ISOLATION, CHARACTERIZATION, AND DISTRIBUTION OF ACID MUCOPOLYSACCHARIDES IN RABBIT LEUCOCYTES*

By MARTHA E. FEDORKO,‡ M.D. and STEPHEN I. MORSE, M.D.

(From The Rockefeller Institute)

(Received for publication, August 17, 1964)

The leucocytes of human peripheral blood contain significant quantities of acid mucopolysaccharides. In addition to a type of chondroitin sulfate, in certain instances hyaluronic acid has been detected (1, 2). The relative distribution of these acid mucopolysaccharides among the various leucocyte types as well as their subcellular localization is unknown.

The cytoplasmic granules of the rabbit polymorphonuclear leucocyte are now recognized to be lysosomal elements containing hydrolytic enzymes and antibacterial factors (3, 4). Indirect evidence suggests the presence of acid substances in these organelles since they have an affinity for weak basic dyes. More recently, studies to demonstrate a sulfated mucopolysaccharide in the rabbit polymorphonuclear leucocyte granule by histochemical and autoradiographic methods have been performed (21).

This report describes studies in which acid mucopolysaccharides from rabbit polymorphonuclear leucocytes and their granules have been isolated and characterized.

Materials and Methods

Preparation of Polymorphonuclear Leucocytes.—Peritoneal exudates were harvested from rabbits by the method of Hirsch (3). Representative differential counts were: 93 per cent polymorphonuclear leucocytes, 5 per cent monocytes, 1 per cent eosinophils, 1 per cent lymphocytes. Contaminating red blood cells were removed by hypotonic lysis. The washed leucocytes were then suspended in approximately 50 ml cold acetone per one-half billion cells, centrifuged, and then similarily treated with 50 ml cold ether. The pellet was allowed to air dry and was then stored in a desiccator.

Preparation of Polymorphonuclear Leucocyte Granules.—Leucocyte granules were prepared by the method of Cohn and Hirsch (4). After the granule pellet obtained from one billion cells had been centrifuged in 0.25 M sucrose, the granules were washed two times in cold isotonic saline, then once in 15 ml cold acetone, and once in 15 ml cold ether. The pellets were dried and stored as were the cells.

Preparation of BCG-Induced Alveolar Macrophages.—The cells were collected by a modifi-

* This investigation was supported in part by Research Grants 01831 and AI-0648-02 from the National Institute of Allergy and Infectious Diseases, Public Health Service.

‡ Fellow of the Leukemia Socity.

cation of the procedure of Myrvik as described by Cohn and Wiener (5). The differential count showed 66 to 85 per cent macrophages, and from 10 to 19 per cent polymorphonuclear leucocytes. The remainder of the cells were lymphocytes.

Extraction and Isolation of Acid Mucopolysaccharides.—The method of Mathews and Hinds was utilized with several modifications (6). One hundred to 200 mg samples of whole cells or 20 to 40 mg of granules were suspended in 5 ml of 0.4 M NaOH and the mixtures were stirred for 24 hours at room temperature. The suspensions were then neutralized with 0.5 M HCl and the insoluble residue removed by centrifugation. The supernatant fluid was diluted fivefold to achieve a final salt concentration of 0.04 M. Ten ml of 1 per cent cetylpyridinium chloride (CPC) in 0.04 M NaCl was then added. After standing for 24 hours at room temperature the precipitated CPC-acid mucopolysaccharide complexes were removed by centrifugation. The precipitated complexes were then dissolved in varying salt concentrations (see Results), and the mucopolysaccharides were isolated by precipitation with 3 volumes of ethanol.

Paper Electrophoresis of Acid Mucopolysaccharides.—Two methods were employed: (*a*) Whatman No. 1 paper, sodium acetate buffer (pH 5, 0.14 M), 400 v for 3 hours at 4°C. The dried paper was dipped in a 1 per cent aqueous solution of cetyltrimethylammonium bromide and then washed for 10 minutes in hot tap water. The paper was dried at 100°C and sprayed with a 0.04 per cent ethanolic solution of bromcresol purple, and heat-dried again (7); (*b*) Whatman No. 3 paper, phosphate buffer (pH 7.2, 0.025 M), 400 v for 3 hours at 4°C. The paper was dried and stained with a 0.08 per cent solution of toluidine blue in 30 per cent ethanol, and decolorized with a 30 per cent solution of ethanol containing 0.5 per cent acetic acid (8). With both procedures a 4 per cent solution of acid fuchsin was used as a marker.

Standard solutions employed were hyaluronic acid (Worthington Biochemical Corporation, Freehold, New Jersey) and chondroitin sulfate A, B, and C, kindly supplied by Dr. Karl Meyer.

Enzymatic digestion was performed on solutions of polysaccharide containing 5 mg/ml of hexuronic acid mixed with an equal volume of: (*a*) testicular hyaluronidase at 1 mg/ml in 0.1 M acetate buffer pH 5 in 0.15 M NaCl; (*b*) streptococcal hyaluronidase (kindly supplied by Dr. Maclyn McCarty) in 0.2 M sodium acetate pH 6.

Paper Chromatography.—The solution of acid mucopolysaccharide was hydrolyzed at 100°C for 6 hours in 2 M HCl, evaporated to dryness, and taken up in water. Descending chromatograms were performed on Whatman No. 1 paper for 40 hours at room temperature. The solvent system was butanol, ethanol, water (4:1:1). Monosaccharides and oligosaccharide were identified by the silver nitrate method of Trevelyan (9). Amino sugars in the hydrolysates were differentiated by the method of Jeanloz and Stoffyn (10).

Quantitative Chemical Determinations.—Hexuronic acid was determined by the carbazole color reaction of Dische (11) and the orcinol method of Brown (12).

Hexosamine content was determined by the Elson-Morgan reaction after hydrolysis for 12 hours in 4 M HCl at 100°C (13).

Protein was estimated by the method of Lowry, using crystalline egg white lysozyme as a standard (14).

RESULTS

Hexuronic Acid Content of Polymorphonuclear Leucocytes and Polymorphonuclear Leucocyte Granules.—In initial experiments the content of hexuronic acids in the acid mucopolysaccharide isolated from whole cells was measured. As shown in Table I, leucocytes contained 3.4 μg hexuronic acid/mg protein, and 1.6 μg/mg dry weight. The yield of hexuronic acid was approximately 0.05 mg per billion cells.

Hexuronic acid determinations were next performed on acid mucopolysaccharides prepared from isolated polymorphonuclear leucocyte granules. The findings, also presented in Table I, demonstrated that the content of hexuronic acid for isolated granules was 12.0 μg/mg protein and 4.1 μg/mg dry weight. The total acid mucopolysaccharide hexuronic acid content of the granules compared to that in whole cells was 2.6 times greater on a dry weight basis and 3.5 times greater on a protein basis. These results clearly showed that acid mucopolysaccharide was concentrated as well as present in the granule.

TABLE I

Hexuronic Acid Content of Acid Mucopolysaccharides in Polymorphonuclear Leucocytes and Polymorphonuclear Leucocyte Granules

Exp. No.	Hexuronic acid	Protein	Dry wt.	Hexuronic acid per mg protein	Hexuronic acid per mg dry wt.
			Whole cells		
	μg	mg	mg	μg	μg
1	55	24	65.6	2.3	0.8
2	389	103	215.9	3.8	1.8
3	239	89.5	200.8	2.7	1.2
4	515	144	223.3	3.6	2.3
5	109	28.75	49.2	3.8	2.2
6	277	93	212.2	2.9	1.3
Average				3.4	1.6
			Granules		
7	150	9.5	33.4	15.8	4.5
8	143	8.5	39.1	16.8	3.7
9	170	19	38.5	8.9	4.4
10	137	20.75	34.7	6.6	3.9
Average				12.0	4.1

Isolation of Various Mucopolysaccharides from Polymorphonuclear Leucocytes and their Granules.—In the process of isolation of the acid mucopolysaccharides the solubility properties of different cetylpyridinium chloride (CPC)–mucopolysaccharide complexes were utilized to obtain separation of various groups of polysaccharides. Each group of CPC-polysaccharide complexes has a characteristic solubility in sodium chloride. Hyaluronic acid and chondroitin are soluble in 0.4 M sodium chloride whereas chondroitin sulfate A, B, and C, as well as heparin are insoluble at this molarity (15). It was, therefore, possible to prepare 2 fractions. Fraction 1 consisted of those mucopolysaccharides whose CPC complexes were soluble in 0.4 M sodium chloride. Mucopolysaccharides

whose CPC complexes were soluble at higher salt concentrations, 2 M, were designated fraction 2. The ratio of fraction 1 to fraction 2 based on hexuronic acid content in various whole cell preparations was not constant, ranging from 0.6 to 2.0. In the granules the ratio of fraction 1 to fraction 2 varied from 12.5 to 16.8 (Table II). From these data it was concluded that in the whole cell and granule two types of polysaccharides were present. The excess of fraction 1 in the granules indicated that either hyaluronic acid, chondroitin, or a related compound was concentrated in this organelle.

During processing of the two fractions, it was noted that the physical characteristics of each fraction after alcohol precipitation differed. Fraction 1 appeared translucent and gelatinous whereas fraction 2 was white and flaky.

TABLE II

Relative Hexuronic Acid Content of Acid Mucopolysaccharide Fractions in Polymorphonuclear Leucocytes and Polymorphonuclear Leucocyte Granules

	μg hexuronic acid/mg protein		
	Fraction 1	Fraction 2	Fraction 1/Fraction 2
Whole cells	1.5	0.75	2
	1.4	2.3	0.6
	1.4	1.3	1.1
	1.9	1.7	1.1
Granules	8.4	0.5	16.8
	6.1	0.5	12.5

It was of interest to determine whether the polysaccharide extracted from whole cells with sodium hydroxide was dialyzable. Half of the neutralized whole cell extract was dialyzed in 0.2 M sodium chloride overnight and the final volume was adjusted to the starting volume. The two samples were processed through precipitation with 3 volumes of ethanol. There was a 27 per cent loss in the dialyzed sample with equal loss from both fractions. From these data it was concluded that only a small fraction of the polysaccharide isolated by this method was dialyzable.

Although the cell preparations were washed twice in normal saline, they had originally been collected in flasks containing heparin. One group of experiments was performed to determine whether detectable heparin remained in the dehydrated sample. A cell preparation containing approximately 1.5 billion cells was collected in flasks containing 70 mg heparin, approximately five times the amount of heparin normally used. A control sample was collected without anticoagulant. The heparinized cells gave a value for hexuronic acid which

was not significantly higher than the control, thus ruling out possible effects of the added heparin on the final mucopolysaccharide determinations.

Paper electrophoresis was performed on the mucopolysaccharides isolated from whole cells in order to define further their characteristics. A slow moving component having mobility similar to hyaluronic acid was demonstrated in fraction 1. The component in fraction 2 had mobility similar to that of chondroitin sulfate. When stained with toluidine blue, fraction 1 was not metachromatic; the mucopolysaccharide of fraction 2, in contrast, had a degree of metachromasia equivalent to that of the standard chondroitin sulfates. No component having mobility or metachromatic properties similar to heparin was demonstrated nor were other polymers detected.

Parallel studies were performed on the 2 fractions isolated from the granules. The electrophoretic mobility of the 2 granule fractions corresponded to those obtained from intact cells.

Observations were also made on the susceptibility of the various mucopolysaccharide fractions to hyaluronidases. Fraction 1 was digested by both bacterial and testicular hyaluronidases, whereas fraction 2 was minimally digested by bacterial hyaluronidase and not at all by testicular hyaluronidase. The behavior of fraction 1 was thus comparable to that of hyaluronic acid or chondroitin. The behavior of fraction 2 after incubation with testicular hyaluronidase was compatible with the presence of a compound similar to chondroitin sulfate B, since chondroitin sulfates A and C are susceptible to testicular hyaluronidase.

Composition of the Mucopolysaccharide Isolated from Polymorphonuclear Leucocytes.—Neither fraction 1 nor fraction 2 contained detectable protein or nucleic acids.

Since all hexuronic acids, more particularly iduronic acid, do not give the same color intensity in the Dische carbazole reaction as does glucuronic acid, it was desirable to determine the quantity of uronic acid present by another method. The orcinol test of Brown was performed with glucuronic acid as a standard. Some indication of the nature of the compounds present could be obtained by calculating the carbazole/orcinol ratios of the different fractions and comparing these ratios to the values obtained with authentic hyaluronic acid, and chondroitin sulfates A, B, and C. The results of these experiments are recorded in Table III. The carbazole/orcinol ratio of fraction 1 coincided with that for hyaluronic acid, whereas that for fraction 2 coincided with either chondroitin A, C, or hyaluronic acid. These results negated the possibility that significant amounts of chondroitin sulfate B were present in fraction 2 despite the findings in the enzymatic studies, since the hexuronic acid present in chondroitin sulfate B is iduronic acid.

Hexuronic acid and hexosamine content were determined on weighed samples of fractions 1 and 2 prepared from whole cells. Fraction 1 prepared by the

usual method contained 26.9 per cent hexuronic acid and 37.4 per cent hexosamine. Fraction 2 yielded 36.3 per cent hexuronic acid and 28.6 per cent hexosamine. Molar ratios are listed in Table IV.

TABLE III

Carbazole/Orcinol Ratios of Mucopolysaccharide Fractions from Polymorphonuclear Leucocytes and of Standards

Acid mucopolysaccharide	Carbazole/orcinol ratio
Chondroitin sulfate A	1.6
Chondroitin sulfate B	0.33
Chondroitin sulfate C	1.7
Hyaluronic acid	1.5
Fraction I	1.5
Fraction II	1.6

TABLE IV

Properties of Mucopolysaccharides Isolated from Rabbit Polymorphonuclear Leucocytes Compared to Those of Hyaluronic Acid and Chondroitin Sulfate C Standards

Characteristic	Fraction 1	Fraction 2	Hyaluronic acid	Chondroitin sulfate C
CPC complex soluble in 0.4 M NaCl	+	−	+	−
Metachromasia	−	+	−	+
Digestion by bacterial hyaluronidase	+	Slight	+	−
Digestion by testicular hyaluronidase	+	−	+	+
Amino sugar	Glucosamine	Galactosamine	Glucosamine	Galactosamine
Uronic acid other than iduronic	+	+	+	+
Molar ratio, hexosamine/hexuronic acid	1.7	0.9	1.0	1.2

Information was obtained concerning the amino sugar present in each fraction by making use of the degradation of glucosamine and galactosamine by ninhydrin to their corresponding pentose derivatives of arabinose and lyxose respectively. After ninhydrin transformation of the amino sugar in fraction 1 a spot having mobility of arabinose appeared indicating that glucosamine was present. Similar oxidation by ninhydrin was performed on fraction 2 and yielded a monosaccharide with the mobility of lyxose, indicating that the amino sugar constituent was galactosamine.

Thus these studies have characterized two types of polysaccharides isolated

from both whole cell and granule preparations of rabbit polymorphonuclear leucocytes (see Table IV). The component of one fraction (fraction 1) was soluble in 0.4 M sodium chloride when complexed with CPC. Its sodium salt was not metachromatic and had an electrophoretic mobility similar to that of hyaluronic acid. This fraction was hydrolyzed by both bacterial and testicular hyaluronidase. The uronic acid component did not give the color intensity of iduronic acid, and the amino sugar present was glucosamine. All observations point to the presence of hyaluronic acid in this fraction.

The other fraction (fraction 2) was insoluble in 0.4 M sodium chloride when complexed with CPC; as a sodium salt it was metachromatic. This fraction was attacked minimally by bacterial hyaluronidase and not at all by testicular hyaluronidase. Iduronic acid was not present and the amino sugar was galactosamine. The precise chemical nature of this fraction remains unknown, but it is most likely related to the chondroitin sulfates.

Acid Mucopolysaccharide Content of Rabbit BCG-Induced Alveolar Macrophages.—To determine whether acid mucopolysaccharides were constituents of lysosomes of other cell types, studies were performed on whole cell extracts of BCG-induced alveolar macrophages of the rabbit. One sample of these cells, which consisted of 66 per cent macrophages, 19 per cent polymorphonuclear leucoctyes, and 15 per cent lymphocytes, was dried with acetone-ether and carried through the same steps of digestion and precipitation as was utilized with the polymorphonuclear leucocytes. Two hundred mg of dried, defatted whole cells contained 116 mg protein and yielded only 0.052 mg hexuronic acid. This hexuronic acid value was approximately 10 per cent of that obtained from the same weight of polymorphonuclear leucocytes. Since the alveolar macrophage preparation contained 19 per cent polymorphonuclear leucocytes its hexuronic acid content could be attributed entirely to the polymorphonuclear leucocytes present. Efforts to extract acid mucopolysaccharides from other alveolar macrophage preparations gave similar results; no evidence was therefore obtained for the existence of these compounds in macrophages.

DISCUSSION

These studies confirm that rabbit polymorphonuclear leucocytes contain acid mucopolysaccharides, and further demonstrate that these substances are concentrated in the granule. Both whole cells and granules contain two types of acid mucopolysaccharides as distinguished by the differing solubilities of their CPC complexes in solutions of sodium chloride.

Fraction 1, whose CPC complex is soluble in 0.4 M sodium chloride, has an electrophoretic mobility and metachromatic properties identical with authentic hyaluronic acid, and contains glucosamine, but no iduronic acid. The susceptibility of this fraction to both bacterial and testicular hyaluronidases is typical of hyaluronic acid. Quantitative analyses show a relatively high content of

amino sugar in fraction 1, as reflected by a molar ratio of 1.7/1 for glucosamine/hexuronic acid. It can be concluded that fraction 1 is hyaluronic acid or a very closely related compound. It is this compound that is particularly abundant in the granule where its concentration is 3.2 to 6.3 times greater than in the whole cell.

The exact nature of fraction 2, whose CPC complex is insoluble in 0.4 M sodium chloride, is less clear; it is strikingly metachromatic, equivalent to that of chondroitin sulfate, and contains galactosamine but no iduronic acid. It is digested slightly by bacterial hyaluronidase, but not by testicular hyaluronidase. Its insusceptibility to digestion by testicular hyaluronidase resembles the behavior of chondroitin sulfate B, but the carbazole/orcinol ratio shows that chondroitin sulfate B is not present. Fraction 2 gives on quantitative analysis a molar ratio for galactosamine/hexuronic acid of 0.9/1, a result comparable to that given by a sample of chondroitin sulfate A under the analytical conditions employed. The data presented here do not identify completely the mucopolysaccharide of fraction 2. All results point to the presence in this fraction of chondroitin sulfate A or C, except for the pattern of response to hyaluronidases. This behavior with the hyaluronidases may be due to the presence of chondroitin sulfate A or C which has a unique molecular arrangement, contains different substituents, is incompletely sulfated, or has been partially degraded (16, 17).

The results presented above do not exclude the presence of small amounts of other acid mucopolysaccharides, such as keratosulfate, or chondroitin.

Infrared studies by Clausen on the mucopolysaccharides isolated from human peripheral blood leucocytes indicated that a compound identical with chondroitin sulfate C was present (2). In some instances the pattern suggested the presence also of hyaluronic acid. The quantitative and qualitative studies by Kerby also led to the conclusion that chondroitin sulfate of some type was present in leucocytes (1). Both Clausen and Kerby employed a starting material (mixed human blood leucocytes) and extraction methods somewhat different from those employed by us, thus perhaps explaining minor differences in results obtained. No previous study of acid mucopolysaccharides in isolated polymorphonuclear leucocyte granules has been reported.

The concentration of one type of mucopolysaccharide, namely hyaluronic acid, in the polymorphonuclear leucocyte granule suggests a special function, but this function remains unknown. It has been proposed by de Duve that the sole factor involved in latency and release of lysosomal enzymes is the integrity of the lysosome membrane (18). However Koenig has proposed that latency of lysosomal enzymes may also be due to their binding to a ground substance or matrix of acid nature; hyaluronic acid might well function as such a ground substance in rabbit polymorphonuclear leucocyte granules (19). Especially likely is a reaction between acid groups of hyaluronic acid and the basic groups of the

cationic proteins of the granules (20). Hyaluronate in the granules might also influence the state of hydration within the granule, and thereby influence activity of the hydrolases.

No acid mucopolysaccharides could be extracted from rabbit alveolar macrophages, which are also rich in lysosomes. Thus the findings with polymorphonuclear leucocytes do not indicate the general occurrence of hyaluronic acid or a similar compound in all lysosomes. Perhaps acid polymers of an entirely different nature occur in lysosomes from other cell types.

SUMMARY

Acid mucopolysaccharides have been extracted from whole rabbit polymorphonuclear leucocytes and from the cytoplasmic granules of these cells.

The leucocyte acid mucopolysaccharides can be separated into two fractions by the solubility of their CPC complexes in solutions of differing salt concentration. One of these fractions appears to be identical with hyaluronic acid; the other appears to be an atypical chondroitin sulfate.

On both a dry weight and total protein basis the polymorphonuclear leucocyte granule contains approximately 2.6 times as much acid mucopolysaccharide as does the whole cell. Hyaluronic acid is concentrated in the granules in particular; its function is unknown.

These results do not indicate that all lysosomes contain abundant acid mucopolysaccharides, for no detectable carbohydrate of this class could be extracted from lysosome-rich alveolar macrophages.

The authors wish to thank Dr. James G. Hirsch for his kind encouragement and advice during this investigation.

BIBLIOGRAPHY

1. Kerby, G., The occurrence of acid mucopolysaccharide in human leucocytes and urine, *J. Clin. Inv.*, 1955, **34,** 1738.
2. Clausen, J., and Andersen, V., Acid mucopolysaccharides in human leucocytes, *Clin. Chim. Acta*, 1963, **8,** 505.
3. Hirsch, J., Phagocytin: a bactericidal substance from polymorphonuclear leucocytes, *J. Exp. Med.*, 1956, **103,** 589.
4. Cohn, A., and Hirsch, J., The isolation and properties of specific cytoplasmic granules of rabbit polymorphonuclear leucocytes, *J. Exp. Med.*, 1960, **112,** 983.
5. Cohn, Z., and Wiener, E., The particular hydrolases of macrophages, *J. Exp. Med.*, 1963, **118,** 991.
6. Mathews, M., and Hinds, L., Acid mucopolysaccharides of frog cartilage in thyroxine-induced metamorphosis, *Biochim. et Biophysica Acta*, 1963, **74,** 198.
7. Bera, B., Foster, A., and Storey, M., Observations on the properties of cetyl-

trimethylammonium salts of some acidic polysaccharides, *J. Chem. Soc.*, 1955, **77**, 3788.

8. Dziewiatkowski, D., Some aspects of the metabolism of chondroitin sulfate-S^{35} in the rat, *J. Biol. Chem.*, 1956, **223**, 239.
9. Trevelyan, W., Procter, D., and Harrison, J., Detection of sugars on paper chromatograms, *Nature*, 1950, **166**, 444.
10. Stoffyn, P., and Jeanloz, R., Identification of amino sugars by paper chromatography, *Arch. Biochem. and Biophysics*, 1954, **52**, 373.
11. Dische, Z., A new specific color reaction of hexuronic acids, *J. Biol. Chem.*, 1947, **167**, 189.
12. Brown, A., Determination of pentose in the presence of large quantities of glucose, *Arch. Biochem.*, 1946, **11**, 269.
13. Elson, L., and Morgan, W., A colorimetric method for the determination of glucosamine and chondrosamine, *Biochem. J.*, 1933, **27**, 1824.
14. Lowry, O., Rosebrough, N., Farr, A., and Randall, R., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 1951, **193**, 265.
15. Schiller, S., Slover, G., and Dorfman, A., A method for the separation of acid mucopolysaccharides: its application to the isolation of heparin from the skin of rats, *J. Biol. Chem.*, 1961, **236**, 983.
16. Davidson, E., and Meyer, K., Chondroitin, a new mucopolysaccharide, *J. Biol. Chem.*, 1954, **211**, 605.
17. Hadidian, Z., and Pirie, N., The effects of serum and of hyaluronic acid derivations on the action of hyaluronidase, *Biochem. J.*, 1948, **42**, 266.
18. de Duve, C., General properties of lysosomes, *in* Lysosomes, Ciba Foundation Symposium, (A. de Reuck and M. Cameron, editors), London, J. & A. Churchill, Ltd., 1963, 1.
19. Koenig, H., Histological distribution of brain gangliosides: lysosomes as glycolipoprotein granules, *Nature*, 1962, **195**, 782.
20. Zeya, H., and Spitznagel, J., Antibacterial and enzymic basic proteins from leucocyte lysosomes: separation and identification, *Science*, 1963, **142**, 1085.
21. Horn, R., and Spicer, S., Sulfated mucopolysaccharide and basic protein in certain granules of rabbit leukocytes, *Lab. Inv.*, 1964, **13**, 1.