HUNTER'S SYNDROME: A DEFICIENCY OF L-IDURONO-SULFATE SULFATASE

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Received August 22, 1973

SUMMARY

[$^{35}SO_4$]Dermatan sulfate, isolated from normal Hurler and Hunter fibroblasts was degraded by chondroitinase A, B, C to yield mono-and disaccharides. The products were separated by ion exchange chromatography and those arising from the non-reducing terminus were characterized by paper electrophoresis. The position of sulfate substituents was established by periodate oxidation and partial acid hydrolysis. Normal dermatan sulfate terminates with GalN-SO_4 whereas IdUA-SO_4 was a prominent terminus in Hunter dermatan sulfate but not in Hurler dermatan sulfate. It is concluded that Hunter's syndrome is due to a deficiency of L-idurono-sulfate sulfatase.

INTRODUCTION

Previous studies have defined the enzymic defects in several mucopolysaccharidoses including the Hurler (1-3), Scheie (3), Sanfilippo A (4, 5) and Sanfilippo B syndromes (6, 7) and a deficiency of β-glucuronidase (8). Like Hurler's syndrome, Hunter's syndrome is characterized by excretion of dermatan sulfate and heparan sulfate (9). These two glycosaminoglycans share α-L-iduronide and L-idurono-sulfate linkages (10, 11). Since α-L-iduronidase activity is present in fibroblasts of patients with the Hunter syndrome (2), it seemed possible that a deficiency of a sulfatase which specifically cleaves the ester sulfate of iduronic acid might be responsible for the defect in Hunter's syndrome. Evidence for such a deficiency has been obtained by a study of the non-reducing termini of dermatan sulfate accumulated in fibroblasts derived from a patient with Hunter's syndrome. While this work was in progress, similar

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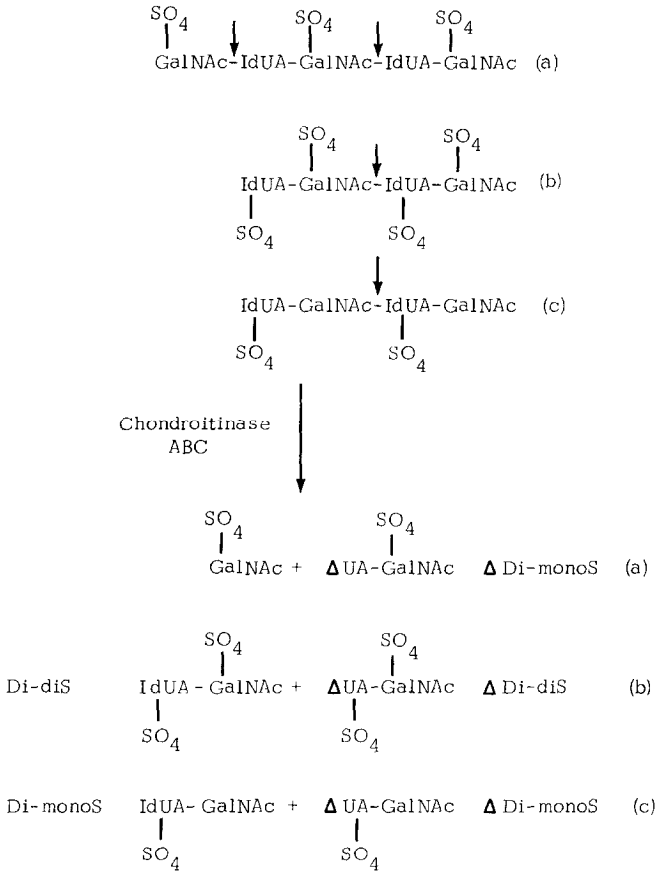

Fig. 1. The sites of action of chondroitinase ABC on dermatan sulfate are illustrated. The products formed from the non-reducing ends of the molecule are shown in lower left while those from interior portion of the molecule are in the lower right. The unsaturated disaccharides are indicated as Δ UA since the same product is formed from L-iduronic acid and D-glucuronic acid.

conclusions have been reached by Bach et al (12) and Coppa et al (13).

MATERIALS

Enzymes. Crystalline papain was isolated from a crude preparation as described by Kimmel and Smith (14). DNAse and RNAse from bovine pancreas as well as a bacterial protease (subtilisin) were purchases from Sigma Chemical. Chondroitinase-ABC from Proteus vulgaris (15) was a product of Seikagaku Fine Biochemicals, Tokyo, Japan, and purchased from Miles Laboratories, Elkhart, Indiana.

Carbohydrates. Oversulfated chondroitin sulfate (type CS-D) from shark cartilage was obtained from the Seikagaku Kogyo Co., Tokyo, Japan. N-Acetyl-

galactosamine (GalNAc) was purchased from Calbiochem. Chondroitin 4-sulfate N-acetylchondrosine (Di-OS), saturated monosulfated disaccharide (Di-monoS), 4, 5-unsaturated monosulfated disaccharide (Δ Di-monoS) and N-acetyl-galactosamine 4-or 6-sulfate (GalNAc-S) were the same preparations as described previously (16). Unsulfated, 4, 5-unsaturated disaccharide (Δ Di-OS) and disulfated, 4, 5-unsaturated disaccharide (Δ Di-diS) were obtained after chondroitinase-ABC digestion (15) of chemically desulfated chondroitin sulfate (17) and oversulfated chondroitin sulfate (11) respectively.

Miscellaneous. Omnifluor was obtained from New England Nuclear and Instagel from Packard Instruments.

METHODS

Tissue culture. Skin fibroblasts from normal, Hunter patients and Hurler patients were grown as previously described (18). Fibroblasts used in these experiments had undergone 4-6 trypsinizations. Cells were grown in 100mm Falcon plastic plates until they reached confluency (1.6 x 10^7 cells/plate) and then 40 plates were incubated for 4 days with $SO_4^=$-free medium to which 150μc $^{35}SO_4$ were added per plate. The medium was removed and cells were washed three times with balanced salt solution, removed from the plates by scraping with a rubber policeman, and lyophilized.

Preparation and degradation of glycosaminoglycans from fibroblasts. The dry residue of acetone extracted (9 vols.) fibroblasts was suspended in 20ml of 0.10 M NaCl-0.015 M Na_2EDTA-0.01 M cysteine hydrochloride-0.05 M phosphate buffer, pH 6.9. After digestion with 2.25mg of twice crystallized papain at 65° overnight, the mixture was dialyzed against 3 x liter of distilled H_2O. The retentate was lyophilized and dissolved in 20ml of 0.05 M $CaCl_2$-0.05M, Tris-acetate, pH 7.5 and digested overnight with 1 mg of subtilisin at 37°. The solution was concentrated on a rotary evaporator and desalted on a column (1.1 x 200 cm) of Sephadex G-50, superfine, eluted with 0.02 M pyridine acetate, pH 5.0 at a rate of 12 ml/h. The excluded material was recovered, lyophilized and dissolved in 5 ml of 0.05 M $MgSO_4$-0.05 M phosphate buffer, pH 7.3. Digestion overnight was carried out with RNAse and DNAse (0.5 mg each) at 37°. Glycosaminoglycans and oligonucleotides were separated by gel chromatography on Sephadex G-50 as described above. The excluded material was recovered, concentrated and dissolved in 1 ml of 0.5 M Tris, pH 8.0. The dermatan sulfate was degraded with chondroitinase-

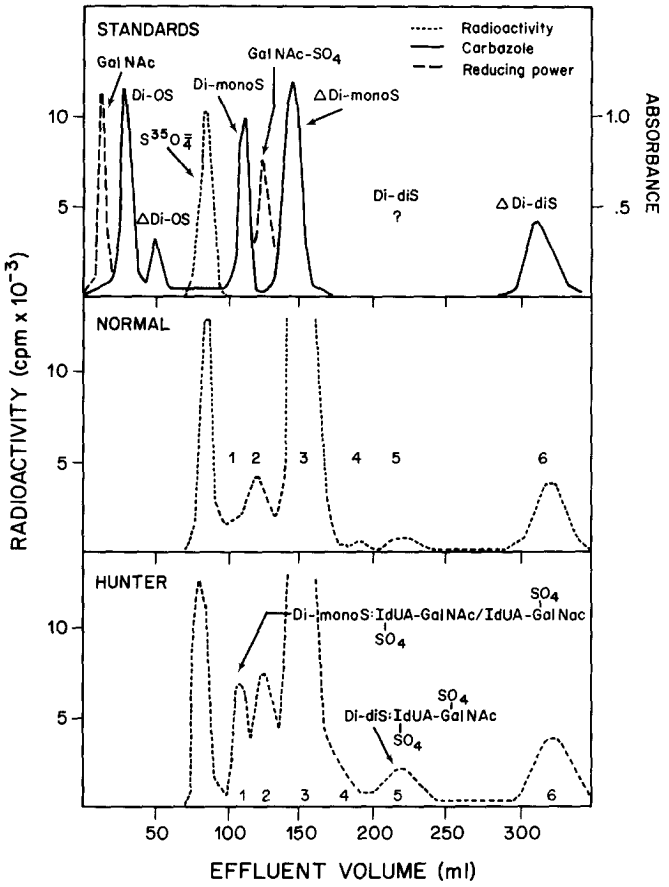

Fig. 2. Ion exchange chromatography of various mono- and disaccharides. A chromatogram of standard compounds is shown in the upper section. The position of Di-diS may be deduced from the positions of Δ Di-OS, Di-monoS and Δ Di-diS in relation to those of Di-OS and Di-monoS. Chromatograms of chondroitinase-ABC digests of radioactively labelled dermatan sulfate from normal and Hunter cells are shown in the middle and lower graphs, respectively. The samples were partially desulfated on standing in concentrated solutions, presumably due to radiolysis. The amount of free sulfate was approximately 10% of the total radioactivity. The various radioactive components were desalted on Sephadex G-10, lyophilized, dissolved in 70% (v/v) ethanol, and stored in the deep-freezer.

ABC (0.5 units) at 37° for 24 h. The split products were separated from undegraded polysaccharides (primarily heparan sulfate) by gel chromatography on Sephadex G-25. All of the radioactivity in the included fraction was recovered and subjected to ion exchange chromatography as described below.

Chromatographic and electrophoretic techniques. The non-reducing terminal sugar moiety of dermatan sulfate was characterized as follows.

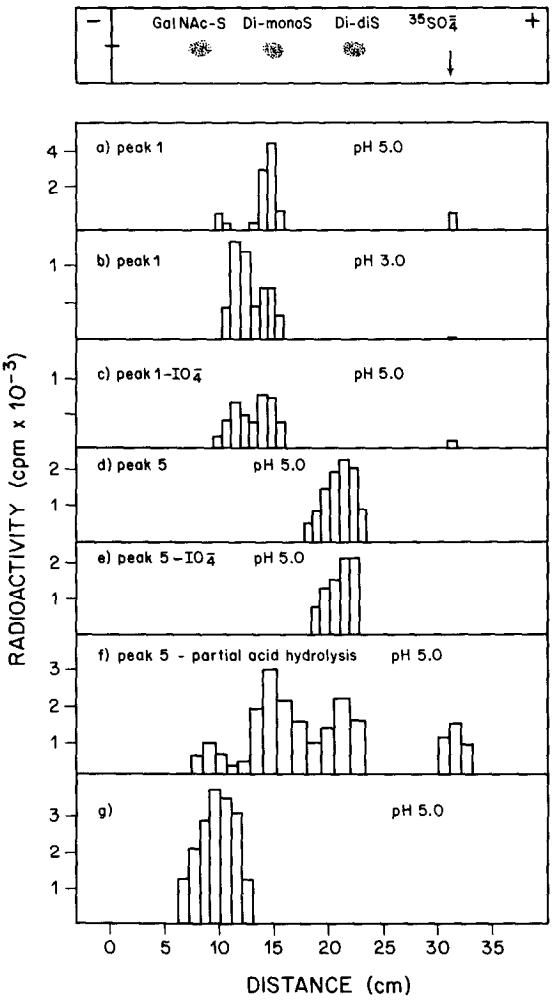

Fig. 3. Paper electrophoresis of various non-reducing terminal disaccharides at pH 3.0 or 5.0. In the top chromatogram the mobilities of various standards are depicted. It should be noted that saturated and unsaturated disaccharides have the same mobility in this system ($M_{Di\text{-}monoS} = M_{\Delta\ Di\text{-}monoS}$; $M_{Di\text{-}DiS} = M_{\Delta\ Di\text{-}diS}$). Oxidations were performed in 0.1 ml of 0.05M acetate buffer, pH 4.5 containing $NaIO_4$ (0.04M) at 37°C for 1 h. After terminating the reaction by addition of 0.2 ml of 3% (w/v) mannitol the solution was concentrated and spotted on paper. Partial acid hydrolysis was carried out in 0.5 ml of 0.04 M HCl at 100°C for 1 h. After evaporation the samples were spotted on paper. In one case (Fig. 3b) the electrophoresis was performed at pH 3.0. In this system the fast-moving component has the mobility of a chondroitin 6-sulfate disaccharide (GlcUA-GalNAc-6-SO_4). Furthermore, the slow-moving component is contaminated with GalNAc-SO_4 which has the same mobility under these conditions. The paper electrophoretograms were cut and the strips were counted for radioactivity in Omnifluor/toluene.

After exhaustive treatment with chondroitinase-ABC (15), the split products were resolved by ion exchange chromatography on a column (1.2 x 40 cm) of AG 1-X8 (Cl^- form) and eluted with a linear LiCl gradient (0-3.0 M) as previously described (18). High voltage paper electrophoresis was carried out in buffer A, 0.1 M pyridine acetate, pH 5.0 and B, 1.6 M pyridine acetate, pH 3.0 (16).

Analytical methods. Uronic acid was determined by the carbazole method (20). Reducing power was estimated by the method of Park and Johnson (21). Radioactivity was measured in a TriCarb liquid scintillation spectrometer using either Omnifluor in Toluene (4 g/l) or Instagel (4 ml of sample and 5 ml of gel) as scintillators.

RESULTS

The non-reducing terminal portion of dermatan sulfate is released by digestion with chondroitinase-ABC (Fig. 1). When GalNAc-SO_4 is terminal, this monosaccharide is liberated, whereas terminal uronosyl moieties are recovered in the form of various saturated disaccharides. As illustrated in Fig. 2 these mono- and disaccharides can be resolved by ion exchange chromatography. The chromatogram of standards (upper graph) shows that the various compounds are separated primarily according to charge density. In addition, disaccharides containing Δ 4,5-unsaturated uronosyl moieties are eluted later than their saturated counterparts (19). The principal non-reducing sugar moiety of normal dermatan sulfate was GalNAc-SO_4 (Fig. 2, middle graph), whereas dermatan sulfate from Hunter fibroblasts yielded, in addition, two non-reducing terminal disaccharide peaks (1 and 5 in lower graph of Fig. 2). These disaccharides, which were eluted in the positions of Di-monoS and Di-diS, respectively, were absent in a chondroitinase-ABC digest of Hurler dermatan sulfate. The charge densities of these disaccharides were confirmed by paper electrophoresis at pH 5.0 (Fig. 3a and d). However, at pH 3.0, peak 1 was resolved into two electrophoretically distinct components (Fig. 3b). Furthermore, periodate oxidation of peak 1 yielded two components, one migrating slower than the starting material (Fig. 3a and c). In contrast, the electrophoretic mobility of peak 5 (Di-diS) was unaffected by periodate oxidation (Fig. 3d and e). The latter disaccharide was subjected to partial acid hydrolysis which yielded GalNAc-SO_4 and Di-monoS in addition to unhydrolyzed material (Fig. 3f). The Di-monoS fraction obtained after

hydrolysis (Fig. 3f, middle peak) was isolated by preparative electrophoresis and subsequently oxidized with periodate. The bulk of the oxidized material was retarded on electrophoresis (Fig. 3g). This indicates that the Di-monoS fraction was mainly composed of IdUA-GalNAc-SO_4 [1].

The above results are interpreted to indicate that peak 5 consisted of a saturated disulfated disaccharide, the structure of which is shown in Fig. 2 (lower graph). Peak 1 is thought to be a mixture of, at least, two saturated, monosulfated disaccharides. The proposed structures of these isomeric compounds [2] are also shown in Fig. 2.

DISCUSSION

If the dermatan sulfate chain is eroded stepwise from the non-reducing end by the action of sulfatases and exoglycosidases, a number of intermediates with different termini can be envisaged. An enzymic defect at any step would cause accumulation of the intermediate prior to this step. The identification of IdUA(-SO_4)-GalNAc(-SO_4)- as a terminal unit in dermatan sulfate from Hunter fibroblasts is consistent with a lack of L-idurono-sulfate sulfatase in this metabolic disorder. IdUA(-SO_4)-GalNAc- was also present as a terminal unit in Hunter dermatan sulfate. Whether this reflects the activity of a hexosaminyl-sulfate sulfatase or structural heterogeneity within the polysaccharide chain cannot be settled at present.

GalNAc-SO_4 and UA-GalNAc-SO_4 were also present in the chondroitinase-ABC digest of the Hunter polysaccharides [3]. Since the former was more prominent than the latter, it is conceivable that they represent the terminal units of newly synthesized, undegraded galactosaminoglycan. It should be added that the dermatan sulfate which accumulates within the lysosomes might inhibit glycosidases operating in earlier steps of the degradative pathway.

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[1] Non-reducing terminal GlcUA would be resistant under these conditions of periodate oxidation (22).

[2] It should be noted that the presence of the disaccharide GlcUA-GalNAc-SO_4 in peak 1 cannot be excluded. Further work is necessary to clarify this point.

[3] UA-GalNAc-SO_4 may terminate with either IdUA or GlcUA.

fatty acyl CoA (8) since the system is not sufficient to generate such a metabolite.

Although *in vitro* inactivation by ATP and Mg^{++}, in accordance with phosphorylation of the protein, can readily be demonstrated, it has not yet been possible to consistently demonstrate the reverse reaction, the activation expected in the presence of a high concentration of magnesium (up to 20 mM). The failure of high concentrations of Mg^{++} to activate the enzyme in crude preparations suggests that the protein phosphatase may become very labile when the cells are broken. The lability of the phosphatase was previously observed in the case of rat liver preparations (1). Data presented in this communication establish that acetyl CoA carboxylase activity is directly regulated by insulin and epinephrine without involving protein synthesis. Further experiment must be carried out to determine whether the hormones regulate the transformation between the phosphorylated and dephosphorylated forms of the enzyme.

ACKNOWLEDGEMENT

We wish to thank Mrs. Ai-Yu Tsai for her skillful, technical assistance.

This investigation was supported in part by the United States Public Health Service Research Grant AM 12865 from National Institute of Arthritis and Metabolic Diseases.

*Journal paper No. 5113 of Purdue Agricultural Experiment Station.

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