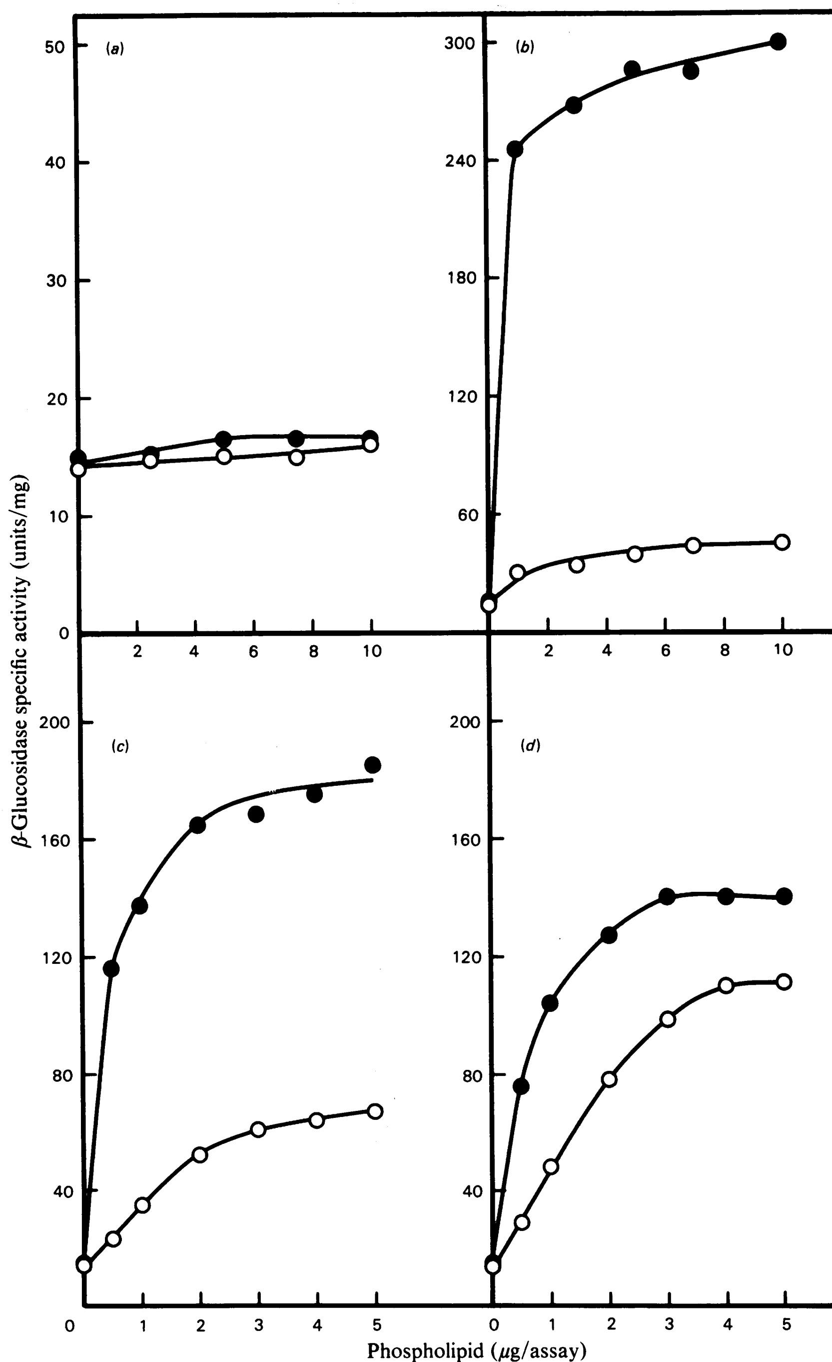

Fig. 2. *Comparison of the effect of heat-stable factor on the activation of β-glucosidase by various phospholipids*
β-Glucosidase activity was determined as described under 'Materials and methods' in the presence (●) or absence (○) of HSF (1.1 μg) over the indicated concentration range of 18:1 PtdCho (*a*), bovine brain PtdSer (*b*), 18:1 PtdGro (*c*), and 12:0 PtdGro (*d*).

activator substances on the active site of glucocerebrosidase, we compared the rates of inactivation of the enzyme by 0.25 mM-CBE in the presence of various combinations of phospholipids and HSF. The data followed first-order kinetics and from plots of log (activity remaining) versus time, we

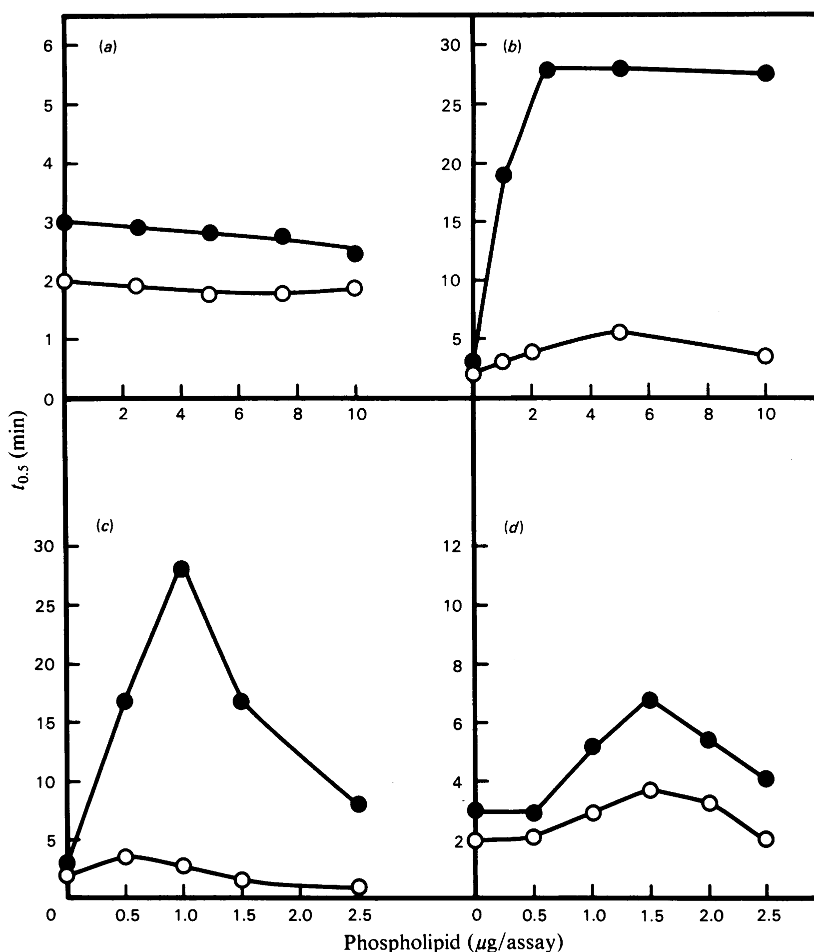

Fig. 3. *Effects of phospholipids and heat-stable factor on the rate of inactivation of β-glucosidase by heat*
Aliquots (10μl) of the β-glucosidase preparation (0.5mg of protein/ml) was preincubated at 60°C with or without various phospholipids in the presence and absence of HSF for 0–30min. At 3min intervals tubes were plunged into an ice bath and assayed for β-glucosidase activity at 37°C using the standard taurodeoxycholate assay. In all cases a plot of log (residual activity) versus time yielded a straight line from which we determined the time required for 50% inactivation ($t_{0.5}$). The $t_{0.5}$ was plotted in the presence (●) or absence (○) of HSF (1.1μg) with increasing amounts of 18:1 PtdCho (*a*), bovine brain PtdSer (*b*), 18:1 PtdGro (*c*), or 12:0 PtdGro (*d*).

estimated the time required for inactivation of half of the original enzyme activity ($t_{0.5}$). The results of this experiment are summarized in Table 1. Consistent with our previous observations (Figs. 2 and 3), HSF and 18:1 PtdCho, either separately or in combination, did not affect the rate of inactivation of the enzyme by CBE. Although PtdSer, 18:1 PtdGro and 12:0 PtdGro increased the rate of inactivation, the effects are more pronounced in the presence of HSF.

Effect of PtdSer and HSF on the pH-dependency of the β-glucosidase reaction

With regard to kinetic constants, in general, the effect of the activator substances is to decrease the K_m for MUGlc and increase the $V_{max.}$ of β-glucosidase (Table 2). This lowering of K_m by these effectors was also observed with glucocerebrosidase from bovine (Berent & Radin, 1981*a*) and human spleen (Basu *et al.*, 1984).

In an effort to learn more about the functional groups on the enzyme that are involved in catalysis and which might be affected by the various activator substances, we analysed the pH-dependency of the kinetic parameters K_m and $V_{max.}$ (Fig. 4). The $\log(V_{max.}/K_m)$ versus pH profile determined in the presence of the most effective combination of β-glucosidase activators, namely PtdSer and HSF, appeared as a bell-shaped curve. The slope at low pH is close to 2.0, suggesting that

Table 1. *Effect of various phospholipids and heat-stable factor on the rate of inactivation of β-glucosidase by conduritol B epoxide*

β-Glucosidase was preincubated at 20°C with 0.25mM-CBE and the indicated concentrations of various phospholipids in the presence or absence of 1.1 μg of HSF/assay. At 3min intervals, 10 μl aliquots were removed from the preincubation medium and assayed for β-glucosidase activity using 0.3ml of assay medium containing 5mM-MUGlc, 0.2M-sodium acetate buffer, pH5.25, and 0.2% sodium taurodeoxycholate. The time required for CBE to inactivate 50% of the initial activity ($t_{0.5}$) under the conditions is indicated in the two right-hand columns.

Phospholipid	Final concn. in the preincubation medium (μg/assay)	Rate of inactivation by CBE ($t_{0.5}$) (min) −HSF	+HSF
None	–	11.6	12.4
PtdSer	10	8.1	4.1
18:1 PtdCho	10	13.0	14.0
18:1 PtdGro	2.5	6.9	2.8
12:0 PtdGro	5.0	6.5	2.8

Table 2. *Effect of phosphatidylserine and heat-stable factor on K_m and $V_{max.}$ of β-glucosidase*

β-Glucosidase activity was determined in the presence of the substances indicated in the left-hand column under 'Effector'. K_m and $V_{max.}$ values were calculated from Lineweaver–Burk plots obtained by linear regression analysis of results of 10 determinations carried out over a 20-fold range of MUGlc concentrations, as described by Segel (1975).

Effector	K_m (mM)	$V_{max.}$ (units/mg)
None	24	78.7
HSF (1.3 μg)	12	57.0
PtdSer (10 Mg)	6.3	89.3
PtdSer (10 μg)+HSF (1.3 μg)	2.7	400

the ionization of at least two groups affects $V_{max.}/K_m$ (Schimerlik & Cleland, 1977); both groups appear to have pK values of 4.5. Above pH 5.0 the slope is unity and a group on the enzyme with a pK of 5.1 is revealed. Thus, at least three ionizable groups appear to play a role in the catalytic reaction when the enzyme is optimally activated by HSF and PtdSer.

When the same kind of study was carried out under conditions where β-glucosidase was activated only by exogenous PtdSer (10 μg/assay), a different result was obtained. First, the $V_{max.}/K_m$ values were lower over the entire pH range when

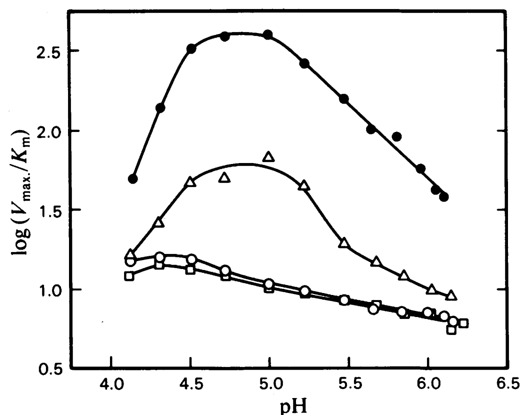

Fig. 4. *Effects of phosphatidylserine and heat-stable factor on the pH-dependence of the hydrolysis of 4-methylumbelliferyl-β-D-glucopyranoside by β-glucosidase*

β-Glucosidase activity was determined as a function of MUGlc concentration either in absence of any effector (○), or in the presence of 1.1 μg of HSF (□), 10 μg of PtdSer (△), or 1.1 μg of HSF plus 10 μg of PtdSer (●) using 0.2M-sodium acetate buffer at the indicated pH. The kinetic constants, K_m and $V_{max.}$, were determined by using double-reciprocal plots. The log ($V_{max.}/K_m$) values were plotted as a function of pH (Dixon & Webb, 1979).

compared with values obtained when a mixture of PtdSer and HSF served as activators. Second, the slope of the log ($V_{max.}/K_m$) profile on the acid side of the curve approached a value of 1.0, indicating that omission of HSF from an assay medium containing PtdSer eliminates the participation of one ionizable group in the catalytic process. The slope at the high-pH end of the curve remains unity, and the pK of 5.1 is nearly the same as it was when the enzyme was activated by PtdSer and HSF.

The belief that the activators PtdSer and HSF act at least in part by recruiting ionizable groups in β-glucosidase to participate in the catalytic process is further supported by the finding that when the pH study was performed without PtdSer, using HSF alone or no exogenous activators whatsoever, a nearly straight line was obtained (Fig. 4, open squares and open circles, respectively).

Effect of HSF and PtdSer on the inhibition of β-glucosidase by glucocerebroside-related compounds

HGS and GS have structural features in common with glucocerebroside, the natural substrate of glucocerebrosidase, and both compounds are effective inhibitors of the enzyme (Erickson & Radin, 1973). We performed a kinetic study of the effect of these inhibitors on β-glucosidase in the

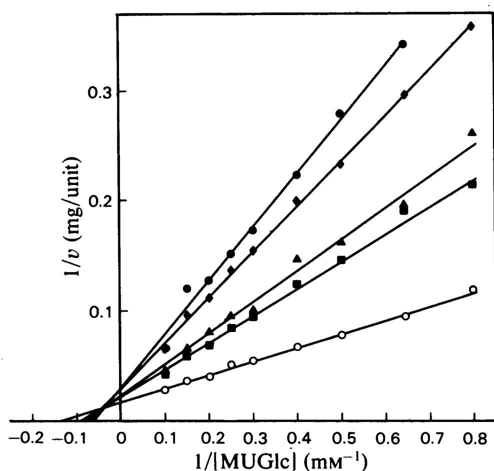

Fig. 5. *Lineweaver–Burk analysis of N-hexyl-O-glucosylsphingosine inhibition of β-glucosidase in presence of phosphatidylserine*

β-Glucosidase activity was determined in presence of 10 μg of PtdSer using varying amounts of MUGlc as the substrate in the absence (○) or presence of 0.68 μM- (■), 1.36 μM- (▲), 2.73 μM- (◆), or 4.09 μM- (●) HGS.

presence of HSF, PtdSer and a combination of the two effectors.

Fig. 5 shows the results of a typical experiment in which the inhibition of β-glucosidase by HGS was evaluated in the presence of 10 μg of PtdSer/assay. The finding of mixed-type inhibition kinetics suggests that, in the presence of PtdSer, HGS inhibits β-glucosidase by binding at some site other than the substrate binding site (Segel, 1975). Qualitatively, the same type of inhibition kinetics was obtained when the experiment was performed in the absence of PtdSer and HSF, in the presence of HSF alone, or in the presence of a combination of HSF and PtdSer (results not shown). As shown in Table 3, the inclusion of HSF or PtdSer alone in the β-glucosidase assay had only a modest effect on the K_i values. However, the combination of HSF and PtdSer had a dramatic effect on K_i, decreasing the K_i value 20-fold, indicating that HSF and PtdSer acting together greatly increase the binding of HGS to a site on the enzyme which is distinct from the substrate-binding site.

A similar kinetic study was performed with GS and a typical result is presented in Fig. 6. It is apparent from this set of Lineweaver–Burk plots and the slope-versus-GS-concentration replot (inset) that the nature of inhibition of β-glucosidase caused by GS differs from that obtained with the more hydrophobic HGS. In every case, the slope-versus-inhibitor-concentration plots yielded parabolic curves. These results indicate the complex nature of the inhibition of β-glucosidase by GS. The non-linear character of the replots compelled us to express the effects of activator substances in

Table 3. *Effect of phosphatidylserine and heat-stable factor on inhibition of β-glucosidase by N-hexyl-O-glucosylsphingosine and glucosylsphingosine*

The K_i value for HGS was determined under the various conditions indicated in the left-hand column under 'Effector' as described by Segel (1975). The concentration of GS required to inhibit β-glucosidase activity by 50% ($I_{0.5}$) was determined from the plot of percentage of enzyme activity remaining versus GS concentration.

	Inhibitor	
Effector	HGS (K_i) (μM)	GS ($I_{0.5}$) (μM)
None	2.12	89.4
HSF (1.3 μg)	3.08	21.8
PtdSer (10 μg)	1.47	29.4
PtdSer (10 μg) + HSF (1.3 μg)	0.11	7.6

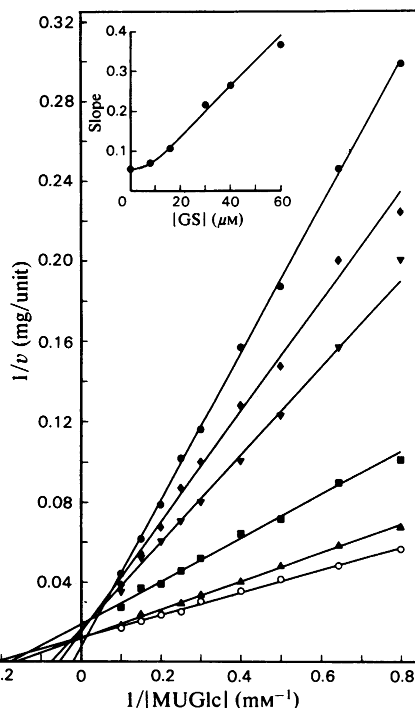

Fig. 6. *Lineweaver–Burk analysis of glucosylsphingosine inhibition of β-glucosidase in presence of phosphatidylserine*

β-Glucosidase activity was determined in the presence of 10 μg of PtdSer using varying amounts of MUGlc in the absence (○), or presence of 8 μM- (▲), 16 μM- (■), 30 μM- (▼), 40 μM- (◆), or 60 μM- (●) GS.

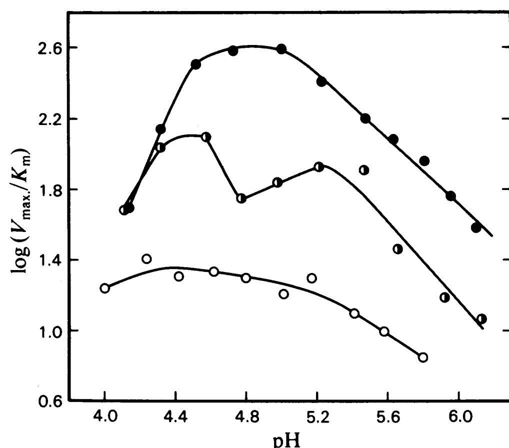

Fig. 7. *Effect of N-hexyl-O-glucosylsphingosine on the pH-dependence of MUGlc hydrolysis by β-glucosidase in the presence of phosphatidylserine and heat-stable factor*
β-Glucosidase activity was determined as a function of substrate concentration in 0.2M-sodium acetate buffer at different pH values in the presence of 10 μg of PtdSer and 1.1 μg of HSF and (●), no inhibitor, (◑) 0.273 μM- and (○) 0.455 μM-inhibitor HGS. The log ($V_{max.}/K_m$) versus-pH plots were determined as described in the legend to Fig. 4.

terms of $I_{0.5}$ values, which represent the concentration of inhibitor required to inhibit activity by 50% (Table 3). The fact that HSF by itself decreased the $I_{0.5}$ for GS 4-fold provides kinetic evidence that the factor interacts with β-glucosidase. Of the three conditions where activator substances were compared, the combination of HSF and PtdSer proved most effective in decreasing the $I_{0.5}$ for GS.

Finally, we examined the interaction of the more potent inhibitor, HGS, with β-glucosidase by determining its effects on $V_{max.}$ and K_m at different pH values. As shown in Fig. 7, increasing the concentration of HGS profoundly alters the log($V_{max.}/K_m$) versus pH profile, first modifying the pK values of ionizable groups involved in catalysis, and finally obliterating these groups. These last results indicate that the binding of HGS to the enzyme modifies the ionization of functional groups which influence the catalytic process.

Discussion

Our study of the lipid requirement of rat liver β-glucosidase has documented the importance of both acidic phospholipids and a heat-stable protein for the reconstitution of activity to a cholate/butanol-extracted preparation of the enzyme. Phospholipids that support the highest specific activities are the ones with a net negative charge (e.g. PtdGro, PtdSer). Unlike the corresponding enzyme from human tissues (Basu *et al.*, 1984), rat liver β-glucosidase was completely unresponsive to PtdCho, a neutral phospholipid. However, for both the rat liver enzyme, which is activated by PtdGro, and the human enzyme, which is moderately stimulated by PtdCho (Basu *et al.*, 1984), the most effective activators are the phospholipids containing short chain or polyunsaturated fatty acids.

With PtdSer, the most effective lipid activator of rat liver β-glucosidase tested in this study, β-glucosidase activity is markedly dependent on the inclusion of HSF in the assay medium. The mechanism whereby the HSF/phospholipid mixture reconstitutes β-glucosidase activity is obscure. It is not known if the active form of the enzyme is a binary complex composed of enzyme and acidic phospholipid, or if it is a ternary complex composed of enzyme, phospholipid and HSF. In the first case, HSF could be acting by facilitating the binding of phospholipid to the enzyme. In this regard, there are at least two roles that the HSF could play. First, it could function as a lipid-transfer protein, accelerating the movement of phospholipid from a micellar form to a binding site on β-glucosidase. Alternatively, the factor could act by binding to the enzyme, thereby inducing a conformational change which either creates a phospholipid-binding site on β-glucosidase or greatly enhances the affinity of a pre-existing site for acidic phospholipids. There is evidence (Berent & Radin, 1981*b*) in the literature which suggests that HSF binds directly to β-glucosidase, even when phospholipids are absent. First, β-glucosidase will bind to an affinity column composed of HSF. Second, HSF protects β-glucosidase against inactivation by *N*-ethylmaleimide and chloromercuriphenylsulphonate. Furthermore, in the present study we have shown that HSF, in the absence of phospholipid, markedly affects the K_m for the substrate MUGlc (Table 2), and decreases the $I_{0.5}$ for GS 4-fold (Table 3). It is conceivable that if the HSF influences the reactivity of β-glucosidase to thiol reagents and the binding of inhibitors and substrates to the enzyme, it could also create the acidic phospholipid-binding site. Once the phospholipid-binding site is occupied, the HSF could remain in association with the enzyme, thereby giving rise to a ternary complex, or it could dissociate from the enzyme, leaving behind the active phospholipid–enzyme binary complex.

The HSF/PtdSer pair has a profound effect on the active site of rat liver β-glucosidase; the activators: (1) increase the $V_{max.}$, (2) decrease the K_m, (3) increase the rate of inactivation of the enzyme by CBE and (4) bring into place at least three ionizable groups which influence catalysis. These observations are consistent with the view that

PtdSer and HSF induce a conformational change in β-glucosidase such that key functional groups are now in proper juxtaposition so as to create the enzyme's active centre.

Thus far, the Discussion has alluded to three kinds of binding sites on β-glucosidase: a catalytic centre where substrate is bound, a phospholipid-binding site which prefers acidic phospholipids, and a third domain which is involved in binding the HSF. These hypothetical sites could be distinct or they may be constructed from overlapping domains. The fact that rat liver β-glucosidase is inhibited by low concentrations of HGS and GS and that the inhibition is of mixed type suggests the presence of another binding site on the enzyme for these sphingolipids. The slope-versus-inhibitor-concentration replots for HGS and GS reveal some differences between these two inhibitors. The straight line replot seen with HGS suggests that: (1) one inhibitor molecule binds to both the free enzyme and the enzyme–substrate complex, (2) the enzyme–inhibitor complex can still bind substrate but the resultant enzyme–substrate–inhibitor complex is nonproductive (Segel, 1975). On the other hand, the parabolic nature of the slope-versus-inhibitor-concentration replot for GS suggests the binding of more than one GS molecule to β-glucosidase. As evident from Fig. 7, the binding of the inhibitor HGS abolishes the active conformation created by the PtdSer/HSF pair.

Although evidence is accumulating that HSF plays a role in creating binding sites for substrate, sphingolipid inhibitors and acidic phospholipid activators, a definitive characterization of these sites remains to be accomplished. Since most of the work on the phospholipid requirement of β-glucosidase has involved reconstitution experiments that have employed selected pure lipids, there is a need for information about the composition and distribution of phospholipids in purified lysosome preparations. The goal of such studies should be to define the phospholipid(s) which activate β-glucosidase *in situ*.

It should be emphasized that, because a water-soluble nonphysiological β-glucosidase substrate was employed in these studies, one should not draw firm conclusions from our results about the metabolism of glucocerebroside. In addition, the pronounced dependency of PtdSer stimulation on HSF raises a question about the existence of a corresponding activator protein in rat liver lysosomes (Mraz *et al.*, 1976); more specifiically, was an activator protein separated from β-glucosidase during preparation of the enzyme used in the present study?

This work was suported by grant AM-31357 from the United States Public Health Service, National Institutes of Health.

References

Basu, A., Glew, R. H., Daniels, L. B. & Clark, L. S. (1984) *J. Biol. Chem.* **259**, 1714–1719

Berent, S. L. & Radin, N. S. (1981*a*) *Arch. Biochem. Biophys.* **208**, 248–260

Berent, S. L. & Radin, N. S. (1981*b*) *Biochim. Biophys. Acta* **664**, 572–582

Beutler, E., Kuhl, W., Trinidad, F., Teplitz, R. & Nadler, H. (1971) *Am. J. Hum. Genet.* **23**, 62–66

Blonder, E., Klibansky, C. & de Vries, A. (1976) *Biochim. Biophys. Acta* **431**, 45–53

Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254

Brady, R. O., Kanfer, J. N. & Shapiro, D. (1965*a*) *Biochem. Biophys. Res. Commun.* **18**, 221–225

Brady, R. O., Kanfer, J. N. & Shapiro, D. (1965*b*) *J. Biol. Chem.* **240**, 39–43

Dale, G. L., Villacorte, D. G. & Beutler, E. (1976) *Biochem. Biophys. Res. Commun.* **71**, 1048–1053

Daniels, L. B., Glew, R. H., Diven, W. F., Lee, R. E. & Radin, N. S. (1981) *Clin. Chim. Acta* **115**, 369–375

Daniels, L. B., Coyle, P. J., Glew, R. H., Radin, N. S. & Labow, R. S. (1982) *Arch. Neurol.* **39**, 550–556

Dixon, M. & Webb, E. C. (1979) in *Enzymes* (Dixon, M., Webb, E.C., Thorne, C.J.R. & Tipton, K. F., eds.), pp. 138–164, Academic Press, New York

Erickson, J. S. & Radin, N. S. (1973) *J. Lipid Res.* **14**, 133–137

Furbish, F. S., Blair, H. E., Shiloach, J., Pentchev, P. G. & Brady, R. O. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3560–3563

Ginns, E. I., Brady, R. O., Pirruccello, S., Moore, C., Sorrell, S., Furbish, F. C., Murray, G. J., Tager, J. & Barranger, J. A. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5607–5610

Glew, R. H., Daniels, L. B., Clark, L. S. & Hoyer, S. W. (1982) *J. Neuropathol. Exp. Neurol.* **41**, 630–641

Hara, A. & Radin, N. S. (1979) *Biochim. Biophys. Acta* **582**, 412–422

Ho, M. W. (1973) *Biochem. J.* **136**, 721–729

Ho, M. W. & Light, N. D. (1973) *Biochem. J.* **136**, 821–823

Ho, M. W. & O'Brien, J. S. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2810–2813

Ho, M. W., O'Brien, J. S., Radin, N. S. & Erickson, J. S. (1973) *Biochem. J.* **131**, 173–176

Hultberg, B. & Öckerman, P. A. (1970) *Clin. Chim. Acta* **28**, 169–174

Kanfer, J. N., Legler, G., Sullivan, J., Raghavan, S. S. & Mumford, R. A. (1975) *Biochem. Biophys. Res. Commun.* **67**, 85–90

Legler, G. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 25–31

Maret, A., Salvayre, R., Negre, A. & Douste-Blazy, L. (1981) *Eur. J. Biochem.* **115**, 455–461

Matoth, Y., Zaizov, R., Hoffman, J. & Klibansky, Ch. (1974) *Isr. J. Med. Sci.* **10**, 1523–1529

Mraz, W., Fischer, G. & Jatzkewitz, H. (1976) *FEBS Lett.* **67**, 104–109

Mueller, O. T. & Rosenberg, A. (1979) *J. Biol. Chem.* **254**, 3521–3525

Patrick, A. D. (1965) *Biochem. J.* **97**, 17C–18C

Pentchev, P. G., Brady, R. O., Hibbert, S. R., Gal, A. E. & Shapiro, D. (1973) *J. Biol. Chem.* **248**, 5256–5261

Pentchev, P. G., Brady, R. O., Blair, H. E., Britton, D. E. & Sorrell, S. H. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3970–3973

Peters, S. P., Lee, R. E. & Glew, R. H. (1975) *Clin. Chim. Acta* **60**, 391–396

Peters, S. P., Coyle, P. & Glew, R. H. (1976) *Arch. Biochem. Biophys.* **175**, 569–582

Peters, S. P., Coyle, P., Coffee, C. J., Glew, R. H., Kuhlenschmidt, M. S., Rosenfeld, L. & Lee, Y. C. (1977*a*) *J. Biol. Chem.* **252**, 563–573

Peters, S. P., Glew, R. H. & Lee, R. E. (1977*b*) in *Practical Enzymology of the Sphingolipidoses* (Glew, R. H. & Peters, S. P., eds.), pp. 71–100, Alan R. Liss, New York

Radin, N. S. (1982) *Prog. Clin. Biol. Res.* **95**, 357–383

Raghavan, S. S., Topol, J. & Kolodny, E. H. (1980) *Am. J. Hum. Genet.* **32**, 158–173

Schimerlik, M. I. & Cleland, W. W. (1977) *Biochemistry* **16**, 576–583

Segel, I. H. (1975) *Enzyme Kinetics*, pp. 100–160 and 161–226, Wiley-Interscience, New York

Stephens, M. C., Bernatsky, A., Burachinsky, V., Legler, G. & Kanfer, J. N. (1978) *J. Neurochem.* **30**, 1023–1027

Svennerholm, L., Håkansson, G. & Dreborg, S. (1980) *Clin. Chim. Acta* **106**, 183–193

Wenger, D. A. & Olson, G. C. (1981) in *Lysosomes and Lysosomal Storage Diseases* (Callahan, J. W. & Lowden, J. A., eds.), pp. 157–171, Raven Press, New York

Wenger, D. A. & Roth, S. (1982) *Prog. Clin. Biol. Res.* **95**. 551–572