Excretion patterns of glycosaminoglycans and glycoproteins in normal human urine

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SYNOPSIS Glycosaminoglycans and glycoproteins in the urine of 100 healthy, active, human subjects were examined by cellulose acetate electrophoresis and salt gradient, ion-exchange, column chromatography. The cetylpyridinium chloride (CPC) turbidity and uronic acid : creatinine ratio was also studied. Fractions were identified by electrophoretic mobility, staining reactions, susceptibility to enzyme digestion, identification of amino- and neutral sugars, hexosamine, uronic acid, and sulphate assays, and optical rotation.

The CPC turbidity is relatively high in childhood, falling to lower levels in adults, but rising again to relatively high levels in old age. The uronic acid : creatinine ratio is high in children, falling to a low level in adult life, and rising only slightly in old age.

Three major electrophoretic fractions, corresponding with glycoprotein, heparan sulphates, and chondroitin sulphates, were identified in every urine sample. Hyaluronic acid was identified in some samples. A small amount of keratan sulphate was present in the 'heparan sulphate' fraction.

Chondroitin sulphate excretion is high in children. Adults excrete relatively less chondroitin sulphate and more heparan sulphate. In old age, the proportion of glycoprotein increases. The excretion pattern in the first few days of life resembles that of the adult. It is stressed that extreme caution must be exercised in interpreting the urinary glycosaminoglycan pattern of a child.

In view of the increasing importance of urinary glycosaminoglycans in diagnosis, it was decided to examine the excretion patterns of these substances in normal human urine at various ages.

MATERIALS AND METHODS

MATERIAL Complete 24-hour urine samples, preserved with 1:10,000 w/v merthiolate, were collected from 100 healthy, active subjects, not in hospital, with ages ranging from 1 day to 94 years. Cellulose acetate electrophoresis strips were obtained from the Oxoid division of Oxo Ltd. Ovine testicular hyaluronidase and clostridial neuraminidase were obtained from British Drug Houses Ltd. Visking dialysis tubing was obtained from Hudes Merchandising Corporation Ltd.

CETYLPIRIDINIUM CHLORIDE TURBIDITY This was determined as described by Manley and Hawksworth (1966).

URINE URONIC ACID CONTENT Total uronic acid was measured by the method of Segni, Romano, and Tortorolo (1964) with one modification. The chlorine content of Oxford tap water interfered with the carbazole

reaction for uronic acid, so all samples were dialysed against distilled water. The CPC-precipitable uronic acid was determined by the method of Manley and Hawksworth (1966).

CPC : CREATININE RATIO Urinary creatinine was measured by the alkaline picrate method of Bonsnes and Taussky (1945). The CPC : creatinine ratio was obtained by dividing the CPC turbidity by the urinary creatinine, expressed as g/100 ml.

URONIC ACID : CREATININE RATIO This figure was obtained by dividing the CPC-precipitable uronic acid (mg/100 ml) by the urinary creatinine (g/100 ml).

ELECTROPHORETIC SEPARATION OF URINARY GLYCOSAMINOGLYCANS AND GLYCOPROTEINS Samples of urine (50 ml) were concentrated by vacuum dialysis as described by Manley and Hawksworth (1966). The residues were dried *in vacuo* and taken up in Michaelis' veronal acetate buffer pH 9.2, allowing 80 μ l buffer for each increment of 0.1 in the CPC turbidity of the original urine sample. The glycoproteins and glycosaminoglycans contained in 20 μ l of each sample were separated by electrophoresis on cellulose acetate, stained with alcian blue, and scanned

in the Joyce Chromoscan, as described by Manley and Hawksworth (1966). A marker sample containing chondroitin sulphate, heparan sulphate, and hyaluronic acid, prepared from human aorta (Manley, 1965), was applied alongside each urine sample.

ENZYME DIGESTION OF URINARY GLYCOPROTEINS AND GLYCOSAMINOGLYCANS Urine samples (50 μ l) were dialysed against phosphate-citrate buffer pH 6.0 (Matthews, 1961) for 24 hours. Ovine testicular hyaluronidase (1.0 mg) or clostridial neuraminidase (200 units) was added, and digestion carried out at 37°C for 24 hours. The samples were then concentrated and examined by electrophoresis, as described above.

EXAMINATION OF SUGAR COMPONENTS OF ELECTROPHORETIC FRACTIONS Electrophoresis of urinary concentrates (40 μ l) was carried out as described above. Alcian blue positive fractions were located by staining narrow strips cut from the edges of the cellulose acetate membrane, and the areas of the unstained strip corresponding to each fraction were cut out, shredded, and transferred to $\frac{1}{8}$ in. Visking tubing that had been previously heated to reduce pore size (Callanan, Carrol, and Michael, 1957), containing 0.5 ml water. Dialysis was carried out for 24 hr against six changes of distilled water at 4°C. The solutions were centrifuged and the clear supernatants dried *in vacuo*. The residues were taken up in 0.5 ml 4 N or 0.1 N HCl, sealed in Pyrex tubes, and hydrolysed at 100°C for eight hours. HCl was removed *in vacuo* over KOH. The dried residues were taken up in 50 μ l H₂O and applied to Whatman 3 mm paper. Descending chromatography was carried out with n-butanol: pyridine: 0.1 M HCl 5:3:2 as solvent, for 24 hr at 20°C. Reducing sugars were located by the silver method of Trevelyan, Procter, and Harrison (1950).

PREPARATION OF GLYCOSAMINOGLYCANS FROM POOLED URINE Pooled urine (5 l) from healthy, active adults was preserved with a little merthiolate. Fifty ml 10% w/v CPC in water was added slowly with stirring. The white flocculent precipitate was collected by centrifugation and washed with 100 ml 0.1% w/v CPC. The precipitate was dissolved in 2 M NaCl (50 ml) and the solution was centrifuged to remove insoluble material. Glycosaminoglycans were precipitated as their sodium salts from the clear supernatant by the addition of 200 ml ethanol. The precipitate was collected by centrifugation, washed with 100 ml ethanol and dried *in vacuo*.

SEPARATION OF GLYCOSAMINOGLYCANS BY COLUMN CHROMATOGRAPHY An aqueous solution of the glycosaminoglycan preparation from pooled urine, containing 5 mg uronic acid in 5 ml, was applied to the top of a 1.8 $\times$ 55 cm column containing Dowex 1 $\times$ 2 - 400 resin in chloride form. The column was surrounded by a water jacket at 25°C. The column flow rate, controlled by a roller pump, was 10 ml per hour. After application of the sample, the column was washed with 100 ml 0.1 M NaCl. A steadily increasing concentration of sodium chloride was then applied to the column, rising from 0.1 M NaCl to 2.5 M NaCl over 12 hr (120 ml). The NaCl molarity

of the column effluent was measured by platinum electrodes set into the tip of the column, connected to a conductivity bridge. The out-of-balance potential from the conductivity bridge was converted to current and fed into a recording microammeter. Thus a constant recording of the NaCl molarity of the column effluent was obtained. The effluent was collected in fractions of 1.2 ml and uronic acid assays were carried out on 0.2 ml aliquots of effluent diluted to 1.0 ml with water. Fractions corresponding to uronic acid peaks were pooled, and dialysed in previously heated Visking tubing against glass-distilled water at 4°C (40 hr). The solutions were centrifuged, and the clear supernatants freeze-dried.

URONIC ACID This was measured by the method of Bitter and Muir (1962).

HEXOSAMINE Boas' (1953) modification of the Elson-Morgan reaction was used, after hydrolysis of the samples in 4 N HCl at 100°C for eight hours.

SULPHATE Samples of glycosaminoglycans (1.0 mg) were hydrolysed in 0.1 N HCl at 100°C for four hours. 'Acid hydrolysable' sulphate was measured by the method of Belcher, Bhasin, Shah, and West (1958), modified to give maximum sensitivity in the 50 to 200 μ g range.

OPTICAL ROTATION This was measured in a Bellingham and Stanley Polarimeter at 25°C and 589 m μ , using 1 cm cells and solutions containing 1 mg glycosaminoglycan per ml of water.

TERMINOLOGY The nomenclature of 'mucopolysaccharides' is a little confused at present, so the suggestions of Jeanloz (1960) are followed in this paper.

RESULTS

The CPC turbidity of the normal urines fell within the range 0.025-0.50 in 99% of cases. Values were high in children (mean 0.30, standard deviation 0.09 in the age group 0-15 yr) falling to relatively low levels (mean 0.11, SD 0.06, 15-65 yr). In old age, the CPC turbidity tended to rise (mean 0.25, SD 0.13, 65-95 yr). The CPC : creatinine ratio showed less scatter within individual age groups than the CPC turbidity alone, though due to the low urinary creatinine in childhood, the effect of the high CPC turbidity in early life was accentuated. The uronic acid : creatinine ratio showed a similar pattern to the CPC : creatinine ratio, though the elevation in old age was less marked. These results are shown in Figure 1. The 24-hour excretion of CPC-precipitable uronic acid was higher in adult males (mean 4.3 mg) than in adult females (mean 3.0 mg).

The uronic acid content of dialysed urine was considerably higher than, and showed only a rough correlation with the CPC-precipitable uronic acid, probably due to the presence of non-dialysable,

FIG. 1. The CPC : creatinine ratio (a); the uronic acid: creatinine ratio (b); the proportion of chondroitin sulphates expressed as a percentage of the total alcian blue positive material (c); and the proportion of heparan sulphates expressed as a percentage of the total alcian blue positive material (d) in 100 normal urine samples, plotted against age.

non-mucopolysaccharide glucuronides, and non-specific chromogens.

Electrophoresis of urinary concentrates resulted in three major alcian blue positive fractions in every case, numbered in order of decreasing electrophoretic mobility, E1, E2, and E3 (Fig. 2).

Fraction E1 corresponded in mobility with chondroitin sulphate, failed to stain with lissamine green and the periodic acid-Schiff (PAS) technique, and was reduced to about 10% of its original density by testicular hyaluronidase. It was unaffected

by neuraminidase digestion. After elution and hydrolysis of this fraction, paper chromatography showed that galactosamine was the only amino sugar present. Traces of glucose were also detected, probably derived from the cellulose acetate.

Fraction E2 corresponded in mobility with heparan sulphate, failed to stain with lissamine green and PAS, and was resistant to both testicular hyaluronidase and neuraminidase. Chromatography showed the presence of glucosamine as the major reducing sugar, together with smaller quantities of

FIG. 2a. Salt gradient, ion-exchange chromatogram of glycosaminoglycans from pooled urine, showing the two major uronic acid-containing fractions, C1 and C2. The dotted line . . . shows the NaCl molarity of the column effluent, and the unbroken line — shows the uronic acid content of the fractions.

FIG. 2b. Chromoscan record of the alcian blue-positive fractions obtained by electrophoresis of the same sample showing the three electrophoretic fractions E1, E2, E3. The horizontal scale is expanded $\times 3$ to correspond with the chromatogram. Electrophoretic migration is from left to right, as indicated by the arrow. The spot beneath the baseline shows the position of a marker sample of hyaluronic acid. Technical details are given in the text.

galactosamine and galactose. Traces of glucose were also present. This fraction could be separated by careful electrophoresis into two distinct subfractions, apparent in Figure 3c.

Fraction E3 migrated more slowly than hyaluronic acid and stained with lissamine green and PAS. It was resistant to testicular hyaluronidase but was reduced to about 25% of its original density by neuraminidase. Acid hydrolysis of the eluted fraction resulted in marked humin formation, and chromatography showed the presence of glucosamine as the major amino sugar, together with traces of galactose and glucose. This fraction usually migrated as two closely related subfractions.

A minor alcian blue positive fraction, corresponding in mobility with hyaluronic acid, failing to stain with lissamine green or PAS, and completely removed by hyaluronidase digestion, was present in some samples.

From 5 l of pooled adult urine, 75 mg crude glycosaminoglycan was obtained which dissolved in 50 ml H_2O to produce a slightly viscous, straw-coloured solution containing 500 μg uronic acid/ml. Salt gradient, Dowex 1-Cl column chromatography of this material resulted in two major uronic acid-containing peaks, one (fraction C2) leaving the

FIG. 3. Chromoscan traces of alcian blue-positive fractions obtained by cellulose acetate electrophoresis of urinary concentrates from (a) a neonate (M, 1 day); (b) a child (F, 6 yr); (c) an adult (F, 22 yr); and (d) an old person (M, 84). Direction of electrophoretic migration from left to right. The spot beneath the baseline marks the position of a marker sample of hyaluronic acid. Technical details are given in the text.

column in 1.0 M NaCl, the other (fraction C1) in 1.5 M NaCl (Fig. 2). Electrophoresis of the isolated uronic acid peaks showed fraction C1 to correspond in mobility with fraction E1, and fraction C2 to correspond in mobility with fraction E2. A minor uronic acid-containing fraction leaving the column in 0.5 M NaCl corresponded in mobility with hyaluronic acid and was contaminated with material corresponding with fraction E1.

TABLE I

CHARACTERISTICS OF THE THREE ALCIAN BLUE-POSITIVE ELECTROPHORETIC FRACTIONS (E1, E2, AND E3) AND THE TWO URONIC ACID-CONTAINING COLUMN CHROMATOGRAPHIC FRACTIONS (C1 AND C2) OBTAINED FROM HUMAN URINE

Fraction	Electrophoretic Mobility Corresponding with	Susceptibility to Enzyme Digestion		Major Amino Sugar	Molar Ratios Hexosamine: Uronic Acid: Sulphate	Optical Rotation [α] ²⁵ D
		HYAL	NEUR			
E1	Chondroitin sulphate	90%	0	Galactosamine		
C1	Chondroitin sulphate	90%	0	Galactosamine	1.0:1.0:1.1	-24°
E2	Heparan sulphate	0	0	Glucosamine		
C2	Heparan sulphate	0	0	Glucosamine	1.1:1.0:0.6	+40°
E3	Serum α_1 acid glycoprotein	0	75%	Glucosamine		

Molar ratios of hexosamine, uronic acid, and sulphate, together with the specific optical rotation, of fractions C1 and C2 are shown in Table I. All the analytical data, summarized in Table I, suggest that fractions E1 and C1 are identical, being composed entirely of a mixture of chondroitin sulphates. Fractions E2 and C2 are also identical, being composed mainly of heparan sulphates. Fraction E3 is composed of glycoproteins, rich in sialic acid.

The electrophoretic pattern of urinary glycosaminoglycans and glycoproteins showed interesting alterations with age. In the first few years of life, the proportion of chondroitin sulphates was high, obscuring insignificant amounts of heparan sulphate (Fig. 3b). The proportion of chondroitin sulphates decreased with increasing age throughout childhood, to reach adult values at about the age of 20 years. The proportion of heparan sulphate increased with age, again reaching adult values at about the age of 20 yr (Fig. 3c). In the first few days of life, however, the proportion of heparan sulphates was higher than at any other time in early childhood, and a fraction corresponding with hyaluronic acid was detectable (Fig. 3a). In old age, the proportion of glycoprotein increased (Fig. 3d). The changes in the relative proportions of chondroitin sulphates and heparan sulphates in the urine throughout life are illustrated in Figures 1c and 1d.

No clear sex difference in excretion pattern could be established in this study. An occasional distortion of the pattern was due to bacterial contamination, or to the presence of autogenous hyaluronidase in samples of male urine.

DISCUSSION

The CPC turbidity provides a quick and reliable method of screening 24-hour urine samples for excess mucopolysaccharide, the value being less than 0.5 in 99% of normal cases and less than 0.6 in 100% of normal cases. The one 'normal' case showing a CPC turbidity in excess of 0.5 (0.54) in this study was a woman of 77 yr who showed a high

urinary glycoprotein content. In cases of gargoylism, the CPC turbidity is always above 0.5 and above 0.6 in 94% of cases (Manley and Hawksworth, unpublished data). In addition, the CPC turbidity provided a rough quantitative measure of the urine glycosaminoglycan content, as shown by the similarity between the CPC : creatinine ratios and the uronic acid : creatinine ratios (Figs. 1a and 1b). The CPC turbidity is, however, influenced by the urinary glycoprotein, and this accounts for the divergence between the CPC : creatinine and the uronic acid : creatinine curves in later life; the CPC : creatinine ratio shows a more marked rise in old age due to the increasing proportion of urinary glycoprotein, which does not contain uronic acid. The figures obtained for the uronic acid : creatinine ratio in this study are in agreement with those obtained by Teller, Burke, Rosevear, and McKenzie (1962).

The figures obtained for the 24-hour uronic acid excretion in this study are very similar to those obtained by Kerby (1954). Although measurement of the CPC-precipitable uronic acid underestimates the true mucopolysaccharide uronic acid (Muir, Mittwoch, and Bitter, 1963) this is probably of little consequence in clinical work, providing one is aware of the fact, and the method, with its normal range, is made clear.

The analytical data suggest that the most rapidly migrating electrophoretic fraction (E1) is composed entirely of chondroitin sulphates, of which dermatan sulphate forms only a small proportion. This fraction is the major glycosaminoglycan component of children's urine and was the only fraction identified by Di Ferrante and Rich (1956) in their study of normal human urine. The electrophoretic fraction of intermediate mobility (E2), although composed mainly of heparan sulphates, is not a homogeneous fraction. It is completely resistant to the action of testicular hyaluronidase, but the slight molar excess of hexosamine in relation to uronic acid, and the presence of small amounts of galactose, suggest that some keratan sulphate is present. This fraction could be separated by careful electrophoresis

into two closely related subfractions (Fig. 3c). A similar separation could not be achieved by column chromatography, so it was not possible to examine the components of the two subfractions separately. However, both were resistant to testicular hyaluronidase, and the proportion of galactose was higher in the more slowly migrating electrophoretic fraction. Teller (1967), using the column chromatographic method of Schiller, Slover, and Dorfman (1961), to examine the glycosaminoglycans in the urine of eight normal subjects, found proportions of chondroitin sulphates and heparan sulphates that correspond approximately to those found in adults in this study, and he also detected traces of keratan sulphate.

The slowly migrating electrophoretic fraction (E3) contained protein, glucosamine, small amounts of galactosamine, galactose and glucose, together with sialic acid, but no uronic acid. Therefore this fraction is not apparent in the chromatogram shown in Figure 2a. It was detected, however, in the column effluent between 0.15 and 0.4 M NaCl. This fraction could be resolved by careful electrophoresis into two subfractions, which were both reduced, but not abolished, by neuraminidase. This incomplete susceptibility to neuraminidase is similar to that of the Tamm-Horsfall glycoprotein, where only three of the total of four sialic acids per heterosaccharide are readily available substrates for neuraminidase (Maxfield, 1966). The Tamm-Horsfall glycoprotein has been shown to exist in two molecular forms, a tetramer and a monomer (Maxfield, 1961), and it is possible that the two urinary glycoprotein subfractions revealed by electrophoresis in this study may represent a similar difference in physical state rather than separate chemical entities. However, prior precipitation of the Tamm-Horsfall glycoprotein from the urine with 0.58 M NaCl (Tamm and Horsfall, 1952) caused only slight reduction of this glycoprotein fraction, so that it would be inaccurate to refer to this fraction as 'the Tamm-Horsfall glycoprotein'.

One interesting point regarding this glycoprotein fraction is that its staining intensity increased markedly after testicular hyaluronidase digestion of the urine, and it is possible that this phenomenon may be due to the binding of the products of digestion by the glycoprotein. The binding, or adsorbing, properties of the Tamm-Horsfall glycoprotein have been discussed by Maxfield (1966). It is also of interest that Keutel and King (1965) found exceedingly little 'uromucoid' in the urine of newborn children using an immunophoretic technique, whereas the methods employed in this study showed no significant reduction of glycoprotein in the urine of neonates.

The three electrophoretic fractions discussed in this paper probably correspond to the three fractions detected, but not characterized, by Heremans, Vaerman, and Heremans (1959).

As to the origin of these fractions, ultracentrifuge studies showed that the urinary chondroitin sulphates are polydisperse, with an average molecular weight in the region of 10,000, so that they would be easily filtered by the renal glomerulus. The glycoprotein, on the other hand, is a much larger, non-dialysable molecule, and it seems unlikely that this component enters the urine by filtration through the glomerulus. Keutel (1965) has obtained evidence to suggest that 'uromucoid' is produced by the epithelial cells of the proximal segments of the renal tubules.

The changes demonstrated in the excretion patterns with age are interesting. The high proportion of chondroitin sulphate in the urine of children may be related to rapid skeletal growth. The similarity of the neonatal excretion pattern to that seen in the adult rather than the child was an unexpected finding and may be due to a trans-placental passage of maternal glycosaminoglycans. The hyaluronic acid present in neonatal urine may be related to the high hyaluronic acid content of the umbilical cord or young connective tissue (Manley, 1965).

The urinary pattern of glycoproteins and glycosaminoglycans remains relatively constant throughout adult life, but in old age many cases show an increase in the proportion of glycoprotein. The possibility of an association between urinary glycoprotein and stone formation has aroused interest (MacLagan and Anderson, 1958) but no causal relationship has been demonstrated. The rising urinary glycoprotein in old age may be a phenomenon analogous to the increasing mucus-producing cough seen in many elderly patients, perhaps related to infection. This possibility is being explored. One result of the rising proportion of glycoprotein in old age is an increase in the proportion of high CPC turbidity readings, with an increase in the standard deviation, but from the diagnostic aspect this is of less importance than the relatively high CPC turbidity in young children, for a diagnosis of 'gargoylism' is unlikely to be made in the 65-95 age group.

The high chondroitin sulphate content of entirely normal children's urine presents a very real danger of a mistaken diagnosis of 'gargoylism' in this age group. Such a diagnosis should only be made on biochemical examination of the urine when the CPC turbidity is above 0.6, the uronic acid: creatinine ratio is above 50, and electrophoresis of the urine concentrate shows an excess of chondroitin sulphate and/or heparan sulphate. It is stressed that extreme

caution must be exercised in interpreting the urinary glycosaminoglycan pattern of a child.

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REFERENCE

- Belcher, R., Bhasin, R. L., Shah, R. A., and West, T. S. (1958). *J. chem. Soc.*, 4054.
- Bitter, T., and Muir, H. M. (1962). *Analyt. Biochem.*, **4**, 330.
- Boas, N. F. (1953). *J. biol. Chem.*, **204**, 553.
- Bonsnes, R. W., and Taussky, H. H. (1945). *Ibid.*, **158**, 581.
- Callanan, M. J., Carroll, W. R., and Mitchell, E. R. (1957). *Ibid.*, **229**, 279.
- Di Ferrante, N., and Rich, C. (1956). *Clin. chim. Acta*, **1**, 519.
- Heremans, J. F., Vaerman, J. P., and Heremans, M. T. (1959). *Nature (Lond.)*, **183**, 1606.
- Jeanloz, R. W. (1960). *Arthr. and Rheum.*, **3**, 233.
- Kerby, G. P. (1954). *J. clin. Invest.*, **33**, 1168.
- Keutel, H. J. (1965). *J. Histochem. Cytochem.*, **13**, 155.
- , and King, J. S., Jr. (1965). *Clin. chim. Acta*, **11**, 341.
- MacLagan, N. F., and Anderson, A. J. (1958). In *Ciba Foundation Symposium on the Chemistry and Biology of Mucopolysaccharides*, edited by G. E. W. Wolstenholme and M. O'Connor, p. 268., Churchill, London.
- Manley, G. (1965). *Brit. J. exp. Path.*, **46**, 125.
- , and Hawksworth, J. (1966). *Arch. Dis. Childh.*, **41**, 91.
- Mathews, M. B., and Inouye, M. (1961). *Biochim. biophys. Acta (Amst.)*, **53**, 509.
- Maxfield, M. (1961). *Ibid.*, **49**, 548.
- (1966). In *Glycoproteins, Their Composition, Structure and Function*, edited by A. Gottshalk. Vol. 5, pp. 455 and 458. BBA Library, Elsevier, Amsterdam.
- Muir, H., Mittwoch, U., and Bitter, T. (1963). *Arch. Dis. Childh.*, **38**, 358.
- Segni, G., Romano, C., and Tortorolo, G. (1964). *Lancet*, **2**, 420.
- Schiller, S., Slover, G. A., and Dorfman, A. (1961). *J. biol. Chem.*, **236**, 983.
- Tamm, I., and Horsfall, F. L., Jr. (1952). *J. exp. Med.*, **95**, 71.
- Teller, W. M. (1967). *Nature (Lond.)*, **213**, 1132.
- , Burke, E. C., Rosevear, J. W., and McKenzie, B. F. (1962). *J. Lab. clin. Med.*, **59**, 95.
- Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950). *Nature (Lond.)*, **166**, 444.