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Note

Dermatan sulfate of porcine mucosal tissue. N.m.r. observations on its separation from heparin with the aid of heparinase, and its degradation by chondroitinase

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Dermatan sulfate is found[1] in many mammalian tissues where it occurs in common with other glycosaminoglycans, as a proteoglycan macromolecule. It is constituted[2–4] mainly of residues of α-L-idopyranosyluronic acid (**1**) (which is 4-*O*-linked) and 2-acetamido-2-deoxy-β-D-galactopyranosyl 4-sulfate (**2**) (which is 3-*O*-linked). Minor residues are β-D-glucopyranosyluronic acid (**3**) and 2-acetamido-2-deoxy-β-D-galactopyranosyl 6-sulfate (**4**), present in proportions that may be distinctive[4,5] for dermatan sulfates from different species or tissues, or both.

Pharmaceutical heparin prepared from porcine mucosal tissue often contains small quantities of dermatan sulfate[6,7]. Depending, presumably, on the purification procedure employed in the manufacture of the heparin, dermatan sulfate contents of up to 15% are found[8]. Its presence in the heparin preparations was detected by n.m.r. spectroscopy, as well as by tests of its susceptibility to degradation by chondroitinase. Nevertheless, we considered it worthwhile to isolate the material and examine it in greater detail, because its seeming affinity for association with the heparin raised the possibility that it is an atypical, minor, form of dermatan sulfate.

Procedures employed[1,5] in the isolation of dermatan sulfate from mammalian tissue entail sequential extraction and purification by selective precipitation or gel electrophoresis. We describe the use of heparinase[9,10] from *Flavobacterium heparinum* to degrade the preponderant heparin component into fragments, from which the dermatan sulfate was readily separable by gel-filtration chromatography. The polymer recovered, which is presumed to have suffered little or no change during its isolation, was characterized with respect to known preparations of dermatan sulfate, by comparing n.m.r. spectra of the intact glycosaminoglycans, as well as those of their products[11,12] formed by enzymolysis with chondroitinase ABC.

The action of *Flavobacterium heparinum* heparinase on a pharmaceutical heparin containing about 15% of dermatan sulfate was examined by n.m.r. spectroscopy. As shown in Fig. 1A, the 300-MHz ^{1}H-n.m.r. spectrum of this preparation contains several signals attributable[7,13] to the dermatan sulfate constituent. Most prominent is that, at δ 2.1, due to the methyl group of residues of 2-acetamido-2-deoxy-β-D-galactopyranosyl

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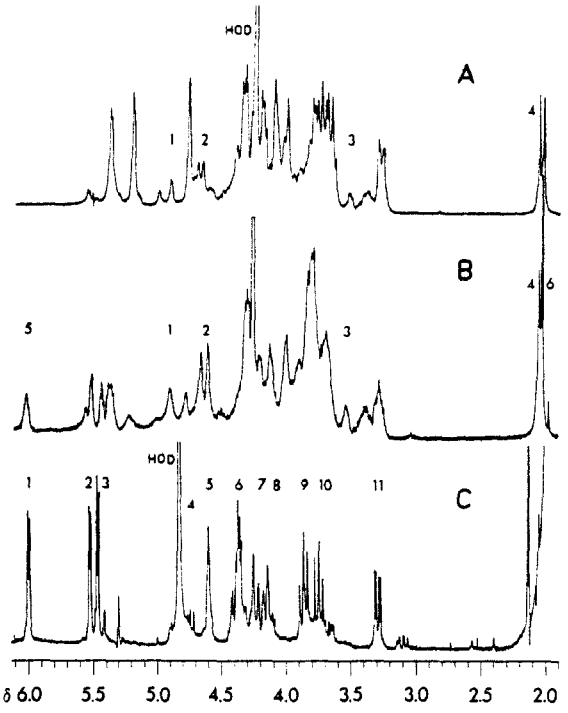
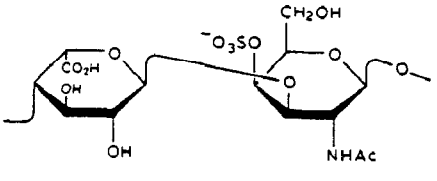


Fig. 1. ^{1}H-N.m.r. spectra (300 MHz; solvent, D_2O) of: (A) A pharmaceutical porcine mucosal heparin containing 15% of dermatan sulfate (temp. 75°); signal designations for the dermatan sulfate component, (1) U-1, (2) overlapping group for U-5, A-1, A-4, (3) U-2, and (4) acetamidomethyl. (B) Nondialyzable material recovered following the enzymolysis of the heparin–dermatan sulfate mixture by *F. heparinum* heparinase (temp. 75°); signal designations for the dermatan sulfate component, as under (1–4) in (A), and (5) vinylic H-4 of unsaturated uronosyl end groups. (C) Disaccharide **5** obtained as the dialyzate from the mixture of products formed on enzymolysis (temp. 30°); signal designations, (1) U-4, (2) U-1, (3) A-1(α), (4) A-1(β), (5) U-2, (6 and 7) U-3 and A-6,6′, (8) A-5, (9) A-4, (10) A-3, and (11) A-2 (assignments according to ref. 14, confirmed here with a COSY spectrum). The broad band at $\delta \sim 2.0$ is due to acetate salts in the dialyzate. (U) L-Iduronic or 4-enohexuronic acid and (A) acetamidodeoxyhexose unit.

4-sulfate (**2**), although other identifiable resonances are those due to H-1 (U-1) and H-2 (U-2) of the α-L-iduronosyl residues (**1**), at δ 5.0, and 3.6, respectively, and an overlapping cluster in the δ 4.7–4.8 region attributable to H-1 (A-1) and H-4 (A-4) of **2**, and H-5 (U-5) of **1**. Following exhaustive enzymolysis with the heparinase, dialysis was found to separate out the major disaccharide[14] **5**, produced during the elimination process, from the higher-molecular-weight material. The ^{1}H-n.m.r. spectrum of the dialyzate (Fig. 1C) indicated, in fact, that **5** was virtually the only product detectable, its identity being confirmed by reference to an earlier spectral characterization[14] of the disaccharide.

It is apparent from the spectrum of the nondialyzable material (Fig. 1B), which shows an enhancement in the relative intensities of the signals due to dermatan sulfate, that the content of the latter had been enriched. Also, the dermatan sulfate was accompanied by fragments that are terminated by an unsaturated glycosyluronic acid



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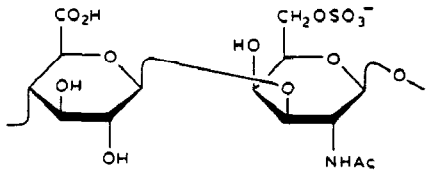

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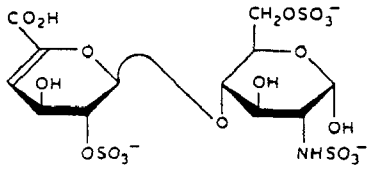
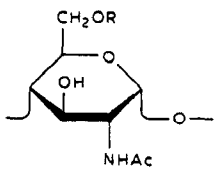
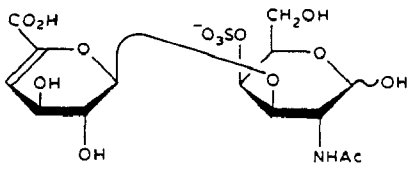


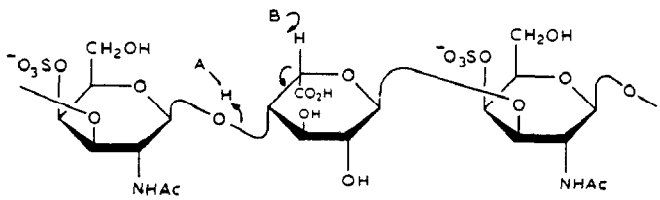
5 (α-anomer)



6 R = H or SO_3^-



7a = α anomer
7b = β anomer



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group analogous to that in **5**, as shown by a downfield resonance at δ 6.0 (Figs. 1B and 1C) due to an olefinic H-4. These fragments account for regions of the heparin that are not susceptible to the heparinase, and contain the residues of 2-acetamido-2-deoxy-α-D-glucopyranosyl (**6**) and β-D-glucopyranosyluronic acid (**3**) in the original heparin, which are represented[13] in the ^{1}H-n.m.r. spectrum by a prominent singlet for the methyl protons of **6** (δ 2.0), and the H-2 multiplet for **7** (δ 3.4).

Chromatography of the nondialyzable material on a column of Sephadex G-75 afforded a fraction that had an elution profile closely similar to that of a specimen of dermatan sulfate from porcine skin. Also the eluted polymer gave a ^{1}H-n.m.r. spectrum (Fig. 2A) that was closely similar to that of the skin specimen, as well as to the spectrum of a purified dermatan sulfate from porcine mucosal tissue, shown in Fig. 2B. The only apparent differences are a few minor resonances shown in Fig. 2A (at δ 5.0 and 3.6). An additional basis for demonstrating the close structural affinity between these two polymers is a comparison of the products formed[11,12] by enzymic degradation of them

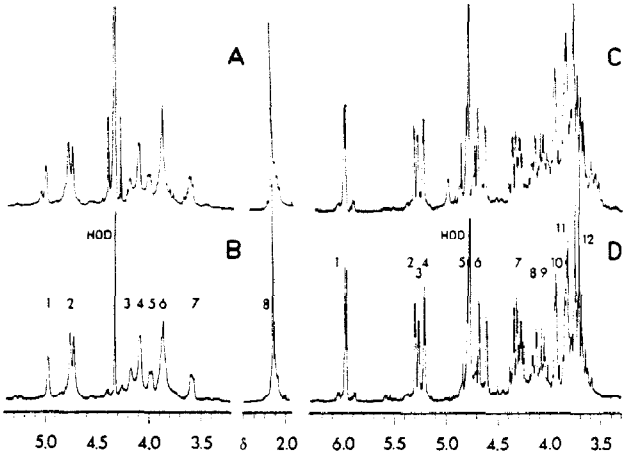

Fig. 2. ^{1}H-N.m.r. spectra (300 MHz; solvent, D_2O) of: (A) Dermatan sulfate isolated from the pharmaceutical porcine mucosal heparin represented by Fig. 1A, and purified by gel chromatography (temp. 75°). (B) A "normal" specimen of dermatan sulfate (HEPAR) from porcine mucosal tissue (temp. 75°) (virtually indistinguishable from the spectrum of porcine skin dermatan sulfate (Sigma); signal designations: (1) U-1, (2) overlapping group for U-5, A-1, and A-4, (3) U-4, (4) A-2, A-3, (5) U-3, (6) A-5, A-6,6', (7) U-2, and (8) acetamidomethyl. (C) Product of the enzymolysis of dermatan sulfate [represented by (A)] with chondroitinase ABC (temp. 35°). (D) Product of the enzymolysis of dermatan sulfate [represented by (B)] with chondroitinase ABC; signal designations for disaccharide 7 (a = α; b = β): (1) U-4, (2) U-1α, (3) U-1β, (4) A-1α, (5) A-1β, (6) A-4α,β, (7) A-2α, -3α, -5α, (8) A-3β, (9) A-2β, (10) U-3, (11) U-2, and (12) A-6,6'α,β, A-5β (the acetamidomethyl signals at $\delta \sim 2.1$ are not shown). (U) 4-Enohexuronic acid and (A) acetamidodeoxyhexose unit.

with chondroitinase ABC. As seen in Figs. 2C and 2D, the ^{1}H-n.m.r. spectra of the two enzymolysis products showed that there is an almost exact superimposition of the well-resolved resonances far larger in number than in the two spectra of the intact polymers. In each instance, the pattern of signals corresponded to the presence of a large preponderance of the expected unsaturated disaccharide **7**; individual resonances in the ^{1}H-n.m.r. spectrum illustrated in Fig. 2D were accounted for, based on an analysis of the COSY spectrum of **7**. (Additional comments on the spectroscopic characteristics of **7** are presented later.) The extra, minor peaks, at δ 5.0 and 3.6, already shown in Fig. 2A appear to be reproduced intact in the spectrum shown in Fig. 2C, which suggests that they are due to some nondialyzable component that is chemically distinct from the material that is susceptible to degradation by chondroitinase.

This evidence indicated that the dermatan sulfate present in some pharmaceutical heparin preparations is not substantially different from the "normal" dermatan sulfate of porcine mucosal tissue, as well as other dermatan sulfates. Presumably, any role that it may play, when the heparin is administered clinically, would be comparable to functions now recognized specifically for dermatan sulfate, such as the activation of heparin cofactor II[15], or antithrombotic activity[16]. The possibility[17] that its presence can moderate the effect of a combination of heparin and cortisone on angiogenesis activity[18] remains to be determined.

Observations on the enzymolysis of dermatan sulfate by chondroitinase. — The

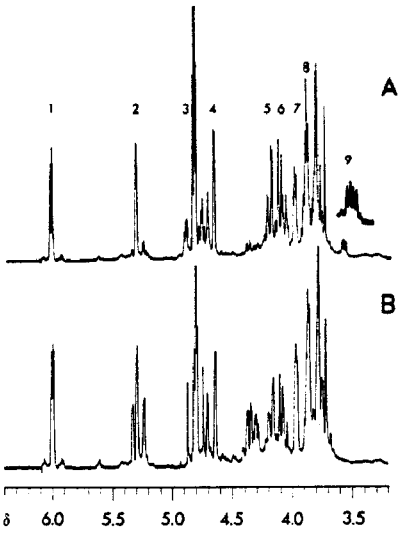

Fig. 3. ^{1}H-N.m.r. spectra (300 MHz; solvent, D_2O; 35°) of: (A) The products of the enzymolysis of dermatan sulfate by chondroitinase ABC at 10-min reaction time, showing the selective formation of the β anomer of disaccharide 7; signal designations: (1) U-4, (2) U-1, (3) A-1β, (4) A-5β, (5) A-3β, (6) A-2β, (7) U-3, (8) U-2, and (9) (and expansion) U-2 of residual L-iduronic acid units. (B) The enzymolysis reaction mixture 50 min later, showing the emergence of the α anomer of disaccharide 7, attributable to anomeric tautomerization, *en route* to the equilibrium mixture represented by Fig. 2D.

spectrum of disaccharide 7, illustrated in Figs. 2C and 2D, showed the presence of signals due to both anomers (**7a**, **7b**). Three signals clustered in the δ 5.2–5.3 region account for the two nonequivalent anomeric protons (H-1, H-1′) of the uronic acid unit of each disaccharide, as well as the anomeric proton (A-1) of the α-aldose residue in **7a**. By contrast, only one anomer is prominently represented at the outset of the enzymolysis reaction. As seen in Fig. 3A, the disaccharide formed during the first 10 min was accounted for (see ref. 19) almost entirely by the H-1 signal of the glycosyluronic acid group of the β anomer (**7b**); the corresponding doublet for the anomeric proton of the 2-acetamido-2-deoxy-β-D-galactopyranosyl residue was detectable at δ 4.8 ($J_{1,2} \sim$ 8.0 Hz) where, at the temperature of the enzymic reaction, it was partly obscured by the HOD peak (also see Figs. 2C or 2D). This finding is in accord with views (see Ref. 20) as to how chondroitinase catalyzes the elimination process, *e.g.*, as in **8**, because the separation of residue **2** from **1** liberates an aldose having the *same* configuration as **2**.

At 1 h into the enzymic reaction, the α-disaccharide **7a** became a prominent component (Fig. 3B) due to mutarotation occurring as the degradation proceeded, leading to the equilibrium state depicted at 100 h in Fig. 2D. It is worth noting that, according to this latter figure, the ratio of α to β anomers of disaccharide **7** is ~1.3:1, which contrasts with an equilibrium composition of 2:1 for the parent aldose, 2-acetamido-2-deoxy-D-galactose[21]. A more striking difference is found (see Fig. 1C) in the tautomeric equilibrium of disaccharide **5** produced from heparin, in which the α anomer is preponderant by a factor of about 8[14,22]. Such large differences appear to merit further consideration.

Also noted among the spectra of the enzymolysis reaction was a small signal at δ 3.6 (Fig. 3A) attributable to U-2 of a still-intact residue of L-idopyranosyluronic acid (**1**). As the latter is such a minor constituent of the enzymolysis product, neither dermatan sulfate nor intermediate oligosaccharide fragments that would have been formed from it were present in substantial amount at 10-min reaction time. The well-resolved splittings for the U-2 signal of **1**, which are not evident in spectra of dermatan sulfate (see Fig. 2A) gave $J_{1,2}$ 3.5 Hz and $J_{2,3}$ 6.5 Hz. These values are close to the corresponding couplings (3.0 and 6.0 Hz, respectively) estimated[23] from a convolution-difference spectrum of dermatan sulfate, and provide an additional example of coupling patterns for residues of **1** and model compounds[24,25], which are consistent only[25] with an equilibrating mixture of conformers of the pyranose ring.

Additional observations deal with the characteristics of the acetamidomethyl protons of **7**, when this disaccharide is produced by chondroitinase acting on dermatan sulfate *admixed with heparin*. For the intact polymer mixture, the methyl resonance due to residues of **2** is largely resolved[6,7] from that of the residues of the *gluco* epimer present in the heparin as a minor constituent. This is illustrated by the pair of signals in the δ 2.0–2.2 region of Fig. 1A, and also in Fig. 4A with a pharmaceutical heparin that is less contaminated ($\sim$ 5%) with dermatan sulfate. Ten minutes after the latter preparation had been treated with chondroitinase ABC, when as already noted most of the dermatan sulfate had been converted into disaccharide **7b**, the methyl signal (D) was found to be strikingly narrower (Fig. 4B). Further narrowing was observed at 1-h reaction time (Fig. 4C). Subsequently, however, the shape of the signal was changed to that shown in Fig. 4D, at 5 h, and then to the equilibrium state at 24 h represented by Fig. 4E. The polymer mixture depicted in Fig. 1A gave closely analogous results although, in that experiment, the excessively tall methyl (D) signal observed at the outset was less suitable for presentation as in Fig. 4.

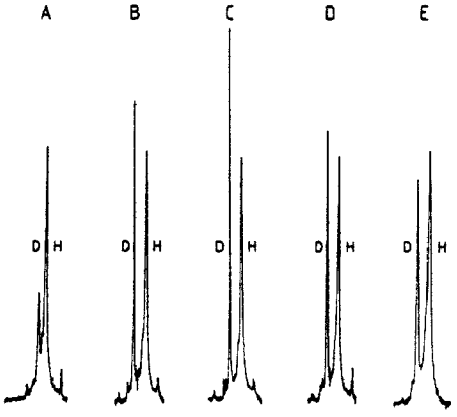

Fig. 4. Acetamidomethyl ^{1}H resonances in the region δ 2.0–2.1 of 300 MHz spectra (solvent, D_2O; 30°) depicting the action of chondroitinase ABC on dermatan sulfate (5%) contained in a pharmaceutical heparin preparation. Signals D and H are those, respectively, of 2-acetamido-2-deoxy-D-galactosyl 4-sulfate (in dermatan sulfate or disaccharide **7**, or both) and 2-acetamido-2-deoxy-D-glucosyl (**6**, a minor constituent of the heparin) residues. (A) Before the introduction of chondroitinase; (B) to (E), 10 min, 60 min, 5 h, and 24 h, respectively, after the enzyme was added.

A controlled enzymolysis of dermatan sulfate in the *absence* of heparin showed the similarly marked decrease in line width anticipated for the acetamidomethyl signal, during the first 10 min while the polymer was being converted into **7b**. In this instance, however, the appearance of the signal did not change subsequently during the next 24 h.

The initial narrowing of the acetamidomethyl signal during the initial stages of the enzymolysis, whether in the presence of heparin (Figs. 4A→B→C) or in its absence, may be ascribed to an increase in the T_1 of the methyl protons from its value in **2** to that in **7b**, a more mobile molecule. Then, while **7b** slowly anomerized into its preponderant α form (**7a**) during the period represented by Figs. 4C→D→E, a reduction in T_1 of the methyl protons occurred, leading to signal broadening. As these changes were observed only in the *presence* of heparin (and the chondroitinase), it appears possible that the molecular motion of **7a** became restricted owing to an interaction with the heparin. Self-association has been demonstrated[26] for the polymeric chains of dermatan sulfate, and aggregates of heparin have also been reported[27,28]. In admixture, however, the two glycosaminoglycans appear[28] not to interact appreciably. The present observations suggested that disaccharide **7** can associate in some manner with heparin, although they interact slowly.

The ^{13}C-n.m.r. spectrum of disaccharide **7**. — Having analyzed the ^1H-n.m.r. spectrum of **7** with the aid of a COSY experiment, the corresponding ^{13}C data (Table I) were then obtained by ^1H–^{13}C correlation (HETCOR). They are fully consistent with the ^{13}C data[29] for the isomeric disaccharide produced by the action of chondroitinase on chondroitin 6-sulfate, which differs from **7** in having a 6- instead of a 4-sulfate group. Consequently, the only substantial differences are in the chemical shifts of the C-4 and C-6 signals which, for **7**, are located further downfield and upfield, respectively, owing to the influence of the sulfate substituent. For the unsaturated uronic acid unit of both

TABLE I

^{13}C-Chemical shifts (δ) and $^1J_{C\text{-}H}$ (Hz)[a] for disaccharide **7**

2-Acetamido-2-deoxyhexosyl residue				*Unsaturated uronosyl group*	
A-1(β)	97.5 (162.3)	A-5(β)	77.2	U-1	102.8 (170.5)
A-1(α)	94.0 (171.6)	A-5(α)	73.1	U-2	71.2 (150.3)
A-2(β)	55.9 (146.1)	A-6(α,β)	63.8 63.7	U-3	67.2 (151.3)
A-2(α)	53.4 (143.6)			U-4	109.4 (169.0)
		CO	177.6		
A-3(β)	78.6		177.3	U-5	146.8
A-3(α)	76.0	CH_3	25.0 24.9	U-6	172.0
A-4(α,β)	80.1 79.0				

[a] In parentheses.

disaccharides, as well as disaccharide **5**, the value of $^1J_{C,H}$ is ~ 170 Hz, which is consistent[29,30] with a *quasi*-axial orientation of the anomeric C–O bond, and a $^1H_2$ conformation for the 4-enopyranose ring. Additional evidence favoring this conformation is the presence of a long-range coupling (~ 0.7 Hz) between vinylic H-4 and H-2 (*e.g.*, see Fig. 3A), a characteristic described earlier[14,29,30] for molecules in this series.

In conclusion, the objective of this study was to determine whether or not the dermatan sulfate contained in many pharmaceutical preparations of porcine mucosal heparin, at a level of 10–15% in some instances[6–8], is a distinct form of the polymer. Accordingly, the dermatan sulfate accompanying one of these heparins has now been isolated. This entailed the use of heparinase to selectively depolymerize the heparin, which facilitated a chromatographic purification of the dermatan sulfate component by gel filtration. High-field ^{1}H-n.m.r. spectra of this isolated material and of the products formed from it by enzymolysis with chondroitinase ABC were found to be so closely similar to the corresponding pair of spectra for the "normal" dermatan sulfate of porcine mucosal tissue that the polymers must have virtually the same structure. This also suggested that it should be feasible to remove most of the dermatan sulfate retained in a pharmaceutical heparin by the use of conventional purification procedures.

While utilizing chondroitinase ABC for characterization of the dermatan sulfate isolated and examining the enzymolysis products, we obtained ^{1}H-n.m.r. data showing an early stage at which the polymer had been converted almost quantitatively into the β anomer of the expected unsaturated disaccharide (**7b**). This is definitive evidence that the elimination reaction catalyzed by the enzyme is accompanied by *retention* of the configuration of residues of 2-acetamido-2-deoxy-β-D-galactosyl 4-sulfate (**2**) within the aldose end-units liberated. Hence, it supports current views about the mechanism of the elimination process.

Also noteworthy is another n.m.r. spectral observation made when the chondroitinase treatment was applied to the dermatan sulfate *prior* to its isolation from the pharmaceutical heparin. In that instance, the acetamidomethyl signal of the newly-formed disaccharide (**7b**) exhibited a striking decrease in line-width during the anomerization to the more stable α form (**7a**), an effect that could not be reproduced in the absence of heparin. As this implies that the mobility of the disaccharide is affected by heparin, it is suggestive of some form of association between these two molecules.

EXPERIMENTAL

General. — The n.m.r. spectra were recorded with a Varian XL-300 spectrometer operating at 300 MHz for ^{1}H, and are referenced with respect to the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (δ 0.0). Deuterium exchange of samples (as sodium salts) was effected by repeatedly dissolving the materials in D_2O and then evaporating the solutions prior to the n.m.r. analysis, which was conducted with D_2O solutions containing 3–4% (w/w) of polysaccharide. Solutions were evaporated under diminished pressure at 40°.

The heparins were representative of several pharmaceutical preparations, of

porcine mucosal origin, from various suppliers, and were kindly furnished by G. A. Neville. Heparinase from *Flavobacterium heparinum*, devoid of enzymic acitivity on dermatan sulfate, was obtained from IBEX Technologies, Montreal, Que., through the courtesy of K. R. Holme. Dermatan sulfate from porcine mucosa was supplied by Hepar Industries Inc. (Franklin, OH), and porcine skin dermatan sulfate as well as chondroitinase ABC (from *Proteus vulgaris*) by Sigma Chemical Co. (St. Louis, MO). The Sephadex G-75 was a product of Pharmacia LKB (Uppsala, Sweden), and the dialysis membranes (SPECTRAPOR) were obtained from Spectrum Medical Industries (Los Angeles, CA).

Enzymolysis of pharmaceutical heparin (containing 15% of dermatan sulfate) with heparinase. — To a stirred solution of heparin (0.5 g; 150 USP units/mg) in acetate buffer (50 mL, pH 7.0, containing 250 mmol of sodium acetate and 2.5 mmol of calcium acetate) at 30° was added *Flavobacterium heparinum* heparinase (150 units) in phosphate buffer (150 μL, pH 7.0). After 3 h, additional enzyme (200 units) was introduced and then, after 18 h, another 100 units. By monitoring the formation of elimination products as an increase in absorption A_{230}, the enzymolysis reaction was found to be 80% complete within 2 h and 90% complete within 6 h. At 24-h reaction time, the solution was heated at 100° for 5 min and subjected to dialysis repeatedly against distilled water using a membrane with a mol. wt. cut-off of 3500. The combined dialyzate was lyophilized and, following deuterium exchange, was examined by n.m.r. spectroscopy (see Fig. 1C). The solution inside the membrane was then transferred to a second sac having a mol. wt. cut-off of 6000–8000, and the dialysis against distilled water was resumed. This dialyzate was found to contain very little product. The material retained inside the membrane was then recovered by lyophilization, and also examined by n.m.r. spectroscopy (Fig. 1B); the yield of the nondialyzable product was 114 mg.

Isolation of dermatan sulfate by gel chromatography. — A column (60 cm × 2.5 cm) of Sephadex G-75 gel was prepared with 0.05M phosphate buffer (pH 7.0) as the mobile phase. Operating at a flow rate of 0.6 mL/min, the column was determined, by use of Dextran Blue (10 mg; mol. wt. ~ 10^6), to have a void volume of 40 mL. When a test was then performed with dermatan sulfate of porcine skin (33 mg), which was monitored colorimetrically at A_{620} with Azure A dye, all of the polymer was found to be eluted shortly after the void volume. Under the same conditions, chromatography of the nondialyzable product (50 mg) afforded, initially, a fraction (8 mg) that closely mirrored the elution pattern in the dermatan sulfate test, and did not absorb significantly at A_{230}. The ^{1}H-n.m.r. spectrum of this product is shown in Fig. 2A. Further elution of the column afforded fractions that absorbed strongly at A_{230}, and produced a strong olefinic ^{1}H signal (δ 6.0) corresponding to those in Figs. 1B and 1C.

Enzymolysis of a pharmaceutical heparin (containing 5% of dermatan sulfate) with chondroitinase. — The ^{1}H-n.m.r. spectrum of a solution of heparin (20 mg; deuterium-exchanged) in 0.05M deuterated phosphate buffer (0.5 mL; pD 8.4) was recorded at 30°. Powdered chondroitinase (~ 1 mg) was introduced, and the spectrum was rerecorded at intervals, as shown in Fig. 4.

Enzymolysis of dermatan sulfate with chondroitinase. — A sample of dermatan

sulfate (10 mg) was dissolved in 0.05M phosphate buffer (1.0 mL; pH 8.0), the solution was concentrated, the residue was subjected to deuterium exchange, and redissolved in D_2O (0.5 mL). The ^1H-n.m.r. spectrum was recorded at 30°, following which powdered chondroitinase (~ 10 mg) was added, and the spectrum was rerecorded at intervals, as shown in Figs. 2B and 2C, and Fig. 3.

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