MAROTEAUX-LAMY DISEASE (MUCOPOLYSACCHARIDOSIS VI), SUBTYPE A: DEFICIENCY OF A N-ACETYLGALACTOSAMINE-4-SULFATASE

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SUMMARY: The non-reducing terminal moiety of $^{35}SO_4$-dermatan sulfate accumulating in fibroblasts cultured from the skin of patients with one form of Maroteaux-Lamy disease was found to be N-acetylgalactosamine-4-sulfate. This end group accounted for about 3 % of the total radioactivity. Using both $^{35}SO_4$- and ^{14}C-N-acetylgalactosamine-labeled dermatan sulfates from the patients fibroblasts as substrates, it was found that homogenates of Maroteaux-Lamy fibroblasts, but not of normal, Hurler and Sandhoff fibroblasts fail to cleave inorganic sulfate from the non-reducing termini. We conclude, that deficiency of N-acetylgalactosamine-4-sulfatase is the biochemical basis for this form of Maroteaux-Lamy disease.

Maroteaux-Lamy disease (mucopolysaccharidosis VI) is an autosomal recessive disorder of glycosaminoglycan metabolism (1,2). The patients accumulate in their tissues and excrete in their urine excessive amounts of dermatan sulfate (1,2). In fibroblasts cultured from the patients skin, an impaired catabolism of sulfated glycosaminoglycans due to lack of a specific protein factor has been demonstrated (3). Recently, it was shown that arylsulfatase B activity is markedly diminished in tissues of some Maroteaux-Lamy patients (4,5). A role for this sulfatase in the degradation of dermatan sulfate, however, has not been demonstrated. Studies in other mucopolysaccharidoses (e.g. 6-8) have shown that in cultured fibroblasts, glycosaminoglycans are degraded sequentially from the non-reducing terminal by lysosomal exoglycosidases and sulfatases.

This report demonstrates that the non-reducing terminal moiety of the dermatan sulfate accumulating in fibroblasts from patients with Maroteaux-Lamy disease is N-acetylgalactosamine-4-sulfate, and that Maroteaux-Lamy fibroblasts are severely deficient in a sulfatase for such residues.

EXPERIMENTAL: Materials: D-glucosamine-1-^{14}C·HCl and carrier free $H_2{}^{35}SO_4$ were obtained from NEN Chemicals, Dreieichenhain, Germany. Ecteola cellulose was purchased from Schleicher and Schüll, Dassel, Germany. Chondroitinase ABC, chondro-4-sulfatase, 2-acetamido-2-deoxy-3-O- (ß-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose (ΔDi-4S) and its 6-sulfate isomer (ΔDi-6S) were purchased from Miles Laboratories, Slough, England. N-acetylgalactosamine and Dowex 1X2 were from Serva, Heidelberg, Germany. N-acetylgalactosamine-4-sulfate (GalNAc-4S) was prepared by acid hydrolysis of ΔDi-4S (9). AG 1X8 resin, 100-200 mesh, was purchased from Bio-Rad, Munich, Germany.

Fibroblast lines: The maintenance of cell cultures has been previously described (10). Assignment of fibroblast lines to a particular genetic disease was based on clinical data and determinations of arylsulfatases A and B (11), -L-iduronidase (6), ß-glucuronidase, ß-galactosidase and ß-N-acetylhexosaminidase (12). Fibroblast lines from patients K. and S. with Maroteaux-Lamy disease had undetectable arylsulfatase B activity.

Preparation of labeled dermatan sulfate: ^{14}C-N-acetylgalactosamine- and $^{35}SO_4$-labeled glycosaminoglycans were prepared by adding either 125 µc of D-glucosamine-1-^{14}C·HCl or 1 mc $H_2{}^{35}SO_4$ along with 20 ml of medium to a 75 cm^2 Falcon flask of cultured fibroblasts (ca. 5 mg protein) obtained from patients with Maroteaux-Lamy disease. After incubation for 6 days, the cells were harvested by trypsinization (10), washed twice with 0.15 M NaCl and frozen and thawed 5 times in 1 ml of 0.15 M NaCl. After centrifugation, the supernatant was applied to a microcolumn of Ecteola cellulose (OH^- form) equilibrated with 0.15 M NaCl. The column was washed with 25 ml 0.15 M NaCl and the labeled glycosaminoglycans were eluted in 4 x 0.5 ml portions of 2.5 M NaCl. The pooled eluate was dialyzed overnight against 3 x 2 liters of distilled water and concentrated in a collodion bag to ca. 0.5 ml for the ^{14}C-labeled glycosaminoglycans (ca. 2 x 10^5 cpm/ml) and 2 ml for the ^{35}S-labeled glycosaminoglycans (6.8 x 10^6 cpm/ml). The ^{14}C-labeled dermatan sulfate was further purified on a column (0.6 x 5.5 cm) of Dowex 1X2 (13), dialyzed and concentrated as above. The radioactivity of the sample was 3.1 x 10^5 cpm/ml.

Chondroitinase ABC digestion: 135,000 cpm of ^{35}S-labeled and ca. 20,000 cpm of ^{14}C-labeled dermatan sulfate were digested with chondroitinase ABC, as described (14), except that NaCl was omitted from the incubation buffer.

Chromatographic and electrophoretic techniques: Descending paper chromatography was carried out for 18 hours using sheets of 50 x 11 cm of Selecta filter paper no. 2040 a (Schleicher and Schüll, Dassel, Germany) which were both prewashed and developed with the solvent system 1-butanol-acetic acid-0.1 N NH_3 (2:3:1) (7). After drying, lanes containing the standards were sprayed with the aniline phthalate reagent (E. Merck, Darmstadt, Germany), whereas lanes with labeled hydrolysis products were cut in 0.5 cm sections, each of which was counted in 0.5 ml H_2O and 10 ml of scintillation fluid (10).

Ion exchange chromatography was done using a column of AG 1X8 (0.6 x 18 cm) which was eluted with 140 ml of a linear gradient of 0 - 2.5 M LiCl, as described (8). LiCl concentration of eluted fractions was determined by conductivity measurements.

Paper electrophoresis was done essentially as described (7).

Incubations of labeled Maroteaux-Lamy dermatan sulfate with fibroblast homogenates: 20 µl (135,000 cpm) of ^{35}S-labeled or 50 µl (15,730 cpm) of ^{14}C-labeled dermatan sulfate were incubated with 100 µl of fibroblast homogenates (homogenized in water in a small Teflon-glass homogenizer and containing from 3 - 6 mg of protein/ml) and 10 µl of 1 M sodium acetate buffer, pH 4.8. After 18 hours at 37°C, the incubations were stopped by adding 100 ul of 1 M $NaHCO_3$ containing 1 mg of chondroitin sulfate (Carl Roth, Karlsruhe, Germany). After mixing, 1 ml of absolute ethanol was added, the mixture was heated to boiling, cooled, and centrifuged. The ethanol soluble radioactivity in 0.5 ml of the supernatant was determined.

RESULTS: 1. The structure of dermatan sulfate in Maroteaux-Lamy fibroblasts.

Digestion of dermatan sulfate with chondroitinase ABC gives rise to unsaturated, sulfated disaccharides (14) and releases the non-reducing terminal mono- or disaccharide. When a uronic acid is terminal, saturated disaccharides are found, whereas if sulfated N-acetylgalactosamine is terminal, it is recovered as such (8).

$^{35}SO_4$-labeled dermatan sulfate isolated from Maroteaux-Lamy fibroblasts was so digested and the resulting products identified by paper chromatography. 95 % of the total radioactivity were converted to the products shown in Fig. 1. The end group N-acetylgalactosamine-4-sulfate accounted for 2.6 % of the total radioactivity, which is roughly equal to that found by Sjöberg et al.

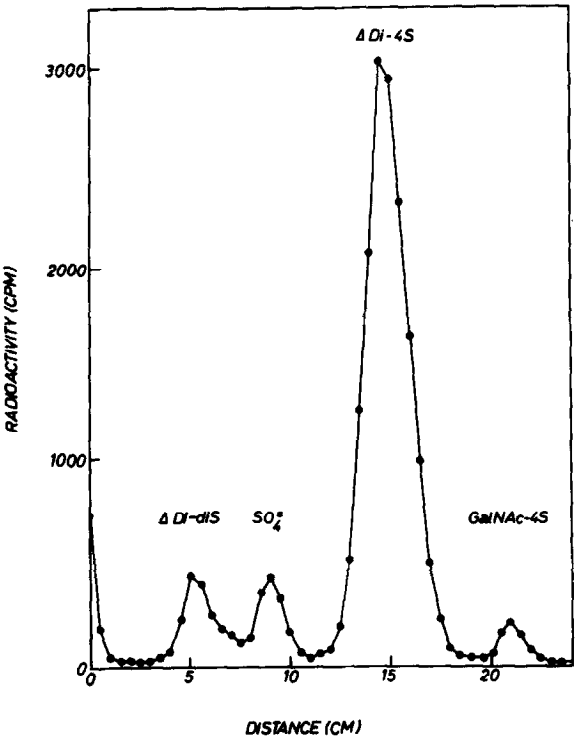

Fig. 1: Paper chromatography of the chondroitinase ABC digestion products of $^{35}SO_4$-labeled dermatan sulfate from fibroblasts of a patient with Maroteaux-Lamy disease. The identity of ΔDi-diS (2-acetamido-2-deoxy-3-O- (2 or 3 -O-sulfo-ß-D-gluco-4-enepyranosyluronic acid) -4-O-sulfo-D-galactose) was inferred from its reported mobility on chromatograms using the same solvent system (14), and its elution position when rechromatographed on the AG 1X8 column shown in Fig. 2. The proportion of $^{35}SO_4^=$ was very low initially and increased upon storage (-20°C) of the dermatan sulfate, possibly due to radiolysis (8).

(8) for the dermatan sulfate in normal fibroblasts. Since in our chromatographic system GalNAc-6S has the same mobility as ΔDi-4S, it was possible that part of the end groups consist of the moiety. This was ruled out by elution of the ΔDi-4S area and digesting it with chondro-4-sulfatase (14). Upon rechromatographing the incubation mixture, all of the radioactivity migrated with inorganic sulfate.

Chondroitinase ABC digestion of ^{14}C-N-acetylgalactosamine-labeled Maroteaux-Lamy dermatan sulfate showed about 3 % GalNAc-4S and less than 0.5 % GalNAc, confirming our above findings and ruling out GalNAc as a significant end group.

Chondroitinase ABC digestion products of $^{35}SO_4$-dermatan sulfate obtained from two Maroteaux-Lamy fibroblast lines were also chromatographed on a column of AG 1X8 resin. Both cultures showed identical patterns, one of which is presented in Fig. 2. All of the radioactivity eluted in positions corresponding to the $^{35}SO_4^=$, GalNAc-S, ΔDi-4S and ΔDi-diS standards. Since no saturated di-

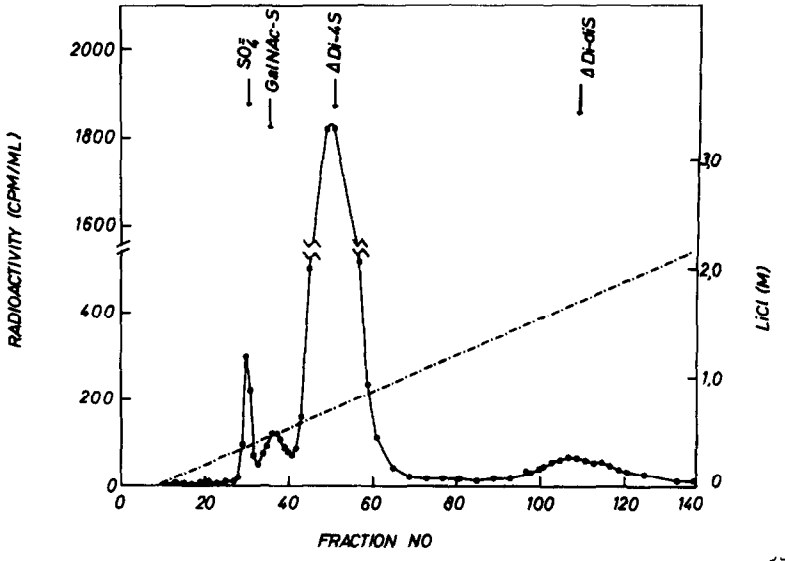

Fig. 2: Ion exchange chromatography of chondroitinase ABC degraded $^{35}SO_4$-labeled Maroteaux-Lamy dermatan sulfate. To 50 µl of the digest, containing 19,400 cpm was added 200 µl of H_2O and the sample applied to the column. Radioactive standards of ΔDi-4S and ΔDi-diS were obtained from paper chromatograms; GalNAc-4S was prepared as described in Materials. The elution positions of the standards are indicated by arrows.

saccharides could be found, GalNAc-4S is the only sulfated end group.

Further evidence for a GalNAc-4S end group was obtained by preincubating $^{35}SO_4$-dermatan sulfate with a homogenate of Sandhoff fibroblasts, which lacks a ß-hexosaminidase for N-acetylgalactosamine residues of dermatan and chondroitin sulfates (15-17). Paper chromatographic analysis of the chondroitinase ABC digestion products showed that the percentage of GalNAc-4S had been reduced from 2.6 to 0.9, with a corresponding increase in the $^{35}SO_4^=$ fraction from 7.2 to 8.7 %. No significant change in the disaccharide fractions was found. The percentage of GalNAc-4S was not changed by preincubation with Maroteaux-Lamy homogenate.

These results suggest that the dermatan sulfate accumulating in Maroteaux-Lamy fibroblasts has the following structure (IdUA=iduronic acid, some of which is sulfated):

$$\text{GalNAc-4S} - \left[\text{IdUA(S)} - \text{GalNAc-4S}\right]_n -$$

2. Deficiency of a N-acetylgalactosamine-4-sulfatase.

$^{35}SO_4$- and ^{14}C-N-acetylgalactosamine-labeled dermatan sulfates isolated from Maroteaux-Lamy fibroblasts were used as substrates in incubations with homogenates of Maroteaux-Lamy and other fibroblast lines. Table I shows that upon incubation of the $^{35}SO_4$-labeled dermatan sulfate with normal fibroblast homogenates, and with homogenates of Hurler (deficient in ∝-L-iduronidase (6)) and Sandhoff fibroblasts, there is release of ethanol-soluble radioactive fragments (5.6 % of the total radioactivity for the normal homogenate), which behave as inorganic sulfate by paper electrophoresis. Very little sulfate is released, however, when fibroblast homogenates from two Maroteaux-Lamy patients are examined. Similar experiments with ^{14}C-N-acetylgalactosamine-labeled dermatan sulfate show (Table I) that normal and Hurler fibroblast homogenates release ethanol-soluble radioactive products (8.5 % of the total radioactivity for the normal homogenate) which chromatograph as N-acetylgalactosamine on paper. Homogenates of Maroteaux-Lamy and Sandhoff fibroblasts, on the other hand, show very little cleavage of the N-acetylgalactosamine moiety.

Table I: Release of ethanol-soluble radioactivity from $^{35}SO_4$- and ^{14}C-galactosamine-labeled dermatan sulfate by fibroblast homogenates.

Fibroblast line	$^{35}SO_4^=$ released cpm/mg protein	^{14}C-N-acetylgalactosamine released cpm/mg protein
Normal	6,945	2,035
Hurler	1,824	948
Sandhoff	1,629	69
Maroteaux-Lamy		
patient K.	264	59
patient S.	192	68

Values represent the mean of duplicate determinations after subtraction of ethanol-soluble counts determined in incubations with boiled normal homogenates. Blank values were: 505 cpm/mg protein for $^{35}SO_4^=$ release and 66 cpm/mg protein for ^{14}C-N-acetylgalactosamine release.

Thus, whereas a Sandhoff homogenate cleaves sulfate from terminal GalNAc-4S but does not liberate the GalNAc moiety, Hurler homogenates cleave both before stopping at an iduronic acid residue. The sulfatase deficiency of Maroteaux-Lamy fibroblasts causes a block in the degradation of the dermatan sulfate with no release of either sulfate or hexosamine.

DISCUSSION: Our findings strongly suggest that deficiency of a N-acetylgalactosamine-4-sulfatase (EC 3.1.6.-.) required for the complete degradation of dermatan sulfate is the biochemical basis for one form of Maroteaux-Lamy disease, which we arbitrarily designate subtype A. This deficiency is coincident with a depression of arylsulfatase B activity described by Stumpf et al. (4), and with deficiency of the Maroteaux-Lamy factor in fibroblasts of patient S. (3) and patient K. (18). The identity of these three proteins, however, has not been proven. It seems possible that N-acetylgalactosamine-4-sulfatase is also specific for chondroitin-4-sulfate. A sulfatase acting on

Table 3. Effect of m.o.i. on the change of intracellular and total activity of various enzymes

Enzymes \ m.o.i. / Fractions	0		1.0		10		50		100	
	Cell	Total	Cell	Total	Cell	Total	Cell	Total	Cell	Total
α-D-Glucosidase	390	390	316	316	257	341	225	440	213	484
β-D-Glucosidase	242	243	219	219	159	159	159	159	150	150
β-D-Galactosidase	1601	1674	1325	1476	1159	1425	1044	1369	918	1332
α-D-Mannosidase	974	1042	767	990	706	903	762	997	778	1002
N-Acetyl-β-D-glucosaminidase	19125	20360	15940	17970	13980	16440	13840	16550	14130	16780
Acid phosphatase	9716	9891	9367	9671	8612	9147	8399	9132	7908	9095
Alkali phosphatase	1849	1849	1477	1590	1005	1147	1034	1235	1020	1348
β-Glucuronidase	95.2	95.2	91.8	91.8	83.6	83.6	77.4	77.4	76.8	76.8
Cathepsin D	4.47	4.47	4.23	4.23	4.16	4.16	4.17	5.14	4.13	5.09

Results expressed as n moles/hr/bottle.

Viral infection was allowed to proceed for 40 min at 37°C. Each figure represents the average of triplicated experiments.

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