

The Glycosaminoglycans of Human Plasma

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ABSTRACT A method is proposed for the measurement of glycosaminoglycans (GAG) on 5–10 ml of plasma. It is based on the adsorption of GAG on small ECTEOA columns followed by measurement of the hexuronic acid in the NaCl eluates. Routine use of the method has indicated the presence of a GAG fraction that adsorbs readily on ECTEOA ("free" GAG) and of another that adsorbs on it only after treatment with papain ("bound" GAG). "Free" and "bound" GAG have been measured in normal adults, normal children, and children affected by mucopolysaccharidosis type I; the results obtained are in good agreement with those previously reported in the literature.

Various analyses performed on purified "free" and "bound" GAG have confirmed that chondroitin-4-sulfate is the main glycosaminoglycan of normal human plasma where it occurs both free and bound to protein and at various levels of sulfation. The presence of small amounts of heparan sulfate and keratan sulfate has also been demonstrated.

Metabolic experiments performed in rabbits have indicated that plasma GAG derive from peripheral tissues and increase sharply after papain injection. In young animals the "free" GAG have a faster turnover than the "bound," possibly a reflection of active processes of remodeling and calcification. The synthesis of the "free" and "bound" GAG, as measured with ^{35}S -sulfate incorporation, seems to proceed at the same rate, and the hypothesis has been advanced that as a result of the action of tissue proteases, part of the "bound" GAG may be transformed into "free" GAG, the latter being immediately extruded from the tissues into the circulatory system.

INTRODUCTION

The presence of glycosaminoglycans (GAG) (1)¹ in human plasma has been demonstrated in various labora-

Received for publication 15 August 1968.

¹The nomenclature suggested by Balazs and Jeanloz (1) has been used.

tories. In 1955 Badin, Schubert, and Vouras (2) isolated material containing hexuronic acid and hexosamine in equimolar amounts from the "euglobulin" fraction of human plasma. With their procedure, the average values for normal individuals and for patients with active rheumatoid arthritis were found to be, respectively, 206 and 388 μg of hexuronic acid/100 ml of plasma.

In the same year, Bassiouni (3) extracted from 420 ml of normal human blood a material which on paper electrophoresis could be separated into a component moving at the same rate as cartilage chondroitin sulfate and another with lower mobility.

In 1957 Bollet, Seraydarian, and Simpson (4), using a modification of Badin's method, studied 34 normal adults and obtained values ranging from 215 to 385 μg of hexuronic acid/100 ml of plasma. No apparent differences were found between males and females or between individuals of different age. The material isolated had a hexosamine:hexuronic acid ratio of 1.3 (72% galactosamine, 28% glucosamine) and could be separated by paper chromatography with various solvents into two components, both depolymerized by testicular hyaluronidase; one behaved like chondroitin sulfate and had a hexosamine:hexuronic acid ratio of 1.1, whereas the other was not conclusively identified and had a hexosamine:hexuronic acid ratio of 1.5.

Working with purified plasma protein fractions, Kerby (5) demonstrated in 1958 that plasma GAG are present only in Cohn fractions III and IV-1, where they seem to be bound to the protein rather than to the lipid components of the fractions. After tryptic digestion and precipitation with cetyltrimethyl ammonium bromide, the GAG could be separated by paper chromatography into two fractions, corresponding to those described by Bollet et al. (4).

Isolation and purification of GAG from human plasma or "euglobulin" fraction provided Schiller (6) with material that could be separated electrophoretically into two fractions. The faster moving one was identified as chondroitin-4-sulfate on the basis of analytical data, infrared pattern, specific rotation, and sensitivity to testic-

ular hyaluronidase. The slower moving one (containing N, hexosamine, hexuronic acid, and ester sulfate in 1.00, 0.99, 0.71, 0.54 ratio) was scarcely sensitive to testicular hyaluronidase and remained unidentified. A concentration of 310-440 μg of hexuronic acid/100 ml of normal human plasma was found in this study.

Quite recently, the method of Bollet et al. (4) was employed by Friman and Brunish (7) to isolate plasma GAG that were subsequently analyzed by electrophoresis on cellulose acetate strips. The major component, representing 44-100% of the total GAG, was found to behave like chondroitin sulfate; occasionally slower components, with the mobility of hyaluronic acid or with intermediate mobilities, were also found. The results, although essentially qualitative, indicated that a great variability of plasma GAG may exist among different individuals.

The present study has been undertaken for the following reasons:

(1) to devise a simple method which could be of value in routine clinical determination of plasma GAG.

(2) to investigate the nature of the association between GAG and plasma proteins. In the method of Badin et al. (2), tryptic digestion of the "euglobulin" fraction was emphasized as a preliminary step to the precipitation of GAG. In the method of Bollet, however, the "euglobulin" fraction was treated for 30 min with 0.4 M perchloric acid at room t° , a treatment that could hardly break covalent bonds between protein and GAG. Nevertheless, the values obtained in the two laboratories are quite compatible, a fact that seems to indicate either that plasma GAG are not linked covalently to proteins or that the protein moiety does not affect the solubility of the complex in perchloric acid.

(3) to attempt separation and identification of the various fractions of plasma GAG which have been demonstrated with various techniques in different laboratories. To this extent, we have used for measurement of plasma GAG a modification of a method independently described by Wessler (8) and by one of us (9) for the measurement of urinary GAG.

METHODS

Blood samples were obtained from medical students and laboratory personnel who were free from acute illness and without history of chronic disease. Blood samples from patients with mucopolysaccharidosis type I (10) and from young, mentally retarded patients were obtained through the kindness of Dr. Buford Nichols, Department of Pediatrics, Baylor University College of Medicine, and Dr. Gordon Farrell, Texas Research Institute of Mental Sciences.

Measurement of plasma GAG. The technique used for duplicate measurement consists of the following steps:

(a) 20 ml of blood is added to 0.20 ml of aqueous EDTA solution (EDTA, tetrasodium salt, 7.98 g, EDTA, 2.05 g/100 ml; final pH 7.10). After mixing, plasma is obtained by centrifugation. Four 0.7×2 cm columns of ECTEOLA²

²ECTEOLA cellulose, the reaction product of cellulose with epichlorohydrin and triethanolamine, can be obtained

in the OH^- form (8) are prepared as described (see *Standardization of ECTEOLA and preparation of columns*, below) and labeled Nos. 1-4.

(b) Two equal aliquots of each plasma sample (5 ml each or less) are passed through columns 1 and 2 at a flow rate of 5 ml/hr. Each column is washed with 15 ml of 0.9% NaCl and then closed to prevent dryness. The effluent from each column (plasma plus washing) is collected in a 25 ml Erlenmeyer flask, to which cysteine HCl 0.5 M and EDTA solution (0.20 ml each) are added. The pH is adjusted to 6.20-6.40 with 2.5 N acetic acid, and 2 mg of twice crystallized papain³ are added to each flask before incubation at 56°C overnight.

(c) The content of each flask is centrifuged for 15 min at $8000 \times g$, and the supernatants are applied to columns 3 and 4 at a flow rate which allows complete drainage in about 1 hr. Each sediment remaining after centrifugation is washed once with 3-ml of 0.9% NaCl, twice with 1.5 ml of 1.0 M NaCl, and eventually with 5 ml of 0.9% NaCl. The washings from each sediment are pooled, diluted to final 0.15 M NaCl concentration (0.9% NaCl), and applied to the respective column. The four ECTEOLA columns are then washed with 100-ml of 0.9% NaCl, at a flow rate of 40-50 ml/hr, and the flow is stopped as soon as the top layer of the cellulose becomes exposed.

(d) For the elution, 3 ml of 2.0 M NaCl is delivered onto each column, and the effluent is collected into calibrated Shevsky-Stafford tubes in two approximately equal fractions. The borate-carbazole reaction is performed in duplicate on aliquots from each tube, using an aqueous solution of glucuronolactone as a standard. As previously stated (9), despite the high salt concentration of the eluting solution, no depression of color formation was observed. The values obtained are expressed as μg of hexuronic acid/10 ml of plasma (Tables I and II). Whereas in normal individuals all the plasma GAG are found in the first 2.0 M NaCl fraction, in patients with high levels of plasma GAG the second fraction is also positive.

Standardization of ECTEOLA and preparation of columns. ECTEOLA "standard type" was used. A lot with a stated capacity of 0.40 meq/g is desirable because it allows the use of short columns which require small volumes of eluant. However, since it is difficult to obtain ECTEOLA of such high capacity, it is necessary to standardize the type available in order to establish the smallest volume compatible with a complete retention of the GAG present in the plasma sample.

Two methods of standardization have been used. 200-300 μg of chondroitin sulfate in 0.9% NaCl are passed through a column of known dimensions, according to the procedure described under (b). The amount of material which is not retained on the column and appears in the washings or the amount which is retained on the column and is eluted with 2.0 M NaCl is measured with the borate-carbazole reaction. Alternatively, 5-ml of plasma digested with papain are passed through a column of known dimensions, following the procedure described under (c). The effluent and column washings are passed through a second column of identical dimensions. Upon elution of the columns, the presence or absence of hexuronic acid-containing material in the eluate of the second column indicates whether or not the retention of the first column was adequate and if its dimensions could be further reduced.

from Schleicher and Schuell Co., Keene, N. H., or Brown Paper Co., Berlin, N. H.

³Papain $\times 2$ crystalline, Nutritional Biochemicals Corp., Cleveland, Ohio.

New ECTEOLA is initially washed with 1 N HCl and distilled water. Before use it is washed with 1 N NaOH and distilled water, and suspended in 0.9% NaCl. Used ECTEOLA is regenerated with 1 N NaOH but it should be standardized periodically to insure against loss of retentive capacity. The ECTEOLA suspension is poured into columns consisting of a 15 ml centrifuge tube sealed to a 10 cm long Pyrex tube having an internal diameter of 7-mm (Fig. 1). The end of the tube is fitted in the large opening of a Teflon Multifit connector⁴ which contains a porous polyethylene disc for support of the cellulose and a lower opening which accommodates a 5 cm length of polyethylene tubing,⁵ 1.0-mm internal diameter. The column flow is regulated by means of a screw compressor clamp applied to the polyethylene tubing.

Recovery tests. Recovery tests were performed by applying to 0.7 × 2 cm ECTEOLA columns 200 μg of chondroitin-4-sulfate, 200 μg of chondroitin-4-sulfate, and 200 mg of crystalline bovine albumin, or a papain digest of 200 μg of chondroitin-4-sulfate and 200 mg of crystalline bovine albumin. The papain digestion was performed as specified under (b) and (c). The columns were washed and eluted as described under (d), and the recoveries were calculated in terms of hexuronic acid (Table IV).

Isolation and identification of GAG. Isolation was achieved by a large scale modification of the method described. Duplicate 634-ml aliquots of pooled plasma (containing 50 μg of total GAG, as hexuronic acid/10 ml⁶) were applied to 2 × 15 cm columns of ECTEOLA in the OH⁻ form (columns P1 and P2), which were washed with 0.9% NaCl until the absorbance at 278 mμ was negative. The columns were then eluted with 4.0 M NaCl, and the hexuronic acid-positive peaks from the two columns were combined, desalted, and concentrated by ultrafiltration through a UM-1 Diaflo membrane,⁷ and eventually lyophilized. Redissolved in a small volume of water, the material was passed through several 2 × 14 cm columns of SE Sephadex C-50 medium (11), equilibrated, and eluted with a pH 4.0 citrate-phosphate buffer, 0.0062 M. The ratio between absorption at 278 mμ and hexuronic acid content of the effluent from each column was measured, and the GAG were considered to be free of contaminating protein when such a ratio could not be reduced further. At this point, electrophoresis on acetate cellulose strips (0.05 M borate buffer, pH 7.6, 150 v for 30 min at room temperature) indicated two rapidly migrating, poorly resolved areas which could be stained with Alcian blue (0.05% in 0.2 M MgCl₂) and absence of material which would stain with Thiazine red (0.1% in 1% acetic acid). The material thus obtained, referred to as "free GAG" (see Discussion), was used for the identification procedures.

The effluents and washes from columns P1 and P2, adjusted to pH 6.2-6.4, were separately digested with 1.5 g of crude, activated papain (12) for 68 hr at 56°C. The digests were

⁴Multifit Teflon connector No. 3020-3/8" to B, or the entire column is available from Glass Engineering Co., Inc., Houston, Texas.

⁵PX039 Polyethylene, Becton, Dickinson and Co., Rutherford, N. J.

⁶The amounts of GAG found in blood bank plasma are consistently higher than those found in freshly drawn blood. These higher values might be due either to destruction of white cells, with liberation of GAG (3), or to variation of the level of circulating GAG in relation to food intake (4). Our subjects were bled either in mid-morning or mid-afternoon and were not fasting, whereas the blood donors were fasting and were bled in the early morning.

⁷Amicon Corp., Lexington, Mass.

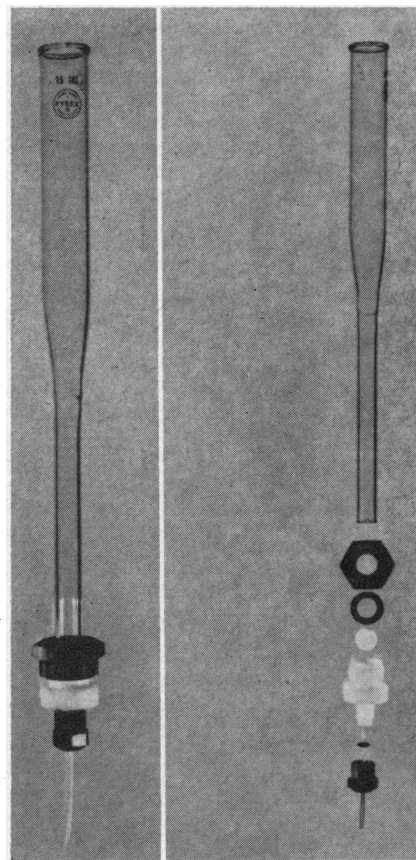


FIGURE 1 Details of the column used for chromatography of plasma GAG on ECTEOLA. The internal diameter of the cylindrical part of the glass tube is 0.7 mm.

cleared by centrifugation at 8000 *g*, and the residues were washed and extracted with 1.0 M NaCl as described under (c). The two digests and their diluted washings were passed through 2 × 15 cm ECTEOLA columns (columns P3 and P4), were washed, and eluted as described for columns P1 and P2; the hexuronic acid-positive peaks from the two columns were combined, desalted, concentrated by ultrafiltration, and lyophilized. Electrophoresis on acetate cellulose strips demonstrated two fast-migrating, well-separated Alcian blue-positive areas and a slow-moving, Thiazine red-stainable area. The material thus obtained, referred to as "bound GAG" (see Discussion), was used for the identification procedures.

The total amount of "free GAG" (1210 μg of hexuronic acid) was divided into two equal aliquots, and one of them was incubated with 100 μg (30 USP U) of bovine testicular hyaluronidase for 20 hr at 37°C in 0.1 M acetate buffer, pH 4.85, 0.1 M in NaCl. Thereafter, the digested and non-digested aliquots were each applied to a 0.9 × 3 cm column of Dowex 1 × 2, 200-400 mesh, Cl⁻ form, and each column was eluted with increasing concentrations of NaCl (13). Elution with a given concentration of NaCl was continued as long as hexuronic acid-containing material was eluted from the column. The recovery of hexuronic acid from the two columns was quantitative, and the patterns of elution obtained are illustrated in Fig. 2. Each pattern of elution was divided

TABLE I
*Plasma GAG of Normal Adults, Expressed as μg of Hexuronic Acid/10 ml of Plasma**

	Age	Free	Bound	Total
	yr			
Normal males				
1 C. A.	25	4.4	16.4	20.8
2 B. H.	23	5.8	28.3	34.1
3 G. C.	33	4.2	9.1	13.3
4 J. E. C.	23	3.8	12.9	16.7
5 C. K.	23	3.6	7.6	11.2
6 R. E. N.	24	6.3	17.2	23.5
7 R. B.	25	4.9	10.1	15.0
8 S. R. A.	22	6.7	27.0	33.7
9 T. M. B.	24	6.3	22.7	29.0
10 R. O.	23	5.5	32.8	38.3
11 P. K. N.	23	5.3	46.6	51.9
12 J. K.	23	6.6	32.5	39.1
13 G. J. L.	22	8.9	32.0	40.9
14 D. P.	24	9.6	34.5	44.1
15 T. W.	25	9.0	36.8	45.8
16 L. M.†	33	5.9	14.8	20.7
Mean \pm SD		6.05 \pm 1.82	23.8 \pm 11.61	29.8 \pm 12.85
Normal females				
1 P. D.	34	7.0	10.0	17.0
2 M. B.	26	6.0	9.3	15.3
3 H. S.	28	4.5	15.1	19.6
4 L. C.	27	2.4	15.4	17.8
5 W. B.	26	5.0	18.7	23.7
6 D. P.	21	9.7	16.0	25.7
7 T. G.	22	5.0	25.6	30.6
8 B. D.	19	5.3	23.0	28.3
9 S. T.	22	5.3	32.2	37.5
10 B. M.	23	4.5	5.3	9.8
11 J. M.	42	7.5	31.5	39.0
12 J. B.	33	7.2	9.4	16.6
13 J. H.	24	4.5	17.6	22.1
14 S. S.	22	7.4	12.8	20.2
15 P. K.	23	5.9	26.5	32.4
16 J. L.	23	5.7	19.8	25.5
17 L. S.	21	5.8	19.8	25.6
18 F. M.†§	20	10.0	19.2	29.2
19 F. B.†§	19	9.4	31.0	40.4
		6.9	19.0	25.9
20 S. F.†§	17	8.2	29.0	37.2
		5.3	24.6	29.9
21 C. W.†§	19	4.6	13.2	17.8
		5.4	11.5	16.9
22 A. F.†	26	8.3	21.3	29.6
Mean \pm SD		6.27 \pm 1.86	19.07 \pm 7.50	25.34 \pm 8.14

* Each value represents the mean of duplicate determinations.

† Mentally retarded but without known metabolic defect.

§ Repetitive measurements were performed on different days or weeks.

into several peaks (Fig. 2), and each peak was desalted, lyophilized, and assayed for its hexuronic acid content.

The total amount of "bound GAG" (5176 μg hexuronic acid) was dissolved in 10 ml of distilled water. 1-ml aliquot was incubated with 100 μg of testicular hyaluronidase under the conditions described. Thereafter, the digest and another equivalent, nondigested sample were fractionated on Dowex 1 \times 2 columns as described for the "free GAG." The recoveries were quantitative, and the patterns of elution, as well as the division into various peaks, are illustrated in Fig. 3.

The material present in each peak was used for the following analytical procedures: (1) hexuronic acid, performed with the carbazole method of Dische (14), the

borate modification of Bitter and Muir (15), and the naphthoresorcinol method of Pelzer and Staib (16); (2) total amino sugars and glucosamine:galactosamine ratio performed after hydrolysis in 4 N HCl for 8 hr at 100°C with the Technicon amino acid analyzer, using a 0.6 \times 30 cm cation exchange column and the ninhydrin reagent; (3) 2-deoxy-2-sulfoamino-hexose, with the indole reaction of Dische and Borenfreund (17), performed after hydrolysis and deamination with nitrous acid for 80 min, according to Lagunoff and Warren (18). The possible presence of 2-deoxy-2-amino-hexose was assessed by performing acetylation of the material before hydrolysis with nitrous acid; (4) neutral sugar, measured with the anthrone method (19); (5) total sulfur, measured according to a modification of

TABLE II
*Plasma GAG of Normal Children and Patients with Mucopolysaccharidosis Type I,
Expressed as μg of Hexuronic Acid/10 ml of Plasma**

	Age	Free	Bound	Total
	yr			
		Normal children		
Males				
N. A.	8	8.0	34.5	42.5
S. L.†	14	8.6	30.5	39.1
		9.7	29.5	39.2
G. W.	8	7.3	29.3	36.6
Mean \pm SD		8.40 \pm 1.02	30.95 \pm 2.42	39.35 \pm 2.42
Females				
K. L.†	7	13.6	23.5	37.1
		10.9	39.5	50.4
F. A.	8	9.5	24.1	33.6
G. V.	14	12.4	35.4	47.8
Mean \pm SD		11.60 \pm 1.78	30.62 \pm 8.06	42.22 \pm 8.14
		Patients with mucopolysaccharidosis type I		
Males				
T. C.	7 months	23.0	38.2	61.2
H. T.†	4	24.4	46.0	70.0
		23.3	38.0	61.3
J. F.†	10	15.0	41.5	56.5
		13.6	62.4	76.0
		21.0	52.5	73.5
		29.5	71.0	100.5
		23.0	61.0	84.0
		23.5	61.1	89.6
		27.3	62.8	90.1
Mean \pm SD		22.32 \pm 4.87	53.95 \pm 12.30	76.27 \pm 14.55
Females				
C. H.†	5	14.9	29.1	44.0
		12.7	29.8	42.5
G. C.†	10	31.0	38.0	69.0
		27.0	49.0	76.0
		5.3	28.0	43.3
Mean \pm SD		18.18 \pm 10.59	36.78 \pm 8.06	54.96 \pm 16.21

* Each value represents the mean of duplicate determinations.

† Repetitive measurements were performed on different days or weeks.

TABLE III
Recovery of Chondroitin-4-Sulfate Applied to a 0.7 × 2 cm ECTEOLA Column, OH⁻ Form, Either Alone or with Bovine Albumin*

Chondroitin-4-sulfate	Crystalline bovine albumin	Recovery
μg hexuronic acid	mg	%
47.40	0	105.0
47.40	0	103.0
48.10	200	99.2
51.60	200	98.8
48.10	200 + 1 mg crystalline papain†	100.5
48.10	200 + 1 mg crystalline papain†	98.0
48.10	200 + 1 mg crystalline papain†	99.6

* The columns were washed and eluted as specified in Methods under (d).

† Incubation with papain was performed as described in Methods under (b) and (c).

the method of Antonopoulos (20); (6) infrared analysis performed with the Beckman IR-9 spectrophotometer, using 10–20 μg of material dried on an Irtran[®] plate.

The results of the various analyses are presented in Tables V and VI.

Metabolic experiments. 500 μc of carrier-free sodium sulfate-³⁵S in water were injected i.m. into two New Zealand white rabbits (Nos. 1 and 2, 6–8 wk old, 1.36 kg body wt) which were bled by cardiac puncture at various intervals after the injection. The injections of radioactive isotope were repeated 54 and 108 hr after the first injection. 72 hr after the last ³⁵S injection, the rabbits were injected intravenously with 1 ml of 1% crude papain in water (21), and they were bled 6, 24, 48, and 72 hr later. All the blood samples were processed for the measurement of plasma GAG with the method described, using 0.9% NaCl containing 0.005 M Na₂SO₄ for the preparation and washing of the columns. The specific activity (cpm ³⁵S/μg hexuronic acid) of the “free” and “bound” GAG was measured as follows: duplicate 100 μl aliquots of each eluate were added to 5-ml of 2-methoxyethanol and 10-ml of toluene containing 15 g of 2,5-diphenyloxazole (POP) and 50 mg of 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP)/liter. The radioactivity was measured in a Packard Tri-Carb Liquid Scintillation spectrometer, Model 3003. Corrections for decay of the isotope were made with appropriate standards, and absence of quenching was assessed with an external standard device. The results obtained are presented in Table VI. The same experiment was performed on two older rabbits (Nos. 3 and 4, 10–12 wk old, 1.7 and 2.1 kg body wt) using 250 μc of carrier-free sodium sulfate-³⁵S for each injection. The results obtained are presented in Table VII.

RESULTS

Tables I and II present the values for plasma GAG (expressed as μg of hexuronic acid/10 ml of plasma) obtained from normal adults, normal children, and children affected by mucopolysaccharidosis type I.

^a Beckman Instruments, Inc., S. Pasadena, Calif.

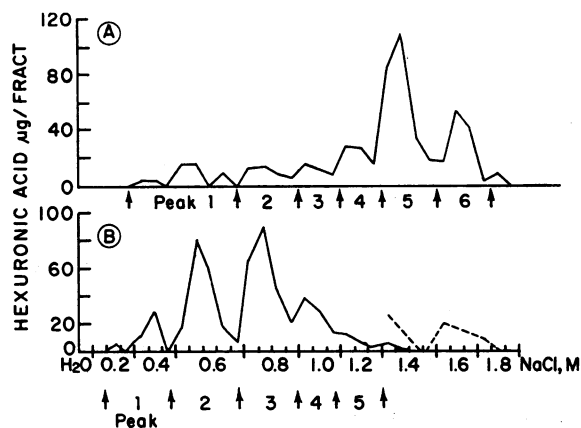


FIGURE 2 Fractionation of “free” plasma GAG on Dowex 1 × 2 columns. (A) Before treatment with hyaluronidase; recovery 98.5%. (B) After treatment with hyaluronidase; recovery 97.1%. (—) = hexuronic acid; (---) = galactose.

The results of the recovery tests (Table III) indicate that chondroitin-4-sulfate is quantitatively retained on, and eluted from, ECTEOLA columns independently from the presence of the enzyme papain, bovine albumin, or its products of proteolytic digestion. The fractionation of the “free” and “bound” GAG on Dowex 1 × 2, before and after incubation with testicular hyaluronidase, is illustrated in Figs. 2 and 3. It is evident that the “free” GAG are resolved into a more complicated fractionation pattern than the “bound” GAG and that the

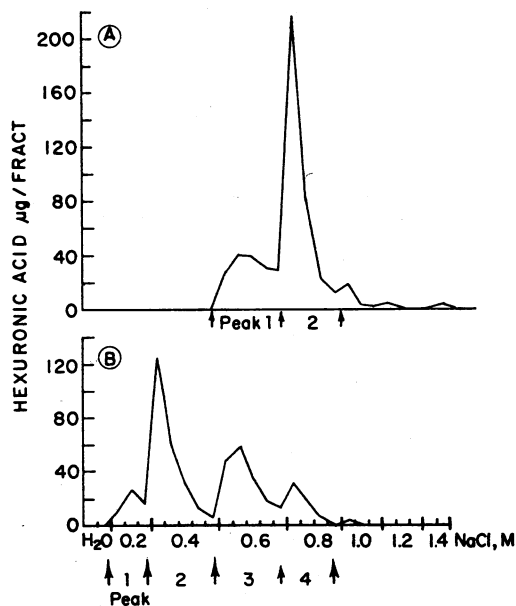


FIGURE 3 Fractionation of “bound” plasma GAG on Dowex 1 × 2 columns. (A) Before hyaluronidase treatment; recovery 100%. (B) After hyaluronidase treatment; recovery 101%. (—) = hexuronic acid.

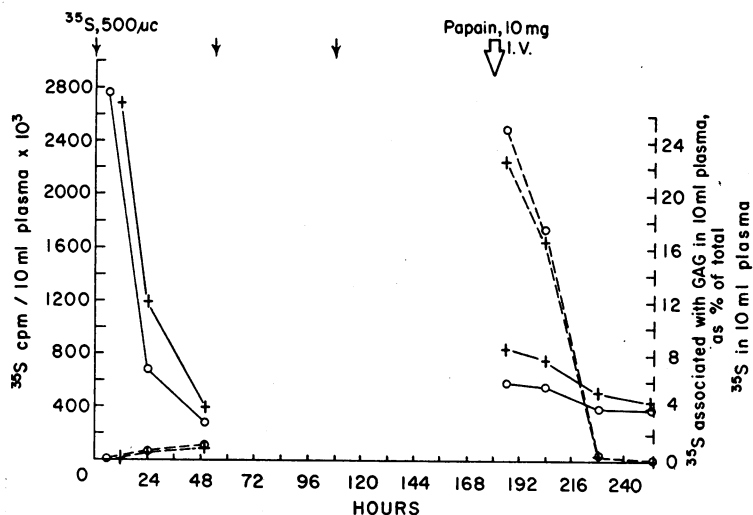


FIGURE 4 Disappearance of ^{35}S from rabbit plasma after injection of $500\ \mu\text{c}$ intramuscularly; (\bigcirc — \bigcirc) = rabbit 1; (+—+) = rabbit 2. Incorporation of ^{35}S into plasma GAG as per cent of total ^{35}S present in plasma; \bigcirc --- \bigcirc = rabbit 1; (+---+) = rabbit 2.

latter are eluted at NaCl molarities lower than those required for the elution of the "free" GAG.

The analytical data of the "free" and "bound" GAG, before fractionation, after fractionation, and before and after treatment with testicular hyaluronidase are presented in Tables IV and V. The results of the metabolic studies on rabbits may be summarized as follows: 6, 12, and 24 hr after the first injection of sulfate- ^{35}S into young rabbits (Table VI), the "free" and "bound" plasma GAG show approximately the same specific activity. 50 hr after the injection, however, the specific

activity of the "free" GAG increases sharply, whereas that of the "bound" continues to decline. After repetitive injections of sulfate- ^{35}S , depletion of tissue GAG with papain is followed by a sharp rise in the level of plasma GAG (the "bound" being more affected than the "free") and by a rapid decrease in the specific activity of both "free" and "bound" plasma GAG. Similar experiments performed in more mature animals produced essentially the same results, with the exception that the specific activity of the "free" GAG did not increase 50 hr after the ^{35}S -labeled pulse-dose (Table VII).

TABLE IV
Fractionation on Dowex 1×2

	Hexuronic acid (borate)	Glucosamine	Galactosamine	Galactosamine		Galactose (anthrone)
				Glucosamine	Sulfur	
Before fractionation	0.87 (15.3)	0.23 (4.1)	0.76 (13.4)	3.27	0.88 (2.7)	0.37 (6.1)
Dowex 1×2 fractions						
0.4, 0.6 M NaCl	0.27	0.45	0.54	1.2		
0.8 M	0.58	0.37	0.62	1.67	1.45	
1.0 M	0.63	0.32	0.67	2.06	0.91	
1.2 M	0.98	0.16	0.83	5.08	1.28	
1.4 M	1.02	0.07	0.92	12.60	1.31	
1.6 M	1.04	0.16	0.83	5.17	1.08	
After hyaluronidase treatment:						
Dowex 1×2 fractions						
0.2, 0.4 M NaCl	0.65	0.31	0.68	1.92		
0.6 M	0.64	0.23	0.76	3.30		
0.8 M	0.97	0.12	0.87	6.80		
1.0 M	0.94	0.19	0.80	4.00		
1.2 M	0.58	0.39	0.60	1.50		1.70
1.4, 1.6, 1.8 M	0.30	0.68	0.31	0.46		0.95

* Values are molar or atomic ratio per mole of total hexosamine. Figures in parentheses are per cent values by weight.

† Per cent of total material applied to column, as hexuronic acid.

‡ Pink rather than violet color appeared with naphthoresorcinol reaction.

Fig. 4 illustrates the amount of radioactivity incorporated into plasma GAG (free plus bound) as per cent of the total amount of radioactivity present in plasma at the time of the bleeding. After the pulse-dose of sulfate-³⁵S, the amount of radioactivity present in rabbit plasma decreases precipitously, while the amount of radioactivity incorporated into GAG increases slowly, to reach a maximum value of 1%, 50 hr after the isotope injection. After additional injections of isotope, mobilization of tissue GAG by i.v. papain produces the appearance in the circulation of considerable amounts of radioactivity, 23% of the total being represented by ³⁵S-labeled GAG. The percentage of radioactivity bound to GAG decreases rapidly, to approach zero values 42 hr after papain injection, as a probable reflection of a rapid rate of GAG excretion and new GAG synthesis from nonlabeled precursors.

DISCUSSION

The average values of total GAG for normal adults are in good agreement with those reported previously by other authors (2, 4, 6). In view of the close agreement between duplicate measurements, the great variability found when repetitive analyses were performed on the same subject on different days should not be considered as due to limitations of the proposed technique, but rather as a possible indication that plasma GAG reflect fairly active metabolic processes taking place at the tissue level.

Our data indicate that significant quantitative differences exist between plasma GAG of normal adults and children ($P < 0.05$), but not between normal males and females, either adults or children. The plasma GAG of male children affected by mucopolysaccharidosis type I are significantly higher than those of normal male children ($P < 0.01$) and those of affected female children ($P < 0.05$).

The results of the recovery tests indicate that in the method described, ECTEOLA readily displaces albumin and papain that may be electrostatically bound to chondroitin sulfate and forms electrostatic bonds of its own with the polyanions. However, a fraction of plasma GAG cannot be retained on ECTEOLA before proteolytic digestion, and it is likely that this fraction is "bound" to protein through bonds other than electrostatic. Evidence provided by previous authors (2, 5, 6, 22) seems to indicate that at least a part of plasma GAG may be covalently linked to proteins. The possibility that the "bound" GAG may be linked to plasma proteins by a larger number of electrostatic bonds does not seem to be supported by the results of the fractionation on Dowex 1 × 2 columns, nor by the analytical and infrared data. In fact, on fractionation of "free" and "bound" GAG on Dowex columns (before hyaluronidase treatment, Figs. 2 and 3), the latter are eluted at NaCl molarities lower than those required for elution of the "free" GAG, and this reflects either a lower charge density or a smaller molecular weight (23). That a lower charge density is the

of "Free" Plasma GAG*

2-Deoxy- 2-sulfoamino- hexose	2-Deoxy- 2-amino- hexose	Ratio OD:OD		Infrared absorbancy, cm^{-1}		Per cent‡
		Borate: Carbazole	Carbazole: Naphtho- resorcinol	860; 920 (4-SO ₄)	775; 820; 992 (6-SO ₄)	
		2.0	5.6	+ + +; + +		
		1.9	5.5	+ +; +	+; +	8.1
				+ +;	+; + +	7.0
				+ +; +	+; + +	6.3
		2.0	5.2	+ + +; +		12.3
		2.0	5.2	+ + +; + +	+	39.0
		2.2	3.3§	+ + +; + +	+	20.3
				+ +; +	+; + +	9.0
Neg	Neg	2.0	7.8	+ + +; + +		30.8
0.01	0.007			+ + +; + +		38.0
Neg	Neg			+ + +; + +		14.2
				+ +;	+; +	4.0
				+ +;	+ +; +	1.5

TABLE V
Fractionation on Dowex 1 × 2

	Hexuronic acid (borate)	Glucosamine	Galactosamine	Galactosamine Glucosamine
	Before fractionation	0.99 (15.3)	0.05 (0.7)	0.94 (13.3)
Dowex 1 × 2 fractions 0.6 M NaCl	0.97	0.11	0.88	7.87
0.8 M	0.99	0.04	0.95	21.30
After hyaluronidase treatment:				
Dowex 1 × 2 fractions 0.2 M NaCl	0.81	0.07	0.93	12.5
0.4 M	0.87	0.05	0.94	17.6
0.6 M	1.11	0.04	0.95	20.3
0.8 M	1.06	0.04	0.95	21.8

* Values are molar or atomic ratio per mole of total hexosamine. Figures in parentheses are per cent values by weight.

† Per cent of total material applied to column, as hexuronic acid.

determining factor is demonstrated by the sulfur analyses (Tables IV and V) and by the infrared data which show a lower absorbance in the SO_4^- region (1230 cm^{-1}) for the "bound" GAG. Thus, the number of electrostatic bonds with plasma proteins should be less for the "bound" than for the "free" GAG. If it is assumed that the "bound" GAG are covalently linked to protein, the

source of this protein-GAG complex and its relationship to the "free" GAG remain to be considered.

The incorporation of sulfate- ^{35}S into GAG may represent either sulfation of an already existing acceptor or an event during the "de novo" synthesis of GAG (24). The labeling of rabbit plasma GAG obtained 6, 12, and 24 hr after a single dose of sulfate- ^{35}S probably

TABLE VI
Labeling of Rabbit Plasma GAG with Sulfate- ^{35}S , before and after Intravenous Injection of 10 mg Crude Papain (Young Rabbits)

Time	Treatment	Rabbit 1 (1.36 kg)				Rabbit 2 (1.36 kg)			
		Free		Bound		Free		Bound	
		Amt*	SA†	Amt*	SA†	Amt*	SA†	Amt*	SA†
hr									
0	Sulfate- ^{35}S 500 μc								
6		12.3	99.5	38.2	98.5				
12						11.7	141.0	34.1	120.0
24		9.3	91.2	34.5	97.0	27.2	91.7	34.7	103.0
50		8.8	141.0	32.8	63.0	14.4	126.0	45.9	34.0
54	Sulfate- ^{35}S 500 μc								
108	Sulfate- ^{35}S 500 μc								
180	Crude papain i.v. 10 mg								
186		250.4	86.5	1380.0	92.7	256.8	101.0	1660.0	100.0
204		209.5	71.0	983.0	85.0	261.2	81.4	1810.0	57.4
228		24.0	3.7	38.9	1.5	14.8	12.0	37.0	1.4
252		24.5	1.9	36.5	1.0	15.0	4.5	30.3	0.77

* μg Hexuronic acid/10 ml plasma.

† cpm/ μg Hexuronic acid.

of "Bound" Plasma GAG*

Sulfur	Galactose (anthrone)	2-Deoxy- 2-sulfoamino hexose	2-Deoxy- 2-amino hexose	Ratio OD:OD		Infrared absorbancy cm^{-1}		Per cent‡
				Car- Borate: Car- bazole	Car- Naphtho- bazole: resorcinol	860; 920 (4-SO ₄)	775; 820; 992 (6-SO ₄)	
0.48 (1.16)	0.20 (2.9)			2.02	6.6	+++; +		
0.59		Neg	Neg	2.00		+++; +		32.2
0.53				2.04	8.4	+++; +		63.8
						+++;		10.8
						+++;		45.6
						+++; +		33.9
				2.04	5.7	++++; ++		10.5

represents sulfation of polymers that have already left the cellular environment and are in the process of being either excreted or metabolized. In younger rabbits, 50 hr after the single injection of sulfate-³⁵S the specific activity of the "free" GAG increases sharply, whereas that of the "bound" continues to decline. These data indicate that GAG that were synthesized in the peripheral tissues in the presence of a high level of sulfate-³⁵S begin to appear in the blood at this time.

Since the specific activity of the "bound" GAG does not increase, one must conclude that at the tissue level the "free" have a faster turnover and are released into the blood stream at a faster rate than the "bound." The limitation of this phenomenon to very young rabbits is in line with the views and results of various authors related to the role of GAG-protein complexes in the process of calcification. The experimental results of Weinstein, Sachs, and Schubert (25) indicate that di-

TABLE VII
Labeling of Rabbit Plasma GAG with Sulfate-³⁵S, before and after Intravenous Injection of 10 mg
Crude Papain (Older Rabbits)

Time hr	Treatment	Rabbit 3 (1.7 kg)				Rabbit 4 (2.1 kg)			
		Free		Bound		Free		Bound	
		Amt*	SA‡	Amt*	SA‡	Amt*	SA‡	Amt*	SA‡
0	Sulfate- ³⁵ S 250 μ c								
6		11.6	128.0	35.5	128.0				
12						8.6	48.0	32.3	52.0
24		23.0	89.0	30.5	96.0	7.0	44.0	28.0	49.0
50		7.1	64.0	31.0	61.0	9.4	26.0	31.5	15.0
54	Sulfate- ³⁵ S 250 μ c								
108	Sulfate- ³⁵ S 250 μ c								
180	Crude papain, i.v. 10 mg								
186		158.4	98.0	688.0	88.0	183.7	74.0	965.0	73.0
204		26.0	97.0	45.0	59.0	96	57.0	652.0	52.0

* μ g Hexuronic acid/10 ml plasma.

‡ cpm/ μ g Hexuronic acid.

gestion of the protein moiety of protein-GAG complexes allows the precipitation of calcium phosphate from metastable solutions. On the other hand, Gerber and Schubert have also pointed out that protein-free GAG are immediately extruded from cartilage because of the "excluded volume" effect caused by the presence therein of protein-GAG complexes (26). The experimental results of Hirschman and Dziewiatkowski (27) indicate that the protein moiety of the protein-GAG complexes is indeed decreased or modified in calcifying tissues through the action of proteases (28). One would expect that these processes, and the release of "free" GAG into circulation, would be more evident in very young animals who undergo an almost constant process of remodeling and calcification.

In view of the well-known effects of papain on cartilage and connective tissue (21, 29), our data indicate that both "free" and "bound" GAG are present in peripheral tissues; since the latter are more abundant, they are mobilized to a greater extent and constitute a larger aliquot of the plasma GAG. The fact that the specific activities of the "free" and "bound" GAG decline almost at the same rate after papain injection indicates that both pools are being diluted equally by the newly synthesized, nonradioactive tissue GAG. It may be concluded that the metabolic events transforming part of the "bound" GAG into "free" GAG must be fairly rapid, to the extent that the two pools are practically in equilibrium. One may speculate that both the "free" and "bound" GAG derive from tissue protein-GAG complexes and represent the products of different tissue metabolic activities, the former being mainly the product of proteolytic digestion (28) and the latter of depolymerization and partial desulfation. These two pools possess more than a superficial resemblance to those recently proposed by Fratantoni, Hall, and Neufeld (30) on the basis of studies on cultures of normal and abnormal fibroblasts.

Once in the blood stream, the "free" GAG could establish electrostatic linkages with plasma proteins, while the "bound" would remain covalently linked to fragments of tissue proteins that have not been completely digested by tissue proteases. Some observations made in our laboratory confirm the latter point. If an antiserum to bovine protein-GAG complex is absorbed with polymerized, whole bovine serum (31), its ability to agglutinate red cells coated with the bovine protein-GAG complex is completely lost. This is compatible with the presence in bovine serum of a component having the immunological properties of the bovine protein-GAG complex. It is of interest, in this connection, that the presence of collagen-like components, immunologically related to human skin collagen, have been found recently in human serum (32).

The analytical data indicate that the "bound" GAG

consist essentially of chondroitin-4-sulfate with low sulfate content. The preponderant presence of galactosamine in every fraction, the infrared pattern, and the effect of testicular hyaluronidase support this conclusion (Fig. 2, Table V). Despite the extensive purification, the "free" GAG still seem to be contaminated by a glucosamine-containing material, which is eluted from Dowex 1×2 columns with 0.4–0.8 M NaCl. The presence in the latter eluate of small amounts of 2-deoxy-2-sulfoamino-hexose and 2-deoxy-2-amino-hexose indicates that some heparan sulfate may be present. The presence of glucosamine and galactose in fractions 1.2–1.8 M NaCl after treatment with testicular hyaluronidase is strongly suggestive of the presence of keratan sulfate. The infrared pattern of this material demonstrates the presence of sulfate esters in position 6 and the absence of carboxylic groups, thus confirming the analytical data. The larger amount of the "free" GAG is, however, represented by galactosamine-containing, fully sulfated compounds that are eluted from Dowex 1×2 columns and are affected by testicular hyaluronidase like a standard of skeletal chondroitin sulfate. The infrared patterns indicate a great preponderance of ester sulfate in position 4 and only a few in position 6 (Fig. 3, Table IV). The possible presence of dermatan sulfate in the two pools seems to be excluded by the values of the ratios of borate:carbazole and carbazole:naphthoresorcinol (15, 33).

These results are in good agreement with those obtained by previous authors (3–6) and confirm that chondroitin-4-sulfate is the main glycosaminoglycan of normal human plasma. The reported presence of components resistant to the action of testicular hyaluronidase (6) may be due to the small amounts of heparan sulfate and keratan sulfate demonstrated, or to the possible presence of excessive amounts of protein in some crude preparations. Certainly, the latter factor, added to variations in charge density of the different GAG fractions, must have contributed to the electrophoretic and chromatographic heterogeneity of plasma GAG which has been previously reported (7).

The demonstration that plasma GAG consist of two pools, quite similar in structure but reflecting different metabolic events, may be of importance in the clinical investigation of diseases involving an abnormal turnover of glycosaminoglycans and may very well complement studies performed on "in vitro" systems.

ACKNOWLEDGMENTS

We wish to thank Mrs. Nancy Wilson for excellent technical assistance.

This project was carried out while one of the authors, Dr. Di Ferrante, was an Established Investigator of the American Heart Association.

This investigation was supported by grant AM 10811 from the U. S. Public Health Service and grant Q-236 from

the Robert A. Welch Foundation; and by grants-in-aid from the American Heart Association (66-639) and the Texas and Houston Heart Associations. Statistical evaluation of the data was performed by the Common Research Computer Facility, supported by U. S. Public Health Service grant FR 00254.

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