Glucuronate-2-sulphatase activity in cultured human skin fibroblast homogenates

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The optimization of the assay conditions to detect glucuronate-2-sulphatase (GS) activity present in cultured human skin fibroblast homogenates towards a heparin-derived disaccharide substrate *O*-(β -D-glucuronic acid 2-sulphate)-(1 $\rightarrow$ 4)-D-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate (GSMS) has shown that a complex relationship exists between pH, buffer composition, ionic strength and the influence of added BSA and salts (NaCl, Na₂SO₄, CuCl₂ and ZnCl₂) to achieve maximum sulphatase activity. Whereas albumin stimulated GS activity by more than 2-fold over the pH range 2.7–5.7, CuCl₂ stimulated GS activity over the narrow pH range 3.0–4.2, and inhibited GS activity at higher pH. ZnCl₂ stimulated GS activity more than 3-fold at pH 3.0 and by more than 10-fold at pH 4.8. NaCl inhibited GS activity at pH 3.0, while activity between pH 4.2 and 4.8 was stimulated by up to 10-fold, resulting in a shift in the observed pH optimum from 3.0 to 4.8 in the presence of 315 mM-NaCl. Skin fibroblast GS activity toward GSMS had apparent *K_m* values of 0.5–1.2 μ M at pH 3.0, and 27.0–33.2 μ M at pH 4.8. Albumin stimulated GS activity at both low and high pH by an increase in the apparent *V_{max}* values without significant alteration in the respective *K_m* values. At pH 4.8, NaCl stimulated GS activity as a result of an increase in *V_{max}* values. These observations raise the possibility that two forms of GS activity are present in skin fibroblast homogenates: a low-*K_m* form that has a pH optimum of 3.0 and is stimulated by BSA and a high-*K_m* form with a pH optimum of 4.8 which is stimulated by NaCl.

INTRODUCTION

The structurally related sulphated glycosaminoglycans heparin and heparan sulphate are degraded from their non-reducing end by the sequential action of five highly specific lysosomal sulphatases and four other *exo*-enzyme activities [1,2]. A deficiency in humans of any one of these lysosomal enzyme activities results in the accumulation of that enzyme's substrate in the tissues and can lead to one of the mucopolysaccharidosis (MPS) group of lysosomal storage disorders [3]. Recently it was shown that a small percentage of glucuronic acid residues present in the highly sulphated regions of heparan sulphate were 2-*O*-sulphated [4]. Chick-embryo chondrocytes and cultured human skin fibroblasts were shown to contain a specific sulphatase activity, glucuronate-2-sulphatase (GS), which acted towards glucuronic acid 2-sulphate (GlcA2S) residues present in a heparin-derived disaccharide substrate, *O*-(β -D-glucuronic acid 2-sulphate)-(1 $\rightarrow$ 4)-D-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate (GSMS). It was shown that the same enzyme activity could also desulphate the chondroitin 6-sulphate-derived disaccharide substrate, *O*-(β -D-glucuronic acid 2-sulphate)-(1 $\rightarrow$ 3)-D-*O*-2,5-anhydro[1-³H]talitol 6-sulphate (GSTS) [5].

We recently reported the isolation of a single form of GS present in human liver which was purified two million-fold to homogeneity and was shown to be active toward both GSMS and GSTS [6]. Once purified, GS was unstable and required the addition of BSA to incubation mixtures used to determine and characterize GS activity. The low abundance of GS (3 μ g of protein isolated from 1 kg of human liver) may be related to the low abundance of GlcA2S residues reported to be present in heparan sulphate and chondroitin 6-sulphate [5,7].

Patients with lysosomal storage disorders deficient in GS activity have yet to be described. The diagnosis of a deficiency in GS activity would be based on the demonstration of a gross deficiency of enzyme activity in patient leucocytes or cultured skin fibroblasts. We now report our evaluation of GSMS as a diagnostic substrate for the detection of GS activity in cultured human skin fibroblast homogenates. A complex relationship was shown to exist between pH, buffer composition, ionic strength and the influence of added BSA and salts (NaCl, Na₂SO₄, CuCl₂ or ZnCl₂) to achieve optimal GS enzyme activity.

MATERIALS AND METHODS

GSMS and GSTS were prepared as previously described [6,7]. Sigma Chemical Co. supplied crystalline BSA (globulin-free grade) and D-saccharic acid 1,4-lactone. Beckman Instruments Inc. (Gladesville, Australia) supplied Ready Solv EP scintillation cocktail, and Promega Corp. (Rozelle, Australia) supplied acetylated BSA. Protein was determined as described [8], with BSA as standard.

Enzyme preparation

Fibroblast cultures were established from skin biopsies taken in, or provided to, this hospital and were harvested as previously described [9]. Typing of MPS patients was consistent with the extent and type of glycosaminoglycanuria, clinical phenotype and nature of the lysosomal enzyme deficiency [9,10]. Cells were suspended at a concentration of 2–10 mg of protein/ml in aqueous Triton X-100 (1 mg/ml), and were disrupted by rapid freezing and thawing six times in a solid-CO₂/ethanol mixture. Homogenates were dialysed against 150 mM-NaCl for 16 h at 4 °C.

Abbreviations used: GSMS, *O*-(β -D-glucuronic acid 2-sulphate)-(1 $\rightarrow$ 4)-D-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; GMS, *O*-(β -D-glucuronic acid)-(1 $\rightarrow$ 4)-D-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; MS, 2,5-anhydro[1-³H]mannitol 6-sulphate; ISMS, *O*-(α -L-iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-D-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; GSTS, *O*-(β -D-glucuronic acid 2-sulphate)-(1 $\rightarrow$ 3)-D-*O*-2,5-anhydro[1-³H]talitol 6-sulphate; GlcA2S, glucuronic acid 2-sulphate; GS, glucuronate 2-sulphatase; MPS, mucopolysaccharidosis.

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Enzyme assay

The pH-enzyme activity profiles were determined by the incubation of 4 μ M-GSMS in sodium formate or glycine/HCl (pH 2.4–4.5) or sodium acetate (pH 2.7–6.6) at the indicated ionic strength, with 1 μ l of dialysed cell homogenate (adjusted with dialysis buffer to 2 μ g of protein) and any additive in a total volume of 10 μ l. All assays therefore contained 15 mM-NaCl and were incubated at 37 °C for a time-interval of up to 8 h to give between 5 and 25% conversion of substrate into product. The reaction was stopped by freezing in a solid- CO_2 /ethanol bath and the substrate separated from the product by high-voltage electrophoresis on Whatman 3 MM chromatography paper in 0.74 M-formic acid, pH 1.7, at 45 V/cm for 40 min in a Shandon Southern model L-24 system (Shandon Southern Products, Runcorn, Cheshire, U.K.). Areas of radioactivity were detected by scanning of the strips on a Packard model 7201 radiochromatogram scanner (Packard, Chicago, IL, U.S.A.) and were cut from the strip, then placed in 20 ml glass scintillation vials along with 5 ml of water and 10 ml of scintillation cocktail, and their radioactivities were determined in a Searle model 6868 Isocap ambient-temperature liquid-scintillation system (Searle Analytic, Des Plaines, IL, U.S.A.). Enzyme activity was determined from the percentage breakdown of substrate to product and was expressed as pmol of product/min per mg of homogenate protein. K_m and V_{max} values were obtained from Lineweaver–Burk plots using the standard assay, with GSMS assayed within a concentration range of 0.8–40 μ M.

The standard enzyme assay mixture for the routine detection of GS activity in cultured human skin fibroblast homogenates contained 4 μ M-GSMS, BSA (1 mg/ml), 50 μ M-D-saccharic acid 1,4-lactone in 20 mM-sodium formate buffer, pH 3.3, and 1 μ l (1–4 μ g of protein) of dialysed cell homogenate in a total volume of 10 μ l incubated at 37 °C for 90 min.

RESULTS

High-voltage electrophoresis of the whole incubation mixture was routinely used to assess the level of de-O-sulphation of the substrate by enzyme preparations. Fig. 1 shows the electrophoretic separation of GSMS from GMS (product 1) and MS (product 2, which resulted from the subsequent action of β -D-glucuronidase activity toward GMS) in an incubation mixture containing skin fibroblast homogenate. The addition of the β -D-glucuronidase inhibitor, D-saccharic acid 1,4-lactone, resulted in the complete inhibition of β -D-glucuronidase activity towards GMS (see below). Dialysis of the skin fibroblast homogenates against 150 mM-NaCl increased the sulphatase activity by up to 2-fold over the pH range 3.0–5.7 (results not shown). A similar increase in sulphatase activity after dialysis was reported for the detection of iduronate-2-sulphatase and glucosamine-6-sulphatase activities toward their disaccharide substrates [11,12]. All assays therefore contained an initial concentration of 15 mM-NaCl.

Effect of buffer composition and ionic strength on GS activity

The pH-activity profile for GS activity towards GSMS was dependent on both the buffer composition and the ionic strength. Whereas maximal activity was observed at pH 3.3 in each of the sodium formate, sodium acetate and glycine/HCl buffers at 60 mM concentration, sulphatase activity at pH 3.3 was 24% higher in 60 mM-sodium acetate (Fig. 2a). GS activity in each of the buffers at pH greater than 3.6 was similar; however, the buffer composition and ionic strength had a marked effect on GS activity, particularly at lower pH. For example, sulphatase activity in 60 mM-glycine/HCl buffer at pH 2.7 was less by 73% than the activity observed in sodium formate.

GS activity was inhibited by increased sodium formate buffer concentration (Fig. 2a). Maximal GS activity was observed in 4 or 20 mM-sodium formate buffer at pH 3.0 and was up to 48% higher than GS activity observed in 60 mM-sodium formate buffer at pH 3.3. The conversion of the sulphatase product GMS into MS from the subsequent action of β -D-glucuronidase was similarly influenced by the buffer composition and concentration.

Fig. 1. Separation by electrophoresis of an incubation mixture containing normal fibroblast homogenate and GSMS incubated in 20 mM-sodium acetate, pH 3.3 (....), or 20 mM-sodium acetate, pH 3.3, containing 50 μ M-D-saccharic acid 1,4-lactone (—)

Fig. 2. Hydrolysis of (a) 4 μ M-GSMS and (b) 4 μ M-GMS by a normal fibroblast homogenate as a function of buffer composition and incubation pH

Fibroblast homogenate was incubated in 4 mM-sodium formate (○), 20 mM-sodium formate (●), 60 mM-sodium formate (□), 20 mM-sodium acetate (■), 60 mM-sodium acetate (△) and 60 mM-glycine/HCl (▲).

Table 1. Hydrolysis of 4 μM-GSMS by a normal fibroblast homogenate incubated in sodium formate and sodium acetate, pH 3.0

Buffer	Concentration (mM)	Product (pmol/min per mg of protein)		
		GMS	MS	Total (GMS + MS)
Sodium formate	60	8.7	0.2	8.9
	20	12.3	1.2	13.5
	4	6.4	8.2	14.6
Sodium acetate	60	4.1	8.2	12.3
	20	4.0	9.8	13.8
	20	13.6	0.0	13.6
Sodium formate + D-saccharic acid 1,4-lactone	20	13.9	0.0	13.9

Skin fibroblast activity towards GSMS incubated in the presence of 4 mM-sodium formate, pH 3.0, resulted in the conversion of 56 % of the GMS product into MS, whereas incubation in 60 and 20 mM-sodium formate at pH 3.0 resulted in the conversion of only 2 and 9 % of the sulphatase product GMS into MS respectively (Table 1). Incubation in 20 and 60 mM-sodium acetate, pH 3.0, resulted in the conversion of 71 and 67 % of the GMS product into MS respectively. The addition of the β-D-glucuronidase inhibitor, D-saccharic acid 1,4-lactone, had no significant effect on the total amount of product formed for each of the buffers tested (Table 1); therefore the stimulation of the hydrolysis of GSMS at pH 3.0 observed in the presence of lower buffer concentration was independent of β-D-glucuronidase activity. Skin fibroblast homogenate β-D-glucuronidase activity was itself similarly influenced by buffer concentration because activity toward 4 μM-GMS was increased 2-fold at pH 3.0 when the sodium formate concentration was decreased from 20 to 4 mM, and this resulted in an apparent shift in the observed pH optimum from 3.3 to 3.0 (Fig. 2b).

Homogenates were incubated in 20 mM-sodium formate or acetate buffers to ensure adequate buffering capacity over the period of incubation. There was no significant difference in GS activity assayed in 20 mM-sodium formate or acetate buffer between pH 3.0 and 4.5 (Fig. 2a). GS activity was linear with

Fig. 3. Hydrolysis of 4 μM-GSMS by a normal fibroblast homogenate as a function of CuCl₂, ZnCl₂ and EDTA concentration and incubation pH

Fibroblast homogenate was incubated in 20 mM-sodium formate (●) and 20 mM-sodium formate containing 1 mM- (▲), 5 mM- (○), 10 mM- (■) and 15 mM-CuCl₂ (□), 5 mM-ZnCl₂ (◇) and 2 mM-EDTA (△).

respect to time for up to 12 h for incubations at 37 °C containing between 0.7 and 7 μg of homogenate protein and for up to 35 % conversion of substrate into product. Lineweaver–Burk plots of GS activity present in three control cultured skin fibroblast homogenates toward GSMS at pH 3.0 resulted in apparent K_m values of 0.7–1.2 μM with corresponding V_{max} values of 11.9–22.2 pmol of product/min per mg of homogenate protein (Table 2). Substrate inhibition was observed when GSMS was assayed at substrate concentrations above 10 μM.

Effect of salts on GS activity

The stimulation of skin fibroblast sulphatase activity by the addition of CuCl₂ was observed to be pH-dependent. CuCl₂ at 1 and 5 mM concentrations stimulated maximal GS activity by 1.7- and 2.3-fold respectively and shifted the observed respective pH optima from 3.0 to 3.3 and 3.6 (Fig. 3). GS activity was stimulated

Table 2. K_m and V_{max} values for normal fibroblast homogenate GS activity toward GSMS

Four fibroblast homogenates (1–4) were incubated in (a) 20 mM-sodium formate buffer, pH 3.0; (b) 20 mM-sodium formate buffer, pH 3.3, containing 1 mg of BSA/ml; (c) 20 mM-sodium acetate buffer, pH 4.8; (d) 20 mM-sodium acetate buffer, pH 4.8, containing 1 mg of BSA/ml; (e) 20 mM-sodium acetate buffer, pH 4.8, containing 315 mM-NaCl. K_m and V_{max} values are expressed in units of μM and pmol/min per mg of protein respectively.

pH	Additive	Fibroblast-cell homogenate ...	(1)		(2)		(3)		(4)	
			K _m	V _{max}	K _m	V _{max}	K _m	V _{max}	K _m	V _{max}
(a) 3.0	None		0.9	11.9	0.7	22.2	—*	—	1.2	17.4
(b) 3.3	BSA		1.0	29.4	0.8	43.4	—	—	1.3	39.2
(c) 4.8	None		33.3	6.6	—	—	27.0	5.9	—	—
(d) 4.8	BSA		32.2	12.2	—	—	27.0	12.5	—	—
(e) 4.8	NaCl		32.2	35.7	—	—	—	—	33.1	28.6

* —, Not done.

Fig. 4. Hydrolysis of (a) 4 μ M-GSMS and (b) 40 μ M-GSMS by a normal fibroblast homogenate as a function of NaCl concentration and incubation pH

Fibroblast homogenate was incubated in either 20 mM-sodium formate (pH 2.4–4.5) or 20 mM-sodium acetate (pH 3.6–5.7) containing 15 mM- (●), 45 mM- (○), 115 mM- (□), 165 mM- (▲), 315 mM- (△) and 465 mM-NaCl (◆).

within the pH range 3.0–4.2, but activity was inhibited by CuCl_2 at pH higher than 4.5. At pH 2.4 GS activity was inhibited 50% by 5 mM- CuCl_2 , but this may be due to inhibition by Cl^- since a similar inhibition occurred after the addition of 10 mM-NaCl (see below). Inhibition of GS activity by Cl^- may also explain why the stimulation of GS activity by 15 mM- CuCl_2 was less than that observed for 5 and 10 mM- CuCl_2 . Unlike GS activity, β -D-glucuronidase activity toward the product GMS was completely inhibited by 5 mM- CuCl_2 , since no conversion of the GMS product into MS was detected within the pH range 2.4–4.5. EDTA at 2 mM concentration inhibited GS activity by approximately 75% across the pH range 2.4–4.5 (Fig. 3). ZnCl_2 at 5 mM concentration stimulated maximal GS activity by 3.6-fold to give a broad pH-activity profile with maximal activity at pH 3.3. Whereas CuCl_2 completely inhibited GS activity at pH 4.8, ZnCl_2 stimulated sulphatase activity by 10-fold. At pH 3.3, 5 mM- MgCl_2 or - MnCl_2 had no effect on GS activity.

Fig. 4(a) shows the effect of increasing the concentration of NaCl from 15 to 465 mM on skin fibroblast GS activity toward 4 μ M-GSMS. GS activity observed in 15 mM-NaCl at pH 3.0 was not significantly altered when the concentration of NaCl was reduced to 5 mM. However, GS activity was inhibited 57% by incubations containing 45 mM-NaCl. Inhibition of GS activity was most profound on the acidic side of the pH profile. NaCl stimulated GS activity at pH greater than 3.3 resulting in an

Fig. 5. Hydrolysis of 4 μ M-GSMS by a normal fibroblast homogenate incubated in the presence of (a) 15 mM-NaCl and (b) 315 mM-NaCl as a function of Na_2SO_4 concentration and incubation pH

Fibroblast homogenate was incubated with (a) 20 mM-sodium formate containing 0 mM- (●), 20 mM- (○), 50 mM- (■) and 100 mM- Na_2SO_4 (□) and (b) 20 mM-sodium acetate containing 0 mM- (●), 40 mM- (▲) and 200 mM- Na_2SO_4 (△).

apparent shift in maximal activity from 3.0 to 3.6. Increasing the salt concentration from 45 to 165 mM inhibited GS activity at pH less than 4.5 and shifted the observed pH optimum from 3.6 to 4.2. Maximal activity at pH 4.2 in the presence of 165 mM-NaCl was half that originally observed at pH 3.0. Further increasing the salt concentration to 315 mM-NaCl resulted in an inhibition of activity at pH 4.2, while activity was increased at pH 4.8 resulting in a further shift in maximal activity from pH 4.2 to 4.8. GS activity at pH 3.0 was completely inhibited by 315 mM-NaCl, while activity at pH 4.8 was stimulated 17-fold compared with previous activity in 15 mM-NaCl. Maximal sulphatase activity at pH 4.8 was less than half of that previously observed at pH 3.0. Increasing the salt concentration from 315 to 465 mM decreased GS activity at pH 4.8 by 64%. The presence of 315 mM-NaCl completely inhibited β -D-glucuronidase activity over the pH range 3.0–5.7, and GMS was the only product observed after fibroblast homogenate activity toward GSMS. The influence of 5 mM- CuCl_2 and - ZnCl_2 on GS activity in the presence of 315 mM-NaCl was complex. Sulphatase activity stimulated by NaCl at pH 4.8 was completely inhibited by CuCl_2 , whereas ZnCl_2 had no effect. Both CuCl_2 and ZnCl_2 were unable to stimulate GS activity at pH 3.6 in the presence of 315 mM-NaCl.

Skin fibroblast GS activity at pH 4.8 in the presence of 15 mM-NaCl had apparent K_m values of 27.0 and 33.3 μM , up to 47 times higher than observed at pH 3.0, while the corresponding V_{max} values were less than half (Table 2). The addition of 315 mM-NaCl to incubations at pH 4.8 did not significantly alter the apparent K_m value, but NaCl stimulated GS activity as a result of a 5-fold increase in the V_{max} value (Table 2). GS activity towards 40 μM -GSMS was maximal at pH 3.0 in the presence of 15 mM-NaCl (Fig. 4b). However, because of substrate inhibition (which was also observed for purified human liver GS activity [6]), GS activity towards 40 μM -GSMS at pH 3.0 was less than that observed for activity toward 4 μM -GSMS. The influence of added NaCl was similar for sulphatase activity towards both 4 and 40 μM -GSMS. GS activity toward 40 μM -GSMS in the presence of 165 mM-NaCl at pH 3.0 was almost completely inhibited and resulted in a shift in maximal activity from pH 3.0 to 4.2 (Fig. 4b). Maximal activity in the presence of 315 and 465 mM-NaCl was observed at pH 4.8 and 5.1 respectively.

Skin fibroblast GS activity toward 4 μM -GSMS was inhibited 50% by 30 μM - Na_2SO_4 in 20 mM-sodium formate buffer at pH 3.0. The inhibition of GS activity by Na_2SO_4 was pH-dependent and had greatest influence on the acidic side of the pH profile (Fig. 5a). Whereas 20 μM - Na_2SO_4 inhibited GS activity by 63% at pH 2.4, it had no significant effect at pH 3.9, whereas GS activity at pH 3.0 was inhibited 95% by 100 μM - Na_2SO_4 , but was not decreased at pH 4.2. In the presence of 315 mM-NaCl, GS activity was also inhibited by Na_2SO_4 in a pH-dependent fashion (Fig. 5b). At pH 4.8, 40 μM - Na_2SO_4 inhibited GS activity by 38%, whereas 200 μM - Na_2SO_4 inhibited GS activity by 57% at pH 4.8, but at pH 5.4 GS activity was decreased by only 39%.

Effect of BSA on GS activity

The influence of increasing amounts of BSA on GS activity as a function of the incubation pH is shown in Fig. 6. At all of the concentrations evaluated, BSA stimulated GS activity present in skin fibroblast homogenates over the entire pH range 2.4–5.7. Maximal GS activity was observed at pH 3.3 in the presence of 1 mg of BSA/ml, whereby GS activity was stimulated 2.1-fold. GS activity was increased by 7.2-fold at pH 4.2, while at pH 4.8 it was stimulated 2-fold. However, GS activity at pH 3.3 and 4.8 was not stimulated by the addition of 1 mg of acetylated BSA/ml (results not shown). Whereas both 5 mM- CuCl_2 and 1 mg of BSA/ml stimulated GS activity at pH 3.6, BSA did not further enhance stimulation by CuCl_2 at pH 3.6, nor did it have any influence on CuCl_2 -induced inhibition of activity at pH 4.8. Similarly, BSA had no effect on GS activity determined in the presence of 315 mM-NaCl at pH 4.8, whereas GS activity at pH 3.3 in the presence of BSA was completely inhibited by NaCl. The addition of 1 mg of BSA/ml to the incubation mixture did not significantly affect the respective apparent K_m values for skin fibroblast GS activity toward GSMS at pH 3.3 or at pH 4.8, but increased the corresponding apparent V_{max} values by up to 2.5-fold for activity at both pH 3.3 and 4.8 (Table 2).

Detection of GS activity in skin fibroblast homogenates from normal controls and from MPS patients

Skin fibroblast homogenates were assayed for GS activity toward 4 μM -GSMS in the presence of the β -D-glucuronidase inhibitor, D-saccharic acid 1,4-lactone, to simplify the detection of only one product (GMS) (Fig. 1). β -D-Glucuronidase activity toward 4 μM -GMS was completely inhibited by the addition of 50 μM -D-saccharic acid 1,4-lactone over the pH range 2.4–5.7, but the total GS activity toward GSMS was unaffected. D-Saccharic acid 1,4-lactone at a concentration of 1 mM inhibited GS activity at pH 3.0 by 32%. Lineweaver-Burk plots indicated that the β -D-glucuronidase inhibitor at a concentration of 50 μM

Fig. 6. Hydrolysis of 4 μM -GSMS by a normal fibroblast homogenate as a function of BSA concentration and incubation pH

Fibroblast homogenate was incubated with 20 mM-sodium formate (pH 2.4–4.5) and 20 mM-sodium acetate buffer (pH 4.2–5.7) containing 0.0 (●), 0.02 (○), 0.1 (▲), 0.5 (△), 1.0 (■), 3.0 (□) and 6.0 (◆) mg of BSA/ml.

Fig. 7. Hydrolysis of 4 μM -GSMS by fibroblast homogenates from normal controls (lane 1) and from MPS and other patients with lysosomal storage disorders (lane 2)

The lysosomal storage disorders were I-cell (ML-III), I-cell (ML-II), multiple sulphatase deficiency (MSD) and Sandhoff (S). Homogenates were incubated in (a) 20 mM-sodium formate buffer, pH 3.0, and (b) 20 mM-sodium formate buffer, pH 3.3, containing 1 mg of BSA/ml.

had no significant effect on either the apparent K_m or V_{max} values of GS activity toward GSMS, either in the presence (at pH 3.3) or absence (at pH 3.0) of 1 mg of BSA/ml (results not shown).

Fig. 7 shows that GS activity in normal control skin fibroblast homogenates at pH 3.0 in the absence of BSA was between 9.5

and 22.7 pmol of product/min per mg of homogenate protein. GS activities in fibroblasts from patients with lysosomal storage diseases lay within the normal range with the exception of a mucopolidosis II (I-cell), two clinically severe MPS I (α -L-iduronidase-deficient), an MPS VII (β -D-glucuronidase-deficient) and a Sandhoff (β -N-acetylhexosaminidase-deficient) patient each of whom had GS activities significantly below the normal range. GS activities in fibroblasts from a multiple sulphatase deficiency and from an MPS II (iduronate-2-sulphatase-deficient) patient lay just below the normal range. At pH 3.3 in the presence of 1 mg of BSA/ml, GS activity for the normal controls and most other patients with lysosomal storage disorders was stimulated by up to 6-fold to increase the range of GS activities from between 9.5 and 22.7 to between 17.6 and 129.7 pmol of product/min per mg of protein. GS activities present in the skin fibroblast homogenates from the patients with MPS I, MPS II, MPS VII, Sandhoff and multiple sulphatase deficiency were increased by up to 10-fold to lie just within the normal range, but fibroblast GS activity in the presence of BSA from the mucopolidosis II patients, whilst increased, was well below the normal range.

GS activity toward GSTS

Skin fibroblast sulphatase activity toward the chondroitin 6-sulphate-derived disaccharide substrate GSTS was 19.6 pmol/min per mg of protein at the pH optimum of 2.7 assayed in 20 mM-sodium formate buffer. The same skin fibroblast homogenate had activity toward GSMS (at pH 3.0) of 22.1 pmol/min per mg of protein. GS activity toward GSTS was stimulated 1.8-fold by the addition of 1 mg of BSA/ml (pH optimum 3.0) and 1.7-fold by the addition of 5 mM-CuCl₂ (pH optimum 3.3).

DISCUSSION

We have evaluated the heparin-derived disaccharide substrate GSMS for detection of GS activity in cultured skin fibroblast homogenates. It is important for diagnostic purposes to use substrates that match as closely as possible the structures of the substrates *in vivo* to minimize errors in diagnosis which have been reported for other lysosomal enzyme activities [13]. GlcA2S residues have only been shown to occur in highly sulphated regions of heparin and heparan sulphate (adjacent to glucosamine N-sulphate and glucosamine N-6-disulphate residues) [4,14] and chondroitin 6-sulphate (adjacent to N-acetylgalactosamine 6-sulphate residues) [7]. Studies of the interaction of other heparan sulphate-degrading enzymes towards their substrates have shown that aglycone structure has a considerable influence on both the binding of the substrate to the enzyme and its catalytic turnover [2,15–19]. A detailed study of the effect of aglycone structure on the catalytic properties of purified or tissue homogenate GS activity has not been reported. It has been shown that α -L-iduronidase, iduronate-2-sulphatase and β -D-glucuronidase each require the presence of a C-6 carboxy group for activity towards heparin-derived substrates, and that disaccharide substrates containing an aglycone C-6 sulphate ester are acted on with increased catalytic efficiency [2,15,18,19]. Therefore it is expected that GSMS, which contains these aglycone structures, would be a most efficient substrate for the detection of GS activity.

The optimization of the assay conditions to detect GS activity present in cultured skin fibroblast homogenates has shown that a complex relationship exists between pH, buffer composition, ionic strength, and the influence of added BSA and salts to achieve maximal GS activity. Skin fibroblast GS activity at pH 3.0 has been shown to have similar kinetic properties for activity toward GSMS, and to be stimulated by BSA, CuCl₂ and ZnCl₂, and inhibited by NaCl and Na₂SO₄, as was reported for

the purified human liver enzyme [6]. Studies of the effect of additives on skin fibroblast homogenate and purified lysosomal sulphatase activities are generally determined at the enzyme's pH optimum towards a particular substrate. However, in the present study the influence of each of the additives was shown to be pH-dependent and their presence can dramatically alter the apparent pH optimum. Purified bovine testicular hyaluronidase activity was shown to be stimulated across the pH-activity profile by the addition of albumin or NaCl and by increased buffer ion concentration with a resultant shift in the pH optimum to a more acidic value [20,21]. Skin fibroblast N-acetylgalactosamine-6-sulphatase activity has been reported to be stimulated by up to 3-fold by the addition of albumin [22]. The mechanism of stimulation by BSA is unknown but may involve ionic interactions since acetylated BSA did not stimulate skin fibroblast GS activity. GS activities determined in the presence of 315 mM-NaCl at pH 3.3 and pH 4.8 similarly were not influenced by the addition of BSA. It is not known whether albumin, CuCl₂ or ZnCl₂ exert their effects directly on the enzyme and/or the substrate GSMS. Both Cu²⁺ and Zn²⁺ were shown to bind strongly to heparin, particularly from pH 3.0 to 3.5 [23], while purified human liver GS was observed to bind tightly to a Cu²⁺-chelating Sepharose 6B column (C. Freedman, unpublished work). Purified human liver α -L-iduronidase, which was inhibited by CuCl₂ but not by ZnCl₂, was shown to have distinct Cu²⁺- and substrate-binding sites [18]. EDTA was shown to inhibit both human skin fibroblast homogenate and purified human liver GS activity, which further suggests a requirement for an endogenous cation cofactor. Since both Cu²⁺ and Zn²⁺ stimulate GS activity at pH 3.3, while at pH 4.8 they have opposite effects, there exists the possibility of a mechanism for the regulation of enzyme activity. Alternatively, there may be two or more forms of GS activity which differ in their cationic requirements.

Glaser & Conrad [24,25] demonstrated that partially purified chick-embryo liver and highly purified rat preputial-gland β -D-glucuronidase exhibited multiple kinetic forms which existed in an equilibrium and could be shifted by varying the assay conditions. A low- K_m form, which was stabilized by BSA, predominated at pH 3.0, and a high- K_m form, stimulated by NaCl, predominated at pH 5.5. At intermediate pH values, both forms were present. Addition of 200 mM-NaCl shifted the equilibrium toward the high- K_m form with inhibition of activity at pH 3.0 [25]. The observed shift in the apparent pH optima for GS activity towards GSMS from 3.0 (a low- K_m form, stimulated by albumin) to 4.8 (a high- K_m form, stimulated by NaCl) in the presence of 315 mM-NaCl is consistent with this proposal. Human liver GS activity was shown to have a close relationship with β -D-glucuronidase, including copurification over a number of chromatographic steps [6]. Whereas purified human liver GS activity was shown to exist in a form with an apparent pI value greater than 7.5, skin fibroblast GS activity was shown to be composed of this form and at least four other lower-pI forms [6]. The possibility therefore exists that some of these lower-pI forms may have different physical and catalytic properties from the purified liver GS activity.

For the detection of GS activity toward GSMS in cultured skin fibroblast homogenates, it is recommended the assay be performed in the presence of BSA since skin fibroblast GS activities from MPS I, MPS VII and Sandhoff patients were increased to be within the normal range by the addition of BSA. To date, no patients have been described with a deficiency in GS activity. The relative amounts of GlcA2S residues in heparin, heparan sulphate and chondroitin 6-sulphate are very low; therefore it is not known if standard urinary screens for stored glycosaminoglycans [10] would be sensitive enough to detect a GS-deficient patient. The expected low level of stored material in

a GS-deficient patient may be reflected in the patient having a very mild clinical phenotype which may not present as a classical-lysosomal-storage-disorder phenotype. Surprisingly, despite the relative difference in the amount of endogenous substrate, skin fibroblast homogenate GS activity toward GSMS was similar in substrate turnover reported for iduronate-2-sulphatase activity toward ISMS (69–38 pmol/min per mg) [26]. The influence of pH, BSA, NaCl, CuCl₂, ZnCl₂ and Na₂SO₄ on GS activity toward GSMS and GSTS may allow a characterization of the enzyme defect when a GS-deficient patient is detected.

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