EVIDENCE FOR DEGRADATION OF HEPARAN SULFATE BY ENDOGLYCOSIDASES: GLUCOSAMINE AND HEXURONIC ACID ARE REDUCING TERMINALS OF INTRACELLULAR HEPARAN SULFATE FROM HUMAN SKIN FIBROBLASTS

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Summary: Intracellular heparan sulfate was isolated from fibroblasts cultured from the skin of patients with Sanfilippo disease type B (mucopolysaccharidosis III B) and with Scheie's disease (mucopolysaccharidosis I S). In both diseases, the stored heparan sulfate exhibits an average molecular size of about one sixth of that of extracellular heparan sulfate. Upon reduction with [^{3}H]$NaBH_4$ [^{3}H]glucosaminitol and [^{3}H]aldonic acid were identified as the [^{3}H]sugar derivatives at the reducing terminal of intracellular heparan sulfate. These results suggest the participation of an endoglucosaminidase and an endohexuronidase in the degradation of heparan sulfate.

Structural and enzymic studies on intracellular heparan sulfate are rendered difficult since this glycosaminoglycan is present only in low amounts in normal cells (1). Cultured skin fibroblasts from patients with certain genetic mucopolysaccharidoses, however, accumulate excessive amounts of heparan sulfate due to the inability for degradation of heparan sulfate. So far four enzyme defects with impaired catabolism of heparan sulfate have been described: Deficiency of α-L-iduronidase, of sulfoiduronide sulfatase, of heparan sulfate sulfamidase and of α-N-acetylglucosaminidase as found in mucopolysaccharidoses I, II, III A, and III B, respectively (2). Each of these deficiencies results in the storage of a heparan sulfate, which contains at the nonreducing end predominantly that linkage to be split by the enzyme missing. Since the catabolism of heparan sulfate is thought to proceed by the action of exoglycosidases and sulfatases, the stored heparan sulfate should bear the carbohydrate-peptide linkage at its reducing end and it should exhibit a molecular weight similar to that of undegraded carbohydrate chains.

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In this paper we demonstrate that intracellular heparan sulfate preparations from Scheie and Sanfilippo B fibroblasts, respectively, have a molecular weight of about one sixth of that of extracellular heparan sulfate and bear either glucosamine or uronic acid at the reducing terminal, thus suggesting the participation of an endoglucosaminidase and an endohexuronidase in the degradation of heparan sulfate.

Materials and Methods: Fibroblast cultures from skin biopsies were established and maintained as described (3).

Preparation of extracellular and intracellular [^{35}S] heparan sulfate: Sanfilippo B and Scheie fibroblasts grown to confluency in 75 cm^2 Falcon flasks were incubated for 48 h in the presence of 5 µCi [^{35}S] Na_2SO_4 (carrier free, Amersham Buchler, Braunschweig)/ml medium and then harvested by trypsinisation. The medium was dialyzed against $(NH_4)_2SO_4$ and NaCl as described (1) and than precipitated with 80 % ethanol/1 % potassium acetate (final concentration). The ethanol precipitate and the cell pellet were subjected to an alkaline degradation and a cetylpyridinium chloride fractionation as described in ref. (1) for the isolation of [^{14}C] labeled intracellular glycosaminoglycans. The mixture of sulfated glycosaminoglycans was chromatographed on a 0.5 x 5 cm column of DOWEX 1 X 2 using a linear NaCl gradient. Fractions eluted between 0.9-1.3 N NaCl were pooled and digested with 0.2 U Chondroitinase ABC (Miles Laboratories, Stoke Court) for 16 h in a TRIS buffer, pH 8.0 (4). 0.1 mg DNase I and 0.1 mg RNase A (Boehringer Mannheim) were added to the digest of intracellular material. After stepwise elution of the digests on a DOWEX 1 X 2 column (0.5 x 5 cm) the material appearing between 0.5 and 2 N NaCl was pooled and subjected to gel filtration on a 0.5 x 126 cm Sephadex G-10 column (Pharmacia, Uppsala) in 0.2 M pyridine-acetic acid, pH 5.5. Material appearing in the void volume was brought to dryness by evaporation.

From bovine aorta a heparan sulfate free of galactosamine was isolated as described (5) and thereafter subjected to digestion with chondroitinase ABC, RNase A and DNase I and to chromatography on DOWEX 1 X 2 as above. The subsequent gelfiltration was performed on Sephadex G-100 (see Fig. 2). This preparation served as a control of the various procedures used during the preparation of heparan sulfate and during the isolation of the reducing terminals. Hyaluronic acid prepared from bovine aorta (5) was reduced with $NaBH_4$ (see below) and digested either with leech hyaluronidase (kindly provided by Dr.E.A. Balazs) or with testes hyaluronidase (Serva, Heidelberg) for 16 h (6). Material precipitable with 80 % ethanol/1 % potassium acetate (final concentration) was collected.

Intracellular, extracellular and aortic heparan sulfate, partially degraded hyaluronic acid, uronosyl anhydromannose (prepared as described in ref. (7)), glucosamine, galactosamine, glucuronic acid (Serva), and xylose (Merck, Darmstadt) were reduced with [^{3}H] $NaBH_4$ (Amersham Buchler) or with $NaBH_4$ (Merck) exactly as described (6).

[^{3}H] Xylitol (8) and [^{3}H] glucosaminitol (see preparation of hexosamine fraction in ref. (5) were isolated and characterized by paper chromatography on Selecta filter paper No 2043 b (Schleicher and Schüll, Dassel) in 1-butanol-acetone-water (2:7:1, v/v/v) (8). [^{3}H] glucosaminitol was chromatographed on DOWEX 50 W X 12 according to Gardell (9). After evaporation the fractions were dissolved in water and analysed for radioactivity and glucosaminitol using a periodate-benzidine reagent for a semiquantitative estimation of glucosaminitol (10). Glucosaminitol and galactosaminitol did not separate under these conditions. [^{3}H] Aldonic acid was identified as described (6).

For radioactivity measurements 10 ml Instagel(Packard, Frankfurt) were added to up to 0.5 ml of liquid samples or 1 x 2 cm paper strips and counted in a 3390 Packard liquid scintillation spectrophotometer.

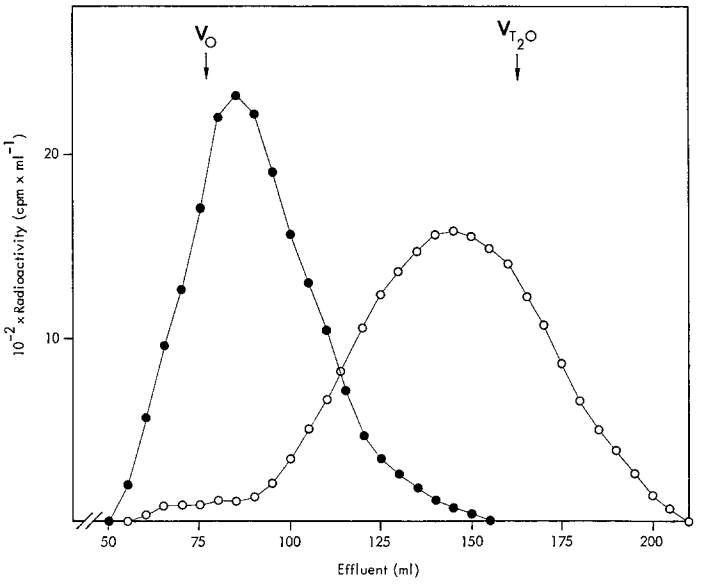

Fig. 1: Elution profile of intracellular (o—o) and extracellular (●—●) [^{35}S]heparan sulfate from Sanfilippo B fibroblasts after chromatography on Sephadex G-200. Alkaline degraded and chondroitinase ABC digested [^{35}S] heparan sulfate was obtained as described (1) and after exhaustive dialysis against 1 N NaCl 0.5 ml were loaded on a 1.6 x 86 cm column of Sephadex G-200, equilibrated with 1 N NaCl and eluted at a rate of 6.7 ml/h with 1 N NaCl. For the calculation of the molecular weights the calibration curve of Wasteson (13) was used.

Results: 1. Molecular weight of heparan sulfate. [^{35}S] heparan sulfate was isolated from cells and culture medium of Sanfilippo B and Scheie fibroblasts and compared with fully analysed heparan sulfate from bovine aorta. According to the elution behaviour on DOWEX 1 X 2, the resistance towards the action of chondroitinase ABC and the susceptibility to nitrous acid degradation it behaved like authentic heparan sulfate. By gel filtration on Sephadex G-200 an average molecular weight of 38000 was found for the heparan sulfate chains secreted by fibroblasts, whereas the intracellular heparan sulfate from Sanfilippo B fibroblasts and Scheie fibroblasts (not shown) exhibited an average molecular weight of 6500 and of 5000, respectively (Fig. 1).

After reduction with [^{3}H] sodium borohydride heparan sulfates were submitted to gel filtration on Sephadex G-100 (Fig. 2). The [^{3}H] / [^{35}S] ratio of intra- and extracellular heparan sulfate and the [^{3}H] / uronic acid ratio of arterial heparan sulfate increase with decreasing

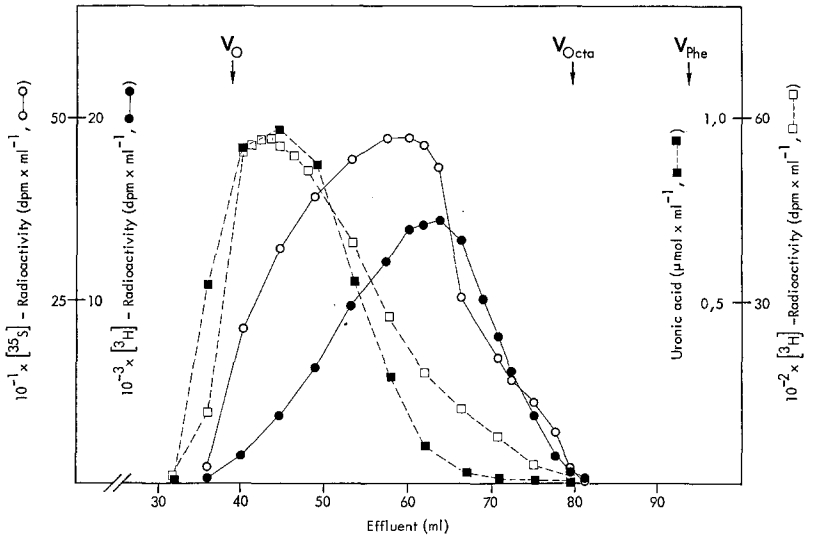

Fig. 2: Elution profile of $[^{35}S]$heparan sulfate obtained from Sanfilippo B fibroblasts (o—o,●—●) and of heparan sulfate from bovine aorta (□- -□,■- -■) on Sephadex G-100, each after reduction with $[^{3}H]$sodium borohydride. 0.5 ml of the samples were applied on a 1.5 x 35 cm column of Sephadex G-100, which was equilibrated and eluted with 0.2 M pyridine-acetic acid, pH 5.5 (0.2 M with respect to pyridine), at a rate of 8 ml/h. V_{Octa} and V_{Phe} are the elution volumes of an octasaccharide obtained from chondroitin 4-sulfate after digestion with testes hyaluronidase and of phenol red, respectively.

chain length. Extracellular (not shown) and arterial heparan sulfate had a similar $[^{3}H]$ elution profile.

2. Identification of $[^{3}H]$ xylitol as derivative from the reducing terminal of heparan sulfate. Alkaline degradation as used for the isolation of heparan sulfate chains liberates from the protein backbone single polysaccharide chains with xylose at the reducing terminal (11). Subsequent treatment with $[^{3}H]$ sodium borohydride should therefore result in the formation of $[^{3}H]$ xylitol. Whereas $[^{3}H]$ xylitol was the only labeled compound present in the neutral sugar fraction from $[^{3}H]$ heparan sulfate from the culture medium and from bovine aorta, no label was found in that fraction from intracellular heparan sulfate.

3. Identification of $[^{3}H]$ glucosaminitol as derivative from the reducing terminal of heparan sulfate. Heparan sulfate chains terminating with N-acetyl- or N-sulfonyl-

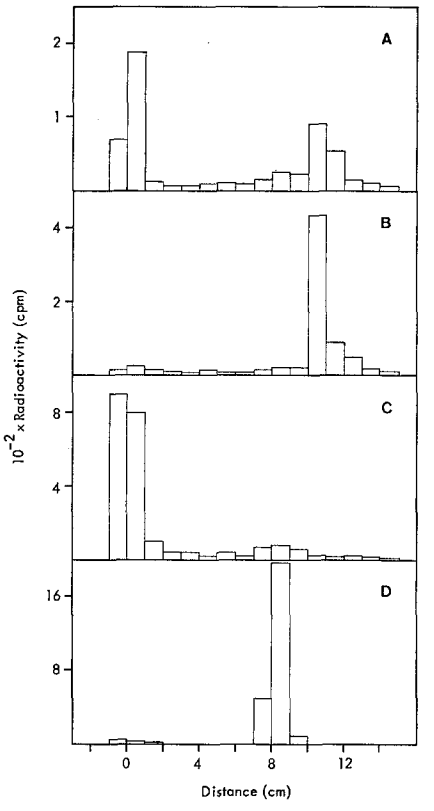

Fig. 3: Paper electrophoresis of [^{3}H] labeled compounds derived from intracellular [^{35}S] heparan sulfate from Sanfilippo B fibroblasts (A), from hyaluronic acid digested with leech hyaluronidase (B), from hyaluronic acid digested with testes hyaluronidase (C), each saccharide being reduced with [^{3}H] $NaBH_4$, and of [^{3}H] uronosylanhydromannitol (D). Preparations of hyaluronic acid partially digested with leech hyaluronidase or testes hyaluronidase and then reduced with [^{3}H] $NaBH_4$, were used as standards for polysaccharides exposing [^{3}H] gulonic acid or [^{3}H] N-acetyl-glucosaminitol at the reducing terminal. After hydrolysis and delactonization (6) the samples were applied to Selecta filter paper No 2043 b (Schleicher and Schüll) and electrophoresis was performed at 75 V/cm for 25 min in 0.08 M pyridine-acetic acid, pH 5.3 (6). Under these conditions the migration distance for [^{35}S] SO_4^{--} was 27 cm.

glucosamine would be reduced by treatment with [^{3}H] sodium borohydride to the corresponding [^{3}H] hexosaminitol. After acid hydrolysis of intracellular [^{3}H] heparan sulfate from Sanfilippo B and Scheie fibroblasts 50.6-53.6 % of the total [^{3}H] radioactivity were recovered in the hexosamine/hexosaminitol fraction, whereas only trace amounts (0.5 % of total [^{3}H] radioactivity) were detectable in that fraction from extracellular and aortic

heparan sulfate. All radioactivity of that fraction cochromatographed with glucosaminitol on paper chromatography and ion exchange chromatography on DOWEX 50 W X 12 (see Methods). It should be noted that 15 % of the glucosamine are lost during hydrolysis.

4. Identification of [^{3}H] aldonic acid as derivative from the reducing terminal of heparan sulfate. Heparan sulfate contains two kinds of uronic acids, D-glucuronic acid and L-iduronic acid. Exposed at the reducing terminal, these uronic acids would be reduced to [^{3}H] L-gulonic acid and [^{3}H] L-idonic acid, respectively, by [^{3}H] sodium borohydride treatment. The [^{3}H] aldonic acid content of hydrolysates from reduced intracellular heparan sulfate from Sanfilippo B and Scheie fibroblasts was 15.6-24.9 % of the total [^{3}H] radioactivity (Fig. 3). Under the conditions used for the detection of [^{3}H] aldonic acids [^{3}H] glucosaminitol would be liberated either as neutral monosaccharide or as non-sulfated disaccharide (for detailed discussion see ref. (6)). 9.2-9.9 % of the radioactivity were found in the position of a nonsulfated disaccharide and 47.7-66.7 % as non migrating material. The [^{3}H] radioactivity recovered in the latter two fractions corresponds well to the value given above for the [^{3}H] glucosaminitol content. The [^{3}H] aldonic acid content of extracellular and aortic heparan sulfate was below 1.0 and 2.5 % of total radioactivity, respectively.

Discussion: Heparan sulfate chains secreted by fibroblasts are attached to a protein core by an alkali labile linkage. Therefore only xylose was found at the reducing terminal of alkaline degraded heparan sulfate chains isolated from the medium. Intracellular heparan sulfate, however, does not terminate with the polysaccharide protein linkage region. As summarized in Table 1, glucosamine and uronic acid instead of xylose appear to be the reducing terminals of that material. It seems unlikely that degradation during isolation is responsible for the appearence of glucosamine and uronic acid at the reducing terminal of intracellular heparan sulfate, since extracellular heparan sulfate chains and almost all heparan sulfate chains from bovine aorta, which are thought to derive predominantly from the extracellular space, contain the polysaccharide-protein linkage

Table 1: Distribution of [^{3}H] Sugar Derivatives at the Reducing End of Heparan Sulfates

Source of Heparan Sulfate	[^{3}H] Xylitol	[^{3}H] Glucosaminitol	[^{3}H] Aldonic Acid
		% of [^{3}H] Radioactivity*	
Bovine Aorta	> 96.5	< 0.5	< 2.5
Control**	n.d.***	n.d.	n.d.
Sanfilippo B Fibroblasts			
Medium, Preparation A+	> 98	< 0.5	< 1.0
Cells, Preparation A	n.d.	71	29
Preparation B+	n.d.	80	20
Scheie Fibroblasts			
Medium, Preparation A	> 98.5	< 0.5	< 1.0
Cells, Preparation A	n.d.	77	23

*[^{3}H] Radioactivity recovered as [^{3}H] xylitol, [^{3}H] glucosaminitol and [^{3}H] aldonic acid accounted for 82-87% of total [^{3}H] radioactivity and was taken as 100% for the calculations of this table. Since the uronic acid and the hexosamine moieties were lost at equal proportions during hydrolysis for the [^{3}H] aldonic acid determination (up to 35% each), only the [^{3}H] glucosaminitol content was corrected for the loss during hydrolysis assuming a similar recovery as found for hexosamine (85%).

** Aortic heparan sulfate reduced with unlabeled sodium borohydride prior treatment with [^{3}H] sodium borohydride.

*** n.d.: not detectable

+ Preparations A and B were obtained from fibroblasts grown in 20 and 60 culture flasks, respectively.

region (Table 1). Experimental data for the synthesis of heparan sulfate chains devoid of the linkage region are not available in the literature. It seems therefore reasonable to assume that the intracellular heparan sulfate chains are products of the action of at least two different endoglycosidases, an endoglucosaminidase and an endohexuronidase. The low molecular weight of the storage material supports this hypothesis.

Short heparan sulfate chains containing the carbohydrate-peptide linkage have been isolated from urine and liver of patients with mucopolysaccharidoses (12). The lack of such chains in our material might be explained by the action of an endoglycosidase near to the carbohydrate-peptide linkage region. Such a split product would differ from others in charge and size and could have been lost during the isolation procedure.

Preliminary experiments showing the liberation of oligosaccharides from Sepharose-linked [^{35}S] heparan sulfate peptides by fibroblast homogenates give further evidence for the concept that endoglycosidases participate in the degradation of heparan sulfate. So far two mammalian endoglycosidases are known which degrade glycosaminoglycans: The lysosomal hyaluronidase (EC 3.2.1.35.), which is an endo-ß-N-acetylhexosaminidase and the endoglucuronidase, degrading exclusively macromolecular heparin (6). The characterization of the heparan sulfate degrading endoglycosidases and of the [^{3}H] aldonic acid(s) is subject of further investigations.

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