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Research Article

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The quantity of heparan sulfate in fibroblasts derived from patients with Sanfilippo A, Hurler's and Hunter's diseases was found to be 7-10%, while it was about 1.25% of the total glycosaminoglycans in fibroblasts of normal controls.

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Sanfilippo A Syndrome

SULFAMIDASE DEFICIENCY IN CULTURED SKIN FIBROBLASTS AND LIVER

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ABSTRACT The Sanfilippo A syndrome is an autosomal recessive mucopolysaccharidosis characterized clinically by severe mental retardation and biochemically by storage in tissue and excretion in urine of excessive amounts of heparan sulfate. Since sulfamide groups are present in heparan sulfate, a sulfamidase deficiency could explain the impaired degradation of this polysaccharide. To investigate the enzymic basis of this disease, assays for sulfamidase were performed. Extracts of cultured fibroblasts and post-mortem liver were prepared by suspension of tissues in acetate: NaCl buffer, pH 4.5, containing Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.), sonication, and centrifugation at 10,000 *g*. The supernatant fluid was incubated with [^{35}S]-*N*-sulfated heparin. The release of inorganic sulfate after 18 h of incubation was determined by chromatography on Sephadex G-25. The liver and fibroblast extracts of patients with the Sanfilippo A syndrome showed a deficiency of sulfamidase.

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INTRODUCTION

The Sanfilippo syndromes are autosomal recessive mucopolysaccharidoses characterized by severe mental retardation, mild skeletal deformities, and deposition in tissues and excretion in urine of heparan sulfate. The accumulation of glycosaminoglycans (GAG)[1] in cultured skin fibroblasts from patients with the Sanfilippo syndromes was reported by Matalon and Dorfman (1). In that report, heparan sulfate was not detected in cultured normal skin fibroblasts and in fibroblasts obtained from patients with these mucopolysaccharidoses. However, Kresse and Neufeld (2) indicated the presence of a GAG with properties of heparan sulfate in fibroblasts from Sanfilippo A disease. The development by Cifonelli (3) of the nitrous acid method for the degradation of *N*-sulfated glucosamine-containing GAG has made possible the identification of small amounts of such substances in complex mixtures of GAG. Using this method, Kraemer (4, 5) has shown the presence of heparan sulfate in a number of cultured cell lines. Dorfman and Ho (6) demonstrated the synthesis of heparan sulfate by cultured rat glial tumor cells (RG-C6). All of these findings suggest that our earlier observation was in error and led us to re-examine the question of the presence of heparan sulfate in cultured human skin fibroblasts. This substance was found to represent 1.25% of the GAG in normal fibroblasts and 7–10% in fibroblasts derived from patients with Hurler's, Hunter's, and Sanfilippo diseases.

This report also deals with the enzymic defect in Sanfilippo A syndrome. On the basis of cross-correction studies, Kresse, Weissmann, Cantz, Hall, and Neufeld (7) demonstrated the existence of two genetically distinct Sanfilippo syndromes, A and B. O'Brien (8) has reported the deficiency of α-*N*-acetylglucosaminidase in Sanfilippo B syndrome. This observation was confirmed by Von Figura and Kresse (9).

Kresse and Neufeld (2) have indicated the probability that Sanfilippo A results from a deficiency of an enzyme that hydrolyzes the *N*-sulfate linkages. In order to demonstrate more directly the enzymic defect in Sanfilippo A syndrome, sulfamidase activity in fibro-

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[1] *Abbreviation used in this paper:* GAG, glycosaminoglycans.

Table I
Heparan Sulfate Isolated from Cultured Skin Fibroblasts

Cell strain	Total GAG*	Heparan‡ sulfate	UA§/hex	N-Sulfate/hex	GlcN/GalN	$[\alpha]_D^{25}$‖
	mg	%				
Normal (H. P.)	1.6	1.25	1.6	0.27	11.1	—
Hurler (B. H.)	23.0	8.5	1.4	0.40	20.0	+50°
Hunter (C. H.)	9.0	7.9	1.5	0.41	20.0	+45°
Sanfilippo A (M. G.)	5.0	10.4	1.5	0.37	25.0	+51°
Sanfilippo A (D. D.)	3.7	9.4	1.6	0.30	14.3	—

* Isolation from 40 tissue culture plates (100 mm). Each plate contained approximately 1.5×10^7 cells with dry wt of 15 mg. Total GAG is based on 33% hexosamine (hex).
‡ The 1.0 M and 1.25 M fractions, as based on *N*-sulfated hexosamine.
§ UA, uronic acid.
‖ The optical rotation ($[\alpha]_D^{25}$) is based on 33% hexosamine.

blasts and liver was assayed by the method of Friedman and Arsenis (10). Dietrich (11) and Friedman and Arsenis (10) have shown the existence of heparin sulfamidase in various mammalian tissues by using [^{35}S]-*N*-sulfated heparin as substrate. The latter investigators showed that sulfamidase differs from arylsulfatases A and B. In a preliminary communication, we have reported the deficiency of sulfamidase in Sanfilippo A fibroblasts and liver (12). Subsequently, Kresse (13) reported the deficiency of sulfamidase in fibroblasts of affected patients and showed that leukocytes can be used to diagnose affected patients and heterozygotes.

METHODS

Source of cultures. All skin fibroblasts utilized in these studies, with exception of P. L. and S. V. (Tables II and III), were isolated from biopsies obtained in this laboratory. Fibroblasts from P. L. and S. V. were obtained from Dr. John O'Brien. All patients designated as Sanfilippo disease showed typical clinical findings and excreted heparan sulfate in their urine. Patients C. B. and A. B. were siblings. The Hurler patient, S. F., was diagnosed on the basis of clinical features, urine studies, and absence of α-L-iduronidase in fibroblast extracts. The Hunter patients (D. R. and C. H.) were diagnosed on the basis of clinical characteristics, genetic pattern, urine studies, and demonstration of absence of idurono-sulfate sulfatase, based on end group analyses of dermatan sulfate. The liver (L. L.) was obtained from Drs. F. K. Yoosufani and Jay F. Lewis.

Culture conditions. Skin fibroblasts, which had undergone three to six transfers, were cultured as previously described (14) in modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with ascorbic acid (100 mg/liter), streptomycin (75 mg/liter), penicillin (100,000 U/liter), 10% fetal calf serum, and 10% calf serum. Under the conditions of these experiments, by using 100 mm Falcon tissue culture dishes under CO_2 concentration of 10% at 37°C, cell density of approximately 1.6×10^7 was achieved in 3 wk. During this time, pH fluctuation of the medium was between 7.2–7.4. Routine monitoring for mycoplasma infection was performed in mycoplasma agar medium (Grand Island Biological Co.). Before harvesting, cells were washed three times with 0.15 M NaCl, scraped from the plate with a rubber policeman, and suspended in 0.05 M acetate buffer in 0.15 M NaCl, pH 4.5.

Isolation of GAG. GAG were isolated from the harvested cells after papain digestion in 0.1 M acetate buffer (pH 5.5) containing 0.005 M disodium ethylenediaminetetraacetate (Fisher Scientific Co., Pittsburgh, Pa.) and 0.005 M cysteine HCl. Digestion was carried out for 18 h at 60°C followed by 24 h dialysis against H_2O. Under these conditions, the GAG remain intact, although there is a possibility of a loss of small oligosaccharides during dialysis. The crude GAG mixture was precipitated with cetylpyridinium chloride (Sigma Chemical Co., St. Louis, Mo.), as previously described (14). The GAG was fractionated by chromatography on Dowex 1×2 Cl^- columns (18×1 cm) (Dow Chemical Co., Midland, Mich.) with stepwise NaCl elution (14). Only fractions eluted with 1.0 and 1.25 M NaCl contained nitrous acid-reactive material. Gel filtration was carried out with Sephadex G-25, superfine (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), on 180×1.0-cm columns.

Analysis and identification of GAG. Uronic acid was estimated by the method of Dische (15). *N*-Sulfated hexosamine was determined by a micromodification of the method of Lagunoff and Warren (16) by utilizing a final volume of 1.0 ml instead of 6.2 ml. This scaling down of volume leads to more sensitive assay, so that 1.0 μg of *N*-sulfated hexosamine can be detected. Amino sugars were determined by a Technicon amino acid analyzer (Technicon Instruments Corp., Tarrytown, N. Y.). Electrophoresis was performed on Sepraphore III (Gelman Instrument Co., Ann Arbor, Mich.) at pH 3.0 by using formic acid: pyridine buffer, as previously described (14). The purified heparan sulfate fractions were subjected to digestion with chondroitinase ABC (Seikagaku, Tokyo, Tokyo, Japan) by using 0.2 U/ml in 0.01 M Tris-acetate buffer, pH 8.0, for 16 h at room temperature. Protein concentration was determined by the method of Lowry, Rosebrough, Farr, and Randall (17).

Enzyme assays. Extracts for sulfamidase assays were prepared from harvested fibroblasts suspended in 0.05 M acetate buffer in 0.15 M NaCl, pH 4.5. The cells were disrupted by sonication for 60 s at 10,000 cycles/s. After sonication, a portion was centrifuged at 10,000 *g* at 4°C for 10 min, and the supernatant solution was used for enzyme assays. Frozen liver samples, obtained at autopsy 2–4 h after

death, were extracted in the same way. For comparative enzyme assays, the protein concentration was equalized by the addition of acetate: NaCl buffer. Sulfamidase activity was determined with the [^{35}S]-*N*-sulfated heparin, 12.7 mCi/g, (Amersham/Searle Corp., Arlington Heights, Ill.) as a substrate. Before use, commercially obtained [^{35}S]-*N*-sulfated heparin was purified by chromatography on Sephadex G-25. Approximately 1% of the radioactivity was removed as free sulfate. The radioactive heparin eluted in the void volume was used as substrate. Treatment of the radioactive heparin with nitrous acid released quantitatively the labeled sulfate, proving that the radioactivity is entirely in *N*-sulfate. Each incubation mixture contained 2 mg of unlabeled heparin as carrier in addition to 100,000 or 250,000 cpm of the labeled substrate. After incubation for 18 h at 37°C (in the presence of toluene), 0.1 ml of sodium sulfate (0.01 M) was added to each tube and allowed to equilibrate for 2 h at 37°C. The pH was adjusted to 7.0, and each sample was centrifuged at 10,000 *g* for 20 min.

In earlier experiments, high voltage electrophoresis according to Friedman and Arsenis (10) was used. The released radioactivity migrated as free sulfate. However, chromatography on Sephadex G-25 was found to be more reproducible. An aliquot was chromatographed on a Sephadex column, G-25 (180 × 1.0 cm), by using 0.15 M NaCl in 15% ethyl alcohol as eluent. The included fractions, which contained inorganic sulfate, were counted in a Packard Tricarb Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The scintillation mixture contained 0.5 ml of the sample with 10 ml of Permafluor (Packard Instrument Co.) and 1 ml of Bio-Solv BBS-3 (Beckman Instruments, Inc., Fullerton, Calif.).

α-*N*-Acetylglucosaminidase was assayed with the phenyl-α-*N*-acetylglucosamine (a gift from Dr. B. Weissmann) as a substrate. β-*N*-Acetylhexosaminidase and β-galactosidase were assayed with *p*-nitrophenyl derivatives (Sigma Chemical Co.) as described by Weissmann, Rowlin, Marshall, and Freiderici (18).

RESULTS

Isolation of heparan sulfate. In experiments with normal fibroblasts, 1.6 and 2.0 mg of GAG were isolated from 40–60 plates, each plate containing about 1.6 × 10^7 cells with a dry wt of about 12–15 mg. Heparan sulfate comprised 1.25% of the total GAG. This estimation is based on the data in Table I indicating that the *N*-sulfated hexosamine in the purified heparan sulfate is about 40% of the total hexosamine samples which contained sufficient material for adequate analyses. The amounts of purified heparan sulfate isolated from normal fibroblasts were insufficient for determination of optical rotation. The medium collected from a 3-wk period of biweekly feeding of the cells contained small quantities of GAG, 0.3 mg/plate. The GAG isolated from the medium migrated, as does hyaluronic acid on electrophoresis with Sepraphore III. No *N*-sulfated hexosamine was demonstrable in this fraction. The medium supplemented with fetal calf serum and calf serum contains small amounts of hyaluronic acid and chondroitin sulfate but no detectable heparan sulfate (6).

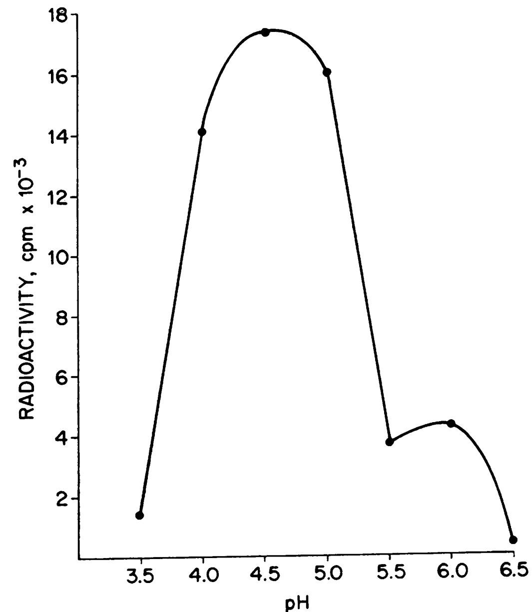

FIGURE 1 Effect of pH on the activity of heparin sulfamidase. Fibroblast extracts were incubated with 250,000 [^{35}S]-*N*-sulfate heparin at 37°C in acetate: NaCl buffer (0.05 M acetate: 0.15 M NaCl) for 18 h. The released radioactive sulfate was isolated by chromatography on Sephadex G-25.

Hurler, Hunter, and Sanfilippo A fibroblasts yielded larger quantities of GAG than the normal control. On separation by chromatography on Dowex 1 × 2 Cl^-, heparan sulfate in the amounts shown in Table I was obtained. All the heparan sulfate was eluted from Dowex-1 with 1.0 and 1.25 M NaCl. After digestion of the heparan sulfate fractions with chondroitinase ABC and subsequent filtration on Sephadex G-25, all of the *N*-sulfated hexosamine-containing GAG were recovered in the void volume. Analysis of this material (Table I) showed that glucosamine was the predominant amino sugar. The optical rotations presented in Table I are characteristic of heparan sulfate. On electrophoresis, the heparan sulfate fractions migrated as single spots, slower than a standard heparan sulfate containing 1 mol of sulfate (isolated by Dr. J. A. Cifonelli from beef lung heparin by-products), but faster than umbilical cord heparan sulfate which has less than 1 mol of sulfate.

Sulfamidase activity. Fibroblasts and liver extracts were assayed with [^{35}S]-*N*-sulfated heparin as substrate. The pH optimum was pH 4.5 as illustrated in Fig. 1. Addition of 2 mg of carrier heparin to the incubation mixture enhanced the release of radioactive sulfate by 12%. Under these conditions, optimal protein concentration of crude extract was 300 μg/ml of reaction mixture. Increasing the amount of enzyme

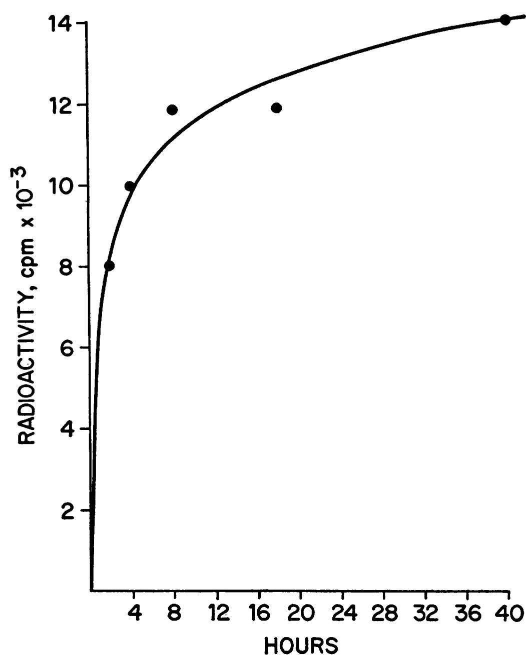

FIGURE 2 Effect of incubation time on the release of $^{35}SO_4$ from [^{35}S]-*N*-sulfated heparin. Fibroblast extracts were incubated with 250,000 cpm radioactive heparin at 37°C in acetate: NaCl buffer (0.05 M: 0.15 M), pH 4.5. The released $^{35}SO_4$ was isolated by chromatography on Sephadex G-25.

extract beyond that level did not increase the amount of $^{35}SO_4$ released after 18 h of incubation (Fig. 2).

Extracts of fibroblasts and liver from patients with Sanfilippo A syndrome demonstrated less than 10% of the activity of extracts of normal fibroblasts and fibroblasts of patients with other mucopolysaccharidoses (Table II). When the same extracts were assayed for α-*N*-acetylglucosaminidase (Table III) and other lysosomal glycosidases (Table IV), the activities were found to be normal except for the expected deficiency of α-*N*-acetylglucosaminidase in Sanfilippo B samples.

DISCUSSION

Several studies (19–21) have indicated that heparan sulfates are a family of GAG which contain *N*-sulfated and *N*-acetylated glucosamine D-glucuronic acid and L-iduronic acid in varying proportions (22). Recent studies (23, 24) have demonstrated that some of the L-iduronic acid groups are sulfated. Although chemically similar, the heparan sulfates differ from heparin in their lack of anticoagulant properties, lower *N*-sulfate, and total sulfate content. Certain fractions of heparan sulfates show lipoprotein-lipase-releasing activity and prevent dietary-induced atherosclerosis in rabbits (25). The exact function of the heparan sulfates is unknown, though reports indicate their presence in many cell types (4–6) which might suggest a general function in cell physiology. A role in the division of cells for heparin and "heparin-like" compounds has been suggested by Heilbrunn (26) and Kinoshita (27). Lipmann (28) has noted an effect of heparin on division of cells in culture.

In contrast to the excretion of both dermatan sulfate and heparan sulfate in Hurler's and Hunter's syndromes, only heparan sulfate is excreted in the Sanfilippo syndromes. Heparan sulfate and heparin have two unique linkages not present in dermatan sulfate, α-*N*-acetylglucosamine and glucosamine *N*-sulfate. The fact that deficiency of the two hydrolases concerned with degradation of these linkages produces a similar phenotype is, therefore, fully consistent with structural considerations. The Sanfilippo syndromes are characterized by severe mental retardation. Kapan (29) has suggested that accumulation of heparan sulfate is correlated with mental retardation. Perhaps this is related to the relatively high synthesis of this GAG by glial cells (6).

TABLE II
Sulfamidase Activity of Fibroblast and Liver Extracts

Extract	$^{35}SO_4$ released
	cpm/mg protein/18 h
Fibroblasts	
Normal (J. T.)	5,700
Normal (R. A.)	3,250
Hurler (S. F.)	3,073
Hunter (D. R.)	5,046
Sanfilippo B (P. L.)	1,015
Sanfilippo B (S. V.)	2,642
Sanfilippo B (K. P.)	2,592
Sanfilippo A (C. B.)	10
Sanfilippo A (A. B.)	13
Sanfilippo A (S. M.)	16
Liver	
Normal (N. L.)	1,316
Sanfilippo A (L. L.)	33

Fibroblasts and liver samples were suspended in acetate:NaCl buffer (0.05 M:0.15 M), pH 4.5, sonicated, and centrifuged at 10,000 *g* for 10 min. The supernatant fluid was incubated at 37°C for 18 h with [^{35}S]-*N*-sulfated heparin, 100,000 cpm/incubation mixture. 2 mg of nonradioactive heparin was added to the incubation mixture. Fibroblasts and liver extracts were added at a protein concentration of 300 μg/tube. The vol in each tube was 0.5 ml. The free $^{35}SO_4$ released was isolated after chromatography on Sephadex G-25.

The extent of hydrolysis demonstrated by normal extracts of sulfamide linkages is relatively low. A similar observation has been made by Friedman and Arsenis (10). These results suggest that the enzyme, under the conditions studied, is unable to hydrolyze all of the sulfamide groups. The low percentage suggests that perhaps only *N*-sulfates on nonreducing terminal glucosamine groups are hydrolyzed. Of interest is the further fate of the molecule, since hydrolysis of the *N*-sulfate group would leave a nonreducing terminal glucosamine. Whether this can be hydrolyzed by an α-*N*-acetylglucosaminidase or requires yet another hexosaminidase has not yet been determined. If heparan sulfate in fibroblasts is sequentially degraded only by exoenzymes, low activity toward degradation of other linkages under the conditions of the experiments may account for the relatively low sulfate yield.

The results reported here add to the accumulating knowledge that the mucopolysaccharidoses result from genetically determined diminished activity of specific hydrolases required for degradation of GAG. Hurler's and Scheie's syndromes show a deficiency of α-L-iduronidase (30–32); Sanfilippo A syndrome, a deficiency of heparan sulfate *N*-sulfamidase (12, 13); Sanfilippo B, a deficiency of α-*N*-acetylglucosaminidase (9, 18); Hunter's syndrome, a deficiency of idurono-sulfate sulfatase (33–35); and Morquio's syndrome, a deficiency of chondroitin sulfate *N*-acetylhexosamine sulfate sulfatase (36). An additional syndrome has been reported to demonstrate absence of β-glucuronidase (37). There is evidence of a diminished arylsulfatase B activity in Maroteaux-Lamy syndrome (38), although the defect in degradation of the accumulated dermatan sulfate in this syndrome remains to be elucidated.

TABLE III
α-N-Acetylglucosaminidase Activity of Fibroblast Extracts

Extract	Activity
	μmol phenol/mg protein/18 h
Normal (J. T.)	0.340
Sanfilippo A (C. B.)	0.234
Sanfilippo A (A. B.)	0.161
Sanfilippo A (S. M.)	0.150
Sanfilippo B (P. L.)	0.006
Sanfilippo B (S. V.)	0.020
Sanfilippo B (K. P.)	0.020

The 10,000 *g* supernate of sonicated fibroblasts was incubated with phenyl-α-*N*-acetylglucosamine, 5 mM, for 18 h in citrate:NaCl buffer (0.05 M:0.15 M), pH 4.5. Incubation was at 37°C with 200 μg of protein in final volume of 0.5 ml. The hydrolysis was terminated by addition of 1 vol of Folin-Ciocalteu reagent. The release of phenol was measured as described by Weissmann et al. (18).

TABLE IV
Lysosomal Enzymes of Fibroblasts and Liver

	β-*N*-Acetylglucosaminidase *p*-nitrophenol	β-Galactosidase *p*-nitrophenol
	μmol/mg protein/h	*μmol/mg protein/h*
Fibroblasts		
Normal (J. T.)	2.50	0.70
Sanfilippo A (A. B.)	1.50	0.50
Liver		
Normal (H. L.)	2.25	0.40
Sanfilippo A (L. L.)	1.70	0.30

The 10,000 *g* extracts from fibroblasts and liver were assayed with 5 mM of the *p*-nitrophenylglucosides. 200 μg of protein extracts was incubated at 37°C for 2 h. The buffer for β-hexosaminidase was citrate:NaCl (0.05 M:0.15 M), pH 4.5, and for β-galactosidase it was acetate:NaCl (0.05 M:0.1 M), pH 4.5. Final incubation mixture was 0.5 ml. The hydrolysis was stopped by the addition of 0.4 M glycine, pH 10.7, and the release of *p*-nitrophenol measured spectrophotometrically at 410 nm.

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