The Nature of the Non-Ultrafilterable Glycosaminoglycans of Normal Human Urine

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1. The non-ultrafilterable acidic glycosaminoglycans from pooled urine of normal men, aged about 20, were isolated and characterized. The isolation procedure included digestion with sialidase and pronase, and fractionation by stepwise elution from an ECTEOLA-cellulose column. The glycosaminoglycans in each fraction were separated from each other by preparative electrophoresis in sodium barbital buffer and in barium acetate. 2. Approximate relative amounts of the different glycosaminoglycans were: chondroitin sulphate 60%, chondroitin 2%, hyaluronic acid 4%, dermatan sulphate 1%, heparan sulphate 15% and keratan sulphate 18%. Chondroitin sulphate-dermatan sulphate hybrids seemed to occur in trace amounts. 3. Chondroitin sulphate, heparan sulphate and keratan sulphate were heterogeneous with respect to degree of sulphation. Two distinct groups of chondroitin sulphate fractions were found, with sulphate/hexosamine molar ratios of about 0.5 and 1 respectively. The sulphate/hexosamine molar ratios in the heparan sulphate fractions varied from 0.5 to 0.9; the *N*-sulphate/hexosamine ratio was about 0.5 in all fractions. The sulphate/hexosamine molar ratios in the keratan sulphate fractions varied from 0.2 to 0.7.

All of the types of glycosaminoglycan that occur in human tissues, with the exception of heparin, have also been found in normal urine (Mörner, 1895; Di Ferrante & Rich, 1956; Berggård, 1962*b*; King, Fielden & Boyce, 1962; Di Ferrante, 1963; Kelley, Poncet & Di Ferrante, 1963; Linker & Terry, 1963; Berenson & Dalferes, 1965; Varadi, Cifonelli & Dorfman, 1967; Wessler, 1967; Orii, 1968). This fact together with the marked charge and molecular-weight polydispersity has, thus far, precluded the complete separation of the various glycosaminoglycan species. Consequently, most of the urinary glycosaminoglycans described have been mixtures of polysaccharides. The analytical data for such mixtures are difficult to interpret. This may be one reason why quantitative values given by different authors do not agree.

A detailed knowledge of the different glycosaminoglycans present in normal urine is a prerequisite for detecting and evaluating minor deviations in their normal excretion in cases with defective glycosaminoglycan metabolism.

The present paper gives methods for the quantitative isolation and separation of the glycosaminoglycans from the non-ultrafilterable material of normal human urine. It has been possible by using proteolytic digestion, followed by chromatography on ECTEOLA-cellulose and preparative electrophoresis, to isolate the urinary glycosaminoglycans

as apparently pure fractions. About 75% of the total amount of urinary glycosaminoglycans, corresponding to 2–3 mg of uronic acid/litre of urine, are non-ultrafilterable (Wessler 1970*a*).

EXPERIMENTAL

Urine. This was collected from healthy males aged about 20. Phenylmercuric nitrate was added as a preservative to give a final concentration of 1:50000. The urine was cooled to +4°C and passed through Munktell filtering paper no. 1F to remove insoluble matter, including the Tamm-Horsfall glycoprotein (Berggård, 1962*a*).

Glycosaminoglycans. Hyaluronic acid from human umbilical cord and chondroitin 4-sulphate from bovine nasal septum were kindly provided by Dr G. Blix of this Institute. Chondroitin 6-sulphate from a human chondroma and dermatan sulphate were gifts from Dr Karl Meyer and Dr S. Roseman respectively to Dr G. Blix. Heparan sulphate was donated by Dr L. Rodén.

Enzymes. Pronase with an activity of 45000 proteolytic units (Kaken)/g was purchased from Kaken Chemical Co. Ltd., Tokyo, Japan. Sialidase (neuraminidase, *N*-acetylneuraminidase glycohydrolase, EC 3.2.1.18) with an activity of 100 units/ml was obtained from Behringwerke A.G., Marburg/Lahn, Germany. Hyaluronidase (hyaluronate glycanohydrolase, EC 3.2.1.35) with a specific activity of 20000 international units/mg was obtained through the courtesy of Dr B. Högborg, AB Leo, Hälsingborg, Sweden.

Cetylpyridinium chloride of analytical grade was a gift from the Recip Company, Stockholm, Sweden.

ECTEOA-cellulose (Serva Entwicklungslabor, Heidelberg, Germany, 0.38 mg-equiv./g) was washed with 1M-NaOH, 3M-NaCl in 0.1M-HCl, water, ethanol, and ethyl ether and dried. Before use it was suspended in water and de-aerated in a vacuum. The columns were packed under slight hydrostatic pressure, washed with 3M-NaCl in 0.1M-HCl (to produce the Cl⁻ form) and then with water until the effluent was neutral.

Methods

Concentration of urinary macromolecules. Samples (5–10 litres) of filtered urine were concentrated 500–1000 times by low-pressure ultrafiltration as described by Everall & Wright (1958). The ultrafiltration was performed at +4°C at a rate of 1 litre/day. Visking dialysis tubing (Union Carbide Corp., Chicago, Ill., U.S.A.) of 23/32 in inflated diameter, encased in tube gauze no. 01 (The Scholl Manufacturing Co. A/B, Stockholm, Sweden) was used at a pressure-difference of 660 mmHg. This tubing, when used as ultrafiltration membrane, retains completely proteins with molecular weights above 10 000–15 000 (Berggård, 1962a). The lower-molecular-weight limit for retention of glycosaminoglycans is not known.

Enzymic digestion. A suitable quantity of concentrated urinary macromolecules was centrifuged. The precipitate was washed with a few ml of water until free from uronic acid and then discarded. The combined supernatant and water washings were adjusted to pH 7 with NaOH and the solution was heated for 30 min on a boiling-water bath to facilitate the subsequent proteolysis (Gardell, 1952). The pH did not change during the heating. To each ml of sample was added 0.5 ml of sialidase solution, CaCl₂ to 0.01 M concentration and a few drops of ether. The solution was incubated at 37°C for 24 h. After sialidase treatment, pronase was added to a concentration of 50 proteolytic units/ml and the digestion continued for 24 h at 55°C.

After completed digestion, the mixture was centrifuged. The precipitate was washed with water until free of uronic acid and then discarded. The supernatant, including the aqueous washings, was dialysed for 2 days against water.

Fractionation on ECTEOA-cellulose. Fractionation on ECTEOA-cellulose was performed essentially as described by Ringertz & Reichard (1959, 1960). Elutions were carried out either by gradient or stepwise procedures.

Preparative electrophoresis. Preparative zone electrophoresis was performed in blocks (Kunkel, 1954) (1 cm × 26 cm × 100 cm) of Pevikon C 870 (Stockholms Superfosfat Fabriks AB, Stockholm, Sweden) (Müller-Eberhard, 1960). Barbitol-NaOH buffer, pH 8.6, I 0.1, was used at a potential gradient of 3.5 V/cm. The separation time was 42–55 h depending on the mobilities of the polysaccharides being separated. When 0.1M-barium acetate (Wessler, 1968) was used, a potential gradient of 3 V/cm was maintained for 90 h. Sodium formate buffer, pH 3.0 (Mathews, 1961), I 0.4, was used at a potential gradient of 1.5 V/cm. The separation time was 7 days. Samples of 10–20 ml volume were applied. After electrophoresis, the blocks were generally cut into 12 mm segments, which were eluted with 12 ml portions of 0.9% NaCl.

Alcohol fractionation. Ethanol fractionation was carried out as described by Gardell (1957). About 3 ml of polysaccharide solution, which had been dialysed against 0.3% barium acetate, was applied to a cellulose column (30 cm × 2 cm) equilibrated with 0.3% barium acetate in 80% (v/v) ethanol. After washing with the same ethanol solution, the polysaccharides were eluted with a gradient solution, obtained by using a closed mixing chamber (250 ml) containing the barium acetate-ethanol solution and a reservoir vessel with 0.3% barium acetate. Fractions of 15 ml were collected at a rate of 20 ml/h.

Concentration of fractions. Polysaccharide fractions obtained by the various separation procedures described above were prepared for further experiments by thorough dialysis against water followed by concentration in a rotary evaporator at a temperature not exceeding 40°C. Acidic eluates were neutralized before dialysis.

Analytical electrophoresis of glycosaminoglycans. This was performed on cellulose acetate strips in the following solutions: sodium barbitol buffer, pH 8.6, I 0.1 (Näntö, 1963); 0.1M-barium acetate (Wessler, 1968); 0.1M-HCl (Wessler, 1970b). The last electrolyte is the medium of choice for the separation of glycosaminoglycans according to sulphate content. Separation of polysaccharides differing with respect to structure of the carbohydrate backbone is preferentially achieved by use of barium acetate. Glycosaminoglycans were detected by staining with Alcian Blue.

Hexosamine. This was determined essentially by Blix's (1948) modification of the Elson & Morgan (1933) procedure. The samples were hydrolysed at 100°C with 6M-HCl for 8 h (Gardell, 1958). The HCl was removed by evaporation *in vacuo* over NaOH pellets, and the residue was taken up in 2 ml of water. Glucosamine hydrochloride was used as standard.

Glucosamine and galactosamine were separated and determined by the ion-exchange method of Gardell (1953).

Hexosamines were also identified by paper chromatography of the pentoses obtained after degradation with ninhydrin (Gardell, Hejkenkjöld & Roch-Norlund, 1950; Støffyn & Jeanloz, 1954).

Further identification of hexosamines was accomplished by paper electrophoresis in 25 mm-sodium tetraborate (Ohkuma & Shinohara, 1963) after *N*-acetylation with acetic anhydride (Wheat, 1966). The papers were dried at room temperature, sprayed with water-saturated butan-1-ol, dried at 95°C for 5 min and finally sprayed with *p*-dimethylaminobenzaldehyde (Partridge, 1948).

Uronic acid. This was assayed by the carbazole reaction in the presence of borate (Gregory, 1960; Bitter & Muir, 1962) as well as by the original procedure of Dische (1947). In both reactions 0.5 ml of sample was heated with 3 ml of the H₂SO₄ reagent in a boiling-water bath for 20 min. After cooling to room temperature, *E*₅₃₀ was read before and 2.5–3.5 h after the addition of 0.1 ml of carbazole reagent. Under these conditions the molar extinction of neutral sugars is approx. 5% of that of glucuronolactone. D-Glucuronolactone was used as standard. The ratio of the uronic acid contents, as determined by the Dische (1947) procedure and by the modified Bitter & Muir (1962) method respectively, served as a preliminary identification of glycosaminoglycans. This ratio was found to be 1.00 for hyaluronic acid, chondroitin 4-

sulphate and chondroitin 6-sulphate, 0.6 for dermatan sulphate and 1.13 for heparan sulphate.

Qualitative identification of uronic acids was performed by paper chromatography in 2-methylbutan-2-ol-propan-2-ol-water (4:1:2, by vol.) after hydrolysis with formic acid at 100°C for 6 h (Radhakrishnamurthy & Berenson, 1963).

Neutral sugar. This was determined with the anthrone reaction (Scott & Melvin, 1953) with galactose as standard. Glycosaminoglycuronans interfere with the anthrone reaction. However, since it is impossible to evaluate the interference accurately, no corrections were generally applied. The original anthrone method cannot be used in the presence of appreciable amounts of Ba^{2+} , since the BaSO_4 formed makes the solution turbid. To overcome this difficulty, the H_2SO_4 concentration was increased. The temperature was lowered to counteract the interference by glycosaminoglycuronans, which increases when the H_2SO_4 concentration is increased. The reaction was performed in the following way: 0.5 ml of sample in an ice bath was mixed with 2.5 ml of anthrone reagent (2 g of anthrone in 1 litre of conc. H_2SO_4); the mixture was then heated for 16 min at 60°C, cooled, and its E_{620} measured.

Qualitative analysis of the neutral sugars in glycosaminoglycans was performed after hydrolysis in 1 M-HCl at 100°C for 3 h. Dowex 3 (OH^- form) was added to raise the pH of the hydrolysate to about 5. The resin was removed by filtration, and the filtrate was passed through a small column of Dowex 50 (H^+ form). The effluent was concentrated by rotary evaporation and applied to Whatman no. 1 paper, which was developed with butan-1-ol-pyridine-water (6:4:3, by vol.) for 24 h or with butan-1-ol-formic acid-water (8:2:1, by vol.) three times for 24 h. The last-mentioned solvent, which is a modification of that of Fuleki & Francis (1967), was found to separate galactose, glucose, mannose, arabinose, xylose, lyxose, fucose, rhamnose and glucuronic. The spots were detected with aniline hydrogen phthalate (Partridge, 1949).

Sulphate. This was determined by the benzidine method of Antonopoulos (1962). Before analysis, the samples were passed through small columns of Dowex 50 (H^+ form) to remove Ba^{2+} . The glycosaminoglycuronans were freed from inorganic sulphate by precipitation with cetylpyridinium chloride (1 ml of sample containing 40–140 μg of uronic acid + 0.2 ml of 5% cetylpyridinium chloride in 0.05 M-sodium citrate) (Scott, 1960) and the keratan sulphate likewise by chromatography on Sephadex G-25 (fine grade).

N-Sulphate. The disappearance of hexosamine, as measured by the Elson-Morgan method (Boas, 1953), after reaction of the polymer with nitrous acid (Dische & Borenfreund, 1950) for 80 min (Lagunoff & Warren, 1962), was regarded as a measure of N-sulphated hexosamine.

Protein. This was determined by the Folin method as described by Lowry, Rosebrough, Farr & Randall (1951) with human albumin as standard. It should be noted that the Lowry *et al.* (1951) method may not be very accurate for proteins in proteoglycans.

Chloride. Concentrations of chloride were determined by a mercurimetric method (Schales & Schales, 1941).

Digestion with hyaluronidase. This was performed essentially as described by Thunell (1967). To 3 mg of polysaccharide in 1 ml of McIlvaine buffer, pH 7.0, was added

0.5 mg of enzyme. After incubation at 37°C for 24 h, another 0.5 mg of enzyme was added and digestion was continued for a total of 48 h. The extent of degradation was determined by chromatography on Sephadex G-50.

Optical rotation. This was measured at 20°C in a Perkin-Elmer model 141 polarimeter by using the 589 nm filter. Glycosaminoglycans were converted into the sodium form by passage through small columns of Dowex 50 (Na^+ form) and analysed at concentrations ranging from 0.3 to 25 mg/ml.

Infrared spectra. These were recorded on a Unicam SP.200 spectrophotometer by using the KBr-pellet technique.

RESULTS

Fractionation on ECTEOLA-cellulose of the urinary macromolecules after treatment with sialidase and pronase. Concentrated urinary macromolecules were digested with sialidase and pronase, dialysed and subjected to chromatography on ECTEOLA-cellulose. By washing the column with water, followed by 0.01 M-hydrochloric acid, large amounts of peptide- and neutral sugar-containing material were removed whereas the glycosaminoglycuronans remained on the column. This procedure was much more efficient for eliminating protein than fractionation of the undigested material on ECTEOLA-cellulose, in which case the rapidly eluted glycosaminoglycan fractions were grossly contaminated with peptide-containing material. Elution with a

Fig. 1. Chromatography on a column (7.5 cm $\times$ 0.9 cm) of ECTEOLA-cellulose of digested (sialidase, pronase) urinary macromolecules from 3 litres of urine. The column was first washed with 300 ml of 0.01 M-HCl. The polysaccharides were then eluted with a concave HCl gradient obtained with the aid of two closed mixing chambers containing 1 litre each of 0.01 and 0.1 M-HCl respectively. The reservoir contained 6 M-HCl. Fractions of volume 10 ml were eluted at a rate of 5 ml/h. —, Uronic acid (carbazole-borate reaction); ----, neutral sugar (anthrone reaction); ·····, gradient of Cl^- .

Table 1. *Composition of urinary glycosaminoglycan fractions obtained by chromatography on ECTEOLA-cellulose*

Uronic acid was measured by the carbazole (C) and the carbazole-borate (B) methods. Values in column 6 are molar ratios. The material was obtained from 85 litres of urine.

ECTEOLA-cellulose fraction	Eluent	Uronic acid (B) (mg)	Hexosamine (mg)	Protein (mg)	Uronic acid (B)	Uronic acid (C)
					Hexosamine	Uronic acid (B)
I	0.04M-HCl	13.1	50.8	18.9	0.24	1.15
II	0.05M-NaCl	7.0	9.3	1.7	0.70	1.05
	0.05M-HCl					
III	0.25M-NaCl	23.9	22.5	2.2	0.98	1.03
	0.05M-HCl					
IV	0.45M-NaCl	15.0	14.7	1.2	0.94	1.06
	0.05M-HCl					
V	0.65M-NaCl	31.3	28.0	1.3	1.03	1.04
	0.05M-HCl					
VI	0.85M-NaCl	43.7	37.9	1.0	1.06	1.07
	0.05M-HCl					
VII	1.05M-NaCl	28.2	23.1	0.6	1.13	1.06
	0.05M-HCl					
VIII	3M-NaCl	7.3	6.0	0.5	1.11	0.96

gradient of 0.01–3M-hydrochloric acid revealed the presence of three partially resolved uronic acid-containing peaks (Fig. 1). All three fractions contained both glucosamine and galactosamine. Analyses of the fractions obtained on stepwise elution are given in Table 1. Only fraction I contained appreciable amounts of protein. The uronic acid/hexosamine molar ratio was low in the first two fractions, indicating the presence of uronic acid-free hexosamine-containing substances, such as keratan sulphate or glycoproteins or both. In the remaining fractions this ratio was about 1 as expected for pure glycosaminoglycuronans. Further information as to the composition of the various fractions was deduced from the ratios between the uronic acid values obtained by the carbazole reaction without and with borate. The ratios for fractions II–VII were slightly higher than unity, suggesting the presence of mixtures of chondroitin sulphate and heparan sulphate. The high ratio for fraction I probably reflects the presence of an appreciable amount of neutral sugar, whereas the low ratio for fraction VIII may indicate the presence of some dermatan sulphate. Qualitative hexosamine analysis by the method of Stoffyn & Jeanloz (1954) and by electrophoresis in borate buffer after *N*-acetylation showed that all fractions contained glucosamine and galactosamine. The sulphate/uronic acid molar ratios gradually increased from less than 0.08 in fraction I to 0.95 in fraction VIII.

Zone electrophoresis of glycosaminoglycan fractions obtained by chromatography on ECTEOLA-cellulose.

(a) Analytical electrophoresis. The glycosamino-

glycan fractions obtained by chromatography on ECTEOLA-cellulose were further analysed by electrophoresis on cellulose acetate strips in barbital buffer, pH 8.6 (Plate 1). The mobilities of the isolated materials were found to increase progressively from fraction I to fraction VIII. Fractions IV–VIII produced two spots. Fraction I showed the same mobility as hyaluronic acid, whereas the fastest-migrating component of fraction VIII moved slightly faster than chondroitin sulphate. These findings are in accordance with the sulphate contents of the eight fractions.

(b) Preparative electrophoresis. The fractions obtained by chromatography on ECTEOLA-cellulose were separated by zone electrophoresis on Pevikon blocks in barbital buffer, pH 8.6 (Fig. 2). Fractions I–IV gave two major peaks. The slower-moving peak of each fraction contained neutral sugars and glucosamine with traces of galactosamine, except for fraction I, in which about one-third of total hexosamine was galactosamine. The faster-migrating peaks of fractions I–IV contained glycosaminoglycuronans. The glycosaminoglycuronans did not separate (fractions I–III) or were incompletely separated (fraction IV) from the galactosaminoglycuronans. The latter tended to have a higher mobility.

Sugar analyses by the anthrone method after allowance for non-specific colour due to glycosaminoglycuronans established that fractions V–VIII contained only minimal amounts of hexose. The galactosaminoglycuronans moved ahead of and were partly separated from the glucosaminoglycuronans. In fraction VIII, the carbazole/

EXPLANATION OF PLATE I

Analytical electrophoresis in sodium barbital buffer, pH 8.6, of fractions I-VIII obtained by chromatography on ECTEOLA-cellulose (Table 1). The anode is at the top.

Fig. 2. Preparative electrophoresis in sodium barbital buffer, pH 8.6, of fractions I–VIII obtained by chromatography on ECTEOLA-cellulose (Table 1). The separation time was 42–55 h, depending on the mobility of the sample to be separated. —, Uronic acid (carbazole–borate reaction); ----, neutral sugar (anthrone reaction); ———, carbazole/carbazole–borate uronic acid ratio.

carbazole–borate uronic acid ratio for the slow-moving part of the galactosaminoglycuronan peak was low indicating partial separation of dermatan sulphate from chondroitin sulphate.

Purification and analysis of uronic acid-containing fractions obtained by chromatography on ECTEOLA-cellulose and preparative electrophoresis. Fraction I. The glycosaminoglycuronan constituent of fraction I, isolated by preparative electrophoresis, contained both glucosamine and galactosamine in about equal amounts. The sulphate/uronic acid molar ratio was 0.06. Further fractionation was achieved by

chromatography on Dowex 1, as shown in Fig. 3, yielding two incompletely separated peaks. The main peak, which constituted about 6% of the total amount of glycosaminoglycans excreted, was found to contain glucosamine and galactosamine in the molar ratio of about 2:1. The uronic acid/hexosamine molar ratio was 1.08 and the carbazole/carbazole–borate uronic acid ratio was 1.04. The sulphate/hexosamine molar ratio was 0.02. The mobility of the material on electrophoresis on cellulose acetate in sodium barbital buffer, pH 8.6, sodium formate buffer, pH 3.0, 0.1 M-barium acetate

Fig. 3. Chromatography on a column (22 cm $\times$ 1 cm) of Dowex 1 X2; 200–400 mesh; Cl^- form) of the electrophoretically purified (Fig. 2) glycosaminoglycuronans in fraction I of the eluate from ECTEOLA-cellulose (Table 1). The column was eluted with a HCl gradient, as described in the legend of Fig. 1. The fractions were combined as indicated by the arrows. —, Uronic acid (carbazole-borate reaction); ----, gradient of chloride ions.

Fig. 4. Preparative electrophoresis in 0.1 M-barium acetate of the glycosaminoglycuronans in fraction IV. The sample had been obtained by chromatography on ECTEOLA-cellulose (Table 1), followed by preparative electrophoresis in sodium barbital buffer, pH 8.6 (Fig. 2). —, Uronic acid (carbazole-borate reaction); ----, carbazole/carbazole-borate uronic acid ratio.

or 0.1 M-hydrochloric acid was identical with that of hyaluronic acid. The $[\alpha]_D^{20}$ was -56° , which is consistent with a mixture of two-thirds hyaluronic acid and one-third chondroitin and excludes the presence of significant amounts of non-sulphated heparan, for which a specific rotation of $+60^\circ$ has been reported (Cifonelli & Dorfman, 1960). Digestion with hyaluronidase resulted in almost complete conversion of the material into low-molecular-weight fragments.

The small fraction eluted from Dowex 1 (Fig. 3) at higher concentrations of hydrochloric acid contained galactosamine and only traces of glucosamine. This material, with a sulphate/uronic acid molar ratio of 0.09, migrated as far as hyaluronic acid when subjected to electrophoresis on cellulose acetate strips in sodium barbital buffer, pH 8.6, sodium formate, pH 3.0, or 0.1 M-barium acetate. In 0.1 M-hydrochloric acid it migrated slowly, in contrast with hyaluronic acid which was immobile. This fraction was not further analysed.

Fractions II–IV. The glycosaminoglycuronan components of fractions II–IV, obtained by preparative electrophoresis in sodium barbital buffer, were almost completely separated into galactosaminoglycuronans and glucosaminoglycuronans by preparative electrophoresis in barium acetate, as illustrated for fraction IV in Fig. 4. The analytical results for the galactosaminoglycuronans (Table 2) are consistent with that for low-sulphated chondroitin sulphates. The carbazole/carbazole-borate uronic acid ratios are the same as for chondroitin sulphate. The optical rotation values are similar to those of chondroitin 4-sulphate. The i.r. spectrum of fraction III CS (Fig. 5), which is the main low-

sulphated fraction with a sulphate/hexosamine molar ratio of 0.5, shows a comparatively weak S=O band at 1240 cm^{-1} . Weak bands, characteristic of chondroitin 4-sulphate (Mathews, 1958), are seen at 928 cm^{-1} , 852 cm^{-1} and 725 cm^{-1} . All fractions were incompletely precipitated by cetylpyridinium chloride.

Analytical results for the sulphated glucosaminoglycuronans (Table 3) indicate the presence of low-sulphated heparan sulphates in normal human urine. The identification is based on the presence of glucosamine and *N*-sulphate, the high carbazole/carbazole-borate ratios and the positive optical rotation. The fractions were incompletely precipitated by cetylpyridinium chloride.

Fractions V–VII. The galactosaminoglycuronans and glucosaminoglycuronans in fractions V–VII were completely separated by repeated electrophoresis in sodium barbital buffer of the overlapping portions of the peaks shown in Fig. 2. The purity of the fractions was assessed by qualitative hexosamine analysis, by determination of carbazole/carbazole-borate uronic acid ratios and by analytical electrophoresis in barbital buffer, pH 8.6, and in 0.1 M-barium acetate. The carbazole/carbazole-borate uronic acid ratios and the electrophoretic mobility in barium acetate indicated that the slower-moving galactosaminoglycuronan portion of fraction VII contained a minute amount of dermatan sulphate.

The galactosaminoglycuronans in fractions V–VII had a high sulphate content (Table 2). The carbazole/carbazole-borate uronic acid ratios were the same as for chondroitin sulphate. The optical rotation values are consistent with the presence of mixtures of chondroitin 4-sulphate and chondroitin

Table 2. *Analytical results for the galactosaminoglycuronan constituents [chondroitin sulphates (CS) and dermatan sulphate (DS)] of fractions II–VIII obtained by chromatography on ECTEOLA-cellulose*

Column 2 denotes percentage of total urinary glycosaminoglycan hexosamine. The composition is given as molar ratios with hexosamine as 1.0. C/B denotes the ratio between uronic acid determined by the carbazole reaction without borate (C) and with borate (B). Values for optical rotation are calculated on the basis of polysaccharide concentration determined from hexosamine and sulphate values. The value in parentheses is uncertain owing to low polysaccharide concentration. The last column denotes the proportion of uronic acid in each fraction precipitable with cetylpyridinium chloride.

Fraction	%	Molar ratio			$\frac{C}{B}$	$[\alpha]_D^{20}$	% precipitated
		Galactosamine	Uronic acid (B)	Sulphate			
II CS	2.9	1.0	1.13	0.2	1.00	−36°	70
III CS	10.1	1.0	1.13	0.5	1.00	−35°	84
IV CS	5.3	1.0	1.02	0.8	1.00	−31°	93
V CS	12.2	1.0	1.14	1.2	1.00	−21°	98
VI CS	16.9	1.0	1.15	1.3	1.00	−23°	100
VII CS	9.2	1.0	1.17	1.3	0.98	−25°	100
VIII CS	2.2	1.0	0.99	1.3	0.98	−37°	100
VIII DS	0.7	1.0	0.98	1.2	0.67	(−100°)	100

Fig. 5. Infrared spectra of fractions III CS, VI CS and VII HS (Tables 2 and 3).

6-sulphate. The i.r. spectrum of fraction VI CS (Fig. 5) showed a strong S=O band in the 1240 cm^{-1} region. Bands characteristic of chondroitin 4-sulphate (Mathews, 1958) are seen at 928 cm^{-1} , 852 cm^{-1} and 725 cm^{-1} . Bands characteristic of chondroitin 6-sulphate (Mathews, 1958) are found at 1000 cm^{-1} and 820 cm^{-1} .

From the analytical results, the glucosamino-

glycuronans in fractions V–VII (Table 3) could be identified as heparan sulphates with various sulphate contents. The i.r. spectrum of fraction VII HS (Fig. 5) is similar to those published for pure heparan sulphate (Schiller, 1959).

Fraction VIII. Complete separation of galactosaminoglycuronans and glucosaminoglycuronans was obtained by repeated preparative electrophoresis in sodium barbital buffer of the overlapping portions of the peaks shown in Fig. 2. The galactosaminoglycuronan fraction was found to contain both glucuronic and iduronic acid, thus confirming the presence of both chondroitin sulphate and dermatan sulphate. A partial separation of these polysaccharides was obtained by electrophoresis in formate buffer, pH 3.0, on a Pevikon block (Fig. 6). Five fractions from this electrophoresis (a–e, Fig. 6) were subjected to analytical electrophoresis in 0.1 M-barium acetate (Fig. 7), which is known to separate dermatan sulphate from chondroitin sulphate (Wessler, 1968). The results of the separation experiments are compatible with the presence of dermatan sulphate, dermatan sulphate–chondroitin sulphate hybrids, chondroitin 4-sulphate and chondroitin 6-sulphate. The identity of the small, fast-moving spot in fraction (e) is unknown. Analytical results for the chondroitin sulphate, dermatan sulphate and heparan sulphate fractions obtained by preparative electrophoresis in 0.1 M-barium acetate of fraction VIII (Fig. 3, Wessler 1968) are given in Tables 2 and 3.

Purification and analysis of neutral sugar-containing uronic acid-free fractions obtained by chromatography on ECTEOLA-cellulose and preparative

Table 3. *Analytical results for the glucosaminoglycuronan constituents [heparan sulphates (HS)] of fractions III–VIII obtained by chromatography on ECTEOLA-cellulose*

Column 2 denotes the percentage of total urinary glycosaminoglycan hexosamine. The composition is given as molar ratios with hexosamine as 1.0. C/B denotes the ratio between uronic acid determined by the carbazole reaction without (C) and with borate (B). Values for optical rotation are calculated by using the polysaccharide concentrations determined from hexosamine and sulphate values. Values in parentheses are uncertain owing to low polysaccharide concentration. The last column denotes the proportion of uronic acid in each fraction precipitable with cetylpyridinium chloride.

Fraction	%	Molar ratio				$\frac{C}{B}$	$[\alpha]_D^{20}$	% precipitated
		Glucosamine	Uronic acid (B)	Sulphate	N-Sulphate			
III HS	1.5	1.0	1.09	0.5		1.09	(+73°)	71
IV HS	1.9	1.0	1.13	0.5	0.5	1.10	+80°	85
V HS	2.9	1.0	1.15	0.6	0.6	1.11	+86°	96
VI HS	4.2	1.0	1.12	0.7	0.6	1.10	+87°	98
VII HS	4.4	1.0	1.14	0.9	0.6	1.12	+87°	100
VIII HS	0.7	1.0	1.11	0.9		1.12	(+51°)	100

Fig. 6. Preparative electrophoresis in sodium formate buffer, pH 3.0, of the galactosaminoglycuronans obtained from the ECTEOLA-cellulose fraction VIII (Table 1) by preparative electrophoresis in sodium barbital buffer, pH 8.6 (Fig. 2). Complete separation from the glucosaminoglycuronans had been obtained by repeated electrophoresis in the same buffer of overlapping parts of the peaks. —, Uronic acid (carbazole–borate reaction); —, carbazole/carbazole–borate uronic acid ratio. Eluates from the electrophoresis in formate buffer were combined into fractions (a to e) as shown by the vertical lines.

electrophoresis. Further purification of the slow-moving neutral sugar-containing material in fractions I–IV (Fig. 2) was achieved by alcohol fractionation. Each fraction emerged from the column as a sharp peak with the elution properties of keratan sulphate. The analytical results for the fractions (Table 4) correspond to about equimolar amounts of hexosamine and neutral sugar. The hexosamine was glucosamine, except in fraction I, which also contained traces of galactosamine. Galactose and traces of mannose and fucose were the only neutral sugars present in all fractions. All fractions contained peptide material. It is concluded that the

Fig. 7. Analytical electrophoresis in barium acetate of fractions obtained by preparative electrophoresis at pH 3.0 (a to e, Fig. 6) of the galactosaminoglycuronans from ECTEOLA-cellulose fraction VIII. A reference mixture of chondroitin 6-sulphate (C-6-S), chondroitin 4-sulphate (C-4-S) and dermatan sulphate (DS) was separated on the same cellulose acetate strip.

glucosaminoglycan isolated is apparently keratan sulphate of various degree of sulphation.

In summary, chondroitin sulphates of various

Table 4. *Composition of keratan sulphate constituents (KS) of fractions I-IV obtained by chromatography on ECTEOLA-cellulose*

Column 2 denotes the percentage of total urinary glycosaminoglycan hexosamine. The compositions are given as molar ratios with hexosamine as 1.0.

Fraction	%	Molar ratio		
		Glucosamine	Hexose	Sulphate
I KS	14.6	1.0	1.01	0.2
II KS	1.1	1.0	1.15	0.4
III KS	1.7	1.0	1.12	0.5
IV KS	0.5	1.0	1.02	0.7

sulphate content amounted to about 60% of the total amount of urinary glycosaminoglycans. The sulphate/hexosamine molar ratios in the main fractions were about 0.5 and 1 respectively. Heparan sulphates constituted about 15% of the total amount of glycosaminoglycans. They showed different degrees of sulphation with a sulphate/hexosamine molar ratio of 0.5-0.9. The degree of *N*-sulphation did not seem to vary between the different fractions. Of the glycosaminoglycans about 18% were keratan sulphates with a sulphate/hexosamine molar ratio of 0.2-0.7. Small amounts of hyaluronic acid (4%), chondroitin (2%) and high-sulphated dermatan sulphate (1%) were also found.

DISCUSSION

Owing to the lack of adequate separation methods, only a few of the glycosaminoglycans excreted in normal human urine have hitherto been isolated in a reasonably pure form. The sequence of methods used in the present investigation, including proteolytic digestion, chromatography on ECTEOLA-cellulose, followed by preparative electrophoresis in sodium barbital buffer and in barium acetate, enabled the separation of most polysaccharide species present.

Chondroitin sulphate was first isolated from urine by Mörner (1895). Identification was based on elementary analyses and sulphate determination. This finding has been confirmed (Di Ferrante & Rich, 1956) and extended to include both chondroitin 4-sulphate and chondroitin 6-sulphate (Berggård, 1962b; Berenson & Dalferes, 1965; Varadi *et al.* 1967). The presence of chondroitin sulphate with varying sulphate content in urine (Di Ferrante, 1963; Kelley *et al.* 1963; Berenson & Dalferes, 1965; Varadi *et al.* 1967) has now been confirmed by analyses of pure chondroitin sulphate fractions. The results of the present study indicate that the urinary chondroitin sulphates do not form a continuous spectrum with regard to sulphate content but rather three partly overlapping main fractions consisting of chondroitin sulphate, half-sulphated chondroitin sulphate and chondroitin.

Excretion of a talosamine-containing compound has been reported (Berenson & Dalferes, 1965) but could not be confirmed in the present study.

There has been some controversy as to the presence of hyaluronic acid in normal urine. The present findings are in accordance with those of Varadi *et al.* (1967), who reported a small amount of this polysaccharide.

The presence of dermatan sulphate in normal urine has been inferred from indirect evidence, such as the presence of iduronic acid (Di Ferrante, 1963; Berenson & Dalferes, 1965; Orii, 1968), carbazole/orcinol ratios, paper chromatography and the presence of galactosamine in glycosaminoglycan material resistant to testicular hyaluronidase (Linker & Terry, 1963). Only Varadi *et al.* (1967) have succeeded in obtaining pure dermatan sulphate; the amount isolated (1% of total urinary glycosaminoglycans) was similar to that isolated in this work. Other investigators (Linker & Terry, 1963; Berenson & Dalferes, 1965; Teller, 1967) claim much higher values, that were, however, based on analyses of polysaccharide mixtures. Evidence is presented in this paper for the excretion of trace amounts of chondroitin sulphate-dermatan sulphate hybrids. This material might be identical with the dermatan sulphate, migrating rapidly on paper chromatography, found by Linker & Terry (1963).

The excretion of glucosaminoglycans in urine was first suggested from the finding of glucosamine in urinary mucopolysaccharides (Kobayasi, 1938). The presence of heparan sulphate was inferred from the presence of glucosamine in a glycosaminoglycan material resistant to testicular hyaluronidase (King *et al.* 1962; Di Ferrante, 1963) and further from carbazole/orcinol ratio, paper chromatography and the products obtained by digestion with *Flavobacterium* heparinase (Linker & Terry, 1963). Finally, urinary glycosaminoglycans have been shown to contain sulphamino groups (Varadi *et al.* 1967). The sulphate/hexosamine molar ratio in purified urinary heparan sulphate has been given as 0.65 (Berenson & Dalferes, 1965). However, heparan sulphate was obtained in several fractions

together with chondroitin sulphate when urinary glycosaminoglycans were fractionated by ion-exchange chromatography, thus suggesting variability in the sulphate content (Di Ferrante, 1963; Kelley *et al.* 1963; Varadi *et al.* 1967). This variability is conclusively established in the present work, which shows that pure heparan sulphate fractions exhibit sulphate/hexosamine molar ratios ranging from 0.5 to 0.9 (Table 4). The presence of non-sulphated heparan in urine has been suggested (Orii, 1968), but could not be confirmed in the present investigation. The fact that urinary heparan sulphate and chondroitin sulphate are eluted together over several fractions on ion-exchange chromatography invalidates quantitative determination methods based merely on the comparison of the elution profile of urinary glycosaminoglycans with that of 'standard' glycosaminoglycans. However, since about half of the hexosamine in the urinary heparan sulphate is *N*-sulphated (Table 3), it seems justified to use the content of *N*-sulphated hexosamine as a measure of urinary heparan sulphate in normal (Varadi *et al.* 1967) and pathological (Lagunoff, Pritzl & Scott, 1967) states. In disorders of glycosaminoglycan metabolism, a certain caution is warranted, since fractions of urinary heparan sulphate from Hurler patients have been reported to display varying degrees of *N*-sulphation (Knecht, Cifonelli & Dorfman, 1967). The quantitative value for the heparan sulphate excretion found in this work (15% of the total glycosaminoglycans) is in fair agreement with values obtained by others from analyses of polysaccharide mixtures. The values previously reported vary from 8% (Varadi *et al.* 1967) to 27% (Berenson & Dalferes, 1965) of the total amount excreted.

The presence of keratan sulphate in normal urine has been suggested by Kelley *et al.* (1963) and Linker & Terry (1963). Purified urinary keratan sulphate has been reported to have a sulphate/hexosamine molar ratio of 0.63 (Berenson & Dalferes, 1965), 1.70 (Varadi *et al.* 1967) or varying from 0.31 to 0.83 (Wessler, 1967). Although the presence of small amounts of highly sulphated material cannot be excluded, the main part of the urinary keratan sulphate has a low sulphate content. This material, which may also be regarded as sulphated glycoprotein, since the length of the carbohydrate chains has not been determined, was separated into fractions differing in degree of sulphation (Table 4). The low-sulphated keratan sulphate has probably been overlooked by investigators using quaternary ammonium salt in their purification procedure, as this material would be soluble in excess of the precipitating agent (Antonopoulos, Borelius, Gardell, Hamnström & Scott, 1961; Cifonelli, Saunders & Gross, 1967).

Besides glucosamine and galactose, keratan sul-

phates from different sources have been found to contain small amounts of galactosamine (Mathews & Cifonelli, 1965), fucose (Gardell, 1957; Bray, Lieberman & Meyer, 1967) and mannose (Bhavanandan & Meyer, 1968). The presence of glucose, rhamnose, arabinose and xylose in keratan sulphate preparations has also been reported (Kelley *et al.* 1963; Balduini, Brovelli & Castellani, 1968; Greiling, Stuhlsatz, Kisters & Plagemann, 1968). The urinary keratan sulphate fractions isolated in this work were free of uronic acid and contained glucosamine and galactose, and traces of galactosamine, fucose and mannose. No other hexosamines or neutral sugars were detected.

The origin of the urinary glycosaminoglycans is not unequivocally established. They have been shown not to derive to a significant degree from the lower urinary tract (Kerby, 1954). Admixture of glycosaminoglycans from the kidney cannot be excluded. On the other hand, normal serum contains glycosaminoglycans which display an electrophoretic pattern (Friman & Brunish, 1966) similar to that of urinary glycosaminoglycans. The main glycosaminoglycan in both blood plasma (Schiller, 1958) and urine is chondroitin sulphate. The presence of small amounts of heparan sulphate and keratan sulphate in human plasma has also been reported (Calatroni, Donnelly & Di Ferrante, 1969). Increased plasma contents and increased urinary excretion of glycosaminoglycans have been found to occur concomitantly in several diseases. It therefore seems reasonable to conclude that most of the urinary glycosaminoglycans originate from the blood. The source of the glycosaminoglycans in blood plasma is unclear. They probably originate from the connective-tissue ground substance but the vessel walls and the leucocytes and blood platelets may contribute.

The significance of the variations in sulphate content of the chondroitin sulphates, the heparan sulphates and the keratan sulphates is obscure. Desulphation may have been accomplished by sulphatases. A chondroitin sulphatase has been purified from mammalian tissue (Held & Buddecke, 1967). Keratan sulphatase activity has been reported to occur in liver and kidney lysosomes (Greiling *et al.* 1968). On the other hand, variations in sulphate content need not necessarily be ascribed to degradation but may be an inherent property of the newly synthesized polysaccharide. Chondroitin sulphates with varying sulphate contents have been isolated from several tissues (Meyer, Linker, Davidson & Weissmann, 1953; Meyer, Davidson, Linker & Hoffman, 1956; Bettelheim & Philpott, 1960; Anseth & Laurent, 1961; Laurent & Anseth, 1961; Greiling, Herbertz & Stuhlsatz, 1964). It has been reported that tissue heparan sulphate comprises a family of substances with varying *O*- and *N*-

sulphate content (Cifonelli & Dorfman, 1960). Keratan sulphate fractions with various sulphate content have been isolated from different tissue sources (Cifonelli *et al.* 1967). Finally the possibility exists that the low-sulphated polymers are incompletely sulphated intermediates in glycosaminoglycan synthesis.

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