Increased Urinary Excretion of Acidic Mucopolysaccharides in Exophthalmos

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ABSTRACT The excretion of mucopolysaccharides normally found in urine (chondroitin, chondroitin sulfates A and C, keratosulfate, and heparitin sulfate) is increased approximately twofold in patients with progresive exophthalmos. A threefold elevation of total serum mucopolysaccharides is also found. These increases are unrelated to thyroid function.

INTRODUCTION

Mucopolysaccharide deposition in retrobulbar tissue of patients with endocrine exophthalmos was demonstrated histochemically several years ago (1, 2). Metachromatic substance is also found in connective tissue, lacrymal glands, and muscles under the sarcolemnal membrane. Similar changes are found in the affected skin of patients with localized myxoedema, a condition often associated with infiltrative exophthalmos (3, 4). Increased mucopolysaccharide measured chemically is found in the area of the lesion and also in clinically uninvolved skin (5).

These changes have prompted the present investigation of the mucopolysaccharide content of urine and blood in normal subjects and patients with progressive exophthalmos.

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METHODS

Materials

24-hr samples of urine were collected from 10 patients with progresive exophthalmos and 10 normal adults and preserved with toluene (25 ml).

Chondroitin sulfate C was kindly donated by Dr. K. Meyer while the other purified acidic mucopolysaccharides (AMPS) were gifts from Dr. E. Davidson. Papain (14 units/mg) and hyaluronidase from bovine testes (300 USP units/mg) were obtained from Worthington Biochemical Corp., Freehold, N. J.; Crystallized trypsin (9500 BAEE units/mg) was obtained from Mann Research Labs. Inc., N. Y.

Colorimetric analysis

Hexosamine was determined by the Morgan Elson reaction as modified by Boas (6), uronic acid by the method of Dische (7), glucosamine and galactosamine by the method of Davidson (8), and total sulfate by the method of Antanopoulos (9).

Electrophoresis and chromatography

Electrophoresis of AMPS was performed on cellulose acetate (Sepraphore, Gelman Instrument Company, Ann Arbor, Mich.) at 10 v/p cm for 1 hr. Two types of buffer were used: 0.1 M sodium acetate, pH 3.5, and 0.15 M zinc sulfate adjusted to pH 2 with 1 N HCl. The latter gives a good separation of chondroitin sulfate B from the isomers A and C. The strips were stained by immersion for 30 min in an 0.5% solution of Alcian blue in 0.5 N acetic acid and destained in 0.2 N acetic acid.

AMPS were separated by ion exchange chromatography on a DEAE-Sephadex A-50 column (2 × 20 cm) equilibrated with 0.1 M sodium chloride. Elution of the AMPS was performed with a linear gradient of NaCl to a final concentration of 4 M (2 liters).

Descending paper chromatography (Whatman No. 3) was carried out with the following solvents: 1-butanol: acetic acid: water 50:15:35 (solvent I), pyridine: ethyl

acetate : water : acetic acid 5 : 5 : 3 : 1 (solvent II), pyridine : ethyl acetate : water 1.0 : 3.6 : 1.15 (solvent III). Sugars were located on paper with $AgNo_3$ reagent (10).

RESULTS

Isolation of urinary mucopolysaccharides

The procedure followed was a modification of the method of Perlman, Telser, and Dorfman (11) for the isolation of AMPS from cells. A typical isolation is as follows. A urine sample (1.2–2.0 liters) was dialyzed overnight against running tap water and concentrated to 100 ml under vacuum. Insoluble material was removed by filtration through fluted filter paper (The Eaton-Diekeman Company, Mt. Holly Spring, Pa. Grade 513). To the clear filtrate were added 85 mg cysteine, 1.36 g sodium acetate, and 186 mg EDTA and the pH was adjusted to 5.5 with either 1 N HCl or 1 N NaOH. Papain, 20 mg, was then added and the solution incubated for 14 hr at 50°C. Trichloroacetic acid, 10 g, was added and the resulting precipitate removed by centrifugation. The supernatant solution was then dialyzed against 10 liters of deionized water at 4°C.

Sodium chloride was added to the dialyzed solution to a concentration of 0.03 M and the mucopolysaccharides were precipitated by the addition of 20 mg of cetyl pyridinium chloride. After 1 hr at 37°C, the AMPS—cetyl pyridinium complex was centrifuged at 15,000 *g* and the precipitate dissolved in 10 ml of 2 M NaCl and 1 ml of methanol. The AMPS were then precipitated by the addition of 2 volumes of ethanol. After 1 hr at 4°C, the suspension was centrifuged for 30 min at 15,000 *g* and the precipitate dried by washing twice with 5-ml aliquots of 80% ethanol, absolute ethanol and finally with ether. The residue was then stored in a desiccator under vacuum.

The dry weight of residues obtained from the urine of 10 patients with progressive exophthalmos, 5 patients with hyperthyroidism without exophthalmos, and 10 normal adults, is given in Table I. On the average twice as much material was obtained from the urine of the patients as from the normal controls. The increase did not seem to be related to thyroid function or the presence of long-acting thyroid stimulator (LATS) in serum.

A partial characterization of the mucopolysaccharides in the material isolated from urine is shown in Table II. There is a proportional increase in hexosamine, uronic acid, and sulfate. The ratio of glucosamine to galactosamine also remains the same. Similar electrophoretic patterns were obtained from normal and exophthalmic urinary AMPS. Electrophoresis of both samples in acetate buffer revealed several components, the major ones corresponding in mobility to chondroitin and chondroitin sulfates; no hyaluronic acid was detected. No chondroitin sulfate B was observed after electrophoresis in zinc sulfate solution.

TABLE I

Excretion of AMPS in Urine

Normal			Hyperthyroidism			Exophthalmos			
Subject	Sex	AMPS	Subject	Sex	AMPS	Subject	Sex	AMPS	LATS in serum*
		mg/24 hr			*mg/24 hr*			*mg/24 hr*	
1	M	2.7	11	M	3.0	16	F	6.4	Pos.
2	M	1.0	12	M	3.0	17	M	11.4	Pos.
3	M	1.2	13	M	3.2	18	F	7.9	Pos.
4	M	5.3	14	F	0.5	19	M	7.5	Neg.
5	M	5.3	15	F	2.8	20	F	8.5	Pos.
6	F	4.4				21	F	10.7	Neg.
7	F	4.5				22	M	14.5	Neg.
8	F	4.2				23	M	7.4	Pos.
9	F	5.4				24	F	6.5	Neg.
10	F	5.4				25	F	6.8	Neg.
	Average	3.94		Average	2.5		Average	8.78	

Clinical information is given in Addendum.

* LATS detection was performed by the method of McKenzie (12).

TABLE II

Analysis of AMPS in Urine

Sample	Dry weight	Hexosamines			Uronic acid	Sulfate	Sulfate-hexosamine molar ratio
		Total	Galactosamine	Glucosamine			
	mg		*mg*		*mg*	*mg*	
Pooled normal	39.4	11.0	9.4	1.7	12.5	4.1	0.67
Pooled exophthalmos	87.8	22.5	18.7	3.8	27.5	9.0	0.72

Characterization of urinary mucopolysaccharides

The pooled material, the partial analysis of which is seen in Table II, was dissolved in H_2O (10 mg/ml) and analyzed for various AMPS as follows.

Chondroitin sulfate B and hyaluronic acid. Chondroitin sulfate B and hyaluronic acid are known to be selectively precipitated by Benedict's reagent (13). The addition of Benedict's reagent to a solution (10 mg/ml) of AMPS from normal or exophthalmos urine did not result in a precipitate, indicating the presence of little if any of these two polymers (artificial mixtures of AMPS containing 1% of each of these two polymers gave visible precipitates). Further evidence for a low level of chondroitin sulfate B was obtained by analysis for L-iduronic acid, a characteristic component of this polymer. Only traces of L-iduronic acid and its lactone were found in hydrolysates of both samples of AMPS (0.1 N HCl for 10 min at 100°C [14]) as revealed by paper chromatography with solvent I.

Chondroitin, chondroitin sulfate A, and chondroitin sulfate C. Aliquots (5 mg) of urinary AMPS were chromatographed on a DEAE-Sephadex column as described in Methods. Elution of AMPS by a gradient of NaCl was followed by means of the carbazole reaction (7). Most of the carbazole-positive material was eluted as a single peak at about 0.8 M NaCl. This material was pooled and an aliquot analyzed for amino sugar by hydrolysis in 4 N HCl for 6 hr at 100°C and subsequent paper chromatography with solvent II. Only galactosamine was detected, indicating the possible presence of chondroitin, chondroitin sulfate A, and chondroitin sulfate C and eliminating other glucosamine-containing AMPS (chondroitin sulfate B, the only other AMPS that contains galactosamine, was eliminated as a possibility by the results presented in the previous section). If it is assumed that this fraction contains only these three AMPS, their relative concentration can be determined as follows: galactosamine determination gives the total AMPS; sulfate determination gives chondroitin sulfate A + chondroitin sulfate C (assuming one sulfate/hexosamine) and the Morgan Elson reaction after hyaluronidase treatment measures chondroitin and chondroitin sulfate C (15) (assuming complete enzymatic hydrolysis) as the disaccharides from chondroitin sulfate A do not react in the Morgan Elson test. By using the above methods and assumptions the ratios of the three AMPS in the two samples were found to be quite similar. For the normal exophthalmic AMPS fraction the values were, respectively, 25 and 27% chondroitin, 28 and 25% chondroitin sulfate A, and 30 and 30% chondroitin sulfate C.

Keratosulfate. As keratosulfate is the only mucopolysaccharide that contains galactose instead of uronic acid, its level could be estimated from the galactose content of the pooled material. Samples of AMPS, 2 mg, were hydrolyzed in 1.0 ml of 1 N sulfuric acid at 100°C for 14 hr (17). The solution was neutralized with saturated $Ba(OH)_2$, the precipitate of $BaSO_4$ removed by centrifugation, and the supernatant solution deionized by passage through a small column of Amberlite MB-3. The sugars in the effluent were then separated by paper chromatography with solvent III. The galactose area of the chromatogram was eluted with H_2O and galactose quantitated by the anthrone reaction (18).[1] Using purified keratosul-

[1] Other mucopolysaccharides contain galactose in the linkage region with protein. With the described technique galactose represents 1% of the sugars in chondroitin sulfate.

TABLE III

Type of AMPS in Urine

Mucopolysaccharide	Normal	Exophthalmos
	% of total	*% of total*
Hyaluronic acid	<1	<1
N-sulfated mucopolysaccharides (calculated as heparitin sulfate)	8	13
Keratosulfate	5	5
Chondroitin sulfate B	<1	<1
Chondroitin, Chondroitin sulfate A, Chondroitin sulfate C	85	80

fate carried through the same procedure as a standard, we estimated that the AMPS of both normal and exophthalmos urine was 5% keratosulfate.

N-Sulfated mucopolysaccharides. *N*-sulfated mucopolysaccharides in the pooled samples were estimated by the method of Lagunoff and Warren (19) using purified heparitin sulfate as a standard (heparitin sulfate is the only *N*-sulfated AMPS found in normal urine [16]). With this assumption, the data show that 8% of the normal urinary AMPS and 13% of the exophthalmic urinary AMPS is present as heparitin sulfate.

The results of these analyses are summarized in Table III. The distribution of AMPS in the urine of exophthalmic patients is similar to that found in normal urine.

Blood serum mucopolysaccharides

5 ml of 10 sera from the patients with exophthalmos, 5 ml of 5 sera from patients with hyperthyroidism without exophthalmos and 5 ml of 10 sera from 10 control adult volunteers were analyzed for their content of AMPS. Sera were heated to 100°C in a water bath for 10 min. 20 mg of trypsin, 15 ml of distilled water, and 0.2 ml of toluene were added and the samples were incubated at 37°C for 24 hr. Residual protein was then removed with 10% trichloroacetic acid. Further steps in the isolation of the AMPS with NaCl, cetyl pyridinium chloride, alcohol, and ether were identical to those described under "Isolation of Urinary Mucopolysaccharides" except for the addition of 5 mg instead of 20 mg of cetyl pyridinium chloride. The dry residue was dissolved in 1 ml of distilled water and the uronic acid content determined.

As shown in Table IV, the amount of AMPS in the serum of exophthalmic patients is three times the amount found in the normal controls and in the patients with hyperthyroidism without exophthalmos.

DISCUSSION

An increase in urinary excretion of AMPS has been described in studies of rheumatoid arthritis (20), lupus erythematosus (21), some types of cancer (22, 23), Marfan's syndrome (24), and Hurler's syndrome (25, 26). In patients with

TABLE IV

Total Amount of AMPS in Blood Serum

	Uronic acid (μg) per 100 ml serum				
Subject	Normal	Subject	Exophthalmic	Subject	Hyperthyroidism
1	220	11	600	21	230
2	180	12	630	22	400
3	210	13	455	23	160
4	250	14	1350	24	200
5	340	15	330	25	275
6	170	16	340	26	120
7	205	17	770		
8	230	18	630*		
9	220	19	1380*		
10	250	20	570		
	Average 227		Average 750		Average 232

* These patients also had pretibial myxedema.

Hurler's syndrome only chondroitin sulfate B and heparitin sulfate are increased, while in Marfan's syndrome there is an increase in all mucopolysaccharides. As described in this paper, all urinary mucopolysaccharides are also increased when exophthalmos is present. In this condition mucopolysaccharides containing sulfoamino groups (heparin and/or heparitin sulfate) may be increased to a proportionately greater amount than other AMPS.[2]

Greater than normal amounts of plasma mucopolysaccharides have been found in patients with rheumatoid arthritis, and in those with inflammatory conditions secondary to bacterial infection or traumatic injuries (27, 28). The increase of serum AMPS in exophthalmos described in this communication is greater than the increase thus far described in other diseases.

The increased AMPS in the serum and urine of patients with exophthalmos is unrelated to thyroid function or plasma LATS in the patients described in this communication. It appears, therefore, that whatever the etiology of exophthalmos, its appearance in severe form is accompanied by increased mucopolysaccharide in blood and urine.

ADDENDUM

Subjects 1 through 10 are normal adult volunteers of both sexes.

Subjects 11 through 15 have untreated hyperthyroidism without exophthalmos.

Subject 16, aged 43, had the onset of hyperthyroidism 9 yr ago and was treated with propylthiouracil, after which he developed exophthalmos. Signs of hyperthyroidism persisted until treatment with 25 mc of ^{131}I 8 yr ago. Severe, fluctuating exophthalmos has continued to the present with proptosis (with a Hertel ophthalmometer) measuring 22 mm, right eye (R.E.) and 25 mm, left eye (L.E.).

Subject 17, aged 43, has a 2 yr history of hyperthyroidism with exophthalmos treated with two doses of ^{131}I (5 mc) after which exophthalmos progressed and pretibial myxoedema developed. Proptosis measures 20 mm (R.E.) and 23 mm (L.E.).

Subject 18, aged 47, has had an 8 yr history of hyperthyroidism treated with 5 mc of ^{131}I with disappearance of clinical and laboratory signs of hyperthyroidism but exophthalmos has progressed in severity since treatment. Proptosis measures 26.5 (R.E.) and 26 mm (L.E.).

Subject 19, aged 42, had the onset of hyperthyroidism 5 yr ago, and at that time was treated with subtotal thyroidectomy but exophthalmos and pretibial myxoedema developed subsequently. Normal thyroid function returned 4 yr ago after two doses of 5 mc of ^{131}I. Exophthalmos and localized changes of the pretibial skin has persisted until the present. Proptosis measures 24 mm bilaterally (R.E. and L.E.).

Subject 20, aged 43, has had severe exophthalmos and hypothyroidism for 14 yr, and pretibial myxoedema for 10 yr. Treatment has been 3 g of thyroid powder a day. The patient has a high level of circulating thyroglobulin antibodies (red cell agglutination test is positive at a dilution of 1/25,000). Proptosis measures 29 mm bilaterally (R.E. and L.E.).

Subject 21, aged 54, has had a 4 wk history of hyperthyroidism and progressive exophthalmos without treatment. Proptosis measures 28 mm (R.E.) and 29 mm (L.E.).

Subjects 22 and 23, aged 47 and 31, respectively, have had untreated hyperthyroidism and progressive exophthalmos for 5 wk. Proptosis in subject 22 measures 28 mm (R.E.) and 27 mm (L.E.), and in subject 23 24 mm bilaterally (R.E. and L.E.).

Subject 24, aged 35, has a 1 yr history of hyperthyroidism treated with 5 mc of ^{131}I. Exophthalmos appeared after treatment. Proptosis measures 22 mm (R.E.) and 21 mm (L.E.).

Subject 25, aged 50, had hyperthyroidsm 30 yr ago, treated with subtotal thyroidectomy. 4 months ago he had recurrences of symptoms of hyperthyroidism and in addition exophthalmos. Proptosis measures 23 mm (R.E.) and 21 mm (L.E.).

[2] Iversen and Asboe Hansen (1) found that mast cells (which produced heparin) accumulate in the retrobulbar tissues of exophthalmic patients.

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