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A potentiometric titration study of the interaction of heparin with metal cations

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Potentiometric titrations, at ionic strengths (I) ranging from 0.0057 to 0.336, suggest that Ca^{2+}, Cu^{2+}, Li^+ and Na^+ bind to heparin in a manner that depends on cation identity. These interactions were less affected by the value of I than those of heparin with Mg^{2+}, which binds weakly below I0.050 and with K^+, which binds weakly at I0.0057. Of the interactions studied, that of heparin with Cu^{2+} was the least readily reversible.

INTRODUCTION

There is considerable uncertainty regarding the mechanistic details and biological relevance of heparin–metal-ion interactions (Nieduszynski, 1989). Some debate has arisen over whether the binding occurs at specific localized sites or in a de-localized non-specific manner. The latter possibility is often discussed in terms of the theories put forward by Manning (1969*a*,*b*,*c*, 1977, 1978). These consider that a critical charge density exists on a polyelectrolyte like heparin and that, if the net charge density, which includes the charge of the counter-ions, exceeds this, then cations condense on the anionic sites in a de-localized non-specific fashion. However, a dependence on Na^+ concentration of the diffusion rates of these ions in aqueous solutions of heparin lacking additional electrolyte (Ander & Lubas, 1981; Ander & Kardan, 1984), and an apparent polyanionic charge of heparin smaller than that predicted by electrostatic theory (Tivant *et al.*, 1983), suggest that all heparin–cation interactions may not be explicable in terms of simple electrostatic interactions. Moreover, in situations in which small amounts of particular metal ions together with the polyelectrolyte exert specific effects, as may be the case, for example, with Cu^{2+}–heparin complexes in angiogenesis (Raju *et al.*, 1982; Alessandri *et al.*, 1983), in anti-tumour activity (Bergendal *et al.*, 1988) and in the fractionation (Shing, 1988) and modulation of the activity (Folkman & Klagsbrun, 1987) of growth factors, a more specific type of binding might be suspected.

Further, analysis of the optical-rotation changes that occurred on addition of Ca^{2+} or Cu^{2+} ions to heparin revealed curves, analogous to adsorption isotherms, which were linear at [metal ion]/[heparin disaccharide] ratios of less than 0.5 (Grant *et al.*, 1991*c*,*d*). This linearity, together with a near-coincidence of the linear plots obtained in the presence of various heparin concentrations, suggests that, under the conditions used, metal-ion–heparin interaction occurred by a mechanism that cannot be described adequately in terms of conventional solution-phase reversible equilibrium thermodynamics.

Potentiometric titration studies, by providing information complementary to that available from experiments involving i.r. spectroscopy (Grant *et al.*, 1987), n.m.r. spectroscopy (Grant *et al.*, 1991*a*,*b*,*c*,*d*), equilibrium dialysis (Grant *et al.*, 1986; Woodhead *et al.*, 1986) and optical rotation (Grant *et al.*, 1991*b*,*d*), might enhance an understanding of the processes involved in the binding of cations to heparin. The technique is accurate and rapid, and has been used elsewhere in the study of other oligo- and poly-anions (Van Wazer & Campanella, 1950; Lambert & Watters, 1957; Rinaudo & Milas, 1975; Aplincourt *et al.*, 1990) and, in a preliminary way, of heparin (Villiers *et al.*, 1980).

EXPERIMENTAL

The source, preparation and properties of the heparin used in most of the experiments have been described previously (Grant *et al.*, 1987). The experiments reported in Fig. 1 (below) involved an Na^+-heparin from Evans Pharmaceuticals, Speke, Liverpool, U.K. (lot no. 184113); it showed similar potentiometric titration properties before and after percolation through an Amberlite IR-120 cation-exchange column, indicating the absence of cations other than Na^+. An average heparin disaccharide unit was taken to be tetrasodium or tetralithium 2-*O*-sulphatoiduronosyl 6-*O*-sulphatoglucosamine-2-*N*-sulphate. The sodium-containing and the lithium-containing disaccharide units were taken to be hexadecahydrated and decahydrated respectively (Grant *et al.*, 1990). It is acknowledged that polysaccharide heterogeneity means that the assumed structure and hydrations allow only 'conventional' M_r values, of average disaccharide units, to be deduced. M_r values so deduced were used to calculate the ratios plotted as ordinates in Figs. 1–4 (below). Other reagents used were of Analar grade, except for tetramethylammonium chloride, which was of reagent grade; all were from BDH Ltd., Poole, Dorset, U.K.

pH changes were measured by using a Pye–Unicam 291 Mark 2 pH-meter fitted with an electrode from Russell pH Ltd., Auchtermuchty, Fife, Scotland, U.K. Glass-distilled water used in the preparation of all the solutions was boiled to remove CO_2 and then cooled under O_2-free N_2. Titrations were conducted in thermostatically controlled polypropylene containers.

RESULTS AND DISCUSSION

In the equilibrium binding of cations to polyanions as described in eqn. (1):

$$x\mathrm{M} + \text{polyanion} \overset{k}{\rightleftharpoons} \text{complex} + y\mathrm{H} \quad (1)$$

the association constant, k, defined in eqn. (2):

$$k = [\text{complex}]\cdot[\mathrm{H}]^y/[\mathrm{M}]^x\cdot[\text{polyanion}] \quad (2)$$

may be derived directly from potentiometric titration curves (Van Wazer & Campanella, 1950), provided that the values of x, y and the activities of the other components of the equilibrium can be experimentally determined. However, in the case of heparin–cation interaction, it is doubtful whether the process may be properly regarded as involving simple reversible equilibrium thermodynamics (Grant *et al.*, 1991*c*,*d*). In this case, the use of a selectivity constant, rather than an equilibrium constant, is probably more appropriate. Elsewhere, changes in optical-rotation values observed during cation–heparin interaction have been used to derive selectivity constants for cation binding to heparin, which varied in the order: tetramethylammonium

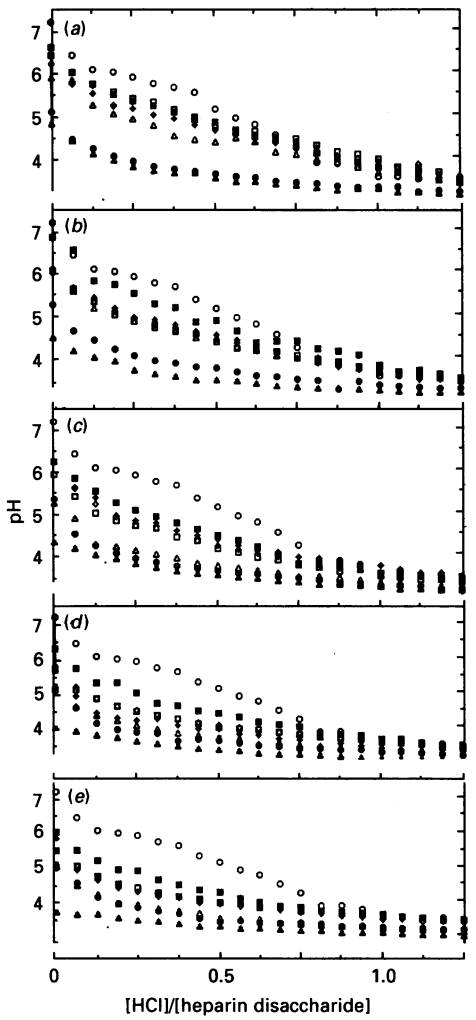

Fig. 1. Potentiometric titration of Na^+-heparin with HCl in the presence of electrolytes at set (initial) values of *I*

Aliquots (0.025 ml) of HCl (0.05 M) were added to 30 ml of salt-containing heparin (0.059 M) solutions. Solutions were continuously mixed by sparging with N_2. Values of *I* were: (*a*) 0.0057; (*b*) 0.017; (*c*) 0.050; (*d*) 0.160; (*e*) 0.336. *I* was generated by use of the following salts: ◆, NaCl; □, KCl; ◇, LiCl; △, $MgCl_2$; ▲, $CuCl_2$; ●, $CaCl_2$; ■, tetramethylammonium chloride; ○, no added salt. Titrations were carried out at 37 °C.

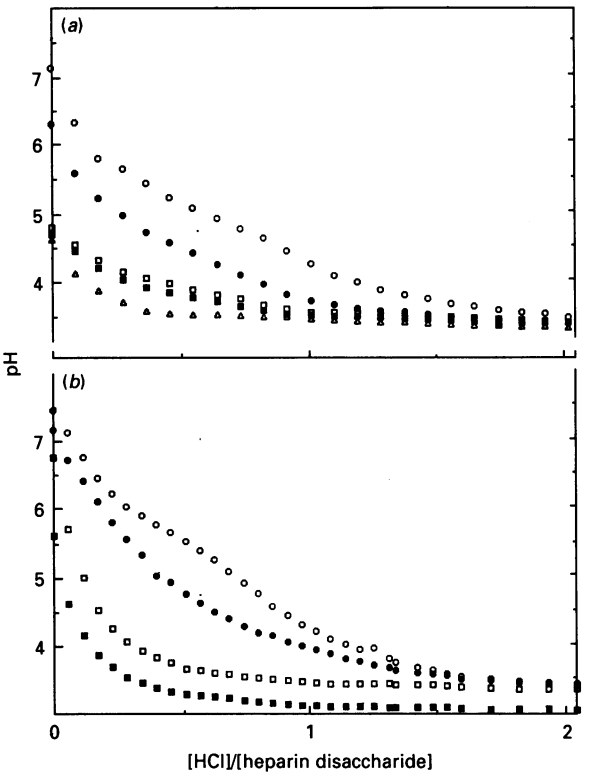

Fig. 2. Potentiometric titration with HCl of (*a*) Li^+-heparin in the presence of $CuSO_4$ and (*b*) Na^+-heparin in the presence of $CaCl_2$

(*a*) $CuSO_4,5H_2O$ solution in water was added to 30 ml of a solution of Li^+-heparin (0.25 mg/ml) in water, to give the following final Cu^{2+}/heparin disaccharide ratios: ○, 0; ●, 0.29; □, 2.20; ■, 4.30 (in a volume of 30 ml). To each mixture, 0.01 M-HCl in water was then added in 0.1 ml portions. Mixing was as described in the legend to Fig. 1. The △ curve was generated as described in Fig. 1, except that 1 ml of a 7.5 mg/ml solution of Li^+-heparin was added to 30 ml of a 0.3507 mg/ml solution of $CuSO_4,5H_2O$ to give a final [Cu^{2+}]/[heparin disaccharide] ratio of 4.30. (*b*) Experimental details were as described above, except that $CaCl_2,2H_2O$ was added to a solution of Na^+-heparin (2.5 mg/ml), to give the following final [Ca^{2+}]/[heparin disaccharide] ratios: ○, 0; ●, 0.28; □, 2.8; ■, 21.0 (in a volume of 30 ml).

ion < (K^+, Li^+, Mg^{2+}, Na^+) < Ca^{2+} < Cu^{2+} (D. Grant, W. F. Long & F. B. Williamson, unpublished work). Small soluble aliphatic quaternary ammonium cations do not readily bind to heparin or to other polyanions (Van Wazer & Campanella, 1950; Van Wazer & Callis, 1958; Dais *et al.*, 1988) and are usefully employed as control counter-cations in binding studies.

The work of Dunstone (1962) suggested the paramount importance of the value of solution ionic strength (*I*) in determining the strength of cation binding to glycosaminoglycans. It was therefore of interest to determine the effect of a variation of *I* on the potentiometric titration of heparin in the presence of a variety of cations; a strong dependence of binding on *I* would accord with an important role for electrostatic interaction in the binding, as predicted by ion-condensation concepts (Manning, 1969*a*,*b*,*c*, 1977, 1978).

The potentiometric titration of Na^+-heparin, at (initial) *I* values of 0.336, 0.160, 0.050, 0.017 and 0.0057, generated with solutions of $CaCl_2$, $CuCl_2$, KCl, LiCl, $MgCl_2$, NaCl and tetramethylammonium chloride, and carried out at 37 °C, are shown in Fig. 1. Similar results (not shown) were obtained at 25 °C. The results suggest that the binding of cations to heparin is less dependent upon the value of *I* than on the individual binding characteristics of the cation. Of the cations studied, Ca^{2+} and Cu^{2+} had the greatest effect upon the potentiometric titration curves at all the values of *I* studied. However, the curves produced in the presence of these cations were less affected by a change in *I* than were curves obtained when the *I* values were generated by tetramethylammonium chloride. Binding of Mg^{2+} appeared to be weaker at the lower values of *I* studied, in the presence of which the effect of this cation on the curves was similar to that of a univalent cation. This may have biological relevance: heparin and related glycosaminoglycans may retain Mg^{2+} at high values of *I*, but release the cation in biological compartments in which the value of *I* is lower. For the binding of K^+, a similar situation was observed at the lowest of the *I* values studied.

For all of the salts added, and at all of the values of *I* examined, the S-shaped part of the curve associated with the process of carboxylate protonation was flattened. This indicates that the protonation equilibrium had been perturbed, perhaps, for example, through a diminished ability of protons to bind to individual carboxylate groups. The *I* value at which this apparent

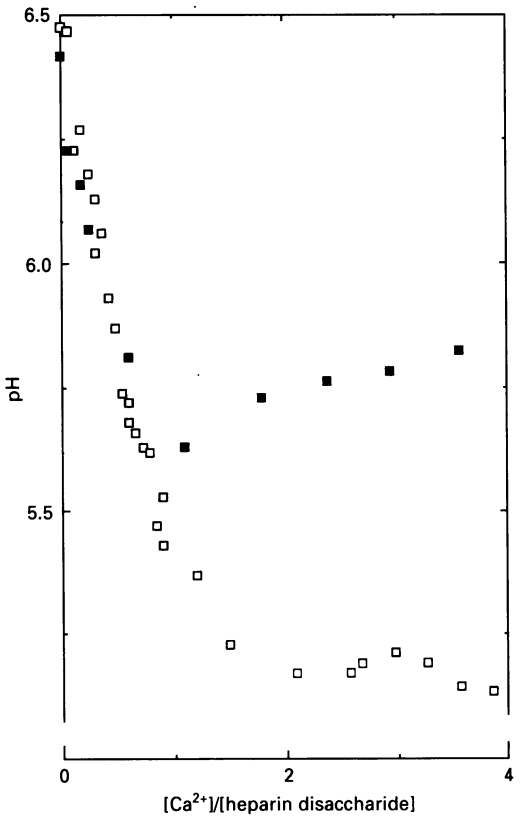

Fig. 3. pH on addition of $CaCl_2$ solution to Na^+-heparin as a function of [total Ca^{2+}]/[heparin disaccharide]

Portions (0.005 ml) of $CaCl_2$ (0.4 M) solution were added to 2 ml of Na^+-heparin (0.05 M). After the addition, solutions were mixed by: □, gentle inversion of the mixing vessel; ■, vigorous sparging with N_2, followed by vigorous stirring.

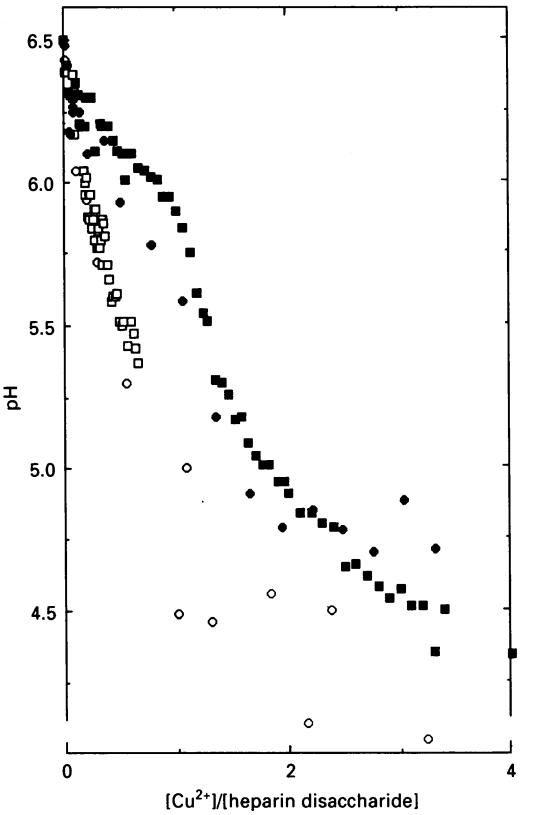

Fig. 4. pH on addition of $CuSO_4$ solution to Li^+- or Na^+-heparin as a function of total [Cu^{2+}]/[heparin disaccharide]

Experimental conditions were as described in the legend to Fig. 3, except that $CuSO_4$ was added to Li^+- (○, ●) or Na^+- (□, ■) heparin with gentle (○, □) or vigorous mixing (●, ■).

change in carboxylate-group–proton interaction occurred was, of course, that of the micro-environment in the immediate vicinity of the heparin molecule itself, and this is unlikely to be identical with the average value of I throughout the entire solution. A saturation of added electrolyte at such heparin surface environments may occur, since, for particular cations, similar perturbations were generally observed, regardless of the value of I. This situation differs from the usual 'salt effect' as described, for example, by Netter (1969), in which salt addition decreases carboxylate pK_a values without otherwise altering the proton–carboxylate binding equilibrium.

Fig. 2(*a*) shows the results of an experiment in which an Li^+-heparin solution was titrated with standard HCl, either alone or in the presence of one or the other of several different concentrations of $CuSO_4$. Fig. 2(*b*) shows the results of a similar experiment in which Na^+-heparin was titrated in the presence of $CaCl_2$. The results in Fig. 1 suggest that differences between the individual curves in Fig. 2(*a*) and in Fig. 2(*b*) do not arise because of differences in I within the different mixtures; the different shapes of the curves within Fig. 2(*a*) and within Fig. 2(*b*) accord with this. Instead, the variations in the curves appear to arise from differing abilities of heparin carboxylates to undergo proton exchange at different [metal ion]/[heparin disaccharide] ratios. The results obtained by Villiers *et al.* (1980) led them to a similar conclusion.

During the study of Cu^{2+}-heparin, an alteration in the curve observed when the order of addition of the components of the mixture in solution was reversed (as is the case for the results represented by the symbols ■ and △ in Fig. 2*a*) indicated that cation binding under these conditions may not be completely reversible.

Fig. 3 shows the decrease in pH observed upon addition of $CaCl_2$ solution to Na^+-heparin in water initially at pH 6.5. Fig. 4 shows a similar experiment, in which $CuSO_4$ solution was added to Li^+-heparin in water initially at pH 6.5. (The identity of the univalent counter-cation used did not affect the resultant curves.) In Figs. 3 and 4 (open symbols), at low [metal ion]/[heparin disaccharide] ratios, a linear relationship appears to exist between the pH change and the [metal ion]/[heparin disaccharide] ratio. Linearity in analogous polarimetry-derived plots has been interpreted in terms of the existence, at such [metal ion]/[heparin disaccharide] ratios, of a cation–polyanion complex that may resemble a colloid-like phase and is not subject to simple aqueous solution equilibrium processes (Grant *et al.*, 1991*c*,*d*).

The shape of the curves obtained upon addition of salt to heparin was dependent upon the precise details of the experimental technique used. Under defined conditions (gentle mixing of the salt with the heparin), graphs of which Fig. 3 (open symbols) and Fig. 4 (open symbols) are representative, were reproducibly observed. Under other experimental conditions (which included more vigorous mixing), graphs of which Fig. 3 (closed symbols) and Fig. 4 (closed symbols) are representative, were reproducibly observed. The patterns of pH change suggest that, particularly for Cu^{2+} under these conditions, a series of transitions between several discrete metal ion–heparin complexes occurs, as is suggested by the work of Burger *et al.* (1984).

We have already suggested that, if particular cation–heparin interactions indeed result in the partition of cations between

more than one phase, exchanges between which do not occur through simple reversible thermodynamic equilibrium processes, then the kinetics of phase formation will be critical, and only very precise techniques, in which, for example, nucleation events are controlled, will allow reliable results to be achieved. It is likely that the incompletely reversible Cu^{2+}-binding pattern revealed in Fig. 2(*a*), and the non-identical Cu^{2+} binding patterns reproducibly produced under slightly different experimental conditions (Fig. 4), are manifestations of this dependence of cation binding on the kinetics of the molecular events leading to complex-formation.

Melting and diffusion of ions across hydrate barriers and the nucleation of specific cation–heparin phases may, therefore, be rate- and process-determining in the experiments reported here. Bernal (1965) suggested that ion penetration of biopolymer surfaces might be hindered by surface hydration. Such effects may be related to the different amounts of heparin-associated water molecules seen in the presence of different cations, and to the dependency on cation identity of the vibrational characteristics of these water molecules (Grant *et al.*, 1987, 1990, 1991*b*); they may also be related to perturbation of the equilibrium that exists between different conformational forms of the heparin iduronate residues. A cation-dependent 'hard water' barrier is therefore an attractive model in the context of which the hints of thermodynamic irreversibility revealed by these and earlier experiments might be discussed.

The source of the protons released during cation binding in neutral solutions of heparin is not clear. In the experiments described here, protons may be generated by a direct or indirect replacement of covalently bound protons by cations (Grant *et al.*, 1988). Some of these protons may be present in hydrogen-bonded structures, including those occurring in heparin-associated water molecules. Proton release might also occur by an alteration, in the presence of cations, of the pK_a values of unhindered or previously occluded carboxy groups. Further, proton release may occur as a consequence of cation hydrolysis and/or polymerization. This latter possibility accords with the results shown in Figs. 2(*a*) and 4, which may be due in part to the effect of locally high [cation] in the vicinity of the polymer: in these circumstances, Cu^{2+} polymerization in particular may be favoured. The formation of Cu^{2+} polymers in solutions containing Cu^{2+} and heparin may also provide an explanation for the observed induction by protons of paramagnetic interactions between Cu^{2+} and the ^1H or ^{13}C atoms of heparin through a disruption by H^+ of Cu–O–Cu bridges (Rej *et al.*, 1990; Grant *et al.*, 1991*d*). Figs. 3 and 4 suggest that, whereas curves generated under gentle mixing conditions may result from proton release upon cation complexation with the polyanion, under more vigorous mixing conditions a combination of both proton release and proton absorption may occur, perhaps because heparin carboxylates which are otherwise occluded become more amenable to proton exchange.

In summary, these results suggest that cation binding to heparin cannot be described adequately in terms of simple electrostatic concepts; nor can it be described in terms of simple equilibrium processes. The occurrence of possible inflections in curves obtained by potentiometric titration of heparin supplements similar results from equilibrium dialysis (Grant *et al.*, 1986) and polarimetry (Grant *et al.*, 1991*c*) experiments, and accords with the suggestion (Burger *et al.*, 1984) that multiple discrete cation–heparin complex phases may exist, the nature and proportions of which depend upon the conditions under which the interactions occur. These are likely to be, at least in part, dependent upon the nucleation of specific forms of cation–heparin structures.

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