THE ISOLATION OF CHONDROITINSULFURIC ACID FROM NORMAL HUMAN PLASMA*

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Several reports have suggested that acid mucopolysaccharides are normal constituents of blood but these have not been supported by adequate chemical evidence. The presence of a uronic acid-containing polysaccharide in the "euglobulin" fraction of human plasma was demonstrated by BADIN *et al.*[1]. BASSIOUNI[2] reported that an extract of 420 ml normal human plasma contained traces of two acid polysaccharides which could be detected by paper electrophoresis. One of the substances migrated at the same rate as chondroitinsulfuric acid (CSA) of cartilage while the other possessed a slower mobility. This finding was confirmed recently by BOLLET *et al.*[3] using paper chromatographic techniques. They obtained two components which stained metachromatically with toluidine blue: The chromatographic behavior of one resembled CSA of cartilage. DEUTSCH[4] isolated hyaluronic acid (HA) from the sera of two patients, one with a reticulum-cell sarcoma and the other with a neuroblastoma, but was unable to demonstrate the presence of HA in serum of normal individuals.

The present paper is concerned with the characterization of a sulfated mucopolysaccharide isolated from normal human plasma. The mucopolysaccharide appears to be identical with CSA-A[5] of cartilage. Limited observations are reported on a second mucopolysaccharide component, also present in normal plasma. A preliminary account of this investigation has been reported elsewhere[6].

EXPERIMENTAL AND RESULTS

Initially, the starting material was whole plasma. However, later trials revealed the acid mucopolysaccharides to be associated with a water-insoluble or globulin fraction. This finding is in accord with the report by BADIN *et al.*[1] who could demonstrate no characteristic color for uronic acid, by the carbazole reaction of DISCHE[7], in globulin-free plasma. Consequently, more recent attempts to isolate mucopolysaccharides from plasma have been concerned entirely with the globulin fraction.

Normal human plasma** was dialyzed against running tap water for 48 to 72 h. The precipitate which formed in the dialysis sac was collected by centrifugation, washed 3 times with water and suspended in 0.1 *M* phosphate buffer, pH 7.8 (300–310 ml of buffer for each l of original plasma). Crystalline trypsin*** (2.5 mg/g protein) was added and the mixture was incubated at 38° in cellophane casings and dialyzed

* This investigation was aided by grants from the Chicago Heart Association and the Variety Club of Illinois.

** The plasma was obtained from the blood bank, Billings Hospital, University of Chicago.

*** The 1 × crystallized trypsin was purchased from Pentex Incorporated, Kankakee, Illinois.

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simultaneously against the buffer for 5 days. Precipitation of residual protein with trichloroacetic acid and precipitation of the crude polysaccharides with alcohol was performed as described previously for isolating mucopolysaccharides from skin[8].

Paper electrophoresis of a crude mucopolysaccharide preparation was carried out in a 0.025 *M* phosphate buffer, pH 7. A potential gradient of 8 to 9 V/cm was applied across Whatman No. 1 filter paper (1 × 20 inches) for 3 h. After staining with toluidine blue, two spots could be demonstrated. Their mobility and staining characteristics were similar to those of authentic samples of CSA-A and HA, respectively.

The crude mucopolysaccharide extract was subjected to zone electrophoresis on Celite filter-aid according to the method described in a previous publication[8]. 1 cm portions of the slab were eluted with 15 ml of water in coarse sintered-glass funnels. Analysis of the eluates by the carbazole reaction disclosed 2 peaks. The result of an electrophoretic separation of 82 mg of crude mucopolysaccharide from the globulin fraction of 8 l of plasma (Table I, E-101) is demonstrated in Fig. 1. The eluates from each peak were combined, dialyzed against distilled water at 5°, and concentrated under reduced pressure.

The slower migrating fractions from extracts E-101, E-128 and P-10 (Table I), representing 51 l of plasma, were combined, concentrated under reduced pressure, and made up to 25 ml. Molar ratios of 1.00, 0.99, 0.71 and 0.54, respectively, were found for nitrogen, hexosamine, uronic acid[7] and ester sulfate[10]. Incubation with testicular hyaluronidase caused a 13% reduction in turbidity, as measured by the method of DORFMAN AND OTT[11]. The presence of both glucosamine and galactosamine was demonstrated by the method of STOFFYN AND JEANLOZ[12]. Insufficient material pre-

TABLE I

ANALYSIS OF CHONDROITINSULFURIC ACID FROM HUMAN PLASMA

No.	*Type of preparation*	*Plasma extracted (l)*	*CSA isolated (mg)*	*N**	*Hexosamine*** (*μequivalents per ml*)	*Uronic acid*[7] (*μequivalents per ml*)	*S*[10]
E-82	Whole plasma	2.28	1.7	1.90	0.34	0.39	+
E-87	Whole plasma	3.14	2.6	0.91	0.51	0.53	
E-101	Globulin fraction	8.00	12.1	1.71	0.96	0.97	1.16
E-128	Globulin fraction, alkali-treated	25.1	12.6	1.61	0.85	1.00	
P-10	Globulin fraction, alkali-treated	17.9	14.3	2.21	1.14	1.12	
P-11	E–128 + P–10			2.21	1.69	1.67	2.10

* By the micro-Kjeldahl method.

** By a modification of the ELSON AND MORGAN method[9] following hydrolysis in 4 *N* HCl for 14 h at 100°.

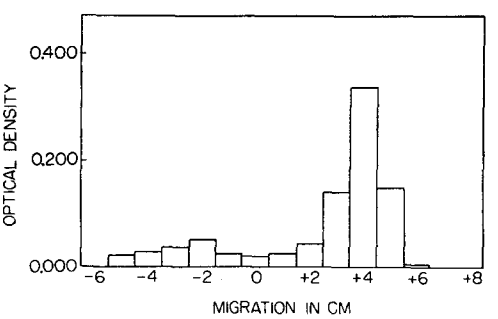

Fig. 1. Distribution of uronic acid after slab electrophoresis of mucopolysccharides from normal plasma.

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vented further characterization of this fraction. The low-ester sulfate to hexosamine ratio would indicate that it is composed of a sulfated and nonsulfated mucopolysaccharide.

Analyses of the faster migrating component from extracts of globulin fractions are shown in Table I and compared with the same component from extracts of whole plasma. In an attempt to reduce the nitrogen content, crude mucopolysaccharide extracts were dissolved in 2% NaOH, dialyzed, redigested with trypsin and precipitated as described above. The alkali treatment did not alter the molar ratio of nitrogen to hexosamine (Table I, E-128 and P-10).

The faster migrating fractions from extracts E-128 and P-10 (Table I) were combined and precipitated with 15 volumes of glacial acetic acid. The gelatinous material was collected by centrifugation, washed repeatedly with 95% ethanol and finally with ethyl ether. After drying, the precipitate was dissolved in water and made up to a volume of 25 ml (Table I, P-11). Nitrogen hexosamine, uronic acid[7] and ester sulfate analyses indicated molecular ratios of 1.30, 1.00, 1.00, and 1.24. The hexosamine was identified as galactosamine by the method STOFFYN AND JEANLOZ[12].

Like CSA-A of cartilage, the compound is hydrolyzed completely by testicular hyaluronidase and is resistant to streptococcal hyaluronidase.

In neutral solution, the sulfated mucopolysaccharide has a specific rotation of $-31.6° \pm 0.5$ which agrees with the published values of MEYER *et al.*[13] for CSA-A.

The infra-red spectrum* of the preparation, in the free acid form, is identical with that published by ORR[14] for CSA-A, with the exception that the intensity of the band at 1560 cm^{-1}, attributed to the N-acetyl group[14], is greater than at 1736 cm^{-1}.

A modification of the WAUGH-RUDDICK test[15] was utilized to examine the anticoagulant activity of the sulfated mucopolysaccharide isolated from plasma. 10 to 200 μg of the mucopolysaccharide were added to whole blood and the anticoagulant effect compared with similar quantities of CSA-A from cartilage, β-heparin and heparin. Like CSA-A, the sulfated mucopolysaccharide of plasma possessed no anticoagulant activity.

DISCUSSION

The faster migrating component isolated from the globulin fraction of normal human plasma appears to be identical with the CSA-A of cartilage. While the nitrogen value is in excess of theory, ultraviolet examination of E-101 and P-11 (Table I) failed to disclose the presence of protein. Small amounts of amino acids were detected in acid hydrolysates by paper chromatography, but were not identified. The difficulty of removing excess nitrogen from the CSA of plasma by methods which are adequate for this purpose in other tissues indicates an extremely strong association with plasma protein. Of interest in this connection is the report of DZIEWIATKOWSKI AND DI FERRANTE[16], demonstrating that the major portion of administered $Na_2{}^{35}SO_4$ is strongly bound as sulfate, presumably as a sulfated carbohydrate, with the serum proteins.

The results of this study throw no light on the identity of the slower migrating fraction found in plasma. Unlike component II of BOLLET *et al.*[3], only a minor portion of this fraction is degraded by testicular hyaluronidase. It seems likely, however,

* Infra-red analysis was performed by ROBERT HART, Argonne Cancer Hospital, University of Chicago.

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that the serum component I, described by BOLLET *et al.*[3] as resembling CSA of cartilage in chromatographic characteristics, is identical with the sulfated mucopolysaccharide investigated in the present study.

The actual concentration of CSA in plasma cannot be inferred from the data. The quantity of circulating acid mucopolysaccharides appears to be small even when losses inherent in the method employed for isolation are taken into consideration. At most, approximately 1.5 mg of CSA was isolated per l of plasma. This agrees well with average uronic acid levels of 206[1] and 277[13] μg%, reported by others for plasma mucopolysaccharide extracts. We have found crude preparations of acid mucopolysaccharides to contain 310 to 440 μg of uronic acid per 100 ml of normal human plasma prior to zone electrophoresis.

The source of acid mucopolysaccharides in normal plasma is not known. It is conceivable that mucopolysaccharides enter capillaries supplying ground substance, or that they originate in the walls of the blood vessels, themselves. Since their presence has been indicated in leucocytes[2,17] and blood platelets[18], the possibility that these cells provide a source of plasma mucopolysaccharides cannot be excluded.

ACKNOWLEDGEMENTS

The author wishes to express her appreciation to Dr. ALBERT DORFMAN for his interest and encouragement, to Dr. BURTON J. GROSSMAN for the anticoagulant assays, and to Mrs. KATHRYN F. DEWEY for valuable technical assistance.

SUMMARY

Two uronic acid-containing components have been demonstrated in a globulin fraction of normal human plasma by zone electrophoresis. The slower migrating fraction appears to be a mixture containing uronic acid, hexosamines and ester sulfate. The faster migrating fraction behaves in all respects like the chondroitsinsulfuric acid-A isolated from cartilage.

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Received November 11th, 1957

Addendum in proof: Since the manuscript was submitted, an acetyl determination was carried out on P-11 (Table I). It contained 2.00 μequivalents of acetate per ml, as determined by a method to be published (J. LUDOWIEG).