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A RELATIONSHIP BETWEEN ^{13}C-CHEMICAL-SHIFT DISPLACEMENTS AND COUNTERION-CONDENSATION THEORY, IN THE BINDING OF CALCIUM ION BY HEPARIN

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ABSTRACT

Characteristics of the interaction between heparin and calcium ion in the presence of sodium ion have been examined by monitoring the ^{13}C-chemical shift changes as a function of the calcium ion concentration and the total ionic strength. The results indicated that the association between the polyanion and the divalent cation is a delocalized process, as opposed to one involving specific binding. The correspondence found between chemical shift and the number of Ca^{2+} ions bound per charged group, as derived from the Manning counterion-condensation model, showed that the stoichiometry is not a constant quantity but, rather, varies throughout the titration, and approaches a limiting value of 2 at high dilution. Additional measurements of T_1 and line-width were consistent with an intramolecular order–disorder conformational process induced by the binding of calcium ion. Moreover, binding does not occur or is relatively weak with *N*-desulfated heparin, or chondroitin 4-sulfate and 6-sulfate, each of which possesses fewer sulfate groups than heparin. These differences serve to emphasize the importance of the charge–density parameter in the control of counterion condensation according to the Manning model, and suggest that the spacing between the negatively charged groups is an associated factor.

INTRODUCTION

The influence of heparin and calcium ion on the activity of thrombin in blood clotting has stimulated extensive studies[1–26] on the interaction between the metal ion, as well as other cations, and heparin and related anionic polysaccharides. Changes in the ^{1}H-chemical shifts of heparin due to the presence of cations[22–26] have been used, along with other types of measurements, to monitor the interaction and to assess[25,26] the influence of such factors as the presence or absence of specific sulfate groups on the polymer.

As the ^{1}H-n.m.r. observations have been confined, for experimental reasons,

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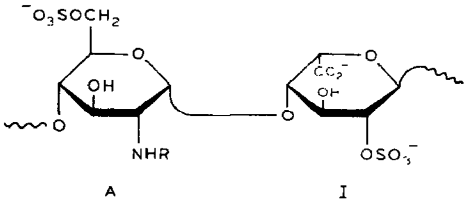
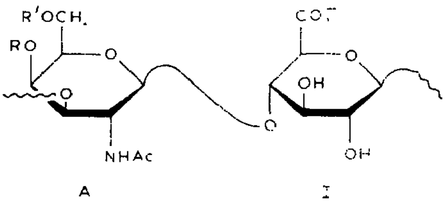


1 R = SO_3^-
2 R = H



3 R = SO_3^-, R' = H
4 R = H, R' = SO_3^-

largely to the anomeric protons and H-5 of L-iduronic acid residues, ^{13}C-n.m.r. spectroscopy has been used in the present study to provide a more extensive set of data. In addition, however, two other aspects of the problem are addressed here. One of these concerns the heparin–calcium ion interaction in the presence of sodium ion, which more closely resembles the *in vivo* environment of heparin when administered as an anticoagulant. The other involves attempts to interpret the ^{13}C-n.m.r. data in the context of polyelectrolyte theory. Accordingly, ^{13}C-chemical shift changes have been recorded for each nucleus of the main disaccharide repeat unit of heparin[22] (**1**) as a function of calcium ion concentration and total (Ca + Na) ionic strength, and assessed relative to recent theoretical treatments of polyelectrolyte theory based on the principle of counterion condensation[27–29]. This approach should distinguish between such alternatives as to whether the affinity of heparin for calcium ions depends on a classical type of interaction governed by charge density[6,13,14,19], or on the formation of a well-defined complex[17,25,26]. As to the latter possibility, it has been suggested[25,26] that the carboxylic acid group strongly binds calcium ions, and the sulfamino group of the adjacent residue helps to stabilize the complex by a weaker electrostatic interaction. To test this possibility, the binding of calcium ions to chemically-modified heparin devoid of an *N*-sulfate group (**2**) also has been examined by ^{13}C-n.m.r. spectroscopy. In addition, ^{13}C-spin–lattice relaxation times and line-width measurements, before and after the addition of calcium ions, were evaluated as a complementary set of data for elucidation of the binding to heparin. Finally, the ^{13}C-n.m.r. chemical shift measurements have been extended to include chondroitin 4-sulfate (**3**) and chondroitin 6-sulfate (**4**) in the presence of calcium ions.

EXPERIMENTAL

Samples of sodium heparin, extracted from beef lung or hog mucosa, were furnished by Upjohn Co. of Canada. The samples were purified by dialysis against distilled water brought to pH 7 upon addition of NaOH, and freeze-dried. Equivalent weights were determined by passage through a column of Amberlite IR-120 (H^+) resin, and subsequent conductimetric titration of the acidic eluate with 0.1M NaOH according to Casu and Gennaro[30]. The equivalent weights for beef lung and hog mucosal heparins were found to be 178 and 190, respectively, whereas the sulfate to carboxylate ratio was 2.4 and 2.2, respectively. The reaction conditions used for the removal of the relatively labile *N*-sulfate group of heparin have already been described[25,26]. Calcium heparin salt was prepared from heparinic acid solution by neutralization with a 0.1M solution of $Ca(OH)_2$, and further purification by dialysis against distilled water, and freeze drying.

^{13}C-N.m.r. experiments were conducted with a Varian XL-300 spectrometer operating at 75.4 MHz. Spectra were recorded for solutions in D_2O at 70° and ^{13}C-chemical shifts (reproducibility, ±2 Hz) were referenced with respect to internal sodium 4,4-dimethyl-4-sila(2,3-$^2H_4$)pentanoate (TSP). The concentrations of polymer solutions, calculated on the basis of the experimentally determined equivalent weights, were 0.1M (0.4 equiv.$\cdot L^{-1}$) and 0.05M (0.20 equiv.$\cdot L^{-1}$), and the calcium or sodium ions were introduced into the n.m.r.-tube in the form of solid $CaCl_2$ or NaCl, in appropriate amounts. ^{13}C-Spin–lattice relaxation times were measured with the same spectrometer under conditions of full ^{1}H-noise decoupling, using the IRFT technique. The pulse duration for a 180° flip angle was 34 μs. Typically, 8–10 τ values were used, averaging approximately a $2 \times T_1$ range and a total of 3000 scans was accumulated for each partially relaxed spectrum. The probable error in the three-parameter fit, in the T_1 calculations, was ±10% or better, and the reproducibility ±5–10%.

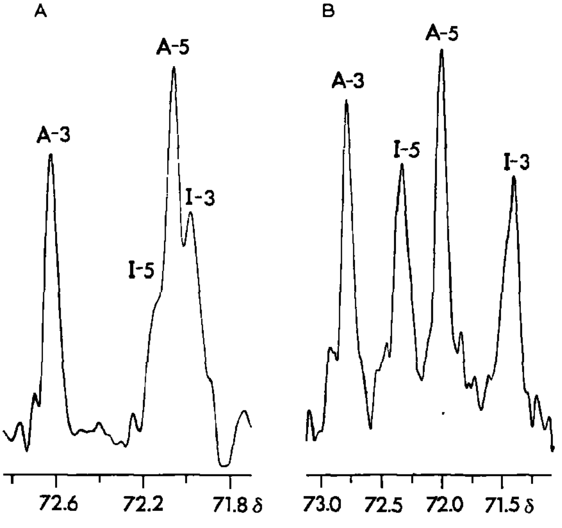


Fig. 1. Partial ^{13}C-n.m.r. spectra of beef lung heparin in D_2O at 70°, showing the I-3, I-5, A-3, and A-5 resonances: (A) $X = 0$, and (B) $X = 1$.

TABLE I

^{13}C-CHEMICAL SHIFT DISPLACEMENTS[a] (IN Hz) INDUCED BY INTERACTION OF CALCIUM IONS[b] WITH HEPARIN, *N*-DESULFATED HEPARIN, AND CHONDROITIN 4-SULFATE AND 6-SULFATE[c]

Carbon atom[d]	*Beef lung heparin*	*Hog mucosal heparin*	N-*Desulfated*[e] *heparin*	*Chondroitin 4-sulfate*	*Chondroitin 6-sulfate*
I-1	-17.2	15.1	-3.5	-10.1	-6.1
I-2	29.7	-23.4	-3.1	-8.0	-5.3
I-3	-42.7	-37.6	-1.5	-7.2	-5.3
I-4	+2.0	+0.5	-3.3	-8.0	-14.2
I-5	+13.4	+14.7	-2.3	-4.5	-6.3
I-6	+29.6	+32.3	+3.2	+2.5	+5.5
A-1	+27.7	+18.5	-2.1	-8.7	-10.6
A-2	-0.5	-3.2	-2.5	-2.1	+1.8
A-3	+12.1	+9.9	-2.4	-9.6	-13.3
A-4	+13.1	+9.8	-3.1	-16.1	-4.8
A-5	-5.2	-8.0	-3.8	-4.9	-5.0
A-6	-4.1	+2.4	-1.0	-4.0	-4.8

[a] Negative values denote an upfield shift. [b] At X = c_2/c_p = 1.0, with c_p 0.1M and a total ionic strength I 0.4M, where c_2 is [Ca] and c_p is [Na-HEP]. [c] For solutions in D_2O at 70°. [d] **I**, L-iduronic or D-glucuronic acid residue; **A**, 2-acetamido-2-deoxy-D-glucose or -D-galactose residue. [e] Only the I-1, I-6, and A-1 resonances have been assigned

RESULTS

Interaction of the sodium heparin (Na-HEP) with calcium ions is clearly evident in the 75.4-MHz spectrum, from the chemical-shift displacements of ^{13}C-nuclei induced upon addition of the calcium ions. As illustrated by the partial spectra in Fig. 1 and data in Tables I and II, all resonances experienced shielding effects of variable magnitude. Pronounced shielding is observed for carbon atoms I-1, I-2, and I-3, and deshielding for I-6 of L-idopyranosyluronic acid residues, whereas the anomeric carbon atom of the 2-amino-2-deoxy-D-glucopyranosyl residues (A-1) is strongly deshielded. The overall effect of calcium binding to heparin, as reflected in chemical-shifts displacements, is illustrated in Fig. 2, as a function of calcium ion concentration at a given (0.4M) total ionic strength. The magnitude of shielding effects for these carbon atoms is closely similar for both beef lung and hog mucosal heparin (Tables I and II). Chemical-shift changes for the corresponding proton resonances, I-1, I-5, and A-1, have been observed[25,26] as well in ^{1}H-n.m.r. experiments, although the shielding effects were the converse of those in the ^{13}C spectra, *i.e.*, I-1 and I-5 were deshielded and A-1 more shielded.

A noteworthy feature of the partial spectra in Fig. 1 is the clear differentiation between resonances I-3, I-5, A-3, and A-5 in the δ 69–72 region (which have been assigned unambiguously by hetero decoupling), in the presence of calcium, which contrasts with the extensive overlap found[22,31] in the absence of a heparin–

TABLE II

^{13}C-CHEMICAL SHIFT DISPLACEMENT[a] (IN Hz) INDUCED BY INTERACTION OF HEPARIN WITH CALCIUM IONS AS A FUNCTION OF CALCIUM ION CONCENTRATION AND IONIC STRENGTH[b]

Heparin source

X[c] *Carbon atom*

	I-*1*[d] *Ionic strength*					I-*2*[d] *Ionic strength*					I-*3*[d] *Ionic strength*					I-*6*[e] *Ionic strength*					A-*1*[e] *Ionic strength*			
	0.4	*0.6*	*0.8*	*1.0*	*1.2*	*0.4*	*0.6*	*0.8*	*1.0*	*1.2*	*0.4*	*0.6*	*0.8*	*1.0*	*1.2*	*0.4*	*0.6*	*0.8*	*1.0*	*1.2*	*0.4*	*0.6*	*0.8*	*1.0*
Beef lung																								
0.2	7.0	7.6	6.7	2.0	8.5	9.0	7.6	6.3	2.0	7.0	11.6	10.5	8.0	5.6	6.6	7.0	7.3	6.1	10.1	3.0	6.0	4.4	5.6	5.0
0.5	11.7	13.7	11.6	8.7	10.6	16.1	14.6	11.7	7.8	10.1	23.7	21.6	18.2	12.7	14.8	18.0	11.8	12.0	12.1	5.9	16.6	12.0	10.6	9.3
0.7	16.2	18.7	15.7	11.5	14.6	23.1	21.2	16.4	11.2	12.2	34.1	31.2	24.6	19.5	18.9	23.2	15.7	15.0	14.9	10.2	21.0	14.4	15.2	12.8
1.0	17.2	20.2	16.1	13.3	15.1	29.7	24.8	18.9	13.1	14.0	42.7	36.7	29.6	23.2	22.9	29.6	20.2	20.2	18.1	12.7	27.7	20.2	19.0	16.4

Hog mucosa

X[c] *Carbon atom*[f]

	I-*1*[d]	I-*2*[d]	I-*3*[d]	I-*6*[e]	A-*1*[e]
0.2	5.0	1.8	3.6	5.6	0.9
0.5	9.6	7.8	11.0	22.7	9.1
0.7	13.6	19.4	31.1	25.7	14.9
1.0	15.1	23.4	37.6	32.3	18.5

[a] Negative values denote upfield shifts. [b] For solutions in D_2O at 70°. Total ionic-strength was kept constant by adding NaCl. [c] Ratio of calcium ion to heparin concentration (c_2/c_p, c_p 0.1M). [d] Negative values. [e] Positive values. [f] At ionic strength I 0.4M.

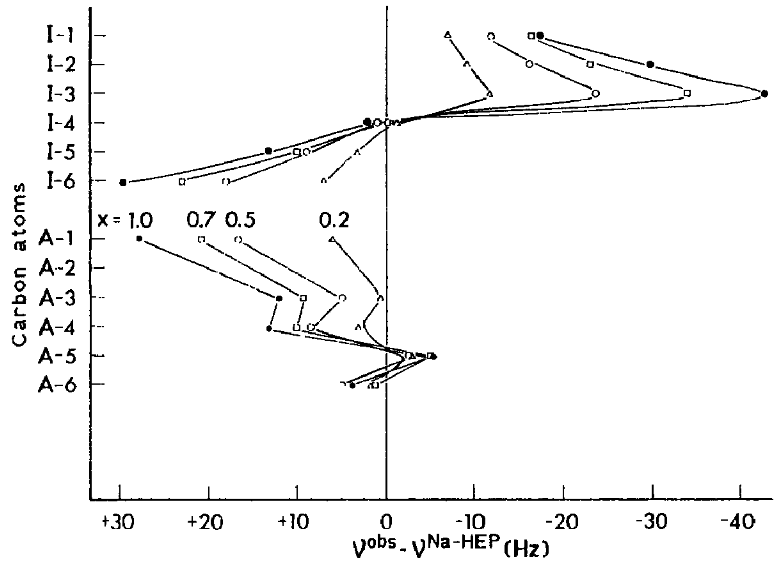


Fig. 2. ^{13}C-Chemical-shift displacement ($\nu^{obs} - \nu^{Na\text{-}HEP}$) for all carbon atom resonances of beef lung heparin as a function of calcium ion concentration ($X = c_2/c_p$).

calcium interaction. Further inspection of Table II and Fig. 2 shows that, (a) chemical shift displacements generally increase with increasing concentration of calcium ions at a constant total ionic strength, and (b) the direction of these variations in shielding are not the same for all carbon atoms, *i.e.*, some of the induced shifts are upfield and some downfield. Similar trends, although of different

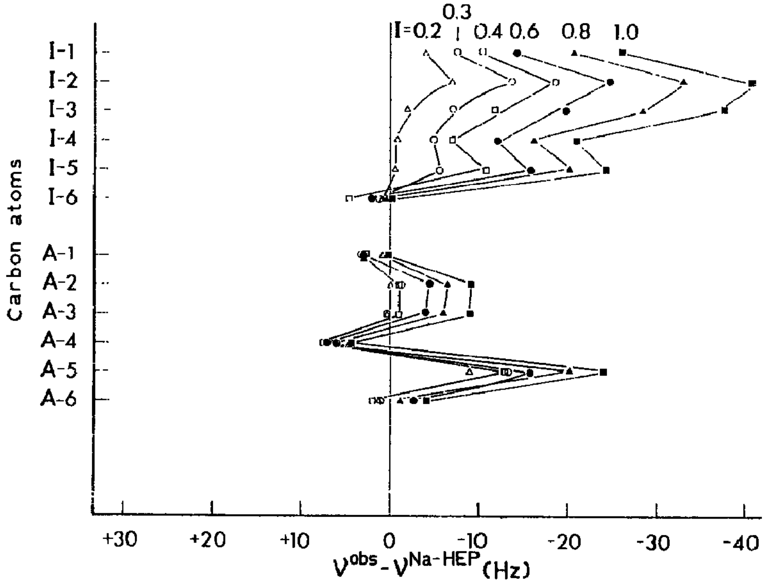


Fig. 3. ^{13}C-Chemical-shift displacement ($\nu^{obs} - \nu^{Na\text{-}HEP}$) for all carbon atom resonances of beef lung heparin as a function of ionic strength (I).

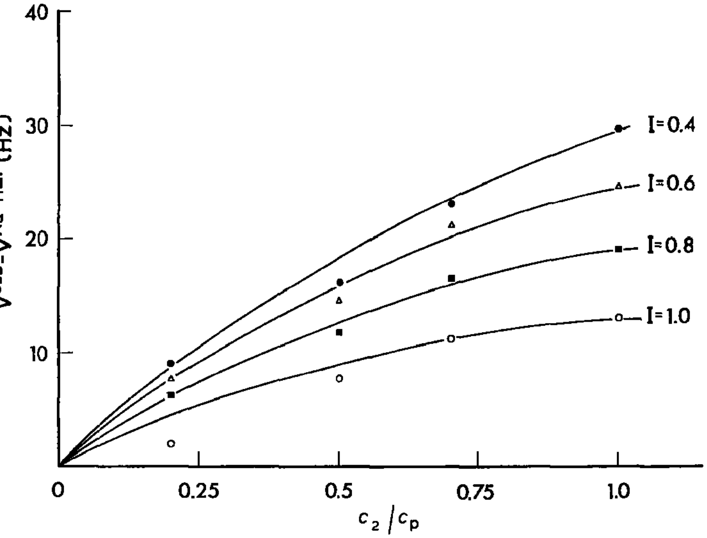


Fig. 4. ^{13}C-Chemical-shift displacements ($\nu^{obs} - \nu^{Na\text{-}HEP}$) for the I-2 resonance of beef lung heparin as a function of calcium ion concentration ($X = c_2/c_p$) at ionic strength, I = 0.4, 0.6, 0.8, and 1.0M.

magnitude, are observed in Fig. 3, where chemical-shift displacements for all carbon atoms of the main disaccharide unit are plotted for different concentration (or ionic strength) of sodium chloride.

The effect of ionic strength is given in Table II, and illustrated specifically for

TABLE III

^{13}C-CHEMICAL-SHIFT DISPLACEMENTS[a] (in Hz) FOR BEEF LUNG HEPARIN (CALCIUM, SALT)[b] IN THE PRESENCE OF INCREASING AMOUNTS OF NaCl[c]

Carbon atom	*Ionic strength*					
	0.4	*0.6*	*0.8*	*1.0*	*1.2*	*1.4*
I-1	−3.4	−5.2	−6.6	−8.5	−10.7	−12.5
I-2	+4.7	+7.8	+8.3	+5.3	+6.3	+5.8
I-3	+5.2	+7.0	+9.2	+8.7	+8.2	+7.6
I-4	−4.5	−9.2	−13.3	−16.1	−19.6	−22.2
I-5	+1.7	−2.8	−5.3	−9.3	−13.1	−15.6
I-6	−5.1	−8.1	−10.8	−11.6	−14.8	−13.1
A-1	−7.1	−13.6	−18.3	−21.6	−25.6	−28.3
A-2	+1.4	+2.2	+1.1	+1.6	+0.9	+1.1
A-3	−3.5	−8.9	−13.5	−18.2	−21.0	−23.0
A-4	0.0	−1.5	−2.7	−3.5	−5.0	−3.5
A-5	−0.5	−2.8	−3.0	−4.8	−5.1	−5.1
A-6	+1.2	+0.5	−0.3	+0.7	−1.5	0.0

[a] Negative values denote upfield shifts. [b] c_p 0.1M. [c] For solutions in D_2O at 70°.

TABLE IV

^{13}C-SPIN LATTICE RELAXATION TIMES (IN S) AND LINE WIDTHS $\Delta\nu_{0.5}$ (in Hz) FOR BEEF LUNG HEPARIN[a]

Carbon atom	T_1 values			$\Delta\nu_{0.5}$ values		
	X[b]	*0.0*	*1.0*	*X*	*0.0*	*1.0*
I-1		0.21	0.21		8.1	6.3
I-2		0.20	0.19		9.4	5.9
I-3		0.20	0.22		10.1	5.7
I-4		0.24	0.22		7.4	5.2
I-5		0.21	0.20			
A-1		0.21	0.20		6.3	5.4
A-2		0.21	0.19		6.2	4.6
A-3		0.23	0.19		8.4	5.9
A-4		0.19	0.18		8.5	5.7
A-5		0.20	0.20		6.6	5.1
A-6		0.12	0.11		7.9	6.5

[a]For solutions in D_2O at 70°, c_p 0.1M at ionic strength 0.4M. [b]Ratio of c_s/c_p.

carbon atom I-2 in Fig. 4, which shows that at a given concentration of calcium ions, the chemical-shift displacements decrease with increasing ionic strength.

To investigate the converse process, *i.e.*, the binding of sodium ion to heparin in the presence of calcium ions, the effect of sodium chloride on the ^{13}C-chemical shifts of the calcium salt of heparin (Ca-HEP) was studied (Table III). In contrast to the calcium chloride–Na-HEP system, the addition of sodium chloride to Ca-HEP caused less pronounced chemical-shift changes, even at ionic strengths as high as 1.4M.

Chemical-shift displacements for *N*-desulfated heparin, and the two glycosaminoglycuronans, chondroitin 4-sulfate and chondroitin 6-sulfate, upon addition of calcium ions, are summarized in Table I. *N*-Desulfated heparin showed practically no chemical shift changes, whereas the observed chemical-shift displacement for the two chondroitin sulfates were much smaller than those for heparin, at the same ratio of calcium ion to polyion concentration ($X = C_s/C_p = 1$) and ionic strength (0.4M).

Table IV compares the T_1 and T_2 (line widths in Hz) values for all the main carbon resonances in the ^{13}C-n.m.r. spectrum of beef lung heparin, before and after the addition of calcium ions. From this Table, it can be seen that the apparent spin–spin relaxation time (T_2^*), or line width, is far more sensitive to changes accompanying the binding of calcium than is T_1.

DISCUSSION

^{13}C-N.m.r. chemical shifts and relaxation data. — The complexation of calcium ions at a specific carbon atom site in heparin should show a shift dis-

placement for the carbon nucleus due to diamagnetic effects. For example, carbon atoms I-5 and I-6 should be sensitive to specific calcium binding by the carboxyl group. Indeed, although chemical-shift displacements are observed for these carbon atoms (Tables I and II, and Fig. 2), almost all carbon atoms of the main disaccharide unit share this property. Furthermore, the most pronounced chemical-shift displacements occur for carbon atoms near the glycosidic bond. These observations appear to be less consistent with the formation of a chemically- and conformationally-defined complex, than a situation in which heparin is closely surrounded by calcium ions, which induce chemical-shift displacements by electrostatic interactions. According to this model, the calcium ions are free to travel along the polyanionic surface. Marked changes in ^{1}H- and ^{13}C-n.m.r. spectra of heparin have been observed[31] by varying the pH. These pH-induced changes have been ascribed to electrostatic effects of the counterion on the magnetic shielding of nuclei close to the ionization site, as well as to conformational changes of the polyelectrolyte chain. X-Ray diffraction studies[32] of glycosaminoglucans have addressed the role of cations in conformation and packing. It was found that among the various allomorphs of glycosaminoglucan helices, univalent and divalent cations support actively distinct conformations. For instance, in chondroitin 4-sulfate, the 3_2 allomorph is the one stable in the presence of Na^+ ions, whereas in the presence of Ca^{2+} ions transformation into the 2_1 allomorph occurs. Analoguous conformational changes have been observed[32] for chondroitin 6-sulfate and sodium hyaluronate fibers.

Other physicochemical studies, in particular viscosity measurements, suggest[1,2,5–7,14,15] that the conformation of heparin in solution is altered by changes in pH, ionic strength, and the type of cation. The observation that divalent ions, including calcium, affect viscosity more drastically than do monovalent ions, suggests that the extent of the conformational change is related to the binding affinity of the cation to heparin. It also appears from those studies that the preferentially-bound cations are more capable of shielding the negative charges along the polymer backbone, so that the intramolecular, electrostatic repulsion is reduced. Under these circumstances, the macromolecule has a less extended conformation and exhibits increased chain flexibility[1,2,7]. In any event, if a molecular conformational change occurs as a result of increasing ionic strength, or the presence of preferentially-bound calcium ions, it must be manifested in part by the pronounced chemical shift displacements for carbon atoms near the glycosidic bond[33], as illustrated in Fig. 2.

Other results that may be considered in terms of a change in the conformation of heparin induced by calcium binding are the ^{13}C-T_1 values and line widths measured for the protonated carbon resonances, summarized in Table IV. The decrease in line widths upon addition of calcium ions indicated a transition into a more mobile conformation, in accord with earlier investigations[1,2,7]. However, there were no significant changes in the T_1 values. This suggested that segmental motions within the polymer backbone, which largely determine the spin–lattice relaxation times, occur on the same time scale in both Na-HEP and Ca-HEP systems. T_2 values, by contrast, which measure mainly the slower, large amplitude motions, *e.g.*, transition from a rather rigid to a more flexible conformation, are very sensitive to molecular rearrangements within the polymer.

Figure 3 illustrates the variation in chemical shifts for all carbon atoms of beef lung heparin with increasing amounts of sodium chloride. These chemical-shift displacements follow a trend notably different from those in Fig. 2 for the mixture of calcium chloride–Na-HEP. In particular, I-6 showed no significant change, and also the majority of the carbon resonances of the amino sugar residue were shifted upfield, rather than downfield, as in Fig. 2. Tentatively, these differences may be attributable to such factors as (a) an effect of ionic strength, (b) preferential surrounding of sulfate groups by sodium ions[15], or (c) a different type of alteration in the chain conformation induced by the binding of sodium ions. Nevertheless, the larger chemical-shift displacements observed for all carbon atoms in Fig. 2 than for those in Fig. 3, at the same total ionic-strength (0.4M), support other indications[3,9] that the binding affinity of calcium ions to heparin is stronger than that of sodium ions. Further evidence that calcium ions bind more strongly to heparin than do sodium ions, is reported in Table III. The small chemical-shift displacements upon addition of the latter ions to calcium heparin (Ca-HEP) indicated that the replacement of bound Ca^{2+} by Na^+ ions occurs with great difficulty, and that an ionic strength higher than 1.4M is required to reach in magnitude the changes characteristic of the system calcium chloride–Na-HEP.

Polyelectrolyte condensation model. — In an attempt to quantify these n.m.r. data, and to present a comprehensive picture of the binding of calcium to heparin, Manning's condensation theory for polyelectrolyte solutions[27–29] has been employed. According to this theory, the polyanion is modelled as a uniformly charged line of infinite length, interacting with a solution of mobile ions. A fundamental quantity involved in this theory is the charge–density parameter, ξ, defined

$$\xi = \frac{e\sigma}{\varepsilon kT} = \frac{e^2}{b\,\varepsilon kT} \qquad (1)$$

by Eq. (*1*) where, e is the magnitude of the electronic charge, ε is the bulk dielectric constant, and σ the charge density of the polyion, which is equal to e/b (where b is the spacing between singly charged groups along the axis of the linear polyelectrolyte chain). The principal feature of this theory is the fact that the charge density has a definite maximum, *i.e.*, $\xi \leq N^{-1}$ (Eq. *2*), where N is the counterion valence. If ξ is initially greater than 1 for univalent cations (or 0.5 for divalent cations), the condensation hypothesis requires that a sufficient number of counterions "condense" on the polyion surface to reduce ξ effectively to 1 (or 0.5); if ξ is less than 1 (or 0.5) initially, no counterion condensation occurs. Further addition of counterions to the solution does not lead to an increase in ion binding, and the charge fraction (fraction of the polyion charged-groups uncompensated for by bound ions) is stable to increases in ionic strength. This is defined in Eq. (*3*), where

$$r = 1 - N\theta_N = (N\xi)^{-1} \tag{3}$$

$\theta_N = c_{N\mathrm{b}}/c_\mathrm{p}$ is the number of *N*-valent ions bound per polyion fixed charge, $c_{N\mathrm{b}}$ is the concentration of bound *N*-valent ions, and c_p is the polyion concentration.

It is apparent from Eq. (*3*) that the charge fraction, and hence the number of bound counterions, does not depend on the ionic strength nor the concentration of free counterions. Thus, the conventional mass-action formulation does not hold for counterion binding in polyelectrolyte solutions described by the condensation model, and calculations of binding constants[24] based on the mass-action law may require re-evaluation.

Another implication of Eq. (*3*) is that the charge fraction does not depend on specific properties of the counterion and polyion, other than the valence of the former and the linear charge density of the latter. This leads to the conclusion that the binding mechanism must involve only long-range electrostatic interactions between constituents in the solution. In this event, the counterion is said to be "delocalized" or "territorial", retaining the fully hydrated state characteristic of a pure aqueous environment, while moving in an unrestricted and random way along the length of the polyion chain. A deviation of the charge fraction from these empirical properties should indicate the existence of site (or specific) binding determined by short-range interactions.

For the mixed uni–divalent counterion system, application of these condensation principles would predict that the number of divalent ions bound per polyion fixed charge, $\theta_2 = c_{2\mathrm{b}}/c_\mathrm{p}$, is given by Eqs. (*4*) and (*5*), where c_2 is the divalent ion

$$\theta_2 = c_2/c_\mathrm{p} \text{ for } c_2 \leqslant 0.5\,(1 - 0.5\xi^{-1})c_\mathrm{p} \tag{4}$$

$$\theta_2 = 0.5\,(1 - 0.5\xi^{-1})c_\mathrm{p} \text{ for } c_2 \geqslant 0.5\,(1 - 0.5\xi^{-1})c_\mathrm{p} \tag{5}$$

concentration. Eqs. (*4*) and (*5*) are valid when $\xi \geqslant 1$. Also, Eq. (*5*) represents the situation where no more divalent counterions are bound, and ξ has been reduced to the critical value of N^{-1} according to Eq. (*2*).

According to this treatment, an explanation can be offered for the observation[25,26] that the *N*-desulfation of heparin eliminates chemical-shift displacements (Table I) due to calcium ions, as mentioned earlier. Hydrolysis of the *N*-sulfate bond exposes an amino group, which can either be linked by a hydrogen bond with remaining sulfate groups, or form a zwitterion[5,19]. In either event, these intramolecular effects should increase the original spacing, *b*, between the negatively-charged groups of heparin and, hence, decrease the charge-density parameter (through Eq. *1*), to a level smaller than the critical value (Eq. *2*) at which condensation of calcium ions occurs. Consequently, in the context of the counterion condensation model, the present results support earlier experimental evidence[5,19] based on viscosity measurements and acid–base properties of *N*-desulfated heparin, and suggest that the participation of the *N*-sulfate group in the binding of calcium to heparin is delocalized, rather than of the specific type proposed previously[25,26].

The effect of the charge–density parameter of the polyanion on calcium ion condensation can be seen additionally in the chemical-shift displacements for the carbon atoms of chondroitin 4-sulfate (**3**) and chondroitin 6-sulfate (**4**) at a total ionic strength of 0.4M (Table I). The much smaller magnitude of chemical-shift changes (Table I) for the chondroitin sulfates, as compared with those for unmodified heparin, reflects the smaller charge–density parameter of the former polyions[34] (ξ ~1.20) relative to the latter[17] (ξ 2.43). According to Eq. (*3*), the fraction of the charged groups of a chondroitin sulfate, uncompensated for by calcium ion at the critical value ξ 0.5, is 0.42, which is twice as large as the corresponding value of 0.21 for heparin. This finding is in agreement with earlier reports[35,36] in which the binding affinity of these polysaccharides for Ca^{2+} and $Co(NH_3)_6^{3+}$ ions decreases in the order heparin > chondroitin 6-sulfate > chondroitin 4-sulfate. Also, it is worth mentioning that no binding was observed[37] between Zn^{2+} ions and chondroitin 4- or 6-sulfate, whereas heparin does bind zinc ions.

The counterion-condensation theory has been extended by Manning[27,28] to account for the delocalized nature of the bound counterion, as evidenced by the ionic-strength dependence of the binding process. In this two-variable theory, a new parameter, V_p, is defined by Eq. (*6*) within which the delocalized counterions

$$V_p = 1000e\,(1 - \xi^{-1})\,(kb)^2 c_1^{-1} \tag{6}$$

are free to move, and both θ_2 and θ_1 (θ_1 = number of M^+ ions bound per ionic group = c_{1b}/c_p) parameters are considered variables in the binding process. In Eq. (*6*), k^2 is the Debey–Hückel screening parameter (Eq. *7*) where L_{AV} is Avogrado's

$$k^2 = \frac{(8\pi\,10^{-3}\,L_{AV}\,e^2)\,\Sigma_i c_i N_i^2}{\varepsilon k T} \tag{7}$$

number and $I = \Sigma_i c_i N_i^2$ is the ionic strength. Minimization of the sum of the free energies of the charging of the polyion, and the mixing of ions in both bound and free states with respect to θ_1 and θ_2, leads[27,28] to the two Eqs. (*8*) and (*9*) where K_2

$$1 + \ln(1000\,\theta_1\,V_p^{-1}\,c_1^{-1}) = -2\xi(1 - \theta_1 - 2\theta_2)\ln(1 - e^{kb}) \tag{8}$$

$$\ln K_2 = \ln(\nu_p/1000e) + 2\ln(1000e\,\theta_1\,V_p^{-1}\,c_1^{-1}) \tag{9}$$

$$K_2 = \frac{\theta_2}{c_{2f}} \tag{10}$$

TABLE V

NUMBER OF BOUND COUNTERIONS (θ_1 AND θ_2) PER CHARGED GROUP OF BEEF LUNG HEPARIN, ASSOCIATION CONSTANTS (K_2), AND STOICHIOMETRY (n), AS A FUNCTION OF CALCIUM ION CONCENTRATION (c_2) AND IONIC STRENGTH (I)[a]

c_2 *(mol/L)*	I *(mol/L)*	θ_1	θ_2	K_2	n	$n\theta_2$
	0.005[b]					
0.0007		0.104	0.311	800	1.561	0.485
0.0005		0.135	0.286	1340	1.590	0.454
0.0002		0.299	0.174	6700	1.673	0.291
	0.04[b]					
0.001		0.378	0.142	165	1.477	0.210
0.0007		0.417	0.117	205	1.463	0.172
0.0005		0.452	0.095	235	1.439	0.137
0.0002		0.527	0.049	325	1.274	0.062
	0.2[c]					
0.05		0.344	0.215	5.5	1.136	0.244
0.035		0.389	0.181	7.0	1.106	0.200
0.025		0.430	0.150	8.5	1.054	0.158
0.010		0.531	0.079	13.1	0.725	0.057
	0.4[d]					
0.10		0.444	0.185	2.3	0.785	0.145
0.07		0.487	0.151	2.8	0.673	0.102
0.05		0.526	0.121	3.2	0.515	0.062
0.02		0.610	0.060	4.3		
	0.6[d]					
0.10		0.556	0.137	1.6	0.240	0.033
0.07		0.593	0.107	1.8		
0.05		0.627	0.083	2.0		
0.02		0.685	0.039	2.4		
	0.8[d]					
0.10		0.641	0.106	1.2		
0.07		0.673	0.081	1.3		
0.05		0.698	0.062	1.4		
0.02		0.743	0.028	1.6		
	1.0[d]					
0.10		0.713	0.085	0.9		
0.07		0.740	0.064	1.0		
0.05		0.760	0.047	1.0		
0.02		0.795	0.021	1.2		

[a] Two-variable approach from Eqs. (*8*) and (*9*). [b] c_p 1mM. [c] c_p 0.05M. [d] c_p 0.1M.

(Eq. *10*) is the association constant for binding of Ca^{2+}, and c_{2f} is the concentration of free divalent ions. Eqs. (*8*) and (*9*) can be solved by numerical iteration[27] to obtain K_2, θ_2 and hence θ_1. By use of ξ 2.43 (ref. 17), theoretical values of K_2, θ_2, and θ_1 were obtained for c_p^{*} 0.001, 0.05 and 0.1M, and total ionic strengths of 0.005, 0.04, 0.2, 0.4, 0.6, 0.8, and 1.0M, and are summarized in Table V. These data showed that the binding affinity (θ_2 or K_2 values) of heparin decreases with increasing sodium ion concentration, *i.e.*, ionic strength, as the two-variable theory predicts. This phenomenon is nicely reflected in the chemical-shift displacements, *e.g.*, of carbon atom I-2 of beef lung heparin (Fig. 4). For a given concentration of calcium ions, the chemical-shift displacement decreased with increasing ionic-strength, and the same effect of the ionic strength on the chemical-shift displacements for the remaining carbon atoms is found as well in Table II, as mentioned earlier. However, it should be noted that, at high ionic-strength ($I \geqslant 0.6$M), the situation is less ideal than the two-phase counterion-condensation model predicts. The consistency of the polyion composition in the polyelectrolyte phase may no longer be valid[38] owing to significant imbibition of the polyanion by sodium chloride. This phenomenon was clearly manifested at the ionic strength of 1.2M (Table II) where deviation from the decreasing order of the chemical-shift displacements is observed. In this range of high ionic-strengths, the higher counterion density of the condensed volume must lead to a Donnan potential, as introduced by Marinsky[39], that is resistant to further entry of calcium ions.

From these observations, the following question arose: what is the relationship between chemical-shift displacement and the number of calcium ions bound to heparin? We begin with the stoichiometric ion-exchange equilibrium (Eq. *11*) in

$$n\mathrm{Na}_b^+ + \mathrm{Ca}_f^{2+} \rightarrow n\mathrm{Na}_f^+ + \mathrm{Ca}_b^{2+} \qquad (11)$$

which Na_b^+ and Ca_b^{2+} represent bound states of heparin with respect to Na^+ and Ca^{2+} ions, respectively, whereas Na_f^+ and Ca_f^{2+} are the ion-free states of heparin. The quantity n can be defined[40,41] as the ratio of the total number of sodium ions that have been displaced by calcium ions to the total number of associated calcium ions at any point of the titration, *i.e.*, Eq. (*12*) where θ_1° is the fraction of the bound

$$n = \frac{\theta_1^\circ c_p - c_{1b}}{c_{2b}} = \frac{\theta_1^\circ - \theta_1}{\theta_2} \qquad (12)$$

sodium ions before the titration and, according to the counterion-condensation theory[27], is invariant, or moderately different, for high ionic-strengths up to 1.0M; c_{1b}, c_{2b}, θ_1, and θ_2 have been defined previously. For the present system, θ_1° equals 0.59, as calculated from Eq. (*3*) for $N = 1$. Although n may not be constant throughout the titration, it can be interpreted as a stoichiometric coefficient in Eq. (*11*). Calculations based on the counterion-condensation model indicated[40] that $n \leqslant N$, N being, as previously, the metal valency. Therefore, if the binding of calcium to heparin can be described satisfactorily by the counterion-condensation model, then the stoichiometry of the bound calcium ions should not be a constant quantity, as has been implied elsewhere[22-24], but should vary with the calcium ion concentration, to a maximum value of 2. Calculated n values from Eq. (*12*), summarized in Table V, support this view.

The observed chemical shift, ν^{obs}, in the competition between calcium and sodium ions, is the population-weighted average of the chemical shifts of the bound and free states of heparin, ν^{b} and ν^{f}, respectively, *i.e.*, Eq. (*13*) where P^{b} and P^{f} are

$$\nu^{\text{obs}} = P^{\text{b}}_{\text{Na}}\,\nu^{\text{b}}_{\text{Na}} + P^{\text{f}}_{\text{Na}}\,\nu^{\text{f}}_{\text{Na}} + P^{\text{b}}_{\text{Ca}}\,\nu^{\text{b}}_{\text{Ca}} + P^{\text{f}}_{\text{Ca}}\,\nu^{\text{f}}_{\text{Ca}} \tag{13}$$

the fractions of the bound and free states of heparin; here Eqs. (*14*). From relations

$$P^{\text{b}}_{\text{Na}} + P^{\text{f}}_{\text{Na}} + P^{\text{b}}_{\text{Ca}} + P^{\text{f}}_{\text{Ca}} = 1 \text{ and } \nu^{\text{f}}_{\text{Na}} = \nu^{\text{f}}_{\text{Ca}} = \nu^{\text{f}} \tag{14}$$

(*14*), and after rearrangement of Eq. (*13*), we obtain Eq. (*15*)

$$\nu^{\text{obs}} = \nu^{\text{f}} + P^{\text{b}}_{\text{Na}}\,(\nu^{\text{b}}_{\text{Na}} - \nu^{\text{f}}) + P^{\text{b}}_{\text{Ca}}\,(\nu^{\text{b}}_{\text{Ca}} - \nu^{\text{f}}) \tag{15}$$

where

$$P^{\text{b}}_{\text{Na}} = \theta_1 \quad \text{and} \quad P^{\text{b}}_{\text{Ca}} = n\theta_2 \tag{16}$$

Substituting Eq. (*16*) and the equivalent of θ_1 from Eq. (*12*) into Eq. (*15*) we obtain Eq. (*17*).

$$\nu^{\text{obs}} = \nu^{\text{f}} + (\nu^{\text{b}}_{\text{Na}} - \nu^{\text{f}})\,\theta_1^{\circ} + (\nu^{\text{b}}_{\text{Ca}} - \nu^{\text{f}})\,n\theta_2 \tag{17}$$

The quantity $\nu^{\text{Na-HEF}}$, defined in Eq. (*18*), is the chemical shift of heparin before the

$$\nu^{\text{Na-HEP}} = \nu^{\text{f}} + (\nu^{\text{b}}_{\text{Na}} - \nu^{\text{f}})\,\theta_1^{\circ} \tag{18}$$

addition of calcium ions. Hence, Eq. (*17*) becomes Eq. (*19*). It appears from

$$\nu^{\text{obs}} - \nu^{\text{Na-HEP}} = (\nu^{\text{b}}_{\text{Ca}} - \nu^{\text{b}}_{\text{Na}})\,n\theta_2 \tag{19}$$

Eq. (*19*) that the observed chemical-shift displacements depend not only on θ_2, but also on the stoichiometry of the calcium ion binding. Provided that the quantity $(\nu^{\text{b}}_{\text{Ca}} - \nu^{\text{b}}_{\text{Na}})$ is constant, the chemical shift displacements $(\nu^{\text{obs}} - \nu^{\text{Na-HEP}})$ will be a linear function of the product $n\theta_2$. This, indeed, is observed in Fig. 5 for c_{p} 0.05M, which also illustrates the nonlinear behavior of the chemical-shift displacement *vs.* θ_2 alone predicted by Eq. (*19*).

Substituting into Eq. (*19*), $\theta_2 = c_{2\text{b}}/c_{\text{p}}$, and multiplying the right hand side by $c_2/c_{\text{p}} \cdot c_{\text{p}}/c_2$, we obtain the relationship between chemical shift displacement and the $X = c_2/c_{\text{p}}$ ratio, as shown in Eq. (*20*) with $p_2 = c_{2\text{b}}/c_2$. Eq. (*20*) appears to account

$$\nu^{\text{obs}} - \nu^{\text{Na-HEP}} = np_2\,(\nu^{\text{b}}_{\text{Ca}} - \nu^{\text{b}}_{\text{Na}})\,c_2/c_{\text{p}} \tag{20}$$

well for the chemical-shift data as a function of c_2/c_{p} in Fig. 4. That is, the downward curvature with increasing calcium ion concentration is a result expected on

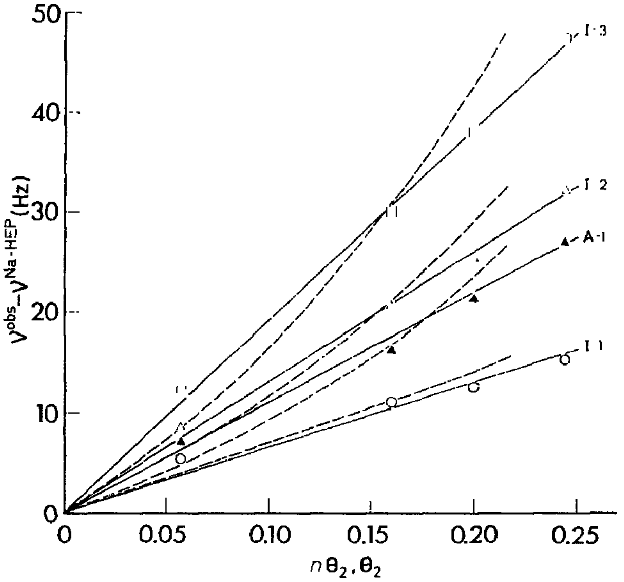


Fig. 5. ^{13}C-Chemical shift displacements ($\nu^{obs} - \nu^{Na\text{-}HEP}$) for I-1, I-2, I-3, and A-1 resonances of beef lung heparin (c_p 0.05M) *vs.* $\eta\theta_2$, according to Eq. (*19*). The non-linear behavior of the chemical shift displacement *vs.* θ_2 alone is illustrated by the dotted lines.

decreasing n or P_2 (or both), in accord with Manning's condensation model. Hence, the observed effect of ionic strength on chemical shift is described implicitly by Eq. (*20*), since both the n and P_2 parameters decrease with an increasing amount of sodium chloride (Table V). The counterion-condensation theory of Manning yields a soundly analytical description of calcium binding to heparin as studied here. However, since this theory takes into account, in its mathematical treatment, only long-range electrostatic interactions consistent with territorial binding, specific binding cannot be assessed in a quantitative manner, nor can such polyanions as heparin, which bear charged groups of different types.

In summary, the binding affinity of heparin for calcium ions in the presence of sodium ions has been examined by observing the ^{13}C-chemical-shift displacements induced as a function of the calcium ion concentration and ionic strength. The results are consistent with the counterion-condensation model[27-29] and, therefore, they indicate that the association between the polyanion and the divalent cation is a delocalized process, as opposed to one involving specific binding. With *N*-desulfated heparin and chondroitin 4-sulfate and 6-sulfate, each of which possesses fewer sulfate groups than heparin, binding does not occur or is relatively weak, which emphasizes the importance of the charge-density parameter in controlling the condensation of calcium ions. These variations, as well as the binding differences found[26] among other modified forms of heparin, suggest that the spacing between the negatively-charged groups is an associate factor in differentiating between polyanions of the glycosaminoglycan type.

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