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Research Article

PLASMA POLYSACCHARIDE FRACTION CONTAINING URONIC ACID, IN NORMAL SUBJECTS AND IN PATIENTS WITH RHEUMATOID ARTHRITIS [1]

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Recent work on the ground substance of connective tissue has stressed the importance of mucopolysaccharide components which contain glucuronate such as hyaluronate and chondroitin sulfate. In different tissues these polysaccharides appear to vary widely in degree of polymerization and in relative quantity (1–3). Little is known about the catabolism of the ground substance polysaccharides and their possible elimination through blood and urine.

The total polysaccharide content of plasma has frequently been studied and found to contain mannose, galactose, fucose, and glucosamine (4–7). In human plasma the polysaccharides are contained mainly in the globulins (8). Albumin normally contains much less (9). Of the total polysaccharide about 10 per cent is in the mucoprotein fraction and is not precipitated by high concentration of ethanol or by 6 per cent perchloric acid (10–13). In these studies no significant amount of hexuronate has been found in any plasma polysaccharide fraction, perhaps due to the unreliability of uronic acid determination in the presence of large amounts of protein, and the drastic methods of protein hydrolysis used. The glucuronate of tungstic acid treated plasma filtrates (14) is not part of a polysaccharide. The only report of glucuronate bound to serum proteins is that of Stary and Yuvanidis (15). They precipitated serum proteins with trichloracetic acid, hydrolyzed them by boiling four hours in concentrated HCl and used Tollens' naphthoresorcinol reaction on the hydrolysates. They claimed this reaction was specific for glucuronic acid and reported good recovery of free glucuronic acid added to the precipitated protein. They found about 100 to 168 μg. glucuronic acid bound to 100 mg. protein. However they did not study the recovery of added polysaccharide such as chondroitin sulfate and did not point out that the color developed in a serum protein hydrolysate differed from that developed by glucuronic acid. In the present work, difficulties, to be described later, were found with Tollens' method when applied to plasma fractions containing polysaccharides.

Polysaccharides of urine, on the contrary, do contain glucuronate. Tayeau, Biserte, Montreuil, and Marqueville (16) reported a mucoprotein in urine containing mannose, fucose, galactose, acetylhexosamine and hexuronic acid. Kerby (17) found a polysaccharide in urine which contained hexuronic acid and hexosamine and which on paper electrophoresis behaved as chondroitin sulfate. This polysaccharide was not derived from the urinary tract or the prostatic gland.

In the present work a method has been developed which separates from plasma a material which contains polysaccharide with glucuronic acid as a component. In this form the glucuronic acid can be quantitatively determined. The method involves two novel procedures. The first is based on the previous observation (18) that chondroitin sulfate added to plasma is recovered in the euglobulin fraction. The glucuronate-containing material of plasma is also found entirely concentrated in the euglobulin fraction, since examination of the euglobulin-free plasma revealed no glucuronate. The second new procedure is the separation, after protein digestion, of the glucuronate-containing material by the relatively

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selective action of lysozyme in precipitating high molecular weight anionic materials.

EXPERIMENTAL

The hexuronate-containing fraction of plasma was isolated in the following six steps.

1) Blood (30 to 40 ml.) was collected with addition of sodium citrate (1 ml., 10 per cent solution). The sample was centrifuged, and the plasma separated and diluted with 3 vol. of aqueous sodium citrate (0.1 per cent).

2) Using Sandor, Sabetay, and Vargues' procedure (19) the diluted plasma was dialyzed in a cellophane bag against 2 L. of buffer (citric acid, Na_2HPO_4, M/150, pH 5.8 ± .1). After dialysis for 3 days at 4° C. euglobulins were centrifuged and then washed with 5 ml. buffer, then with ethanol and ether, dried and weighed (column 3, Table I).

3) The only suitable digestion procedure found was as follows. A solution of crystalline pepsin (3 mg. per ml. in .1 M HCl) was added to the protein in the ratio of 6 mg. pepsin for 100 mg. protein. Samples were gently shaken at room temperature for 3 days and then neutralized with 1 M NaOH. A solution of crystalline trypsin (3.5 mg. per ml. in HCl-sodium barbital buffer, M/7, pH 8.4) was then added to give 2 mg. trypsin for 100 mg. original protein. This gave a pH of about 8 and a sodium barbital molarity of about M/35. Toluene (1 ml.) was added and digestion continued 3 days at about 30° C.

4) The hydrolysates were neutralized with acetic acid and made up to 10 ml. with water. Perchloric acid, 60 per cent, was added to give a final concentration of 6 per cent. After standing an hour, precipitated proteins were centrifuged, washed with ethanol, dissolved in 1 ml. alkaline saline and protein determined with the biuret reaction. Deduction of this residual protein from the initial sample gave the amount of protein digested (column 4, Table I).

5) The solution of protein hydrolysate containing $HClO_4$ was dialyzed in cellophane bags against 3 L. potassium acetate solution, 0.1 per cent, for 24 hours and then added to 10 vol. of a mixture of ethanol (8 parts) and ether (2 parts) containing potassium acetate (0.5 per cent) in flasks. After 48 hours at room temperature the polysaccharides adhered to the glass and the fluid was poured off. Flasks were dried 1 hour at 40° C. and polysaccharides were dissolved in 10 ml. water.

6) To each solution was added a freshly made, clear solution of lysozyme, 15 mg. in 5 ml. water. Turbidity appeared immediately and a flocculent precipitate soon formed which was centrifuged easily after 3 hours and washed with water.

The material precipitated by lysozyme was dissolved in 1 ml. saline alkalinized with a trace of NaOH and used for the carbazole test as described below. All samples developed the characteristic pink color as did glucuronic acid. Seven samples of normal human euglobulins gave an average of 57 μg. glucuronic acid for 100 mg. protein digested (column 5, Table I). Recovery of

TABLE I

Results of analysis, by the procedure described, of the euglobulin of plasma of normals and of subjects with rheumatoid arthritis

	Euglobulins			Hexuronic acid	
	Per 100 ml. plasma (mg.)	Used for determ. (mg.)	Digested by enzyme (mg.)	Per 100 mg. euglobulin (μg.)	Per 100 ml. plasma (μg.)
Rheumatoid subjects					
1. Ben.	431	95	92	75.0	323
2. Der.	905	145	137	58.0	525
3. Ga.	393	51	48	152.0	597
4. In.	785	126	120	51.7	406
5. Po.	694	111	106	53.8	373
6. Rot.	540	54	51	68.6	370
7. Wo.	533	96	92	67.5	359
8. Av.	1,470	280	276	23.1	339
9. Man.	500	100	98	34.6	173
10. Mas.	1,210	230	221	34.1	412
Average	746			61.8	388
Normals					
1. No. 3934	384	300	295	44.8	172
2. MacL.	430	300	294	35.7	153
3. Cap.	331	133	126	66.7	221
4. Ken.	340	137	130	54.1	184
5. Cruz.	425	162	154	65.0	276
6. Sant.	340	137	130	46.2	157
7. Sla.	325	131	124	86.5	281
Average	361			57.0	206

potassium chondroitin sulfate (1 to 5 mg.) added to the original protein samples was between 66 and 100 per cent.

The proteins of the diluted plasma, which remained after removal of the euglobulins, were precipitated with ten volumes of ethanol and then treated by the six steps described. In no case did the products develop the characteristic pink color in the carbazole reaction.

For further characterization, larger amounts of the product containing hexuronic acid were required. The same procedure was used starting with 1 to 2 gm. euglobulin. The lysozyme precipitate was centrifuged and dissolved in 5 ml. alkalinized saline. On this larger sample glucuronic acid, total polysaccharides, hexosamine and protein could be determined.

ANALYTICAL METHODS

Tollens' naphthoresorcinol method has been considered to be specific for the determination of glucuronic acid (20). In this test a deep violet color develops in the ether layer whose intensity is proportional to the amount of glucuronic acid. The method as described by Ratish and Bullowa (21) was found unsuitable for our purpose. Addition of chondroitin sulfate to serum protein gave less than 50 per cent recovery of hexuronic acid. From plasma protein hydrolysates, which contain large quantities of other sugars, the colored complex was not extracted with ether. In the presence of added hexoses, complexes seemed to be formed with naphthoresorcinol and hexuronic acid which were not soluble in ether. By adding pyridine, these complexes became soluble in ether and a deep purple color developed which was the sum of the colors due to glucuronic acid and the added hexoses. Attempts to separate the violet complex due to glucuronic acid from the purple complexes due to other sugars by chromatography using toluene and toluene-glycol systems failed.

The carbazole reaction of Dische (22), on the contrary, was found very sensitive and specific for uronic acids. This reaction did not give good results on biological materials since some amino acids and high concentrations of serum lipids interfere with the development of color. Mannose, galactose, fucose, and glucose do not give a pink color, as do hexuronic acids, but rather a pale brown-yellow color with a completely different absorption spectrum (Figure 1). This reaction has been considered to measure uronic acids only when the color developed was bright pink or purple with a peak at 520 mμ. For determinations on polysaccharide-lysozyme complexes it was found important to have controls in the presence of a quantity of lysozyme similar to that in the precipitates. Figure 2 shows the standard curve for glucuronic acid and its modification by added lysozyme. Lysozyme when heated alone with H_2SO_4 developed a faint brown-yellow tint that deepened slightly on addition of carbazole but never turned pink. This color limits the sensitivity of the method but never corresponds to more than 5 μg. of glucuronic acid. The Dische reaction was carried out as follows.

To a sample (10 to 120 μg. uronic acid in 1 ml.) in a calibrated Klett tube, 6 ml. H_2SO_4 were added dropwise, with shaking in an ice bath; the fluid was thoroughly mixed by bubbling air through it with a fine pipette. The tubes were heated for 30 minutes in a boiling water bath, then chilled in ice water. The brownish color which developed was read with a Klett-Sumerson photometer (filter S540) and was usually less than 60 divisions. If the reading was greater, the mixtures were diluted with a solution (H_2SO_4 6 vol., water 1 vol.) to make the reading less than 60, and then read. Carbazole (0.2 ml., 0.1 per cent in alcohol) was added to each tube and the fluids mixed by bubbling air through them. The carbazole used was twice crystallized from ethanol and sublimed *in vacuo*. The tubes were set at 4° C. for 12 to 15 hours, then a second set of readings was made and the first set deducted. Samples not developing the characteristic pink color were considered as totally lacking in hexuronic acid.

For hexosamine Shetlar, Foster, Kelly, and Everett's micro method (23) was modified. The sample to be analyzed (1 ml.) was hydrolyzed 4 hours with HCl (1 ml., 8M) at 100° C. The mixture was decolorized with Darco (50 mg.), centrifuged, the Darco washed with water and the washings added to the hydrolysate. The method, continued as described by Shetlar, allowed determination of 20 to 100 μg. hexosamine with about 10 to 15 per cent error.

Total sugars determined with the modified tryptophane method (24, 25) gave results with an error of about 10 per cent.

Proteins estimated with the biuret method were reliable when solutions were colorless and no turbidity developed with the reagent (26).

The larger samples of precipitate obtained with

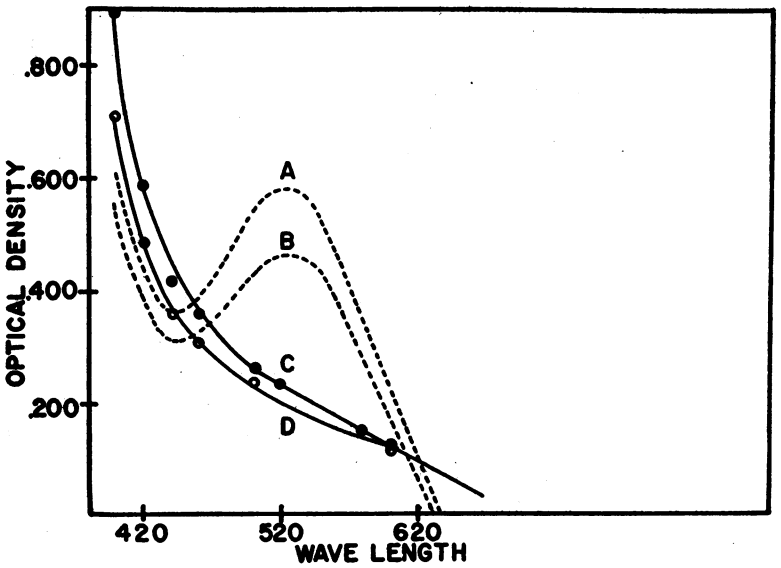

FIG. 1. SPECTRA OF CARBAZOLE REACTION PRODUCTS IN SULFURIC ACID WITH DIFFERENT SUGARS

A, galacturone (60 μg.); B, glucurone (50 μg.); C, mannose (100 μg.); D, galactose (100 μg.). Glucose, glucosamine, and fucose give spectra almost identical with those of mannose and galactose.

lysozyme from 1 to 2 gm. of normal human euglobulin as described above were analyzed for hexuronate, hexosamine, total sugars, and protein. The analytical results were subjected to several corrections. The lysozyme (Worthington Biochemical Corp.) was similarly analyzed and found to contain 164 μg. total sugars and 52 μg. hexosamine per 100 mg., but no detectable

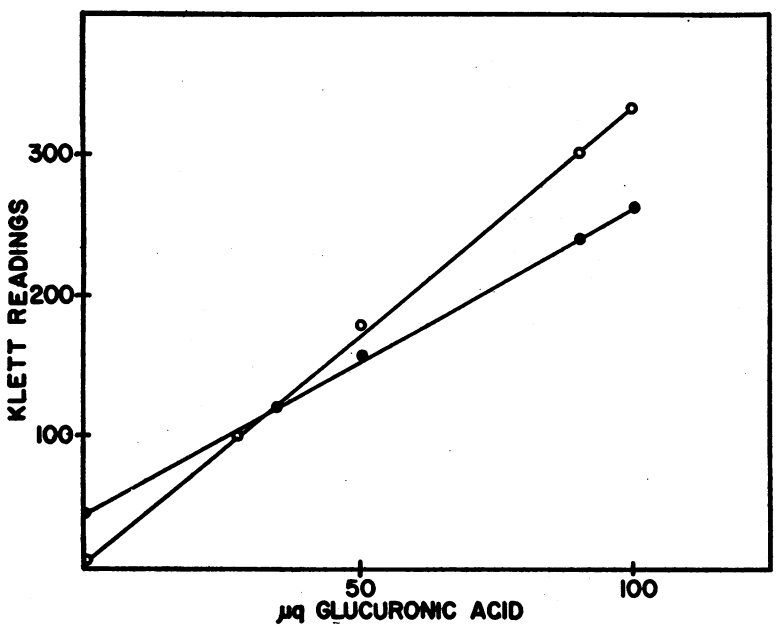

FIG. 2. INFLUENCE OF LYSOZYME ON DISCHE'S CARBAZOLE REACTION

○, glucuronic acid; ●, glucuronic acid in the presence of a constant amount (5 mg.) of lysozyme.

TABLE II

Results of analysis of material precipitated with alcohol and the polysaccharide material precipitated with lysozyme *

Starting materials (*1 gm.*)†	Total sugars *mg.*	Hexuronic acid *mg.*	Hexosamine *mg.*	Protein (Biuret) *mg.*
Total polysaccharides precipitated in step 5				
Euglobulins	13.50	No pink color develops‡	7.35	42.0
Lysozyme precipitates produced in step 6				
Euglobulins	.408	.480	.483	20.8
Total plasma proteins (including euglobulins)	.043	.072	.191	14.4
Total plasma proteins with added chondroitin sulfate (1.33 mg.)		.342	.400	17.5
Lysozyme precipitate of potassium chondroitin sulfate carried through steps 1–6				
Chondroitin sulfate (1 mg.)		.270	.209	
Calculated		.266	.250	

* No attempt was made to calculate the percentages of the several components since the products were never isolated in a dry form.

† Euglobulins were prepared as in steps 1 and 2. Total plasma proteins were prepared by the addition to plasma of ten volumes of ethanol, and the product was washed with ethanol and ether.

‡ The failure of a pink color to develop in this material, although glucuronic acid must be present, is probably due to the presence of the large excess of hexoses and also a larger proportion of protein.

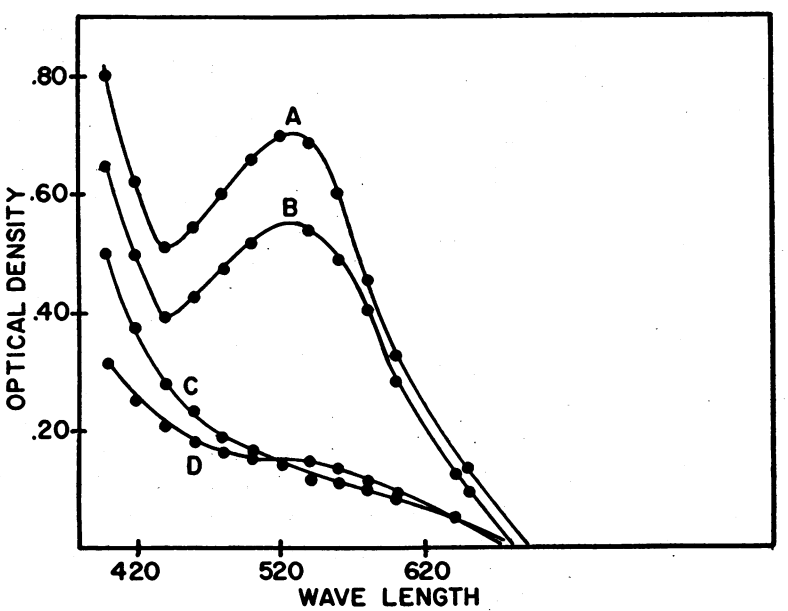

FIG. 3. SPECTRUM OF CARBAZOLE REACTION PRODUCT IN SULFURIC ACID WITH THE ACID POLYSACCHARIDE FROM SERUM EUGLOBULINS AND SOME CONTROLS

A, euglobulin polysaccharide precipitated with lysozyme; B, 5 μg. of glucurone and 8 mg. of lysozyme; C, 8 mg. of lysozyme; D, polysaccharide from dialyzed serum (without euglobulins) and 8 mg. of lysozyme. Spectra measured with Beckman spectrophotometer.

hexuronate. Estimations on lysozyme-polysaccharide precipitates were corrected by deducting the amounts of sugar and hexosamine present in the quantity of lysozyme developing the same color with the biuret reaction. The color of the tryptophane reaction for total polysaccharides is due to hexoses, pentoses, and uronic acids. To measure the sugars, the color corresponding to uronic acids was deducted, 100 μg. of glucuronic acid developing color corresponding to 70 μg. mannose (25). Table II contains data on analysis of the material precipitated with lysozyme in step 6. If the total polysaccharides of step 5 are examined with the Dische reaction, no glucuronic acid can be detected because the pink color of the carbazole reaction does not develop with this product. Only after the selective precipitation with lysozyme in step 6 does hexuronic acid appear in the Dische reaction with the characteristic pink color. The euglobulin fraction was found to contain all the hexuronic acid of the total plasma proteins. The figures for the material isolated from euglobulins with lysozyme show the following proportions: 1.00 mol. hexuronic acid, 1.09 mol. hexosamine, .91 mol. sugar. These figures cannot be interpreted as indicating that the product isolated is a single polysaccharide.

Figure 3 shows that the spectrum of the color produced by a mixture of glucuronic acid and lysozyme cannot be distinguished from that of the complex of lysozyme and the polysaccharide isolated from euglobulins. This is the evidence that the product isolated with lysozyme contains uronic acid.

RESULTS

Plasma samples of ten patients with active rheumatoid arthritis and of seven normal subjects were compared. All rheumatoid patients had a positive sheep cell agglutination test and all except No. 9 had a high erythrocyte sedimentation rate. All had marked clinical symptoms except No. 1 (Ben.) and No. 9 (Man.). Their ages ranged from 30 to 60. All normal subjects were healthy donors at the Hospital Blood Bank. Results in Table I show no difference in the glucuronic acid calculated per 100 mg. euglobulins between normals and rheumatoids (column 5, Table I). When calculated per 100 ml. of plasma (column 6, Table I) there seems to be a difference; all but one of the patients had a higher plasma polysaccharide glucuronate level than the normals.

DISCUSSION

The presence of the glucuronate containing polysaccharide exclusively in the euglobulin fraction is an expression of its marked anionic properties. It behaves as free chondroitin sulfate does when added to plasma *in vitro* (18). Dried on a glass slide the lysozyme complex, prepared as described from euglobulin, gives with toluidine blue 0 the characteristic deep violet metachromatic color. Lysozyme alone does not do so. The identity of the material isolated from the euglobulin is not certain. It has an unexpectedly large protein component. Table II shows that the ratio of protein to combined sugar is about 15 in the lysozyme precipitate derived from euglobulin while in the precipitate produced by lysozyme and chondroitin sulfate the ratio is about 5 (18). The material isolated from euglobulin appears to contain protein not completely removed by enzyme digestion. There is no evidence that the material described is a single substance.

There appears to be an increase in the glucuronate containing polysaccharide in the plasma of patients with rheumatoid arthritis. Only one (No. 9) of ten patients studied had a glucuronate level within the range of the normals. This patient had a normal erythrocyte sedimentation rate and the disease was not active. It is possible that the increase in glucuronate containing polysaccharide in rheumatoid arthritis plasma is related to cartilage destruction known to occur in the active disease.

The increase in glucuronate containing polysaccharide in the plasma of rheumatoid arthritis may be the cause of the larger amount of euglobulin formed in this plasma, just as chondroitin sulfate added to plasma would cause the appearance of a larger amount of euglobulins (18).

SUMMARY

1. A material behaving as an anionic polysaccharide containing uronic acid, hexosamine and one or more sugars has been found in human euglobulin. The components are in the ratio, 1.09 mol. hexosamine, 1.00 mol. glucuronic acid,

.91 mol. sugar. No trace of this material has been found in euglobulin-free plasma.

2. An increase in this material has been found in the plasma of ten patients with rheumatoid arthritis. This increase appears as an increased amount of euglobulin and not as euglobulin with an increased amount of polysaccharide.

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