7. McCorkindale, J., Edson, N. L., *ibid.*, 1954, v57, 518.

8. Sice, J., *J. Am. Chem. Soc.*, 1954, v76, 1661.

9. Racker, E., *J. Biol. Chem.*, 1950, v184, 313.

10. Kornberg, A., *ibid.*, 1950, v182, 805.

11. Gutcho, S., Stewart, E. B., *Anal. Chem.*, 1948, v20, 1185.

12. Loewus, F. A., Westheimer, F. H., Vennesland, B., *J. Am. Chem. Soc.*, 1953, v75, 5018.

13. Dische, Z., Borenfreund, E., *J. Biol. Chem.*, 1951, v192, 583.

14. Roe, J. H., *ibid.*, 1934, v107, 15.

15. Warburg, O., Christian, W., *Biochem. Z.*, 1941, v310, 384.

16. Horecker, B. L., Kornberg, A., *J. Biol. Chem.*, 1948, v175, 385.

17. Talalay, P., Marcus, P. I., *ibid.*, 1956, v218, 675.

18. Williams-Ashman, H. G., Banks, J., Wolfson, S. K., Jr., *Arch. Biochem. Biophys.*, 1957, v72, 485.

19. Hers, H. G., *Biochim. Biophys. Acta*, 1956, v22, 202.

20. 1957, *Le Metabolisome du Fructose*, Editions Arscia (Bruxelles).

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Chemical Studies of Mucopolysaccharides of Rat Blood Platelets. (24494)

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Isolation of a sulfated mucopolysaccharide from blood platelets of rats has been reported (1). Subsequent investigation on larger quantities of extracts of rat platelets (200 to 500 μg) revealed another mucopolysaccharide-rich fraction that is not sulfated. This report describes the separation and further chemical characterization of these two fractions by paper electrophoresis, hydrolysis, and paper chromatography.

Materials and methods. Male Sprague-Dawley rats weighing 272-477 g were used as platelet donors. Platelets labeled *in vivo* with $Na_2S^{35}O_4$ and harvested by the procedure described(1) were lyophilized or frozen for storage. Mucopolysaccharides (MPS) were extracted from platelets by alkali treatment(1) or by trypsin digestion. The trypsin digestion medium was made by adding 0.1 ml of a 0.1% trypsin† solution to 0.9 ml of M/10 $NaHCO_3$ buffer (pH 8.3, M/1000 with respect to $CaCl_2$). One milliliter of this mixture was added for each 13.3 x 10^9 platelets and reacted for 1 week at 34°-38°C. The undissolved material was removed by centrifugation at 2190 x g for about 30 minutes. The supernatant was neutralized with 1 N HCl, diluted to 10 ml with water, and repeatedly treated with chloroform-amyl alcohol reagent (2) to remove protein. The resulting supernatant was dialyzed and lyophilized(1). In addition to the method of electrophoresis used before [essentially that of Rienits(3)], we also used as the electrolyte a 35% solution of propanol in M/15 phosphate buffer (pH 6.4) a solvent system similar to that used by Kerby(4) for chromatographic separation of heparin and chondroitin sulfate(CSA). The extract was electrophoresed on strips of Whatman #2 filter paper (3.0 x 30.5 cm) for 5 hours at 10.8 V/cm. The two MPS-rich fractions were located on paper strips by staining with Alcian blue. Each MPS-rich fraction was eluted from unstained paper electrophoresis strips run by the Rienits procedure, dialyzed, lyophilized, and hydrolyzed by modification of the method of Glegg and Eidinger (5). Two hundred to 500 μg (estimated by comparing staining density of small samples on paper strips with that of known amounts of CSA) of each fraction and 12 mg of Dowex 50 cation exchange resin‡ in the hydrogen form and 0.1 ml of water were heated at 100°C for 33 or 40 hours in a sealed tube with a volume of about 3 ml. In this procedure hexosamines released during hydrolysis adsorb to the resin, and sugars and uronic acids

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† The trypsin was a crystallized, lyophilized, salt-free preparation (TL 550) obtained from Worthington Biochemical Sales Co.

‡ Dowex-50 resin of analytical grade, 50 to 100 mesh, obtained from Bio-Rad Laboratories.

remain in solution. The hydrolysis was carried out on 2 different batches of each fraction as well as in control samples of CSA,§ of paper electrophoresis strips, and of resin alone. Sugars and uronic acids were chromatographed on Whatman #1 filter paper. Each paper was stapled together to form a cylinder and placed in glass jar with one of the following solvents: (1) butanol-acetic acid-water (4:1:5), bottom layer used to saturate the atmosphere; (2) phenol-water (71:29); (3) butanol-pyrodine-water (5:3:2) of Yosizawa (6); (4) pyridine-ethyl acetate-acetic acid-water (5:5:1:3), and (5) pyridine-ethyl acetate-water (11:40:6) used to saturate the atmosphere(7). The solvent was allowed to rise to the top of the paper 3 times. Material from fraction 1 (faster moving fraction) was chromatographed in all 4 systems, but fraction 2 material was run only in the last 2. The papers were sprayed with aniline hydrogen phthalate to reveal sugars and uronic acids. Hexosamines were eluted from the resin with 0.5 N HCl and chromatographed in water-saturated collidine by Woodin's procedure (8). These papers were sprayed either with ninhydrin or with the Elson-Morgan reagent (9) to reveal hexosamines. Once when ninhydrin was used, the pentose sugars formed as oxidation products of hexosamines(10) were eluted and chromatographed in the pyridine-ethyl acetate-acetic acid-water system

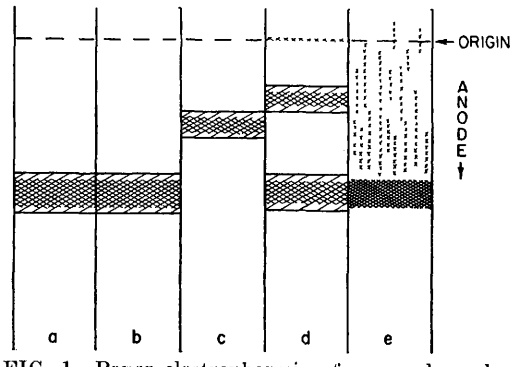

FIG. 1. Paper electrophoresis of mucopolysaccharides and platelet extract by modified Rienits procedure (conditions: M/10 phosphate buffer, pH 6.6; 3 hr; 5°C; 10.8 V/cm). a, chondroitin sulfate; b, heparin; c, hyaluronic acid; d, platelet extract; e, autoradiogram of d.

§ The chondroitin sulfate was obtained from Nutritional Biochemicals Corp.

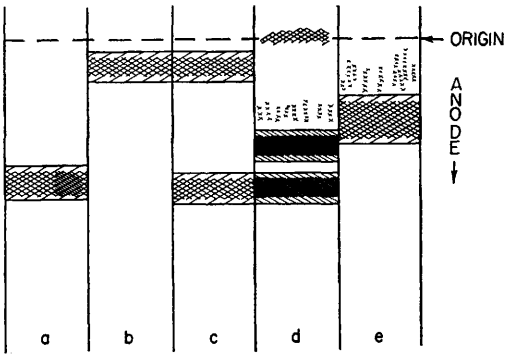

FIG. 2. Paper electrophoresis of mucopolysaccharides and platelet extract in propanol-phosphate buffer system (conditions: 35% propanol, 65% M/15 phosphate buffer, pH 6.4; 5 hr; 5°C; 10.8 V/cm). a, chondroitin sulfate; b, heparin; c, mixture of chondroitin sulfate and heparin; d, platelet extract; e, hyaluronic acid.

described to confirm identification of the hexosamines. Hydrolysis was carried out on 2 different batches of each fraction as well as on control samples of CSA, of paper electrophoresis strips, and of resin alone.

Results. Two fractions that stained with Alcian blue were observed on paper electrophoresis strips that were run either in phosphate buffer or in 35% propanol-phosphate buffer (Figs. 1 and 2). In both systems fraction 1 had the same electrophoretic mobility as CSA. Autoradiograms of paper electrophoresis strips of extracts of $S^{35}O_4^{--}$-labeled platelets showed that fraction 1 was sulfated (Fig. 1). In the phosphate buffer system, fraction 2 (the slower moving fraction) moved slightly behind hyaluronic acid (HA)‖ but overlapped it (Fig. 1), whereas in the 35% propanol-phosphate buffer that separates CSA and heparin,¶ fraction 2 moved in front of and overlapped the position of HA (Fig. 2). Neither fraction had a mobility corresponding to that of heparin.

Chromatograms of the hexosamines sprayed either with ninhydrin or with Elson-Morgan reagent showed the presence of galactosamine in fraction 1, whereas fraction 2 contained glucosamine. When the hexosamine spots that were oxidized with ninhydrin were eluted and

‖ The HA from vitreous humor was obtained from Mann Research Labs.

¶ The heparin was Liquaemin Sodium from Organon.

TABLE I. Chromatographic Detection of Sugars and Uronic Acids in Mucopolysaccharides.

	Fraction 1			Fraction 2			CSA			Resin blank		
	Amt*	Frequency†	Solvent systems	Amt	Frequency	Solvent systems	Amt	Frequency	Solvent systems	Amt	Frequency	Solvent systems
Uronic acid	++	5/5	4	—	0/2	2	++	7/10	4	—	0/6	2
Glucose	++	3/4	4	+	2/2	2	+	7/8	4	+	4/5	2
Galactose	++	3/4	4	++	2/2	2	trace	6/8	4	—	0/5	2
"Mannose"	+	1/4	4	+	1/2	2	trace	1/8‡	4	+	1/5	2
Pentose sugar	++	3/4	4	++	2/2	2	+	1/8‡	4	—	0/5	2

* Symbols are used as follows: —, none; +, small amount; ++, moderate amount.
† Frequency indicates No. of times material was present over total No. of relevant chromatograms.
‡ The one sample of CSA that showed a trace of mannose and pentose sugar had been paper electrophoresed and eluted before hydrolysis and chromatography.

rechromatographed, xylose was present in the material from fraction 1 and arabinose in material from fraction 2, confirming the presence of galactosamine and glucosamine in the respective fractions.

The results of chromatographic analysis for sugars and uronic acids from fraction 1 (4 solvent systems) and fraction 2 (2 solvent systems) are summarized in Table I. A uronic acid with same mobility and same color as glucuronic acid after aniline hydrogen phthalate staining and differing from galacturonic acid was present in hydrolyzates from fraction 1. The Dische procedure(11) confirmed the presence of uronic acid in fraction 1. No uronic acid was seen on chromatograms of fraction 2. The results of application of the Dische procedure to fraction 2 were somewhat equivocal; in a sample of fraction 2 several times larger than that of fraction 1, there was a small peak in absorption curve at 530 mμ, the peak for uronic acids. However, at no time during the procedure was the color of this sample like that of any other samples tested, which included fraction 1, CSA, and sugars. If uronic acid was present, the compound of which it was a part must have been only a minor component of fraction 2.

Small amounts of glucose were found in all samples, including the resin blank. (A strong glucose spot was present in samples obtained by subjecting a few fibers of Kleenex to the hydrolytic and chromatographic procedures). About twice as much galactose was seen in fraction 2 as in fraction 1; it was sometimes present in small amounts in CSA but never in the resin blank. A faint spot with the R_f value of mannose or arabinose was seen on chromatograms of fractions 1 and 2 run in the butanol-pyridine-water solvent system but not in the Fischer-Dörfel system. Another sugar with the R_f value of lyxose or xylose and giving the red color reaction to aniline hydrogen phthalate characteristic of pentose sugars was always present in samples of fractions 1 and 2. It was not found among hydrolysis products of CSA, except in one case when CSA was electrophoresed prior to hydrolysis. An eluate of a large amount of paper that had been carried through the electrophoresis procedure, was hydrolyzed, and the products chromato-

graphed. There was a barely perceptible spot at the xylose position. Therefore, the pentose sugar found in the mucopolysaccharide hydrolyzates does not come from the paper but is either a component of the mucopolysaccharides or a breakdown product.

Discussion. The major constituents of fraction 1 were glucuronic acid, galactosamine, and sulfate, the components of CSA. In addition, fraction 1 behaved like CSA when electrophoresed and chromatographed. The reason for presence of small amounts of other sugars has not been explained, but they were also sometimes found in commercial samples of CSA. It seems likely that at least the major part of fraction 1 is chondroitin sulfate.

Attempts to identify fraction 2 have been less successful, partly because very small amounts of this fraction have been available for study. In addition to its staining with Alcian blue, the presence of glucosamine in fraction 2 identifies it as a mucopolysaccharide. Its paper-electrophoretic mobility did not coincide with that of CSA, heparin, or hyaluronic acid, although it did migrate just in front of hyaluronic acid in one system and just behind it in another. Galactose appeared prominent, and some other sugars may be present in small amounts. Polysaccharides without uronic acid containing glucosamine, sugars, and no uronic acid have been reported in calf bone(12,13), in calf and steer bone (14), and in connective tissue(15). Their presence in human costal cartilage has also been suggested(16). The blood group polysaccharides have a similar composition. The sugars most often found in these substances are galactose, mannose, and fucose.

The functional significance of the fractions described in this account is not known, but several possibilities have been suggested(1).

Summary. Two mucopolysaccharide fractions have been extracted from blood platelets of rats. Paper electrophoresis and paper chromatography of the intact MPS of fraction 1 and paper chromatography of its hydrolysis products show it to be similar to chondroitin sulfate. Fraction 2 is probably a uronic acid-free mucopolysaccharide.

1. Odell, T. T., Jr., Anderson, B., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 151.
2. Sevag, M. G., *Biochem. Z.*, 1934, v273, 419.
3. Rienits, K. G., *Biochem. J.*, 1953, v53, 79.
4. Kerby, G. P., *J. Clin. Invest.*, 1954, v33, 1168.
5. Glegg, R. E., Eidinger, D., *Anal. Chem.*, 1954, v26, 1365.
6. Yosizawa, Z., *Tôhoku, J. Exp. Med.* 1950, v53, 125.
7. Fischer, F. G., Dörfel, H., *Hoppe-Seyler's Z. physiol. Chem.*, 1955, v301, 224.
8. Woodin, A. M., *Biochem. J.*, 1952, v51, 319.
9. Partridge, S. M., with a note by R. G. Westall, *ibid.*, 1948, v42, 238.
10. Gardell, S., Heijkenskjöld, F., Rochnorlund, A., *Acta Chem. Scand.*, 1950, v4, 970.
11. Dische, Z., *J. Biol. Chem.*, 1947, v167, 189.
12. Eastoe, J. E., Eastoe, B., *Biochem. J.*, 1954, v57, 453.
13. Meyer, K., *Bone Structure and Metabolism* (Ciba Fn. Symposium), Little, Brown & Co., Boston, 1955, p65.
14. Dische, Z., Danilchenko, A., *Proc. Am. Assn. Cancer Research,* 1957, v2, 196.
15. Consden, R., Bird, R., *Nature,* 1954, v173, 996.
16. Stidworthy, G., Masters, Y. F., Shetlar, M. R., *J. Gerontol.,* 1958, v13, 10.

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