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Marked stereoselectivity in the binding of copper ions by heparin. Contrasts with the binding of gadolinium and calcium ions.

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ABSTRACT

Heparin forms a complex with cupric ion (Cu^{2+}) at a level of $\leqslant 10^{-3}$ mol of the metal ion per dimeric unit of the polymer, as evidenced by paramagnetic relaxation effects on its ^{1}H- and ^{13}C-n.m.r. spectra. No interaction occurred with heparin derivatives modified either by desulfation of the residues of α-L-iduronic acid 2-sulfate, or by hydrolysis of the sulfamino group of the residues of 2-deoxy-2-sulfamino-α-D-glucose 6-sulfate, although binding was induced by *N*-acetylation of the latter derivative. Under the same experimental conditions, no alternative type of glycosyluronic acid structure tested, including the other glycosaminoglycans, showed significant relaxation exhancement by Cu^{2+}. These results are in contrast to those obtained with gadolinium ion (Gd^{3+}), another paramagnetic probe, or with calcium ion (Ca^{2+}), which promotes chemical-shift displacements. The binding selectivities of those two cations are much broader than that of Cu^{2+}, although they also differ notably in their relationship to the structure of heparin.

INTRODUCTION

Complexes formed between copper ions (Cu^{2+}) and glycosaminoglycans such as heparin attract attention because of their demonstrated, or potential, biological significance. In the presence of Cu^{2+}, heparin has been found[1,2] to promote the new formation of capillaries ("angiogenesis"), and also[2] chemotactic activity on capillary endothelium. As there is an apparent correlation[3,4] between the anticoagulant activity of heparin and its copper-binding capacity, the latter has been regarded[4] as a possible basis for bioassay. Another biological role for Cu^{2+} in relation to glycosaminoglycans concerns[5] its catalysis of the degradation of hyaluronic acid by OH radicals.

By equilibrium-dialysis and gel-permeation measurements, it has been found[3,4] that binding between heparin and Cu^{2+} entails a stoichiometry ranging from 1 to 5 disaccharide-repeating sequences per cation, depending on such factors as pH. Metal-polymer complexes of similar stoichiometry have been detected in chiroptical[6] and polarographic[7] investigations of Cu^{2+} with hyaluronic acid, although effects on its ^{13}C- and ^{1}H-relaxation times are evident[8] in the presence of lower proportions of the cation.

We report herein that heparin is exceptionally sensitive to the paramagnetic-relaxation characteristics of Cu^{2+}. In this regard, it is reminiscent of gadolinium ion (Gd^{3+}) which, in trace proportions, causes[9] similarly marked changes in the spectra of heparin. The Cu^{2+} ion, however, exhibits much greater selectivity than the Gd^{3+} ion towards other types of uronic acid units, an observation that is emphasized by a

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comparison of the two cations in their interactions with several chemically-modified forms of heparin. The latter derivatives also serve to illustrate certain structural constraints on the ability of heparin to bind these cations, as well as on the polymer's affinity for Ca^{2+}, with which Cu^{2+} has been extensively compared[4,10–12].

RESULTS AND DISCUSSION

Both the ^1H- and ^{13}C-n.m.r. spectra of heparin (Fig. 1) are affected by the introduction of Cu^{2+} at a level of 10^{-3} mol of the cation per disaccharide repeating-unit (based on **1** as the major type). Most striking (Fig. 1A) is the reduction in height of ^1H resonances I-1 and I-5 of the α-L-iduronic acid unit (I), and of its carboxyl and anomeric ^{13}C signals (Fig. 1B).* Less readily apparent is the loss, effectively, of the iduronic acid

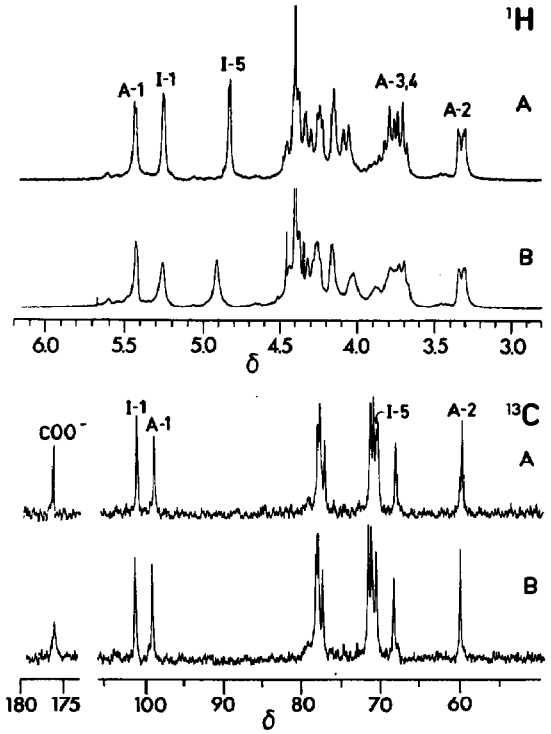


Fig. 1. ^1H-N.m.r. spectra (300 MHz, 65°) and ^{13}C-n.m.r. spectra (75 MHz, 25°) of beef lung heparin in D_2O solution (A), and the spectra (B) following the introduction of 1.1 mmol of copper sulfate/mol of disaccharide repeating unit (1) (see Table II). Signals designated A-1, etc. are due to the corresponding nuclei of the aminodeoxyhexose unit, and I-1, etc. to those of the iduronic acid unit.

* Pharmaceutical preparations of heparin occasionally show n.m.r. characteristics similar to these, attributable to contamination with traces of paramagnetic metal ions. In such instances (*e.g.*, see ref. 13), the introduction of ethylenediaminetetraacetic acid causes the reappearance of the normal heparin spectrum; this effect was duplicated in the present study with Cu^{2+}.

TABLE I

Effect of Cu^{2+} on some ^1H-spin–lattice relaxation times of heparin[a]

H-1 of unit	*Relaxation time (s)*	
	Heparin	*Heparin + Cu^{2+}*[b]
A-1	1.12	0.57
A-2	1.22	0.56
I-1	0.82	0.15
I-5	1.21	0.21

[a] For solutions in D_2O, 0.8M with respect to NaCl, at 65°; error ±0.02 s. [b] $1.1 \cdot 10^{-3}$ mol per disaccharide unit (1).

^{13}C signal attributable[14] to C-5, another notable change reflecting enhanced relaxation rates due to the cation. There is relatively little impact on the resonances due to the amino sugar unit (A). This is also evident in the spin-lattice relaxation data of Table I, which show that the T_1 values of protons A-1 (anomeric) and A-2 (H-2) of this unit are decreased by a factor of two upon the introduction of Cu^{2+}, whereas there is a 5–6 fold decrease in the T_1 values of glycosyluronic protons I-1 (anomeric) and I-5 (H-5). At 3–4 times this concentration of Cu^{2+}, however, not only were the aforementioned nuclei of the uronic acid more strongly affected, but many of the other spectral lines were broadened. This is analogous to what had been observed[9] with Gd^{3+} at comparably high heparin-to-cation levels.

Another similarity was found in the effect of differences in pH. As shown in Fig. 2, no interaction of Cu^{2+} with heparin was detected at pD 7.5, whereas its impact on the line-width of ^1H signals was apparent, and continued to increase over the range of pD

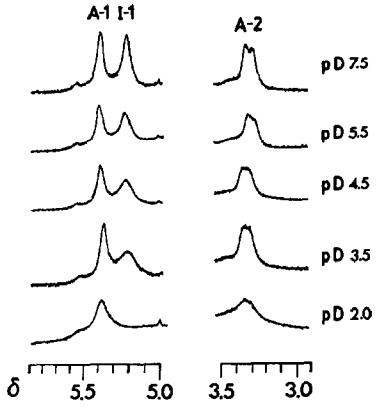


Fig. 2. The influence of changes in hydrogen-ion concentration (pD) on the line widths of ^1H-resonances of heparin in the presence of Cu^{2+} [2 mmol/mol of disaccharide repeating unit (1)]. Spectra recorded at 200 MHz and 25°: A-1 and A-2 are H-1 and H-2 of the aminodeoxy hexose unit, respectively; and I-1 is H-1 of the iduronic acid unit).

6.5–2.5. By far the most striking change evident in Fig. 2 is the broadening of the I-1 signal (δ 5.2) due to enhancement of its spin–spin relaxation rate. Resonances due to the aminodeoxyhexose unit (A), at δ 5.4 (A-1) and δ 3.3 (A-2), are affected comparatively little above pD 3.5. Accordingly, a pD value of $\sim$5 was selected for the experiments described herein. This effect of pH is the converse of that observed[4] in studies involving the much higher Cu^{2+}-to-heparin ratios, which found that binding is 50% lower at pH 3 than at pH 7. Consequently, the present observations may concern a different type of interaction between the metal ion and the polymer.

In its sensitivity towards Cu^{2+} (Table II), hog mucosal heparin proved to be closely equivalent to the beef lung heparin represented by Fig. 1. Also strongly affected were heparin fractions having a slightly higher value, and about one-third, respectively, of the USP anticoagulant activity of these heparins. A similar proportion of Cu^{2+} was required to induce comparable changes in the ^{1}H-n.m.r. spectra of two partially-depolymerized heparin preparations having both a relatively low molecular weight and USP potency (Table II).

On examining several chemically-modified forms of heparin available from other studies, there was evidence in only one instance for the occurrence of binding with Cu^{2+}

TABLE II

Interaction of heparins, modified heparins, and related glycosaminoglycans with Cu^{2+}[a]

Heparin and derivatives[b]	*USP potency (U/mg)*	*Effective*[c] *concentration of* Cu^{2+} *(mmol/structural unit) (mM)*
Beef lung heparin (**1**)	135	1
Hog mucosal heparin (**1**)	146	1
Fraction (high affinity) (**1**)	278	0.5
Fraction (low affinity) (**1**)	50	1
Low-molecular weight[d] (**1**)	38	2
Low-molecular-weight[e] (**1**)	50	1
Modification **2**	$\leqslant$5	[g]
Modification **3**	[f]	[g]
Modification **4**	$\leqslant$5	[g]
Modification **5**	$\leqslant$5	1
Other glycosaminoglycans		
Heparan sulfate[h]		1
Dermatan sulfate		[g]
Chondroitin 4-sulfate		[g]
Chondroitin 6-sulfate		[g]
Related compounds		
Disaccharide **6**		[g]
D-Glucuronic acid		[g]
D-Galacturonic acid		[g]

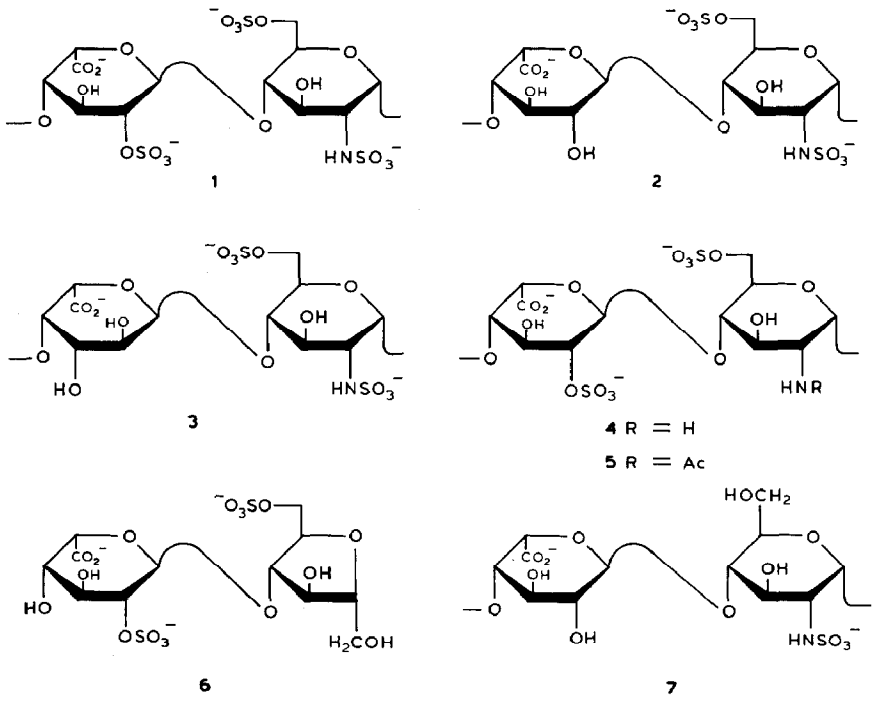
[a] Sodium salts in D_2O solution at pD $\sim$5 and 65°. [b] Major disaccharide repeating-unit. [c] Concentration of Cu^{2+} required to induce changes in the uronic acid unit H-1 and H-5 signals visually comparable to those shown in Figs. 1A and 1B. [d,e] Prepared commercially through partial chemical degradation (Sandoz and Wyeth, respectively). [f] Anti-Xa potency. [g] No activity. [h] Fraction giving a spectrum containing minor I-1 and I-5 signals; the effective conc. listed applies only to those signals.

at the concentration levels involved in the foregoing experiments. In these heparin derivatives, either the L-iduronic acid 2-sulfate unit (**1**, I) had been modified[15–17] by selective removal of its sulfate group (giving **2**), or by its transformation into a unit of α-L-galacturonic acid (as in **3**). Alternatively, the sulfamino group of **1** had been selectively hydrolyzed to give **4** which then had been converted into the *N*-acetyl derivative (**5**). As shown in Table II, the sensitivity of **5** towards Cu^{2+} was equal to that of heparin. Among the other derivatives, a response was detected only when the Cu^{2+} concentration was 3-times that required to induce the type of response shown in Fig. 1, at which level the ^{1}H-n.m.r. spectrum of **4** underwent a weak overall broadening in a nonselective manner, and the I-1 signal of **2** showed a slight reduction in height. The ^{13}C nuclei of **2** proved to be no more sensitive than its protons, as their various signals, including that of the carboxyl group, were barely affected.

These observations suggested that, in functioning as a paramagnetic relaxation agent towards heparin, Cu^{2+} requires the 2-sulfate group at the L-iduronic acid unit*, although not necessarily the sulfamino group. In addition to the total sulfate content, the latter group was found[3,4] to be important for promoting the binding of Cu^{2+} at high metal-to-heparin ratios. This observation that a reduction in the number of sulfate groups leads to reduced anticoagulant activity was the source of the proposition[4] that such activity might be assayed chemically through measurements of copper binding. As the anticoagulant potencies of polymers **2**–**4** are all very low, the present findings are not inconsistent with that proposal. However, when the major structural features (*e.g.*, **1**) of the heparin molecule are intact, and normal or even moderate anticoagulant activity is present (Table II), then the polymer effectively binds Cu^{2+}. The *N*-acetyl derivative **5** is a striking exception to this. Its reactivity raises the possibility that the total charge density of the polymer may be critical, because **5** bears one more anionic charge than does its amino precursor, **4**, which exists[4] as a zwitterion. Nevertheless, as its *net* negative charge of three is equal to that of polymers **2** and **3**, which do not bind Cu^{2+}, it is also feasible that the spatial arrangement of the anionic groups is important. These factors are again considered later in dealing with some observations on the binding of calcium ion by polymers **2**–**5**.

Other glycosaminoglycans were also tested at these low Cu^{2+}-to-polymer ratios. Dermatan sulfate, which contains a unit of α-L-iduronic acid (nonsulfated), was found to give a barely perceptible response towards the metal ion, in this respect complementing that of the analogous type of residue in **2**. Nor was binding detected for chondroitin 4- and 6-sulfates, each of which contains a unit of β-D-glucuronic acid. Hyaluronic acid also contains units of β-D-glucuronic acid, although we did not examine the polymer because its ^{1}H- and ^{13}C-n.m.r. signals are unsuitably broad. However, information is available[8] about the influence of Cu^{2+} on the ^{13}C relaxation rates of a slightly-depolymerized specimen; corresponding data for D-glucuronic acid show[18] analogous characteristics. In those experiments, the metal-to-glycosyluronic ratios were some two orders

* However, when present at the molecular level of disaccharide **6** (obtained by deaminative degradation of heparin), the α-L-iduronic acid 2-sulfate unit showed no complex formation (Table II).



of magnitude greater than in ours. By contrast, we could detect no interaction between Cu^{2+} and sodium D-glucuronate (nor with sodium D-galacturonate) (Table II). Consequently, these findings indicate that heparin binds Cu^{2+} far more readily than does hyaluronic acid.

In addition to heparin, the only other example of a glycosaminoglycan showing a response to a trace of Cu^{2+} was heparan sulfate. Some fractions of this polymer gave ^{1}H-n.m.r. spectra[19] containing minor signals attributable to I-1 and I-5 of the α-L-iduronic acid 2-sulfate unit (I in **1**). It is noteworthy, therefore, that line-broadening of these signals was observed when Cu^{2+} was introduced, whereas the other resonances in the spectrum were not appreciably affected. Hence, the β-D-glucuronic acid units in this polymer, as did those of the chondroitin sulfates and hyaluronic acid, exhibited no binding of Cu^{2+} under the experimental conditions used.

Observations on the binding of gadolinium ion. — Although, as already noted, Gd^{3+} and Cu^{2+} show close similarities as relaxation agents in their interaction with heparin, these negative results with Cu^{2+} and the aldohexuronic acids (and also with dermatan sulfate) sharply distinguish the binding affinities of the two paramagnetic ions. This difference includes the group of modified polymers, **2–4**, which do not bind Cu^{2+}. At a molar ratio of substrate-to-Gd^{3+} in the vicinity of $1:1\cdot10^{-4}$, a level at which aldohexuronic acids and heparin showed a marked response, each of these polymers experienced enhanced relaxation-rates for glycosyluronic H-1 and H-5 (Table III). Also noteworthy are variations observed in the relative sensitivities of these two protons (Fig. 3), inasmuch as both protons of heparin are affected similarly (Fig. 3A), whereas either

TABLE III

Interaction of heparin and modified heparins[a] **2–4** with Gd^{3+}

Heparin and derivatives[b]	*Effective*[c] *concentration of* Gd^{3+} *(mmol/structural unit) (0.1mM)*
Beef lung heparin (**1**)	0.20
Modification **2**	0.35
Modification **3**	0.58
Modification **4**	0.20
α-D-Galacturonic acid[d]	0.10

[a] Sodium salts in D_2O solution at pD ~5 and 65°. [b] Major disaccharide repeating unit. [c] Concentration of Gd^{3+} required to induce changes in the uronic acid unit H-1 or H-5 signals (or both), as shown in Fig. 3. [d] See ref. 9.

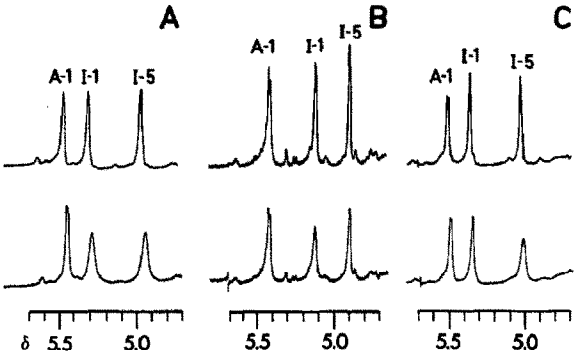


Fig. 3. The influence of gadolinium ion (Gd^{3+}) on the ^{1}H-n.m.r. spectra (300 MHz, 65°; δ 4.8–5.6 region) of: (A) beef lung heparin, (B) modified heparin **2**, and (C) modified heparin **4**. The upper spectra were recorded prior to the introduction of Gd $(NO_3)_3$, and the lower spectra, after its addition (see Table III); A-1 is H-1 of the aminodeoxyhexose unit; and I-1 and I-5 are H-1 and H-5 of the iduronic acid unit, respectively.

H-1 or H-5 showed more pronounced broadening in the spectra of **2** and **4** (Figs. 3B and 3C, respectively). These variations are assumed to reflect slight differences in the proton–Gd^{3+} internuclear distances within the individual complexes, the geometry of which has already been discussed[9].

Overall, then, Gd^{3+} binds strongly to the α anomers of a variety of aldohexopyranuronic acid units, and appears to be diagnostic for that configuration. By contrast, Cu^{2+} shows far greater selectivity in its uniquely high affinity for components of the heparin structure.

Observations on the binding of calcium ion. — Extensive studies on the interaction of Ca^{2+} with heparin include some instances[12,20–22] in which chemically-modified heparin derivatives have been examined. In general, the removal of sulfate groups led to a decrease in the amount of Ca^{2+} bound. Although this parallels the observations on Cu^{2+}, these two cations need not engage in analogous kinds of coordination with the polymer. Based on the observation that the only resonances strongly affected by Cu^{2+} are those of the carboxyl-group ^{13}C atom, and the I-1 and I-5 ^{13}C- and ^{1}H-nuclei, it

TABLE IV

Displacement (ΔHz) of ^{1}H- and ^{13}C-n.m.r. signals of modified heparins **2** and **3**, and heparin (**1**) by Ca^{2+}

Heparin and derivatives	*^{1}H-Signals (ΔHz)*[b]			*^{13}C-Signals (ΔHz)*[c]					
	A-1	*I-1*	*I-5*	*A-1*	*A-3*	*I-1*	*I-4*	*I-5*	*I-6*
Beef lung (**1**)[d]	−2.4	29	24	27	13	17	2	13	29
Hog mucosal (**1**)	−2.3	29	25						
Hog mucosal[e]		41							
Modification **2**	−10	45	32	7	7	−14	−30	− 3	13
Modification **3**	−2.3	25	9						
Modification **7**[f]	−4	24	16						

[a] At 2.0 mol of Ca^{2+}/repeating unit **1**, **2**, or **3** (using as the unit wt. of Na salt of **1** = 665, and that of **2** or **3** = 563). [b] At 65°; referenced with respect to the A-2 signal of **1**. [c] At 25°; referenced with respect to the A-2 signal of **1**. [d] See ref. 22. [e] For the minor, nonsulfated, unit of α-L-iduronic acid. [f] At 1.2 mol of Ca^{2+}/repeating unit **7**; pD 8.5 (see ref. 20).

appears that binding is confined largely to coordination between the cation and CO_2^-, O-1, and O-5 of the L-iduronic acid unit of **1**. By contrast, calcium binding is characterized[21] by a widespread pattern of chemical-shift changes involving electrostatic influences on most of the nuclei of both residues (I and A) of sequence **1**, in a delocalized association with the polyanion. That the sulfamino group of heparin may be an important participant in Ca^{2+} binding has already been suggested[12,20], based on the observed inactivity of *N*-desulfated heparin (**4**), as well as of its *N*-acetyl derivative **5**. It is now seen, by contrast, that when the glycosyluronic acid residue is desulfated to give **2**, Ca^{2+} promotes chemical-shift displacements (Table IV) comparable in magnitude to those of heparin itself, although the pattern is different for some individual nuclei*. Also exhibiting Ca^{2+}-binding is polymer **3**, in which the uronic acid unit is the α-L-*galacto* diastereomer of that in **2**. Yet another modified form of heparin shown to bind Ca^{2+} is **7** (ref. 20) (Table IV), which bears only one sulfate group; in this polymer, the sulfamino group has been restored.

Although charge density has an important bearing[4,21,22] on the interaction between heparin and Ca^{2+} and, as noted earlier, may also influence its binding of Cu^{2+}, this factor cannot solely account for the variations in reactivity observed among the various heparins. Hence, binding is observed where the polyanion possesses either a negative charge of three (**2** and **3**), or two (**7**), whereas three negative charges (**5**) need not impart activity, which can also apply for a *net* negative charge of two (**4**; zwitterion form). Consequently, these results reinforce the earlier suggestion[21] that, in addition to the requirement of a minimum charge-density level at which the condensation of Ca^{2+} on the polyanion can occur, the spatial arrangement of the negatively-charged groups of heparin with respect to each other is an important factor.

* It is noteworthy that the downfield displacement of the anomeric signal (I-1) of **2** is close to that[20] for the analogous unit of α-L-iduronic acid (nonsulfated) which is a minor constituent of hog mucosal heparin.

TABLE V

Comparative selectivity in the binding of cations by heparin and modified heparins

Polymer	*Binding affinity for*		
	Cu^{2+}	Gd^{3+}	Ca^{2+}
Heparin	+	+	+
Modification **2**		+	+
Modification **3**		+	+
Modification **4**		+	
Modification **5**	+	+	

In summary. — The high sensitivity and marked selectivity of heparin in forming a complex with cupric ion appears to be commensurate with the possibility of their combined stimulatory role in angiogenesis, inasmuch as effective binding is now known to occur when only a trace proportion of the metal ion is present. Although the major type of heparin structure represented by **1** is associated, almost uniquely, with the observed paramagnetic relaxation effect of Cu^{2+} on its n.m.r. spectra, this interaction is not structurally related to anticoagulant activity. Such a possibility is negated by the fact that the 2-acetamido-2-deoxy analog of heparin (**5**), which exhibits little anticoagulant potency, readily binds the metal ion.

Gadolinium ion (Gd^{3+}), a paramagnetic-relaxation agent that gives a response with heparin comparable in sensitivity to that of Cu^{2+}, is far less selective than the latter cation. It interacts strongly with several modified forms of heparin, as well as a variety of other aldohexopyranosuronic acid compounds, although all these polymers have uronic acid units in the α-anomeric form (O-1 axial).

Calcium ion is intermediate in selectivity between Cu^{2+} and Gd^{3+}. It forms a complex with heparin and some, though not all, of the modified forms of heparin examined, inducing chemical-shift changes that are detectable at cation levels far higher than those feasible with the paramagnetic ions. Structural constraints on Ca^{2+}-binding with heparin differ most notably from those of Cu^{2+}, in that the latter appears to require an intact unit of α-L-iduronic acid 2-sulfate, as in **1**, although the 2-amino-2-deoxyhexose unit of **1** may be modified, whereas the required contact sites are reversed for the Ca^{2+} ion.

A list of the various combinations of selectivity that heparin and its modified forms exhibit in their binding interactions with these three cations is presented in Table V to emphasize the diversity and distinctive characteristics that have been encountered in this study.

EXPERIMENTAL

Samples of sodium heparin were furnished by the Upjohn Co. of Canada. The modified heparins were available from earlier studies: **2** and **3** (refs. 15–17), **4** and **5** (refs.

20,21), as was also heparan sulfate (ref. 19). The low-molecular-weight heparins were provided by G. A. Neville. N.m.r. spectra were recorded with a Varian XL300 spectrometer operating at 300 MHz for ^1H and 75 MHz for ^{13}C, or with a Varian XL200 spectrometer (^1H at 200 MHz). Chemical shifts are given with respect to the signal for internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (δ 0.0). The polysaccharide solutions consisted of ~20 mg per 0.5 mL of D_2O, and the appropriate quantity of a given cation was introduced into the n.m.r. tube as a solution (generally 2–10 μL) of either $CuSO_4 \cdot 5H_2O$, $Gd(NO_3)_3 \cdot 5H_2O$, or $CaCl_2 \cdot 2H_2O$ of the required molarity in D_2O. The adjustment of pH was carried out initially with an aqueous solution of the carbohydrate sample, which was then evaporated. Deuterium-exchange was carried out subsequently by repeatedly dissolving the residue in D_2O, followed by distillation of the solvent under reduced pressure.

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