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Note

N-Deacetylation of keratan sulphate by alkali

SHIGEHIRO HIRANO, YOSHINOBU AKIMOTO, AND MAKOTO ISHIGAMI
Department of Agricultural and Biological Chemistry, Tottori University, Tottori 680 (Japan)
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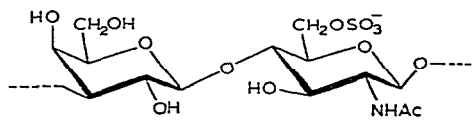
The main repeating-unit of keratan sulphate (KS) is *O*-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**1**), polymerized *via* β-(1→3)-linkages Some of each type of monosaccharide residue is sulphated[1–3] at position 6 Much is known about the action of alkali on the carbohydrate–protein linkages in KS and proteoglycans[4–9] Corneal KS is stable[10] in 2M sodium hydroxide at room temperature for at least 120 h, and the 3,6-anhydro derivative of the hexosamine moiety is formed[11] from KS by the action of 0 5M sodium hydroxide in the presence of sodium borohydride at 80° for 7 h Alkaline fusion[12–14] or treatment[15–16] with 40% sodium hydroxide at 115° for 6 h produces chitosan from chitin



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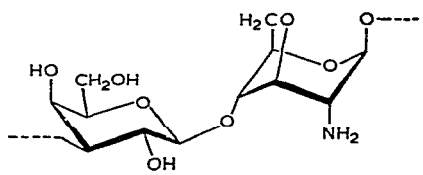

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Arising from a study of the action of alkali on glycosaminoglycans[17], we now report on the *N*-deacetylation of KS by alkali and the preparation of some new derivatives of the *N*-deacetylated KS

As shown by n m r spectroscopy, treatment of KS with 0 5M sodium hydroxide at 80–83° for 5 h gave an incompletely *N*-deacetylated product, and treatment with 2M sodium hydroxide gave a completely *N*-deacetylated product in a relatively low yield (42%) However, treatment with 1 5M sodium hydroxide gave 79% of a completely *N*-deacetylated product (anhydro-KS, **2**) involving 2-amino-3,6-anhydro-2-deoxy-D-glucose (3,6-anhydro-GlcN) in place of 2-acetamido-2-deoxy-D-glucose residues Sampson and Meyer[11] reported the formation of 3,6-anhydro derivatives from KS and heparin by the action of alkali

The molar ratios (Table I) of Gal GlcN 3,6-anhydro-GlcN SO_4 were 1 00.0 85 0 00 0 95 for KS and 1 00 0 17 1.00 0 25 for **2** Amino acid analysis (Table I) and the carbazole reaction showed that serine, threonine, and uronic acid, present in small

proportions in KS, almost disappeared on the formation of **2**. This indicates[17] that contaminated glycosaminoglycan moieties are linked glycosidically to hydroxyl groups of serine and threonine. On the other hand, asparagine and glutamine present in KS were almost completely unaffected by the alkali-treatment. Corneal KS is stable to alkali, and presumably contains 2-acetamido-2-deoxy-D-glucose residues linked to the amide groups of asparagine and glutamine moieties, whereas cartilaginous KS is labile to alkali and contains[4,5] 2-acetamido-2-deoxy-D-galactose residues attached to the hydroxyl groups of serine and threonine. The present data reveal that the glycosylamine linkage at the reducing end of the polysaccharide chain in corneal KS is stable to 1.5M sodium hydroxide in the presence of sodium borohydride at 80–83° for 5 h.

TABLE I

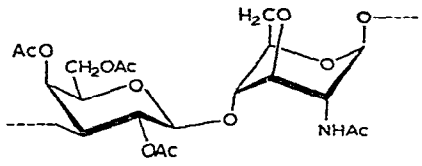
COMPONENT ANALYSIS OF KERATAN SULPHATE AND *N*-DEACETYLATED KERATAN SULPHATE[a]

Component	*Keratan sulphate*	*N-Deacetylated keratan sulphate*[c]
Gal	1890 (34.0%)	2350 (42.3%)
GlcN	1610 (28.9%)	406 (7.3%)
3,6-Anhydro-GlcN	0[b]	2360 (38.0%)
GalN	trace	0
SO_4	1790 (17.2%)	594 (5.7%)
GlcU	92 (1.8%)	0
Peptide	(0.7%)	(0.65%)
Asp	27.4	28.2
Thr	3.9	0.9
Ser	3.1	0.4
Glu	6.5	10.7
Pro	0	0
Gly	2.2	1.7
Ala	2.6	1.3
Val	6.5	6.4
Met	0.0	0.0
Ileu	0.9	0.4
Leu	trace	0.4
Tyr	trace	trace
Phe	trace	trace
Lys	trace	0
His	trace	0
Arg	trace	0

[a] In μmoles/g. [b] A preparation contained a trace of 3,6-anhydro-GlcN, and analysis of various preparations is progressing in our laboratory. [c] N-content (4.0%, calculated from GlcN, 3,6-anhydro-GlcN, and peptide) and S-content (1.9%, calculated from sulphate) are slightly lower than the observed values of N, 4.58 and S, 2.38%, but the difference is within the limit of experimental error.

Treatment of **2** with acetic anhydride and pyridine in formamide gave peracetylated anhydro-KS (**3**), $[\alpha]_D^{21}$ −97° (water). The n.m.r. spectrum of **3** indicated four acetyl groups per disaccharide unit, in contrast to five acetyl groups [δ 2.17 (2Ac),

2 10 (2Ac), 1 95 (1Ac)] for peracetylated KS, and six [δ 2 15 and 2 05] for peracetylated keratan *O*-Deacetylation of **3** with 0 1M sodium hydroxide at room temperature overnight gave *N*-acetyl-anhydro-KS (**4**), $[\alpha]_D^{15}$ −23° (water)



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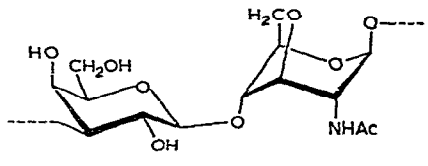

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On formation of the 3,6-anhydro rings, there is inversion of the hexosaminide moiety from the $^4C_1$ to the $^1C_4$ conformation and this is reflected by the n m r data and the $[\alpha]_D$ values The signal for H-1 of the 3,6-anhydro-GlcN moiety in **2**, **3**, and **4** appeared at δ 5 03–5 43 as a doublet ($J_{1,2}$ 0 0–1 0 Hz) Compounds **2**, **3**, and **4** showed $[\alpha]_D^{21}$ values of −62°, −97°, and −23°, respectively, whereas KS, keratan, peracetylated KS, and peracetylated keratan showed values of +1.7°, +1°, +7°, and +3°, respectively

Anhydro-KS **2** is an excellent material for preparation of *N*-substituted derivatives Much more severe treatment (7–10M NaOH) was employed for the *N*-deacetylation of chondroitin sulphates and hyaluronate, which was accompanied by considerable degradation[17] 3,6-Anhydrohexosamine moieties were not found in chondroitin sulphates treated with alkali[11].

On methanolysis, the 3,6-anhydroglycoside moiety of seaweed polysaccharides is converted into the dimethyl acetal derivative and there is less destruction than in acid hydrolysis[18] Methanolysis was therefore used in the amino acid analysis (*cf* ref 11) The retention times are shown in Table II

TABLE II

RETENTION TIMES OF 2-AMINO-3,6-ANHYDRO-2-DEOXY-D-GLUCOSE AND ITS DIMETHYL ACETAL BY AN AMINO ACID ANALYZER[a]

Compound	R_{GLCN}
2-Amino-3,6-anhydro-2-deoxy-D-glucose	1 71[b]
2-Amino-3,6-anhydro-2-deoxy-D-glucose dimethyl acetal	1 85
Methyl 2-amino-2-deoxy-α-D-glucopyranoside[c]	1 20
Methyl 2-amino-2-deoxy-β-D-glucopyranoside[c]	1 02
Ammonia	2 38

[a]Using a 50-cm column eluted with 0 35M citrate buffer (pH 5 09) [b]Reported value[11] = 1 65, with the use of 0 35M citrate buffer, pH 5 28 [c]These methyl glycosides were prepared from the corresponding *N*-acetyl derivative by the action of alkali The detailed experiment will be reported elsewhere

EXPERIMENTAL

General methods — Specific rotations were recorded on aqueous solutions using a cell of path length 1 cm with a Yanagimoto automatic polarimeter (OR-50) N m r. spectra were recorded at 60 MHz with a Hitachi spectrometer (R-24) on solutions in D_2O with sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate as internal standard. I r spectra were recorded with a Hitachi grating spectrometer (Type 215) D-Galactose was analysed by the anthrone method[19], amino acids and hexosamine by a Hitachi KLA-5 amino acid analyser, D-glucuronic acid by the carbazole method[20], and sulphate by the barium chloroanilate method[21]

2-Amino-3,6-anhydro-2-deoxy-D-glucose was determined as its dimethyl acetal derivative in the amino acid analyser 2-Amino-3,6-anhydro-2-deoxy-D-glucose dimethyl acetal was prepared by refluxing **2** in 10% methanolic hydrogen chloride for 30 h

The reaction mixture was concentrated to dryness (*in vacuo*) and the residue was dissolved in distilled water. A part of this solution was applied to the analyser, which involved a column (50 cm) of Amberlite CG-120 (Type III) resin and elution with 0 35M citrate buffer (pH 5 09)

Materials — KS, conventionally isolated as the sodium salt from bovine corneas[22], had $[\alpha]_D^{21}$ +1 7° (*c* 1), and the analytical data are shown in Table I Keratan, prepared[1] from corneal KS had $[\alpha]_D^{15}$ +1° (*c* 0 7) (Found SO_4, 2 7%) Peracetylation of KS and keratan was performed with acetic acid and pyridine, by prior swelling in formamide[23] Peracetylated KS(Ba) (66%) had $[\alpha]_D^{21}$ +7° (*c* 1)· ν_{max}^{Nujol} 3350w (NH), 1760–1700s (C=O in OAc), 1240s (C–O in OAc and S=O), 820w cm^{-1} (equatorial C–O–S) The ratio of signals for acetate methyl/methine and methylene groups in the n m r spectrum was 1 06 (Calc 1 07) (Found N, 2 4%) Peracetylated keratan (45%) had $[\alpha]_D^{21}$ +3° (*c* 0 79), ν_{max}^{Nujol} 3400–3300w (NH), 1760–1730s (C=O in OAc), 1240s cm^{-1} (C–O in OAc) The ratio of signals for acetate methyl/methine and methylene groups in the n m r spectrum was 1 19 (Calc 1 28) (Found N, 2 6%)

N-*Deacetylation of KS* — A solution of KS (200 mg) in 10 ml of 1 5M sodium hydroxide containing 200 mg of sodium borohydride was heated at 80–83° for 5 h and then allowed to attain room temperature Excess borohydride was decomposed with 6M hydrochloric acid The solution was concentrated to dryness *in vacuo* at <40°. Boric acid was removed from the residue by repeated distillation of methanol therefrom The residue was then eluted from a column (1.6×86 5 cm) of Sephadex G-25 with 10% ethanol Fractions (4 g) were assayed by the anthrone method[19] A minor peak (fractions 30–37) was discarded, and the fractions (19–29) containing the major peak were concentrated *in vacuo* at <40° to a small volume and then lyophilized to give anhydro-KS (**2**, 158 mg, 79%), $[\alpha]_D^{21}$ −62° (*c* 1), ν_{max}^{KBr} 3400–3300s (OH, NH), 1240s cm^{-1} (S=O)

Anal Calc for $\{C_{12}H_{18}NO_8(H)_{0\,8}(SO_3Na)_{0\,2}\}_n$ N, 4 17, S, 1 91 Found N, 4 58, S, 2 38

N m r data δ 5 10 (d, 1 proton, $J_{1,2}$ ~1 Hz, H-1 of 3,6-anhydro-GlcN); there was no acetamido methyl signal (δ 1–3) A ratio of 3,6-anhydro-GlcN/GlcN of 5 8 was obtained by the amino acid analyser. Other analytical data are shown in Table I

Peracetylation of anhydro-KS (**2**) — After treatment of **2** (70 mg) with acetic anhydride and pyridine in formamide at room temperature[23], the reaction mixture was poured into ice–water (~30 ml) After ~1 h at room temperature, the solution was concentrated *in vacuo* to ~10 ml, then applied to a column (1 6 × 86 5 cm) of Sephadex G-25, and eluted with 10% ethanol Fractions which gave a positive anthrone reaction were combined and eluted from a column (1 7 × 10 cm) of Dowex-50(H^+) resin with distilled water The eluate produced was concentrated *in vacuo* at <40° and lyophilized to give peracetylated anhydro-KS (**3**, 58 mg, 81%), $[\alpha]_D^{21}$ −97° (*c* 1 6), ν_{max}^{KBr} 1740s (C=O in OAc), 1230s cm^{-1} (C–O in OAc and S=O)

Anal Calc for $\{C_{18}H_{24}NO_{11}(COCH_3)_{0\,8}(SO_3H)_{0\,2}\}_n$: N, 2 90; S, 1 32 Found N, 2 80, S, 1 45.

N m r data: δ 2 09 and 2 20 (s, 12 protons, 4Ac), 5 43 (d, 1 proton, $J_{1\,2}$ ~0 Hz, H-1 of 3,6-anhydro-GlcN)

O-Deacetylation of **3** — A solution of **3** (40 mg) in 5 ml of 0 1M sodium hydroxide was kept at room temperature overnight, then neutralized with 0 1M hydrochloric acid, and eluted from a column (1 6 × 86 5 cm) of Sephadex G-25 with 10% ethanol. Fractions which gave a positive anthrone reaction were combined and eluted from a column (1 7 × 10 cm) of Dowex-50(H^+) resin as described above The eluate was concentrated *in vacuo* at <40° and lyophilized to give *N*-acetyl-anhydro-KS (**4**, 23 mg, 58%), $[\alpha]_D^{15}$ −23° (*c* 0 69), ν_{max}^{KBr} 3350–3450s (OH, NH), 1240s (S=O), 1040–1090 cm^{-1} (C–O–C)

Anal Calc for $\{C_{14}H_{20}NO_9(H)_{0\,8}(SO_3H)_{0\,2}\}_n$ N, 3 86, S, 1 76 Found N, 4 14; S, 1.94

N m r data δ 2 06 (s, 3 protons, Ac), 5 03 (d, 1 proton, $J_{1,2}$ ~0 Hz, H-1 of 3,6-anhydro-GlcN)

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