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AN ESSENTIAL ROLE FOR THE 2-SULFAMINO GROUP IN THE INTERACTION OF CALCIUM ION WITH HEPARIN

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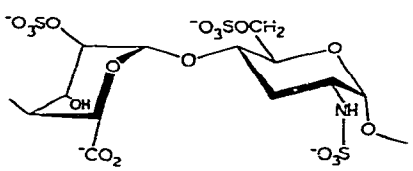
ABSTRACT

Differences in the circular dichroism and n.m.r. spectra of the sodium and calcium salts of heparin concur in suggesting that a chelate complex is formed between 1 mol of calcium ion and a disaccharide repeating segment of the polymer. Upon selective hydrolysis of the *N*-sulfate group of heparin, this specific binding of calcium is no longer observable. It is proposed, among other possibilities, that the carboxylic group strongly binds the cation, and that the sulfamino group of an adjacent sugar residue simultaneously engages in a weaker electrostatic interaction, so as to stabilize the complex. Replacement of the *N*-sulfate by an *N*-acetyl group also eliminates the specific calcium-binding capability of heparin.

INTRODUCTION

Heparin is a member of the glycosaminoglycan class of biopolysaccharides. A major proportion of the macromolecule consists[1,2] of the repeating unit **1**, in which alternating 2-deoxy-2-sulfamino-α-D-glucopyranosyl 6-sulfate and α-L-idopyranosyluronic acid 2-sulfate residues are linked at O-4, although β-D-glucopyranosyluronic acid and several other sugar residues are present as minor constituents.



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Heparin is endowed with unique biological properties, the best known being its anticoagulant and lipolysis-stimulating activities[3].

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Studies[4,5] on the interaction of heparin with metal ions have provided information regarding the nature of the interaction, and the conformation of the polymer. The extent of binding by cupric ion, for example, has been proposed as a basis for the assay of anticoagulant activity. More pertinent to heparin's biological role is its interaction[6] with calcium ion, inasmuch as the latter appears to affect the retarding action of heparin on the rate of thrombin formation. The interaction of heparin with calcium has been studied by n.m.r. spectroscopy[7-10], and also by chiroptical methods[10-12]. Both c.d. and n.m.r. spectroscopy have been utilized as well in the present study. As discussed hereafter, however, some of our conclusions are not in full accord with those of the earlier reports. In addition, we have broadened the subject by examining the effects of chemical modifications of heparin on its ability to interact with calcium ion. As a result, we propose herein that the sulfamino group of heparin plays a critical role in the relationship between this strongly anionic polymer and the cation.

EXPERIMENTAL

Materials. — Sodium and calcium salts of heparin (Na-HP and Ca-HP, respectively)* having activities of 170 USP units were obtained from Sigma Chemical Co (St Louis, MO 63178), and Na-HP (hog mucosal) having an activity of 150 USP units from the Upjohn Co. (Kalamazoo, MI 49001). Ca-HP also was prepared by dialyzing Na-HP against a large excess of mM calcium chloride or, for n.m.r. experiments, by the addition of calcium chloride to a solution of Na-HP contained in the sample tube. *N*-Desulfated heparin (DNSHP) was prepared[13] by hydrolysis of the pyridinium salt of heparin in 19:1 (v/v) dimethyl sulfoxide–water for 2 h at 50°. Selective *N*-acetylation of DNSHP was effected with acetic anhydride in the presence of Dowex 1-X8 ion-exchange resin (CO_3^{2-}). To prepare the methyl ester[14] of heparin, Na-HP was suspended in methanol containing 0.5% (v/v) of acetyl chloride.

Methods. — For c.d. measurements, standard stock solutions of the various materials were prepared by dissolving known amounts of samples in glass-distilled water. Solutions of different pH values were obtained by mixing various proportions of two appropriate solutions having the same metal–polymer concentration, but different pH values. Deuterium oxide was the solvent in the n.m.r. experiments.

Circular dichroism spectra were recorded with a Cary-60 spectropolarimeter equipped with a c.d. attachment, for solutions at room temperature in cells of 0.05–1.0-cm path-length. The concentrations of the solutions were determined by a modified carbazole reaction[15], and calibrated with standard solutions. The molar ellipticity values were expressed in terms of a disaccharide repeating-unit, which was assumed to have an average weight of 563. No correction was made for the refractive index

*Abbreviations: Na-HP, sodium heparinate; Ca-HP, calcium heparinate; and DNSHP, *N*-desulfated heparin.

of the solvent. ^{1}H-N.m.r. spectra were recorded with a Varian XL-200 spectrometer operating at 70°, for solutions in 99.96% deuterium oxide, and with sodium 3-(trimethylsilyl)propionate-d_4 as the internal standard.

RESULTS

Previous c.d. measurements[16–18] have shown that Na-HP gives one minimum at 210 nm and one maximum at 190 nm. When sodium was replaced by calcium, both bands showed significant changes in magnitude and position (Fig. 1A): the 210-nm band decreased to half its original intensity and showed a red shift of 2–3 nm, whereas the 190-nm band, which also exhibited a red shift, decreased slightly in intensity. On titration with calcium chloride (Fig. 1B), ~1 mol of calcium was bound per disaccharide repeating-unit. In view of the high dilutions used, it is unlikely, as already pointed out by others[10,11], that the calcium ion is engaged in interchain interactions.

The c.d. spectra of the sodium and calcium salts of DNSHP (Fig. 2) showed no difference in the 210-nm band. Similarly, the c.d. spectrum of the methyl ester of heparin, which is closely similar to the curves shown in Fig. 2 (see figure caption), was unaffected when the sodium counter-ions of the sulfate groups were replaced by calcium. These data clearly indicate that both the sulfamino and carboxyl groups are required for an interaction between heparin and calcium ion.

Variations in the 210-nm c.d. band of Ca-HP with pH obeyed the same empirical equation as did[17] Na-HP, giving a pK_a of 3.6 for Ca-HP. This decrease from the

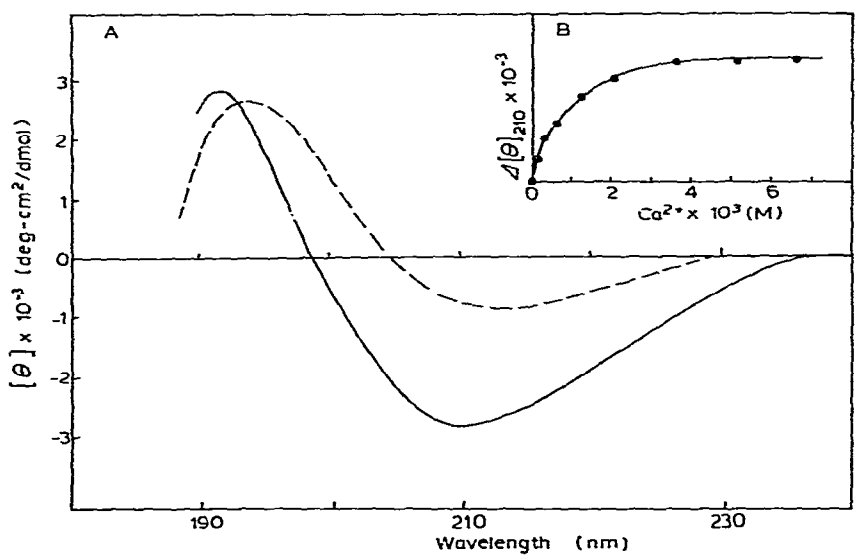

Fig. 1. (A) C.d. spectra of sodium heparinate (——) and calcium heparinate (– – –) solutions at pH 5.5–6.0, HP concentration 1.2 mg/mL, pathlength of cell 5 mm. (B) Titration of Na-HP by $CaCl_2$ as measured by c.d. change at the wavelength 210 nm. Concentration of Na-HP 3.8mM, pH 5.5–6.0.

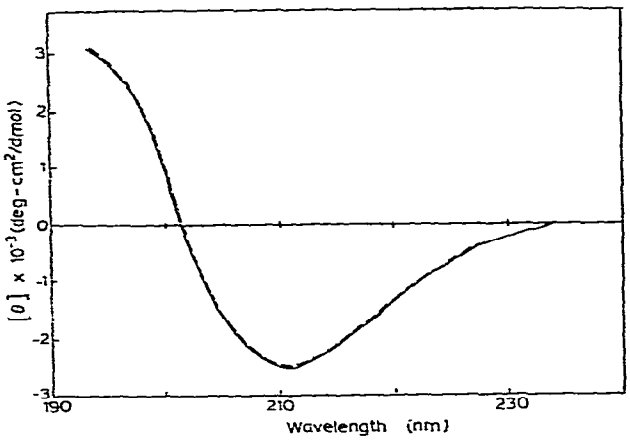

Fig. 2. C.d. spectra of Na-DNSHP (——) and Ca-DNSHP (– – –) solutions at pH 6.0, concentration of DNSHP 1.2 mg/mL, pathlength of cell 5 mm. The c.d. spectrum of the methyl ester of heparin (Na or Ca salt) closely parallels the curves shown, differing principally in that the band is shifted to 212 nm.

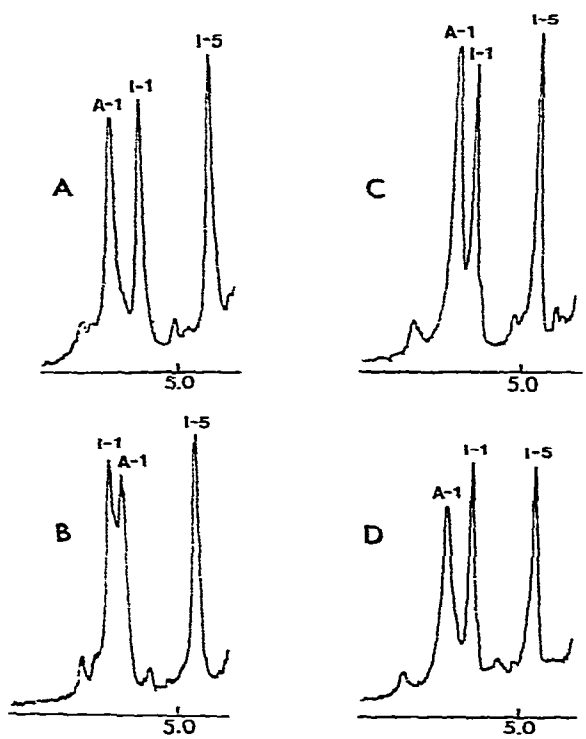

Fig. 3. Partial ^{1}H-n.m.r. spectra (200 MHz, 70°) showing signals downfield of DOH peak of: (A) sodium heparinate (NaHP); (B) NaHP, following addition of 1.25 mol Ca^{2+}/disaccharide sequence; (C) sodium salt (pD ~7) of *N*-desulfated heparin (DNSHP); and (D) material represented by (C) (DNSHP), following *N*-acetylation. Spectra (C) and (D) remained virtually unchanged upon the addition of calcium chloride.

TABLE I

^{1}H-N.M.R. DATA FOR HEPARIN AND CHEMICALLY MODIFIED HEPARINS[a]

Protons	*Chemical shifts (δ) of compounds*[b]		
	Heparin	N-*Desulfated*	N-*Desulfated and* N-*acetylated*
A-1	5.42	5.42 (0)[b]	5.45 (6)[b]
I-1	5.42	5.31 (14)	5.31 (14)
I-5	4.82	4.94 (24)	4.93 (22)
A-2	3.30	3.36 (12)	3.42 (24)

[a]Materials were in the form of sodium salts. [b]Displacement in parentheses (in Hz) from corresponding signal of heparin.

pK_a value of 5.1 obtained[17] for Na-HP is in agreement with the findings of Villiers *et al.*[11].

In the presence of Cu^{2+}, Ca-HP showed a new c.d. band at 235 nm and a decrease in the 210-nm band, changes similar to those observed in previous studies[5] with Na-HP, which had indicated that Cu^{2+} chelates with the carboxyl group of the L-idopyranosyluronic acid residue and the sulfamino group of the adjacent sugar residue. Also, comparative measurements on the titration of Ca-HP with Cu^{2+} and of Cu-HP with Ca^{2+} indicated that Cu^{2+} has a higher affinity for the polymer than does Ca^{2+}. According to the results of other workers[8] relating the ability of various cations to displace sodium ion from Na-HP, Ca^{2+} and Cu^{2+} exhibit comparable affinities for the polymer, and both are substantially more effective than a variety of other cations that have been examined. Although the comparison of affinities between these two metal ions may be of questionable validity on the ground that Cu^{2+} is likely to form a charge-transfer complex[5], such studies are of considerable biological interest, irrespective of the nature of the interaction.

The interaction of Na-HP with calcium ion was clearly evident, in the 200-MHz ^{1}H-n.m.r. spectrum, from several chemical-shift displacements that were induced. As illustrated by the partial spectra shown in Fig. 3 and data in Table I, the H-1 and -5 protons of the L-idopyranosyluronic acid residues (I-1 and I-5) became strongly deshielded, whereas H-1 protons of the 2-amino-2-deoxy-D-glucopyranosyl residues (A-1) experienced somewhat greater shielding, in agreement with earlier reports[7,9,10]. From the variations in chemical-shift displacements accompanying an increase in the concentration of calcium ion (Table I), the stoichiometry of the interaction corresponded to at least 1 mol of Ca^{2+} per disaccharide repeating-unit.

DISCUSSION

The difference in chiroptical properties observed between the sodium and calcium salts of heparin is unusual, in that none of the other glycosaminoglycans

examined[19,20] show such a significant difference. In view of the fact that the methyl ester of heparin failed to show a c.d. change, that the ellipticity of the 210-nm band for Ca-HP is pH-dependent, and that the major effects of calcium ion on chemical shift were observed for I-1 and I-5, it is apparent that the carboxyl group is a chelating ligand. On this, there is general agreement. However, there is disagreement about the stoichiometry of the interaction. Although the present c.d. and n.m.r. data, as well as earlier n.m.r. data[7,9], indicate that at least 1 mol of calcium ion is engaged in interaction with the (major) disaccharide repeating-unit (*i.e.*, **1**), Boyd *et al.*[10] concluded, on the basis of equilibrium-dialysis measurements, that 1 mol of calcium binds to a tetrasaccharide sequence.

It has been proposed[11,12] that although the heparin polyanion—particularly its carboxyl groups—is closely surrounded by calcium counterions, no chemically and conformationally well-defined complex is formed. By contrast, other workers[10] depict a model in which the carboxyl groups of alternate L-idopyranosyluronic acid residues are situated at precise locations on the polymer backbone, so as to accommodate the formation of an intramolecular calcium chelate. The observation that removal of the *N*-sulfate residue completely suppresses the interactions between heparin and Ca^{2+} that are detectable by c.d. and n.m.r. measurements strongly indicates that a specific complex is, indeed, formed. However, the mechanism whereby the sulfamino group influences complex-formation is not obvious, as none of the spectral data clearly point to its *direct* involvement with calcium ion.

One possible effect is that *N*-desulfation alters the conformation of the polymer sufficiently to weaken the normal interaction between Ca^{2+} and the uronic acid carboxyl group. Evidence of such a conformational change is not apparent from the c.d. spectrum of the sodium salt of DNSHP (Fig. 2), because it differs little from that of intact Na-HP (Fig. 1). The corresponding n.m.r. spectra (Fig. 3) suggest that there are no substantial differences in the conformations of the individual residues, based on a comparison of the linewidths of signals A-1, I-1, and I-5, nor in the rotational conformation of the anomeric linkage of the hexosamine residue, as the chemical shift of A-1 of DNSHP is unchanged (Table I). Nevertheless, I-1 resonates moderately downfield, *i.e.*, by δ 0.07, and, more strikingly, I-5 experiences a downfield displacement of δ 0.12 (Table I). These changes may reflect appreciable differences in rotamer populations about the 1- and 4-glycosidic bonds of the L-idopyranosyluronic acid residue of DNSHP, as compared with those of Na-HP.

Because the amino is much smaller than the sulfamino group and differs from it chemically, the possibility that the size and nature of the *N*-functionality at C-2 influences the spectral differences found between DNSHP and Na-HP was examined—admittedly, not ideally—by *N*-acetylation of DNSHP. As seen in Table I, however, the chemical shifts of I-1 and I-5 were unaffected by this modification, *i.e.*, there was no detectable displacement towards their original values in Na-HP. Particularly noteworthy, also, is the fact that the acetamido derivative did not bind Ca^{2+}, as shown by the absence of a change in its ^{1}H-n.m.r. spectrum when calcium chloride was introduced.

An additional possibility is that the sulfamino group of Na-HP is normally near enough to the adjacent pyranosyluronic acid residue, especially to I-5, to induce an appreciable anisotropic shielding of that proton. The shielding influence is removed when the *N*-sulfate group is hydrolyzed, and a downfield shift is observed. If this were true, then the sulfamino substituent also may be sufficiently close to the carboxyl group, adjacent to I-5, to assist in the formation of a calcium chelate. Hence, the fact that the sulfamino group is essential for the specific interaction with calcium to occur can be accounted for most simply by assuming that, as the pyranosyluronic carboxyl group binds 1 mol of the cation, the sulfamino group of an adjacent sugar residue (as in **1**) simultaneously engages in a weaker electrostatic interaction[7] so as to stabilize the charge complex. Intramolecular binding of charged groups on adjacent sugar residues, in this general manner, appears to be compatible with the conformation of heparin, as determined[21] by X-ray fibre diffraction.

Both the 210-nm c.d. band[17,18] and that <200 nm undoubtedly arise from the carboxyl group. That is, although the n–π^* amide transition is prominent in the c.d. spectra of most glycosaminoglycans[22], it can be of only minor significance here, because of the relatively low *N*-acetyl content of the heparin samples. Furthermore, the band <200 nm must be attributed essentially to a π–π^* carboxyl transition, because it was not appreciably affected when Na-HP was *N*-desulfated; *i.e.*, the possibility of a significant contribution from the sulfamino group is eliminated.

Of these two c.d. bands, that <200 nm is expected to be the more indicative of conformational changes. However, its usefulness may be limited, because π–π^* transitions of both carboxyl and amide groups in glycosaminoglycans are sensitive to a variety of factors[20]. As to the 210-nm band, analyses of c.d. spectra of Na-HP have shown[17] that pH-induced changes in ellipticity are due mostly to acid–base properties of the iduronic acid unit rather than to conformational changes. As the results are similar for Ca-HP, no major change is indicated in the conformation of the polymer in the presence of calcium ion. The observed decrease in the rotational strength of the 210-nm band during chelation is similar to that found for Na-HP when titrated with cupric ion[5]. The latter interaction did not appear[5,23] to involve any major conformational modification of heparin and, on the same basis, the present

TABLE II

DISPLACEMENTS (Hz) INDUCED BY INTERACTION OF HEPARIN WITH CALCIUM ION[a]

Protons	*[Ca]/[Hep]*[b]			
	0.25	*0.4*	*1.0*	*1.25*
A-1	−6	−10	−12	−10
I-1	16	26	27	32
I-5	8	12	14	20

[a]Minus denotes an upfield shift. [b]Mol of calcium/heparin disaccharide repeating-unit.

c.d. data do not suggest substantial changes in geometry in the presence of calcium ion.

By contrast, some of the n.m.r. data are consistent with a conformational change as the calcium complex is formed, as already pointed out by Boyd *et al.*[10]. That is, the induced shifts of signal I-1 downfield, and of A-1 upfield (Table II), have been ascribed to variations in anisotropic shielding as rotamer populations about the inter-residue linkages are altered (although, presumably, the magnitude of these changes is below the level of detection by c.d.). It is worth noting that I-5 also experiences a decrease in shielding (Table II), to which the nearby calcium ion may contribute by an electrostatic interaction. However, if, in fact, the sulfamino group of Na-HP does shield I-5, and engages in the binding with calcium (both of which have been considered above), it is conceivable that the sulfamino group is reoriented in the complex, so that its shielding contribution to I-5 is reduced.

One particular aspect of the present findings bears additional comment. That is, the observation that the interaction of heparin with calcium ion is suppressed by removal of the *N*-sulfate group parallels the well-known observation[24] that this same modification leads to almost total loss of heparin's anticoagulant activity. Whether or not these analogies are independent of each other, or are in some way related, remains to be determined.

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