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COUNTER-ION DEPENDENCE IN SALT-CONTAINING, AQUEOUS SOLUTIONS OF HEPARIN*

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ABSTRACT

The effects of various salts (alkaline and alkaline-earth chlorides) on the 210-nm c.d. band of heparin for various values of the degree of neutralization of sulfate and carboxylic groups, and for different counter-ions in aqueous solution were investigated. Variations of ellipticity were related to the acid–base properties of uronic carboxylic acid groups. Furthermore, the determination of ellipticity at 210 nm appears to be a suitable method for monitoring the counter-ion–exchange at uronic sites of a given heparinate in the presence of salts with different cations. It was shown that sodium heparinate, in the presence of a small amount of Ca^{2+} ions (provided either by calcium heparinate or by calcium chloride), exchanges some of its sodium for calcium counter-ions with redistribution, to yield divalent counter-ions balancing uronic carboxylic negative charges preferentially. This intramolecular affinity of carboxylic groups for divalent cations may be of considerable importance for protein–heparin interactions in salt-containing, body fluids where heparin takes the form of a mixed heparinate.

INTRODUCTION

In a previous paper[1], we reported pH-induced, chiroptical-property changes of heparinic acid, the polyacid form of heparin, for various counter-ions (Na^+, Mg^{2+}, Ca^{2+}, and Ba^{2+}) in salt-free aqueous solutions. For Na^+, pH-induced ellipticity changes at 210 nm were assigned to acid–base properties of uronic carboxylic groups, as already proposed[2,3]. For alkaline-earth counter-ions, we showed that c.d. variations at 210 nm are related to the ionization behavior of CO_2H groups too[1]. This conclusion was drawn from c.d. titration curves, *i.e.*, plots of $[\theta']_{210}^{\ddagger}$ against the degree of neutralization of heparinic acid ($\bar{\alpha}$). On the other hand, differences in magnitude of

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*Acid–base and chiroptical properties of heparinic acid and heparinates, Part II; for Part I, see ref. 1.

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‡Molar ellipticity expressed for one equiv.-gram of heparinic acid. The reason for the use of $[\theta']_{210}$, instead of the usual monomolar ellipticity, has already been given in ref. 1.

the 210-nm c.d. band of heparinates have been considered as reflecting the nature of counter-ions that balance negative charges due to uronic carboxylate groups. Therefore, it was concluded that ellipticity measurements at 210 nm can be used to monitor the ionization state of CO_2H chromophores in the various forms of heparin (heparinic acid or heparinates) in salt-free aqueous solutions, whatever the counter-ion.

When used as an anticoagulant, heparin is actually in the presence of various cationic species, especially mineral cations such as Na^+, Ca^{2+}, Mg^{2+}, etc., at different concentrations. Therefore, it was of interest to investigate acid–base properties of heparinic acid and of its salts in salt-containing aqueous solutions. In this paper, we report on the polyelectrolytic properties of heparinic acid and of some heparinates in salt-containing systems, as monitored by c.d. spectrometry. This technique allowed to distinguish $-CO_2^-$ from $-SO_3^-$ groups, in so far as their ionization and the nature of counter-ions are concerned. The effect of a salt with the same cation as the titrating reagent on c.d. of heparinic acid, at various values of the degree of neutralization, was first investigated, with a special interest for heparinic acid solutions at $\bar{\alpha}$ 0, in the presence of sodium or calcium chloride. Subsequently, the effects of salts with a different cation and finally, the particular case of mixtures of two different heparinates were considered.

EXPERIMENTAL

Chemicals. — Heparin was obtained as calcium salt from Choay Chimie (for characteristics, see ref. 1). Samples of heparinic acid and of the various heparinates were obtained by ion exchange according to a process previously described[1]. The mineral salts and chemical reagents were of analytical grade.

Methods. — Ellipticity was measured with a Jasco J 40 B dichrometer at room temperature by setting the monochromator at 210 nm. The double monochromator and the cell compartment were purged with nitrogen. Data have been expressed in term of equiv. ellipticity $[\theta'] = 10\ (\theta)_{meas.}/l.T_N$, with $(\theta)_{meas.}$ being the measured ellipticity in degrees, l the pathlength in dm, and T_N the normality of the solution as deduced from the potentiometric titration curve of the heparinic acid solution obtained by percolating the heparinate solution through a column of cation-exchange resin (H^+).

Solutions of heparinic acid at various pH, and solutions of mixed heparinates, were obtained from titrated stock solutions combined in suitable proportions to give the desired systems. This method was already proved as suitable since it gave the same results as direct neutralization.

RESULTS AND DISCUSSION

Fig. 1 represents dichroic titration curves of heparinic acid neutralized by sodium and calcium hydroxide in the presence of a salt with the same cation, *i.e.*, sodium and calcium chloride, respectively, at various concentrations. C.d. titration

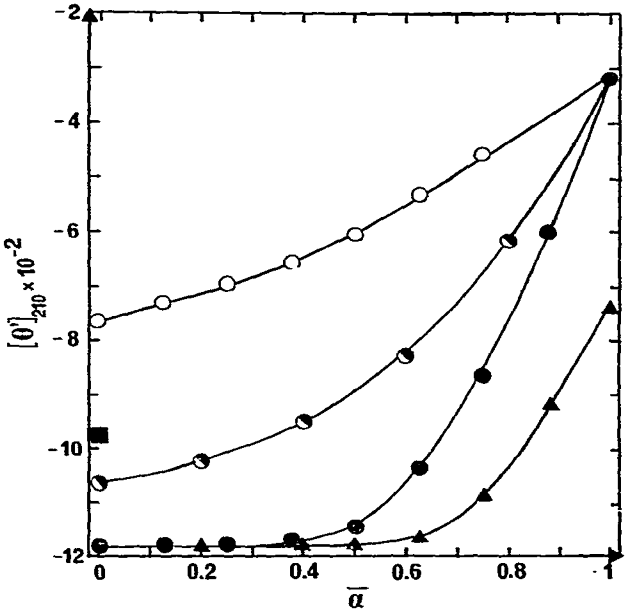

Fig. 1. Effects of salts on circular dichroic titration curves of heparinic acid in aqueous solution (T_N mN): (▲) NaOH in salt-free solution and in 0.1M NaCl; (●) $Ca(OH)_2$ in salt-free solution; (◐) $Ca(OH)_2$ in 2.5 mM $CaCl_2$; (○) $Ca(OH)_2$ in 25 mM $CaCl_2$; and (■) $Ca(OH)_2$ in 2.5 mM $CaCl_2$ (T_N 2.5 mN).

curves in salt-free solutions have been also plotted in Fig. 1. For the Na^+ system, at the selected polymer concentration, a single titration curve was found regardless of the sodium chloride concentration up to 0.1M. It is of value to point out that $[\theta']_{210}$ for $\bar{\alpha}$ 1 (sodium heparinate) was independent of the sodium chloride concentration. This finding contrasts with the rather high difference (20%) observed by Ching Ming Chuong and Ellerton[4] between pure water and 0.1M sodium chloride solutions of heparin (Na^+ form from Weddel Pharm. Ltd.). The discrepancy could not be simply explained through available data.

Contrary to sodium chloride, calcium chloride drastically affected the c.d. titration curve of heparinic acid in salt-free aqueous solution. An increase of the calcium chloride concentration resulted in different c.d. titration curves which nevertheless joined for $\bar{\alpha}$ 1 (calcium heparinate). The circular dichroism value $[\theta']_{210}$, as the whole c.d. spectrum of calcium heparinate, was independent of both the polymer concentration and the calcium chloride concentration up to 0.1M. In contrast, for heparinic acid ($\bar{\alpha}$ 0), both concentrations affected $[\theta']_{210}$ drastically. At a given polymer concentration, lower negative $[\theta']_{210}$ values correspond to higher salt concentrations. At a fixed calcium chloride concentration, lower negative $[\theta']_{210}$ values correspond to lower polymer concentrations. Similar trends were observed for intermediate $\bar{\alpha}$ values, *i.e.*, for partially neutralized heparinic acid. Dilution of calcium chloride–heparinic acid systems with a fixed ratio, $R = T_{N\ CaCl_2}/T_{N\ hep.}$ changed $[\theta']_{210}$ too.

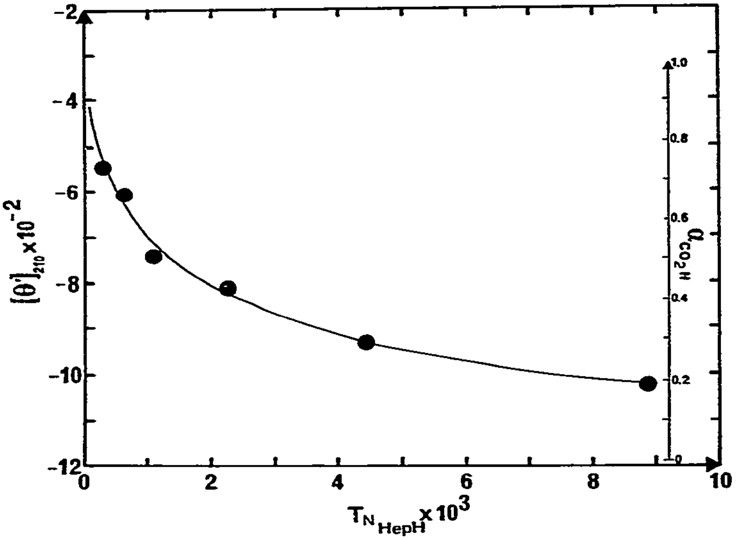

Fig. 2. Variations of equivalent ellipticity $[\theta']_{210}$ with concentration for a constant ratio of equiv. $CaCl_2$ to equiv. heparinic acid, *R* 100.

The lower the concentration, the lower the negative $[\theta']_{210}$ value, as exemplified in Fig. 2 for heparinic acid ($\bar{\alpha}$ 0, *R* 100). It is remarkable that, for infinite dilution, $[\theta']_{210}$ tended to —315°, the value which is characteristic of calcium heparinate. That trend was not fortuitous because solutions of heparinic acid showing the same $[\theta']_{210}$ values presented identical c.d. spectra (based on number, shape, magnitude, and location of c.d. bands). This observation was made regardless of the way the solutions were made (addition of calcium chloride, or modification of the degree of neutralization of salt-free heparinic acid solutions, or both). Since the 210-nm c.d. band indicates the ionization state of CO_2H chromophores for salt-free solutions, the identity of c.d. spectra led us to the conclusion that, in the presence of calcium chloride, heparinic acid is no longer in the polyacid form. This observation and the dependence of the 210-nm c.d. band on polymer- and salt-concentration are in favor of a strong Ca^{2+}–polyanion interaction and of an equilibrium control for calcium chloride-induced c.d. changes. The existence of a chemically and conformationally well defined complex had been previously precluded, on the basis of pH and counter-ion dependences of heparinic acid chiroptical properties in salt-free solutions[1]. However, it is known that carboxylic polymers, such as polyacrylic acid, bind calcium strongly[5]. For example, polyacrylic acid is readily precipitated by the addition of a small concentration of calcium, even at a high concentration of sodium chloride, because of formation of a series of complexes with closely located groups carried by the same macromolecules or by different ones. As a carboxylic polyacid, heparinic acid can also interact with divalent counter-ions. However, sulfate groups impose a high negative fixed charge which prevents macromolecules from precipitating, since the sulfate anion does not bind divalent cations so tightly as the carboxylate anion[6].

Because of these nonspecific interactions, it is possible that some of the uronic carboxylic groups of heparinic acid may be ionized in the presence of calcium chloride, to yield macromolecules which behave, from the chiroptical property viewpoint, like heparinic acid, that is, partially neutralized by Ca^{2+} and showing the same $[\theta']_{210}$ value in salt-free systems. The acid-dependence of calcium chloride-induced c.d. changes of heparinic acid support this interpretation and especially the involvement of H^+ ions in the interaction process. Indeed, addition of hydrochloric acid to a calcium chloride–heparinic acid solution (T_N 0.00111; R 50; $[\theta']_{210}$ −790°) shifted $[\theta']_{210}$ back to −1180°, *i.e.*, the value corresponding to heparinic acid in salt-free solution.

Attempts to correlate interacting species concentrations (heparin, Ca^{2+}, and H^+) through association constants corresponding to 1:1 and 1:2 equilibria failed, as it is usually the case for complexing polyelectrolytes[7]. However, it has been already shown that the degree of ionization of contributing CO_2H chromophores can be evaluated from $[\theta']_{210}$ changes by use of eq. (*1*),

$$\alpha_{CO_2H} = ([\theta']_{210} - [\theta'_o]_{210})/([\theta'_s]_{210} - [\theta'_o]_{210}) \qquad (1)$$

where $[\theta']_{210}$ is the equiv. ellipticity for a given degree of neutralization $\bar{\alpha}$, and $[\theta'_o]_{210}$ and $[\theta'_s]_{210}$ are values of equiv. ellipticity for heparinic acid at $\bar{\alpha}$ 0 in salt-free solution, and at $\bar{\alpha}$ 1 (heparinates), respectively[1].

Equation (*1*), expressed with regard to pH, was shown to be correct for Na^+ counter-ions with the restriction that ellipticity changes at 210 nm are primarily due to ionization of the carboxylic chromophore of L-iduronic acid 2-sulfate residues[2,3]. This restriction was based on two observations: (a) the D-glucuronic carboxyl chromophore does not contribute significantly to the ellipticity at 210 nm, as compared to other glycosaminoglycans, and (b) pK_a values of D-glucuronic and L-iduronic carboxyl groups differ by ~1.7 pK unit, as only the value of the latter group is weakened by the high electrostatic field due to sulfate charges. Actually, both groups are subjected to the polyanion electrostatic field, and they certainly ionize in the same range of pH values. For this reason, and because separate c.d. contributions of D-glucuronic and L-iduronic carboxyl chromophores were not observed in salt-free solutions of our sample of heparin, whatever the counter-ion, the observed c.d. changes have been considered (at least until the predominant contribution of L-iduronic units is conclusively shown) as average ones that reflect the total content in carboxylic groups[1].

Therefore, by use of eq. (*1*) for $[\theta'_o]_{210}$ −1180° and $[\theta'_s]_{210} = [\theta'_{Ca}]_{210} = -315°$, the values of $[\theta']_{210}$ reported in Fig. 2 were converted to α_{CO_2H} values (right-hand side of the inner scale). On the basis of this evaluation, ~50% of the uronic acid groups were ionized for a 1mM heparinic acid solution containing 100mM calcium chloride, whereas none was ionized for salt-free or sodium chloride-containing solutions. The observation that $[\theta']_{210}$ tends to the value characteristic for calcium heparinate when the solution is diluted at a constant R ratio (Fig. 2) qualitatively supports the proposed interpretation of salt-induced c.d. changes for heparinic acid,

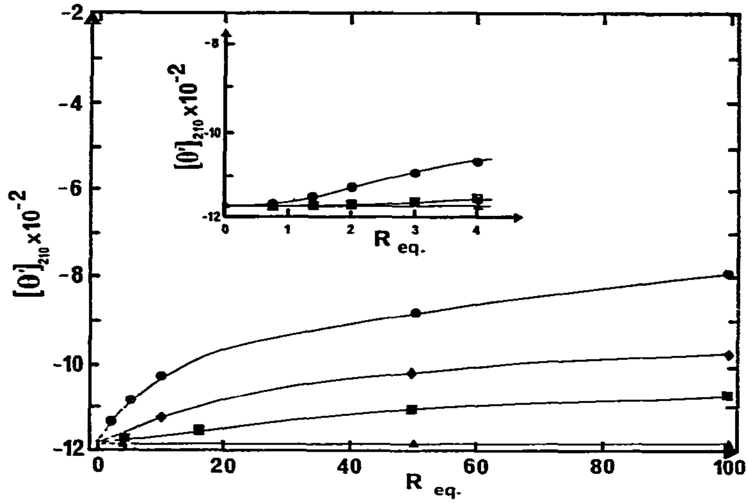

Fig. 3. Variations of $[\theta']_{210}$ for heparinic acid (T_N 2.5 mM) in the presence of various chloride salts (R = equiv. salt to equiv. heparinic acid ratio (▲) Na^+, (■) Mg^{2+}, (◆) Ba^{2+}, and (●) Ca^{2+}.

TABLE I

EQUIVALENT ELLIPTICITY $[\theta_S']_{210}$ OF VARIOUS HEPARINATES FOR SALT-FREE AND 0.1M CHLORIDE-CONTAINING AQUEOUS SOLUTIONS

Cation (M)	$[\theta_S']_{210}$	
	Water	0.1M *MCl*
Li^+	−625 ±20	
Na^+	−740 ±20	−730 ±50[a]
K^+	−800 ±20	−800 ±50
Mg^{2+}	−580 ±20	−550 ±50
Ca^{2+}	−315 ±20	−320 ±50
Sr^{2+}	−460 ±20	
Ba^{2+}	−545 ±20	

[a] The poor accuracy was due to the presence of the salt which shifted the cut-off of the solvent to a higher wavelength.

as dilution lowered the H^+ concentration and, thus, favorized the complex formation, *i.e.*, the ionization of carboxylic groups.

Salt-induced c.d. changes for heparinic acid are cation-dependent, as shown in Fig. 3, where the variations of $[\theta']_{210}$ for increasing amounts of different chlorides ($CaCl_2$, $MgCl_2$, $BaCl_2$, and NaCl) plotted against R· $[\theta']_{210}$ did not change in the presence of sodium chloride whatever the R ratio value. In the presence of barium and magnesium chloride, ellipticity progressively decreased as R increased, in a way that was very similar to that found for calcium chloride. For a given R value, the variations increased according to the series $Na^+ < Mg^{2+} < Ba^{2+} < Ca^{2+}$. This

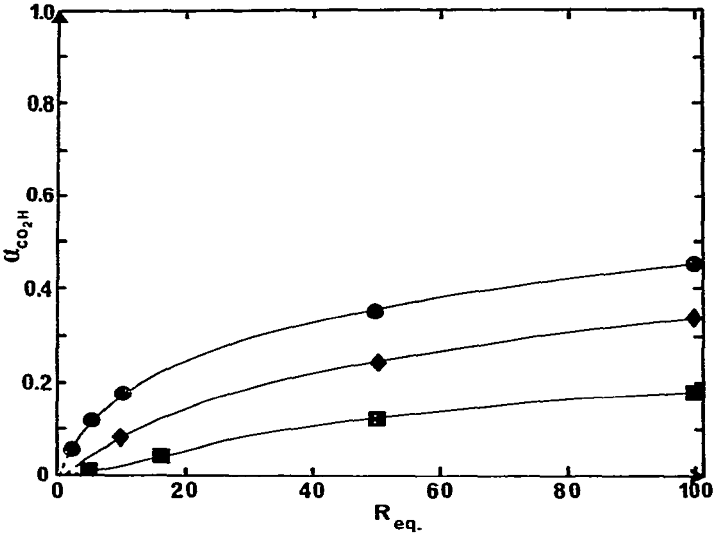

Fig. 4. Values of the degree of ionization α_{CO_2H} of uronic carboxyl groups of heparinic acid (T_N 2.5mN) in the presence of increasing amounts of various chloride salts, as deduced from data in Fig. 3 and eq. (*1*): (■) Mg^{2+}, (◆) Ba^{2+}, and (●) Ca^{2+}.

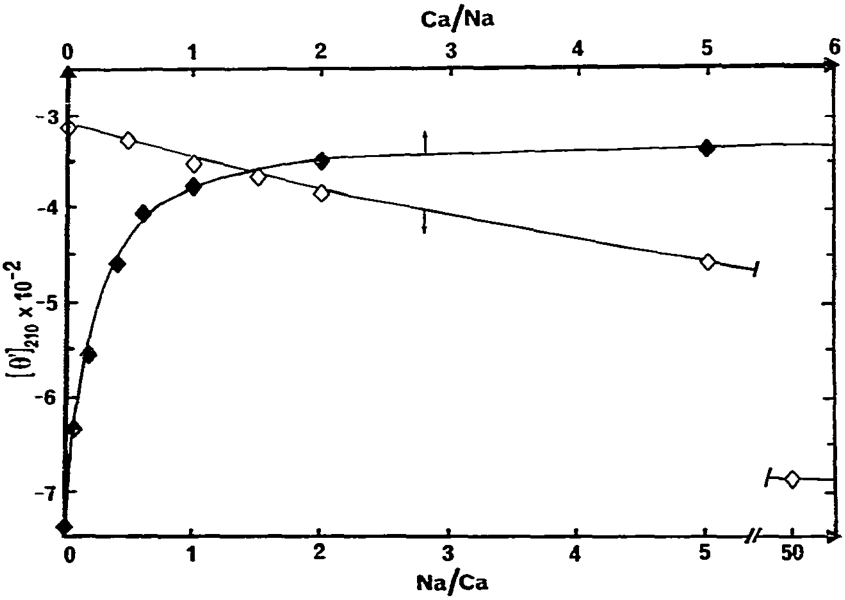

Fig. 5. Variations of $[\theta']_{210}$ for sodium heparinate (T_N 26mN) in the presence of increasing amounts of calcium chloride defined by the normality ratio Ca to Na (◆), and for calcium heparinate (T_N 26mN) in the presence of increasing amounts of sodium chloride defined by the normality ratio Na to Ca (◇).

series was also valid when the minimum R value where a $[\theta']_{210}$-change becomes detectable was considered. Values of α_{CO_2H} corresponding to $[\theta']_{210}$ changes reported in Fig. 3 were evaluated by use of eq. (*1*), and $[\theta'_s]_{210}$ values are given in Table I. The resulting curves are shown in Fig. 4. The series $Na^+ < Mg^{2+} < Ba^{2+} < Ca^{2+}$ agrees well with that deduced from ion-exchange[8] and ^{23}Na-n.m.r.[9] determinations of the affinity of heparin for various cations. Therefore, it is concluded that uronic carboxylic groups play a significant role in the affinity of heparin for divalent cations, and especially for calcium ones.

For salt–heparinate systems with the same cation, $[\theta']_{210}$ was not affected by the salt concentration. In contrast, significant changes were observed when salt and heparinate contained different cations. Fig. 5 represents variations of $[\theta']_{210}$ for sodium heparinate in the presence of increasing amounts of calcium chloride as defined by the ratio Ca^{2+} to Na^+ expressed with regard to normalities. The value of $[\theta']_{210}$, which initially corresponded to the value characteristic of sodium heparinate, decreased very fast and almost linearly as the Ca^{2+} to Na^+ ratio rose up to 0.4–0.5. Then, it tended asymptotically to —315°, the value characteristic for calcium heparinate. A completely different trend was observed for calcium heparinate in the presence of increasing amounts of sodium chloride (Fig. 5). The value of $[\theta']_{210}$, which corresponded initially to the value characteristic for calcium heparinate, increased slowly as the Na to Ca ratio increased. Measurements for high values of that ratio showed that $[\theta']_{210}$ tended asymptotically to $[\theta'_{Na}]_{210}$ ($[\theta']_{210}$ —680° for Na to Ca 50:1).

The ellipticity of sodium heparinate, in the presence of an excess of calcium chloride, and the ellipticity of calcium heparinate, in the presence of a large excess of sodium chloride, tended respectively to values characteristic of Ca^{2+} and Na^+ heparinates (on the basis of the 210-nm c.d. band, the cation-dependence of which has been previously discussed in detail[1]). This observation suggests that the variations of $[\theta']_{210}$ plotted in Fig. 5 reflect $Na^+ \leftrightarrow Ca^{2+}$ ion-exchange, and that intermediate $[\theta']_{210}$ values reflect the cation composition of the resulting sodium and calcium mixed-heparinate macromolecules.

In order to verify this hypothesis, the optical activity of solutions of calcium heparinate containing various amounts of lithium, sodium, or potassium heparinates in the absence of any other salt, and at constant normality in heparin, was determined. Usually, the molar chiroptical parameters (optical rotatory power or ellipticity at a given wavelength) of a mixture of noninteracting, optically active species varies linearly with the composition according to eq. (*2*),

$$Z = X_1 \cdot [Z_1] + X_2 \cdot [Z_2] = X_1 \cdot [Z_1] + (1 - X_1) \cdot [Z_2] \qquad (2)$$

Z is the considered specific parameter, and X_1 and X_2 are the molar fractions of optically active species 1 and 2. For mixtures of different M_1 and M_2 heparinates, the resulting mixed heparinates can be considered, from the viewpoint of the 210-nm c.d. band, as a copolymer of ionized uronic units with either M_1 or M_2 counter-ions.

Accordingly, for mixtures of calcium heparinate with alkaline heparinates, eq. *2* becomes eq. *3*,

$$[\theta'_{\mathrm{Ca,M}}]_{210} = X_{\mathrm{Ca}} \cdot [\theta'_{\mathrm{Ca}}]_{210} + (1 - X_{\mathrm{Ca}}) \cdot [\theta'_{\mathrm{M}}]_{210} \qquad (3)$$

where X_{Ca} is the molar fraction of calcium heparinate in the mixture. Actually, for the three couples of heparinates considered, $[\theta']_{210}$ did not vary linearly with X_{Ca} and, thus, the c.d. changes did not obey eq. (*3*). This discrepancy might be assigned to the dependence of $[\theta'_{\mathrm{Ca}}]_{210}$ and $[\theta'_{\mathrm{M}}]_{210}$ on the composition, because of the neighboring effects of adjacent units, as reported for optically active acrylic acid–chiral acrylamide copolymers[10], and for partially neutralized, optically active carboxylic polyelectrolytes[11]. However, this interpretation is not acceptable for two reasons: (a) the uronic units, which are separated by one co-unit in heparinic compounds, are far from each other, and should not interfere. (b) Constant $[\theta'_{\mathrm{H}}]_{210}$ and $[\theta'_{\mathrm{Na}}]_{210}$ values were successfully used to evaluate the degree of ionization of contributing chromophores in the case of H–Na mixed heparinate[2].

To account for the nonlinearity of the relationship in spite of constancy for $[\theta'_{\mathrm{Ca}}]_{210}$ and for $[\theta'_{\mathrm{M}}]_{210}$, it was assumed that X_{Ca} does not reflect the real Ca^{2+} molar-fraction at uronic carboxylate sites, the only one that are observed by c.d. measurements at 210 nm. This assumption results from the observation that during the mixing of calcium and alkaline heparinates, the Ca^{2+} and M^+ cations exchange to homogenize the ionic content of the macromolecules. However, a further intramolecular rearrangement took place, so that the Ca^{2+} ions neutralized preferentially the carboxylate negative charges, whereas the M^+ ion neutralized the sulfate charges. The molar fraction of Ca^{2+} ions located at the uronic carboxylate sites (N_{Ca}) was thus different from X_{Ca}, the Ca^{2+} molar fraction corresponding to the whole polyanion. Accordingly, eq. (*3*) was expressed in term of N_{Ca} (*4*)

$$[\theta'_{\mathrm{Ca,M}}]_{210} = \mathrm{N}_{\mathrm{Ca}} \,.\, [\theta'_{\mathrm{Ca}}]_{210} + (1 - \mathrm{N}_{\mathrm{Ca}}) \cdot [\theta'_{\mathrm{M}}]_{210} \qquad (4)$$

which could be rearranged to eq. (*5*).

$$\mathrm{N}_{\mathrm{Ca}} = [\theta'_{\mathrm{Ca,M}}]_{210} - [\theta'_{\mathrm{M}}]_{210}/[\theta'_{\mathrm{Ca}}]_{210} - [\theta'_{\mathrm{M}}]_{210} \qquad (5)$$

By use of eq. (*5*), values of N_{Ca} corresponding to data reported in Fig. 6a were calculated, and they were plotted against X_{Ca} (Fig. 6b). A single curve was found for the three couples of heparinates. This finding, which agrees with the observation that heparin has approximately the same low affinity for Li^+, Na^+, and K^+ as compared to its high affinity for Ca^{2+} ions[9], well supports our interpretation based on an intramolecular selectivity for divalent cations.

Independently, the curve $[\theta']_{210} = f(X_{\mathrm{Ca}})$ corresponding to the couple Ca heparinate–Na heparinate (Fig. 6a) was used to evaluate the molar fraction of calcium ions bound to mixed heparinate macromolecules resulting from the ion-exchange observed by mixing sodium heparinate with calcium chloride (Fig. 5). This evaluation was made in term of X_{Ca}, *i.e.*, with regard to the mixture of sodium and calcium heparinates that gives the same $[\theta']_{210}$ value.

The resulting X_{Ca} values were plotted in Fig. 7 together with values deduced from the analysis of mixed heparinates precipitated by addition of ethanol to sodium heparinate solutions containing known amounts of calcium chloride[12,13]. The remarkable agreement between both sets of data provides further support of our interpretation of the changes in optical activity of heparinic acid.

It is noteworthy that for the ratio of Ca to Na < 0.4:1, the slope of the curve indicated that the calcium ions present in the solutions were almost totally bound to the macromolecules. The distribution curve of the counter-ion reported in Fig. 6b asserts further that a large part of these bound Ca^{2+} ions preponderently neutralize the carboxylate negative charges, whereas the Na^+ ions neutralize the sulfate negative charges. The composition of the cations of plasma, and of most of the extracellular liquids, is approximately the following: Na^+ 142 mequiv. L^{-1}; K^+ 5 mequiv. L^{-1}; Ca^{2+} 5 mequiv. L^{-1} (2.5 disposable); and Mg^{2+} 1.75 mequiv. L^{-1}. The ratio of Ca^{2+} to Na^+ of the plasma is ~7:400, based on disposable calcium ions. Given the behavior of heparinates in salt-containing solutions, as shown here from c.d. data,

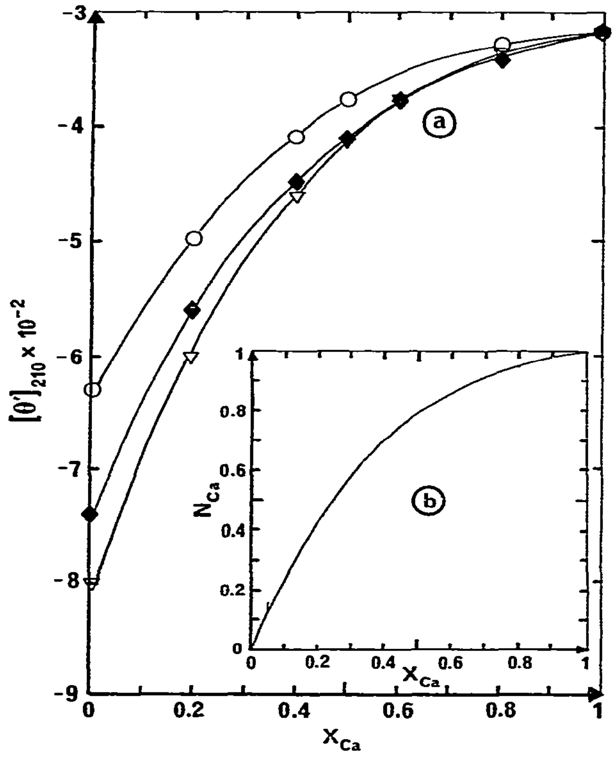

Fig. 6. (a) Variations of $[\theta']_{210}$ with calcium molar fraction X_{Ca} of calcium heparinate–other heparinate mixture at a constant concentration in heparin (T_N 26mN): (○) lithium, (◆) sodium, and (▽) potassium heparinate. (b) Corresponding molar fraction N_{Ca} of calcium ions neutralizing uronic carboxylate negative charges in Li–Ca, Na–Ca, and K–Ca mixed heparinates as a function of X_{Ca}, as deduced from eq. (4).

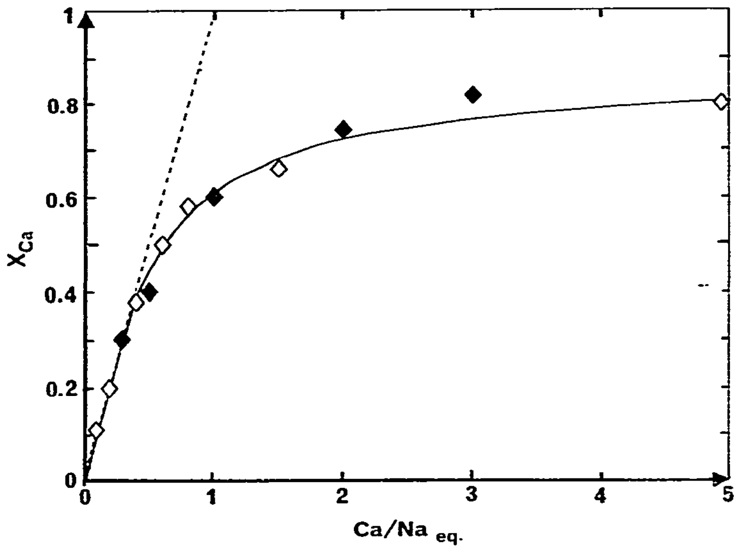

Fig. 7. Molar fraction (X_{Ca}) of calcium ions in mixed heparinates resulting from the ion-exchange between sodium heparinate and increasing amounts of calcium chloride defined by the Ca to Na ratio: (◇) as deduced from c.d. data in Fig. 5 and Fig. 6; and (◆) as determined by complexometric (Ca^{2+}) and by atomic absorption spectrophotometric (Na^+) analyses of ethanol-precipitated mixed heparinates ($T_X \sim$ 100 mN).

it is likely that heparin binds in body fluids, a relatively high proportion of divalent cations, and that most of these divalent cations (and especially the Ca^{2+} ones) are localized at the uronic carboxylate sites. Accordingly, the intramolecular selectivity for M^{2+} counter-ions, which is related to the distribution of uronic residues, and probably to the high charge density of the polyanion, could play a significant role in the biological activity of heparin, especially for heparin–protein interactions.

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