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Selective O-desulphation of heparin in triethylamine

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Alkaline desulphation of heparin has not been so extensively studied as acid hydrolysis or solvolysis. Heparin was treated with 20% triethylamine (TEA) in aqueous solution under two different sets of experimental conditions. After elimination of the base, the products (H-a and H-b) were subjected to gel chromatography, analysed for total and N-sulphate content, and electrophoretic mobility and characterized by ^{13}C-NMR spectroscopy. Selective 2-O-desulphation of L-iduronic acid residues and partial 6-O-desulphation of 2-amino-2-deoxy-D-glucose was observed, while N-sulphoamino groups remained attached. There was also spectroscopic evidence of the formation of 2,3-anhydro-L-iduronic acid moieties.

INTRODUCTION

Heparin, the anticoagulant most frequently employed in clinical therapies, is an alternating copolymer of 2-amino-2-deoxy-D-glucose (D-glucosamine) and hexuronic acid, mainly L-iduronic acid, bearing sulphate substituents either as N-sulphate (at C-2 of the glucosamine residues) or as O-sulphate groups (at C-6 of the glucosamine or at C-2 of the iduronic acid units) (Casu, 1985). The 'sulphation pattern', i.e. the actual location of the sulphate groups along the heparin chain, appears to be at least as important as the absolute content of these groups. In previous studies in the authors' laboratory, a suitable method has been developed for the rapid estimation of sulphated positions in heparin and heparin sulphates (Kovensky *et al.*, 1990).

The functional role of sulphate groups has been mainly studied by desulphation. The rates of acid hydrolysis and/or dimethyl sulphoxide (DMSO) solvolysis are: sulphoamino $\gg$ 6-sulphate of glucosamine $>$ 2-sulphate of iduronic acid (Ayotte & Perlin, 1986). Since sulphoamino groups are essential for the anticoagulant activity of heparin (Inoue & Nagasawa, 1976), the authors looked for experimental conditions that allow the preferential remotion of O-sulphate groups. It has previously been observed that on treatment of heparin with aqueous triethylamine (TEA) at room temperature, the 4-O-glycosidic linkage of the uronic acid moieties did not undergo alkali-catalysed β-elimination, since formation of 4,5-unsaturated 4-deoxypyranuronate residues were not detected in the reaction mixture (Martini Bechech & Fernández Cirelli, 1986). Nevertheless, at higher temperatures, a slight dimunition of molecular weight was observed. Recently, Jaseja *et al.* (1989) reported on novel regio- and stereoselective modifications of heparin in sodium hydroxide solution, evidenced through a complete nuclear magnetic resonance spectroscopy study. Their results prompted the present authors to analyse the modified polysaccharide recovered after TEA treatment, in order to determine if desulphation was operative in this medium.

The present paper now reports on the utility of this method to achieve preferential 2-O-desulphation of L-iduronic acid residues without remotion of N-sulphoamino groups. Organic bases have the additional advantage of being easily eliminated from the reaction mixture by extraction with appropriate solvents.

EXPERIMENTAL

Materials

Heparin from bovine intestinal mucosa (mol. wt 12 000) and FMC (fast moving component) were kindly

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provided by Syntex Argentina S.A. Heparan sulphate (HS) was obtained from heparin by-products (Syntex Argentina S.A.) by alkaline copper-ethanol precipitation (Roden *et al.*, 1972). Sephadex gels and agarose were purchased from Pharmacia Fine Chemicals Inc.; 1,3-diaminopropane from Fluka; triethylamine from Aldrich Chemical Co.

Methods

Sulphate was estimated by the method of Terho and Hartiala (1971); N-sulphate according to Inoue and Nagasawa (1976).

Discontinuous gel electrophoresis was performed with 0·04 M barium acetate (pH 5·8) and 0·1 M 1,3-diaminopropane acetate (pH 9·0) on agarose gel slides. GAGs were detected with toluidine blue (Bianchini *et al.*, 1980).

Gel permeation chromatography was performed on a Sephadex G-50 (superfine) column (65 × 1·5 cm) equilibrated with 0·2 M NaCl in 10% ethanol. V_0 (37 ml) and V_t (95 ml) were estimated with Blue dextran and $CoCl_2$, respectively. Fractions (1 ml) were analysed by the carbazole-borate method (di Ferrante *et al.*, 1971). Experiments were run in triplicate.

^{13}C-NMR spectra were recorded at 25·2 MHz with a Varian XL-100-15 spectrometer (concentration 150 mg/ml) at room temperature, in D_2O–H_2O (1 : 1) solution.

Solvolysis of heparin with DMSO–H_2O (95 : 5) was performed according to Nagasawa *et al.* (1977).

Triethylamine treatment of heparin

Heparin (calcium salt, 250 mg) was purified by ethanol precipitation from its aqueous solution prior to TEA treatment. The purified heparin was dissolved in water (2 ml) and triethylamine (0·4 ml, 20% v/v, pH 12) was added. The reaction mixture was heated either for 5 h at 80 °C (condition a, H-a) or 2 h at 100 °C (condition b, H-b). After cooling, TEA was eliminated through ether extraction (3 × 2 ml) and the resulting product was recovered by ethanol precipitation. After chromatography on Sephadex G-50, fractions corresponding to the main peaks in H-a and H-b respectively, were desalted on Sephadex G-10, evaporated to dryness and subjected to electrophoretic and ^{13}C-NMR analysis. Total sulphate and N-sulphate content were determined.

RESULTS AND DISCUSSION

Triethylamine treatment of heparin was performed under heating, since previous experiments have shown that working at room temperature, the glycosaminoglycan chain was recovered unaltered from the reaction mixture in a 96% yield (Martini Bechech & Fernández Cirelli, 1986). Under these conditions (Fig. 1), gel chromatography of the product recovered after extraction of the base, suggested a slight diminution of molecular weight in comparison with the starting material. Signals of vinylic carbons (120–130 ppm) were not observed in the ^{13}C-NMR spectra (Fig. 2). This fact should suggest that alkali-catalysed β-elimination of 4-O-glycopyranuronosyl linkages with formation of 4,5-unsaturated 4-deoxy pyranuronate residues did not take place or else occurred only to a minor extent.

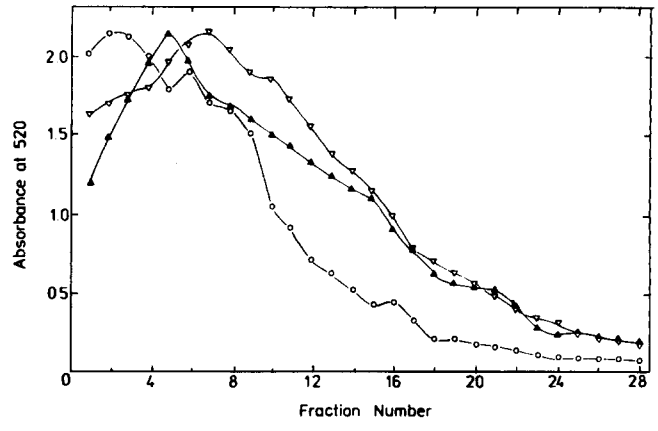


Fig. 1. Gel chromatography of: H (O—O); H-a (▲—▲) and H-b (▽—▽) on Sephadex G-50 (superfine). Fraction No. 0 corresponds to the void volume (37 ml). For more details, see Experimental Section.

Table 1. Sulphur content and anticoagulant activities of H, H-a and H-b

	%S (total)	%S (N-sulphate)	APTT (units/mg)	Anti-Xa (units/mg)
H	15·0	5·3	142	146
H-a	7·1	5·1	75	113
H-b	5·3	4·9	64	ND[a]

[a]ND not determined.

Total sulphate content of treated heparins H-a and H-b (Table 1) was ~50–60% lower than that of the original sample, while the N-sulphate content remained at approximately the same level. This fact should account for the electrophoretic mobility observed for H-a and H-b in diaminopropane-barium acetate buffer. Both samples showed only one spot with mobility comparable to the related glycosaminoglycan heparan sulphate (HS, %S 8) or to the fast moving component of heparin (FMC, %S 10·2). The slow moving component (SMC) of starting material was no longer detected after TEA treatment.

^{13}C-NMR spectra of the products of TEA treatment (H-a and H-b) differed from those of the original sample (H) and the FMC (Fig. 2). The most striking feature in H-a and H-b spectra is the appearance of signals at 96·9 (f); 54·0 (p) and 53·3–53·1 (q) ppm (Table 2), which suggest the formation of epoxide I in the

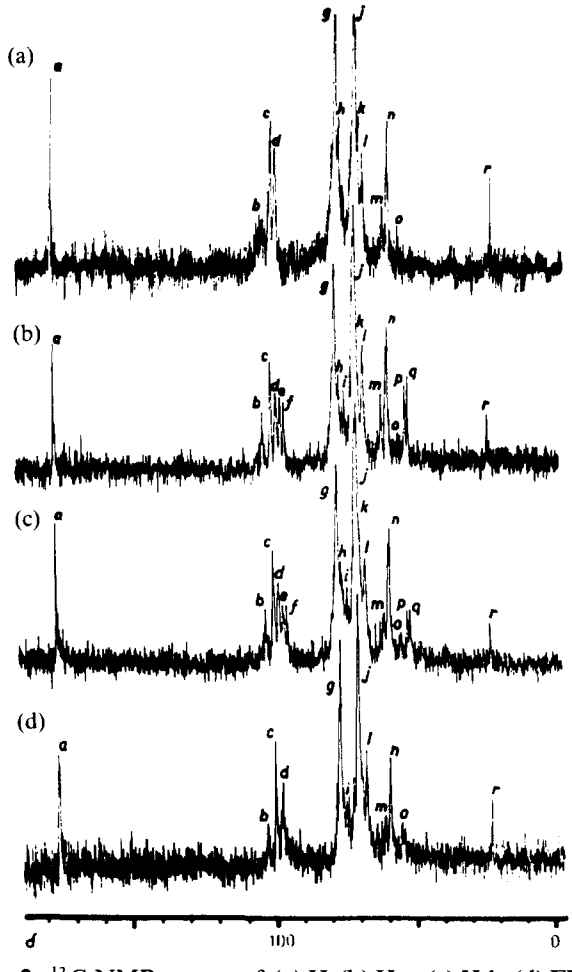
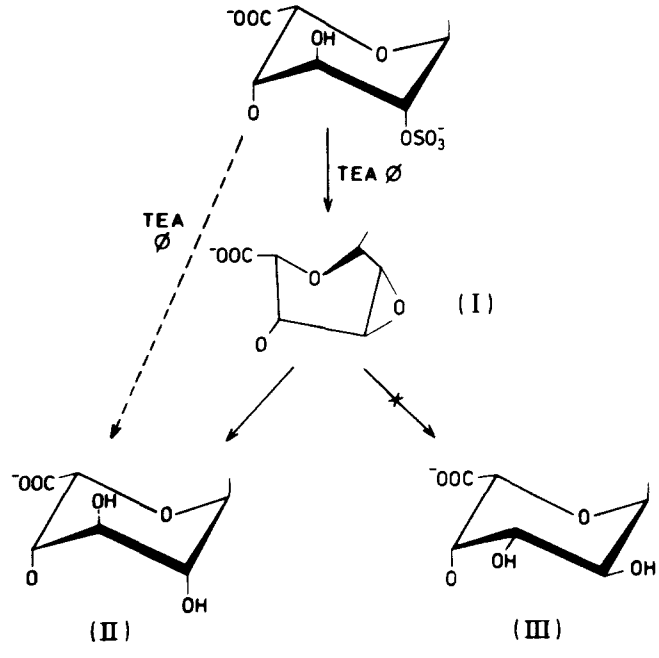


Fig. 2. ^{13}C-NMR spectra of: (a) H; (b) H-a; (c) H-b; (d) FMC.



Scheme 1.

reaction medium (Scheme 1). These signals have been assigned by comparison with those reported by Jaseja *et al.* (1989) for this residue. Besides, signals at 77·4 (h) and 69·9 (k), corresponding to C-2 and C-3 of 2-O-sulphated L-iduronic acid moieties are not clearly observed in H-a and H-b spectra. The signal at 104·2 (b) ppm (C-1 of L-iduronic acid), low in comparison with that at 101·4 (c) ppm (C-1 of 2-O-sulphated L-iduronic

Table 2. ^{13}C-NMR chemical shifts for residues[a] of chemically modified heparins H-a and H-b

Peak	Signal (ppm)			Assignment	Reference
	H	H-a	H-b		
a	176·5	176·5	176·8	I-6	Casu (1985)
b	104·2	104·2	104·2	I-(II)-1	Jaseja *et al.* (1989)
c	101·4	101·4	101·4	I-1	Casu (1985)
d	99·5	99·4	99·5	G-1	Casu (1985)
e		98·3	98·3	G-(I)-1; G-(II)-1	Jaseja *et al.* (1989)
f		96·9	96·9	I-(I)-1	Jaseja *et al.* (1989)
g	78·5	78·5	78·5	I-4	Casu (1985)
h	77·4	77·4	77·5	I-2	Casu (1985)
i		75·1	75·1	I-(II)-2	Jaseja *et al.* (1989)
j	72·0; 71·4	72·1; 71·4	72·1; 71·4	G-3; G-5; I-5	Casu (1985)
k	69·9	69·9	69·9	I-3	Casu (1985)
l	68·5	68·5	68·6	G-6	Jaseja *et al.* (1989)
m	62·1	62·0	62·0	G-6[b]	Ayotte & Perlin (1986)
n	60·2	60·2	60·2	G-2	Jaseja *et al.* (1989)
o	56·6	56·6	56·5	G-2[c]	Ayotte & Perlin (1986)
p		54·0	54·0	I-(I)-2	Jaseja *et al.* (1989)
q		53·3	53·1	I-(I)-3	Jaseja *et al.* (1989)
r	24·3	24·4	24·4	$-CH_3$	Casu (1985)

[a]Major residues.
[b]6-O-Desulphated.
[c]2-N-Desulphated.
I = 2-O-sulphated iduronic acid; G = 6-O-sulphated glucosamine; (I), (II), see Scheme 1.

acid) in the untreated sample (H), was significantly enhanced in H-a and H-b spectra. On the other hand, a signal at 75·1 (i) ppm was clearly observed in H-a and H-b spectra. This signal, which can be assigned to C-2 of non-sulphated iduronic acid moieties (II) was not observed in the spectrum of H, while it was evident in the spectrum of the FMC of lower sulphate content.

The appearance of a signal at 98·6 ppm (e) in H-a and H-b spectra confirmed the presence of I and II since it can be assigned to the anomeric carbon of glucosamine linked to these moieties. Absence of signals at 102·0 and 100·5 ppm, corresponding to C-1 of L-iduronic acid in compound III (Scheme 1), and C-1 of linked hexosamine, respectively, suggested that it was not formed under these conditions. This compound should result from alkaline opening of the epoxide I together with compound II and has been detected by Jaseja *et al.* (1989) under sodium hydroxide treatment. Direct desulphation by attack of the base on the sulphate group should also yield compound II. Partial 6-O-desulphation of glucosamine residues was also evident by enhancement of the signal at 62·0 (m) ppm in condition a (H-a).

No variation was observed for signals at 60·2 (n) and 56·6 (o) ppm confirming that the N-sulphate content remained unaltered. For comparison, heparin was desulphated by solvolysis with DMSO-H_2O (95 : 5) (Nagasawa *et al.*, 1977). The signal at the lower field practically disappeared while enhancement of that at higher fields was observed (spectrum not shown).

Since the sulphoamino group is essential for the anticoagulant activity, preliminary assays *in vitro* were performed. Both fractions (H-a and H-b) possess anticoagulant activities by the APTT (Ostergaard *et al.*, 1987) and anti-Xa (Denson & Bonnar, 1973; Eggleton *et al.*, 1981) clotting assays (Table 1).

In view of these results, the authors can recommend this alkaline treatment to achieve selective O-desulphation of heparin, mainly at C-2 of L-iduronic acid moieties.

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