Colorimetric Measurement of Dermatan Sulphate

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The periodate–Schiff reaction has been adapted for the measurement of dermatan sulphate. The method is specific for this glycosaminoglycan, provided that glycogen and glycoproteins are removed. Measurements of dermatan sulphate present in the urine of patients affected by various mucopolysaccharidoses indicate a good agreement between the values obtained with enzymic methods and those obtained with the colorimetric method described.

Simple reliable methods for the measurement of dermatan sulphate, heparan sulphate and keratan sulphate are desirable. The determination of 2-deoxy-2-sulphoamino-hexose (Lagunoff & Warren, 1962) is well suited for the measurement of heparan sulphate in a mixture of glycosaminoglycans (Lagunoff, Pritzl & Scott, 1967), provided that heparin is not present. Measurements of dermatan sulphate are usually performed after its isolation or separation with methods that depend on the molecular weight and charge density of the polymer (Meyer & Rapport, 1951; Cifonelli, Ludowieg & Dorfman, 1958; Berenson, 1958; Haruki & Kirk, 1967; Wessler, 1968). The results obtained may vary when applied to a mixture of standard glycosaminoglycans or to tissue extracts and biological fluids containing glycosaminoglycans. Saito, Yamagata & Suzuki (1968) described a complex series of enzymic methods suitable for the measurement of dermatan sulphate. Scott & Harbinson (1968, 1969) and Scott & Dorling (1969) postulated that, if the intense negative electrostatic field of glycosaminoglycans is suppressed, either by lowering the pH or by increasing the concentration of cations, the negatively charged periodate ions become capable of oxidizing the C-2 and C-3 glycol groups of the uronic acid portion of the polymers. Under appropriate conditions, polymer-bound L-iduronic acid may be oxidized more easily and rapidly than D-glucuronic acid (Scott, 1968). The technique described below is a modification of the histochemical method of Scott & Dorling (1969) and allows for the direct measurement of dermatan sulphate in various mixtures of glycosaminoglycans.

EXPERIMENTAL

Materials

Glycosaminoglycans. Dermatan sulphate from bovine lungs (preparation R01-2232/715) was a gift from Dr K. von Berlepsch, Hoffmann–La Roche, Basle, Switzer-

land. That from pig mucosal tissues and other standard glycosaminoglycans were provided by Dr J. A. Cifonelli, University of Chicago. Glycosaminoglycans were dissolved in water or in NaCl of different concentrations (from 0.20 to 2.0 M), to a final concentration of 200 µg/ml.

Glycogen and glycoproteins. Rat liver glycogen was prepared by the method of Stetten, Katzen & Stetten (1956). α_1 -Acid glycoprotein was a gift of Dr Edwin A. Popenoe, of Brookhaven National Laboratory, Upton, N.Y., U.S.A. Tamm–Horsfall urinary glycoprotein was prepared as described previously (Di Ferrante & Popenoe, 1957). Human plasma glycoprotein fraction VI was purchased from Miles Laboratories, Elkhart, Ind., U.S.A.

Enzymes, buffers and ion exchangers. Takamine (Diazyme 160; Miles Laboratories) (10 mg) and glucosylase (from *Rhizopus niveus*; Seikagaku Kogyo Co., Ltd., Tokyo, Japan, distributed by Miles Laboratories) (1 mg) were separately dissolved in 10 ml of 0.05 M-sodium acetate–acetic acid buffer, pH 5.50, containing 0.10 M-NaCl. The first enzyme preparation was cleared by centrifugation before use. Testicular hyaluronidase (300 U.S. Pharmacopeia units/mg; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was dissolved in the same acetate buffer to a final concentration of 1 mg/ml. Chondroitinase ABC, chondroitin 4-sulphatase and standard unsaturated disaccharides as described by Yamagata, Saito, Habuchi & Suzuki (1968), Saito *et al.* (1968) and Suzuki *et al.* (1968) were obtained from Miles Laboratories. Pepsin (Worthington Biochemical Corp.) (1 mg) was dissolved in 1 ml of 0.1 M-glycine–HCl buffer, pH 1.90, containing 0.1 M-NaCl. SE- (sulphoethyl-) Sephadex C-50 (medium grade; Pharmacia, Uppsala, Sweden) was swollen and packed either in 0.01 M-glycine–HCl buffer, pH 2.60, containing 0.01 M-NaCl or in 0.05 M-sodium citrate buffer, pH 3.0.

Solutions. Aqueous solutions of 0.1 M-NaIO₄–0.2 M-NaClO₄, 0.5 M-H₂SO₄ and 0.16 M-sodium metabisulphite were prepared. Cold Schiff reagent was prepared as originally described by Lillie (1965). If kept at 4°C, it could be used for 3–4 days.

Methods

To 0.5 ml samples containing increasing amounts of standard glycosaminoglycan preparations (2.5–100 µg) or

to 0.5 ml of unknown samples were added 0.1 ml of 0.5 M-H₂SO₄ and 0.20 ml of NaIO₄-NaClO₄ solution. The tubes were shaken and incubated at 37°C for 1 h under fluorescent cool white light of 99 ft-candle power. The tubes were removed from the water bath and to each tube was added 0.3 ml of sodium metabisulphite solution and, after shaking, 0.5 ml of Schiff reagent. The tubes were incubated again for 20 min at 37°C, after which E_{540} values were measured.

Since glycogen and glycoprotein interfere with the colour reaction, they must be removed before measurement of dermatan sulphate. Although the usual methods of isolation and purification of glycosaminoglycans (Scott, 1960) should eliminate these interfering substances, the following techniques may be employed to assure their removal.

Removal of glycogen. A portion (0.20 ml) of clear Takamine supernatant or glucoamylase solution is added to a portion of unknown sample (0.10–0.30 ml). The tubes, including blanks containing the same amount of enzymes, are incubated at 37°C for 30 min; thereafter they are adjusted to a final volume of 0.50 ml with water and used for the periodate oxidation as described for the purified samples.

Removal of glycoprotein. Because of the large variety of glycoproteins that may be present in crude tissue extracts, no one single technique may be suggested for their removal. The method of Berman (1966) separates dermatan sulphate (100 µg) and α_1 -acid glycoprotein (1 mg) by chromatography on a column (0.5 cm × 7 cm) of SE-Sephadex with 0.05 M-sodium citrate buffer, pH 3.0. The same technique, however, does not separate dermatan sulphate from plasma glycoprotein fraction VI, unless previous digestion with pepsin is performed as described below.

Glycoprotein (1 mg), dermatan sulphate (100 µg) and pepsin (100 µg) are dissolved in 1 ml of 0.1 M-glycine-HCl buffer, pH 1.90, and incubated at 52°C for 3 h. Thereafter, the tubes are incubated at 80°C for 1 h, diluted to 2 ml with water and applied to a column (0.5 cm × 7.0 cm) of SE-Sephadex packed and eluted with 0.01 M-glycine-HCl buffer, pH 2.60. The borate-carbazole reaction (Bitter & Muir, 1962), the periodate-Schiff reaction as described above and the Lowry, Rosebrough, Farr & Randall (1951) reaction are performed directly on samples from the effluent fractions (2 ml each).

Preparation of glycosaminoglycans from urine. The glycosaminoglycans were precipitated with cetylpyridinium chloride from 24 h or 48 h urine specimens as described by Di Ferrante (1967). After removal of the cetylpyridinium chloride, the residual material was dissolved in 0.58 M-NaCl and kept at 4°C overnight. The precipitated glycoprotein from urine was removed by centrifuging at 4°C and 1500 g for 15 min in an International PR-2 centrifuge. The supernatant was dialysed and the non-diffusible material was freeze-dried (crude glycosaminoglycans). Portions of this material were dissolved in water and applied to columns of Dowex 1 resin (X2; 200–400 mesh; Cl⁻ form) (Schiller, Slover & Dorfman, 1961). After the columns had been washed with water and 0.9% NaCl solution (25 ml each), the glycosaminoglycans were eluted with 2.0 M-NaCl. After dialysis and freeze-drying, portions (1 mg) of the purified glycosaminoglycans were used for measurement of total uronic acid (Bitter & Muir, 1962), heparan sulphate (Lagunoff & Warren, 1962) and dermatan sulphate. The last-named measurement was performed by the periodate-Schiff reaction described above, and also with the following enzymic method (Saito *et al.* 1968): portions of purified glycosaminoglycans (containing 50–150 µg of dermatan sulphate) were incubated overnight at 37°C with 0.5 mg of testicular hyaluronidase. The non-depolymerized material was precipitated by centrifugation with ethanol containing 10% (w/v) of potassium acetate, redissolved in water and precipitated again. The final precipitate was dried and dissolved in water. Portions of this solution were incubated with chondroitinase ABC and chondroitin 4-sulphatase (Saito *et al.* 1968), and the amount of desulphated disaccharides thus produced was measured by the Morgan-Elson reaction (Strominger, Park & Thompson, 1959).

The various analytical techniques used have been described in detail previously (Calatroni & Di Ferrante, 1968; Calatroni, Donnelly & Di Ferrante, 1969).

RESULTS

Table 1 gives the analytical results for the glycosaminoglycans used as substrates for the periodate oxidation. Dermatan sulphate is the only compound producing colour with the technique

Table 1. *Chemical composition of the standard glycosaminoglycans used*

Compositions are expressed as percentage of total dry weight.

Compound	Source	N	Uronic acid	Hexosamine	Sulphate	Galactose
Dermatan sulphate	Bovine lungs		26.3*	26.7†, 1.3§	6.5	
Dermatan sulphate	Pig mucosae	2.3	22.8*	23.0†	6.4	
Chondroitin 4-sulphate	Rock sturgeon notochord	2.6	34.1†	27.2†	4.9	
Chondroitin 6-sulphate	Human umbilical cord	2.7	34.6†	26.2†	4.9	
Hyaluronic acid	Human umbilical cord	3.0	47.2†	38.3§	0	
Heparin	Pig mucosae	2.6	38.7†	23.8§	11.7	
Heparan sulphate	Bovine lungs	2.5	44.1†	24.6§	5.0	
Keratan sulphate	Human costal cartilage	3.2		19.8§	6.6	22.5

* Determined by the borate-carbazole method of Bitter & Muir (1962).

† Determined by the carbazole method of Dische (1947).

‡ Galactosamine.

§ Glucosamine.

described. The results presented in Fig. 1 indicate that equal weights of different dermatan sulphate preparations give identical E_{540} values. The inter-

ference by keratan sulphate and chondroitin 6-sulphate is minimal.

The linear relationship between E_{540} and the concentration of dermatan sulphate is maintained up to 100 μg of substrate. Because the E_{540} increases with increasing concentration of sodium chloride (Fig. 1), the unknown samples, their blanks and the standard should be either salt-free or of known salt concentration. The presence of sodium chloride does not influence the behaviour of the other glycosaminoglycans.

The interference by glycogen and three glycoproteins in the colour reaction is demonstrated in Fig. 2. Treatment of a glycogen-containing sample with purified glucoamylase removes the glycogen interference almost completely. However, because of the use of lactose as an enzyme stabilizer, enzyme-containing blanks should be made. Sialic acid, glucose, galactose, glucosamine, galactosamine, glucuronolactone and glucuronic acid do not produce colour with the reaction described above.

Dermatan sulphate, added to α_1 -acid glycoprotein or plasma glycoprotein fraction VI, may be separated from the glycoproteins by chromatography on SE-Sephadex. As indicated in Table 2, the dermatan sulphate is eluted quantitatively from the column, whereas the glycoproteins or the

Fig. 1. Extinctions at 540nm of various glycosaminoglycans that have been treated with periodate-Schiff reagents. ●, Dermatan sulphate from bovine lungs, in water; ▲, dermatan sulphate from pig mucosae, in water; ■, dermatan sulphate from both sources in 0.4–1.0M-NaCl; ○, dermatan sulphate from both sources in 1.2–2.0M-NaCl. The points marked KS and CSC (Δ) indicate the E_{540} of keratan sulphate and chondroitin 6-sulphate.

Fig. 2. (a) E_{540} of rat liver glycogen that has been treated with periodate-Schiff reagent, before and after treatment with glucoamylase. ●, Rat liver glycogen; Δ, after incubation with 0.1mg or, ▲, 0.2mg of purified glucoamylase; ○, after incubation with 1mg of crude glucoamylase. (b) E_{540} values of α_1 -acid glycoprotein (□), plasma glycoprotein fraction VI (○) and urinary glycoprotein (■).

Table 2. Removal of colour interference caused by some glycoproteins

Samples (a) and (c) were applied directly to SE-Sephadex columns (0.5cm × 7.0cm), and samples (b) and (d) were previously digested with pepsin at pH1.9 for 3h and incubated at 80°C for 1h. Hexuronic acid (Bitter & Muir, 1962), iduronic acid (periodate-Schiff reaction) and protein (Lowry *et al.* 1951) were measured and are expressed as μg present in the first 8ml of effluent. See the text for experimental details.

Sample	Hexuronic acid	Iduronic acid	Protein
(a) Dermatan sulphate (100 μg)	23.0	32.0	0
(b) Dermatan sulphate (100 μg) + pepsin (100 μg)	23.1	35.0	106.0
(c) Dermatan sulphate (100 μg) + α_1 -acid glycoprotein (1 mg)	27.0	34.0	25.0
(d) Dermatan sulphate (100 μg) + plasma glycoprotein fraction VI (1 mg)	21.5	34.7	148.4

Table 3. *Total uronic acid, iduronic acid and 2-deoxy-2-sulphoamino-hexose contents of purified urinary glycosaminoglycans*

Uronic acid was determined by the borate-carbazole method of Bitter & Muir (1962), with glucuronolactone as a standard. Iduronic acid values were obtained by dividing by 3 the dermatan sulphate values measured (a) colorimetrically by the periodate-Schiff reaction and (b) enzymically (Saito *et al.* 1968). Dermatan sulphate from bovine lungs was used as a standard. In the determination of 2-deoxy-2-sulphoamino-hexose, no attempts were made to calculate the percentage of acetyl-amino- or amino-hexose present in heparan sulphate. 2-Amino-2-deoxy-hexose was used as a standard. The results are expressed as percentages of the total dry weight of the purified glycosaminoglycans.

Subject and diagnosis		Uronic acid	Iduronic acid		2-Deoxy-2-sulphoamino-hexose
			(a)	(b)	
J.F.	Hurler's syndrome	29.0	19.0	19.0	7.0
J.M.	Hurler's syndrome	29.0	19.0	17.0	3.8
T.R.	Hunter's syndrome	31.0	15.0	17.0	8.3
S.R.	Hunter's syndrome	31.0	14.0	14.0	10.1
J.E.	Hunter's syndrome	21.0	4.1	4.4	0.8
M.H.	Hunter's syndrome	19.5	2.2	1.5	9.3
R.L.	Hunter's syndrome*	12.4	2.1		4.2
D.M.	Maroteaux-Lamy's syndrome*	8.6	6.2	5.0	0.8

* Crude urinary glycosaminoglycans.

interfering sugars present in their proteolytic digests are retained.

Table 3 presents the results of analyses performed on purified urinary glycosaminoglycans from patients affected by various mucopolysaccharidoses and demonstrates the good agreement between the results obtained enzymically (Saito *et al.* 1968) and with the periodate-Schiff reaction described.

DISCUSSION

Scott & Harbinson (1969) were able to oxidize dermatan sulphate preferentially to hyaluronate, chondroitin 4-sulphate and chondroitin 6-sulphate by using 0.01 M-sodium periodate-0.2 M-sodium perchlorate at 30°C in the dark for a period of 10 h. The point of oxidation was considered to be at the C-2-C-3 glycol group of the uronic acid, because of the considerable destruction of carbazole-reacting uronic acid resulting from the treatment with the periodate reagent (Scott & Harbinson, 1969; Scott, 1968).

To effect the oxidation in a shorter time, we have performed it in artificial light at 37°C for 1 h, using 0.1 M-sodium periodate-0.2 M-sodium perchlorate in 0.066 M-sulphuric acid (final pH of the reagent, 1.55). At this concentration of sulphuric acid the colour yield of dermatan sulphate is increased despite the fact that only 15% of the borate-carbazole (Bitter & Muir, 1962)-reacting material present in it is destroyed. Other glycosaminoglycans are not oxidized at this concentration of sulphuric acid and do not interfere with the reaction.

Prolonged periods of incubation or increased con-

centrations of sulphuric acid produce an increased oxidation of uronic acid in dermatan sulphate, but also an appreciable oxidation of uronic acid in other glycosaminoglycans, thus decreasing the specificity of the method. The selective effect of the chosen reagent may be due to the fact that glucuronic acid-containing glycosaminoglycans lactonize easily at a low pH, thus blocking the oxidizable glycol groups (Scott & Harbinson, 1968). The lactone, however, may be partly hydrolysed during prolonged treatment at a low pH (Blake & Richards, 1968), making the polymers sensitive to the oxidizing reagent.

In view of the demonstration (Lindahl & Axelsson, 1971) that iduronic acid is the major uronic acid of heparin, it was surprising to find that this compound does not produce colour with the technique described. However, most of the iduronic acid present in heparin is considered to be sulphated whereas most of the glucuronic acid is not. Thus the conditions chosen for the oxidation produce a limited oxidation of iduronosyl residues and no oxidation of glucuronosyl residues, and so assure the specificity of the colour reaction for dermatan sulphate even in the presence of heparin.

Because of the effect of salt concentration on the colour formation, the various glycosaminoglycan fractions that may be eluted from columns of anion-exchange resin may be accurately analysed only if their sodium chloride content is known. When the values obtained for dermatan sulphate are compared with those of enzymic determinations (Saito *et al.* 1968), the agreement was found to be quite satisfactory. The colorimetric method is faster, simpler and more economical than the enzymic

methods. The enzymic assay cannot be performed by using chondroitinase AC to depolymerize chondroitin 4-sulphate and chondroitin 6-sulphate because of the inhibition of the enzyme in the presence of dermatan sulphate (Saito *et al.* 1968). The use of testicular hyaluronidase allows their depolymerization in the presence of dermatan sulphate, but it is of little advantage when the dermatan sulphate to be measured is of small molecular weight and not precipitable with ethanol. Thus enzymic methods could not measure degradation products of dermatan sulphate in the presence of degradation products of other glycosaminoglycans (Di Ferrante *et al.* 1971).

When used in conjunction with the method of Lagunoff & Warren (1962) for the determination of sulphoaminohexose, this modification of the periodate-Schiff reaction allows a simple and rapid screening of purified urinary glycosaminoglycans obtained from patients with mucopolysaccharidoses.

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