MORQUIO'S SYNDROME: DEFICIENCY OF A CHONDROITIN SULFATE N-ACETYLHEXOSAMINE SULFATE SULFATASE

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The Morquio syndrome is a spondyloepiphyseal dysplasia characterized by excretion in urine of excessive amounts of keratan sulfate and chondroitin sulfate. To investigate the enzymic basis of this disease, assays for sulfatase were performed using chick embryo chondroitin sulfate and rat chondrosarcoma chondroitin 4-sulfate as substrates. The data obtained, using skin fibroblasts as an enzyme source, indicate that Morquio's syndrome is a deficiency of chondroitin sulfate N-acetylhexosamine sulfate sulfatase.

The Morquio syndrome is an autosomal recessive mucopolysaccharidosis, characterized by platyspondyly, epiphyseal dysplasia and dwarfism (1, 2). Patients with this disease resemble other spondyloepiphyseal dysplasias; however, the discovery of excessive excretion of keratan sulfate and chondroitin sulfate in the urine provided a chemical basis for the definition of this entity (3, 4).

Because of the difficulty in obtaining adequate cartilage for metabolic studies cultured skin fibroblasts were utilized in an attempt to elucidate the enzymic defect in this syndrome. The data presented in this report indicate a deficiency of chondroitin sulfate N-acetylhexosamine sulfate sulfatase in cultured skin fibroblasts obtained from patients with the Morquio syndrome.

MATERIALS AND METHODS

All skin fibroblasts utilized in these studies were isolated from biopsies obtained in these laboratories. The patients with Morquio's disease had typical clinical findings and their urines contained increased amounts of keratan sulfate and chondroitin sulfate.

Fibroblast cultures were grown under conditions previously described (5). Cells, grown for three weeks in 100 mm Falcon petri dishes, reached a cell density of 1.6×10^7. The pH was maintained between 7.2 and 7.4. Mycoplasma monitoring was carried out routinely with mycoplasma agar medium (GIBCO).

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Enzyme Preparations

Fibroblast monolayers from three 100 mm petri dishes were washed x3 with 0.05M acetate buffer containing 0.05M NaCl, pH 5.5, harvested with a rubber policeman and suspended in 1 ml 0.05M acetate-0.05M NaCl buffer, pH 4.0. Following disruption by sonication for 60 seconds at 10,000 cycles (power setting 3) in a Branson Sonifier, the samples were centrifuged at 10,000 x g at 4° for 10 min and the supernatant solutions and pellets were used for enzyme assays. For comparative studies, the protein concentrations were equalized.

Each incubation mixture contained enzyme extract (250 μg of protein) and [^{35}S]chondroitin sulfate (100,000 to 200,000 cpm) in the acetate-sodium chloride buffer, pH 4.0, in a final volume of 0.2 ml. Following incubation at 37° for 18h, 0.05 ml of 0.1M sodium sulfate was added to each tube. The released radioactive sulfate was determined by high voltage electrophoresis using as buffer 0.15M pyridine: 0.08M acetic acid (2.5:1, v/v) pH 5.2. Each strip (Whatman No. 3) was run for 15 min at 3,000 volts (High Voltage Electrophoretor, Model D., Gilson Medical Electronics). The strips were cut into 1 inch segments and the regions of substrate and inorganic sulfate were counted in a Packard Tricarb Liquid Scintillation Spectrometer as previously described (6).

β-N-Acetylhexosaminidase, β-galactosidase and β-glucuronidase were assayed with p-nitrophenyl derivatives (Sigma) as described by Weissmann et al. (7). Arylsulfatases A and B were assayed with nitrocatechol sulfate (Sigma) as a substrate (8).

Chick Embryo [^{35}S]Chondroitin 4/6-Sulfates

The femoral and tibial epiphyses of ten 13 day old chick embryos were diced and incubated in 2 ml of Krebs buffer containing 1 mCi/ml of carrier-free $H_2{}^{35}SO_4$ (Chicago Nuclear). After 2h the material was digested with crystalline papain for 18h, dialyzed against sodium sulfate (0.1M) for 2h and then for 24h against H_2O. Glycosaminoglycans were isolated with cetylpyridinium chloride (Sigma) (5). The composition of glycosaminoglycans was determined following digestion with chondroitinase ABC (Seikagaku Kogyo, Tokyo, Japan) and paper chromatography (9). The chondroitin 4- and 6-sulfate represented 59.5%, and 37.1%, respectively, of the total radioactivity. Three percent of the radioactive material remained undigested

Preparation of [^{35}S]Chondroitin 4-Sulfate

[^{35}S]Chondroitin 4-sulfate was prepared from a transplantable rat chondrosarcoma (10). A 2.5 x 2.5 cm piece of tumor was trypsinized and suspended in 40 ml of Eagle's medium to which 1 mCi of $H_2{}^{35}SO_4$ was added. After 24h of incubation, the entire 40 ml were digested with papain for 18h and glycosaminoglycans were isolated as previously described (5).

Preparation of [^{35}S]Heptasaccharide

[^{35}S]Chondroitin sulfate from chick embryo epiphyses (total of 5 x 10^6 cpm) was subjected to digestion with testicular hyaluronidase (A. B. Leo, Sweden) (5) for 18h. The reaction was stopped by heating at 100° for 15 min and the digest was applied to a column of Dowex 1 x 2 Cl^-, 200-400 mesh, (16 x 0.7 cm). Oligosaccharides were separated by linear gradient with 130 ml H_2O and 130 ml of 2M NaCl. The octasaccharide fractions were pooled and concentrated to a final volume of 1 ml and then subjected to digestion by 1 mg of β-glucuronidase (Sigma Type B-3) for two hours. The reaction was stopped by boiling for 2 min. The heptasaccharide was isolated by chromatography on Sephadex G-50.

Preparation of Lysosomal Fraction

Cells from one 100 mm petri dish were suspended in 3 ml of 0.25M sucrose, then sonicated in a Branson Sonifier with two bursts of 1 sec each at power setting of 2. The suspension was centrifuged at 900 x g for 10 min to remove the nuclear fraction. The supernatant solution was again centrifuged at 9,000 x g for 10 min. The sediment, containing the lysosomes and mitochondria, was used as the lysosomal fraction for these assays. Each incubation mixture contained 500 μg protein of the lysosomal fraction in a final volume of 0.1 ml of acetate-NaCl buffer, pH 4.0.

RESULTS

The [^{35}S]chondroitin 4- and 6-sulfates isolated from the chick embryo epiphyses was incubated with fibroblast extracts and pellets for 18h at 37°. Table I sumarizes the results of such experiments using the cell extracts and pellets as enzyme sources. It is evident that the Morquio fibroblasts are deficient in the enzyme that releases sulfate from the chick embryo chondroitin sulfate. The nor-

Table I
The Release of $^{35}SO_4$ from Chick Embryo [^{35}S]Chondroitin Sulfate

Cell Type	$^{35}SO_4$ Released		% of $^{35}SO_4$ Released	
	cpm/250 μg protein/18h			
	Extract	Pellet	Extract	Pellet
Normal	8,410	15,400	7.6	14.4
Normal Boiled	0	0	0.0	0.0
Morquio I	1,005	0	0.8	0.0
Morquio II	1,243	1,017	1.1	0.9
Hurler	11,050	10,900	8.5	8.1
Hunter	12,950	15,875	10.6	13.7
Sanfilippo A	6,520	11,775	5.8	9.0
Metachromatic Leukodystrophy	11,320	14,150	10.0	10.0

All reaction mixtures contained 200,000 cpm [^{35}S] chondroitin-sulfate and 250 μg of protein in 0.2 ml of acetate:NaCl buffer pH 4.0. Percent sulfate released was calculated on the basis of inorganic $^{35}SO_4$ divided by the total radioactivity of undegraded material plus the inorganic sulfate recovered.

Table II
Release of $^{35}SO_4$ from [^{35}S]Chondroitin 4-Sulfate

Cell Type	$^{35}SO_4$ Released		% of $^{35}SO_4$ Released	
	cpm/250 μg protein/18h			
	Extracts	Pellets	Extracts	Pellets
Normal	12,603	4,573	18.0	6.7
Morquio I	4,952	4,643	7.8	6.8
Morquio II	6,790	6,119	9.8	5.4
Hurler	11,806	3,132	19.4	4.9
Hunter	13,355	9,104	20.0	11.4
Sanfilippo A	12,102	4,781	19.8	7.2
Metachromatic Leukodystrophy	15,272	6,036	24.2	10.4

All reaction mixtures contained 100,000 cpm [^{35}S] chondroitin-sulfate and 250 μg of protein in 0.2 ml of acetate:NaCl buffer pH 4.0. Percent sulfate released was calculated on the basis of inorganic $^{35}SO_4$ divided by the total radioactivity of undegraded material plus the inorganic sulfate recovered.

mal fibroblasts and other control cells, such as those obtained from patients with mucopolysaccharidoses other than Morquio's disease and metachromatic leukodystrophy, readily release sulfate.

Since the chick embryo glycosaminoglycan is a mixture of chondroitin 4- and 6-sulfates, the release of sulfate from a substrate containing only chondroitin 4-sulfate isolated from rat chondrosarcoma was also measured. Table II indicates that the Morquio extracts release sulfate from this substrate, as do the pellets. The release of $^{35}SO_4$ from chondroitin 4-SO_4 by the Morquio preparations was somewhat lower than that found with extracts from control samples, but not as low as with the chick substrate.

Additional studies have been carried out with a heptasaccharide derived from chick embryo [^{35}S]chondroitin sulfate. For this purpose lysosomal preparations were used. The results in Table III clearly show a marked decrease of $^{35}SO_4$ release by the Morquio preparations. More detailed studies of the reaction products

Table III
Degradation of [^{35}S]Heptasaccharide by Lysosomal Fractions

Cell Type	$^{35}SO_4$ Released	% of $^{35}SO_4$ Released
	cpm/18h	
Normal Controls	309	22.5
Morquio I	17	1.4
Morquio II	27	2.1

All reaction mixtures contained 3000 cpm of [^{35}S]heptasaccharide and 500 μg protein in 0.1 ml of acetate:NaCl buffer, pH 4.0. Percent of sulfate released was calculated on the basis of inorganic $^{35}SO_4$ divided by the total radioactivity of undegraded material plus the inorganic sulfate recovered.

(11) obtained from this substrate indicate that extracts of Morquio's fibroblasts fail to hydrolyze the N-acetylgalactosamine 6-SO_4 bond of this substrate as indicated by increased recovery of N-acetylgalactosamine 6-sulfate following chondroitinase AC digestion of incubation mixtures treated with Morquio lysosomes. The deficient sulfatase is distinct from arylsulfatases A and B since the levels of the latter were normal when assayed with nitrocatechol sulfate as substrate. Other hydrolases that might be implicated in the degradation of chondroitin sulfate or keratan sulfate, such as β-N-acetylhexosaminidase, β-glucuronidase and β-galactosidase were assayed with the nitrophenol derivatives and found to be of the same level in the Morquio as in the normal fibroblasts.

DISCUSSION

Morquio's syndrome is characterized by the excretion in urine of keratan sulfate and chondroitin 6-sulfate, normal components of cartilage. Fibroblast extracts, from controls and Morquio, did not release measurable radioactivity from [^{35}S]-keratan sulfate prepared from rabbit cartilage (11). However, labeled chondroitin sulfate obtained from chick cartilage which consists of a mixture of 4- and 6-sulfates (probably in the same chains) was found to serve as a satisfactory substrate Extracts of fibroblasts grown from patients with Morquio's disease show a marked deficiency in capacity to cleave this substrate. This was substantiated by the failure to release $^{35}SO_4$ from a heptasaccharide prepared from the same polysac-

charide. More detailed studies of the products of breakdown of the heptasaccharide (11) indicate a failure to hydrolyze the N-acetylhexosamine 6-sulfate linkage. The large amount of sulfate released by extracts of normal fibroblasts suggest that the enzyme acts on internal sulfate linkages or progressive breakdown of the chain occurs by the sequential action of exoglycosidases. The latter explanation seems unlikely since extracts of fibroblasts of a patient with Sandhoff-Jatzkewitz disease (deficient in 3-N-acetylhexosaminidase) releases the same amount of inorganic sulfate from both heptasaccharide and polysaccharides as do normal extracts (11).

In order to determine whether the deficient sulfatase acted on 4- or 6-sulfate linkages, rat chondrosarcoma chondroitin sulfate, which contains only 4-sulfate linkages (10), was used as a substrate. Some decrease in activity of Morquio's extract was observed. This was in contrast to extracts of fibroblasts of Maroteaux-Lamy's disease which fail to act on this substrate (11). Maroteaux-Lamy extracts show a deficiency of arylsulfatase B (12), whereas Morquio's extracts have normal levels of both arylsulfatases A and B. Additionally, Morquio's extracts show normal levels of 3-N-acetylhexosaminidase, β-glucuronidase, and 3-galactosidase.

Since keratan sulfate contains a 6-sulfate linkage, it seems likely that the deficient enzyme in Morquio's disease is an N-acetylhexosamine 6-sulfate sulfatase which may not be entirely specific and exhibit some activity on the 4-sulfate. Arylsulfatase B, however, appears to be specific for N-acetylhexosamine 4-sulfate (11).

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