Acid Mucopolysaccharides in Normal Serum

Alfred Jay Bollet, … , Maria W. Seraydarian, William F. Simpson

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ACID MUCOPOLYSACCHARIDES IN NORMAL SERUM [1]

By ALFRED JAY BOLLET,[2] MARIA W. SERAYDARIAN, AND WILLIAM F. SIMPSON

(From the Medical Service of Wayne State University, College of Medicine and Detroit Receiving Hospital, Detroit, Mich.)

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Mucopolysaccharide which contains uronic acid has recently been described in normal plasma (1, 2). A method for the quantitation of plasma acid mucopolysaccharide was described by Badin, Schubert and Vouras (1), based on the observation that chondroitin sulfate added to plasma is found in euglobulin precipitates (3). These authors reported an increased amount of acid mucopolysaccharide in the plasma of 10 patients with rheumatoid arthritis (1).

The study of acid mucopolysaccharides in blood which are chemically related to those of connective tissue might reveal information of interest concerning the diseases which primarily affect connective tissue. Accordingly, a relatively practical method was devised for measurement of the uronic acid-containing polysaccharide in the presence of large amounts of protein, and studies of the nature of the mucopolysaccharides present in blood were undertaken. Observations made on plasma and serum from normal adults are reported.

METHODS

Quantitation of total uronic acid-containing mucopolysaccharide. The method which was devised for this purpose is based on the method of Badin, Schubert, and Vouras (1), with several modifications. The procedure is as follows:

1. Five 10-ml. samples of plasma or serum were dialyzed for 72 hours at 4° C. against 0.0063 M acid-phosphate buffer of pH 5.8, to precipitate the euglobulins.

2. The contents of the dialysis bag were diluted with 1.5 volumes of citric-phosphate buffer, the precipitate centrifuged, and the supernatant decanted. The precipitate was dissolved in 10 ml. 0.14 M NaCl in 0.02 M NaOH.

3. Three ml. of 1.7 M perchloric acid was then added slowly with constant agitation by a magnetic stirrer. After about 30 minutes, the precipitate was removed by filtration through Whatman No. 5 paper, and a 10-ml. aliquot of the filtrate was dialyzed against running cold tap water overnight.

4. Two-tenths ml. of 1 per cent protamine sulfate was added to the dialyzed material. After 30 minutes in the cold, the precipitate was centrifuged.

5. The precipitate was dissolved in 2.2 ml. of 2 M acetate buffer of pH 5.0 with slight warming, and 1.0-ml. aliquots were used for duplicate uronic acid determinations using the carbazole method of Dische (4). The optical density of each tube was read after heating with sulfuric acid and again 2 hours after adding carbazole; the initial nonspecific color was then subtracted from the final reading.

Paper chromatography. An attempt was made to identify the uronic acid-containing mucopolysaccharides by paper chromatography. The dialyzed material obtained after Step 3 was brought to dryness by lyophilization, dissolved in approximately 0.4 ml. of water, and applied as a spot to Whatman No. 1 paper. Ascending chromatography was done in two solvent systems: 1) ethanol, 52 ml. plus 48 ml. of 0.06 M phosphate buffer of pH 6.6, and 2) propanol, 37 ml. plus 63 ml. of 0.06 M phosphate buffer of pH 6.6, according to the procedure of Kerby (5). After 42 hours, the chromatograms were dried in air, then fixed in 20 per cent formaldehyde in ethanol for 15 minutes, immersed in ether and dried in air. The fixed chromatograms were sprayed with recrystallized toluidine blue solution (40 mg. toluidine blue in 80 ml. acetone and 20 ml. H_2O), rinsed in 2 per cent acetic acid and then distilled water. The staining procedure was a modification of that of Hamerman (6). For chemical determinations, the material obtained from 40 to 80 ml. of normal human plasma was lyophilized and then applied to paper as a six-inch band. After 42 hours, one edge of the paper chromatogram was cut off and stained as a marker, and the corresponding areas containing mucopolysaccharide were eluted from the paper with water. The precipitate obtained following the addition of 0.2 ml. of protamine sulfate to each eluate was divided; part was analyzed for its content of uronic acid as described. An aliquot was taken for the determination of hexosamine by the method of Boas (7). A paper control was eluted and subjected to the same procedure.

Effect of hyaluronidase. The effect of hyaluronidase on the uronic acid containing mucopolysaccharides was determined by adding, to the dialyzed material obtained after Step 3, 200 turbidity reducing units of testicular hyaluronidase (Wyeth Corp.), plus acetate buffer of pH 6.0 and NaCl to a final concentration of 0.1 M acetate

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[2] Markle Scholar in the Medical Sciences.

TABLE I

Recovery of hyaluronic acid and chondroitin sulfate added to 10-ml. samples of normal plasma

Amount of added hyaluronic acid	Amount of added chondroitin sulfate	Recovery
μg.	*μg.*	*per cent*
43		84.6
43		87.1
43		85.2
65		85.8
65		87.7
108		82.3
108		80.8
54		77.5
	21.2	100.1
	21.2	94.7
	12.4	90.9
	12.4	100.4
	Average	88.1

and 0.15 M NaCl. After incubation at 37° C. for 3 hours, perchloric acid was added, the precipitate removed and the supernatant material dialyzed against tap water for 15 hours. For chromatography, dialyzed material was lyophilized, dissolved in 0.3 ml. of water and applied to paper. For quantitation, the dialyzed material was then treated as in Steps 4 and 5.

Determination of the relative amounts of glucosamine and galactosamine was done by the method of Roseman and Daffner (8) and analyses for hexose were done by the anthrone method of Kapuscinski and Zak (9), using a standard containing equal parts of galactose and mannose.

RESULTS

Evaluation of the quantitative method

In order to determine if the method described above allows the detection of small amounts of acid mucopolysaccharide in the presence of large amounts of protein, varying quantities of hyaluronic acid prepared from cock's comb by the method of Boas (10) and commercial chondroitin sulfate were added to normal plasma. Recoveries of these substances (Table I) varied between 77.5 and 100 per cent, averaging 88.1 per cent.

The absorption spectrum between 420 mμ and 620 mμ after the addition of carbazole to the material obtained from plasma was identical with that of pure glucuronolactone. Protamine gave no color with the reagents used in the uronic acid determination and did not interfere with the color development in the reaction. Replicate determinations done on plasma obtained from normal individuals and carried through the entire procedure

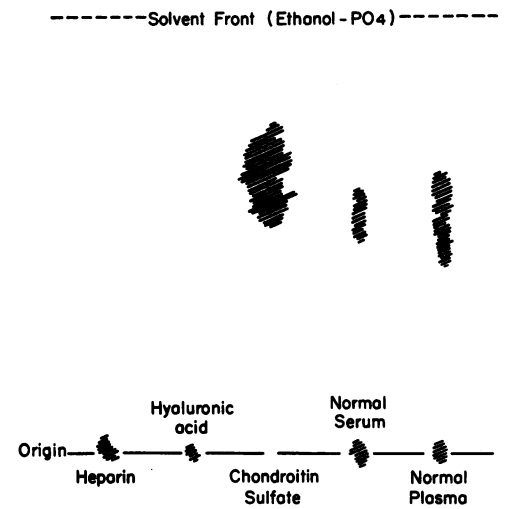

FIG. 1. DIAGRAM OF FILTER PAPER CHROMATOGRAM OF NORMAL PLASMA AND SERUM IN ETHANOL-PHOSPHATE SYSTEM

revealed an error of about 6 to 9 per cent. Anthrone determinations done on the protamine precipitate obtained from plasma by the above procedure revealed the presence of hexose. Although there was insufficient protein to give appreciable color with the sulfuric acid alone, the hexose present gave some color with the carbazole. At 520 mμ, however, hexoses (glucose, galactose and mannose) give relatively little color and it was calculated that the hexose present did not account for more than 10 per cent of the increase in color following addition of carbazole.

Nature of mucopolysaccharides found in normal plasma

The method used established that the material being measured was non-dialyzable, precipitated with the euglobulins under the conditions mentioned, was soluble in perchloric acid and was precipitated by protamine. It was found to contain uronic acid.

Following incubation with hyaluronidase, uronic acid could not be demonstrated in the small amount of precipitate which appeared after addition of protamine.

Filter paper chromatography revealed two components staining metachromatically with toluidine blue. Figure 1 shows the results of paper chromatography in solvent 1 (ethanol-phosphate). Of the polysaccharides tested, only chondroitin

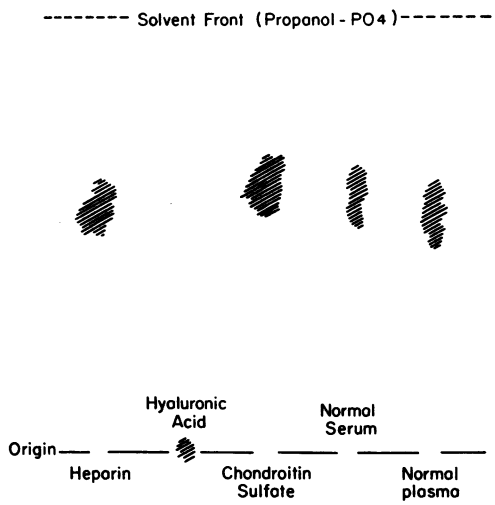

FIG. 2. DIAGRAM OF FILTER PAPER CHROMATOGRAM OF NORMAL PLASMA AND SERUM IN PROPANOL-PHOSPHATE SYSTEM

sulfate (commercial as well as material prepared from bovine nasal septum by the method of Meyer and Smyth [11]) was found to move from the origin in this solvent system. Samples of commercial heparin, β-heparin (chondroitin-sulfate B), and samples of hyaluronic acid (prepared from umbilical cord, cock's comb, vitreous humor and synovial fluid) did not move. The material prepared from plasma was found to give one spot near the solvent front, with an Rf value close to that of chondroitin sulfate (this will henceforth be referred to as component I), and a second spot at the origin.

Figure 2 shows the results of chromatography in solvent 2 (propanol-phosphate). In this solvent system, samples of chondroitin sulfate moved close behind the solvent front, while heparin and β-heparin moved slightly slower. All samples of hyaluronic acid tested remained at the origin. The material obtained from plasma showed no significant amount of staining at the origin, and therefore the component which stayed near the origin in solvent 1 moved in solvent 2; it will henceforth be referred to as component II.

Chromatography after treatment of the material obtained from plasma with hyaluronidase failed to reveal any metachromatic staining material in either solvent system, although some non-metachromatic material could be seen faintly at the origin in ethanol-phosphate and near the solvent front in propanol-phosphate.

Hexosamine to uronic acid molar ratios were determined on the protamine precipitates of Step 4 and of eluates from paper chromatograms. The protamine precipitates of Step 4 were found to have an average hexosamine: uronic acid ratio of 1.3 : 1. The eluates from the ethanol-phosphate system, which separated the two metachromatic components, revealed hexosamine: uronic acid ratios of 1.1 : 1 in the area of component I. The ratio at the origin, corresponding to the area of component II, was 1.5 : 1. These values were calculated after a correction was made for the blank obtained by elution of control samples of paper treated in the same fashion.

The hexosamine in the protamine precipitate of Step 4 was found to be 72 per cent galactosamine and 28 per cent glucosamine.

Level of acid mucopolysaccharide in normal serum

The acid mucopolysaccharide levels of serum from 34 normal adults, obtained at the time of blood donations and from laboratory personnel, revealed uronic acid contents ranging between 215 and 385 μg. per 100 ml. (Table II) measured as glucuronic acid. The mean level was 277 μg. per 100 ml., with a standard deviation of ± 42 μg. per 100 ml. There was no apparent difference between the levels found in males and females, in the individuals studied. No significant change with age was found in the range between 18 and 52 years.

Serum samples taken after 12 hours fasting and one hour postprandial from six chronically ill hospitalized patients with varying initial levels showed an average fall of 7.5 per cent in the concentration of acid mucopolysaccharide after eating. This was not considered to be significant.

DISCUSSION

The method described for the quantitative determination of uronic acid containing mucopolysaccharide in serum is essentially a modification of the method of Badin, Schubert, and Vouras (1). Considerably less starting material is used, and digestion of euglobulin precipitate for three days with pepsin and then three days with trypsin is

omitted, since it was found that perchloric acid removed a sufficient proportion of protein from the euglobulin solution without loss of uronate-containing polysaccharide. A step involving precipitation in alcohol and ether for 48 hours was also found to be unnecessary. Protamine sulfate is used in place of lyzozyme to precipitate the polysaccharide, since it gives quantitative recoveries of hyaluronic acid, chondroitin sulfate and heparin, and is a simple protein of known amino acid composition which contains no neutral sugar or hexosamine to complicate chemical analyses of the precipitate. Since the protamine does not affect the uronic acid determination, internal standards and correction factors are not necessary.

An attempt was made to avoid dialysis after precipitation with perchloric acid, by removing the perchloric acid with potassium hydroxide. Although the pH of the supernatant was adjusted to that found after dialysis (7.35), quantitative recovery of polysaccharides could not be obtained.

Badin, Schubert, and Vouras (1) found the uronic acid content of the mucopolysaccharide isolated from plasma of seven normal adults by their method to range between 153 and 281 μg. per cent, averaging 206 μg. per cent. This is lower than the range which we found in serum, but one possible explanation is that the extra steps in their method might have resulted in some loss.

The present study does not allow definite conclusions to be drawn as to the nature of the two components found in normal serum. The fact that no uronic acid containing polysaccharide was recovered after treatment with testicular hyaluronidase suggests a relationship to the acid mucopolysaccharides of connective tissue. Component I had chromatographic characteristics resembling chondroitin sulfate prepared from cartilage and presumably could have been chondroitin sulfate A or C, or a mixture of the two. It is of interest that Schiller and Dewey (2) have isolated a component from normal plasma by alcohol precipitation after enzyme digestion and trichloracetic acid precipitation of the proteins and identified it as chondroitin sulfate A (12). Component II had chromatographic characteristics resembling heparin and beta-heparin (chondroitin sulfate B) but differed from those substances in that it was digested by testicular hyaluronidase. Most of the hexosamine in the protamine precipitates was found to be galactosamine, suggesting that both components contain that substance, but further study of the nature of component II will be necessary to establish its identity.

TABLE II

Serum acid mucopolysaccharide levels, measured as uronic acid, in normal adults

Age	Sex	Uronic acid	Age	Sex	Uronic acid
		μg. %			μg. %
18	M	219	30	M	268
20	M	234	30	M	265
20	F	256	31	F	273
22	M	279	31	M	248
23	F	324	31	M	251
23	M	324	34	M	385
23	F	282	35	F	351
25	M	246	37	M	288
26	M	271	38	M	330
26	M	259	41	M	277
26	M	247	42	M	330
26	F	247	42	M	275
27	M	215	42	F	275
27	M	290	44	M	222
27	M	241	48	M	352
28	M	253	51	M	223
28	M	281	52	M	346

In view of the presence in serum of polysaccharides containing components and characteristics found in polysaccharides of connective tissue, determination of the serum levels and of the qualitative nature of the material present in various clinical states might reveal information of interest concerning the diseases which primarily affect connective tissue.

SUMMARY

1. Polysaccharide containing uronic acid and hexosamine can be isolated from 5- to 10-ml. quantities of plasma or serum by a method suitable for quantitation.

2. This material contains two components on filter paper chromatography which stain metachromatically with toluidine blue. The chromatographic characteristics of one resembles that of samples of chondroitin sulfate, and the other resembles heparin and beta-heparin, but both components are labile to testicular hyaluronidase.

3. No material with the chromatographic mobility of hyaluronic acid could be found in samples of normal plasma.

4. Sera of 34 normal adults had an average level of mucopolysaccharide of 277 μg. per cent, measured as glucuronic acid.

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