The Alkali-Labile Linkage Between Keratan Sulphate and Protein

By JOHN J. HOPWOOD* and H. CLEM ROBINSON

Department of Biochemistry, Monash University, Clayton, Vic. 3168, Australia

(Received 12 November 1973)

Keratan sulphate was isolated from adult intervertebral disc in 90% yield by sequential digestion of the whole tissue with papain, Pronase and *Proteus vulgaris* chondroitin sulphate lyase. Treatment of this preparation with alkali cleaved a glycosidic bond between *N*-acetylgalactosamine and threonine and produced, by an alkali-catalysed 'peeling' reaction, an unsaturated derivative of *N*-acetylgalactosamine which reacted as a chromogen in the Morgan–Elson reaction, but remained covalently bonded to the keratan sulphate chain. This derivative was reduced and labelled by alkaline NaB^3H_4 . The substituent at position 3 of *N*-acetylgalactosamine in the keratan sulphate–protein linkage was identified as a disaccharide, *N*-acetylneuraminylgalactose, which was isolated from the reaction mixture after alkali treatment.

The sequence of monosaccharides at the linkage between chondroitin sulphate and protein in cartilage proteoglycan is now well defined (Rodén, 1970). Knowledge of the linkage region has enabled study of mechanisms involved in proteoglycan production and biosynthetic control (Telser *et al.*, 1965; Helting & Rodén, 1969*a,b*; Rodén, 1970). Chemical definition of the linkage between keratan sulphate and protein in cartilage proteoglycan has not been published.

Cartilage keratan sulphate preparations contain 0.1–5.0% of *N*-acetylgalactosamine, some of which, together with some serine and threonine, is destroyed on exposure to dilute alkali (Seno *et al.*, 1965; Mathews & Cifonelli, 1965; Bray *et al.*, 1967). Bray *et al.* (1967) further report that alkali treatment of rib cartilage keratan sulphate produces a Morgan–Elson-positive chromogen that remains attached to the keratan sulphate chain, and have proposed that in keratan sulphate, *N*-acetylgalactosamine is linked *O*-glycosidically to a serine or threonine residue, that chromogen is produced by β -elimination of an unknown substituent attached to position 3 of the *N*-acetylgalactosamine and that the keratan sulphate chain is bonded to position 6 of the same *N*-acetylgalactosamine residue.

Besides the *O*-glycosidic link with protein, cartilage keratan sulphate may also be linked to protein through an alkali-stable bond (Seno *et al.*, 1965; Meyer, 1969, 1970; Pedrini, 1969; Heinegård, 1972). The exact nature of this alkali-stable linkage and its relation to the *O*-glycosidic galactosaminyl linkage between keratan sulphate and protein remains to be established.

The aim of the present paper is to identify the unknown substituent attached to position 3 of the alkali-

labile *N*-acetylgalactosamine residue in cartilage keratan sulphate. A preliminary communication of these results has been presented (Hopwood & Robinson, 1973*b*).

Materials and Methods

Materials

Cartilage powder. Whole intervertebral discs from adult steers were collected and milled as described previously (Robinson & Hopwood, 1973).

NaB^3H_4 . Two separate stock solutions were prepared from different batches of borohydride from The Radiochemical Centre, Amersham, Bucks., U.K. The first stock solution contained 100mCi of NaB^3H_4 (502mCi/mmol) and 1.84g of NaBH_4 in 250ml of 0.5M-KOH; the second batch contained 100mCi of NaB^3H_4 (800mCi/mmol) and 0.92g of NaBH_4 in 125ml of 0.5M-KOH. Both stock solutions were prepared at 4°C and stored at –20°C. The specific radioactivities of the first and second stock solutions, assayed as described by Robinson & Hopwood (1973), were 6.65×10^5 d.p.m./ μmol and 17.6×10^5 d.p.m./ μmol respectively.

***N*-Acetylglucosaminitol and *N*-acetylgalactosaminitol.** These were prepared by reducing the corresponding *N*-acetylhexosamines (10mg) with NaBH_4 (16mg) in Tris–HCl buffer, pH 8.0 (0.025M in HCl). After 24h at 4°C each reaction mixture was acidified with acetic acid, applied to a column (20cm $\times$ 1cm) of Dowex 50 (H^+ form) and washed through with water (50ml). The eluate was evaporated under vacuum with repeated additions of methanol to remove boric acid. Finally each preparation was dissolved in water and desalted by passage over a column of Dowex 50 (H^+ form) (20cm $\times$ 1cm) and Dowex 1 (acetate form) (20cm $\times$ 1cm).

* Present address: Department of Pediatrics, University of Chicago, Chicago, Ill. 60637, U.S.A.

Glucosaminitol and galactosaminitol. These were prepared by acid hydrolysis of the corresponding *N*-acetylhexosaminitol (4mg) with 0.05M-HCl (0.8ml) and 250mg of Dowex 50 (H⁺ form) at 100°C for 9h. The hexosaminitols were eluted from the Dowex 50 with 0.5M-HCl and the HCl was removed *in vacuo* over P₂O₅ and KOH.

Other materials. NaBH₄ (assayed at 92% by weight) was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Chondroitin sulphate lyase from *Proteus vulgaris* was prepared as described previously (Robinson & Dorfman, 1969). Neuraminidase (*Vibrio cholerae*) was purchased from Farbwerke-Hoechst B.A.A. (Frankfurt/Main, Germany), Pronase from Calbiochem (Los Angeles, Calif., U.S.A.) and papain from Schwarz/Mann Research Laboratories Inc. (New York, N.Y., U.S.A.).

Sephadex G-25 and G-50 (both fine grade) were purchased from Pharmacia Fine Chemicals A.B., Uppsala, Sweden, and Bio-Gel P-4 (50–100 mesh), Dowex 1 (X4, 200–400 mesh) and Dowex 50 (X4, 100–200 mesh) were from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Whatman Chromedia ET-11 cellulose (ECTEOA) was purchased from W. and R. Balston Ltd., Maidstone, Kent, U.K. (nominal capacity 0.5 mequiv./g). All ion-exchange materials were thoroughly washed and treated to remove fine material before use.

All other reagents were of analytical grade.

Methods

Proteolytic digestion of cartilage. Papain (0.5g) was activated by heating at 60°C for 30min in 0.1M-sodium acetate buffer, pH6.5 (50ml), containing 5mm-EDTA and 10mm-cysteine (Scott, 1960). Milled adult bovine intervertebral disc (6g wet wt.; 1.5g dry wt.) was added to the activated enzyme solution and the mixture incubated at 60°C for 16h. The trace amount of solid material that remained was removed by centrifugation and the supernatant solution applied to a column (28cm×5cm diam.) of Bio-Gel P-4 equilibrated with pyridine-acetic acid buffer, pH5.2 (0.2M in acetic acid and pyridine). The column was eluted with the same buffer at a flow rate of 80ml/h and 12ml fractions were collected and assayed for hexose and hexuronic acid. All of the hexuronate-positive material was eluted close to the void volume of the column and these fractions were combined and freeze-dried (yield 0.8g).

The product of papain digestion was digested at 37°C with 30mg of Pronase in 50ml of Tris-HCl buffer, pH7.9 (0.1M in Cl⁻), containing 0.5mm-CaCl₂ and 2 drops of toluene. After 24h a further 15mg of Pronase was added and the incubation continued for 16h. A sample of the incubation mixture (8.0ml) was applied to a column (30cm×1.5cm diam.) of Dowex 1 (Cl⁻ form), and eluted with a linear gradient of

NaCl in imidazole-HCl buffer, pH5.7 (0.02M in Cl⁻), generated from 500ml of 0.02M-imidazole-HCl buffer and 500ml of 2.0M-NaCl in 0.02M-imidazole-HCl buffer. The column was then washed with 500ml of 5M-NaCl. The flow rate was 40ml/h and 12ml fractions were collected and assayed for hexuronic acid and hexose. All of the hexuronate-positive and 85% of the total hexose-positive material were eluted in a broad peak between approx. 1.0M- and 2.0M-NaCl. The remaining hexose-positive material was eluted with 5M-NaCl (fraction KS-1).

The remaining Pronase digest (42ml) was desalted on a column of Bio-Gel P-4 in pyridine-acetic acid buffer as described above, freeze-dried and digested exhaustively with chondroitin sulphate lyase from *P. vulgaris* in Tris-HCl buffer, pH8.0, as described by Robinson & Dorfman (1969). The digest was concentrated to 8ml by rotary evaporation at 30°C and applied to a column (80cm×2cm diam.) of Sephadex G-50 equilibrated with pyridine-acetic acid buffer, pH5.2. The column was eluted with the same buffer at a rate of 35ml/h and 12ml fractions were collected and assayed for hexose and hexuronic acid. Hexose-positive material was eluted near the void volume (fractions 8–16), well separated from a peak of disaccharide product (fractions 21–26).

The hexose-positive fractions were combined, freeze-dried and digested once more with Pronase as described above. The digest was applied directly to a column (36cm×2cm diam.) of ECTEOA-cellulose (Cl⁻ form) and eluted with a linear gradient of NaCl generated from 500ml of 0.02M-imidazole-HCl buffer, pH5.7, and 500ml of 2.0M-NaCl in imidazole-HCl buffer (0.02M), pH5.7. The hexose-positive material, which was eluted as a single broad peak, was desalted on Bio-Gel P-4 as outlined above and freeze-dried. The yield of this preparation (fraction KS-2) was equivalent to 0.4g/g of original dry tissue.

Alkali treatment of keratan sulphate

(a) *Treatment with 0.2M-NaB³H₄ in 0.5M-KOH.* A solution of 50mg of preparation KS-2 in 5.0ml of the stock borohydride solution (see under 'Materials'; specific radioactivity 6.65×10⁵d.p.m./μmol) was flushed with N₂, and stirred under N₂ at 4°C. Separate reaction mixtures were acidified with acetic acid (0.25ml) after 72h and 336h and freeze-dried. Another reaction mixture was adjusted to pH9.2 after 4h by the addition of Tris-HCl buffer, pH8.0 (2.0M in Tris), and was kept at 4°C for a further period of 48h. This mixture was then acidified with acetic acid and freeze-dried.

Each sample was applied separately to a column (95cm×2.7cm diam.) of Sephadex G-25 equilibrated with 0.2M-pyridine-acetic acid buffer, pH5.2. The products were eluted with the same buffer at a flow

rate of 48 ml/h and 12 ml fractions were collected and assayed for hexose, sialic acid, α -amino groups and radioactivity.

(b) *Treatment with 0.02M-NaB³H₄ in 0.5M-KOH.* Borohydride stock solution (0.5 ml; specific radioactivity 6.65×10^5 d.p.m./ μ mol), 4.5 ml of 0.5M-KOH and 50 mg of preparation KS-2 were stirred under N₂ at 4°C. Separate reaction mixtures were acidified and fractionated (see above) after 72 h and 240 h.

(c) *Treatment with 0.5M-KOH.* Samples of preparation KS-2 (50 mg) were stirred under N₂ in 5.0 ml of 0.5M-KOH at 4°C for 4 h and 72 h. Each reaction mixture was acidified and fractionated as described above except that each fraction was assayed for chromogen reactivity in the Morgan-Elson reaction (see below) instead of for radioactivity.

(d) *Treatment with 1.0M-NaB³H₄ in 0.05M-KOH.* A solution of preparation KS-2 (25 mg in 22.5 ml of water) was stirred with 1.0 g of NaBH₄ and 2.5 ml of borohydride stock solution (specific radioactivity 17.6×10^5 d.p.m./ μ mol) at 47°C for 18 h. The reaction mixture was acidified with acetic acid, freeze-dried and fractionated as described above.

Alkali treatment of 3-O- β -gluc-4-enuronosyl N-acetylgalactosamine 6-O-sulphate (Δ -GluUA-GalNAc-6-SO₄).

The sodium salt of this disaccharide was isolated after digestion of chondroitin sulphate from bovine intervertebral disc with chondroitin sulphate lyase from *P. vulgaris*. This disaccharide (120 mg) was treated with 21 ml of 0.5M-KOH at 4°C under N₂. At certain times, samples (0.5 ml) were removed and assayed for chromogen content with the Morgan-Elson reagent (see below). After 30 h, 2.5 ml of stock borohydride solution (6.65×10^5 d.p.m./ μ mol) was added to yield a final borohydride concentration of 0.02M and the reaction was continued at 4°C. Samples (0.5 ml) were again removed and assayed for chromogen content. After 7 days the reaction mixture was acidified with acetic acid, freeze-dried, dissolved in 5 ml of 0.05M-formic acid and applied to a column (38 cm $\times$ 1.5 cm diam.) of Dowex 1 (formate form). The column was eluted with a linear concentration gradient of formic acid generated from 500 ml of 0.05M-formic acid and 500 ml of 2.5M-formic acid, followed by a linear gradient of ammonium formate generated from 500 ml of 2.5M-formic acid and 500 ml of 2.0M-ammonium formate in 2.5M-formic acid. The column was eluted at a rate of 40 ml/h and 12 ml fractions were collected and assayed for hexuronate content and radioactivity. Two separate peaks of radioactive material were eluted with the ammonium formate gradient. The minor and most retarded peak was identified as reduced chondroitin sulphate linkage oligosaccharide by g.l.c. (Robinson & Hopwood, 1973). The other radioactive peak fractions

were combined, concentrated to about 20 ml by partial freeze-drying and desalted on a column (100 cm $\times$ 2.7 cm diam.) of Sephadex G-25 by elution with 0.2M-pyridine-acetic acid buffer, pH 5.2, at a rate of 56 ml/h. The final material (compound A) was freeze-dried (yield 30 mg).

Half of this product (15 mg) was reduced further with 4 ml of stock borohydride solution (6.65×10^5 d.p.m./ μ mol) for 10 days at 4°C. The final reaction mixture was acidified with acetic acid, freeze-dried and applied to a column (38 cm $\times$ 1.5 cm diam.) of Dowex 1 (formate form) and eluted as described above. Radioactive material was eluted as a single peak, which was desalted on a column of Sephadex G-25 as outlined above, and the radioactive fractions were combined and freeze-dried (yield, 9 mg: compound B).

Each preparation (A and B) was desulphated by acid hydrolysis in 25% (v/v) formic acid for 24 h at 100°C *in vacuo*. Samples were freeze-dried and stored at -20°C.

Reduced Δ -GluUA-GalNAc-6-SO₄ was prepared by mixing 20 mg of the disaccharide, dissolved in 25 ml of 1.0M-(NH₄)₂SO₄ at pH 8.2, with 2.5 ml of borohydride stock solution (6.65×10^5 d.p.m./ μ mol) at 4°C under N₂. The final pH was 9.2. After 4 days the reaction mixture was acidified with acetic acid, freeze-dried and fractionated on Dowex 1 formate form as described above for compound A (yield 15 mg of reduced disaccharide).

Analytical methods

Hexuronic acid was determined by the method of Dische (1947), hexosamine by that of Cessi & Piliego (1960) and total hexose by the method of Trevelyan & Harrison (1952) with galactose as standard. Chromogen was determined by the method of Reissig *et al.* (1955) modified by Bray *et al.* (1967). This modification eliminated the heating step after the addition of potassium tetraborate. *N*-Acetylgalactosamine and *N*-acetylglucosamine, which served as standards, were carried through the entire method as described by Reissig *et al.* (1955). Protein was determined by the method of Lowry *et al.* (1951), with crystalline dry bovine serum albumin (Commonwealth Serum Laboratories, Melbourne, Australia) as standard. Sialic acid was assayed by the resorcinol procedure of Svennerholm (1957) as described by Graham *et al.* (1970), with *N*-acetylneuraminic acid as standard. α -Amino groups were determined by the procedure described by Nitecki *et al.* (1967) with 0.1M-sodium borate buffer at pH 9.0. Sulphate was determined by the benzidine method of Antonopoulos (1962) after hydrolysis of the samples in 25% formic acid for 25 h at 100°C *in vacuo*, (NH₄)₂SO₄ being used as standard.

Amino acid analysis was performed on samples hydrolysed in 6M-HCl for 24 h at 100°C in tubes

sealed under vacuum. A Beckman Spinco automatic amino acid analyser (model 120-C) was used to determine amino acids, total hexosamine, galactosamine, glucosamine and galactosaminitol. Samples (about 1.0mg) of all keratan sulphate fractions were hydrolysed for 16h in 5.0ml of 4M-HCl at 100°C *in vacuo* for assay of hexosamine and hexosaminitol. Hydrolysates were evaporated to dryness at room temperature over P₂O₅ and KOH under vacuum. G.l.c. was performed as described by Robinson & Hopwood (1973). A second method of sample preparation was used to detect the presence of free monosaccharides. In this method 25 µg of inositol was added to each carbohydrate sample (10–100 µg) as an internal standard and the free monosaccharides were converted into trimethylsilyl derivatives without methanolysis. The derivatives were then separated as previously described (Robinson & Hopwood, 1973).

Radioactivity was assayed by using a Phillips PW4510 liquid-scintillation analyser. Aqueous samples (0.5ml) were mixed with 10ml of scintillation mixture prepared as described by Bruno & Christian (1961). Under these conditions ³H radioactivity was counted at 27.0% efficiency.

Paper chromatography was performed on Whatman 3MM paper with descending development in the following solvents: *A*, ethyl acetate–pyridine–water (8:2:1, by vol.); *B*, butan-2-one–acetic acid–water saturated with boric acid (9:1:1, by vol.). Sugars and sugar alcohols were detected by means of alkaline AgNO₃ solution. Papers run in solvent *B* were washed with a solution of HF in acetone (1ml of conc. HF solution in 40ml of acetone) before treatment with alkaline AgNO₃.

Molecular weights were determined from sedimentation–equilibrium experiments by the method previously described (Robinson & Hopwood, 1973). A value of 0.52 was assumed for the partial specific volume of keratan sulphate in this technique. End-group estimation of the molecular weight of keratan sulphate was performed by the method described by Robinson & Hopwood (1973) for chondroitin sulphate.

Results

Isolation and analysis of keratan sulphate from intervertebral disc

Keratan sulphate was isolated from ox intervertebral disc tissue by sequential digestion with papain and Pronase and by ion-exchange chromatography on Dowex 1 as shown in Scheme 1 and Fig. 1. Approx. 15% of the tissue keratan sulphate was obtained as a fraction (KS-1) almost completely free of hexuronate (Table 1). During the isolation of fraction KS-1, however, the bulk of the tissue keratan sulphate (85%) was eluted from Dowex 1 as a single peak of

hexose-positive material coinciding with a peak of hexuronate. The material in this peak sediments as a single component in the ultracentrifuge and has a weight-average molecular weight (M_w) of 33500 determined by equilibrium sedimentation as described under 'Methods'. Separate intact chains of chondroitin sulphate isolated from the same tissue by alkaline cleavage (Robinson & Hopwood, 1973) have a number-average molecular weight (M_n) of only 13500, whereas separate chains of keratan sulphate have a M_n of about 20000 (Hopwood & Robinson, 1973a), so this material probably consists of chondroitin sulphate and keratan sulphate 'doublets' bridged by peptide fragments from the protein moiety of the proteoglycan. This peptide fraction must be stable to exhaustive digestion with papain and Pronase, since all attempts to resolve the two polysaccharides after extended proteolytic digestion were unsuccessful.

A second preparation of keratan sulphate (KS-2) was obtained from the milled tissue by digestion with Pronase and papain followed by an exhaustive digestion with chondroitin sulphate lyase before ion-exchange chromatography on ECTEOLA-cellulose as outlined under 'Methods'. Approximately 90% of the tissue keratan sulphate was isolated by this method, but the results in Table 1 show that preparation KS-2 contains fragments of chondroitin sulphate. Preparation KS-1, however, contains little or no chondroitin sulphate, since only trace amounts of hexuronate or xylose could be detected. The weight per cent composition of preparation KS-2 was as follows: hexosamine 28.0, hexose 33.2,

Fig. 1. Elution profile from Dowex 1 of products obtained after digestion of milled bovine intervertebral disc with papain and Pronase

Fractions (12ml) were eluted with a calculated linear gradient of NaCl (0–2.0M) until fraction 83, when the elution was continued with 5.0M-NaCl. Hexuronate (○) and hexose (●) were assayed as outlined under 'Methods'. KS-1 indicates those fractions combined to form preparation KS-1

Scheme 1. Isolation of keratan sulphate from intervertebral disc and the treatment with alkaline NaB^3H_4

The procedures used for each step are detailed under 'Methods'. Dry-weight values (in parentheses) are for the freeze-dried material in each fraction after the removal of salt by gel filtration on Sephadex G-25 in pyridinium-acetate buffer.

sulphate 18.0, protein (by the method of Lowry *et al.*, 1951) 4.6, hexuronate 3.2 and sialic acid 4.7.

The number-average molecular weight of keratan sulphate in preparation KS-2 was determined by alkaline cleavage with 0.02M- NaB^3H_4 in 0.5M-KOH as outlined by Robinson & Hopwood (1973) for chondroitin sulphate. The value calculated by this

method was 17900, which agreed well with a value (M_w) of 18200 obtained for intact preparation KS-2 by equilibrium sedimentation. This agreement suggests that the NaB^3H_4 reduction method is equally valid for keratan sulphate. A molecular weight of 18000, together with the analytical data shown in Table 1, is consistent with the presence of 3 residues

Table 1. *Keratan sulphate composition determined by g.l.c. analysis*

All samples were treated with methanolic HCl. All values are expressed as mol/mol of fucose. Preparations KS-1 and KS-2 were isolated as described under 'Methods'. Preparations KS-3 and KS-4 were isolated from reaction mixtures of preparation KS-2 and 0.2M-NaB³H₄ in 0.5M-KOH after 4h and 72h at 4°C respectively. Each sample of keratan sulphate was isolated after gel filtration on Sephadex G-25 and Sephadex G-50 (fraction A; Scheme 1) as outlined under 'Methods'. Preparation KS-5 was isolated in the same way after treatment of preparation KS-2 with 1.0M-NaB³H₄ in 0.05M-KOH at 47°C for 18h. The content of total amino acids (mol/mol) was calculated from amino acid analyses of each preparation and is based on the glucosamine content in that assay after correction for loss during acid hydrolysis. N.d., not determined; trace, <0.05 mol/mol of fucose.

Sample	Composition				
	KS-1	KS-2	KS-3	KS-4	KS-5
N-Acetylglucosamine	25.6	24.1	25.0	25.0	26.5
N-Acetylgalactosamine	0.9	5.8	0.8	Trace	0.2
Galactose	28.8	28.2	26.9	26.4	28.3
Mannose	1.1	1.0	1.1	1.1	1.1
Fucose	1.0	1.0	1.0	1.0	1.0
N-Acetylneuraminic acid	3.1	3.0	2.8	2.1	3.2
Glucuronic acid	Trace	4.4	0.4	N.d.	Trace
Xylose	Trace	1.0	0.1	N.d.	Trace
Amino acid	9.5	13.5	7.5	6.1	N.d.

of sialic acid, 1 residue of mannose, 1 residue of fucose and 1 residue of N-acetylgalactosamine per keratan sulphate chain.

The results obtained by g.l.c. analysis of preparation KS-2, shown in Table 1, also indicate the presence of 1 mol of xylose/mol of keratan sulphate. The result is consistent with the presence of about 1 mol of chondroitin sulphate oligosaccharide/mol of keratan sulphate. Each chondroitin sulphate oligosaccharide contains an average of 4–5 residues of galactosamine and 4–5 residues of glucuronic acid, which suggests incomplete attack by chondroitin sulphate lyase (Robinson & Hopwood, 1973).

Effect of reaction time and reaction conditions on the alkali cleavage of preparation KS-2

The specific radioactivity of chondroitin sulphate prepared by alkali cleavage of cartilage proteoglycan with NaB³H₄ in 0.5M-KOH is not markedly dependent on the reaction conditions (Robinson & Hopwood, 1973). Studies with keratan sulphate, however, indicate that the specific radioactivity of this polysaccharide is dependent on the time of the reaction and the concentration of borohydride used. Table 2 shows analytical results for products of alkali cleavage of preparation KS-2 after different periods of time and with different conditions of reaction. Each reaction mixture was separated into three fractions by gel chromatography on Sephadex G-25 and Sephadex G-50.

Table 2. *Reaction of keratan sulphate preparation KS-2 with NaB³H₄ in KOH*

Each reaction mixture was acidified with acetic acid and fractionated by gel filtration on Sephadex G-25 and Sephadex G-50 into fractions A, B and C as shown in Scheme 1. All results are expressed per mol of keratan sulphate ($\bar{M}_w = 18000$) in the original KS-2 preparation. Sialic acid is expressed as equivalents of N-acetylneuraminic acid. N.d., not determined; — indicates that no radioactivity or sialic acid could be detected.

Reaction conditions	Time of reaction (h)	Analysis of products				
		Fraction A		Fraction B		Fraction C
		10 ⁻¹¹ × Specific radioactivity (d.p.m./mol)	Sialic acid content (mol/mol)	10 ⁻¹¹ × Specific radioactivity (d.p.m./mol)	10 ⁻¹¹ × Specific radioactivity (d.p.m./mol)	Sialic acid content (mol/mol)
0.02M-NaB ³ H ₄ in 0.5M-KOH at 4°C	0	—	3.01	—	—	—
	72	5.00	2.41	6.60	5.98	0.66
	240	6.64	2.20	6.70	6.38	0.75
0.2M-NaB ³ H ₄ in 0.5M-KOH at 4°C	4	3.85	2.90	5.98	7.00	0.15
	72	8.13	2.34	6.60	5.86	0.68
	336	9.52	2.12	6.50	6.67	0.80
1.0M-NaB ³ H ₄ in 0.05M-KOH at 47°C	18	N.d.	2.92	N.d.	—	—
0.5M-KOH	4	N.d.	2.92	N.d.	—	0.12
	72	N.d.	2.27	N.d.	—	0.70

Fig. 2. Elution profile from Sephadex G-25 of products obtained by treating preparation KS-2 with 0.2M- NaB^3H_4 in 0.5M-KOH for 72h at 4°C

Fractions were 12ml; radioactivity (○), sialic acid content (●) and α -amino acid content (△) were determined as outlined under 'Methods'. V_0 indicates the void volume of the column and C indicates those fractions combined to form fraction C.

Fig. 3. Elution from Sephadex G-50 of the components in the void-volume peak from Sephadex G-25 shown in Fig. 2

Fractions were 12ml; hexuronate (▼), radioactivity (○) and hexose (●) are shown. V_0 indicates the void volume of the column and A and B indicate the fractions combined to form fraction A and fraction B respectively.

Fig. 2 shows the elution patterns from Sephadex G-25 of preparation KS-2 after cleavage in 0.2M- NaB^3H_4 in 0.5M-KOH for 72h at 4°C. The radioactive material eluted at the void volume of the column (fractions 16–20) was fractionated further on Sephadex G-50, whereas the retarded material (fractions 25–29) was analysed for radioactivity and sialic acid content. This latter material was called fraction C.

Fig. 3 shows the elution pattern from Sephadex G-50 of the void-volume fraction eluted from Sephadex G-25 (described in the preceding paragraph). Two main peaks of radioactivity are seen: a first peak of keratan sulphate (fraction A) and a second peak (fraction B) due to the fragments of chondroitin sulphate present in the original KS-2 preparation. These two peaks were readily resolved on Sephadex G-50, and similar fractions (A, B and C) were isolated from each reaction mixture studied.

The results in Table 2 show clearly that the specific radioactivity of the keratan sulphate in fraction A is not as constant as that of the chondroitin sulphate oligosaccharides in fraction B. In addition, the sialic acid content of fraction A decreases during the cleavage reaction, whereas that of fraction C increases. These findings indicate that the cleavage reaction is more complex with keratan sulphate than it is with chondroitin sulphate.

Chemical nature of the linkage between keratan sulphate and protein

After acid hydrolysis of fraction A (isolated from preparation KS-2, labelled for 4h in 0.2M-NaB³H₄ in 0.5M-KOH and then at pH9.2 as described under 'Methods'), 60% of the label moved with a galactosaminitol standard on paper chromatography in solvent B. A similar result was obtained for fraction A isolated from the reaction performed in 1.0M-NaB³H₄ in 0.05M-KOH at 47°C for 18h. Analysis of the latter sample on the automatic amino acid analyser confirmed the presence of [³H]galactosaminitol and indicated a glucosamine/galactosaminitol molar ratio of about 22.

Acid hydrolysates prepared from other fraction A preparations in Table 2 also contained the same labelled compound, but the proportion of the radioactivity associated with this compound decreased as the time of exposure to 0.5M-KOH increased. Alkali cleavage of preparation KS-2 with 0.5M-KOH at 4°C in the absence of NaB³H₄ produced an unsaturated chromogen in the Morgan-Elson reaction (Kuhn & Kruger, 1956), which remained covalently bonded to the keratan sulphate chain and could be detected in fraction A. After 72h, the amount of this chromogen in fraction A was equivalent to 0.86mol (as molar equivalents of *N*-acetylglucosamine)/mol of keratan sulphate. Concomitant with the loss of *N*-acetylgalactosamine (Table 1) during alkali degradation, there is an increase in the amount of chromogen and the number of labelled components observed after acid hydrolysis. The chemical nature of these labelled components is not known. However, comparison of the elution profile of ³H label obtained by ion-exchange chromatography of these compounds on Dowex 1 (Zill *et al.*, 1953) with that of the components produced when galactosamine is treated with alkaline NaBH₄ suggests that most of the labelled components are derived from galactosamine.

Similar compounds are formed when the disaccharide Δ -GlcUA-GalNAc-6-SO₄ is treated with alkaline borohydride (see under 'Methods'). Fig. 4 illustrates that chromogen formation from galactosamine substituted at position 3 is rapid at 4°C relative to its subsequent reduction with borohydride, which reduction eliminates the chromogen reactivity. The reaction is, however, slow in relation to the destruction of serine and threonine residues from preparation KS-2 in 0.5M-KOH at 4°C (Fig. 5). The latter reaction is 50% complete after 2h, whereas the formation of chromogen from preparation KS-2 under the same conditions is 50% complete only after 15–20h. Fig. 5 also shows that threonine residues are destroyed at a lower rate than serine residues during treatment of preparation KS-2 with dilute alkali.

Fig. 4. Time-course of chromogen production after treatment of preparation KS-2 (○) and Δ -GlcUA-GalNAc-6-SO₄ (●) with 0.5M-KOH at 4°C

Arrows indicate time of addition of NaBH₄ (0.2M) as outlined under 'Methods'.

Fig. 5. Time-course of chromogen production (●) and serine (Δ) and threonine (○) loss by treating keratan sulphate preparation KS-2 with 0.5M-KOH at 4°C

Serine and threonine values shown are the percentage lost of the total lost after 43h of exposure to alkali.

Chromogen formation from substituted *N*-acetyl-galactosamine in 0.5M-KOH at 4°C occurs by a β -elimination to yield an unsaturated derivative of the type shown in Scheme 2, structure III (Kuhn & Kruger, 1956). The preparation of reduced Δ -GlcUA-GalNAc-6-SO₄ and of two reduced chromogen derivatives, compound A and compound B, is described under 'Methods'. The specific radioactivity of each preparation was measured after purification by ion-exchange chromatography. Compound A, which was prepared with 0.02M-NaB³H₄, had a specific radioactivity of 5.96×10^5 d.p.m./ μ mol of ester sulphate and compound B (reduced with 0.2M-NaB³H₄) a specific radioactivity of 8.3×10^5 d.p.m./ μ mol. The reduced disaccharide had a specific radioactivity of 5.26×10^5 d.p.m./ μ mol. It is proposed that this variation in specific radioactivity results from: (a) the reduction of the aldehyde group of the

Scheme 2. Effect of alkaline borohydride on substituted *N*-acetylgalactosamine

Treatment of 3-*O*- β -gluc-4-enuronosyl-*N*-acetylgalactosamine 6-*O*-sulphate (I; $\text{R} = \text{gluc-4-enuronic acid}$, $\text{R}' = \text{SO}_3^-$) with alkaline borohydride can result in reduction to the corresponding galactosaminitol derivative (II) or β -elimination to the sulphated chromogen derivative III ($\text{R}' = \text{SO}_3^-$). In 0.02M- NaB^3H_4 , the latter compound can be reduced to IV ($\text{R}' = \text{SO}_3^-$) but in 0.2M- NaB^3H_4 further reduction to form V ($\text{R}' = \text{SO}_3^-$) appears to occur to a limited extent. A similar structure and reaction sequence is proposed for the linkage region *N*-acetylgalactosamine of keratan sulphate (I; $\text{R} = \text{N-acetyl-neuraminylgalactose}$; $\text{R}' = \text{the main polysaccharide chain of keratan sulphate}$) after an initial β -elimination of the polysaccharide from a threonine residue in the protein 'core' as shown in Figs. 7(a) and 7(b).

Fig. 6. Time-course of reaction of keratan sulphate preparation KS-2 with 0.5M-KOH

At the times shown samples of the reaction mixture were fractionated by gel filtration and analysed for chromogen content and sialic acid content as described under 'Methods'. Sialic acid content in fraction A (○) and in fraction C (●) and the chromogen-reactivity of fraction A (△) are shown.

unsaturated *N*-acetylhexosamine derivative to yield an unsaturated *N*-acetylhexosaminitol (structure IV, Scheme 2) and (b) reduction of the double bond in the unsaturated *N*-acetylhexosaminitol to yield an *N*-acetyldeoxyhexosaminitol derivative (structure V, Scheme 2).

Ketones and aldehydes are generally considered to be the only functional groups reduced with NaB^3H_4 . However, Kadin (1966) has reported that NaB^3H_4 will also reduce $\text{C}=\text{C}$ unsaturation if this group is conjugated with another, such as an ester or an amide group. Dehydroalanyl residues and the proposed chromogen structures (Scheme 2) both contain $\text{C}=\text{C}$ unsaturation conjugated with amide, and are therefore likely to be susceptible to reduction with NaB^3H_4 . Robinson & Hopwood (1973) have reported that aldehydes are reduced with borohydride at a considerably faster rate than are the dehydroalanyl residues.

The specific-radioactivity values recorded above for the two reduced galactosamine chromogen preparations (compounds A and B) suggest that approximately 1.0 and 1.5 sites per chromogen molecule are labelled with 0.02M- and 0.2M- NaB^3H_4 respectively. This result is consistent with a rapid

reduction of the aldehyde group in 0.02M- NaB^3H_4 and a slower and incomplete reduction of the $\text{C}=\text{C}$ double bond in 0.2M- NaB^3H_4 .

The results represented in Table 2 show that, after 336h in 0.2M- NaB^3H_4 and 0.5M-KOH, the specific radioactivity of the material in fraction A isolated from preparation KS-2 is equivalent to the incorporation of 1.45mol of ^3H /mol of keratan sulphate. It is therefore important to note that determination of molecular weight by measurement of specific radioactivity of keratan sulphate is subject to error, but that reasonable values are obtained if the reduction is completed in 0.02M- NaB^3H_4 .

Substituent at position 3 of N-acetylgalactosamine in keratan sulphate

The sialic acid content of the keratan sulphate component (Table 2, fraction A) decreases with time of exposure to alkali at 4°C from about 3.0 to about 2.0 molecules of *N*-acetylneuraminic acid per keratan sulphate chain (Fig. 6). Over a similar period of time the sialic acid content of fraction C increases to an equivalent of 0.8 molecule of *N*-acetylneuraminic acid. The specific radioactivity of this fraction C

Fig. 7. Proposed structures: (a) the alkali-labile linkage between keratan sulphate and protein in cartilage proteoglycan and (b) the likely products from the degradation of structure (a) in alkaline NaB^3H_4 .

Three distinct groups of reaction products are shown (b-I, -II and -III). The b-I group of structures represent the major products from the degradation of (a) by 0.5M-KOH in 0.02M- NaB^3H_4 , whereas the reaction products formed when (a) is degraded by 0.5M-KOH in 0.2M- NaB^3H_4 are represented by the structures shown in both b-I and b-II. After 14 days at 4°C, under these conditions, it is proposed that the reaction mixture contains approximately an equal number of each structure represented in b-I and -II. Finally, it is proposed that the product structures shown in b-III result when (a) is degraded by 0.05M-KOH in 1.0M- NaB^3H_4 .

(b-I)

(b-II)

(b-III)

material, based on the molar content of sialic acid, was constant (Table 2). A molecular weight of 500 for fraction C material was obtained by end-group measurement assuming one reduction with NaB^3H_4 per molecule of *N*-acetylneuraminic acid. Column chromatography of fraction C material on Dowex 1 (formate form) failed to separate the sharp peak of sialic acid from the ^3H label. However, after acid hydrolysis in 0.1M-HCl for 30min at 100°C the sialic acid was retarded on the Dowex 1 (formate form) column and the ^3H -labelled component washed through with water. Paper chromatography of fraction-C material in solvents *A* and *B* showed only one labelled component. After hydrolysis in dilute acid a radioactive compound was observed with mobility similar to that of galactitol and mannitol, which moved identically.

G.l.c. analysis of the sugar components of fraction C, after hydrolysis in dilute acid, show the presence of *N*-acetylneuraminic acid, a trace amount of galactose and a peak corresponding to mannitol or galactitol. Standard mixtures of these two sugar alcohols are not separated under the conditions used for g.l.c. analysis. However, a clear-cut separation of galactose and mannose was possible. Fraction C material derived from alkali degradation of preparation KS-2 in the absence of NaB^3H_4 contains *N*-acetylneuraminic acid and galactose in equimolar proportions as sole components. The absence of mannose in fraction C is confirmed by the fact that the content of this sugar in fraction A is unaltered by treatment of preparation KS-2 with alkaline borohydride (Table 1).

Disaccharides, for example those derived from chondroitin sulphate lyase digestion of hyaluronic acid, are eluted from Sephadex G-25 at the same position as fraction C. This evidence all suggests that the material of fraction C is a disaccharide with the structure *N*-acetylneuraminylgalactose. The glycosidic linkage between these two sugars is likely to be either (2→4) or (2→6) since the disaccharide is stable in dilute alkali at 4°C.

Treatment of this disaccharide with neuraminidase failed to liberate free *N*-acetylneuraminic acid, galactose or galactitol. However, digestion of preparation KS-2 with neuraminidase releases all of its associated sialic acid (Hopwood & Robinson, 1974). The failure of neuraminidase to cleave the disaccharide may be due to isomerization of the sialyl ketosidic bond during the cleavage and isolation of the disaccharide (Yu & Ledeen, 1969).

Discussion

Alkali-catalysed cleavage of the linkage between keratan sulphate and protein occurs by a β -elimination reaction, which releases polysaccharide chains with terminal reducing ends. These chains are

susceptible to alkali-catalysed epimerizations and to degradation by peeling reactions, to produce a series of saccharides of various chain lengths. This peeling reaction has been systematically studied in blood-group substances (Schiffman *et al.*, 1964; Lloyd *et al.*, 1966, 1968). The nature and lengths of the saccharides formed is governed by competition, at each step of the peeling reaction, between reduction to give stable chains and elimination to produce chains one sugar residue shorter (Lloyd & Kabat, 1969).

The presence of galactosamine in the keratan sulphate preparation KS-2, from positions other than the proposed alkali-labile linkage sequence, complicates the interpretation of alkali-degradation experiments where loss of galactosamine is used to measure the extent of β -elimination. Most of the galactosamine in preparation KS-2 originates from the contaminating chondroitin sulphate linkage region, and therefore is cleaved from protein but is not degraded in dilute alkali (Robinson & Hopwood, 1973). G.l.c. analysis of the chondroitin sulphate linkage oligosaccharide released from preparation KS-2 by treatment with 0.2M- NaB^3H_4 in 0.5M-KOH (fraction B) indicated the presence of 4.5mol of galactosamine/mol of xylitol and/or lyxitol. This leaves 1.3 molecules of galactosamine in preparation KS-2 not accounted for as chondroitin sulphate fragments. It seems likely that 1 molecule of galactosamine is involved in the linkage of keratan sulphate to protein, and this is lost by degradation during the alkali treatment.

The present study confirms the work of Bray *et al.* (1967) on the alkali-lability of the keratan sulphate to protein linkage and the generation of chromogen by exposure to diluted alkali. Further, the nature of the moiety giving rise to chromogen and its relation to the linkage region of keratan sulphate has been clarified.

The effect of alkali on *N*-acetylhexosamine substituted at position 3 is well known (Neuberger & Marshall, 1966; Jeanloz & Trémège, 1956). Mayo & Carlson (1970) have reported that *N*-acetylchondrosine undergoes complete β -elimination in 0.05M-NaOH at 37°C. The same disaccharide is rapidly reduced and undergoes little β -elimination when treated with 1.0M- NaBH_4 in 0.05M-NaOH at 50°C. Mayo & Carlson (1970) have proposed that such conditions will reduce the eliminated oligosaccharide chains before significant peeling can occur. After treatment of preparation KS-2 for 18h with 1.0M- NaB^3H_4 in 0.05M-KOH at 37°C, no evidence of peeling was apparent. All of the chondroitin sulphate fragments were absent from the product (KS-5; Table 1) and no loss of sialic acid was evident.

The substituent on the keratan sulphate linkage *N*-acetylgalactosamine is a disaccharide *N*-acetylneuraminylgalactose (Fig. 7a). This disaccharide is

part of the keratan sulphate structure and is not an oligosaccharide with a separate linkage to protein, since: (a) it is released from preparation KS-2 with a concomitant formation of keratan sulphate-bonded chromogen, and (b) under conditions where β -elimination of keratan sulphate from protein is complete but polysaccharide degradation by peeling is minimal, no *N*-acetylneuraminic acid is lost from the keratan sulphate (Fig. 7b). Kathan & Adamany (1967) have presented evidence to show that the presence of sialic acid close to the alkali-labile bond, between oligosaccharide and protein, decreases the rate of β -elimination. Seno *et al.* (1965) found with the alkali cleavage of adult human rib cartilage that the serine residues were destroyed at a faster rate than the threonine residues. Rate studies performed on preparation KS-2 also suggest that threonine is lost at a lower rate than serine in the alkali digestion of keratan sulphate from adult bovine intervertebral disc (Fig. 5). All of these observations are consistent with a proposition that one sialic acid residue is attached to galactose, which in turn is bonded to position 3 of an *N*-acetylgalactosamine residue involved in *O*-glycosidic linkage of the keratan sulphate to threonine (Fig. 7a).

This work was supported by the Australian Research Grants Committee (Project no. D65/15893). J. J. H. thanks Monash University for a Monash Postgraduate Research Scholarship.

References

- Antonopoulos, C. A. (1962) *Acta Chem. Scand.* **16**, 1521–1522
- Bray, B. A., Lieberman, R. & Meyer, K. (1967) *J. Biol. Chem.* **242**, 3373–3380
- Bruno, G. A. & Christian, J. E. (1961) *Anal. Chem.* **33**, 1216–1218
- Cessi, C. & Piliego, F. (1960) *Biochem. J.* **77**, 508–510
- Dische, Z. (1947) *J. Biol. Chem.* **167**, 189–198
- Graham, E. R. B., McKenzie, H. A. & Murphy, W. H. (1970) in *Milk Proteins* (McKenzie, H. A., ed.) p. 277, Academic Press, New York
- Heinegård, D. (1972) *Biochim. Biophys. Acta* **285**, 193–207
- Helting, T. & Rodén, L. (1969a) *J. Biol. Chem.* **244**, 2790–2798
- Helting, T. & Rodén, L. (1969b) *J. Biol. Chem.* **244**, 2799–2805
- Hopwood, J. J. & Robinson, H. C. (1973a) *Biochem. J.* **135**, 631–637
- Hopwood, J. J. & Robinson, H. C. (1973b) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **32**, 2609
- Hopwood, J. J. & Robinson, H. C. (1974) *Biochem. J.* in the press
- Jeanloz, R. W. & Trémège, M. (1956) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **15**, 282
- Kadin, S. B. (1966) *J. Org. Chem.* **31**, 620–622
- Kathan, R. H. & Adamany, A. (1967) *J. Biol. Chem.* **242**, 1716–1722
- Kuhn, R. & Kruger, G. (1956) *Chem. Ber.* **89**, 1473–1486
- Lloyd, K. O. & Kabat, E. A. (1969) *Carbohydr. Res.* **9**, 41–48
- Lloyd, K. O., Kabat, E. A., Layug, E. J. & Gruezo, F. (1966) *Biochemistry* **5**, 1489–1501
- Lloyd, K. O., Kabat, E. A. & Licerio, E. (1968) *Biochemistry* **7**, 2976–2990
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mathews, M. B. & Cifonelli, J. A. (1965) *J. Biol. Chem.* **240**, 4140–4145
- Mayo, J. W. & Carlson, D. M. (1970) *Carbohydr. Res.* **15**, 300–303
- Meyer, K. (1969) *Amer. J. Med.* **47**, 664–672
- Meyer, K. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 1, pp. 5–24, Academic Press, London
- Neuberger, A. & Marshall, R. D. (1966) in *Glycoproteins* (Gottschalk, A., ed.), pp. 235–272, Elsevier Publishing Co., Amsterdam
- Nitecki, D. W., Stoltzenberg, I. M. & Goodman, J. W. (1967) *Anal. Biochem.* **19**, 344–350
- Pedrin, V. (1969) *J. Biol. Chem.* **244**, 1540–1546
- Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955) *J. Biol. Chem.* **217**, 959–966
- Robinson, H. C. & Dorfman, A. (1969) *J. Biol. Chem.* **244**, 348–352
- Robinson, H. C. & Hopwood, J. J. (1973) *Biochem. J.* **133**, 457–470
- Rodén, L. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 2, pp. 797–821, Academic Press, London
- Schiffman, G., Kabat, E. A. & Thompson, W. (1964) *Biochemistry* **3**, 113–120
- Scott, J. E. (1960) *Methods Biochem. Anal.* **8**, 145–198
- Seno, N., Meyer, K., Anderson, B. & Hoffman, P. (1965) *J. Biol. Chem.* **240**, 1005–1010
- Svennerholm, L. (1957) *Biochim. Biophys. Acta* **24**, 604–611
- Telser, A., Robinson, H. C. & Dorfman, A. (1965) *Proc. Nat. Acad. Sci. U.S.A.* **54**, 912–919
- Trevelyan, W. E. & Harrison, J. R. (1952) *Biochem. J.* **50**, 298–303
- Yu, R. K. & Ledeen, R. (1969) *J. Biol. Chem.* **244**, 1306–1313
- Zill, L. P., Khym, J. X. & Cheniae, G. M. (1953) *J. Amer. Chem. Soc.* **75**, 1339–1342