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Impaired degradation of keratan sulphate by Morquio A fibroblasts

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Upon incubation of keratan [^{35}S]sulphate with normal fibroblasts both [^{35}S]sulphate and *N*-acetylglucosamine 6-[^{35}S]sulphate are liberated. From the products obtained after digestion with various mutant fibroblasts and with purified *N*-acetylgalactosamine 6-sulphate sulphatase we suggest that (i) [^{35}S]sulphate is released almost exclusively from galactose 6-sulphate residues; (ii) *N*-acetylgalactosamine 6-sulphate sulphatase exhibits galactose 6-sulphate sulphatase activity; (iii) both sulphatase activities are deficient in Morquio disease type A.

Morquio disease type A (mucopolysaccharidosis IVA; McKusick *et al.*, 1978) is characterized by an intralysosomal storage of chondroitin 6-sulphate and keratan sulphate in cartilage (Pedrini *et al.*, 1962) and visceral organs (Minami *et al.*, 1979) and by an excessive excretion of these glycosaminoglycans in urine. Faulty degradation of chondroitin 6-sulphate is explained by the inactivity of an *N*-acetylgalactosamine 6-sulphate sulphatase (EC 3.1.6.4; Matalon *et al.*, 1974; Singh *et al.*, 1976; DiFerrante *et al.*, 1978; Horwitz & Dorfman, 1978). Since keratan sulphate consists of repeating *N*-acetyllactosamine units which may carry a sulphate ester on the C-6 position of both monosaccharide constituents, it had been suggested that *N*-acetylgalactosamine 6-sulphate sulphatase acts in the degradation of keratan sulphate as galactose 6-sulphate sulphatase. However, this proposal could not be verified due to the lack of appropriate substrates of galactose 6-sulphate sulphatase (Glössl *et al.*, 1979; DiFerrante, 1980).

We observed recently that *N*-acetylglucosamine 6-sulphate residues of keratan sulphate-derived oligosaccharides may be removed *in vitro* as sulphated monosaccharides by β-*N*-acetylhexosaminidase A (Kresse *et al.*, 1981), thus bypassing the action of an *N*-acetylglucosamine 6-sulphate sulphatase (Basner *et al.*, 1979). The present paper provides evidence that, during the degradation of polymeric keratan [^{35}S]sulphate, inorganic sulphate is derived from the hydrolysis of galactose 6-sulphate residues and that *N*-acetylgalactosamine 6-sulphate sulphatase co-chromatographs with galactose 6-sulphate sulphatase activity during a 5500-fold purification. Both activities are absent in Morquio disease type A. In a preliminary communication, Yutaka *et al.* (1981) also described deficient galactose 6-sulphate sulphatase activity of Morquio A fibroblasts by using as substrate a keratan sulphate-derived trisaccharide with a galactose 6-sulphate residue at the non-reducing end.

Experimental

Materials

$Na_2{}^{35}SO_4$ (carrier-free) was purchased from Amersham–Buchler (Braunschweig, Germany). Sepharose 4B coupled with 2-acetamido-ε-aminocaproyl-2-deoxy-β-D-glucopyranoside was kindly provided by Dr. A. Hasilik of this Institute. *N*-Acetylglucosamine 6-sulphate (Kresse *et al.*, 1981). *O*-β-D-6-sulpho-2-acetamido-2-deoxyglucosyl-(1–3)-D-[1-^{3}H]galactitol (Kresse *et al.*, 1980) and other materials (Ludolph *et al.*, 1981) were obtained as described.

Keratan [^{35}S]sulphate (0.13μCi/mg) from bovine cornea was prepared by a procedure analogous to that described for the preparation of [^{3}H]glucosamine-labelled keratan sulphate (Ludolph *et al.*, 1981). [^{3}H]Glucosamine was replaced by $Na_2{}^{35}SO_4$ (10μCi/ml). After treatment with *N*-acetylneuraminidase the material had the following composition: hexosamine, 1.47μmol/mg; galactose, 1.48μmol/mg; sulphate, 1.14μmol/mg; hexuronic acid, not detectable. By using a calibrated Sephadex G-100 column an average M_r of 10000 was estimated.

Assay of keratan [^{35}S]sulphate degrading activity

The incubation mixture contained 175μg of keratan [^{35}S]sulphate (about 25000c.p.m.), 0.02M-sodium acetate buffer, pH4.6, 2mM-mercapto-

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ethanol, 0.3 g of bovine serum albumin/litre and up to 20 μl of a suitably diluted enzyme solution in a final volume of 60 μl. After incubation for 0.5–1 h (purified enzyme), 3–10 h (normal fibroblasts) and up to 20 h (Morquio A fibroblasts) at 37 °C the reaction was stopped by boiling. After concentration to about 20 μl the mixture was spotted on Schleicher and Schüll paper no. 2043 b, and descending chromatography was performed in butan-2-ol/1 M-NH_3/acetic acid (2:1:3, by vol). The paper was cut into 1 cm segments, which were placed in scintillation vials and eluted with 2.0 ml of water before the addition of 4 ml of Instagel (Packard, Frankfurt, Germany). Blanks contained less than 10 c.p.m. of material behaving as inorganic sulphate or as sulphated *N*-acetylglucosamine. Release of inorganic sulphate was linear with time and proportional to the amount of enzyme provided that not more than 0.6% (150 c.p.m.) of the total radioactivity was liberated.

Purification of galactose 6-sulphate sulphatase and N-acetylgalactosamine 6-sulphate sulphatase

N-Acetylgalactosamine 6-sulphate sulphatase and galactose 6-sulphate sulphatase activities were purified from human liver according to the method described for human placenta (Glössl *et al.*, 1979). To remove all contaminant *β*-*N*-acetylhexosaminidase activity a further purification step using Sepharose 4B coupled with 2-acetamido-ε-aminocaproyl-2-deoxy-*β*-D-glucopyranoside (Hasilik & Neufeld, 1980) was included. Keratan [^{35}S]sulphate and a reduced trisaccharide, *N*-acetylgalactosamine 6-sulphate–glucuronic acid–*N*-acetyl-[1-^{3}H]-galactosaminitol 6-sulphate, prepared from chondroitin sulphate (Glössl & Kresse, 1978), were used as substrates.

Other methods

Activity measurement of *N*-acetylgalactosamine 6-sulphate sulphatase (Glössl *et al.*, 1981) and analyses of *N*-acetylglucosamine (Reissig *et al.*, 1955), of other glucosaminoglycan constituents (Ludolph *et al.*, 1981) and of cell protein (Kaltwasser *et al.*, 1967) were performed as quoted earlier.

Skin fibroblasts were maintained in culture (Cantz *et al.*, 1972) and harvested (Glössl & Kresse, 1978) as described. After ultrasonication and centrifugation at 10000 ***g*** for 5 min the supernatant served as enzyme source.

Results and discussion

Degradation of keratan [^{35}S]sulphate by fibroblast homogenates

Incubation of keratan [^{35}S]sulphate with normal fibroblast homogenates resulted in the liberation of two radioactive products upon separation by paper chromatography. One peak ($R_F = 0.23$) behaved chromatographically and on high voltage electrophoresis at pH 1.7 (Glössl *et al.*, 1979) as inorganic sulphate. To identify the second peak which had, with $R_F = 0.40$, the same mobility as *N*-acetylglucosamine 6-sulphate, the following procedure was employed: 2 mg of keratan [^{35}S]sulphate was incubated with a normal fibroblast homogenate for 38 h at 37 °C. After concentration the reaction mixture was separated by paper chromatography. Radioactive bands were cut out, eluted with water, concentrated and analysed for Morgan–Elson-positive material. In the second peak ($R_F = 0.40$) 36 nmol of Morgan–Elson-positive material (expressed as *N*-acetylglucosamine) with a sp. radioactivity of 0.19 Ci/mol were found. Taking into account the chemical structure of keratan sulphate, this product should be *N*-acetylglucosamine 6-sulphate.

Keratan sulphate-degrading activity was measured in fibroblast homogenates from patients with various enzyme deficiencies (Table 1). Cell lines from patients with *N*-acetylgalactosamine 6-sulphate sulphatase deficiency (Morquio disease type A and mucosulphatidosis) did not release significant amounts of inorganic sulphate, whereas cell lines with *N*-acetylglucosamine 6-sulphate sulphatase deficiency (Sanfilippo disease type D) and with deficiencies of other enzymes involved in the degradation of keratan sulphate exhibited sulphatase activity. The ability to liberate *N*-acetylglucosamine 6-sulphate was drastically diminished in fibroblast homogenates from a patient with *β*-*N*-acetylhexosaminidase deficiency (Sandhoff disease) and lowered in *β*-galactosidase-deficient cells (G_{M1} gangliosidosis). These data suggest that in-

Table 1. *Degradation of keratan [^{35}S]sulphate by fibroblast homogenates*
Abbreviation: n.d., not determined.

	Liberation (% of total radioactivity/h per mg of cell protein) of:	
Enzyme source	SO_4	*N*-acetylglucosamine 6-sulphate
Morquio disease type A		
cell line 1	0.02	n.d.
cell line 2	0.01	5.3
cell line 3	0.01	5.5
cell line 4	0.02	6.0
Mucosulphatidosis	<0.01	11.5
G_{M1} gangliosidosis	3.0	2.7
Sandhoff disease	3.4	0.8
Sanfilippo disease type D	3.2	7.5
Normal	4.3	10.2

organic sulphate is released predominantly from galactose 6-sulphate residues and that *N*-acetylglucosamine 6-sulphate is liberated by *β-N*-acetylhexosaminidase A not only from keratan sulphate-derived oligosaccharides (Kresse *et al.*, 1981) but also from the polymer. If at all, *N*-acetylglucosamine 6-sulphate sulphatase appears to play only a minor role in the degradation of polymeric keratan sulphate *in vitro*. Its function in desulphation of the monosaccharide remains to be investigated.

Identity of galactose 6-sulphate sulphatase and N-acetylgalactosamine 6-sulphate sulphatase

To prove the suggested identity of the keratan sulphate degrading galactose 6-sulphate sulphatase and of the chondroitin 6-sulphate-degrading *N*-acetylgalactosamine 6-sulphate sulphatase the activities against both keratan sulphate and a trisaccharide substrate from chondroitin sulphate were measured throughout a purification procedure. The activities against both substrates co-chromatographed in each of the purification steps (results not shown) and the ratio of activities did not alter significantly (Table 2). *N*-Acetylgalactosamine 6-sulphate sulphatase activity and keratan sulphate sulphatase activity were purified about 5500-fold and 4800-fold, respectively. The slight loss of keratan sulphate sulphatase activity compared with *N*-acetylgalactosamine 6-sulphate sulphatase activity during the purification procedure might be due to the removal of *N*-acetylglucosamine 6-sulphate sulphatase. The final preparation was inactive against the keratan sulphate-derived disaccharide *N*-acetylglucosamine 6-sulphate–[1-^{3}H]galactitol, thus being devoid of *N*-acetylglucosamine 6-sulphate sulphatase and of *β-N*-acetylhexosaminidase A.

Properties

Release of inorganic sulphate by G_{M1} gangliosidosis fibroblasts (Fig. 1) and by purified *N*-acetylgalactosamine 6-sulphate sulphatase (results not shown) was optimal at pH 4.6, whereas a chondroitin 6-sulphate-derived trisaccharide is optimally desulphated at pH 4.0 (Glössl *et al.*, 1981). When homogenates of G_{M1} gangliosidosis fibroblasts were incubated with 0.3–1.47 mmol of keratan [^{35}S]sulphate/litre the plot $1/v$ against $1/[S]$ showed a straight-line relationship. An apparent Michaelis constant of 2 mM was found.

On the basis of our results it can be concluded that galactose 6-sulphate sulphatase and *N*-acetylgalactosamine 6-sulphate sulphatase activities reside in the same enzyme protein and that both activities are deficient in Morquio disease type A. Impaired degradation of keratan sulphate in Morquio disease

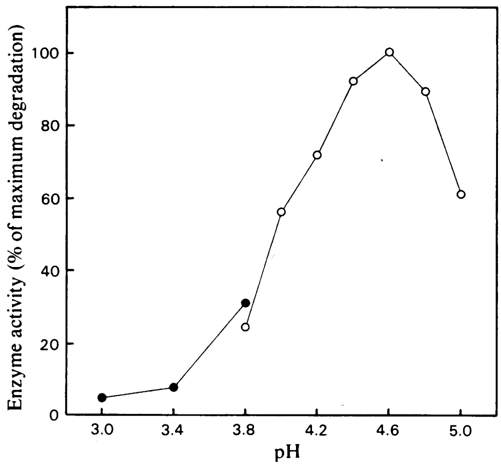

Fig. 1. *Effect of pH on keratan sulphate sulphatase activity from G_{M1} gangliosidosis fibroblasts*
The assays were performed in 20 mM-sodium formate buffer (●) and 20 mM-sodium acetate buffer (○), both buffers containing 2 mM-mercaptoethanol and 0.3 g of bovine serum albumin/litre. The maximum activity was taken as 100%.

Table 2. *Ratio of N-acetylgalactosamine 6-sulphate sulphatase (GalNAc 6-S sulphatase) and keratan sulphate sulphatase (KS sulphatase) activity during purification from human liver according to Glössl et al. (1979)*
GalNAc 6-S sulphatase and KS sulphatase activities are expressed as nmol/h per A_{280} and %/h per A_{280}, respectively. Abbreviation: n.d., not determined.

Purification step	Specific activity of GalNAc 6-S sulphatase (μmol/h per A_{280})	GalNac 6-S sulphatase / KS sulphatase
Crude enzyme solution	0.006	n.d.
Concanavalin A–Sepharose	0.36	6.9
DEAE-cellulose	1.1	7.1
Sephacryl S-200	4.6	7.2
Polyacrylamide-gel electrophoresis	31.9	7.9
β-N-Acetylhexosaminidase affinity column	33.4	7.9

type A is therefore a primary consequence of the enzyme defect. The observation of a patient with 'non-keratan-sulphate excreting Morquio syndrome' (Fujimoto & Horwitz, 1981) may be explained by a mutation affecting the activities towards chondroitin 6-sulphate and keratan sulphate to a different extent.

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