Studies in Immunochemistry

18. THE ISOLATION AND PROPERTIES OF A SIALOMUCOPOLYSACCHARIDE POSSESSING BLOOD-GROUP Le^a SPECIFICITY AND VIRUS-RECEPTOR ACTIVITY*

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Werner (1953) and Odin (1959) examined in considerable detail the different types of carbohydrate-protein complexes occurring in human epithelial mucus, in secretions of the digestive and respiratory tracts, in cervix uteri and especially in ovarian-cyst fluids, and distinguished two main forms of carbohydrate bound to protein, glycoprotein and mucoprotein or sialomucin.

The glycoproteins isolated from these sources were found to be closely similar in chemical and physical properties to the blood-group-specific substances, materials called neutral mucopolysaccharides, which had previously been isolated from human secretions and tissue digests and thoroughly examined (cf. Kabat, 1956; Morgan, 1947, 1959). The blood-group A, B, H and Le^a mucopolysaccharides earlier described each contained four sugars, fucose, galactose, glucosamine and galactosamine, which made up about 80% of each substance, and not less than 11 or 12 firmly bound amino acids, which together comprised the remaining 20% or so of the mucopolysaccharide. The substances sometimes contained a relatively small amount of sialic acid.

The second type of carbohydrate-protein combination called sialomucin, mucoprotein or, more simply, muco-substance, contained much less carbohydrate and a larger amino acid-containing moiety than did the neutral mucopolysaccharide. The sugar components, however, were different, and in place of fucose, which was decreased in amount to about 1% and was possibly there as mucopolysaccharide impurity, sialic acid was present as a major component. These muco-substances were found to be widespread in the body tissues and fluids, more especially in urine (Tamm & Horsfall, 1952; Gottschalk, 1952; Odin, 1952; Tamm, Bugher & Horsfall, 1955), in the pseudomyxomatous water-insoluble gelatinous mucus of ovarian, peritoneal and appendiceal origin (Odin, 1959), and in serum (Weimer, Mehl & Winzler, 1950; Winzler, 1955). The present views on mucopolysaccharides and on sialo- or muco-proteins are reviewed by Bettelheim-Jevons (1959).

* Part 17: Buchanan, Crumpton & Morgan (1957).

Muco-substances which contain fucose and sialic acid as major components were believed to be present in cervical mucus (Shettles, Dische & Osnos, 1951; Bergman & Werner, 1951; Werner, 1953), and in the mucus of certain intermediate-type cystadenoma (Odin, 1959), but, until recently, substantially homogeneous, water-soluble materials containing considerable amounts of both these sugars had not been described. Kawasaki (1959), however, isolated from cancerous and normal gastric mucosae, mucopolysaccharides which were closely similar in composition and blood-group activity to the neutral mucopolysaccharides described above but contained, in addition, up to 10% or so of sialic acid. Gibbons (1959) has also obtained from mid-cycle and from pregnancy cervical mucus of cattle, mucoid materials which contain considerable amounts of sialic acid, but which are otherwise similar in type to the human-blood-group substances.

A mucopolysaccharide which contains fucose and sialic acid as major constituents as well as galactose, both amino sugars and at least 11 or 12 amino acids, has now been isolated from a single human ovarian-cyst fluid, and is described in the present paper. In the absence of any agreed terminology, the substance is described as a sialomucopolysaccharide. A preliminary communication on this material has already appeared (Pusztai & Morgan, 1960).

MATERIALS AND METHODS

Cyst fluid. Obtained from a pseudomucinous ovarian cystadenoma, volume 41. The patient's blood phenotype was A₁Le(a+).

Nitrogen. Kjeldahl nitrogen was determined in the apparatus of Markham (1942) and with bromocresol green-methyl red indicator (Ma & Zuazaga, 1942).

Methylpentose. The method of Dische & Shettles (1948), as modified by Gibbons (1955), was employed. Fucose standards (10–50 µg.) were included in each determination.

Amino sugars. A modification (Rondle & Morgan, 1955) of the method of Elson & Morgan (1933) was used. Glucosamine hydrochloride standards in the range 10–50 µg. were included in each determination. Extinctions were read at 535 mµ and the results expressed as glucosamine base.

Galactosamine gives at this wavelength about 92% of the absorption given by an equal weight of glucosamine.

Individual sugars. The quantitative determination of each sugar was also made by paper chromatography with a procedure closely similar to that described by Wilson (1959). The mixtures of sugars obtained after hydrolysis of the mucopolysaccharide, in sealed ampoules with 0.5N-HCl at 100° for 18 hr., were freed from acid by shaking with Amberlite IR-4B resin in the carbonate form and concentrated *in vacuo*. The individual sugars were separated on paper with, as solvent mixture, ethyl acetate-pyridine-acetic acid-water (7.5:5:1:3) and developed with aniline hydrogen phthalate reagent (Partridge, 1949). Each coloured area was cut out and cut into small strips, and the strips were placed in a test tube and extracted with 8 ml. of 0.7N-HCl in 80% (w/v) ethanol, as described by Leloir (1951). Extinctions were determined in 2 cm. cells in a Unicam spectrophotometer. Fucose and galactose (60–70 µg.) were measured at 390 mµ and the two amino sugars (120–150 µg.) at 420 mµ. These analyses were kindly carried out for us by our colleague, Dr D. Doyle.

Reducing sugars. Determined by the colorimetric technique of Nelson (1944). The reducing values are expressed as glucose.

Sialic acid. Determined by the resorcinol method (Svennerholm, 1957), a specimen of *N*-acetylneuraminic acid, kindly supplied by Professor Blix, being used as standard. Absorption was measured at 450 and 580 mµ to eliminate the interference caused by other sugars in the mucopolysaccharide. A preparation of the mucopolysaccharide which had been derived from the sialomucopolysaccharide by removal of the sialic acid by mild acid treatment was included in the assay, and served as a control for the influence of the sugars present on the sialic acid determination. Extinction values obtained were subtracted from those obtained for the sialomucopolysaccharide. *N*-Acetylneuraminic acid standards from 20 to 100 µg. were included in each determination.

The total sialic acid was also determined by the thio-barbituric acid method (Warren, 1959) after the sialomucopolysaccharide had been heated at 80° in 0.1N-H₂SO₄ for 1 hr. to liberate the sialic acid.

Physical examination. Ultracentrifugal and electrophoretic examinations were carried out at pH 8.0, as described by Kekwick (1950).

Preparative ultracentrifugal experiments were made in a Spinco ultracentrifuge (model L) at 1–2°.

Ultraviolet-light absorption was determined on a Hilger quartz spectrophotometer, with an optical path of 1 cm.

Relative viscosity was measured at 20° at a concentration of 1% (w/v) in water in an Ostwald-type viscosimeter.

The intrinsic viscosities were determined from measurements made at 20° in phosphate buffer (I 0.2) containing 0.15M-NaCl, pH 8.

Determination of serological activity. The specific Le^a activity was measured as described by Grubb & Morgan (1949) and Annison & Morgan (1952), with a human anti-Le^a serum Aalerud, kindly supplied by Dr J. Lundevall, and group O Le^a+ cells. A standard Le^a substance was included in all tests. The substance had no A, B or H activity (Race & Sanger, 1958).

Inhibition of viral haemagglutination. Serial dilutions of 0.1% solution of the substance in 0.9% sodium chloride

solution were mixed with an equal volume of unheated or heated (56° for 30 min.) virus equivalent to about four haemagglutinating doses. The mixtures were allowed to stand at room temperature for 15 min., a 1% (v/v) suspension of fowl cells (0.1 ml.) was added to each tube, and after standing for 75 min. the degree of inhibition was read as described by Belyavin, Westwood, Please & Smith (1951).

EXPERIMENTAL AND RESULTS

Isolation of a sialomucopolysaccharide

A part of the cyst fluid was dried from the frozen state and extracted with 90% (w/w) phenol, as described by Morgan & King (1943) and by Gibbons, Morgan & Gibbons (1955). The phenol-insoluble residue was freed from phenol by prolonged dialysis and a small amount of water-insoluble material which separated during dialysis was removed by centrifuging. The clear viscous solution of the phenol-insoluble, water-soluble material was adjusted to about 1% (w/v) concentration and saturated with (NH₄)₂SO₄. The solution, which remained clear, was warmed with constant stirring in the presence of a slight excess of solid (NH₄)₂SO₄. A sticky precipitate began to form between 45° and 50°, and separated completely after heating at about 60° for 5 min. As the solution cooled, the particles of the precipitate coalesced and changed to a visco-elastic mass which attached itself firmly to the bottom of the vessel and to the stirrer, and could be easily collected. The material that was insoluble in cold saturated (NH₄)₂SO₄ was washed by trituration with saturated (NH₄)₂SO₄ solution and was dissolved in water, and the process of saturating with (NH₄)₂SO₄, heating to 60° and collecting the precipitate formed, was repeated. About 5% only of the original phenol-insoluble water-soluble material was soluble in saturated (NH₄)₂SO₄ at 60°. The material insoluble in saturated (NH₄)₂SO₄ was dissolved in water (2%, w/v), potassium acetate (2%, w/v) was added and the substance was fractionated by the addition of ethanol. Fractions separated between 48 and 55% (v/v) of ethanol (24.6 g.), 55 and 57% (v/v) of ethanol (2.4 g.) and 57 and 65% (v/v) of ethanol (2.8 g.), and accounted for almost the whole of the substance insoluble in saturated (NH₄)₂SO₄. The fractionation process was repeated on each of the materials and the substances finally obtained were thoroughly dialysed against distilled water at 1–3° and dried from the frozen state. Except where otherwise stated, the observations which are described below were made on the material isolated and purified as described, and finally again precipitated from 1% (w/v) solution in water by ethanol between 48 and 55% concentration.

Properties of the sialomucopolysaccharide

General. The material dissolved readily to give 2% (w/v) solutions in water or in 0.9% sodium chloride solution which were viscous, clear and colourless. Qualitative chromatographic examination of the products of hydrolysis with 0.5N-HCl at 100° for 18 hr. showed the preparation to contain fucose, galactose, glucosamine, galactosamine and, in a 6N-HCl hydrolysate, at least 11 or 12 amino acids. The material gave a strong colour reaction (Svennerholm, 1956) for sialic acid; P was absent.

Physical. The mucopolysaccharide that was insoluble in cold saturated (NH₄)₂SO₄ immediately after it had been precipitated at 60° was, after it had been recovered by dialysis and drying from the frozen state, readily soluble in this solvent. The material (0.1% in water) showed no specific absorption bands between the wavelengths 220 and 320 mμ. The optical rotation was *laevo*, [α]_D -24° for the main fraction (Table 1).

The material examined electrophoretically migrated anodically as a single substance (Fig.1a), substantially free from materials with a different

Table 1. Analytical values for mucopolysaccharide obtained from ovarian-cyst fluid (no. 350)

The dried cyst fluid was extracted with (a) 90% phenol, (b) further fractionated with saturated (NH₄)₂SO₄ at 60° and finally precipitated from aqueous solution by ethanol.

(a) Material insoluble in phenol, soluble in water					
Ethanol fraction (%) ...		(b) Insoluble in saturated (NH ₄) ₂ SO ₄ at 60°			Soluble in saturated (NH ₄) ₂ SO ₄ at 60°
		48-55	55-57	57-66	50-55
Nitrogen (%)	4.9	4.8	4.8	4.8	4.5
Fucose (%)	8.3	8.4	7.6	7.2	10.5
Hexosamine (%)*	27.0	26.6	25.7	25.1	28.4
Reducing sugar (%)*	51.0	51.3	50.2	48.4	54.0
Sialic acid (%)†	14.6	19.0	—	—	10.6
Sialic acid (%)‡	14.0	17.9	18.1	19.4	10.1
Relative viscosity (η)	2.02	2.88	2.32	1.64	1.26
[α] _D in water (c, 1%)	-24°	-24°	-17°	-17°	-31°

* After hydrolysis with 0.5N-HCl for 16 hr. at 100°.
† As N-acetylneuraminic acid (resorcinol method).
‡ As N-acetylneuraminic acid [Warren's (1959) method].

Fig. 1. (a) Electrophoretic pattern of sialomucopolysaccharide (ascending boundary) after 120 min. at 5v/cm. of 2% (w/v) solution in phosphate, pH 8, I 0.2. (b) Mucopolysaccharide derived from the sialomucopolysaccharide after hydrolysis with acid at pH 2 and 37° for 24 hr.; conditions of electrophoresis were as in (a). Arrows show direction of migration in (a) and (b). (c) Sedimentation diagram of sialomucopolysaccharide after 90 min. at 270 000 g; 1.1% (w/v) in phosphate-NaCl (phosphate, pH 8, I 0.2; NaCl, 0.15M).

electrophoretic mobility. The rate of migration, determined by Dr R. A. Kekwick, from the position of the descending boundary after 4.5 hr., was $7.5 \text{ cm.}^2 \text{ sec.}^{-1} \text{ v}^{-1} \times 10^{-5}$ at pH 8.

The relative viscosity of a 1% (w/v) solution in water of the phenol-insoluble water-soluble material, and those of the materials insoluble and soluble in saturated $(\text{NH}_4)_2\text{SO}_4$ obtained from it, are given in Table 1. The highest viscosity is shown by the material insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60°.

The intrinsic viscosity of the substance insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$ was determined by a linear extrapolation to zero concentration of $\eta \text{ spec./c}$ values plotted against the concentration (Table 2).

The rate of sedimentation of the material observed in the ultracentrifuge showed (Fig. 1c) the preparation to contain only a single main component, which appeared to be somewhat polydisperse. The dependence of the sedimentation constant (S) on concentration is evident from the values given in Table 3, which were also determined by Dr R. A. Kekwick. The polydispersity of the

preparation was approximately determined from the results of the following experiment, which was made on the preparative ultracentrifuge. The sialomucopolysaccharide (2.4 g.) was dissolved to give a 1.8% (w/v) solution in phosphate buffer (I 0.2, pH 8) which contained 0.15M-NaCl. The solution was centrifuged at 20 000 g (average value) for 1 hr. As there was no deposit the g value was raised first to 40 000, then to 70 000, and each g force was applied for 1 hr. No deposit was obtained, which indicated that the preparation was substantially free from large-sized molecules. The centrifuge was then run at top speed (105 000 g , average value) for 5 hr., after which time a small pellet of substance was obtained. The contents of the centrifuge tubes were then divided into four approximately equal sections, each about 2 cm.

Table 2. *Intrinsic viscosity $\eta \text{ spec./c}$ values of the sialomucopolysaccharide at different concentrations*

c, Concentration measured in g./100 ml.

Concentration (g./100 ml.)	$\eta \text{ spec./c}$
1.00	0.720
0.50	0.656
0.25	0.636
0.125	0.624
0.0625	0.610
Infinite dilution	0.605

Table 3. *Sedimentation constants of sialomucopolysaccharide at various concentrations*

Values corrected to water at 20° (Svedberg & Pederson, 1940).

Concentration (g./100 ml.)	$10^{13} \times S_{20}$
1.10	4.2 ₇
0.875	4.9 ₃
0.55	5.7 ₂

Fig. 2. Plot of intrinsic viscosity $[\eta]$ of materials, obtained by centrifuging at 105 000 g , against amount of substance in each fraction. Concentration was measured as g./100 ml.

Table 4. *Chemical, physical and serological data*

Analytical values obtained on fractions isolated from the sialomucopolysaccharide after centrifuging at 105 000 g in phosphate buffer (pH 8) for 5 hr. *c*, Concentration measured in g./100 ml.

Fraction	Recovery		Sialic acid (%)*	Nitrogen (%)	$\eta \text{ spec./c}$	Le ^a activity†
	mg.	%				
Top	24	1.0	15.2	5.2	0.380	0.025
Second	264	12.4	18.1	4.5	0.455	0.012
Third	520	24.5	17.7	4.6	0.505	0.012
Bottom	1250	60.0	16.8	4.8	0.605	0.025
Sediment	62	2.1	14.0	5.6	0.700	0.012
Original mucopolysaccharide	—	—	17.9	5.0	0.605	0.025

* As *N*-acetylneuraminic acid [Warren's (1959) method].

† Minimum amount ($\mu\text{g.}$) giving inhibition.

long, starting from the top of the cups, and the solution comprising each layer was carefully removed with a Pasteur pipette, the tip of which was bent upwards. The pellet of substance and the material in each of the sections was recovered after removal of the buffer salts by dialysis. The yields and other analytical data obtained for these fractions are given in Table 4, from which it appears that the bulk of the preparation was composed of molecules with closely similar properties. A plot of the intrinsic-viscosity values for each material against the amount recovered gives a typical Gaussian curve for the molecular-size distribution (Fig. 2), and shows that the majority of the mucopolysaccharide entities are of closely similar size.

Chemical. No precipitate was formed when a 1% (w/v) solution of the material was mixed with an equal volume of the following protein precipitants: trichloroacetic acid (20%, w/v), salicylsulphonic acid (20%, w/v), saturated solutions of tannic acid or picric acid, a 10% solution of lead acetate (in dilute acetic acid), mercuric acetate, aluminium sulphate in 5N-H₂SO₄, phosphotungstic acid, acid or neutral copper sulphate. The analytical figures of the materials obtained as a result of differential high-speed centrifuging (Table 4), indicated that the preparation was remarkably homogeneous in terms of N and sialic acid, and that the latter component was present to the extent of about 18%. The material contained 8.4% of fucose (Table 1), a value considerably lower than that usually found for group-specific A, B and H mucopolysaccharides (about 18%) and somewhat lower than that recorded earlier (about 12%) for preparations of Le^a-specific mucopolysaccharide (Annison & Morgan, 1952).

Table 5. *Amino acid composition of the Le^a specific mucopolysaccharide*

	Amino acid (g./100 g. of mucopolysaccharide)
Aspartic acid	0.49
Threonine	4.48
Serine	1.91
Glutamic acid	0.87
Proline	2.04
Glycine	0.64
Alanine	1.36
Cysteine	0.15
Valine	0.89
Methionine	0.09
Isoleucine	0.38
Leucine	0.87
Tyrosine	0.17
Phenylalanine	0.31
Lysine	0.28
Histidine	0.37
Arginine	0.32

A 1% (w/v) solution of the sialomucopolysaccharide was hydrolysed with 0.5N-HCl for 16 hr. at 100° in sealed ampoules, and the products, which showed considerable humin formation, were assayed for total amino sugar and reducing sugar (Table 1). Analysis of the hydrolysis products by quantitative paper chromatography gave 28.8% of galactose, 20% of glucosamine and 6% of galactosamine, but, owing to extensive humin formation when hydrolysis takes place in the presence of so much sialic acid, these values are subject to a considerably larger error than are those obtained when a sialic acid-free specimen of the mucopolysaccharide is used. Hydrolysis of the material with boiling 6N-HCl and examination of the products on columns of sulphonated polystyrene resins (Moore & Stein, 1951, 1954) was kindly carried out by Dr S. Jacobs of the National Institute for Medical Research. The amino acid composition of the sialic acid-free mucopolysaccharide is given in Table 5, the values obtained being calculated from the ninhydrin values of the effluent fractions. The amino acid composition of the material is similar in pattern to that obtained earlier for other blood-group mucopolysaccharides and shows that the amino acids threonine, serine and proline again make up more than 50% of all the amino acids present, and that the amounts of cysteine, methionine and tyrosine are small. Phenylalanine is present, but in small amounts.

The sialic acid is not released by heating an aqueous solution of the sialomucopolysaccharide at 100°, as reported (Odin, 1956) for the sialic acid-containing cervical mucus or hog seminal gel, but is rapidly eliminated by heating in 0.1N-H₂SO₄ at 80° for 1 hr. or in citrate-HCl buffer at pH 2 and 37° for 24–48 hr.

The sialic acid can also be readily removed from the sialomucopolysaccharide by receptor-destroying enzyme obtained from *Vibrio cholerae*. Sialomucopolysaccharide (0.28 g.) was dissolved in 20 ml. of water. A portion (1 ml.) of the enzyme preparation, which had been purified by adsorption on to and elution from human erythrocytes, was added and the mixture was incubated at 37° for 16 hr. The solution was then dialysed, a thoroughly washed cellophan membrane being used, and the diffusate dried. The indiffusible part was heated at 80° for 10 min., centrifuged to remove any coagulated protein of the receptor-destroying enzyme, and the solution was dried from the frozen state. A single treatment with the enzyme liberated about 30% of the total sialic acid. Repeated treatments, however, as shown in Table 6, removed the sialic acid almost completely. The experimental details and analytical values obtained are given in Table 6.

Immunological. The sialomucopolysaccharide did not inhibit the specific agglutination of A, B or O

Table 6. *Removal of sialic acid from the sialomucopolysaccharide by purified receptor-destroying enzyme*

	Amount of enzyme solution added	Sialic acid content of the sialomuco- polysaccharide (%)	Sialic acid in diffusate (mg.)	Indiffusible material* (mg.)
Original	—	19	—	280
After first treatment	1.0	13	20	236
After second treatment	0.6	10	9.9	198
After third treatment	0.8	3.9	13.1	163
After fourth treatment	0.8	0.2	6.0	124

* Uncorrected for material removed for analysis.

erythrocytes by the appropriate group-specific haemagglutinins, but was a powerful inhibitor of the agglutination of Le(a+) cells by a human anti-Le(a+) serum, and the haemagglutination brought about by the action of three or four completely agglutinating doses of human Le^a antibody on an equal volume of a 1% suspension of Le(a+) red cells in 0.9% sodium chloride solution was completely inhibited by 0.025 µg. of the material. The removal of the sialic acid component by receptor-destroying enzyme or by gentle hydrolysis with acid causes no demonstrable change in the capacity of the material to neutralize anti-Le^a serum (Table 7).

The sialomucopolysaccharide, tested in amounts of 100, 10 and 1 µg. in 0.1 ml. of 0.9% sodium chloride solution, precipitated when mixed with an equal volume of anti-pneumococci type XIV horse serum, as did all other preparations of Le^a substance examined. After removal of the sialic acid component with receptor-destroying enzyme, however, each of the test concentrations gave a considerably increased amount of precipitate when examined under similar conditions.

Virus-inhibitor activity of sialomucopolysaccharide

The knowledge that sialic acid acts as a functional group in those mucosubstances which are powerful inhibitors of viral haemagglutination and the ready elimination of this component with receptor-destroying enzyme, made us determine if the sialomucopolysaccharide possessed any activity as an inhibitor of influenza viruses.

The first experiments were made for us by Professor Wilson Smith and Dr A. Cohen of University College Hospital Medical School, to whom we wish to express our thanks. Three types of influenza-virus strains were used: ASH (type A prime), ROB (type B) and A/Sing/W (Asian type), and the tests were done with both unheated and heated viruses at a constant dose of four agglutinating doses. This ensures the detection of inhibitory activity due to any of the known types of virus-haemagglutination inhibitors. Three classical

virus-haemagglutination inhibitors were tested in parallel with the sialomucopolysaccharide, namely, human urinary mucoprotein (Tamm & Horsfall, 1952; Gottschalk, 1958), sheep submaxillary mucoid (McCrea, 1953) and crude egg white.

The results showed that the sialomucopolysaccharide was without appreciable inhibitor activity against unheated ASH and ROB viruses or against A/Sing/W, either heated or unheated. Against heated or 'indicator' ROB virus, however, as little as 0.28 µg. inhibited the haemagglutinating activity of four agglutinating doses of the virus. This result shows that the substance is an extremely potent haemagglutination inhibitor of the α type (Smith, Westwood & Belyavin, 1951), and was similar in this respect to the urinary mucoprotein and the sheep submaxillary mucoid, which had approximately the same degree of activity. The removal of sialic acid from the sialomucopolysaccharide by treatment either with receptor-destroying enzyme or with dilute acid at room temperature resulted in the complete abolition of inhibitory activity (Table 7).

The Le^a activity of the sialomucopolysaccharide is destroyed by an enzyme obtained from *Trichomonas foetus* (Watkins, 1953, and unpublished work). The enzyme, which was largely separated from the neuraminidase (Dr W. M. Watkins unpublished results) present in the crude *T. foetus* extract by zone electrophoresis on a starch column, at pH 6.2 for 24 hr., brought about the destruction of Le^a specificity, whereas the virus-inhibiting capacity of the sialomucopolysaccharide, although decreased owing to the removal of some of the sialic acid, nevertheless remained considerable when tested with the 'indicator' forms of the ASH and ROB strains of influenza virus.

Isolation and identification of N-acetylneuraminic acid

The sialomucopolysaccharide (5 g.) was dissolved in 500 ml. of 0.01 N-H₂SO₄ and adjusted to pH 2 by the addition of 0.1 N-H₂SO₄. The solution was kept at 37° for 24 hr., after which time the acid was neutralized by the addition of BaCO₃. The

BaSO₄ and excess of BaCO₃ were removed and the clear, colourless solution was thoroughly dialysed through a well-washed cellophan membrane against repeated changes of distilled water at 1–2°. The combined diffusates were concentrated to about 30 ml. and treated as a batch operation with Amberlite IR-120 in the hydrogen form to remove cations. The solution was evaporated to dryness, the residue was extracted with methanol (4 ml.) containing a few drops of water, and ethyl ether was added until a slight turbidity developed. After standing overnight at 1–3°, a small precipitate formed and was removed. A few drops of ethyl ether and light petroleum (b.p. range 40–60°) were added to the clear solution to give a faint turbidity and the solution was allowed to stand at 1–3° for several days. A microcrystalline material (80 mg.) separated and was filtered off, washed with a small volume of ice-cold absolute methanol and dried. The mother liquors contained 630 mg. of sialic acid, as determined by Svennerholm's method, and a further amount of microcrystalline material (110 mg.) was later recovered and identified as *N*-acetylneuraminic acid.

A 1% (w/v) solution of the crystalline material had, after one further recrystallization, a *laevo*-rotation $[\alpha]_D^{18} - 31^\circ$, contained 4.4% of N and decomposed between 182° and 190°. The authentic *N*-acetylneuraminic acid had $[\alpha]_D^{18} - 32 \pm 2^\circ$, 4.5% of N and decomposed between 185° and 187°. Under Svennerholm's (1957) conditions the crystalline material gave an extinction (580 m μ) 95.8% of that given by pure *N*-acetylneuraminic acid. The material behaved on paper chromatograms, with solvent mixtures pyridine–ethyl acetate–acetic acid–water (5:7.5:1:3) or butan-1-ol–propan-1-ol–0.1N-HCl (1:2:1), as did *N*-acetylneuraminic acid. The crystalline material was converted into pyrrole- α -carboxylic acid, as described by Gottschalk (1953, 1955), by heating in 0.1N-NaOH at

100° for 15 min., and the resulting product showed a strong absorption maximum at 263 m μ . The material gave no colour on heating with ninhydrin (Moore & Stein, 1954), but an immediate and strong 'direct' colour with a *p*-dimethylaminobenzaldehyde–HCl reagent (Osaki & Turumi, 1947; Hiyama, 1949; Odin, 1952; Gottschalk, 1953). The results indicate that the crystalline material isolated from the sialomucopolysaccharide was *N*-acetylneuraminic acid.

After hydrolysis at pH 2, as described above, the mucopolysaccharide that did not diffuse through cellophan was recovered and its physical, chemical and immunological properties were examined. The material remained electrophoretically homogeneous at pH 8 (Fig. 1*b*), but its mobility, 4.2 cm.² sec.^{–1} v^{–1} $\times 10^{-5}$, was much reduced. The fall in the viscosity found, about 20%, corresponds closely to that which might be expected from the loss of sialic acid equivalent to about a fifth of the material. The mucopolysaccharide contained 28.8% of galactose, 22.8% of glucosamine and 5.8% of galactosamine. Other analytical figures are given in Table 7. The material showed no loss of Le^a activity, but failed completely to inhibit haemagglutination brought about by the two test strains of heated influenza virus.

Isolation of the Le^a-specific mucopolysaccharide from cyst fluid without the use of phenol

The isolation of the mucopolysaccharide from the cyst fluid by the action of pepsin at pH 1.9, to remove the major part of the co-existing but non-specific protein and thus avoid the use of 90% phenol, was carried out as follows. The cyst fluid (500 ml.) was mixed with an equal volume of 0.1M-citrate–HCl buffer at pH 1.9 and digested at 37° with 100 mg. of crystalline pepsin. Toluene was added as a preservative and the digest was frequently shaken during the period of incubation,

Table 7. *Analytical figures of the sialomucopolysaccharide*

Values obtained before and after treatment with (a) receptor-destroying enzyme (R.D.E.) and (b) acid at pH 2 and 37°.

	Original sialomuco- polysaccharide	Mucopolysaccharide	
		(a) After R.D.E.	(b) After hydrolysis (pH 2)
Nitrogen (%)	4.8	5.2	5.3
Fucose (%)	8.4	9.1	9.0
Hexosamine (%)*	26.6	29.6	29.7
Reducing sugar (%)*	51.3	55.6	55.0
Sialic acid (%)†	17.9	1.0	0.5
Viscosity (η , in water)	2.88	2.48	2.60
$[\alpha]_D$ in water (c, 1%)	–24°	–24°	–24°
Le ^a activity (μ g.)‡	0.025	0.025	0.025
Virus activity (μ g.)‡	0.28	> 28	—

* See footnote Table 1.

† By Warren's (1959) method.

‡ Minimum amount giving inhibition.

and readjusted to pH 1.9 each day with HCl. After several days' incubation the digest was thoroughly dialysed to remove salts, amino acids and low-molecular-weight products of peptic hydrolysis, and the indiffusible material was recovered, dissolved in water to give a 2% solution, saturated with $(\text{NH}_4)_2\text{SO}_4$ and heated at 60° for about 5 min. A precipitate formed and was collected, thoroughly washed with saturated $(\text{NH}_4)_2\text{SO}_4$, dissolved in water, the solution was dialysed and the material insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$ was recovered (10.6 g.). The material soluble in hot saturated $(\text{NH}_4)_2\text{SO}_4$ was likewise recovered (1.5 g.). A part of the insoluble fraction (5 g.) was dissolved in dimethylsulphoxide and fractionated by the addition of ethanol. The largest fraction (3.3 g.), which separated between 40 and 60% (v/v) of ethanol concentration, was recovered, dissolved in aqueous 2% potassium acetate to yield a 2% solution and fractionated with ethanol. The main fraction (2.5 g.) separated between 45 and 50% (v/v) of ethanol. The analytical figures (Table 8) obtained for this material and for that precipitated between

50 and 55% (v/v) of ethanol give values (Table 7) similar to those obtained for material purified through phenol and treated with acid at pH 2 and 37° to remove *N*-acetylneuraminic acid.

Action of papain on sialomucopolysaccharide

There is evidence that the α_1 -acid glycoprotein of human plasma is not attacked by the proteolytic enzymes trypsin and papain (Yamashina, 1956) until after the removal of the sialic acid component (10–11%), when digestion readily occurs. It was therefore determined whether the sialomucopolysaccharide, because of its high content of sialic acid, was degraded by papain, it being already known that the blood-group-specific mucopolysaccharides devoid of sialic acid are readily attacked by this enzyme (Pusztai & Morgan, 1958).

Sialomucopolysaccharide (2 g.) was digested with 100 mg. of three-times crystallized papain activated with 2:3-dimercaptopropanol (BAL) at pH 7.6 and 37° for 24 hr., thoroughly dialysed at 1–2° and the indiffusible material was saturated with $(\text{NH}_4)_2\text{SO}_4$ and warmed to 60°. No precipitate formed, although before digestion with papain the substance was completely precipitated under these conditions. The material was recovered from the saturated $(\text{NH}_4)_2\text{SO}_4$ solution, thoroughly dialysed to remove salt and dried from the frozen state. A 2% (w/v) solution of the substance was then fractionated by the addition of ethanol containing 2% of potassium acetate and the following ethanol fractions were collected: 50–55% (1.38 g.), 55–60% (0.04 g.), 60–75% (0.25 g.). The number of fractions separating and the yield of each indicates that the material was split largely into two main components and that no appreciable amount of diffusible material was formed during digestion with papain. The analytical results obtained for the products, and for the original sialomucopolysaccharide, are given in Table 9.

Table 8. *Analytical values of materials isolated from ovarian-cyst fluid by treatment with pepsin*

The dried cyst fluid digested with pepsin at pH 2, saturated with $(\text{NH}_4)_2\text{SO}_4$ at 60°, fractionated from dimethylsulphoxide by ethanol (40–60%) and subsequently precipitated from aqueous solution by ethanol.

Ethanol fraction (%) ...	45–50 (2.5 g.)	50–55 (0.4 g.)
Nitrogen (%)	5.2	5.5
Fucose (%)	8.2	7.4
Hexosamine (%)*	28.4	30.4
Reducing sugar (%)*	55.0	53.0
Sialic acid (%)†	0.8	0.2
Relative viscosity (η)	3.06	1.62
$[\alpha]_D$ in water (c, 1%)	–24°	–26°

* See footnote Table 1.

† Determined by Warren's (1959) method.

Table 9. *Products of the action of crystalline papain on the sialomucopolysaccharide*

Analytical values obtained for materials isolated from sialomucopolysaccharide after digestion with crystalline papain at pH 7.6 and fractionation with ethanol.

Ethanol fraction (%) ...	Original material	Material recovered after digestion with papain		
		0–55	55–60	60–75
Nitrogen (%)	5.0	4.9	4.6	4.7
Fucose (%)	8.4	9.0	8.1	8.8
Hexosamine (%)*	26.5	28.0	24.4	25.2
Reducing sugar (%)*	51.2	53.0	49.2	50.0
Sialic acid (%)†	19.0	18.7	16.6	16.9
Sialic acid (%)‡	17.9	17.9	16.4	16.5
Relative viscosity (η)	2.88	2.10	1.44	1.17
$[\alpha]_D$ in water (c, 1%)	–24°	–26°	–20°	–29°
Le ^a activity ($\mu\text{g.}$)§	0.05	0.40	0.80	1.50
Material recovered (%)	—	81.5	2.5	15

*, †, ‡ See footnotes Table 1. § See † footnote, Table 4.

DISCUSSION

A mucopolysaccharide which contains sialic acid as a major carbohydrate component, as well as the fucose, galactose, glucosamine, galactosamine and amino acids which are usually found in the A, B, H and Le^a specific blood-group substances, has been isolated from a single specimen of human ovarian-cyst fluid. The cyst fluid in this instance was obtained from a person of blood phenotype A₁Le(a+), a so-called non-secretor, and, in consequence, showed no group A serological activity but strong and specific Le^a character. Mucopolysaccharides possessing Le^a activity alone have already been obtained, and their immunological properties recorded (Grubb & Morgan, 1949; Annison & Morgan, 1952), but they differ from the Le^a substance now described in that they contain at most only very small and variable amounts of sialic acid, whereas the present material contains about 18% of this acid. In order to differentiate this type of mucopolysaccharide from the closely similar blood-group-specific mucopolysaccharides obtained earlier and to indicate its high content of sialic acid, it has been called sialomucopolysaccharide.

The sialomucopolysaccharide remains insoluble in 90% phenol when the dried ovarian-cyst contents are extracted with this solvent, but dissolves readily in water after phenol extraction and can be further purified by precipitation at 60° from solution in saturated ammonium sulphate. Additional purification is achieved by repeated fractionation from solution in dimethylsulphoxide or water by ethanol. The material so obtained gives clear, viscous 2% solutions in water or 0.9% sodium chloride solution and behaves as an essentially homogeneous substance on the basis of electrophoretic and ultracentrifugal measurements at pH 8. An estimate of the size distribution of the macromolecules in the preparation, based on the amount and intrinsic viscosity of each of the fractions obtained by high-speed centrifuging, indicates that about 85% of the macromolecules have intrinsic viscosity values between 0.50 and 0.60. The average molecular weight of the sialomucopolysaccharide preparation, calculated from the sedimentation constant and the intrinsic viscosity of the whole preparation by means of the formula

$$M = \frac{4690 \times (S_{20,w})^3 \times [\eta]^3}{(1 - \bar{V}\delta)^3}$$

(cf. Schachman, 1957), where M is the molecular weight, $S_{20,w}$ is the sedimentation coefficient ($S_{20,w}$ 5.72), $[\eta]$ the intrinsic viscosity (0.67), $\bar{V}$ the partial specific volume (0.63) and δ the density of the solution (1.008), gave a value of 237×10^3 . This figure is similar to that obtained earlier, 270×10^3

(Kekwick, 1952), for a preparation of Le^a substance which contained about 1% of sialic acid, the diffusion constant for the substance being used in place of intrinsic viscosity.

The removal of sialic acid from the sialomucopolysaccharide by the action of receptor-destroying enzyme lowers its relative viscosity by an amount which corresponds closely to that which might be expected from the lower molecular size after the removal of about 18% of sialic acid. It seems improbable therefore that, in this instance, the sialic acid is itself responsible for the high viscosity of the sialomucopolysaccharide. This conclusion is at variance with that reached by Odin (1958), who considers that the high viscosity of certain ovarian-cyst preparations is due to their content of sialic acid. Similarly, Gibbons (1959) is inclined to believe that since the sialic acid of oestrous and pregnancy specimens of bovine cervical mucins is the only major chemical distinction between them, it therefore plays a part in determining the degree of molecular expansion which, in turn, is responsible for the consistency of the mucus.

Electrophoretic examination at pH 8 gives the mobility of the sialomucopolysaccharide as $-7.6 \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1} \times 10^{-5}$, which is much greater than the mobility, $-4.2 \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1} \times 10^{-5}$, of the mucopolysaccharide obtained from it by removing the sialic acid enzymically with receptor-destroying enzyme. The true mobility value for the mucopolysaccharide could be somewhat lower than this figure as, after treatment with the enzyme, the material examined still retained about 1% of sialic acid.

The sialic acid was readily split from the sialomucopolysaccharide by treatment with sulphuric acid at pH 2 and 37° and was identified as *N*-acetylneuraminic acid. Although it is probable that *N*-acetylneuraminic acid is present in the sialomucopolysaccharide, one cannot yet be completely certain of this, as it is well known that the *O*-acetyl groups in the *ON*-diacetylneuraminic acids (Blix, 1959) are extremely labile and are frequently lost during the isolation and purification of sialic acid-containing materials. However a sialic acid other than *N*-acetylneuraminic acid does not yet appear to have been described in materials derived from human sources.

It has long been known (cf. Burnet, 1951) that various strains of influenza virus and other closely related viruses, such as those of mumps and Newcastle disease in fowls, agglutinate vertebrate erythrocytes and that the haemagglutination can be inhibited by many different muco-substances (Anderson, Burnet, Fazekas de St Groth, McCrea & Stone, 1948). Recent work has shown that highly purified mucoprotein obtained from urine and from submaxillary glands, the acid α_1 and α_2 muco-

proteins of serum and the ovomucin fraction of egg white are very powerful inhibitors and, on the basis of many earlier observations of Gottschalk (cf. Gottschalk, 1956), Odin (1952) and Klenk & Laurenstein (1952), first identified the essential functional group in these different mucoproteins as sialic acid.

The sialomucopolysaccharide also inhibits the haemagglutination of fowl erythrocytes by the heated or 'indicator' form of two strains of influenza virus, ASH (type A prime) and ROB (type B), and has about the same capacity to inhibit haemagglutination brought about by these viruses as have the highly purified preparations of human urinary mucopolysaccharide and sheep submaxillary mucoid included in the assay. Neither the unheated nor the heated form of an Asian type of influenza virus was inhibited by the sialomucopolysaccharide. The removal of sialic acid from the sialomucopolysaccharide by receptor-destroying enzyme or by incubation at 37° with sulphuric acid at pH 2 gives a mucopolysaccharide which does not inhibit haemagglutination brought about by heated virus.

The sialomucopolysaccharide, or the mucopolysaccharide derived from it after the removal of sialic acid by acid or by receptor-destroying enzyme, are potent inhibitors of Le^a-specific isoagglutination. The ready removal of sialic acid from sialomucopolysaccharide by purified enzyme without the release of other components, and the recovery of a mucopolysaccharide containing all the sugars originally present except sialic acid, indicates that sialic acid units are most probably attached as single end residues or as side units and are not built into the main carbohydrate chains. In any event, it is clear that they in no way demonstrably interfere with the reactivity of the blood-group Le^a-specific carbohydrate structures which are also considered to be present as non-reducing end groups in the Le^a-active mucopolysaccharides (Watkins & Morgan, 1957). The sialomucopolysaccharide obtained after the Le^a-specific structures have been destroyed by an enzyme obtained from *T. foetus* is still active as an inhibitor of virus haemagglutination and largely retains its sialic acid component.

The complete independence of each of the biologically active structures in the sialomucopolysaccharide is emphasized by the ready destruction of each specific character by the appropriate enzyme, with the result that, from a sialomucopolysaccharide which has both activities, a mucopolysaccharide which possesses either Le^a specificity or anti-viral activity alone can be obtained.

The sialomucopolysaccharide precipitates with anti-pneumococci type XIV horse serum, as was observed earlier (Annison & Morgan, 1952) for an Le^a mucopolysaccharide which contained no appreciable amount of sialic acid. This cross-

reactivity is considerably enhanced when the N-acetylneuraminic acid is removed by receptor-destroying enzyme, so that the removal of sialic acid brings about the exposure of structures which react with type XIV antibody just as does the removal of fucose (cf. Kabat, 1956).

The isolation of a mucopolysaccharide free from sialic acid when the Le^a substance was recovered from the crude ovarian-cyst fluid, by incubation with pepsin at pH 1.9 to remove the co-existing protein, is not surprising in view of the rapidity with which this component is removed from the sialomucopolysaccharide, and indeed from all sialic acid-containing mucoproteins, by dilute acid. It is evident that naturally occurring sialomucopolysaccharides cannot be obtained by any procedure which involves acid digestion with pepsin, a method so frequently used to recover the blood-group-specific substances (cf. Kabat, 1956). On the other hand, the recovery of sialomucopolysaccharide by the extraction of the dried crude ovarian-cyst fluid with 90% (w/w) phenol is readily achieved with no demonstrable loss of the sialic acid component. The further purification of the sialomucopolysaccharide by treatment with saturated ammonium sulphate at 60°, to remove similar material of smaller molecular weight and other impurities, likewise does not eliminate the sialic acid component.

In the light of considerable experience with the use of 90% phenol rather than peptic digestion for the removal of contaminating protein for the isolation of the blood-group-specific mucopolysaccharides, it is evident that most native mucopolysaccharides obtained from pseudomucinous cyst fluids contain only a small amount of sialic acid. On the evidence at present available it is not known if the specific substance is secreted as a sialomucopolysaccharide, which then loses all or part of its sialic acid component by the action of tissue neuraminidases during the time needed for the mucopolysaccharide to accumulate *in vivo*, or is in most instances secreted as a mucopolysaccharide which is free from sialic acid. It is observed, however, that many of the mucopolysaccharide preparations recovered by phenol extraction contain small and variable amounts of sialic acid, the presence of which could be considered as the survival of a small part only of an original major component of sialic acid, or the failure of the synthetic step which adds sialic acid. The contamination of a mucopolysaccharide that is free from sialic acid by a sialomucopolysaccharide similar to that described in this paper, would be readily detected by electrophoretic examination.

The changes induced in the sialomucopolysaccharide by papain are similar to those reported earlier for the blood-group-specific mucopolysac-

charides which were devoid of a major sialic acid component, and it appears therefore that the presence of a considerable amount of *N*-acetylneuraminic acid in the macromolecule does not prevent the degradation of the sialomucopolysaccharide by this proteolytic enzyme.

SUMMARY

1. A sialomucopolysaccharide isolated from ovarian-cyst fluid obtained from a person of blood phenotype A Le(a+) is closely similar in chemical composition and physical properties to the blood-group-specific mucopolysaccharides obtained earlier from pseudomucinous ovarian-cyst fluids. The material contains 18% of sialic acid in addition to the fucose, galactose, glucosamine, galactosamine and amino acids usually found.

2. The sialic acid, which is most probably present as *N*-acetylneuraminic acid, is readily removed by acid at pH 1.9 and 37° or by receptor-destroying enzyme.

3. The sialomucopolysaccharide is an active Le^a-specific substance and a potent inhibitor of the haemagglutination by heated type A and B influenza viruses. The removal of the sialic acid component by receptor-destroying enzyme leaves a mucopolysaccharide which has no capacity to inhibit viral haemagglutination, but is fully active as a Le^a substance.

4. The destruction of the Le^a specificity by a Le^a-destroying enzyme obtained from *Trichomonas foetus* leaves a mucopolysaccharide which contains sialic acid and is an inhibitor of viral haemagglutination.

5. The sialomucopolysaccharide precipitates with anti-pneumococci Type XIV horse serum. The degree of precipitation is considerably enhanced after the removal of the sialic acid by receptor-destroying enzyme.

6. The sialomucopolysaccharide is an example of a naturally occurring macromolecule which possesses two biological activities, each of which is associated with a different chemical structure.

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The Enzymic Formation of Haem by Human and Rat Tissues

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Previous studies have shown that chicken erythrocytes and human reticulocytes contain iron-incorporating enzyme or enzymes, which incorporate iron into protoporphyrin to form haem (Goldberg, 1959). In the adult, the tissues predominantly concerned in haem synthesis are the bone marrow and liver. In the foetus, and in pathological states of extramedullary erythropoiesis in the adult, the liver and spleen play a major role in haem synthesis. Gibson, Neuberger & Scott (1955) have shown that the enzyme δ -amino-laevulinic dehydrase is present not only in bone marrow and liver, but also in the kidney and spleen of the rabbit. The experiments described in this paper were undertaken to determine the distribution and activities of the iron-incorporating enzyme in human and rat tissues.

Bessis & Breton-Gorius (1957) have shown by electron microscopy that iron is preferentially located in the mitochondria of the haemoglobin-forming erythroblast. These histological studies have been a stimulus to determine which intracellular component is the richest source of iron-incorporating enzyme. Ascorbic acid and reduced glutathione potentiate the enzyme system when it is derived from chicken blood. Further investigations were therefore carried out to determine the effect of these substances on the iron-incorporating enzyme derived from human and rat tissues.

MATERIALS

Radio iron ($^{59}\text{FeCl}_3$; specific activity 1–3 mc/mg. of iron) was obtained from A.E.R.E., Harwell. Protoporphyrin methyl ester, prepared from blood by the method of

Grinstein (1947), was a gift from Professor C. Rimington, F.R.S. The ester was hydrolysed with 25% HCl for 16 hr., the free protoporphyrin was dried in a desiccator, suspended in 0.142M-sodium bicarbonate and the final solution adjusted to pH 7.4. Ascorbic acid and reduced glutathione (GSH; British Drug Houses Ltd.) were dissolved in water and the resultant solutions were adjusted to pH 7.4 before use. The water used throughout the experiments was purified by passage through an Elgastat deionizer. The glassware was washed with chromic acid, then with 2N-HCl and finally with water.

METHODS

Rat tissues. Young female albino rats, Wistar strain, were anaesthetized with ether and killed by exsanguination. Rat intestine was washed free of its contents by repeated washes with 0.9% sodium chloride solution. Each tissue was washed with water, dried on filter paper, weighed and homogenized with three or seven times its volume of 0.25M-sucrose in an ice-cooled Potter glass homogenizer. To the homogenate was added one-quarter of its volume of 0.45M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7.4. With liver homogenates, intracellular constituents were fractionated (from a 1:8 homogenate), by the method of Schneider (1948), into nuclei, mitochondria and microsomes plus cell sap. Each fraction was suspended in 0.25M-sucrose to the same volume as the original homogenate, and 0.25 vol. of 0.45M-tris buffer, pH 7.4, was added.

Human tissues. Samples of spleen and kidney removed from patients undergoing surgery for splenectomy or nephrectomy were treated similarly. Biopsies of normal liver were obtained from patients undergoing upper abdominal surgery. Bone-marrow samples were obtained in the following way: a 10 ml. sample of mixed bone marrow and blood was withdrawn from the iliac crest by the method of Rheingold, Weifuse & Dameshek (1949) with a heparin-